EXTRACTION, PURIFICATION AND CHARACTERIZATION OF BIOACTIVE POLYSACCHARIDES AND PROTEINS FROM *LENTINULA EDODES* MUSHROOM

ZHAO ZICHEN
PhD

The Hong Kong Polytechnic University
2025

The Hong Kong Polytechnic University
Department of Food Science and Nutrition

Extraction, Purification and Characterization of Bioactive Polysaccharides and Proteins from *Lentinula edodes* Mushroom

ZHAO Zichen

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

June, 2025

CERTIFICATION OF ORIGINALITY

I hereby declare that this thesis is my own work and that, to the best of my knowledge and belief, it reproduces no material previously published or written, nor material that has been accepted for the award of any other degree of diploma, except where due acknowledgment has been made in the text.

_____ (**Signed**)

Zhao Zichen (**Name of student**)

Abstract

Edible fungi, commonly known as mushrooms, are widely recognized for their nutritional and health benefits. Polysaccharides (PS) and proteins are two of the most abundant components in edible mushrooms, contributing significantly to their medicinal and nutritional value. While numerous studies have explored the diverse biological activities of polysaccharides extracted from edible mushrooms, research on their proteins remains limited. *Lentinula edodes*, known as Shiitake in Japanese and Xianggu in Chinese, is one of the most popular edible mushrooms and is widely consumed as a health food. This project aims to develop and implement effective methods for extracting and purifying proteins from *L. edodes*, as well as to investigate their molecular properties, nutritional composition, and immunostimulatory activity.

The first part of this project focused on evaluating the effectiveness of the three-phase partitioning (TPP) method for isolating PS and proteins from *L. edodes* mushrooms. By analyzing molecular properties and biological activities, the study optimized TPP conditions using response surface methodology (RSM), determining that 48.0% (w/v) ammonium sulfate $(NH_4)_2SO_4$), a 1.2 volume ratio of *t*-butanol to crude extract, and a temperature of 52 °C yielded the best results. Under these conditions, maximum yields of 11.64% for PS and 2.06% for protein were achieved. The PS fraction was primarily composed of glucose, galactose, and mannose, with a molecular weight of 7.35 kDa. Both fractions exhibited significant immunostimulatory effects, including increased nitric oxide (NO) production and enhanced phagocytic activity in RAW 264.7 cells. The protein-rich fraction demonstrated particularly strong

efficacy, with a low EC50 value of 0.28 μg/mL. In summary, TPP proved to be a simple and efficient method for isolating bioactive polysaccharides and proteins from mushrooms.

In the second part, protein extracted from *L. edodes* mushrooms using the TPP method, which has previously demonstrated significant immunostimulatory activity, was further investigated in this study. The research focused on fractionating and characterizing proteins to evaluate their nutritional and immunostimulatory properties. Crude protein was obtained from the mushroom aqueous extract and purified using anion exchange chromatography, resulting in two fractions: F1 and F2, with protein contents of 66.1% and 74.0%, respectively. Both fractions primarily exhibited protein structures consisting of β-sheets and random coils, while the crude protein also displayed an α-helix structure. SDS-PAGE analysis showed that F1 contained two molecular weight bands, one below 10 kDa and another at 34 kDa, whereas F2 exhibited multiple bands, including one below 10 kDa and others ranging from 34 to 95 kDa. In terms of nutritional value, as determined by essential and non-essential amino acid profiles, the ranking was F2 > F1 > crude protein. The amino acid ratio coefficient values were 63% for crude protein, 67% for F1, and 72% for F2. The combination of PS and protein fractions exhibited greater immunoactivity than either F1 or F2 alone. F2 demonstrated superior immunostimulatory activity in RAW264.7 cell cultures and contained a higher concentration of easily absorbed high-quality proteins compared to F1. These findings provide important insights into the dietary and

medicinal applications of protein fractions derived from *L. edodes* and other edible mushrooms.

The third part aimed to identify the most effective method for extracting proteins from *L. edodes* mushrooms by comparing water extraction (WE), enzyme-assisted extraction (EAE), ultrasound-assisted extraction (UAE), and various combinations of EAE and UAE, including EAE followed by UAE (EUE), UAE followed by EAE (UEE), and simultaneous EAE and UAE (SEUE). Among these methods, the two-step sequential process of EAE followed by UAE (EUE) yielded the highest protein content compared to UEE and SEUE. The soluble protein yield obtained by EUE (9.4%) was nearly three times higher than that of UEE (3.6%) and about twice as high as EAE (4.9%). Additionally, the protein fraction extracted by EUE had the highest protein content (56.0%) and β-sheet content (55.8%), and exhibited the strongest in *vitro* immunostimulatory activity. Using statistically designed experiments and response surface methodology, the optimal EUE conditions were determined to be 0.28% (w/v) enzyme concentration, 62% ultrasound amplitude, and 69% $(NH_4)_2SO_4$ saturation, resulting in a protein yield of 9.7% and a protein content of 58.4%. The extracted proteins had molecular weights below 10 kDa and between 25-75 kDa. The protein fraction contained essential amino acids and demonstrated significant immunostimulatory activity in vitro. Overall, EUE shows great promise as an efficient method for protein extraction from mushrooms for use in the food industry.

The studies above have shown that protein fractions from *L. edodes* exhibit even stronger immunostimulatory activity than its PSs, closely linked to their composition

and structural characteristics. More efficient extraction methods and optimized conditions have been developed to improve both protein yield and bioactivity. This research is expected to make a significant contribution to advancing knowledge and understanding of the processes required for the efficient extraction, isolation, and purification of protein from edible and medicinal *L. edodes*. Additionally, the project aims to deepen our understanding of the relationship between the molecular properties of these proteins and their immunomodulatory activity. With these findings, this thesis can provide valuable foundational insights for utilizing *L. edodes* protein as a targeted dietary supplement to enhance immune health.

Publications and Presentations from the Thesis

Publications

1. **Zhao, Z. C.,** Zhu, Y. Y., Gu, F. T., Huang, L. X., Liu, X., & Wu, J. Y. (2025). Sequential enzymatic and ultrasonic extraction of *Lentinula edodes* mushroom proteins leading to enhanced yield and significant immunoactivity. *Food and Bioprocess Technology*. (Accepted)
2. **Zhao, Z. C.,** Gu, F. T., Li, J. H., Zhu, Y. Y., Huang, L. X., & Wu, J. Y. (2024). Fractionation, characterization, and assessment of nutritional and immunostimulatory protein-rich polysaccharide-protein complexes isolated from *Lentinula edodes* mushroom. *International Journal of Biological Macromolecules*, 280, 136082.
3. **Zhao, Z. C.,** Huang, L. X., Dong, X. L., & Wu, J. Y. (2024). Evaluation of three-phase partitioning for efficient and simultaneous isolation of immunomodulatory polysaccharides and proteins from *Lentinula edodes* mushroom. *Food and Bioprocess Technology*, 17(8), 2277-2291.
4. Gu, F. T., **Zhao, Z. C.,** Zhu, Y. Y., Huang, L. X., Li, J. H., Liu, X., & Wu, J. Y. (2025). Human fecal fermentation of high/low-molecular weight exopolysaccharides from a medicinal fungus Cs-HK1 and anti-inflammatory protection on gut barrier function. *International Journal of Biological Macromolecules*, 145481.

5. Gu, F. T., Li, J. H., **Zhao, Z. C.,** Zhu, Y. Y., Huang, L. X., & Wu, J. Y. (2025). Metabolic outcomes of *Cordyceps fungus* and Goji plant polysaccharides during in vitro human fecal fermentation. *Carbohydrate Polymers*, 350, 123019.

6. Zhu, Y. Y., Dong, Y. H., Gu, F. T., **Zhao, Z. C.,** Huang, L. X., Cheng, W. Y., & Wu, J. Y. (2024). Anti-inflammatory effects of *Cordyceps Cs-HK1* fungus exopolysaccharide on lipopolysaccharide-stimulated macrophages via the TLR4/MyD88/NF-κB pathway. *Nutrients*, 16(22), 3885.

7. Huang, L. X., Gu, F. T., Zhu, Y. Y., **Zhao, Z. C.,** Li, J. H., & Wu, J. Y. (2024). Bifidogenic properties of polysaccharides isolated from mushroom *Lentinula edodes* and enhanced immunostimulatory activities through Bifidobacterial fermentation. *Food Bioscience*, 62, 105121.

8. Li, J. H., Gu, F. T., Yang, Y., **Zhao, Z. C.,** Huang, L. X., Zhu, Y. Y., ... & Wu, J. Y. (2024). Simulated human digestion and fermentation of a high-molecular-weight polysaccharide from *Lentinula edodes* mushroom and protective effects on intestinal barrier. *Carbohydrate Polymers*, 343, 122478.

Presentations

1. **Zhao, Z. C.,** Huang, L. X., Dong, X. L., & Wu, J. Y. Optimization of three-phase partitioning technique for efficient isolation of bioactive polysaccharides and proteins from *Lentinan edodes* mushroom. The 3rd ABCT Research Postgraduate Symposium in the Biology Discipline, Hong Kong, China, 2022.
2. **Zhao, Z. C.,** Gu F. T., Wu, J. Y. Evaluation of three-Phase partitioning for efficient and simultaneous isolation of immunomodulatory polysaccharides and proteins from *Lentinula Edodes* mushroom. 2nd PolyU Research Student Conference, Hong Kong, China, 2024.
3. **Zhao, Z. C.,** Huang, L. X., Dong, X. L., & Wu, J. Y. Fractionation, characterization, and assessment of nutritional and immunostimulatory proteins extracted from *Lentinula edodes* mushroom. 1st FSN Research Postgraduate Symposium, Hong Kong, China, 2024.
4. **Zhao, Z. C.,** Gu F. T., Wu, J. Y. Fractionation, characterization, and assessment of nutritional and immunostimulatory proteins extracted from *Lentinula edodes* mushroom. 21st Annual Meeting of CIFST, Chongqing, China, 2024.
5. **Zhao, Z. C.,** Wu, J. Y. Isolation, analysis, and evaluation of nutritional and immune-enhancing protein-rich polysaccharide-protein complexes. 10th International Conference on Food Chemistry & Technology (FCT-2024), Valencia, Spain, 2024.

6. **Zhao, Z. C.,** Wu, J. Y. Isolation, analysis, and evaluation of nutritional and immune-enhancing protein-rich polysaccharide-protein complexes. ICPNFF, Hongkong, China, 2025.
7. **Zhao, Z. C.,** Wu, J. Y. Isolation and characterization of protein-rich polysaccharide complexes from *Lentinula edodes*: Nutritional and immunostimulatory Properties. 2nd FSN Research Postgraduate Symposium, Hong Kong, China, 2025.
8. Gu F.T., **Zhao, Z. C.,** Wu, J. Y. Simulated digestion and gut microbial fermentation of *Cordyceps sinensis* polysaccharides and effects on intestinal barrier. 2nd PolyU Research Student Conference, Hong Kong, China, 2024.

Acknowledgments

I would like to thank my chief supervisor, Prof. Jian-yong Wu, and co-supervisors, Dr. Xiaoli Dong, and Dr. Chunyi Wen, for all their guidance. They provided professional advice and encouragement throughout this project and comments on this thesis and all the publications.

I would also like to thank the Department of Applied Biology and Chemical Technology, and the Department of Food Science and Nutrition of The Hong Kong Polytechnic University, all the technicians and staff, and my lab mates in Prof. JY Wu's group for their valuable suggestions and technical support during my PhD study.

Last but not least, I am indebted to my parents Zhao Yunlong and Guo Jie, my grandparents and grandmother, my fiancé, LAM Ka Wai, and my friends for their constant encouragement and support during this work, especially during the hard times.

Table of Contents

List of Figures

List of Tables

List of Abbreviations and Symbols

AA	Amino acid
AAS	Amino acid score
CS	Chemical score
EAE	Enzyme-assisted extraction
EAA	Essential amino acid
HPGPC	High-performance gel-permeation chromatography
LC-MS	Liquid chromatography-mass spectrometry
LPS	Lipopolysaccharides
MW	Molecular weight
NEAA	Non-essential amino acid
NF-κB	Nuclear factor kappa B
NMR	Nuclear magnetic resonance
NO	Nitric oxide
PS	Polysaccharide
RC	The amino acid ratio coefficient
RAA	Ratio of amino acids
RP-HPLC	Reversed-phase high-performance liquid chromatography
ROS	Reactive oxygen species
RSM	Response surface methodology
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
S/L	Solid-to-liquid ratio

SRC	Amino acid ratio coefficient score
TAA	Total amino acid
TNF-α	Tumor necrosis factor-alpha
TPP	Three-phase partitioning
UAE	Ultrasound-assisted extraction
WE	Water extraction

Chapter 1 Background and Objectives

1.1 Introduction

Mushrooms are cultivated extensively around the world because of their high nutritional value as well as medicinal properties (Muñoz-Castiblanco et al., 2022). Edible mushrooms are of significant interest in the food industry as they are recognized as functional foods, appreciated for their sensory qualities (aroma and flavor) and beneficial chemical composition, including carbohydrates, dietary fiber, proteins, vitamins, and minerals. Consuming mushrooms has been linked to the prevention of various cancers and heart disease, among other health benefits (Roncero-Ramos & Delgado-Andrade, 2017).

Lentinula edodes, generally known as Shiitake in Japanese and Xianggu in Chinese, belongs to the family *Agaricales*, *Tricholomataceae*, *Lentinula*. As a medicinal and edible mushroom with excellent nutritional and health benefits, the shiitake mushroom is often referred to as the "food queen" and the "king of mountain treasures" in Asian countries (Xiang et al., 2025). As the second most widely grown edible species in the world, it is prevalently cultivated in Asian countries, including China, Japan, as well as Korea (Sierra-Patev et al., 2023). *L. edodes* mushroom, as a high-quality edible fungi, is widely eaten by people. It is abundant in carbohydrates, protein, dietary fiber, important vitamins, low ash, and low fat and calories (Li et al., 2021). The superior quality and high nutritional value of *L. edodes* have garnered significant scientific interest due to their bioactive compounds and health-promoting properties (Chang & Miles, 2004). Numerous bioactive components, including PS, proteins, polysaccharide-

protein complexes, and small-molecule compounds, were isolated from mushrooms (Wasser, 2004). PS is one of the main active components of edible mushrooms, made up of long-chain carbohydrates. These are formed by the complex polymerization of neutral sugars or uronic acids via glycosidic linkages (Wang et al., 2022). These bioactive ingredients have been shown to have antioxidant, antitumor, antiviral, antimicrobial, and immunomodulatory effects (Wang et al., 2022). They have been used as natural active ingredients in the cosmetic and food industries.

Proteins are the major functional components of *L. edodes*. *L. edodes* mushroom also contains a higher protein content (typically ranging between 15-23%) compared to many other fungi (Das & Prakash, 2022). They are also receiving increasing attention owing to their medicinal potential (Wong et al., 2010). *L. edodes* exhibits abundant essential amino acids (EAA) (Yu et al., 2023), which is beneficial for human health. Mushrooms possess a wide array of proteins exhibiting fascinating biological functionalities, including antitumor, blood sugar-lowering, antioxidant, and antimicrobial properties (Zong, 2022). However, there is limited research on the immunomodulatory activity of *L. edodes* protein compared to its PS. It has only been found that certain proteins, such as lectin and xylose-rich heteroglycan-protein fraction (LAP1) in *L. edodes*, exhibit immunomodulatory effects (Hibino et al., 1994; Singh et al., 2010). As natural nutritional compounds, the proteins of *L. edodes* have the potential to offer new therapeutic options for enhancing immune health. Therefore, to thoroughly explore the immunomodulatory effects of proteins from *L. edodes*, it is essential to perform extraction and purification steps.

The extraction of *L. edodes* protein involves several steps to obtain a high-quality protein extract rich in bioactive compounds. Protein extraction methods are generally categorized into non-mechanical techniques (electrical, physical, chemical, and enzymatic) and mechanical methods (ultrasound-assisted extraction (UAE), microwave as well as high-pressure homogenization (HPH)) (Ahmed et al., 2024). Common chemical extraction methods for protein from *L. edodes* include alkali and organic extraction (Li et al., 2009; Zhao et al., 2021). Alkaline extraction methods mainly depend on the enhanced solubility of proteins in solutions with a pH that differs greatly from their isoelectric point (Meng et al., 2018; Gerliani, Hammami, and Aïder, 2019). Li et al. (2009) found that by using an alkali extraction method, the protein-to-PS-ratio in *L. edodes* extracts was higher at pH 10, leading to better separation. The alkali-soluble acid precipitation method is commonly used in industrial protein production due to its simplicity, established process, high extraction efficiency, and ease of waste liquid disposal (Zhao et al., 2021). Besides, enzymatic treatment typically functions by breaking down the cell wall, thereby releasing the extracted compounds (Gouseti et al., 2023). This method is promising for *L. edodes* protein extraction due to its simplicity and environmental friendliness, as it avoids the use of harsh chemicals. However, additional optimization is needed to improve both its efficiency and effectiveness. Prandi et al. (2023) used papain or protease from *B. licheniformis* to extract protein fractions from *L. edodes* grown on wood, which is efficient compared with aqueous extraction. Apart from the enzyme-assisted extraction (EAE) method, an environmentally friendly three-phase partitioning (TPP) method can also be used to

extract protein (Yan et al., 2018). The TPP method is an innovative and versatile technique used in nonchromatographic bioseparation processes. By adding ammonium sulfate ($(NH_4)_2SO_4$) and *t*-butanol to the stock solution, the solution can be separated into distinct phases. Proteins typically remain in the middle phase, which aids in their isolation and purification (Yan et al., 2018). However, this method is typically used to extract fungal PS rather than proteins (Yan et al., 2018). No studies have reported using the TPP method to extract protein from *L. edodes.*

In addition, mechanical protein extraction methods offer benefits in terms of scalability and cost-effectiveness (Ahmed et al., 2024). UAE is an efficient protein extraction method that facilitates the transport and release of proteins from the matrix by employing mechanical effects like cavitation, agitation, as well as temperature effects. These effects work together to break down cell structures and facilitate the efficient release of proteins (Ahmed et al., 2022). Prandi et al. (2023) found that UAE was more efficient for extracting proteins from *L. edodes* on a medium scale compared to laboratory conditions. UAE combined with alkaline extraction has been found effective for protein extraction from *L. edodes* (Wang et al., 2023). The UAE extract was obtained under alkaline conditions with a pH of 12 to maximize protein yield. However, no research has investigated the combination of EAE and UAE methods for protein extraction from *L. edodes*.

According to the aforementioned background, the effective extraction of *L. edodes* proteins while maintaining their immunostimulatory activity, as well as exploring the relationship between protein characteristics and immunostimulatory effects, has

become both valuable and crucial. The main objective of this study is to implement an effective approach for isolating and purifying protein from *L. edodes.* Furthermore, the project seeks to delve deeper into the molecular properties of the protein, while also examining the correlation between protein properties and their immunomodulatory activities.

1.2 Literature review

1.2.1 Edible fungi

Fungi form their own distinct kingdom, separate from plants and animals. This kingdom includes both large, fruiting bodies of edible fungi and various microscopic forms like molds and yeasts (Schweigert-Weisz et al., 2020). Edible fungi can produce substantial fruit bodies or colloidal sclerotia tissues (Zhang et al., 2021). Edible fungi belong to the *Phylum basidiomycota*, most of which are in the order *Agaricales*, and specifically refer to large edible fungi (Yan, 2018). Because of their nutritional content, therapeutic effects, and pleasant taste and flavor have been widely used as food and folk medicine worldwide. Nature harbors a diverse array of over 2000 edible fungi species, with approximately 200 being cultivated for commercial or experimental purposes (Pérez et al., 2021). Since the 1980s, researchers have investigated edible fungi chemical composition and pharmacological properties. Edible fungi can be categorized into two primary applications: edible species and medicinal species. Some edible species, such as *L. edodes*, *Flamulina velutipes*, and oyster mushrooms, are commonly consumed in our daily diet. On the other hand, medicinal species such as *Ganoderma lucidum* and *Cordyceps sinensis* are widely utilized in herbal medicine

(Gong et al., 2020). Edible fungi are not only abundant in essential amino acids (EAA), lipids, vitamins, and mineral elements crucial for human health but also encompass active constituents such as polysaccharides (PSs), nucleic acids, triterpenes, and sterols (Yan, 2018; Zhang et al., 2021). Among them, the most researched is PS. PS plays an essential role in anti-tumor, immune regulation, anti-bacterial, anti-viral, and prevention and treatment of heart and brain disorders (Lindequist et al., 2005). Some have already begun to be applied in clinical practice. Compared to PS, proteins have received less attention in research. However, the demand for protein is projected to increase substantially by 2050, fueled by the expected growth of the global population to nearly 9 billion people (Scott, 2017). Proteins, which are the main nutrients found in fungi, have various biological activities that have recently gained increased attention.

1.2.1.1 Fungal proteins

The fungal protein is normally prepared from the fruiting body or mycelium of edible fungi (Chen et al., 2021). Edible fungal protein is nutrient-dense and provides EAAs that complement plant protein (Kim et al., 2011). For example, the limiting amino acid in grain protein is lysine, but its content in the edible fungal protein is high (Young & Pellett, 2018; Gmoser et al., 2020). Process-optimized production of edible fungal mycelium protein with protein-corrected amino acid scores was similar to beef (Chan et al., 2018). Partial replacement of chicken with fruiting bodies of *Pleurotus ostreatus* did not affect the apparent protein digestibility of the product, but also increased the protein efficacy ratio (Wan & Solihah, 2014). It was found that using *Aspergillus fungus* to ferment soybeans can improve the protein nutritional quality of

feed after chemical analysis and protein secondary structure analysis (Yasar et al., 2020). Fungal protein can also be used to process imitation meat products because it provides certain elasticity, firmness, and chewiness (Stephan et al., 2018). Many traditional protein sources, like dairy, eggs, nuts, soybeans, as well as wheat (gluten), contain allergens and are common triggers for food allergies. Allergic reactions to edible fungi are extremely rare, making them a safe alternative for consumers with allergies to other foods (Kayode et al., 2020).

In addition to their extensive application in the food industry, edible fungal proteins are known to exhibit a variety of pharmaceutical and biological activities. These consist of lectins, ribonucleases, laccases, antibacterial and antifungal proteins, fungal immunomodulatory proteins (FIP), and other bioactive proteins. These compounds have gained popularity as natural sources of antitumor, antioxidative, as well as immunomodulatory agents (Xu et al., 2011). Given these diverse biological activities, the immunomodulatory properties of fungal proteins, in particular, have gained considerable interest due to their ability to enhance immune system function and regulate immune responses.

Research indicates that *L. edodes* has a higher protein content than other edible mushrooms (Ayimbila & Keawsompong, 2023), which has led to increased scientific attention on this species.

1.2.2 *L. edodes*

Shiitake mushroom (*L. edodes*) (as shown in **Figure 1-1**) is the fruiting body of the genus *Lentinus* in the family *Lentinaceae*, which belongs to the order *Agaricales* of the

phylum *Basidiomycota*. *L. edodes*, also known as a flower mushroom (Ponnusamy et al., 2022), is the second-largest edible mushroom in the world (Ponnusamy et al., 2022). It is commonly referred to as a "mountain delicacy" in folk culture. Biochemical analysis indicates that the composition of dried *L. edodes* extracts is predominantly carbohydrates, accounting for 58-60% of the total content. Proteins constitute 20-23%, while fiber, ash, and lipids make up 9-10%, 4-5%, and 3-4%, respectively (Wasser, 2004). Extensive research has demonstrated that *L. edodes* possesses a wide range of beneficial properties, including antitumor, immune-modulating, antioxidant, and hypotensive effects (Liu et al., 2019; Ponnusamy et al., 2022). PSs and proteins are identified as the primary active components in shiitake mushrooms responsible for eliciting the aforementioned therapeutic effects. PS, in particular, has undergone the most comprehensive research (Hobbs, 2000).

Figure 1-1. Dried fruiting bodies of *L. edodes*.

1.2.2.1 Polysaccharides

Polysaccharide (PS) is the most abundant component in shiitake mushrooms. The extracted PS from *L. edodes* fruiting bodies contains mainly six types of PSs (Zhang, 2019). The principal active component is β-(1,3)-D-glucan, featuring a central backbone of β-(1-3)-glucose residues and branching side chains of β-(1,6)-glucose residues (Mantovani et al., 2008; Yang et al., 2011). Its molecular formula is $(C_6H_{10}O_5)n$,

with a molecular weight (MW) generally above 1.6 kDa. *L. edodes* PS has significant physiological activities in anti-tumor and anti-cancer effects, and there have been numerous studies conducted both domestically and internationally, with thorough research already conducted (Mantovani et al., 2008; Yang et al., 2011). Many research results indicate that *L. edodes* PS with a molecular weight (MW) between 400 kDa and 600 kDa exhibit activity, while PS with a MW below 20 kDa are generally inactive (Siu, 2015; Zhang, 2019). According to a study, PS extracted from *L. edodes*, specifically those with a MW of 500 kDa, demonstrates anti-tumor activity (Zhang et al., 2007). **Table 1-1** outlines the various biological activities associated with PS.

Table 1-1. PSs and their main bioactivities in *L. edodes* medicinal mushrooms.

PS/PSP	Main bioactivities	References
Lentinan (β-glucans)	antitumor	(Bisen et al., 2010)
Mannoglucan	immunomodulation	(Bisen et al., 2010) (Hobbs, 2000)
Heteroglycan	antiviral	(Siu, 2015)
Heteroglucan-protein	antioxidant activities	(Siu, 2015)

1.2.2.2 Proteins

L. edodes mushroom is rich in high levels of protein, making it a quality source of dietary protein and a primary active ingredient. The protein content in the whole mushroom and the mushroom stem is 15.4% and 19.2%, respectively, including albumin, globulin, and glutelin in a ratio of 100:63:2, with a MW of approximately 20kDa-40kDa (Li et al., 2015; Xiao et al., 2015). As a high-quality edible fungus protein, *L. edodes* protein has higher nutritional value than animal and plant proteins (Manzi et al., 1999). Mushrooms have a rich protein content, several times to several tens of times higher than that of typical fruits and vegetables. For instance, 1 kg of dried mushrooms

provides as much protein as 12 kg of milk, 3 kg of eggs, or 2 kg of lean meat. Additionally, the distribution of amino acids (AAs) in mushroom protein is balanced, with essential amino acids (EAAs) accounting for 30% to 50% of the total amino acids (TAAs). As a result, mushrooms serve as an excellent source of EAA, including lysine, methionine, and tryptophan, which are often deficient in grains and legumes (Chen et al., 2014). The protein found in *L. edodes* mushroom comprises 18 AAs, encompassing both essential and non-essential types. The eight EAAs required by the human body play a crucial role in improving dietary quality, facilitating bone growth, and enhancing overall physical strength.

In addition, *L. edodes* and its active peptides are abundant in nutritional value and possess exceptional immune-modulating properties, making them highly valuable in the field of medical and health sciences. They hold significant value in the field of medicine and health (Gaitán-Hernández et al., 2019). Research has shown that lectins found in shiitake mushrooms can regulate the immune system, leading to anti-proliferative, anti-tumor, and antiviral effects (Singh et al., 2010). Additionally, a protein-polysaccharide complex with a MW of 500 kDa, extracted from *L. edodes* mycelium, has been found to modulate the immune response of human peripheral blood mononuclear cells (hPBMCs) (Liu et al., 1999). Furthermore, lentinin lamina-associated polypeptide 1 (LAP1), a protein found in *L. edodes*, has been found to have immunoregulatory functions on mouse splenocytes. It stimulates the production of interferon-gamma and nitrite, leading to anti-tumor immune responses (Hibino et al., 1994). Apart from immunomodulatory effects, *L. edodes* protein has many

physiological functions (as shown in **Table 1-2**). *L. edodes* protein plays an essential role in modern disease prevention and human activities.

Table 1-2. Proteins and their main bioactivities of *L. edodes* medicinal mushrooms.

Protein/peptide	Main bioactivities	References
Lentin	antifungal, antiproliferative, and HIV-1 reverse transcriptase inhibitory	(Ngai & Ng, 2003)
PS-protein complex, PS-peptide complex	immunomodulation	(Bisen et al., 2010) (Hobbs, 2000)
Ledodin,	cytotoxicity	(Citores et al., 2023)
Glycoprotein	antioxidant activities	(Gao et al., 2023; Siu, 2015)
Latcripin-8	antitumor	(Luan, 2017)
Peptide	angiotensin-I converting enzyme inhibitory	(Paisansak et al., 2021)

While the bioactive properties of *L. edodes* proteins, such as their antifungal, antitumor, and antioxidant activities, are well-documented, the broader immunomodulatory potential of fungal proteins represents a significant area of interest. These proteins, particularly those derived from medicinal fungi, play a crucial role in regulating immune responses, offering promising therapeutic applications for immune-related disorders and enhancing the body's defense mechanisms. The following section explores the immunomodulatory activities of fungal proteins, and the structural characteristics underlying their immune-regulating effects.

1.2.3 Immunomodulatory proteins

Immune system dysfunction is the primary cause of inflammation, tumors, as well as cancer. A stable immune system allows the body to effectively defend itself against external threats (Wang et al., 2013). The body has two types of immune responses, specific immunity, and nonspecific immunity, and macrophages play a bridging role in

both types of immune responses. Protein, as a natural immune modulator, can stimulate the immune system and activate macrophages, causing them to release various cytokines, thereby playing a role in immune regulation (Zhao et al., 2020; Ramlal & Samanta, 2022). This property makes fungal proteins a promising candidate for developing novel therapeutics for various immune-related disorders and further improving the body's defense against pathogens. Protein-derived immunomodulatory compounds from medicinal mushrooms can be categorized into specific groups, primarily Fungal immunomodulatory proteins (FIPs) and lectins. FIP differs from lectins in that they do not have any conjugates, while lectins are characterized by specific carbohydrates attached to a polypeptide (Zhao et al., 2020). Moreover, heat shock proteins (HSPs) have been identified as playing a vital role in immunomodulatory activity (Zininga et al., 2018).

1.2.3.1 Lectins

Lectins are a type of protein that do not possess immunogenicity and enzymatic activity. They contain one or more sugar recognition domains and can selectively and specifically bind to various types of monosaccharides and oligosaccharide structures without altering their covalent structure (Sharon & Lis, 1989). Fungal lectins are proteins that exist as monomers or dimers, with a MW spanning from 10 to 100 kDa. They are composed of 2 to 4 identical subunits. These lectin molecules contain at least one sugar recognition domain connecting two structural domains. They can bind to sugars in different ways, either through O-linked or N-linked interactions, resulting in the formation of glycoproteins (Yu et al., 2021). Among various mushroom species, the

highest number of lectins has been discovered in *Lactarius*, followed by *Pleurotus, Agaricus*, *Amanita*, and *Boletus* (Yu et al., 2021). In addition, lectins rely on targeted selection to bind to sugar residues, specifically recognizing foreign cells. As a result, they also possess immunomodulatory activity (Liu et al., 2021). Lectins can modulate immune responses by inducing nitrite production, enhancing pro-inflammatory cytokine expression, activating lymphocytes, and stimulating macrophage-activating factors (Zhao et al., 2020).

1.2.3.2 Fungal immunomodulatory proteins

Fungal immunomodulatory proteins (FIPs) are another biologically active substance isolated from mushrooms after PS and triterpenoids (Li et al., 2011). They are a class of proteins produced by various types of fungi that have been found to modulate the immune system in animals, including humans. FIPs are small, cysteine-rich proteins typically between 10-20 kDa in size (Ko et al., 1995). More than 38 types of FIP have been identified (Liu et al., 2020). For instance, they are produced by many different types of fungi, including *Cordyceps militaris, Ganoderma lucidum*, and *Trametes versicolor* (Tie et al., 2016). FIP can be categorized into five subgroups according to their shared protein characteristics and structure: *Tremella fuciformis* protein (TFP)-like FIP, *Poria cocos* immunomodulatory protein (PCP)-like FIP, Cerato-type FIP, Fve-type FIP, as well as unclassified FIP. Fve-type FIP is the largest subgroup, with a MW of around 13 kDa and consisting of 110-125 AAs (Ko et al., 1995; Liu et al., 2020).

Research has extensively explored the potential therapeutic applications of FIPs

for treating a range of illnesses, including cancer, infectious diseases, as well as autoimmune disorders (Li et al., 2011). The variable portion of an immunoglobulin-heavy chain shares structural similarity with FIP. It also inhibits allergic reactions, stimulates mouse spleen lymphocyte proliferation, promotes nucleic acid and protein synthesis, accelerates metabolism, and enhances the body's immunity (Guo et al., 2007). FIP activates antigen-presenting cells (APCs) to produce substances such as nitric oxide (NO) and IL-12 by interacting with Toll-like receptors (TLRs). This activation encourages helper T cells (Th0) to proliferate and differentiate into Th1 and Th2 cells. Furthermore, it enhances the activation of macrophages and B cells, leading to the production of various cell factors (**Figure 1-2**). In this process, the nuclear transcription factor NF-κB is activated, and p38/MAPK undergoes phosphorylation. In summary, edible mushroom immunoregulatory proteins activate the immune mechanism by binding to TLRs, which triggers the p38/MAPK and NF-κB signaling pathways (Zhao et al., 2020).

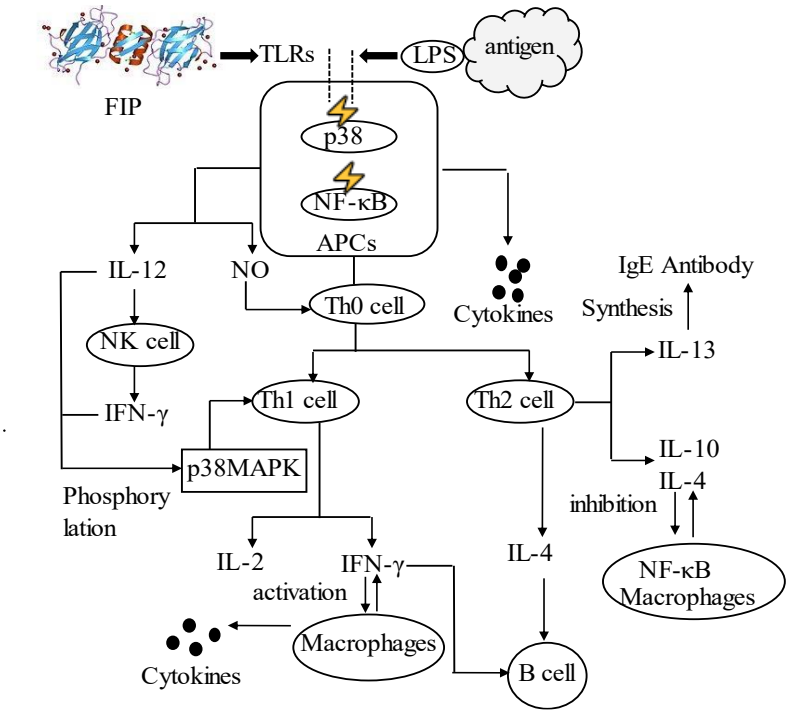

Figure 1-2. Immunomodulatory mechanism of FIPS. LPS: Lipopolysaccharide, NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells, p38MAPK: p38 mitogen-activated protein kinase, IL-12: Interleukin-12, IgE: Immunoglobulin E, Th0 cell - T helper 0 cells, NK cell: Natural Killer cells, Th1 cell: T helper 1 cells, Th2 cell: T helper 2 cells IL-2: Interleukin-2, IFN-γ: Interferon gamma, IL-4: Interleukin-4, IL-13: Interleukin-13, IL-10: Interleukin-10.

1.2.3.3 Heat shock proteins

A class of proteins known as heat shock proteins (HSPs) is produced by cells in response to stressful situations. HSP was initially found by Ritossa, when the temperature goes more than 5°C above the normal level for growth, most regular proteins stop being produced, while some new proteins are quickly made in large amounts, and these are called HSPs (Elizabeth, 1991). In recent years, the immunomodulatory potential of HSPs has drawn considerable interest (Zininga et al., 2018), especially in the context of fungi (Tiwari et al., 2015). HSPs are primarily

categorized into seven families based on their molecular size: small HSPs (approximately 15-30 kDa), Hsp110, Hsp100, Hsp90, Hsp70, Hsp60, as well as Hsp40 (Rappa et al., 2012; Elizabeth, 1991). The fungal HSP9/12 (IPR007250) belongs to the small heat shock protein family, typically ranging from 9 to 12 kDa in size. HSP9 was found in different medicinal mushrooms such as *Grifola frondosa*, *Pleurotus eryngii, Hypsizygus marmoreus,* and *L. edodes*, which was primarily located in the cell wall (Kurahashi et al., 2014). Small HSPs have been found to possess immunostimulatory activity (Van et al., 2012). Other HSPs in fungi have also been identified as possessing immunological activity (Tiwari et al., 2015). The glucose-regulated protein 96 (gp96), HSP60, HSP70, as well as HSP90 are primarily believed to regulate the innate immune system by activating TLR4 and TLR2. This activation leads to the release of pro-inflammatory cytokines (e.g., IL-1β, IL-6, as well as TNF-α), along with the secretion of NO and C-C chemokines by monocytes, macrophages, as well as dendritic cells (Tsan & Gao, 2004; Quintana & Cohen, 2011). Conversely, under certain conditions, HSP60 can also exert anti-inflammatory effects, making it a versatile regulator of immune function (Quintana and Cohen, 2011). This dual capacity to either stimulate or suppress immune activity stresses the therapeutic potential of HSPs in fungi for managing immune-related disorders. Understanding the mechanisms by which HSPs influence the immunomodulatory effects of medicinal mushrooms could provide new insights into their applications in functional foods and immunotherapy.

Proteins from fungi have also been reported to possess immunomodulatory effects. The subsequent section delves into the link between the structural features of fungal

proteins and their ability to modulate immune responses.

1.2.3.4 Structural characteristics of fungal proteins and their immunomodulatory effects

Lectins have been reported that the ricin B chain protein can bind to the specific structure of galactose/N-acetylgalactosamine, regulate immune cell activation, and promote mouse spleen lymphocytes, $CD4^+$ T cells, and mouse RAW264.7 macrophages proliferation and activation (Liu et al., 2021). Several large fungal lectins, structurally similar to the ricin B chain protein, have demonstrated immunomodulatory properties (Mancheñ O et al., 2005; Liu et al., 2021). The ricin-B-like lectin (CNL) from *Clitocybe nebularis*, a 15.9 kDa homodimer, has shown antiproliferative activity targeting human leukemic T cells (Pohleven et al., 2009). Additionally, CNL has been found to induce the maturation and activation of dendritic cells (DCs) and stimulate the production of proinflammatory cytokines such as IL-6, IL-8, and TNF-α (Švajger et al., 2011).

Fungal immunomodulatory proteins (FIPs) can be classified into two groups—domain-type and non-domain-type—based on the presence or absence of conserved domains (Liu et al., 2020). Most of the discovered fungal immunomodulatory proteins exhibit a high degree of homology, and their primary structures have similar amino acid (AA) compositions. Except for *Antrodia antrodia* and *Versicolor versicolor*, most fungi lack histidine (His), cysteine (Cys), and methionine (Met) in their immunomodulatory proteins. Instead, they contain a high abundance of aspartic acid (Asp) and valine (Val), with 28 proteins sharing identical sequences. Among the present fungal immunomodulatory proteins, LZ-8 is a glycoprotein, while the others do not contain

sugar (Tie et al., 2016). The analysis of LZ-8 secondary structure revealed a high abundance of β-sheets. Additionally, based on the secondary structure prediction, LZ-8 is expected to comprise seven β-sheets (Tanaka et al., 1989). The secondary structure of FIP-gts in *Ganoderma lucidum* includes seven β-sheets, one β-turn, as well as two α-helices (Lin et al., 1997). The secondary structure of FIP-lrh is composed of seven β-sheets as well as at least one α-helix (Pushparajah et al., 2016). Ko et al. (1995) found that the immunomodulatory protein FIP-five of *Flammulina velutipes* has similar secondary structures to LZ-8 and FIP-gts, which consist of 3 α-helices, six β-sheets, and one β-turn composition. The only difference is that the β-sheet at the 3' end of FIP-gts becomes an α-helix in FIP-fve (Ko et al., 1997). *Flammulina velutipes* fungal immunity regulatory protein FIP-fve has a dimer structure (Seow et al., 2003). Its molecular structure is similar to a "dumbbell shape" (Paaventhan et al., 2003). Additionally, the N-terminal regions of most FIP proteins are primarily composed of hydrophobic AAs and lack cysteine residues, preventing the formation of disulfide bonds. As a result, hydrophobic interactions play a central role in stabilizing the α-helical structures at the *N*-terminals, enabling the dimerization of two monomers (Paaventhan et al., 2003; Tie et al., 2016). Other studies have shown that the crystal structure of LZ-8 in *Ganoderma lucidum* is also a homodimer formed by two monomers, similar to FNIII of FIP-fve (Huang et al., 2009). It mainly comprises β sheets A-B-E and G-F-C-D to form two sheets, respectively. Another research study discovered that FIP-gmi, a fungal immunomodulatory protein present in the microspore *Ganoderma lucidum*, exists as a homotetramer according to its crystal structure (Wu et al., 2007).

In addition, carbohydrate-binding modules (CBM) structures in glycoside hydrolases are crucial for substrate binding (Boraston et al., 2004). Key residues in FIP-fve, such as W24, D34, as well as T28, are essential for the formation of the CBM34-like structure (Liu et al., 2012). This structure has been associated with hemagglutination activity and the ability to induce IFN-γ secretion in human peripheral blood mononuclear cells (hPBMCs) (Liu et al., 2012). Extensive research has demonstrated that the functional activity of Fve-type FIP is closely linked to its dimerization state. For instance, FIP-gts were found to be inactive in inducing cytokine production on hPBLs when in their monomeric state (Lin et al., 1997). Crucial residues, including L9, F7, L5, and the N-terminal α-helix, which play a vital role in FIP-gts dimerization, are indispensable for triggering IL-2 and IFN-γ secretion (Lin et al., 1997; Ou et al., 2009).

Given the structural sensitivity of these bioactive proteins, the development of optimized extraction and separation methods becomes paramount to preserve their functional integrity. Natural edible fungi contain complex matrices of PSs, lipids, nucleic acids, and polyphenols, which can interfere with protein stability and activity (Yang et al., 2019). Therefore, advancing extraction techniques is essential to isolate high-purity fungal proteins, ensuring their immunomodulatory potential is retained for therapeutic and functional applications.

1.2.4 Methods for extraction and separation of proteins

Common non-mechanical protein extraction methods include physical, electrical, chemical extraction, and enzyme-assisted extraction (EAE) (Zhao et al., 2021). However, some non-mechanical extraction methods may also have several

disadvantages, such as low selectivity, usually necessitating lengthy extraction durations, and substantial organic solvent usage, which pose safety risks to operators and the surrounding environment. Supercritical fluid extraction and three-phase partitioning systems are relatively new, eco-friendly technologies for protein extraction compared to traditional extraction methods (Ketemepi et al., 2024). Additionally, there are several mild mechanical extraction methods, such as bead milling, ultrasonic-assisted extraction, high-pressure homogenization (HPH), microwave-assisted extraction (MAE), and slow freeze-drying for protein extraction (Li, 2015; Yu, 2017; Ahmed et al., 2024).

1.2.4.1 Non-mechanical extraction methods

Non-mechanical methods can be divided into four main categories: physical, electrical, chemical, as well as enzymatic approaches (Pobiega et al., 2024). Details of the non-mechanical extraction methods applied in fungi proteins are shown in **Table 1-3**. Physical extraction methods, like freeze-thawing, disrupt microbial cells by forming large ice crystals during slow freezing, which leads to cell damage. However, this method is usually limited to laboratory-scale applications due to the high operating costs, resulting from the significant time and energy consumption (Pobiega et al., 2024). Electrical methods include pulsed electric field (PEF) and high-voltage electrical discharge (HVED), which work by breaking down or disrupting cells through the application of electric fields (Phong et al., 2018). These methods are typically performed at low temperatures, are environmentally friendly, and energy efficient. PEF entails the application of high-voltage pulses between two electrodes immersed in an

aqueous medium. This process increases the permeability of the cytoplasmic membrane and facilitates the release of intracellular compounds. In contrast, HVED generates an arc discharge by creating an intense electrical field between a needle electrode and a ground electrode (Gautério et al., 2023). This process causes cell damage through bubble cavitation, pressure shock waves, as well as high liquid turbulence. HVED is preferred over PEF due to its higher efficiency and its ability to more effectively break down cell membranes, releasing compounds (Coustets et al., 2015).

Chemical extraction includes water extraction, acid or alkaline extraction, and organic extraction. Chemical extraction is a widely used approach for breaking down the cell wall by dissolving its components (Ahmed et al., 2024), owing to its affordability, operational simplicity, and effectiveness. Water extraction is a method that involves suspending fungal biomass in a buffer solution mainly composed of water. This solution often includes additional components such as ionic detergents (SDS), non-ionic detergents (NP-40 and Triton X100), as well as salts (sodium chloride (NaCl) or potassium chloride (KCl)) (Kumar et al., 2021), to effectively break cell walls and facilitate the release of proteins into the solution. The process is followed by centrifugation to separate the cell debris from the soluble protein extract. Alkaline solutions (sodium hydroxide (NaOH) and potassium hydroxide (KOH)) are commonly used chemicals for protein extraction (Pobiega et al., 2024). Hydrochloric acid (HCl) and sulfuric acid (H_2SO_4) are commonly used for protein extraction. However, their reduced efficiency in cell wall degradation can hinder protein diffusion, making this approach less desirable in some instances (Ketemepi et al., 2024). Additionally, it may

result in gel formation or necessitate extra agitation during scale-up (Ahmed et al., 2024). Alkaline and acid extraction methods exploit the high solubility of proteins in solutions with a pH far from their isoelectric point (Meng et al., 2018; Gerliani, Hammami, and Aïder, 2019). Organic solvents are essential in protein extraction and precipitation, aiding significantly in the purification process. Commonly used solvents include alcohols like ethanol and methanol, along with strong denaturants such as urea, Tris (hydroxymethyl) aminomethane (THAM) hydrochloride (Tris-HCl), and phenol. Additionally, solvents like butanol and acetone are used to extract proteins with lipid-binding properties, non-polar or polar side chains, and aromatic AAs, as they effectively dissolve these compounds (Kumar et al., 2021). The alkali-soluble acid precipitation method is commonly used in industrial protein production due to its simplicity, established process, high extraction yield, and ease of waste liquid disposal (Zhao et al., 2021). However, some harsh chemical methods can negatively impact proteins. Chemical extraction methods can be time-consuming, energy-intensive, and environmentally harmful due to the use of chemical reagents. Meanwhile, factors like the solid-to-liquid ratio (S/L) (g/mL), pH, and extraction time all influence the extraction yield and purity of edible fungi protein during preparation (Chen et al., 2021), which requires further optimization. In recent years, researchers have optimized the alkaline extraction conditions of different edible fungi proteins by controlling different approaches, such as single factor, orthogonality, and response surface methodology (RSM). It was found that the optimal conditions for extracting edible fungi proteins are a pH of 10-12, a temperature range of 10-60 °C, an extraction time of 30-150 min, and

S/L (g/mL) of 1:20 to 1:40. The isoelectric point ranges from 3.5 and 4.5 (**Table 1-3**). Furthermore, numerous studies have indicated that the optimal extraction temperature for fungal protein peaks is between 50-60 °C. Excessive temperatures can compromise the protein structure, leading to increased extract viscosity and reduced extraction efficiency (Cui et al., 2018; Li et al., 2020).

Enzyme-assisted extraction (EAE) presents a promising approach for fungal protein extraction due to its simplicity and avoidance of harsh chemicals. The extraction of cellular proteins is hindered by the presence of a rigid cell wall. EAE primarily targets the disruption of cell wall integrity by enzymatically degrading its key components, including cellulose, as well as pectin, under optimal conditions (Kumar et al., 2021). This mechanism enhances the release of bioactive compounds from diverse natural sources (Franca et al., 2021). Enzymes commonly used for extraction include carbohydrases such as cellulase, pectinase, β-glucanase, and hemicellulase, as well as proteases like papain (Kumar et al., 2021; Ahmed et al., 2024). Proteases increase protein yield by detaching proteins from the polysaccharide matrix, while cell wall degradation helps release the cellular proteins (Kumar et al., 2021). Once released, proteases degrade high MW proteins into smaller, more easily dissolvable fragments, improving the conditions for extraction. Research indicates that EAE achieves higher protein recovery compared to other extraction approaches, such as alkaline and acid. However, alkaline and acid extraction are simpler to operate, cost-effective, well-established, and suitable for large-scale factory production (Hu, 2019; Ahmed et al., 2024). Besides, EAE still faces limitations, such as the high impact of substrate

heterogeneity on enzymatic efficiency and the relatively high cost of enzyme preparations. Future research could focus on the development of heat-resistant/acid-tolerant engineered enzymes or combine mechanical methods (such as ultrasound and HPH) to establish a green and efficient extraction process, meeting the growing demand for high-purity mushroom proteins in the functional food and biopharmaceutical sectors.

Table 1-3. Non-mechanical extraction methods.

Method	*Fungi Species*	Experiment conditions	References
Freeze-thawing	*Neurospora crassa*	Freeze: 60 min at -20 °C, Thawed: 30 min at room temperature	(Stine et al., 1964)
PEF	*Agaricus bisporus*	Electric field strengths: 38.4 kV/cm, Time: 272 μs, Temperature: 85 ºC	(Xue & Farid, 2015)
HVED	*Saccharomyces cerevisiae*	N successive pulses: N=1-500, Amplitude: U=40 kV, Time: 2-1,000 s, Rate: 0.5 Hz,	(Liu et al., 2013)
Chemical extraction	*Pleurotus eryngii*	a. S/L: 1:40, pH: 11, Time: 40 min, Temperature: 40 °C	(Chen et al., 2017)
		b. S/L: 1:25 pH: 11, Time: 70 min, Temperature: 70 °C	(Chen et al., 2021)
		c. S/L: 1:12 pH: 10, Time: 28.6 min, Temperature: 28.2 °C	(Liu et al., 2013)
	Hypsizygus marmoreus	S/L: 1:40, pH: 12, Time: 120 min, Temperature: 61 °C	(Xiao et al., 2020)
	Agricus bisporus	a. S/L: 1:3, pH: 10, Time: 150 min, Temperature: 50 °C	(Zheng et al., 2019)
	Stropharia rugosoannulata	a. S/L: 1:30, pH:12, Time: 45 min, Temperature: 60 °C	(Cui et al., 2018)
EAE	*Agricus bisporus*	Alkaline protease, pH: 8.43, Temperature: 44.32 °C, Time: 3.52 h	(Wang et al., 2022)
	Hericium erinaceus mycelium	Papain, S/L: 1:25, Time: 1.5h, Temperature: 25 °C	(Liu et al., 2020)
	Pleurotus eryngii	Cellulase, pH: 7.2, S/L: 1:35, Time: 4h, Temperature: 60 °C	(Zhang et al., 2015)
	Flammulina velutipes	Compound proteinase, pH: 6.49, S/L: 1:15, Time: 33.03 min, Temperature: 43.92 °C	(Zhang et al., 2015)

1.2.4.2 Mechanical extraction methods

Mechanical approaches are known for their scalability, being environmentally friendly, and low operational cost. These include techniques such as bead milling, ultrasonication, and high-pressure homogenization (HPH) (Scholtmeijer et al., 2023; Ahmed et al., 2024;). Bead milling, or bead beating, involves disrupting cells through shear forces generated by the rotational motion of the cells and the beads (Gautério et al., 2023). This disruption occurs due to the shear forces created by the movement and rotation of the cells and beads, with cells being crushed between them (Nemer et al., 2021). Optimizing protein yield from bead milling requires adjusting factors like bead size, density, filling, agitation speed, as well as lysis agents based on the rigidity of the fungal cell wall (Nemer et al., 2021; Ahmed et al., 2024). It was also observed that the protein yield increased when bead milling was conducted under liquid nitrogen conditions (Taskova et al., 2006). While bead beating is effective, high-speed grinding may cause protein modifications (Liu et al., 2021). HPH is a mechanical method for cell disruption that involves pushing a cell suspension via a narrow nozzle or valve under high pressure (Gautério et al., 2023). In this process, the suspension is pumped into a compression chamber, where it experiences high pressure and significant shear forces (Li et al., 2022). The method relies on fluid impingement and shear forces to rupture cells, releasing their contents into the surrounding liquid medium. The effectiveness of this technique relies on precise control of pressure, temperature, as well as the number of valves passes to achieve efficient cell disruption while maintaining the integrity of intracellular components. HPH was identified as the most efficient technique for breaking down cell walls compared to ultrasonication and enzymatic

treatment, resulting in the release of a larger amount of protein (Zeng et al., 2023). Otero et al., (2000) noted that HPH can cause significant protein denaturation, leading to unfolding and aggregation, which reduces the solubility of extracted fungi proteins to below 40%. Consequently, when using fungal proteins as a source, it's important to consider how different treatment conditions affect their properties to enhance their utilization.

Microwave-assisted extraction (MAE) is another mechanical approach that has been explored for use in industrial-scale processes. Microwaves are electromagnetic fields characterized by frequencies ranging from 300 MHz to 300 GHz, or wavelengths between 1 cm and 1 m (Gartshore et al., 2021). They consist of electric and magnetic fields that are perpendicular to each other. This method is eco-friendly due to its efficiency, requiring minimal time and energy. By heating polar solvents in contact with solid samples, this method increases temperature and pressure, promoting the release of cellular compounds. Microwave heating operates by transforming electromagnetic energy into thermal energy via two main mechanisms: ionic conduction as well as dipole rotation (Sun et al., 2016). It efficiently disrupts cell walls in a short time while using minimal energy (Pobiega et al., 2024). Ion migration and dipole rotation induced by microwave facilitate solvent penetration into the matrix, ultimately breaking the cell wall and leading to more efficient protein extraction than traditional water bath-assisted extraction (Shi et al., 2024). Khotchai et al. (2025) discovered that high temperatures and extended extraction durations can lead to protein degradation, with the most notable reduction occurring at 120 °C. The excessive heat breaks peptide bonds, affecting

protein integrity. Therefore, careful temperature control during MAE is essential to maintain high protein yield.

Ultrasonication is not as commonly used in the industry as the other techniques mentioned earlier (Pobiega et al., 2024). Ultrasound-assisted extraction (UAE) is a powerful technique for extracting components from cells by generating hotspots of elevated temperature and pressure as well as acoustic cavitation (Kumar et al., 2021). The quick generation and implosion of gas bubbles on the cell surface create micro-streaming and shock waves, which exert high shear and mechanical forces, leading to membrane and cell wall disruption (Lupatini et al., 2017). This disruption allows the surrounding solvent to penetrate the cells through the resulting openings, releasing intracellular proteins into it. UAE can enhance extraction yields by helping to disaggregate and disperse proteins in aqueous solutions (Ahmed et al., 2024). In addition to cell disruption, UAE can lead to fragmentation, erosion, pore formation, and enhanced absorption within the solid matrix (Ahmed et al., 2024). To prevent overheating, samples are treated in an ice bath. Key factors influencing the UAE, such as frequency, power, as well as time (Kumar et al., 2021). Using ultrasonic power of 130-756.8 W to treat edible fungus raw materials can effectively accelerate protein entry into the solvent (Chen et al., 2021). Numerous studies on ultrasonication have concentrated on frequency ranges between 20 and 40 kHz. Most findings indicate that 20 kHz produces the most significant cavitation effects while minimizing protein denaturation compared to higher frequencies like 40 kHz (Ketemepi et al., 2024). While the UAE is generally effective, incomplete cell lysis can occur at times, potentially

resulting in decreased protein yields (Rahman & Lamsal, 2021).

To improve protein extraction efficiency while maintaining functionality, a synergistic approach that combines non-mechanical and mechanical methods is increasingly being used (Kumar et al., 2021). Combining ultrasonication with chemical extraction, such as alkali treatment, has been demonstrated to improve cell disruption and boost protein extraction from *Chlorella sorokiniana* (Pobiega et al., 2024), more effectively than using each method alone. By combining the precision of chemical agents with the disruptive power of mechanical forces, this hybrid approach enhances yield and quality, especially for complex structures like fungal biomass. It also addresses the limitations, such as incomplete lysis or excessive degradation, that can occur when using each method separately. Furthermore, enzyme treatment can enhance UAE extraction by significantly increasing protein yields and reducing the risk of protein denaturation (Kumar et al., 2021). **Table 1-4** provides details on mechanical extraction methods and their combination with non-mechanical techniques used for extracting fungal proteins.

Table 1-4. Mechanical and a combination of non-mechanical extraction methods.

Methods	Fungi Species	Experiment conditions	References
Bead Milling	*Pleurotus sapidus and Lepista irina*	0.25-0.5 mm diameter glass beads, Milled: 15 min, Temperature: 4 °C	(Taskova et al., 2006)
HPH	*Filamentous fungal biomass (Mycorena AB)*	HPH: 900 bar, 2 passage, pH: 12	(Zeng et al., 2023)
MAE	*Schizophyllum commune*	S/L: 1:4, Time: 10 min, Temperature: 80 °C	(Khotchai et al., 2025)
	Lactarius volemus	S/L: 1:40, Time: 20 and 60 min, pH: 12	(Shi et al., 2024)

UAE	*Fresh A. bisporus fruiting body*	S/L: 1:5, Time: 40 min, Temperature: 43 °C, Power: 130 W	(Ma et al., 2012)
UAE & Chemical	*M. esculenta*	S/L: 1:30, pH: 11, Time: 60 min, Temperature: 50 °C	(He, 2015)
	Hericium erinaceus mycelium	S/L: 1:25, pH: 10.5, Time: 21.8 min, Temperature: 34.6 °C, Power: 758.6 W	(Liu et al., 2020)
	Grifola frondosa	S/L: 1:95, pH: 10.5, Time: 3 min, Temperature: 35 °C, Power: 600 W	(Huang et al., 2016)
	Cordyceps militaris	S/L: 1:28, pH: 8.5, Time: 3.5 h, Temperature: 25 °C, Power: 100W	(Xu et al., 2023)
UAE & EAE	*Pleurotus eryngii*	Cellulase, S/L: 1:30, Time: 20 min, Power: 400 W	(Xu et al., 2020)

1.2.4.3 Method for protein extraction from *L. edodes*

The commonly used extraction method is alkali immersion and acid precipitation, which involves immersing the mushrooms in an alkaline solution to extract the protein and then collecting the protein using methods such as isoelectric point precipitation and $(NH_4)_2SO_4$ precipitation (Prandi et al., 2023). In addition, EAE, UAE, freeze-assisted, and MAE methods are also used to extract shiitake mushroom protein (Prandi et al., 2023). UAE utilizes mechanical effects to cause strong damage to the raw materials, deforming the cell tissue. Through the utilization of thermal energy, the procedure enhances the dissolution rate of active components, thereby enhancing extraction efficiency and decreasing extraction duration (Shen et al., 2023). Li et al. (2009) conducted a study on the extraction of shiitake mushroom protein, wherein the mushroom powder was combined with a pH 10 NaOH solution at a ratio of 10-20 times the powder volume. The mixture was maintained at 10 °C for 30 min, followed by centrifugation at 3000 r/min for 15 min. The resulting supernatant was concentrated

under reduced pressure, and protein precipitation was achieved by adjusting the ethanol concentration to 83%, yielding a protein recovery of 13.1%. Wang et al. (2023) used UAE to extract protein. The UAE process for extracting mushroom protein was determined to have the optimal extraction conditions of a solid-to-liquid ratio (S/L): 1: 50 (g/ml), pH value of 12, ultrasonic extraction for 16 min at a power of 90 W, and temperature of 50 ℃. The protein extraction yield was 28.56%, accompanied by a protein purity of 61.70% (Wang et al., 2023). Xuan et al. (2014) explored a low-temperature water extraction method for *L. edodes* mushroom protein at 10 °C, using S/L of 1:35 (g/mL). After 20 min of extraction, a protein extraction yield of 9.27% was achieved. Yu (2017) used freeze-assisted extraction, which involved freezing the shiitake mushrooms at -4 to -20 °C and then using alkali immersion and acid precipitation to extract the protein. The principle behind this method is that freezing causes ice crystals to form inside the cells, which helps to rupture the cell walls and facilitate protein dissolution. Prandi et al. (2023) used protease from *B. licheniformis* or papain to extract protein from *L. edodes* grown on wood. Compared to traditional alkaline solubilization and acid precipitation methods, enzymatic extraction of proteins has several advantages, including mild reaction conditions, being environmentally friendly, shorter extraction time, higher extraction efficiency, and fewer by-products (Prandi et al., 2023). The alkali immersion and acid precipitation method, while exhibiting a slightly lower extraction yield compared to the EAE method, offers advantages such as operational simplicity, cost-effectiveness, and technical maturity. These characteristics make it highly suitable for large-scale industrial production (Hu,

2019). However, proteins are prone to denaturation and undergo intense Maillard reactions in a strong alkaline environment, producing brown substances. Additionally, the action of strong alkali can cause dehydration and condensation reactions between lysine and cysteine or alanine in proteins, leading to the formation of harmful substances in the human body (Zhang, 2017). Furthermore, the extraction time is long, and it is easy to cause changes in the structure and properties of active substances (Zhang et al., 2023). To minimize the adverse effects on protein extraction, preserve its biological activity, and increase protein yield, further optimization of the protein extraction process is essential. The extraction yield and purity of edible fungi protein are influenced by factors such as the S/L, pH, temperature, and extraction time during the preparation process (Chen et al., 2021). The conditions for extracting *L. edodes* protein, as used in the previous study, are summarized in **Table 1-5.**

Table 1-5. *L. edodes* protein extraction conditions.

Extraction Methods	Conditions	References
Chemical	S/L: 1:20, pH: 10 Time: 30 min, Temperature: 10 °C	(Li et al., 2009)
Chemical	S/L: 1: 28, pH: 8.5, Time: 300 min, Temperature: 24 °C	(Li, 2015)
Slow-freezing and water	S/L: 1:90 Time: slow freezing 480 min, Extraction: 240 min Temperature: -4-20 °C; 70 °C	(Yu, 2017)
EAE & alkaline	S/L: 1:16, pH: 8.5, Time: 120 min, Temperature: 95 °C	(Wu, 2015)
EAE	Sample to phosphate buffer ratio: 1:5 pH: protease from *B. licheniformis* (6.5-8.5) or papain (6.0-7.0), Time: 2h, Temperature: 60 °C, 65 °C	(Prandi et al., 2023)
UAE	S/L: 1:50, pH: 12, Time: 16 min, Temperature: 50 °C, Power: 90 W	(Wang et al., 2023)

UAE & alkaline	Lab scale: Temperature: 45 °C, Power: 400 W, pH = 13 Medium scale: S/L: 1: 100, Power: 1000 W	(Prandi et al., 2023)

1.2.5 Protein isolation and purification

1.2.5.1 Protein separation

Crude protein extraction methods often result in a mixture containing pigments, PS, and other substances. Therefore, isolating and purifying crude fungi proteins is crucial to achieving a uniform protein composition, understanding structural characteristics, and assessing biological activities. These processes are vital for obtaining high-purity, functionally active proteins suitable for further analysis or applications.

Protein separation methods are mainly based on the solubility of proteins, MW, charge properties, a specific affinity for ligands, and other properties to isolate target proteins from complex mixtures using physical, chemical, and biological means (Wang & Fan, 2001). The commonly used methods to separate protein include salting out, ultrafiltration, gel filtration, two-dimensional electrophoresis, isoelectric point precipitation, as well as ion exchange chromatography (Shi, 2008). Combining these methods is common for the separation and purification of protein.

1.2.5.2 Protein separation method based on solubility

The main protein separation methods for different solubility are the salting out method and the isoelectric point precipitation method. Salting out is the process of precipitation biological macromolecules by adding a neutral salt to the solution. The outstanding advantages of the salting-out method are low cost, simple operation, safety, and stabilization of many biologically active substances without the need for

particularly expensive equipment. The basic principle of protein precipitation with neutral salts is to add a large amount of salt to the protein solution. High concentrations of salt ions, such as SO_4^- and NH_4^- of sulfuric acid, exhibit a potent hydration force capable of capturing and disrupting the hydration layer enveloping protein molecules. It will further cause the protein to lose water and expose the hydrophobic area (**Figure 1-3**) (Novák & Havlíček, 2016). At the same time, it also neutralizes the protein charge, destroying its hydrophilic colloid, so the protein molecules are precipitated. Besides, protein concentration, value, and temperature are the main factors affecting salting out (Wang & Fan, 2001).

The isoelectric point precipitation method utilizes ampholytes with different isoelectric points. The solubility of protein is the lowest when it reaches electrical neutrality. The protein is easy to precipitate to realize the method of protein separation. The electrostatic repulsion between particles is minimal when the protein is in the electrostatic state (seen in **Figure 1-4**) (Novák and Havlíček, 2016). Therefore, the solubility is also the smallest, and the isoelectric points of various proteins differ. Protein can be precipitated by adjusting the isoelectric point of the solution. However, a good separation effect for proteins with close isoelectric points cannot be obtained.

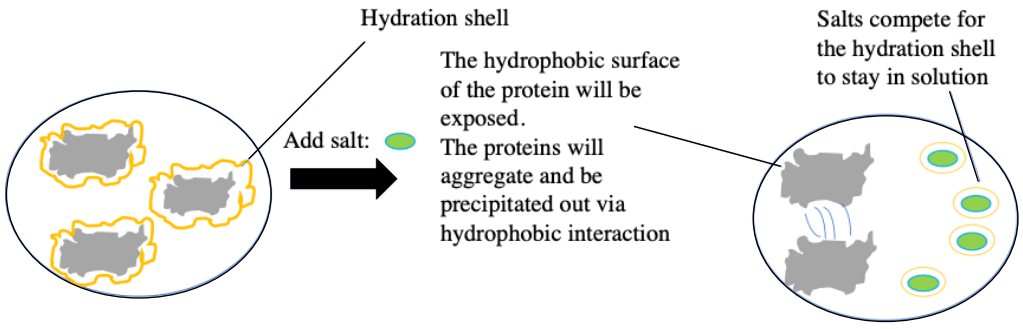

Figure 1-3. The mechanism of protein precipitation by salting-out precipitation.

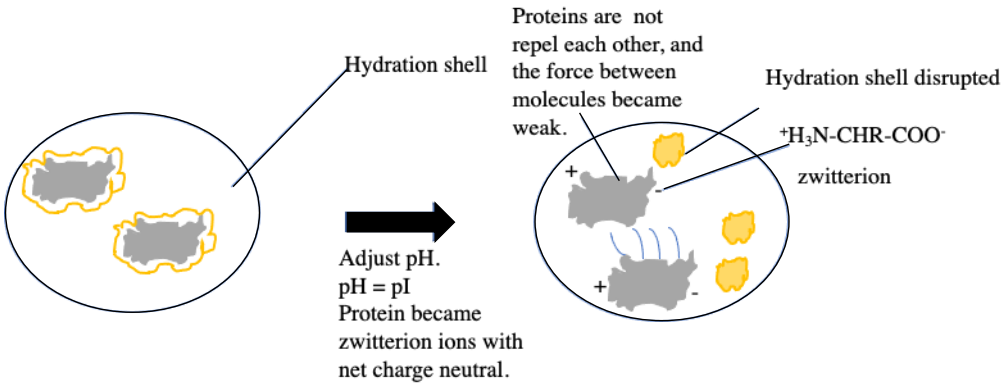

Figure 1-4. The mechanism of protein precipitation by isoelectric point precipitation.

1.2.5.3 Protein separation method based on protein molecule size

Proteins pass through separation media at varying distances based on their sizes, facilitating further separation of the proteins. The separation methods mainly include dialysis, ultrafiltration, and gel filtration (Yang & Bai, 2017). Dialysis uses a semi-permeable membrane to separate proteins of different molecular sizes. Ultrafiltration is a pressurized membrane separation technology. Under specific pressure, a special membrane with defined pore sizes allows small molecule solutes and solvents to pass through while rendering macromolecular solutes impermeable. Macromolecules are retained on one side of the membrane, leading to partial purification of the macromolecular substances (Reis & Zydney, 2010). Ultrafiltration technology offers advantages such as ease of operation, cost-effectiveness, and the absence of the need to introduce any chemical reagents into the process. Furthermore, ultrafiltration technology has mild test conditions and can prevent the denaturation of biological macromolecules (Xiao et al., 2017).

Gel filtration, also known as size exclusion or molecular sieve chromatography, is a technique used to separate molecules based on size. The principle is that the gel is an inert carrier without charge, and the adsorption force is weak. A single gel bead is like a “sieve,” which can allow macromolecular proteins to pass through quickly according to the size of the protein MW, while small molecular proteins stay in the chromatography column for a longer time (Hagel, 1998). This method is very good for separating macromolecular substances and maintaining the separated components' physicochemical properties (Yang & Bai, 2017). It has high resolution and good repeatability. The process operates under relatively mild conditions, can be performed across a broad temperature range, and does not require the use of organic solvents. Commonly used gel fillers include dextran, polyacrylamide, and agarose gel (Shi, 2008).

1.2.5.4 Protein separation method based on protein molecule charge

Protein separation can also be achieved according to the properties of the proteins charged. The methods for separating proteins according to their charged properties mainly include electrophoresis and ion exchange chromatography (Niu, 2010). Electrophoresis involves the migration of charged particles under the influence of an electric field (Rose, 1956). Protein can carry either a positive or negative charge at specific pH values. Due to variations in charge, size, and shape, molecules exhibit distinct mobility rates when subjected to an electric field. Therefore, the molecules can be separated, identified, or purified (Rose, 1956; Niu, 2010). Protein electrophoresis mainly uses polyacrylamide gel as the support medium. Besides, many factors can affect electrophoresis, such as the properties of the biological macromolecules to be

separated, the properties of the buffer, the electric field strength, electroosmosis, and the sieve holes of the supporting medium (Shi, 2008).

Ion exchange chromatography can also be used to separate proteins. Ion exchange chromatography relies on the interplay between the protein and the ion exchange resin as its fundamental principle. A chromatographic technique that separates the components in the mobile phase according to the difference in the binding force of the reversible exchange between the components in the mobile phase and the counter ions on the ion exchanger by selecting different buffers (Jungbauer & Hahn, 2009). The same protein can be exchanged with both anion resins and cations. As the protein solution passes through the ion exchange chromatography column, proteins with opposing charges to the ion exchanger are adsorbed by it. Proteins can be eluted and adsorbed by altering or ionic strength methods. The ion exchange chromatography packing material is cross-linked dextran, cellulose, or resin with a positive (negative) charge (Yang & Bai, 2017). Proteins consist of AAs, each of which bears varying total charges in diverse environments. At physiological pH (6-8), most proteins carry a negative charge, making anion exchange columns commonly necessary for their purification (Shi, 2008). Proteins are prone to denaturation and inactivation at extreme pH levels, so such conditions should be minimized whenever possible. At a specific pH, proteins carry varying numbers of charges, leading to different binding strengths with the resin. By increasing the buffer salt concentration or adjusting the pH, proteins are eluted sequentially based on their binding affinity. The primary chromatographic medium commonly employed for protein separation is DEAE-cellulose 52 within ion

exchange chromatography techniques. For gel filtration chromatography, Sephadex 75 is commonly employed to separate proteins based on their MWs (Fang, 2003; Zhu et al., 2013; Alfred, 2014). **Table 1-6** provides a summary of chromatography methods used for further purification of crude fungal proteins.

Table 1-6. Purification methods of fungal proteins.

Sample	Purification column	Buffer	Eluent	References
Pleurotus eryngii	Superdex 75 SP-Sepharose column (i.d. 1.4 × 25 cm)	5.0 mM Na-phosphate buffer, pH 7.2, containing 0.3 M NaCl	0.3 M NaCl 0-0.15 M NaCl	(Landi et al., 2022)
Agrocybe sp.	DEAE-sepharose FF (1.6 cm × 10 cm) Sephacryl S-200	0.01M PBS (pH 7.8)	0-0.3 M NaCl 0.15 M NaCl	(Fang, 2003)
Sagittaria sagittifolia	CM-Sephadex Dextran G-50	PBS (pH 5.7)	0-1 M NaCl	(Zhu et al., 2013)
	DEAE-52, CM-52 × 30 cm Superdex 75	0.01 M Tris-HCl (pH 8) 0.01 M NH4OAc 0.2M NH4HCO3 (pH 0.5)	0-0.5 M NaCl & 0-0.4 M NaCl	(Alfred, 2014)
Trametes versicolor	Q-Sepharose Fast Flow Column a Superdex G-75	25 mM Tris/HCl (pH 8.0)	0.1, 0.25, 0.4, 0.55, 0.8, and 1.0 M NaCl 0.15 M NaCl	(Li et al., 2011)

1.2.5.5 TPP

Conventional separation techniques like precipitation, membrane separation, crystallization, and ultrafiltration often involve multiple steps, higher maintenance and processing costs, longer processing times, and scaling challenges (Chew et al., 2019).

In contrast, three-phase partitioning (TPP) is an emerging method that addresses these limitations. TPP involves creating three phases using a salt and alcohol mixture, with proteins forming an intermediate phase. Due to its branched structure, *t-butanol* is commonly utilized as an organic solvent since it prevents the penetration of folded protein molecules (Dennison & Lovrien, 1997). When enough $(NH_4)_2SO_4$ is added, *t*-butanol becomes immiscible in the system, separating into two distinct phases: aqueous and organic. Binding to the precipitated proteins enhances their buoyancy, leading them to float above the denser salt-containing aqueous layer and forming an intermediate phase as a result (Garg & Horat, 2014). The principle of this technique involves a combination of processes such as isoionic, cosolvent, osmolytic, and kosmotropic precipitation, as well as the salting out of proteins (Gagaoua & Hafid, 2016). This method is simple, quick, and cost-effective for extracting, purifying, and concentrating proteins. TPP has been successfully applied to purify various protein biomolecules, such as proteases, peroxidases, laccase, and serratiopeptidase, and can be used directly with crude suspensions (Vetal & Rathod, 2015; Pakhale & Bhagwat, 2016; Rajagopalan & Sukumaran, 2018; Patil & Yadav, 2018). It has been found that combining the TPP method with mechanical extraction techniques like microwave and ultrasound treatment can further enhance protein purity and yield. Chew et al., (2019) observed that combining MAE with TPP can increase protein recovery yield from microalgae by over 2.5 times compared to using TPP alone. However, it's important to monitor and control the microwave time to prevent protein denaturation (Patil & Yadav, 2018). Combining UAE with TPP extraction not only reduces the treatment time but also

enhances protein purity (Pakhale & Bhagwat, 2016). Although TPP extraction has been employed to recover proteins from various sources like aloe as well as rice bran (Tan et al., 2015; Wang et al., 2020), its application in extracting proteins from edible fungi is limited, despite their high protein content and nutritional benefits.

1.2.6 Methods for characterization of fungal protein

Proteins derived from fungi, as intricate high molecular polymers, exhibit a diverse and complex array of structures. Understanding these proteins entails a comprehensive examination of their structural features, encompassing MW, AA composition, configuration, as well as spatial conformation. Advanced analytical techniques such as Fourier-transform infrared spectroscopy (FT-IR), Nuclear magnetic resonance (NMR), reversed-phase high-performance liquid chromatography (RP-HPLC), as well as liquid chromatography-mass spectrometry (LC-MS), including liquid chromatography-tandem mass spectrometry (LC-MS/MS), are currently employed to elucidate the properties of proteins.

1.2.6.1 Molecular weight

Molecular weight (MW) is regarded as a crucial factor for protein chemical characteristics. The MW of individual proteins normally ranged from 0.202 to 2546.166 kDa, with predicted isoelectric points (pI) ranging from 1.85 to 13.759, while the average MW of the fungi protein was 50.96 kDa (Mohanta et al., 2021). In the analysis of protein MW, common techniques such as sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), size exclusion chromatography (SEC) and LC-MS are frequently employed. Among these methods, SDS-PAGE is the most commonly

utilized technique for determining protein MW. SDS-PAGE can measure a size range of 5-500 kDa, and SEC can measure a size range of 0.1-10,000 kDa. In contrast, LC-MS can only measure samples with MWs below 100 kDa (Goetz et al., 2004). Furthermore, high-performance gel-permeation chromatography (HPGPC) has been identified as an effective method for determining the MW of proteins. For instance, the MW of *Pleurotus eryngii* was measured to be 63 kDa using HPGPC (Alfred, 2014).

1.2.6.2 Chemical structure

Nowadays, **Figure 1-5** summarizes the numerous techniques (such as HPLC, near-infrared (NIR) spectroscopy, FT-IR spectroscopy, Circular dichroism (CD) spectroscopy, and NMR spectroscopy) used to characterize protein structures (Bolje, 2021; Walport et al., 2021). HPLC analysis is commonly employed for assessing the AA composition of proteins, and LC-MS/MS can further provide information on protein composition (Nehete et al., 2013). FT-IR spectroscopy is utilized to track the conformational stability of proteins both pre- and post-lyophilization. Distinct frequency shifts in the hydrogen bonding patterns of amide bonds provide insights into the type and abundance of secondary structural elements, such as α-helices, β-sheets, and γ-turns (Jiskoot & Crommelin, 2005). These shifts are closely linked to vibrational modes in the mid-infrared range, particularly the amide I region (1650 cm^{-1}), dominated by C=O stretching, and the amide II region (1550 cm^{-1}), characterized by N–H bending (Bolje, 2021). Together, these regions reflect backbone conformation and hydrogen-bonding patterns, enabling the analysis of protein secondary structure. NIR spectroscopy can be used to study the secondary structure of proteins. In contrast to FT-

IR spectroscopy, NIR spectroscopy does not require nitrogen purging since moisture content usually has minimal interference in the NIR spectrum. Additionally, the time needed to acquire NIR spectra for analyzed samples is generally less than 2 min per sample, making it a faster process compared to FT-IR spectroscopy (Miyazawa, 1998). Raman spectroscopy serves as a valuable complement to infrared analysis by offering details on molecular vibrations and aiding in the exploration of diverse aggregation states within biopharmaceutical samples (Bolje, 2021). In Raman spectra, specific regions are typically associated with secondary structural components of proteins. These include the amide I region (1600-1700 cm^{-1}), the amide III region (1230-1340 cm^{-1}) linked to α-helical structures, and the C-C stretching band of the protein backbone (890-1060 cm^{-1}) (Bolje, 2021). Additionally, ultraviolet-visible (UV-Vis) spectroscopy can be utilized to investigate the tertiary structure of proteins and monitor alterations in their conformation. Studying aromatic residues in proteins and their surroundings is possible using the second UV spectroscopy derivative. Within the range of 240 to 300 nm, the primary protein peak comprises multiple spectra that converge in the final broad peak of the zero-order spectrum. The spectral features in this region are predominantly governed by the absorption characteristics of aromatic AA residues, specifically phenylalanine (245-270 nm), tyrosine (265-285 nm), as well as tryptophan (265-295nm) (Ichikawa & Terada, 1979; Kueltzo et al., 2003). Fluorescence spectroscopy serves as a valuable tool for examining protein tertiary structure, with tryptophan fluorescence being the most prevalent method utilized to assess the integrity of such structures (Royer, 2006). CD spectroscopy measures the difference in absorbance of proteins

under left-handed and right-handed circularly polarized light. The difference in absorbance is due to the interaction of the protein chromophore with the chiral environment (Kelly & Price, 2000). CD spectroscopy is widely employed for estimating the content of protein secondary and tertiary structures, as well as for monitoring conformational changes in proteins triggered by environmental alterations, such as variations in pH, temperature, or the presence of excipients and denaturing agents (Bolje, 2021). Proteins can be examined with atomic precision through nuclear magnetic resonance (NMR) spectroscopy, which predominantly utilizes ^{1}H (proton) and ^{13}C (carbon) nuclei due to their widespread applicability. The use of high magnetic fields is generally favored, as they significantly improve the sensitivity and resolution of the analysis. A thermoelectric technique used to analyze the conformational dynamics of proteins is known as dielectric relaxation spectroscopy (DRS). This method can distinguish groups participating in global and local dynamics relatively well. DRS offers insights into protein structure, primary and secondary molecular motion kinetics, as well as water content and its status (bound or free). Hydrogen/deuterium exchange mass spectrometry (HDX-MS) serves as a highly effective approach for investigating protein structure, analyzing interactions with solid-state excipients, and assessing physical stability.

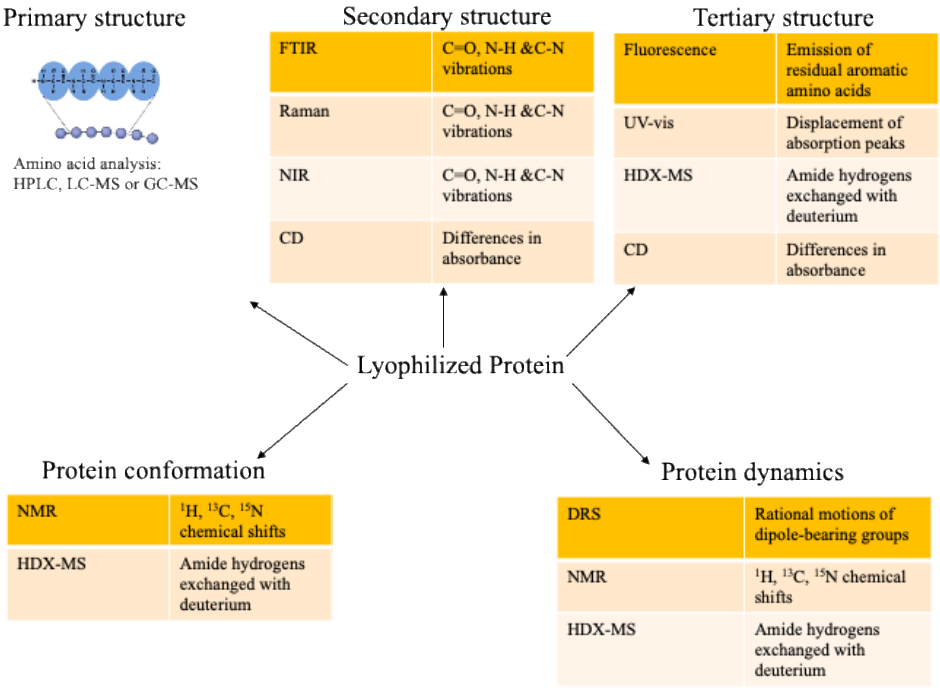

Figure 1-5. Analytical methods are commonly employed for the structural analysis of proteins.

1.3 Objectives and Significance

1.3.1 Objectives

Proteins, which are the primary components of edible and medicinal fungi, possess a wide array of nutraceutical and pharmaceutical functions and have the potential to serve as natural immunomodulators to enhance human health. However, most research has focused on studying the polysaccharide (PS) in *L. edodes*, with inadequate attention to the protein component. This research project aims to fill this gap by isolating and purifying the protein from *L. edodes*. This study will also investigate the chemical composition, molecular properties, and their potential relationship to immunomodulatory activities. The following objectives will be pursued in this study.

1. Isolate and purify protein fractions from *L. edodes* using a three-phase partitioning (TPP) system.
2. Determine the contents and immunomodulatory activities of PS and protein in *L. edodes* using a TPP system.
3. Examine the molecular properties of TPP-extracted protein before and after purification, encompassing chemical composition, distribution of molecular weight (MW), and molecular structure.
4. Assess the relationship between molecular properties and immunomodulatory activities for purified and non-purified protein fractions.
5. Develop and optimize an effective protein extraction method to increase protein yield and content from *L. edodes* and further evaluate the properties and immunostimulatory activities of the extracted proteins to understand their potential health benefits.

1.3.2 Significance

There is growing interest in natural immunomodulators, as immune system modulation is increasingly being recognized as a preventive approach for a wider population, including healthy individuals (Reis et al., 2017; Rathore et al., 2017). While many immunomodulators are synthetic or semi-synthetic, they may pose potential side effects. This has led to a shift toward exploring natural alternatives, such as mushrooms, which are gaining attention for their potential use in dietary supplements.

Earlier research by Chen et al. (2020) showed that PS from *L. edodes* had immunomodulatory properties. However, there is limited research on the

immunomodulatory properties of proteins produced from *L. edodes*. This project aims to develop a method for extracting and isolating protein from *L. edodes*, while also providing evidence of the significant immunomodulatory activity of the protein. Additionally, the project will investigate the relationship between protein fractions and their immunomodulatory effects, aiming to establish a comprehensive understanding of the immunomodulatory mechanism of *L. edodes*-derived protein. This research shows promise for the development of new functional food and dietary supplement products.

1.3.3 Outline of the project

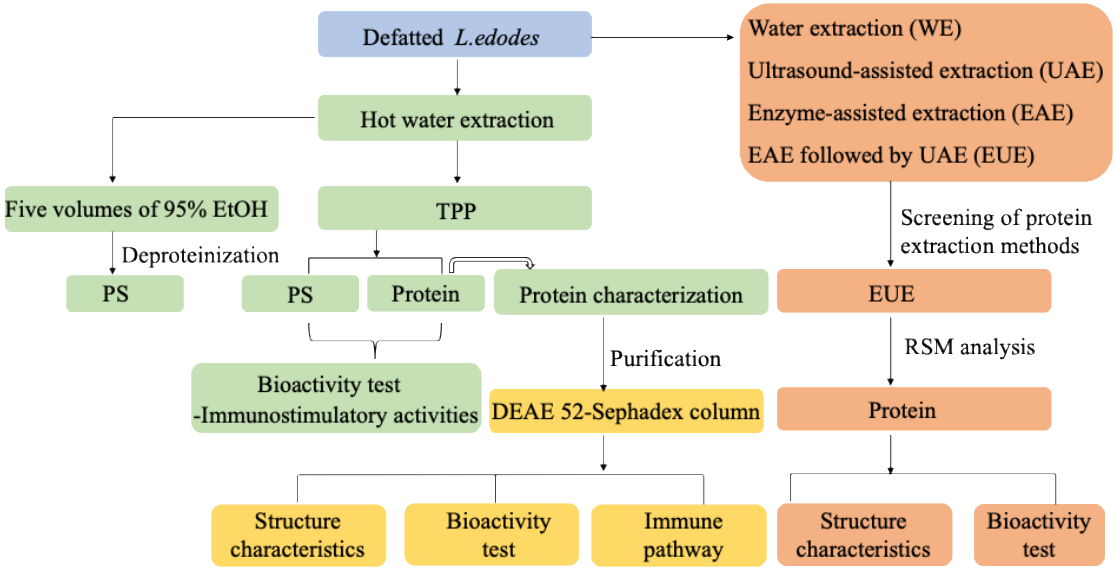

Figure 1-6. Outline of the project.

The project focused on using defatted *L. edodes* mushroom sample powder, divided into three parts: green, yellow, and orange (as shown in **Figure 1-6**). The PS and protein samples were obtained simultaneously using the TPP system after hot water extraction in the green module. In this study, we characterized PS obtained through conventional extraction, PS extracted via TPP, and protein samples, and examined their biological

activities. In the yellow module, the protein extracted via TPP was further purified to identify its active fractions and investigate the potential metabolism related to its immunostimulatory activity. Due to the low protein yield obtained from the TPP method, the orange module was introduced to investigate strategies for enhancing protein yield while preserving its immunostimulatory activity.

1.3.4 Organization of the thesis

The subsequent sections of this thesis are organized as follows:

Chapter 1 provides a comprehensive introduction to edible fungi, covering their immunostimulatory activity in general. It then details the techniques for separating and characterizing fungal proteins, narrowing the focus to the specific proteins of *L.edodes.* The chapter synthesizes this research to identify gaps and clearly outlines the project's objectives.

In **Chapter 2**, the general methods and materials used in this study are detailed, along with an outline of the project.

Chapter 3 evaluates the TPP system for the extraction and isolation of PS and proteins, assessing their biological activities, including antioxidant and immunostimulatory effects. (This chapter has been published in the journal of Food and Bioprocess Technology, 2024, 17(8), 2277-2291).

Chapter 4 explores the relationship between protein structure and immunostimulatory activity. Proteins undergo a purification process, and both purified and non-purified protein structures, nutritional values, and immunostimulatory activities are assessed. (This chapter has been published in the journal of International

Journal of Biological Macromolecules, 2024, 280, 136082).

Chapter 5 discusses different extraction methods, including WE, EAE, UAE, and EUE, to increase protein yield and content while preserving its immunostimulatory activity. The EUE method was selected as the optimal extraction technique, and its conditions were optimized using response surface methodology (RSM) analysis. The extracted protein was then characterized, and the direct immunostimulatory activity of the optimized EUE-extracted proteins was also further evaluated. (This chapter has been accepted in the journal of Food and Bioprocess Technology).

Chapter 6 presents the major conclusions of this research project. Additionally, recommendations for further research are provided to guide future investigations.

Chapter 2 General Materials and Methods

This chapter will provide an overview of the materials and common experimental methods used in the entire project. It is essential to recognize that the experimental conditions may vary depending on the specific materials used. For more detailed information, please refer to the relevant chapters.

2.1 Chemical and biochemical agents

Iron (II) sulfate heptahydrate and iron (III) chloride hexahydrate were sourced from Sigma-Aldrich (St. Louis, MO, USA). Ammonium sulfate ($(NH_4)_2SO_4$) was obtained from AnalaR Normapur (Vienna, Austria), while *T*-butanol was acquired from Macklin Biochemical Co. (Shanghai, China). Ascorbic acid was supplied by Damao Chemical Reagent Factory (Tianjin, China). ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) was procured from Roche (Mannheim, Germany), and Trolox ((S)-(2)-6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid) from Calbiochem (San Diego, CA, USA). Dulbecco's modified Eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Phosphate-buffered saline (PBS) was also obtained from Macklin. The following reagents were provided by Sigma-Aldrich: penicillin, streptomycin, lipopolysaccharide (LPS), sulphanilamide, N-1-naphthyl ethylenediamine dihydrochloride, phosphoric acid, bovine serum albumin (BSA), sodium dodecyl sulfate (SDS), and anthrone. Potassium persulfate and sodium nitrite were sourced from BDH (Poole, England). DEAE cellulose (DE-52) was obtained from Phygene Biotechnology Co., Ltd. (Fuzhou, China). Cellulase (50 U/mg) was from Yuanye Company (Shanghai, China). All other

chemicals were of analytical reagent (AR) grade or higher grade from reliable suppliers.

2.2 Preparation of defatted mushroom powder

The fruit body form of *L. edodes* fungus, obtained from Zhejiang Fangge Pharmaceutical Co., Ltd. (Zhejiang, China), was used in this study. The mushrooms were vacuum-sealed in plastic bags and stored at room temperature (20-25 °C) until further processing. To achieve the final dry weight, the mushrooms were dried in an oven at approximately 60 °C until a constant weight was attained, which was measured using an electronic balance. Subsequently, the dried mushrooms were ground into a fine powder using an electric mill and sieved through an 850 μm mesh sieve. The mushroom powder was treated with absolute ethanol (1:8 w/v) to remove the fat content and stirred continuously overnight at room temperature. The solvent was removed through suction filtration, and the remaining solid was dried in an oven until a constant weight was obtained.

2.3 Preparation of protein by TPP extraction

The defatted mushroom powder was combined with distilled water in a 1:30 (w/v) ratio within a 250-mL Erlenmeyer flask and heated at 90 °C in a water bath for 2 h. After heating, the crude liquid extract was separated from the solid residue via suction filtration. Ammonium sulfate ($(NH_4)_2SO_4$) was gradually added to the extract solution in a plastic sample tube to reach a concentration of 48% (w/v), followed by vigorous vortexing. The pH of the solution was adjusted to 5.5 using either 0.3 M hydrogen chloride (HCl) or 0.3 M sodium hydroxide (NaOH). Subsequently, *t*-butanol was added to the solution at a volume ratio of 1.2 (*t*-butanol to crude extract) and thoroughly mixed.

The sample tube was then placed in a thermostatic water bath equipped with a magnetic stirrer, maintaining a temperature of 52 °C for 30 min. The mixture was centrifuged at 4000 rpm for 20 min, resulting in separation into three distinct layers. The upper organic solvent layer (*t*-butanol) was collected after vacuum evaporation and reused. The middle layer, primarily containing proteins, was collected, redissolved in water, and dialyzed against distilled water using a 3500 Da molecular weight (MW) cutoff membrane for 72 h to remove salt. Finally, the dialyzed solution was freeze-dried to yield partially purified protein fractions. The complete process is illustrated in the diagram presented in **Figure 2-1**.

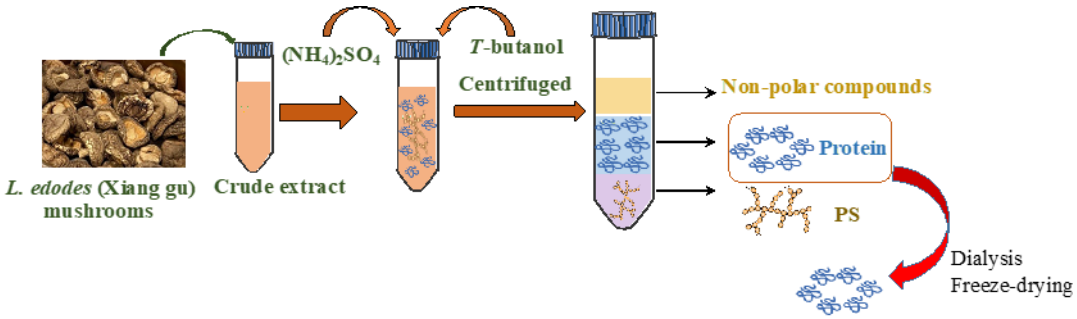

Figure 2-1. Diagram of the TPP extraction process.

2.4 Characterization of protein MW and composition

The carbohydrate content and protein content in *L. edodes* samples were measured using the anthrone and Lowry tests, respectively (Siu et al., 2016). The anthrone test was performed by subjecting samples to acid hydrolysis at 100 °C in the presence of an anthrone reagent (2 g anthrone dissolved in H_2SO_4). The absorbance of the resulting solution was measured at 620 nm using a spectrophotometer, with glucose as the reference standard. Protein content was determined via the Lowry method, with absorbance measured at 750 nm using bovine serum albumin (BSA) as the reference.

The MW of the isolated protein samples was determined using SDS-PAGE analysis based on the method described by Cheung et al. (2012). Protein markers ranging from 10 to 180 kDa (Macklin, Shanghai, China) served as references. The gel was gently agitated at room temperature overnight with Coomassie brilliant blue for staining. Following the staining process, the gel was destained with a mixture composed of Milli-Q water, methanol, and acetic acid in proportions of 6:3:1, respectively. The gel was subsequently scanned using an HP scanner to examine the protein bands and assess their MW.

A Varian Cary 400 spectrophotometer (Varian Co., USA) was used to measure the UV-vis absorbance in the 200-800 nm range at room temperature. The protein was analyzed using Fourier Transform Infrared Spectroscopy (FT-IR) on a Nexus 670 FT-IR spectrometer (Thermo Nicolet Co., Cambridge, UK). Measurements were conducted in the wavenumber range of 500-4000 cm^{-1} using potassium bromide (KBr) pellets, with air as the reference background in the Spectrum 6.1 software (Li et al., 2020).

Circular dichroism (CD) spectra were measured using a Jasco J-1500 spectropolarimeter (Japan) at room temperature (20-25 °C). Protein samples were dissolved in 1 mM phosphate-buffered saline (PBS) and analyzed in a cuvette with a 0.1 cm path length. Spectra were acquired with a scan speed of 100 nm/min, a digital integration time of 1 s, and a bandwidth of 4 nm. Far-UV scans were performed from 190 to 260 nm, with at least two accumulations at 1 nm wavelength intervals. For the analysis of secondary structures, encompassing α-helix, β-sheet, β-turn, and random coil (as shown in **Figure 2-2**), the JWSSE-513 software was employed. This analysis

utilized the Classical Least Squares (CLS) method, with reference spectra derived from Yang's model (Mohapatra et al., 2024).

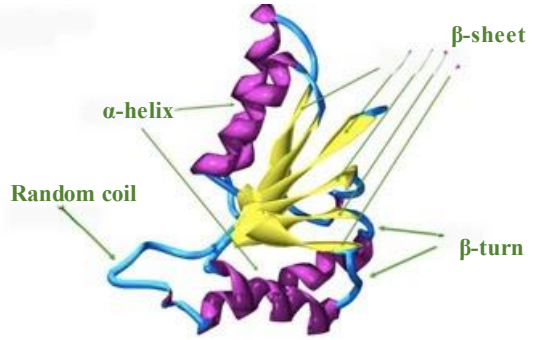

Figure 2-2. Visualization of protein secondary structures: α-helix, β-sheet, β-turn, and random coil.

The amino acid (AA) composition of the protein samples was analyzed using ultra-performance liquid chromatography-electrospray ionization triple quadrupole mass spectrometry (UPLC-ESI-TQMS). Samples were initially purged with nitrogen for 30 s, followed by hydrolysis at 12 M hydrogen chloride (HCl) at 110 °C for 24 h. Post-hydrolysis, neutralization was performed by adding an equal volume of 6 M sodium hydroxide (NaOH). The hydrolyzed product was diluted 16-fold with MilliQ-water and adjusted to 0.1 M using 0.4 M HCl. Derivatization was carried out using the Waters Kairos Amino Acid kit, and the derivatized samples were analyzed using a UPLC-ESI-TQMS system fitted with a CORTECS™ UPLC C18 column (1.6 µm, 2.1 × 150 mm). This approach is consistent with established methodologies reported in prior research (Gray et al., 2017; Guba et al., 2022).

The AA score calculation was performed as follows equations (**2.1-2.5**)

$$AAS\% = AA_{Fi}\,(mg/g)/AA_{Xi} \quad (\mathbf{2.1})$$

$$CS\% = AA_{Fi}\ (mg/g)/AA_{Ei} \quad (\mathbf{2.2})$$

$$RAA = AA_{Si} / AA_{Pi} \quad (\mathbf{2.3})$$

$$RC = RAA/\ \text{average of RAA} \quad (\mathbf{2.4})$$

$$SRC = (1- CV) \times 100\% \quad (\mathbf{2.5})$$

Where, AAS indicates amino acid score, AA_{Fi} represents the concentration of a specific essential amino acid (EAA) in the sample, AA_{Xi} denotes the recommended concentration of that EAA in the FAO/WHO model protein, and AA_{Ei} refers to the EAA concentration in whole egg protein (Li et al., 2022). Additionally, AA_{Si} indicates the AA content in the sample, while AA_{Pi} corresponds to the AA content in the FAO/WHO reference pattern. The ratio of amino acids (RAA) and chemical score (CS) were calculated following established FAO/WHO (2007) methodologies. RAA, the amino acid ratio coefficient (RC), and the amino acid ratio coefficient score (SRC) were determined using the method described by Zhu (1988).

2.5 *In vitro* Immunomodulatory activity

2.5.1 Cell culture conditions

RAW264.7 cells in the logarithmic growth phase were plated in 96-well plates at a concentration of 5×10^4 cells/mL, with 100 μL per well, and allowed to incubate for 24 h. The culture medium consisted of Dulbecco's Modified Eagle Medium (DMEM) enriched with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were kept at 37°C in a CO_2 incubator for optimal growth conditions.

2.5.2 Cell viability measurement

In Chapter 3, the cells were then treated with PS and protein extracts at

concentrations of 25, 50, 100, and 200 μg/mL and incubated for another 24 h. Cell viability was assessed using the MTT assay (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). After adding the MTT solution, samples were incubated for 4 h, and the optical density (OD) at 490 nm was measured with a microplate reader.

Chapters 4 and 5 employed different methods for measuring cell viability. After the initial incubation, cells were treated with protein extracts at a concentration of 4 μg/mL and incubated for an additional 24 h. Following this, 50 μL of 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetra zolium (MTS) solution was added to each well, and the plates were incubated for another 2.5 h. The absorbance of the formazan product was then measured at 492 nm using a CLARIOstar microplate reader (BMG, Germany). The cell proliferation rate was determined by comparing the absorbance of the protein-treated cells to that of the untreated control, expressed as a percentage (Li et al., 2023).

2.5.3 Cell pinocytic assay

Neutral red, a pH-sensitive dye, is taken up by living cells and accumulates in lysosomes, where it forms a red-colored compound (Xu, 2023). Cells in the logarithmic growth phase were plated in 96-well culture plates at a density of 5×10^4 cells/well and incubated for 24 h. After incubation, samples at predetermined concentrations were added to the wells and incubated for another 24 h. The medium was then aspirated, and 100 μL of fresh medium containing 0.1% neutral red was added to each well. The plate was incubated for 30 min, after which the cells were washed five times with PBS to remove any unbound dye. Next, 100 μL of a lysing solution, prepared by mixing ethanol

and pure acetic acid in a 1:1 (v/v) ratio, was added to each well. The plate was placed in a cold room for 2 h, and the optical density (OD) at 540 nm was measured using a microplate reader (Li et al., 2023).

2.5.4 NO assay

During the logarithmic growth phase, cells were seeded into 96-well culture plates at a density of 5 × 10^4 cells/mL, with 100 μL of cell suspension added to each well and incubated for 24 h. Following this, the cells were treated with either LPS (as a positive control) or the designated samples and incubated for an additional 24 h. After incubation, the supernatants were collected, and nitric oxide (NO) concentrations were determined using Griess reagent according to the manufacturer's protocol.

2.5.5 ROS generation assay

Intracellular reactive oxygen species (ROS) levels were quantified using the fluorescent probe DCFH-DA (2′,7′-Dichlorodihydrofluorescein diacetate, Sigma) (Li et al., 2023). Briefly, RAW 264.7 cells were seeded into a 96-well plate at a density of 5 × 10^4 cells/mL, with 100 μL of cell suspension added to each well and incubated for 24 h. After incubation, the cells were washed twice with PBS, and 100 μL of samples, prepared at concentrations consistent with those used for MTS and NO assays, were added to each well. Following a 24-hour incubation period, 10 μM 2'- 7'-dichlorodihydrofluorescein diacetate (DCFH-DA) solution was introduced to each well and incubated for 30 min. The cells were then washed, and 100 μL of lysing solvent was added per well to release intracellular 2,7-Dichlorodihydrofluorescein (DCF). Fluorescence intensity was measured at excitation and emission wavelengths of 488 nm

and 525 nm, respectively, using a microplate reader (Li et al., 2023). (Refer to Chapter 6)

In Chapter 5, a slightly modified method was employed. After 24 h of cell cultivation, 100 µL of EAE, followed by UAE (EUE) extracted protein (4 µg/mL), was added to each well. The plates were incubated for one hour at 37°C, followed by treatment with 10 µM DCFH-DA and further cultured for 23 h at 37°C. Fluorescence intensity was measured at excitation and emission wavelengths of 485 nm and 527 nm, respectively (Jayasinghe et al., 2023).

2.6 Statistical Data Analysis

The results are presented as the mean ± standard deviation (SD) from three independent replicates. Statistical analyses were performed using GraphPad Prism 9 (GraphPad Software Inc., La Jolla, CA, USA) and IBM SPSS Statistics 23 (IBM, New York, NY, USA). For multiple comparisons, a one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test was used, and the Student’s t-test was also applied during the analysis. Statistical significance was determined at $p < 0.05$.

Chapter 3 Evaluation of TPP for Efficient and Simultaneous Isolation of Immunomodulatory Polysaccharides and Proteins from *Lentinula edodes* Mushroom

3.1 Introduction

Lentinula edodes (Agaricomycetes), generally known as Shiitake in Japanese and Xianggu in Chinese, is one of the most popular edible mushrooms worldwide due to their favorable aroma, taste, nutritional, and therapeutic value (Ahmad et al., 2023). Various health effects have been documented of *L. edodes*, such as anticancer, immunomodulatory, antioxidant, and anti-inflammatory activities. Polysaccharide (PS) and proteins are the two most abundant components contributing to *L. edodes*. PS, such as glucans and chitins, are well-known bioactive polymers contributing to the health benefits of *L. edodes* as well as other edible and medicinal fungi (Sheng et al., 2021; Ahmad et al., 2023). In addition to PS, proteins and polypeptides are two other major groups of biopolymers with potential anticancer, antioxidant, and immunomodulatory properties (Rezvani et al., 2020). Proteins from serval mushrooms, such as *Volvariella volvacea, Antrodia camphorata,* and *Ganoderma microsporum* have been reported with immunomodulatory effects (Xu et al., 2011; Arya and Rusevska, 2022). The crude protein extracts from the *L. edodes* fungal mycelia and several other edible/medicinal mushrooms have shown immunomodulatory by stimulating the cytokine production of human peripheral blood mononuclear cells (Jeurink et al., 2008). In addition, some bioactive peptides from edible or medicinal fungi, such as *Grifola frondosa, Cordyceps sinensis, and Agaricus bisporus*, have shown strong antioxidant effects (Jeurink et al.,

2008; Kour et al., 2022). A peptide isolated from *L. edodes* has been found to have an angiotensin-converting enzyme (ACE) inhibitory effect and the potential for the treatment of hypertension (Paisansak et al., 2021).

Separation is an essential step for attaining the desired components from complex mixtures and for assessing their functions. In the laboratory, PS is usually isolated from an aqueous extract of the source material by ethanol precipitation, and the crude PS precipitate is subject to deproteinization by repeated Sevag treatment (Huang et al., 2022). However, Sevag deproteinization is rather tedious and time-consuming. Therefore, alternative separation methods have been recommended to improve the efficiency of protein removal. The three-phase partitioning (TPP) method is a promising non-chromatographic technique for more efficient isolation and purification of bioactive molecules from natural sources (Abdoulaye et al., 2023).

The TPP method was developed several decades ago and has been mainly applied for the separation and purification of proteins (Dennison & Lovrien, 1997). In comparison with the conventional methods, the TPP system is simply formed by adding an inorganic salt (e.g., ammonium sulfate $(NH_4)_2SO_4$) to the aqueous extract solution and then mixing with an organic solvent (usually *t*-butanol). The liquid mixture is then separated into three clear layers, the top organic solvent layer containing the most hydrophobic constituents (Roy & Gupta, 2002). The organic phase of *t*-butanol in the upper layer may serve as a solvent to extract pigments, saving the traditional decolorization step; *t*-butanol can be readily recovered by evaporation and reused. The middle aqueous layer is usually used to extract proteins, substituting the conventional

Sevag method (Roy & Gupta, 2002; Yan, 2021). The bottom aqueous layer contains the most polar components, such as PS. The conventional method for extraction and separation of PS and proteins usually involving ethanol precipitation, deproteinization, and decolorization can be accomplished in a one-step process with the TPP system. Ideally, the TPP method has the advantages of easier solvent recycling, a simpler, more efficient, and more environmentally friendly process. It may also allow for the simultaneous isolation of three constituent groups from a crude extract. The TPP method has been effectively utilized to extract and purify various bioactive compounds from natural sources, such as proteins, polyphenols, and edible oils (Yan, 2021). A few recent studies have also employed TPP for extracting PS from different sources (Xu et al., 2014; Wang et al., 2014; Li et al., 2023). However, only a few previous studies have applied TPP for the simultaneous extraction and purification of PS and proteins, e.g., from aloe (Tan et al., 2015) and rice bran (Wang et al., 2020).

To the best of our knowledge, there is still no reported study on the simultaneous isolation of PS and protein from *L. edodes* by the TPP method. This study was to explore and optimize the TPP method for the simultaneous extraction of PS and proteins from *L. edodes*. Response surface methodology (RSM) was applied to determine the optimal conditions for improving the extraction yield of PS. The physicochemical properties, antioxidants, and immunostimulatory activities of PS and proteins were also evaluated.

3.2 Experiment methods

The pretreatment and preparation of the *L. edodes* sample are detailed in section 2.2. After obtaining the defatted *L. edodes* powder, it was mixed with distilled water at a

ratio of 1:30 (w/v) in a 250-mL Erlenmeyer flask. This mixture was then heated at 90 °C in a water bath for 2 h. The crude liquid extract was separated from the solid residue using suction filtration. Subsequently, the crude extract underwent both conventional and TPP treatments.

The analysis of PS content, protein content, composition, and molecular weight (MW) was conducted using the same methods described in section 2.4.

In this chapter, in *vitro* immune assays, including cell viability (section 2.5), phagocytic activity analysis, and nitric oxide (NO) analysis, were conducted under similar culture conditions and immunomodulatory measurement methods, with slight adjustments. After cell incubation, the cells were treated with PS at concentrations of 25, 50, 100, and 200 μg/mL, and protein samples at concentrations of 0.5, 1, 2, and 4 μg/mL. They were then incubated for 24 h before proceeding with phagocytic activity analysis and NO analysis, as outlined in section 4.5.

3.2.1 Single-factor experiment of TPP

Preliminary experiments were firstly performed to compare different inorganic salts for TPP, including $(NH_4)_2SO_4$, potassium chloride (KCl), sodium chloride (NaCl), and calcium chloride ($CaCl_2$), and $(NH_4)_2SO_4$ was found to be the most favorable for the isolation and purification of PS from the mushroom extract. The following TPP experiments were conducted using *t*-butanol as the organic phase and $(NH_4)_2SO_4$ as the salt phase, as reported by Dennison & Lovrien (1997). The major process factors, including $(NH_4)_2SO_4$ concentration, the volume ratio of *t*-butanol to crude extract solution, temperature, and time were evaluated for their effects on the extraction yield

of PS.

$(NH_4)_2SO_4$ in the concentration range of (10-60%) (w/v) was added slowly to the mushroom extract solution in a plastic sample tube and vortexed vigorously, followed by the addition of 0.3 M hydrogen chloride (HCl) or 0.3 M sodium hydroxide (NaOH) to adjust the solution pH to 5.5. To the crude extract solution containing $(NH_4)_2SO_4$, *t*-butanol was added at a 0.5:1-3:1 volume ratio of *t*-butanol to crude extract solution. The sample tube was placed in a thermostatic water bath (with a magnetic stirrer) to maintain the desired temperature (25-75 °C) for 15-75 min. The initial conditions for experiment factor optimization were as follows, $(NH_4)_2SO_4$ concentration 40%, volume ratio of *t*-butanol to crude extract solution (v/v) 1.5:1.0, and 40 °C for 35 min. The mixture was centrifuged at 4000 rpm for 20 min for phase separation into three layers. The upper organic solvent (*t*-butanol) phase was collected and reused after vacuum evaporation. The middle phase contained primarily proteins, and the middle layer was collected and redissolved in water for analysis of proteins. The bottom aqueous phase contained primarily $(NH_4)_2SO_4$ and PS. The middle and bottom phases were collected and dialyzed against a 3500 Da MW cutoff (MWCO) membrane and distilled water for 72 h to remove salts and then freeze-dried to obtain partially purified PS and protein fractions.

The extraction yield (%) (Y) of PS on the basis of dry mushroom was calculated by $Y = C_L V_L / M_0 \times 100\%$, where M_0 was the initial mass of dry mushroom powder, C_L and V_L were the concentration of PS and liquid volume in the bottom phase at equilibrium. The protein yield from the middle phase was calculated by dividing the

freeze-dried protein fraction by the total mass of the initial *L. edodes* powder.

3.2.2 Optimization of TPP conditions by response surface methodology (RSM)

The TPP conditions for PS extraction were optimized by RSM using a 3^3, three-factor, and three-level factorial Box-Behnken design (BBD). This design generated a total of 17 experiment runs consisting of 12 factorial points and 5 replicates at the center points. The experimental factors and factor levels were chosen based on the above experiments (**Table 3-1**), including the concentration of $(NH_4)_2SO_4$ (% w/v, X_1), the volume ratio of *t*-butanol to crude extract solution (v/v, X_2), and temperature (°C, X_3) as independent variables. The PS yield in the bottom phase was chosen as the response value or optimization object, and the following quadratic polynomial equation represented the response function,

$$Y = A_0 + \sum_{i=1}^{3} A_i X_i + \sum_{i=1}^{3} A_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} A_{ii} X_{ij}$$

Where Y is the predicted PS yield, X_i and X_j are the experimental variables, A_0 is the intercept, and A_i, A_j, A_{ii}, and A_{ij} are the regression coefficients. The model coefficients were computed using the Design-Expert 11 software package (Stat-Ease, Inc., Minneapolis, USA). Statistical analysis was performed using Analysis of Variance (ANOVA) to obtain optimal conditions for TPP.

Table 3-1. Factors and levels of experimental variables.

Factor	Coding factor levels		
	-1	0	1
X_1: $(NH_4)_2SO_4$ (% w/v)	30	40	50
X_2: Temperature (°C)	50	55	60
X_3: volume ratio of *t*-butanol to crude extract solution	1	1.5	2

3.2.3 Conventional method for isolation of PS

The conventional method for PS isolation and partial purification, i.e., ethanol precipitation followed by Sevag treatment (EtOH-Sevag), was applied for comparison. The crude *L. edodes* extract from hot-water extraction was precipitated with five volumes of 95% ethanol at 4 °C overnight. The mixture was centrifuged at 9000 rpm for 20 min (Beckman Coulter Avanti J-26S XPI, Indiana, United States) and then freeze-dried to give the crude PS. Protein was removed from the crude PS by repeated treatment with Sevag reagent (chloroform/*t*-butanol = 4:1) until no precipitation was observed. The deproteinized PS was dialyzed with a 3500 Da MWCO membrane against distilled water for 48 h, followed by lyophilization to obtain the partially purified PS.

3.2.4 Analysis of isolated PS fraction

The molecular weight (MW) of PS samples was analyzed by high-performance gel-permeation chromatography (HPGPC) with the instruments and conditions previously reported by Li et al. (2020). Calibration was performed with dextran MW standards ranging from 1.0 to 670 kDa (Sigma-Aldrich Chemical Co., St. Louis, MO, USA). Data were analyzed with Breeze V3.3 software (Waters Corp., Milford, MA, USA). Monosaccharide composition was analyzed by the PMP-HPLC method. 5 mg PS sample underwent hydrolysis with 2 M trifluoroacetic acid (TFA) at 110 °C for 4 h, then evaporated under vacuum until dryness. The resulting solid was dissolved in DI water, mixed with 0.5 M phenyl-3-methyl-5-pyrazolone (PMP) solution in methanol and 0.3 M NaOH solution in equal volume, and heated at 70 °C for 30 min. The reaction

was halted by adding 0.3 M HCl, followed by three washes with chloroform. The aqueous layer was collected and filtered through a 0.45 μM membrane for HPLC analysis, which was carried out using an Agilent 1100 instrument with an Agilent Zorbax Eclipse XDB-C18 column (150 mm × 4.6 mm) at 25 °C. The mobile phase consisted of solution A (potassium phosphate-buffered saline (0.05 M, pH 6.9) containing 15% acetonitrile and solution B (40% acetonitrile) (Siu et al., 2016). Monosaccharide standards, including mannose (D-Man), rhamnose, glucose (D-Glc), galactose (D-Gal), xylose, and arabinose from Sigma-Aldrich (Buchs, Switzerland), were used for the identification and calibration.

3.2.5 Antioxidant activity assays

Antioxidant activities of PS and protein fractions were determined by three chemical methods, including Trolox equivalent antioxidant capacity (TEAC), the ferric reducing ability of plasma (FRAP), and hydroxyl radical scavenging activity, as reported previously (Cheung et al., 2012). TEAC measures the capacity of a substance to scavenge $ABTS^{\cdot+}$ free radicals generated from the oxidation of ABTS by potassium persulfate, in comparison to Trolox which serves as the antioxidant standard. In this experiment, the measured antioxidant capacity TEAC was the ability of the PS or protein to scavenge or inhibit free radicals ($ABTS^{\cdot+}$). TEAC values were obtained from a calibration curve generated with Trolox (0-120 μM). As for the FRAP assay, low pH caused the reduction of ferric ions to ferrous ions and led to the formation of colored ferrous-tripyridyltriazine complexes. The FRAP values of samples were calibrated using ferrous sulfate (0-1000 μM) as a reference standard. The specific operational

details and methods of the experiment were as previously described (Leung et al., 2009). The TEAC and FRAP activities were expressed in μmol Trolox/g sample and μmol Fe^{2+}/g sample, respectively. For the hydroxyl radical scavenging assay, the measured antioxidant activity is the ability of the PS or protein to scavenge free radicals generated by the Fenton reaction (Wang et al., 2016). Vitamin C (VC) was included as a positive antioxidant reference, and the samples (including crude PS, TPP-PS, and protein) were pre-dissolved in distilled water at different concentrations (0-0.5 mg/mL). The absorbance was measured with a Varioskan LUX Multimode Microplate Reader (Thermo Fisher Scientific).

3.3 Results and discussion

3.3.1 Effects of TPP conditions on PS yield

As stated in a previous section (**3.2.1**), $(NH_4)_2SO_4$ had been compared with a few other salts and identified as the most favorable for the TPP separation of PS and components from the mushroom extract in preliminary experiments (**Figure 3-1**). In the following experiments, four major process factors were assessed one-by-one on the PS yield and separation efficiency, including $(NH_4)_2SO_4$ concentration, the volume ratio of *t*-butanol to crude extract solution, and the TPP process temperature and time, with the results shown in **Figure 3-2**.

Figure 3-2A displays the effect of $(NH_4)_2SO_4$ concentration from 10% to 60% (w/v) on the extraction yield of PS when other conditions such as 1.5:1.0 *t*-butanol to crude extract ratio, 35 min extraction time, and 40 °C, are kept constant. The extraction yield of PS with TPP from the mushroom extract increased with the increase of $(NH_4)_2SO_4$

concentration from 10% to 40% to a maximum PS yield of 6.6 %. The addition of salt at 40% or a lower concentration could cause a "salting in" effect, favoring efficient partitioning of PS in the bottom aqueous phase (Wang et al., 2019). In contrast, the extraction yield also decreased with the higher concentrations, 50% and 60% of $(NH_4)_2SO_4$, which was attributed to the stronger salting-out effect, reducing the availability of water molecules for the solvation of PS (Dutta et al., 2015; Wang et al., 2017). Meanwhile, the protein content gradually decreased (from 1.8% to 0.7%) with increasing $(NH_4)_2SO_4$ concentration, due probably to the competition of excess salt with proteins for water hydration (**Figure 3-3**), causing the protein to aggregate and precipitate (Chan et al., 1986; Arruda et al., 2019). Therefore, the content of soluble protein decreased with increasing salt concentration. The protein content in the lower aqueous phase remained unchanged ($p > 0.05$) after adding 40% $(NH_4)_2SO_4$. Although $(NH_4)_2SO_4$ at 60% favored protein removal from PS, the PS yield decreased. The results suggested that the TPP system containing 40% (w/v) $(NH_4)_2SO_4$ was most favorable for partitioning PS. Similarly, the optimum $(NH_4)_2SO_4$ concentration was 40% (w/v) for isolating PS from *Chlorella spp.* (Zhao et al., 2018). Therefore, 40% was selected as the central point of $(NH_4)_2SO_4$ concentration for the RSM experiments.

Figure 3-2B shows the effect of *t*-butanol to crude extract ratio on the extraction yield of PS by varying *t*-butanol to crude extract ratio (0.5:1 - 3:1) at constant 40% (w/v) $(NH_4)_2SO_4$ concentration, 35 min extraction time, and 40 °C. The PS yield increased as the ratio of *t*-butanol to crude extract was increased from 0.5:1 to 1.5:1, reaching a maximum of 8.0% at 1.5:1. However, the yield of PS decreased when the solvent ratio

exceeded 1.5:1. Similar trend has also been reported by others (Wang et al., 2017; Liu et al., 2019). The reason was that a higher amount of *t*-butanol attracted more water molecules from the aqueous phase, thereby weakening the intermolecular interactions between water molecules and PS in the lower aqueous phase (Dennison and Lovrien, 1997). Besides, the protein content in the lower aqueous phase showed a slight decrease (**Figure 3-2B**). The addition of *t*-butanol can bind to the hydrophobic sites of protein, lowering the density to float above the lower aqueous phase (**Figure 3-2**) (Dennison and Lovrien 1997; Rajeeva and Lele, 2011). Therefore, the protein content in the bottom phase decreased with an increasing amount of *t*-butanol. For a higher PS yield and a lower protein content in the bottom phase, the *t*-butanol to crude extract volume ratio of 1.5:1 was applied in the RSM experiments.

Figure 3-2C shows the variation of PS yield with temperature and the highest yield of 12.4% at 55 °C ($p < 0.05$) while keeping all other parameters, such as 40% (w/v) $(NH_4)_2SO_4$ concentration, 1.5:1.0 (v/v) *t*-butanol to crude extract ratio, and 35 min, at a constant value. Increasing the temperature from 25-55 °C can increase mass transfer and extraction yield (Wang et al., 2017). The temperature increases in the system resulted in an increase in energy, which might facilitate the unfolding of the PS molecule, thereby exposing a greater number of hydroxyl groups. This in turn may promote the formation of more hydrogen bonds, resulting in higher hydrophilicity of the unfolded PS. Consequently, a higher concentration of PS could be achieved in the aqueous phase (Coimbra et al., 2010). However, when the temperature continued to increase over 55 °C, the PS yield showed a notable decline ($p < 0.05$). A possible

explanation was that the volatility of *t*-butanol increased significantly as the temperature rose above 55 °C and reduced its partitioning capacity (Yan et al., 2018). Besides, the soluble protein content in the lower aqueous phase solution did not change much. Based on this set of results, 55 °C was chosen as the central point temperature of the RSM experiment.

As shown in **Figure 3-2D**, the variation in extraction time from 15 min to 60 min had only a minor influence on the PS yield and soluble protein content in the lower phase when the conditions remained constant at 40% (w/v) $(NH_4)_2SO_4$ concentration, 1.5:1.0 (v/v) *t*- butanol to extract ratio, and 40 °C. The PS yield increased with time from 15 min (8.3%) to 30 min (9.4%) but showed a slight decrease from 30-60 min, while the protein content fluctuated in a narrow range. Therefore, the extraction time for RSM experiments was fixed at 30 min.

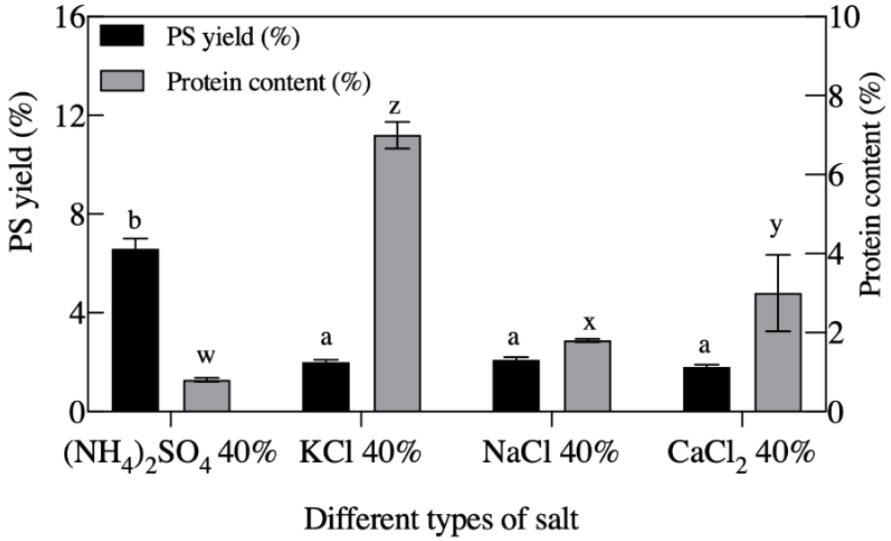

Figure 3-1. Effects of different salts on the extraction yield of PS (%) and protein content (%) in the lower aqueous phases.

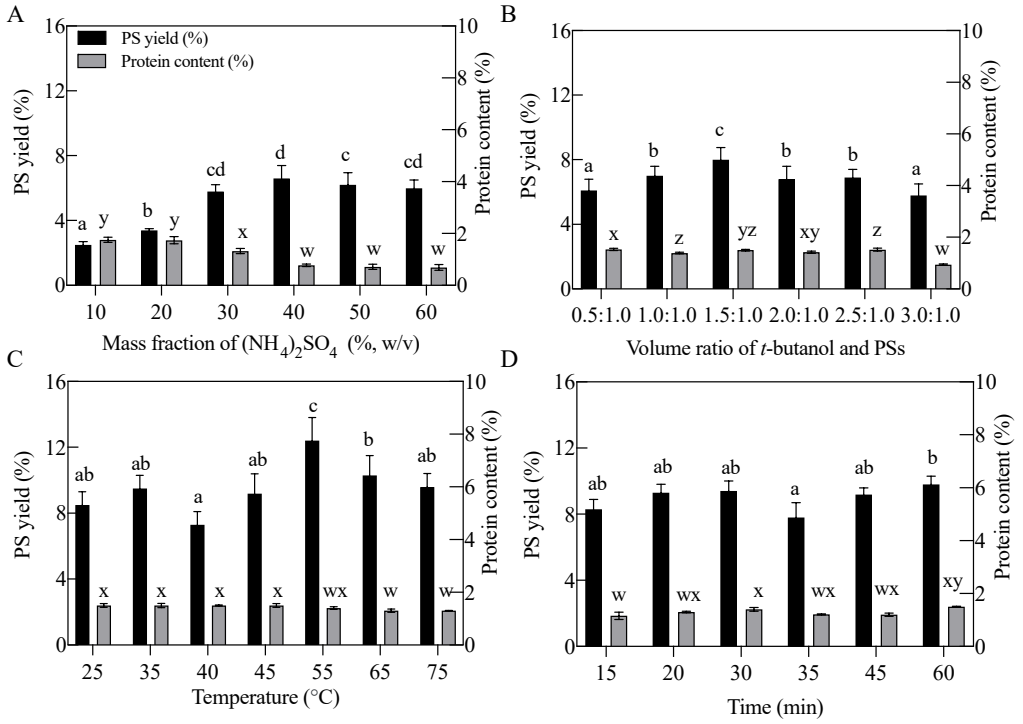

Figure 3-2. Variation of PS yield and protein content (%) in the lower aqueous phase with (A) $(NH_4)_2SO_4$ concentration (%, w/v); (B) volume ratio of *t*-butanol to crude extract solution (v/v); (C) extraction temperature; (D) extraction time. Bars with different letters are statistically different (one-way ANOVA with a Tukey posthoc test, $p < 0.05$). Lowercase letters ranging from (a-d) are used as indicators of significant comparisons between different treatments for PS yield, while lowercase letters ranging from (w-z) indicate significant comparisons of protein content between different treatments. Data with identical letters are deemed insignificant, whereas data that lack the same letter denotes a significant difference.

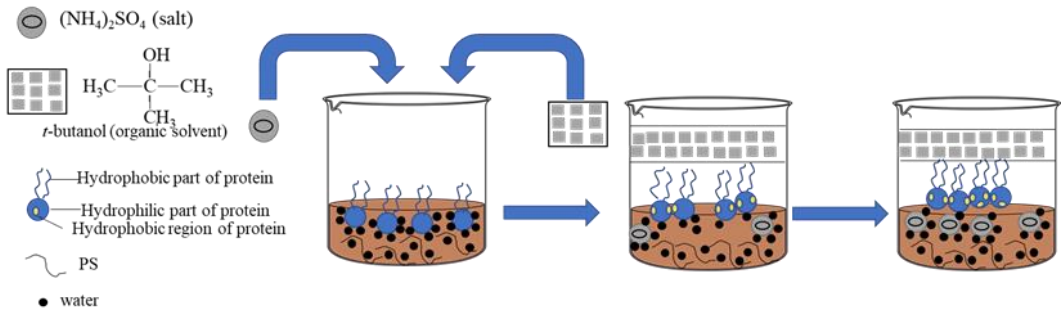

Figure 3-3. Possible mechanisms for protein separation in the TPP system.

3.3.2 Optimization of TPP process by RSM

3.3.2.1 Model fitting and statistical analysis

The above single-factor test results showed that the chief factors of TPP significantly influencing the PS yield were the concentration of $(NH_4)_2SO_4$, the volume ratio of *t*-butanol to crude extract solution, and the extraction temperature, which were included in the RSM optimization experiments. **Table 3-2** shows the 17 runs of experiments and conditions based on a 3^3 Box-Behnken design and the PS yields from the experiments and RSM model prediction. The PS yields were in the range of 9.45% to 11.81% from experiments and 9.3% to 11.72% from the model prediction, respectively. The maximum PS yield was obtained at 50 °C, 50% $(NH_4)_2SO_4$, and the 1.5:1 volume ratio of *t*-butanol to crude extract solution. Multiple regression analysis was carried out with the experimental data to derive the following second-order polynomial equation, in which X_1, X_2, and X_3 represent the actual numerical values used in the analysis.

$$Y = -42.33 + 0.29X_1 + 1.71X_2 + 3.27X_3 + 0.001X_1X_2 + 0.12X_2X_3 - 0.004{X_1}^2 - 0.018{X_2}^2 - 3.12{X_3}^2 \quad (3.1)$$

Table 3-3 shows the ANOVA results on the significance and applicability of the model. The model *F* value of 44.22 at a very low *p*-value (< 0.0001) implies the high statistical significance of the mathematical model. The coefficient of determination (R^2 = 0.9827) suggested that only 1.73% of the total variation was not fitted to the model, confirming the accuracy and good correlation between the response and the independent variables. The adjusted determination coefficient (R^2_{adj} = 0.9605) also suggested the high significance of the model. The lack of fit *F* value of 2.41 meant that lack of fit was insignificant relative to the pure error, though there was a 20.78% lack of fit *F* value due to noise. A non-significant underfit was favorable, indicating a good model fit. A coefficient of variance (CV) value of less than 10% indicated that the model was reproducible.

Besides, a smaller *p*-value indicated a higher significance level of the corresponding coefficient, e.g., the linear coefficient (X_1, X_2, *and* X_3), the interaction coefficient (X_2X_3), and the quadratic term coefficients (X_1^2, X_2^2, and X_3^2) (p < 0.05). Therefore, the ANOVA analysis confirmed the high statistical significance of salt concentration, temperature, and the volume ratio of *t*-butanol to crude extract solution for the efficient extraction of PS by the TPP, and they should be fully considered.

Table 3-2. RSM experimental design (3^3 Box-Behnken) and the PS yield results.

Run	Factor level [a]			*Y*: PS yield (%)	
	X_1: $(NH_4)_2SO_4$	X_2: Temperature	X_3: Volume ratio of *t*-butanol to crude extract	Experimental	Predicted

1	0	1	-1	10.78 ± 0.52	10.93
2	0	0	0	11.74 ± 1.76	11.66
3	0	-1	-1	10.79 ± 0.58	10.86
4	1	0	-1	11.81 ± 0.64	11.72
5	1	0	1	10.77 ± 0.94	10.89
6	0	0	0	11.74 ± 1.31	11.66
7	-1	0	-1	11.00 ± 0.63	10.88
8	-1	1	0	10.40 ± 0.65	10.38
9	0	1	1	10.67 ± 0.99	10.6
10	0	0	0	11.60 ± 0.95	11.66
11	1	-1	0	10.62 ± 1.38	10.65
12	0	0	0	11.48 ± 1.46	11.66
13	-1	-1	0	9.64 ± 0.62	9.69
14	0	-1	1	9.45 ± 0.85	9.3
15	-1	0	1	9.73 ± 0.86	9.82
16	1	1	0	11.38 ± 0.89	11.33
17	0	0	0	11.76 ± 1.52	11.66

a. **Table 3-1** for the actual values.

Table 3-3. Regression coefficient estimation and significant analysis of RSM mathematical model.

Source	Sum of squares	DF	Mean Square	*F*-value	*p*-value
Model	9.36	9	1.04	44.22	< 0.0001
X_1-$(NH_4)_2SO_4$%	1.81	1	1.81	77.12	< 0.0001
X_2-Temperature	1.77	1	1.77	75.11	< 0.0001
X_3-Volume ratio of *t*-butanol to crude extract solution	0.9316	1	0.9316	39.60	0.0004
X_1X_2	0.0132	1	0.0132	0.5621	0.4779
X_1X_3	0.0000	1	0.0000	0.0000	1.0000
X_2X_3	0.3782	1	0.3782	16.08	0.0051
X_1^2	0.5905	1	0.5905	25.10	0.0015
X_2^2	0.8987	1	0.8987	38.20	0.0005
X_3^2	2.56	1	2.56	108.74	< 0.0001
Residual	0.1647	7	0.0235		

Lack of Fit	0.1060	3	0.0353	2.41	0.2078
Pure Error	0.0587	4	0.0147		
Cor Total	9.53	16			
$R^2 = 0.9827$	$R^2_{adj} = 0.9605$		C.V.% = 1.41		

3.3.2.2 Response surface plots

Figure 3-4 presents the 2D contour plots and response surfaces to visualize the relationship of the response function (PS yield) to the process variables and their combinations. As shown in **Figure 3-4A** and **Figure 3-4B**, the PS yield increased rapidly with $(NH_4)_2SO_4$ concentration below 40% (w/v) but slowly later. Interaction effects between variables (X_1X_2 and X_1X_3) on the PS yield were insignificant, as shown by the circular contour plots. The PS yield initially increased but then decreased with an increase in the two variables, X_2 and X_3 (**Figure 3-4C**). The maximum PS yield was obtained at 55 °C, and the ratio of *t*-butanol to extract was 1.5:1. The contour lines tended to be elliptical when the X_2X_3 interacted, which indicated that the interaction was significant.

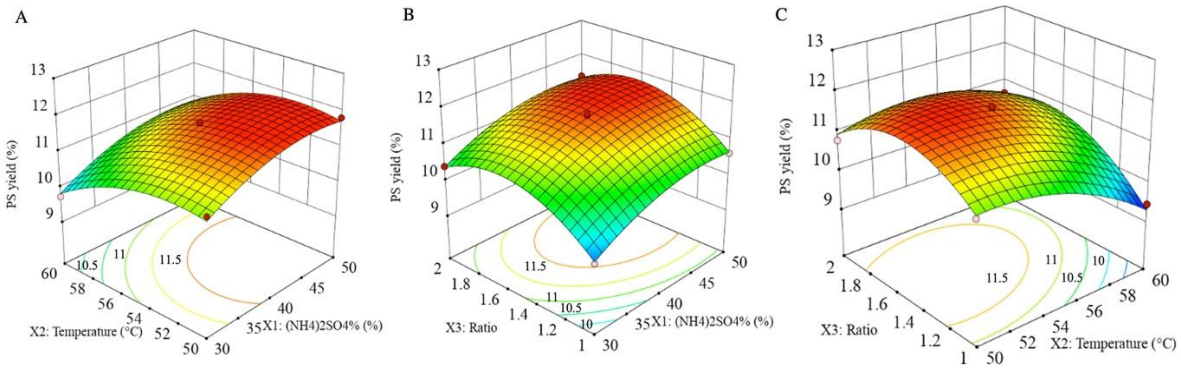

Figure 3-4. Response surface plots of PS yield versus two experimental variables: (A) $(NH_4)_2SO_4$ concentration (X_1); and temperature (X_2); (B) $(NH_4)_2SO_4$ concentration (X_1) and the volume ratio of *t*-butanol to crude extract solution (X_3); (C) temperature (X_2) and the volume ratio of *t*-butanol to crude extract solution (X_3).

3.3.2.3 Verification of model and optimal conditions

According to the ANOVA (**Table 3-3**) and response surface plots (**Figure 3-4**), the optimized conditions for the PS yield by the TPP system were 48% (w/v) $(NH_4)_2SO_4$, 1.2 ratios of *t*-butanol to extract solution, and 52 °C temperature, giving a maximum PS yield of 11.56%. The optimal conditions were confirmed by a verification experiment, resulting in a PS yield of 11.64%, which was close to the model-predicted value. It further indicated that the response model adequately reflected the expected optimization.

3.3.3 Yields and properties of PS via TPP and conventional method

Table 3-4 presents the PS yields physicochemical properties attained using TPP and the conventional method of ethanol precipitation and Sevag treatment. The extraction yield of purified PS from the TPP method (11.64%) was almost two times higher than that obtained via the conventional method (6.28%). The PS from TPP also had a higher purity than from the conventional method, as indicated by a much higher total carbohydrate content (86.01% versus 25.04%) and much lower protein content (1.91% versus 16.05%). Sevag treatment was usually accomplished by adding a solution containing 1-butanol and chloroform (1:4) to crude PS (1:5) and further by centrifugation. This step was repeated several times until no protein layer was visible in the solution. PS obtained from Sevag treatment led to low purity in our study. According to previous studies, when the protein was removed, part of the PS was also removed. Multiple treatments of Sevag led to the loss of PS (Guo et al., 2021). Moreover, proteins were not removed entirely because PS obtained after Sevag

treatment might be covalently bound with proteins. Since phenolic compounds are also present in crude PS, proteins may bind to them through covalent interactions (Sun et al., 2022). In comparison, the TPP process is much simpler with a single step and shorter time but also can achieve much higher purification of PS. Moreover, the PS from TPP and the conventional method were composed mainly of glucose, galactose, and mannose with different molar ratios (**Table 3-4**). The PS from TPP was most abundant with the low-MW fraction (7.35 kDa: 69.90%), and EtOH-Sevag-treated PS was more abundant with the high-MW fraction (> 670 kDa: 52.21%). **Figure 3-5** displays the spectra of HPGPC for PS treated with TPP and PS treated with EtOH-Sevag. This observation is consistent with some other studies that TPP facilitated the extraction of PSs with lower MW (Wang et al., 2017). Since $(NH_4)_2SO_4$ used in TPP was a weakly acidic salt, its $SO4^{2-}$ degree of ionization was greater than the degree of hydrolysis in the solution. PS in an acidic solution was prone to be partially degraded and transferred from high MW to low MW, resulting in a decrease in MW, due mainly to the disruption of hydrogen bonding interactions under acidic conditions.

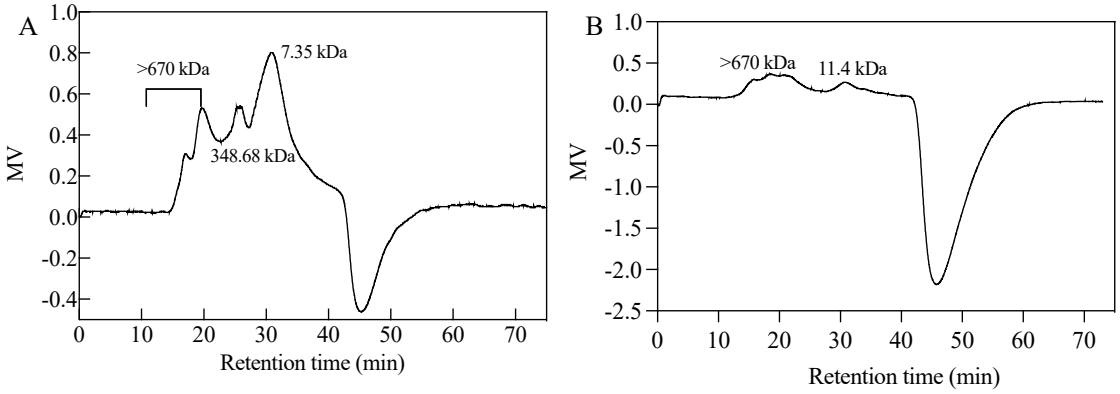

Figure 3-5. The HPGPC profiles of (A) TPP-PS and (B) EtOH-Sevag-PS.

Table 3-4. Yields and properties of PS by TPP and conventional method of ethanol precipitation-Sevag treatment.

Yield and properties	TPP	EtOH-Sevag
Yield (%)	11.64 ± 1.09	6.28 ± 0.23
Carbohydrate (wt%)	86.01 ± 3.77	25.04 ± 2.49
Protein content in the PS (wt%)	1.91 ± 0.23	16.05 ± 2.36
Monosaccharide molar ratio		
D-Glc	1.81	2.27
D-Gal	1.63	1.23
D-Man	1.00	1.00
MW (kDa) (%/Area)	>670.00 kDa (20.97%) 348.68 kDa (9.13%) 7.35 kDa, (69.90%)	>670.00 kDa (52.21%) 11.4 kDa (47.78%)

Figure 3-6A shows a clear peak at 280-300 nm on the UV-spectrum of PS from EtOH-Sevag, indicating the presence of protein residues. In contrast, the corresponding peak for the PS from TPP was barely visible, consistent with its low protein content in **Table 3-4**. These results further suggested that the TPP method was more effective in removing proteins from PS. The remaining protein in the PS could be covalently linked with PS (e.g., as proteoglycans), which could not be completely removed by the physical or chemical methods.

As shown in **Figure 3-6B**, the PS from TPP and EtOH-Sevag had similar FT-IR spectra, indicating their similar chemical structures. The broadband near 3424 cm^{-1} and 3420 cm^{-1} was ascribed to O-H (hydroxyl group) stretching vibration (Balavigneswaran et al., 2013); the weak peak at 2960 cm^{-1} to the stretching vibration of the -CH_2 group (Li et al., 2014). The absorption bands near 1638 cm^{-1} and 1639 cm^{-1} (amid I) were attributed to the presence of protein (Ji et al., 2020), which was in line with the results

shown in **Figure 3-6A**. The band at approximately 1300-1400 cm^{-1} was related to the (C=O symmetric stretching vibrations), which probably indicated the presence of carboxyl groups. Bands near 2800-3400 cm^{-1} and 1200-1400 cm^{-1} represented the characteristic absorption bands of glycan (Cheung et al., 2012), and the peak around 1080 cm^{-1} was attributed to C-O-C (ethers group) stretching vibration (Siu et al., 2016).

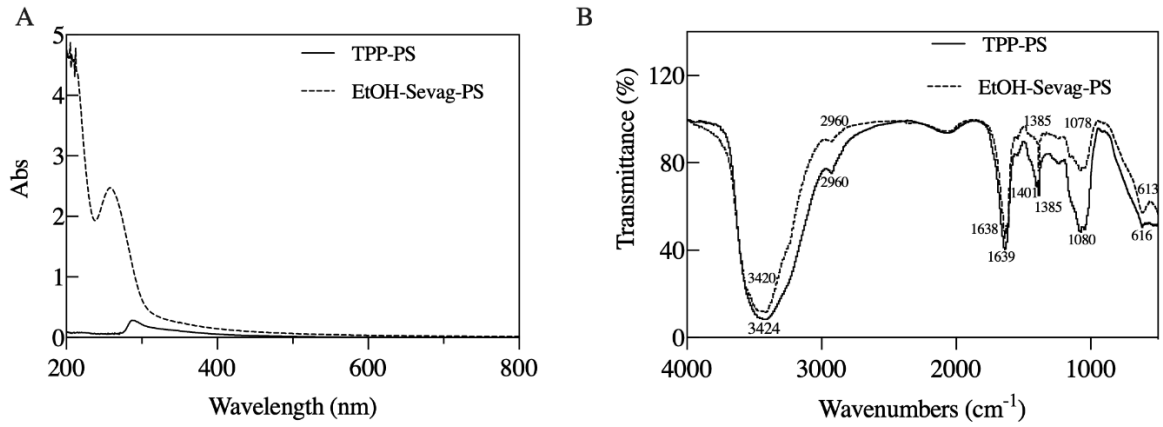

Figure 3-6. Spectral comparison between PS from TPP and EtOH-Sevag method: (A) UV spectra of PS; (B) FT-IR spectra.

3.3.4 Protein yield and properties

The yield of crude protein isolated from the middle layer of the TPP system was 2.06% at the optimal PS extraction conditions with a protein content of 48.82% (**Table 3.5**). Li et al. (2009) used sodium hydroxide combined with ethanol to extract proteins from *L. edodes* and attained a protein content of 41.1% and a carbohydrate content of 24.2%. The protein content was lower than the protein isolated by TPP in the present study. In view of the crude protein isolated as a by-product from *L. edodes*, the TPP method has an advantage.

As shown in **Figure 3-7A**, there was a large absorption peak at 280-300 nm on the

UV spectrum, confirming the presence and abundance of protein in the sample. On the FT-IR spectrum (**Figure 3-7B**), the broadband at 3424 cm^{-1} was ascribed to the O-H (hydroxyl group) stretching vibration, due probably to the presence of PS. The absorption bands at around 1638 cm^{-1} and 1617 cm^{-1} (amide I), and 1541 cm^{-1} (amide II) were attributed to the presence of protein, while no distinct amide II peak was in **Figure 3-6B**. The TPP method could be better for removing relatively unstructured proteins (Deflores et al., 2009). In addition, SDS-PAGE analysis revealed a distinct band between 25 and 130 kDa in the protein sample and a few more intense bands at 25 kDa, 40 kDa, 55 kDa, and 130 kDa (**Figure 3-8**).

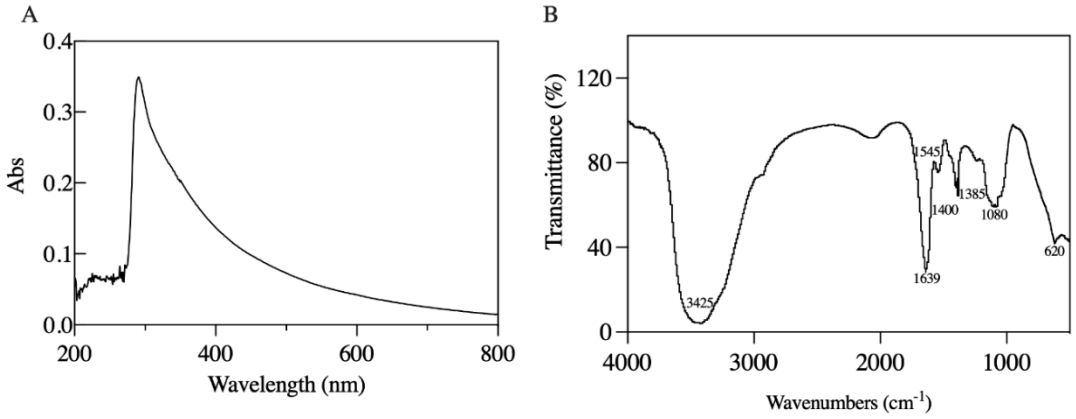

Figure 3-7. Analysis of isolated protein fraction from TPP: A) UV spectrum, B) FT-IR spectrum.

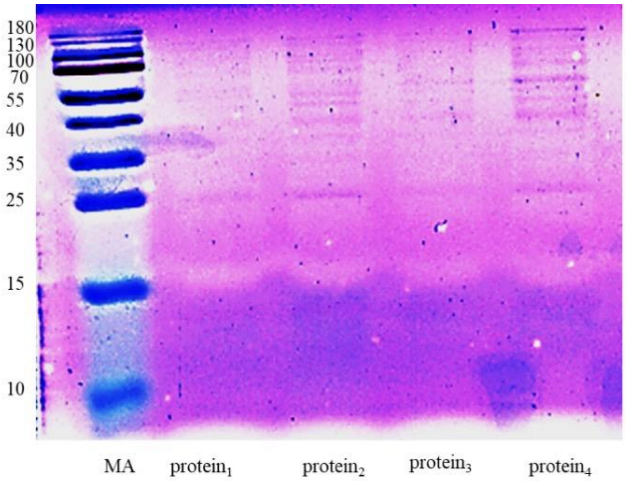

Figure 3-8. SDS-PAGE analysis of protein. MA stands for the marker. $protein_1$, $protein_2$, $protein_3$, and $protein_4$ represented four independent experiment samples.

Table 3-5 shows the AA composition of the crude protein recovered from the middle layer of TPP, including 20 essential and non-essential amino acids (NEAAs). The protein was abundant in both glutamic acid and aspartic acid, suggesting the presence of acidic proteoglycans (Wang et al., 2017). Besides, the protein contained threonine and serine, which might be components of GlcN-α-serine/threonine linkage in the O-glycosidic bonds of PS (Kilcoyne et al., 2009). Therefore, the protein could contain PS because O-glycosidic bonds were not easily removed by TPP treatment, which was consistent with the results in **Figure 3-6B**.

Table 3-5. Yield and composition of crude protein isolated from the middle layer of TPP.

Yield (%)	Total carbohydrate (wt%)	Total protein (wt%)	
2.06 ± 0.32	34.89 ± 2.72	48.82 ± 1.43	
EAA	Content (mg/g crude protein)	NEAA	Content (mg/g crude protein)
Histidine	5.9 ± 0.6	Alanine	21.4 ± 0.8
Isoleucine	13.0 ± 0.5	Asparagine	0.05 ± 0.004
Leucine	21.6 ± 0. 9	Aspartic acid	41.8 ±1.9
Lysine	18.0 ± 0.5	Arginine	12.9 ± 0.5

Methionine	2.1 ± 0. 3	Glutamic acid	39.7 ±1.8
Phenylalanine	14.0 ± 0.8	Glycine	60.6 ± 0.9
Threonine	22.1 ± 0. 6	Serine	26.5 ± 0.9
Tryptophan	0.077 ± 0.003	Tyrosine	9.7 ± 0.3
Valine	17.0 ± 0.6	Cysteine	2.25 ± 0.02
		Glutamine	0. 18 ± 0.04
		Proline	14.7 ± 0.8

3.3.5 Antioxidant activities

Figure 3-9 shows the antioxidant activity results with the crude PS isolated by ethanol precipitation and the PS, and protein isolated by TPP. For both ABTS$^{\bullet+}$ radical scavenging (**Figure 3-9A**) and ferric-reducing activity (**Figure 3-9B**), the activity level was in the order of crude PS > PS > protein. These activity values were mostly higher than the PS from Shiitake mushrooms previously reported by Cheung et al. (2012). Besides, as shown in **Figure 3-9C**, the IC50 values of VC, crude PS, PS, and protein were 0.52 mg/mL, 1.59 mg/mL, 1.73 mg/mL, and 1.84 mg/mL, respectively. Therefore, all three assays showed a consistent downward trend from crude PS to PS and then protein. The higher antioxidant activities in the crude PS were probably attributed to the higher content of small phenolic compounds (Siu et al., 2014), which were removed from the PS to the organic phase of TPP.

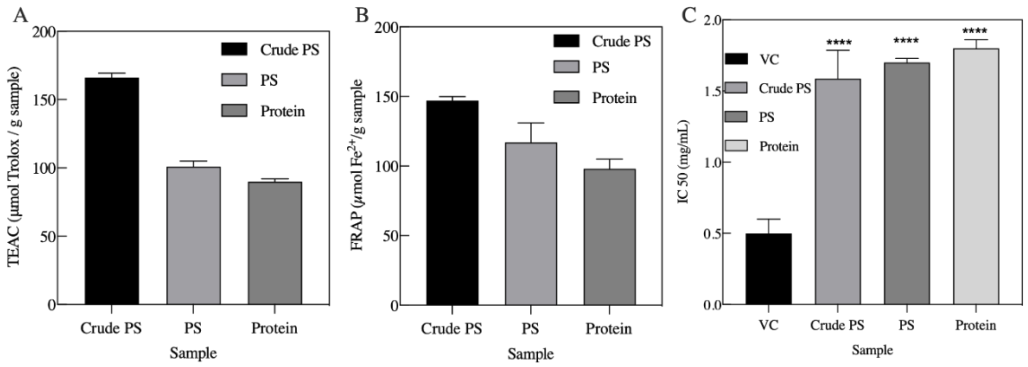

Figure 3-9. Antioxidant activities of isolated crude PS, PS, and protein by A) FRAP

and B) TEAC (assays), C) scavenging ability on •OH. ****: statistically different from VC and sample at $p < 0.0001$. Each value is expressed as mean ± SD (n = 3).

3.3.6 Immunomodulatory activities

Preliminary experiments were first performed to examine the cell viability of crude PS prepared by conventional methods at the same concentration as PS prepared by TPP treatment. However, cell viability with crude PS treatment led to more cell death than TPP-PS. Therefore, crude PS extracted by conventional methods was not used for further immunomodulatory experiments (**Figure 3-10**).

As shown in **Figures 3-11A** and **B**, the phagocytosis-promoting effects of PS or protein on RAW 264.7 cells were measured by neutral red uptake. As the sample concentration increased, more cells took up neutral red, as shown in **Figure 3-11**. Furthermore, the pinocytosis values of cells treated with PS and protein exhibited a statistically significant difference compared to the control group. Conversely, no significant difference was observed in the pinocytosis values of samples treated with LPS ($p > 0.05$). Since a low protein concentration achieved the same effect as PS, it further indicated that protein had a more potent activity to stimulate the phagocytosis of RAW 246.7 cells than PS. As shown in **Figure 3-11C** and **D**, the production of NO in RAW264.7 cells was significantly stimulated by PS and protein in a dose-dependent manner, giving rise to an EC50 value of 23.88 μg/mL for the PS and 0.28 μg/mL for the protein, respectively. NO is a gaseous molecule created by nitric oxide synthase (NOS) from the AA L-arginine and has an important role in immune function (Luiking et al., 2010). Therefore, the results indicated the immunostimulatory activity of PS and

protein fractions isolated by TPP from the mushroom extract. Although PS, including β-glucans (lentinan) and various hetero-PS from *L. edodes,* were found having strong immunostimulatory activity in most previous studies (Xu et al., 2012), the protein from TPP showed much more potent activity to stimulate the NO production in a much lower concentration range, which was consistent with the phagocytic activity analysis observations.

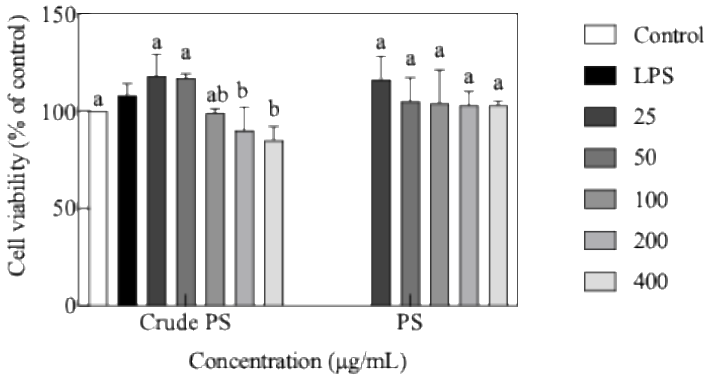

Figure 3-10. Cell viability of RAW246.7 cell treated with PS and crude PS after 24 h incubation. Different letters indicating significant difference ($p < 0.05$).

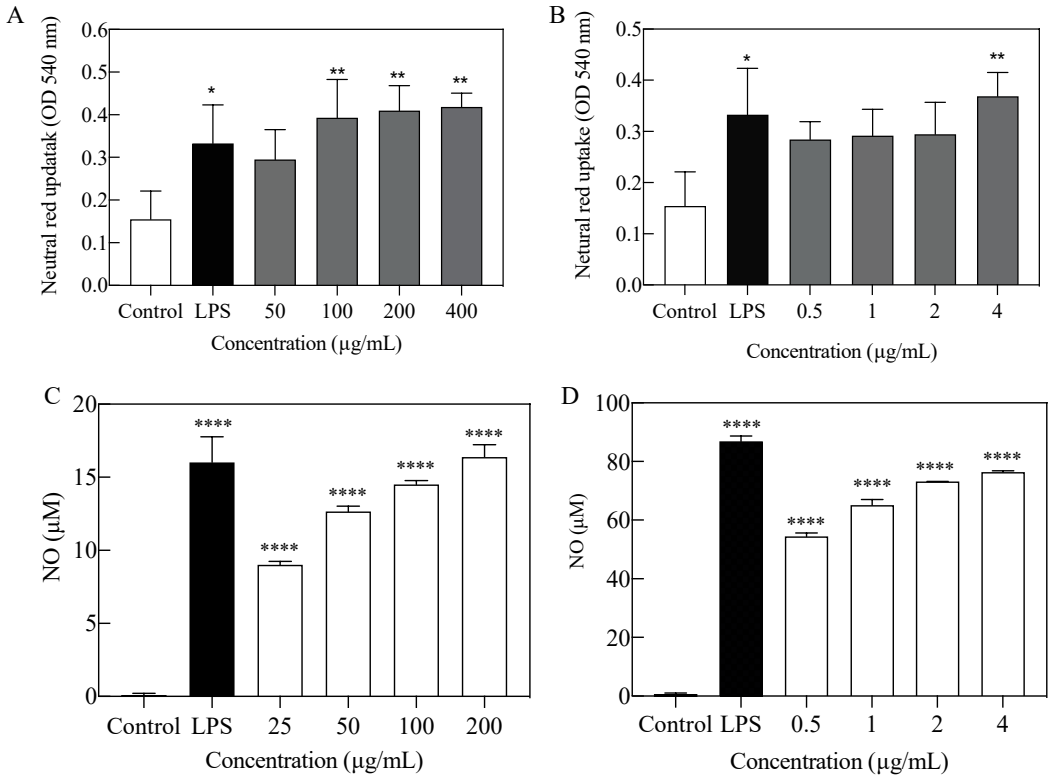

Figure 3-11. Immunostimulatory effects of PS- and protein-rich fractions on phagocytic activity (neutral red uptake) and NO production in RAW246.7 cell cultures: neutral red uptake treated by (A) PS-rich fraction and (B) protein-rich fraction; NO production treated by (C) PS-rich fraction and (D) protein-rich fraction. Data are expressed as the means ± SD (n = 3). The error bar represents the standard deviation. *, ** and ****: statistically significant differences from the control group at $p < 0.01$ and $p < 0.001$, $p < 0.0001$ respectively.

3.4 Conclusions

The present study has demonstrated the efficiency of the TPP method for isolating both PS and protein from *L. edodes* mushroom. The significant factors affecting the PS yield were identified as the concentration of $(NH_4)_2SO_4$, *t*-butanol ratio to crude extract, and extraction temperature. These were optimized by statistical experimental design

and RSM analysis as 48%, 1.2:1.0 *t*-butanol to crude extract ratio, and 52°C to achieve the highest extraction PS yield (11.64%). The protein yield under these conditions was 2.06%. Compared to the conventional extraction methods, the TPP method achieved a higher PS yield and a higher purity. The PS from TPP consisted mainly of D-Glc, D-Gal, and D-Man with three MW fractions (>670 kDa, 348.68 kDa, and 7.35 kDa). The isolated protein fraction contained both EAA and NEAAs and had a MW distribution of 25-130 kDa. The protein exhibited stronger immunostimulatory activity than PS by *in vitro* tests. TPP can serve as a promising and direct method for simultaneously isolating bioactive PS and proteins from edible and medicinal mushrooms. Furthermore, an additional advantage of TPP is the potential for recycling the organic reagents used in the process. By implementing appropriate scaling, TPP has the potential to become a cost-effective industrial method for extracting PS and proteins. Additionally, with a strong immunostimulatory activity, the protein-rich fraction from TPP deserves further research effort to improve the yield and purity of the protein fraction and identify the most active protein structures.

Chapter 4 Fractionation and Characterization of Immunoactivity Proteins Isolated by TPP

4.1 Introduction

Mushrooms, the fleshy fruiting bodies of large fungi, are well known for their high nutritional and medicinal value (Valverde et al., 2015). With the increasing demand for healthy foods and natural therapeutics, edible mushroom products have received special attention. Polysaccharide (PS), as the most abundant component of mushrooms fulfill multiple functions, such as antioxidant, antimicrobial, and anti-inflammatory properties (Araújo-Rodrigues et al., 2024). Proteins constitute another important group of mushroom components contributing to nutrition and bioactivity (Chang & Miles, 2004). Recently, edible mushrooms have been recognized as a promising protein source that differs from conventional animal sources and higher-quality proteins than those from plant sources (Ayimbila & Keawsompong, 2023). In particular, most mushroom proteins or fungi proteins have a complete profile of essential amino acids (EAAs) and a high content of branched-chain amino acids (AAs) (Ayimbila & Keawsompong, 2023). Fungi proteins have shown a number of beneficial functions such as maintaining blood insulin levels, improving blood cholesterol levels, and enhancing immune function (Ahmad et al., 2022). *Lentinula edodes,* is among the most consumed edible mushrooms in the world, due probably to its favorable flavor, aroma, and taste but also to its high nutritional and medicinal value (Singh et al., 2022). *L. edodes* mushroom has claimed numerous benefits because of their high fiber content, nutritional profile, and wide range of functional components (Niego et al., 2021). Among the most abundant

components of *L. edodes* mushroom, proteins make significant contributions to their nutritional value and medicinal functions.

The protein content of *L. edodes* mushroom is typically in the range from 15% to 35% on a dry weight basis, which contributes to various biological activities such as antioxidant, antitumor, antimicrobial, and immunomodulatory (Ngai & Ng, 2003; Gao et al., 2023). However, the complexity and diversity of proteins in *L. edodes* mushroom impose great challenges to their separation and purification for activity studies. *L. edodes* mushroom contains various proteins with differences in chemical composition, molecular structure, physiological function, and cellular location (Fang et al., 2022; Paisansak et al., 2021). Fractionation and purification are essential steps for analyzing and characterizing protein molecules and for a better understanding of the molecular properties and their relationship to the nutritional and medicinal functions of proteins. In a recent study (Zhao et al., 2024), we explored the three-phases portioning (TPP) as an innovative and effective method for the simultaneous extraction of PS and protein components from *L. edodes* mushroom. A protein-rich fraction of polysaccharide-protein (PSP) complex with a total protein content of about 50% (w/w) was attained and showed potent immunostimulatory activity in RAW264.7 macrophage cell culture, and much stronger than the polysaccharide-rich fraction. However, the previously reported protein fraction was a complex mixture containing proteins, PS, and unknown components. It is of significance to further purify and characterize the crude protein fraction for a better understanding of the protein molecular properties and health benefits.

This study aimed to further explore the immunomodulatory properties of proteins before and after purification and the potential for utilizing the natural proteins as immunomodulatory agents and nutritional supplements. Experiments were carried out to fractionate and purify the protein-rich fraction and evaluate and compare the different protein fractions in structure, and nutritional value of *L. edodes* mushroom. The immunological activity was evaluated of the protein fractions before and after purification, the purified protein fractions, and their combinations. The crude protein fraction attained via TPP extraction of *L. edodes* mushroom was separated using DEAE cellulose ion exchange column chromatography (Yuan et al., 2024; Wang, et al., 2024). Analytical experiments were conducted to determine the structures and nutritional value of the protein fractions, and in *vitro,* immunological activity was evaluated in mouse macrophage RAW264.7 cell culture. The observation of Toll-like receptor 4/ nuclear transcription factor κB (TLR4/NF-κB) immune signaling pathways stimulated by purified fractions (Yuan et al., 2024) was also conducted.

4.2 Materials and methods

The majority of chemical and biochemical reagents used in this study were described in section 2.1.

Analysis of protein composition, nutritional value, and molecular weight (MW) were performed using the same methods as described in 2.4.

The cell lines used in this chapter, the culture conditions, and the analysis of immunomodulatory activity were identical to those described in section 2.5. Cell

analysis was conducted using LPS at a concentration of 200 ng/mL, as well as samples including crude-protein, M1 (PS + F1 + F2 at 1:2:5), M2 (F1 + F2 at 1:2.5), F1 and F2 at a concentration of 4 μg/mL.

4.2.1 Protein purification

Protein extraction from *L. edodes* mushroom was performed by the three-phase partitioning (TPP) method as reported previously (Zhao et al., 2024), detailed in section 2.2. The crude protein obtained via TPP extraction of the mushroom, was then purified by anion exchange chromatography in a DEAE-52 column according to a reported method (Li et al., 2010). The column was initially flushed and eluted with 10 mM Tris/HCl buffer (pH 9.0) for overnight to remove neutral-free PS and then eluted with 0.5 M sodium chloride (NaCl) in the elution buffer (Tris-HCl). The protein fractions (F1 and F2) were collected with a fractional collector, and the carbohydrate content was detected at 490 nm by the phenol-sulfuric acid method and protein at 562 nm with a protein assay kit. The purified protein fractions were dialyzed through a 3.5 kDa molecular cut-off bag for 48 h and freeze-dried for further analysis.

4.2.2 Protein structure analysis

The Fourier transform infrared spectroscopy (FT-IR) spectroscopy and Circular dichroism (CD) analysis was outlined in section 2.4. Before nuclear Magnetic Resonance (NMR) analysis, the F1 and F2 fractions were dissolved in D_2O and dried twice by vacuum freeze drying. The ^1H spectra were recorded by a 400 MHz NMR spectrometer (Bruker AVIII500M, Switzerland) with 20 mg of crude-protein, F1 and

F2 in 0.6 mL D_2O at 25 ◦C. Chemical shifts were reported in δ (ppm) with respect to HDO at δ 4.79 ppm (^1H) (Li et al., 2023).

4.2.3 Protein fraction identification

The EasyPep™ Mini MS Sample Prep Kit from Thermo Fisher Scientific (Rockford, Illinois, United States) was utilized to prepare the samples prior to running them in liquid chromatography-mass spectrometry (LC-MS) analysis. 10-100 µg of each protein sample was transferred to a microcentrifuge tube and dissolved in 100 µL of a lysis solution. Then, 50 µL reduction solution and 50 µL alkylation solution were added to the sample, and the samples were incubated at 95 °C for 10 min to reduce and alkylate the protein sample. After this, the Trypsin/Lys-C protease mix was reconstituted by adding enzyme reconstitution solution to each vial. Then, 50 µL of the reconstituted enzyme solution was added to the reduced and alkylated protein sample solution, followed by incubation with shaking at 37 °C for 1-3 h to digest the protein sample. This step allows the enzymes to break down the proteins into smaller peptides, which LC-MS can further analyze. After incubation, 50 µL of digestion stop solution was added to the sample and gently mixed. Besides, to clean up the peptides, the white cap at the bottom of the peptide clean-up column was removed, and the green top cap was loosened before placing the column into a 2 mL microcentrifuge tube. The liquid was removed from the column by centrifugation at 3,000 × g for 2 min. The protein digest sample (~300 µL total volume) was then transferred into the dry peptide clean-up column, and samples were centrifuged at 1,500 × g for 2 min to remove the effluent. Next, 300 µL of wash solution A was added to the column, and samples were

centrifuged at 1,500 × g for 2 min. The effluent was discarded, and the sample was washed twice with wash solution B. 300 µL of wash solution B was added to the column, and samples were centrifuged at 1,500 × g for 2 min before discarding the effluent. These steps were repeated once. The peptide clean-up column was then transferred to a new 2 mL microcentrifuge tube, and 300 µL of elution solution was added to the column. Samples were centrifuged at 1,500 × g for 2 min to collect the clean peptide sample. The peptide sample was dried using a vacuum centrifuge and resuspended in 100 µL of 0.1% formic acid in water for LC-MS analysis. The peptide concentration was adjusted with 0.1% formic acid in a water solution for optimal LC-MS column loading. The LC-MS analysis in this study was performed using the 5mm C18 trap cartridge for heat-trapping and an Acclaim PepMap C18 column (250mm × 75µm, ThermoFisher, USA) for analytical separation in a Vanquish Neo nanoLC platform (ThermoFisher, USA) following the gradient setting described in Tam et al. (2023). The eluents were directly loaded into a Thermo Fisher Orbitrap Fusion Lumos mass spectrometer. The analysis was carried out via data-dependent acquisition (DDA) mode with parameters described in Tam et al. (2023).

The amino acid (AA) constituents of protein fractions (F1 and F2) were identified using the Mascot software and protein entries under the taxonomy "Omphalotaceae" from the UniProt database (access: May 2023). The parameters for Mascot database searches were chosen as precursor tolerance level: 10 ppm, fragment tolerance level: 0.05 Da, maximum miss cleavage: 2, the maximum number of 13X allowed: 1, peptide charge: 2^+ to 4^+. Carbamidomethylation on cysteine residue was set

as a fixed modification, whereas oxidation on methionine residue was set as a variable modification. The expected value cut-off and significant level (*p*-value) for identification were set as 0.05. For identity numbers over 100, the false discovery rate (FDR) of protein identification was set to 0.01 using the decoy database.

4.2.4 Analysis of cytokines by ELISA

The cells were inoculated in 96-well plates (5×10^4 cells/well) and pre-incubated for 24 h. The cells were then stimulated and subjected to lipopolysaccharide (LPS) and two purified protein treatments for 24 h to induce inflammatory cytokines (tumor necrosis factor-alpha (TNF-α)). The conditioned or treated culture medium was collected and centrifuged, and the concentration of cytokines in the supernatant was measured by enzyme-linked immune sorbent assay (ELISA) kits (Jingmei Biological Technology, Jiangsu Province, China) according to the manufacturer's instructions.

4.2.5 Immunofluorescence study

The immunofluorescence assay was performed according to a previous report (Park et al., 2021) to visualize the TLR4/NF-κB immune signaling pathways stimulated by LPS, F1, and F2. Cultured RAW 264.7 cells (2×10^5) at the exponential growth phase were seeded on a glass confocal dish for 24 h and then treated with 0.2 μg/mL of LPS and 4 μg/mL of a protein fraction for 24 h. After the treatment, the cells were fixed with 4% paraformaldehyde for 30 min at room temperature and then with PBS for 10 min for three times. The cells were then permeabilized with 0.2% Triton X-100 in PBS containing 0.1% Triton X for 15 min and then washed three times with PBS and then blocked with FDB (5% goat serum, 2% bovine serum BSA, and 5% fetal bovine serum

in PBS) for 1 h. The cells were incubated at 4 °C for overnight with appropriate primary antibodies including a rabbit anti-TL-R4 (1:1000) and anti-NF-κB antibody and (1:1000) (Sigma-Aldrich). The cells were then incubated with an appropriate secondary antibody Alexa488-conjugated anti-rabbit IgG (1:1000) or DAPI (1:2000) for 1 h (for nuclear staining) in the dark at room temperature. Fluorescence microscopy was performed on a Nikon Eclipse Ti2-E Live-cell Fluorescence Imaging System (Tokyo, Japan) at ×20 magnification.

4.3 Results and discussion

4.3.1 Elution profiles of protein fractions on AEC

The crude protein fraction extracted from *L. edodes* mushroom was loaded to the DEAE-52 anion exchange chromatography (AEC) column and initially eluted with the equilibration buffer (Tris-HCl) without salt addition to remove the PS component. With the addition of salt to the elution buffer, proteins were eluted out due to the surface charges with the AAs (**Figure 4-1**). With the salt concentration in the elution buffer was increased or the pH was altered, the proteins were separated by their respective binding strengths to the anionic column (Culter, 2008). Additionally, as shown in **Figure 4-1**, the most intensive protein peaks were obtained using the 10 mM Tris-HCl buffer (pH = 9.0) containing 0.5 M NaCl. To eliminate free PS, the column was flushed overnight with 10 mM Tris/HCl (pH = 9.0), and the resulting samples were then eluted with a 10 mM Tris-HCl buffer (pH = 9.0) containing 0.5 M NaCl, yielding fractions F1 and F2 as shown in **Figure 4-2**. The PS peaks appearing at the same positions as the protein peaks indicated that the proteins and PS in these two fractions existed in a bound form,

which may also be accounted for the total carbohydrate content of F1 and F2 (**Table 4-1**). Nevertheless, the PS carbohydrate peaks were much lower due to the lower content compared with the protein peaks.

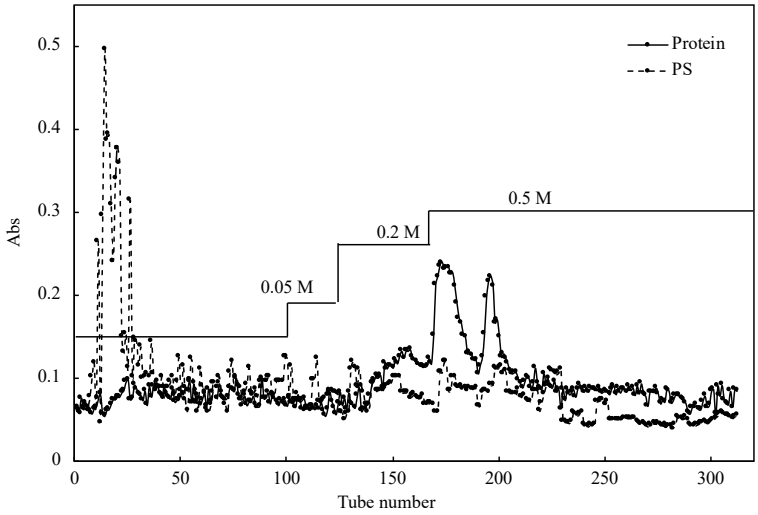

Figure 4-1. The protein fractions of *L. edodes* were eluted using a DEAE-AEC column with different NaCl concentrations (0.05 M, 0.2 M, and 0.5 M) in Tris/HCl buffer (pH 9.0). The elution process was conducted at a 1.1 mL/min flow rate, with each fraction collected in

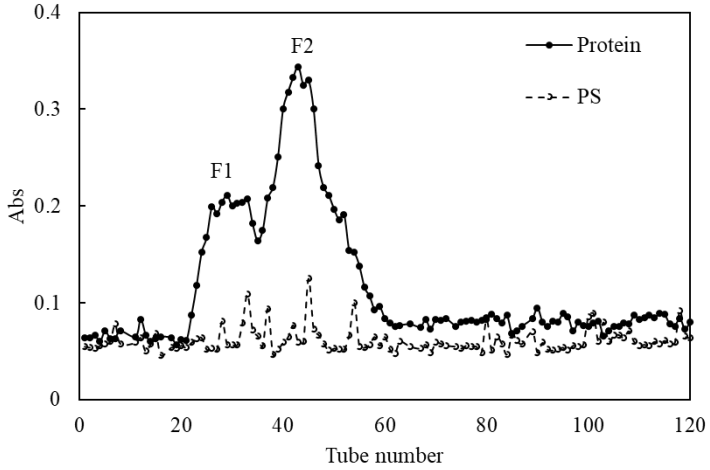

Figure 4-2. Elution profiles of *L. edodes* PS and proteins through a DEAE-AEC column,

eluted with 0.5 M NaCl in Tris/HCl buffer (pH 9.0) (PS carbohydrate detected at 490 nm by phenol-sulfuric acid method and protein at 562 nm with a BCA kit).

4.3.2 Structure characteristics of purified protein

The purified protein fractions F1 and F2 had 66% and 74% total protein, respectively (**Table 4-1**), which were much higher than that of crude protein fraction (49%), and their total carbohydrate contents were 25% and 16%, respectively and much lower than that of the crude protein fraction (35%) (Zhao et al., 2024). The much lower carbohydrate content in F1 and F2 than in the crude protein fraction confirmed the effective purification process, which was consistent with that observed from the elution profiles of **Figure 4-2**. However, AEC was only effective for separation of free PS mixed with but not covalently bonded with the proteins. As observed from the NMR spectra of crude protein fractions (**Figure 4-3**), the crude protein fractions exhibited numerous signals, including signals between 4.4-5.5 ppm attributed to the anomeric protons of PS (Yao et al., 2021). The ^{1}H spectrum of the purified fractions did not show distinct signals for carbohydrates, such as those for the anomeric proton (4.4-5.5 ppm), acetyl (2.0-2.1 ppm), and methyl (1.2 ppm) groups (Bubb 2003). The observation agreed with the analytical results in **Table 4-1**. Besides, in F1, the signal at 2.21 ppm was attributed to glutamic acid, and the signal at about 1.55 ppm was characteristic of alanine (Nord et al., 2004). The signal at about 1.97 ppm was characteristic of arginine. In the F2 fraction, the signal at 3.92 ppm was attributed to alanine, and the peaks at 2.02 ppm and 1.26 ppm were characteristic of isoleucine (Nord et al., 2004; Ritota et al., 2010). The signal at 3.62 ppm could be glutamine (de Falco et al., 2016).

In the FT-IR spectra, protein fractions F1 and F2 exhibited distinct absorption bands, including those associated with amides (**Figure 4-4A**). Characteristic peaks of the amide I band (1600-1700 cm^{-1}) were well-established for the F1 and F2 as well as for the crude protein (Zhao et al., 2024). Furthermore, F1 and F1 both had peaks at 1548 cm^{-1} ascribed to amide II (N-H and C-O binding) as the crude protein (Zhao et al., 2024). The strong and broad absorbance at around 3400 cm^{-1} was attributed to the stretching vibration of O-H in the molecular structure, probably due to the presence of PS. Crude protein, F1, and F2 all exhibited these peaks, indicating they all contained PS. The weak absorption at 2934 cm^{-1} was derived from the stretching vibration of C-H. These two stretching vibrations were both found in F1 and F2. In comparison between F1 and F2, only F2 had a peak at 1240 cm^{-1} peaks, which was assigned to the amide III group (Singh 2000).

As shown in **Figure 4-4B** and **Table 4-1**, the wavenumbers of crude protein and two purified F1 and F2 were primarily in the β-sheet and random coil structure, while the α-helix structure was noticeably absent in F1 and F2 fractions compared to crude protein. The addition of salt during purification probably caused disruption of the α-helix structure, as suggested by Crevenna et al. (2012) that sodium chloride (NaCl) has a significant effect in destabilizing the α-helix. Adding NaCl during purification may also cause the transformation of the α-helical structure into a β-sheet structure, as a reduced level of aggregation favors the formation of hydrogen bonds between proteins as the NaCl concentration increases (Kang et al. 2021). The α-helix structure may also be transformed into a random coil structure (Holtzer and Holtzer, 1992). In contrast, Li

Bo, et al., (2010) showed that a *L. edodes* mushroom protein had a higher content of α-helix structure, which could be attributed to the different protein extraction conditions, such as the temperature of 90°C in our present study, but much lower 10 °C in the previous study. Indeed, the α-helix structure of proteins tends to transform into more extended β-folded structures under heating conditions (Hu, 2019). Moreover, there were also noticeable structural differences between F1 and F2, and in particular, the relative proportion of β-sheets and β-turn were higher in, but the proportion of random coil was lower in F2 than in F1.

F1 and F2 had different MW profiles, as shown by the SDS-PAGE analysis that F1 had a distinct band at < 10 kDa and another around 34 kDa. F2 had more diverse bands than F1, which was saturated at < 10 kDa, 34 kDa, 43-55 kDa, 50-75 kDa, and 75-95 kDa (**Table 4-1, Figure 4-5**). In contrast to the F1 and F2 fractions, crude protein bands were predominantly saturated at 25 kDa, 40 kDa, 55 kDa, and 130 kDa (Zhao et al., 2024). The MW of proteins showed an overall reduction after purification, due probably to a decrease in the protein aggregation caused by the introduction of salt during the AEC elution (Kang et al., 2021).

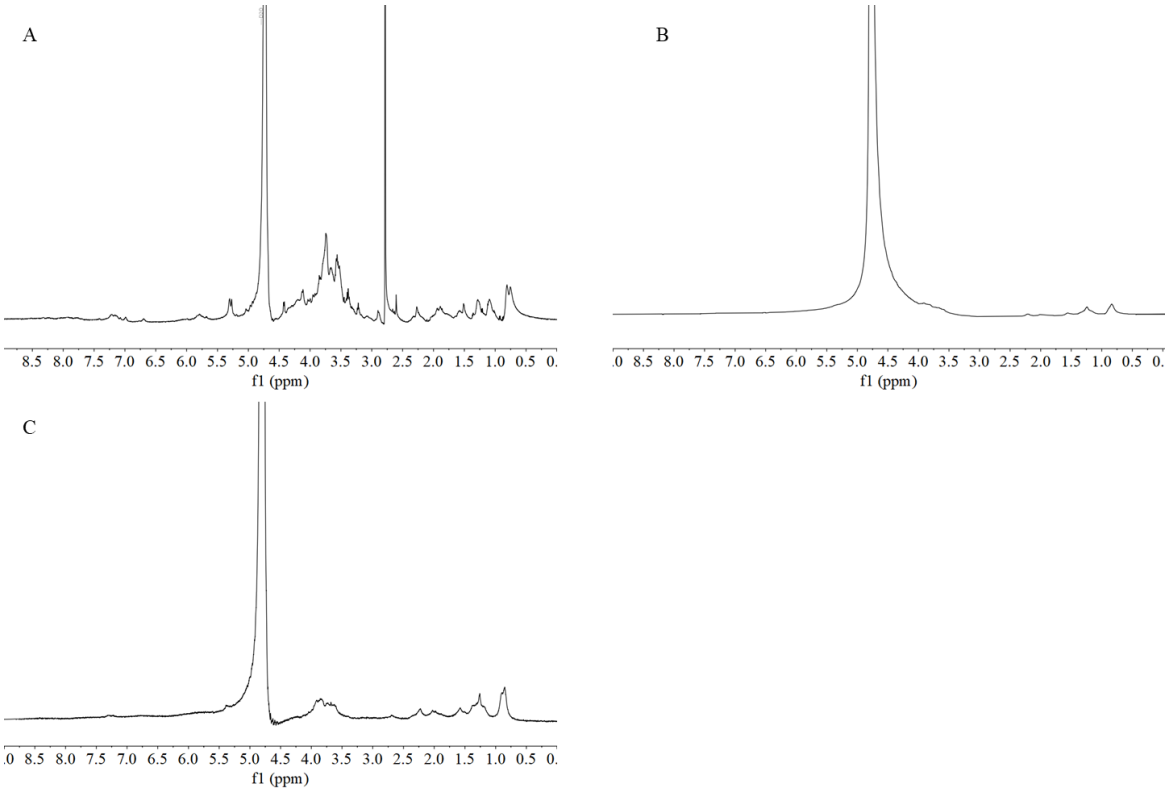

Figure 4-3. NMR 1-H data analysis of A) Crude protein, B) F1, and C) F2.

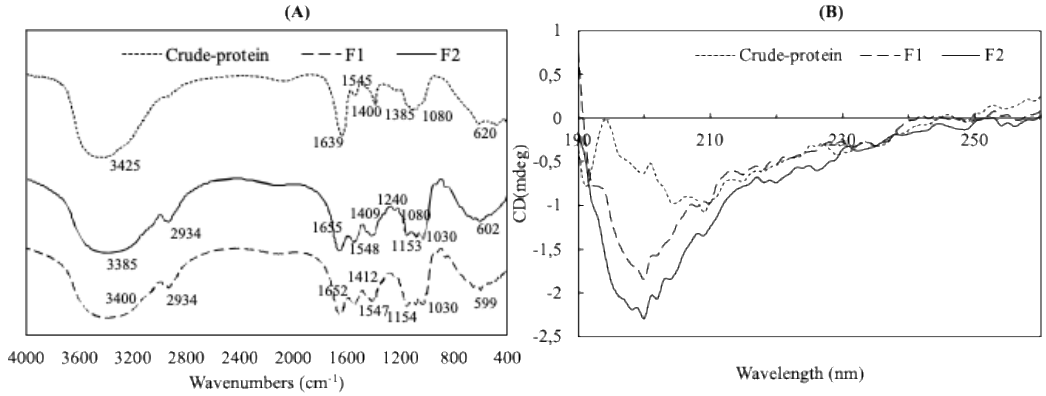

Figure 4-4. The characteristics of crude and purified protein fractions: (A) FT-IR spectra (crude protein from a previous study (Zhao et al., 2024)); (B) CD spectra.

Table 4-1. The structural characteristics of crude protein, F1, and F2 (crude protein previous study (Zhao et al., 2024).

Content and MW	Crude protein	F1	F2
Yield (% w/w)[a]	2.1 ± 0.3	11.3 ± 2.9	28.8 ± 6.3
Total carbohydrate (%)	34.9 ± 2.7	25. 4 ± 2.7	16.8 ± 1.0

Total protein (%)	48.8 ± 1.4	66.1 ± 0.9	74.0 ± 5.4
Mainly, MW of samples analyzed by SDS-PAGE	25 kDa, 40 kDa, 55 kDa, and 130 kDa	<10 kDa and 34 kDa	< 10 kDa, around 34 kDa, 43-55 kDa, 50-75 kDa, and 75-95 kDa

Protein structure				
	Random coil %	α-helix %	β-sheet %	β-turn %
Crude protein	35.5	9.9	39.6	15.0
F1	55.3	0.0	37.5	7.2
F2	50.2	0.0	41.0	8.8

a. Crude protein based on the weight of initial dry mushroom; F1 and F2 on weight of crude protein.

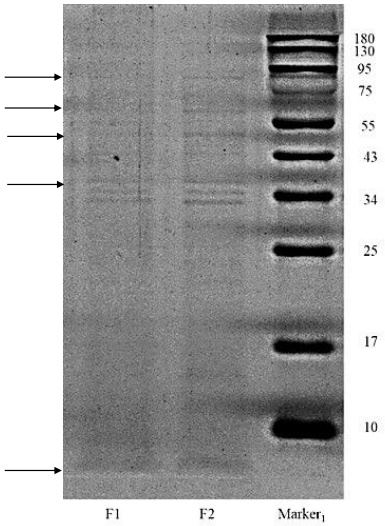

Figure 4-5. In SDS-PAGE analysis of purified target protein, the left and right lanes are the standard marker, and the lane is the protein F1 and F2.

4.3.3 Composition and nutritional evaluation of the amino acids in *L. edodes*

As shown in **Table 4-2**, F1 and F2 contained both essential and non-essential AAs, of which glycine was highest, followed by aspartic and then glutamic acid. The contents of most AAs in F2 were slightly higher than in F1, which was consistent with the higher protein content in F2 than F1. The ratio of glutamic acid plus aspartic acid to the sum of lysine, arginine, and histidine for F1 and F2 were 1.28 and 1.33, respectively, indicating that the two fractions were all acidic proteins. This acidic property has also

been reported by Li et al. (2010) for the purified protein obtained from *L. edodes*. The total negative charge of F2 was higher than F1, so that F1 was more likely to be eluted out earlier than F2 during AEC, as observed in **Figure 4-1**. Besides, the contents of serine and threonine in the two fractions were relatively high, which might be accounted for the persistent sugar content in the purified *L. edodes* protein fractions as these two were among the most active AAs to form o-glycosidic linkages with sugar chains (Li et al., 2010).

In addition to the content and composition of AAs, the nutritional value can be evaluated through analysis of the AA composition and classify the AAs into EAAs and non-essential amino acids (NEAAs) (Li et al., 2022). As shown in **Table 4-2**, F1 contained 367 mg total amino acid (TAA) and 131 mg EAAs, while F2 contained 394 mg TAA and 145 mg EAAs (per gram total protein). All these contents were higher than previously reported (Yu et al., 2023) in the *L. edodes* mushroom 14.4-18.7% (w/w) TAAs and 4.68-6.33% EAAs. Lysine was one of the EAAs with a relatively high content in F1 and F2. Since lysine is the first limiting AA in plant grains such as rice and wheat (Anjum et al., 2005), the *L. edodes* mushroom protein may be a favorable supplement to staple grain foods for a balanced diet. As reported previously (Zhao et al., 2024), the crude protein yield extracted from the dry mushroom by TPP was 2.1%, and the yields of F1 and F2 on the crude protein were 11.3% and 28.8%, respectively (**Table 4-1**). According to the ideal AA pattern established by FAO/WHO, a protein can be considered high-quality if the ratio of EAAs to TAAs is approximately 40% and the ratio of EAAs to NEAAs is close to 60%. Furthermore, the EAAs/TAAs values for F1

and F2 were 36% and 37%, respectively, close to the ideal protein model value (40%). The World Health Organization (WHO)/Food and Agriculture Organization (FAO) suggests that a ratio with approximately 40% EAAs/TAAs is more beneficial for the human body (Li et al., 2022). The EAAs/NAAs values of 57% and 58%, respectively, are also close to the ideal protein standard (60%) (Yu et al., 2023), suggesting that the F1 and F2 proteins provide an excellent source of protein nutrition. Moreover, F2 exhibited a higher content of EAAs than F1 ($p < 0.05$), suggesting that F2 was potentially more beneficial than F1 for human health.

Table 4-2. AAs in F1 and F2 (content in mg/g protein).

EAA	F1	F2
Histidine	9.8 ± 0.3	10.6 ± 2.0
Isoleucine	18.2 ± 0.5	18.7 ± 0.1
Leucine	20.2 ± 1.1	23.1 ± 0.2
Lysine	21.6 ± 0.8	24.1 ± 0.2
Methionine	5.4 ± 0.6	7.3 ± 2.5
Phenylalanine	19.9 ± 0.4	20.4 ± 0.2
Threonine	21.8 ± 0.6	23.1 ± 0.3
Valine	14.3 ± 0.4	17.3 ± 0.03
NEAA		
Alanine	20.4 ± 1.3	21.3 ± 0.2
Asparagine	3.4 ± 0.01	3.5 ± 0.03
Aspartic acid	35.2 ± 2.6	38.9 ± 0.1
Arginine	20.3 ± 0.2	20.4 ± 0.3
Glutamic acid	30.9 ± 2.4	34.4 ± 0.3
Glycine	57.0 ± 0.1	58.9 ± 0.9
Serine	25.2 ± 1.5	26.8 ± 0.02
Tyrosine	12.5 ± 0.2	13.8 ± 0.2
Cysteine	2.3 ± 0.01	2.4 ± 0.1
Glutamine	9.8 ± 0.4	9.2 ± 0.03
Proline	19.0 ± 0.9	19.5 ± 0.6

	Crude protein	F1	F2
TAA	343.5 ± 12.8	367.3 ± 14.3	393.8 ± 8.3
EAA	113.7 ± 4.8	131.3 ± 4.7	144.6 ± 5.5
NEAA	229.8 ± 8.0	236.0 ± 9.6	249.2 ± 2.8

EAA/TAA (%)	33.1 ± 0.2	35.7 ± 0.1	36.7 ± 0.6
EAA/NEAA(%)	49.5 ± 0.4	55.6 ± 0.3	58.0 ± 1.6

Chemical evaluation is a frequently employed approach for assessing food proteins. The AA evaluation comprises a CS and an AAS. AAS was introduced by WHO and FAO in 1973 to assess the nutritional adequacy of protein in food for human consumption (FAO/WHO, 1973), which is quantified on a percentage scale, 100% as the maximum. An AA with an AAS of less than 100 is considered a limiting AA, and the AA with the lowest AAS value is referred to as the first-limiting AA. Additionally, the AA with the lowest CS is referred to as the first limiting AA. It is suggested that the AA pattern in whole egg protein closely matches human needs, and foods with higher CS values are more readily absorbed by the human body (FAO/WHO, 2007). According to the AA scoring standard model recommended by FAO/WHO and the AA pattern of egg protein. AAS and CS calculations refer to Li et al. (2022) research. AAS value and CS of protein fractions were calculated and shown in **Table 4-3**. The AAS, which represents the proportion of an EAA in a given protein sample compared to the corresponding AA in the WHO/FAO reference protein pattern, is a crucial indicator for assessing the nutritional quality of AAs in food. According to the FAO/WHO model, the full AAS score is 100 (Zhao et al., 2023). When AAS was the standard, Methionine and cysteine (Met + Cys) content was the lowest for crude-protein, F1, and F2. The first limiting AA was Met + Cys, and the second limiting AA was leucine for both F1 and F2, which were similar to those observed in the *L. edodes* fungus previously (Yu et al., 2023; Wang et al., 2023).

The AA composition of egg protein has been recommended as a good reference for human consumption and is also frequently utilized as a reference in research studies (Kayode et al., 2015). The CS was represented in the percentage of the EAA content relative to the EAA in the standard whole egg protein (Li et al., 2022). Compared to the egg protein standard, the EAA balance and content of crude-protein, F1, and F2 were slightly lower and may serve as a high-quality protein resource and can be utilized as a supplementary ingredient to complement the nutritional profile of other foods. Besides, the threonine values were close to the AA value in eggs for both crude-protein, F1, and F2. According to the AAS and CS scores, F2 consistently exhibited higher values than F1, indicating that F2 is more nutritionally beneficial.

Furthermore, the AA ratio coefficient method assesses food protein by computing the RAA, RC, and SRC values in the sample. If the RC value exceeds 1, it indicates a relative excess of the AA in the food. Conversely, if the RC value is less than 1, it suggests a relative insufficiency of the AA in the food. SRC indicates the protein relative nutritional value and the discrete degree of RC. A higher SRC signifies a lower dispersion of the AA composition in food, a more balanced ratio, and an increased protein utilization rate (Li et al., 2022). RAA, RC, and SRC are calculated according to the method mentioned by Xu et al. (2022) and Zhao et al. (2023). The RAA and RC values were further evaluated based on the EAA score of crude-protein, F1, and F2 (as shown in **Table 4-3**). According to the RC value, Met + Cys was identified as the first limiting AA for crude-protein, F1, and F2, and leucine the second limiting AA. Isoleucine and lysine were relatively deficient, and Phe + Tyr and

threonine were relatively excess. According to the protein complementation theory, eating should be appropriately done by mixing with other proteins to maximize the nutritional value of mushroom protein effectively. The higher the SRC value, the higher the nutritional value of the protein. In this study, crude-protein F1 had an SRC value of 67 % and F2 72%, which were quite close to soy protein (72.60%), milk (67.31%), and walnut (62.65%) (Wang et al., 2023). This further suggested that both samples had high nutritional value, with F2 having a higher nutritional value than F1.

Table 4-3. AA, chemical scores, RAA, RC, and SRC of EAAs for crude protein, F1, and F2.

	Crude protein (AAS%)	F1 (AAS%)	F2 (AAS %)	Crude protein (CS%)	F1 (CS%)	F2 (CS%)
Isoleucine	32.5 ± 1.3	45.5 ± 1.3	46.8 ± 0.3	24.1 ± 0.9	33.7 ± 0.9	34.6 ± 0.2
Leucine	30.9 ± 1.3	28.9 ± 1.6	33.0 ± 0.3	25.1 ± 1.0	23.5 ± 1.3	26.9 ± 0.2
Lysine	32.7 ± 0.9	39.4 ± 1.5	43.8 ± 0.4	25.7 ± 0.7	31.0 ± 1.2	34.4 ± 0.3
Methionine + cysteine	12.4 ± 0.9	22.0 ± 1.7	27.8 ± 5.3	7.6 ± 0.6	13.5 ± 1.1	17.1 ± 3.2
Phenylalanine + tyrosine	39.5 ± 1.8	54.0 ± 1.0	57.0 ± 0.7	25.5 ± 1.2	34.8 ± 0.6	36.8 ± 0.4
Threonine	55.3 ± 1.5	54.5 ± 1.5	57.8 ± 0.8	47.0 ± 1.3	46.4 ± 1.3	49.1 ± 0.6
Valine	34.0 ± 1.2	29.1 ± 1.6	34.6 ± 0.1	25.8 ± 0.9	22.0 ± 1.2	26.2 ± 0.1
	Crude protein (RAA)	F1 （RAA）	F2 (RAA)	Crude protein (RC)	F1 (RC)	F2 (RC)
Isoleucine	0.33 ± 0.01	0.46 ± 0.01	0.47 ± 0.00	0.96 ± 0.00	1.17 ± 0.01	1.09 ± 0.03
Leucine	0.31 ± 0.01	0.29 ± 0.02	0.33 ± 0.00	0.91 ± 0.00	0.74 ± 0.01	0.77 ± 0.02
Lysine	0.33 ± 0.01	0.39 ± 0.02	0.44 ± 0.00	0.97 ± 0.01	1.01 ± 0.01	1.02 ± 0.02
Methionine + cysteine	0.12 ± 0.01	0.22 ± 0.02	0.28 ± 0.05	0.37 ± 0.01	0.56 ± 0.02	0.64 ± 0.15

Phenylalanine + tyrosine	0.40 ± 0.02	0.54 ± 0.01	0.57 ± 0.01	1.17 ± 0.01	1.38 ± 0.03	1.33 ± 0.03
Threonine	0.55 ± 0.02	0.55 ± 0.04	0.58 ± 0.01	1.63 ± 0.02	1.40 ± 0.01	1.34 ± 0.03
Valine	0.34 ± 0.01	0.29 ± 0.02	0.35 ± 0.00	1.00 ± 0.00	0.74 ± 0.02	0.81 ± 0.02

	Crude protein	F1	F2
SRC	62.6 ± 0.6%	66.9 ± 1.0%	72.2 ± 4.0%

4.3.4 Contents of taste-active amino acids

The study also involves an analysis of the taste-active AA contents and their proportions in relation to TAAs in the F1 and F2, following the methods described in the study by Yu et al. (2023). AAs can be categorized based on their taste characteristics into four groups: umami (aspartic acid and glutamic acid), sweetness (serine, alanine, glycine, and threonine), bitterness AAs (arginine, histidine, tyrosine, leucine, valine, methionine, isoleucine, phenylalanine, lysine, and proline), and tasteless (cysteine) (Nishimura & Kato, 1988; Huang et al., 2022; Yu et al., 2023). **Table 4-4** displays the contents of flavored AAs in crude protein, F1, and F2. In all three protein fractions, the relative contents of taste-active AAs followed the order of bitter > sweet > umami > tasteless. However, the bitter, sweet, and umami AA levels in F2 were all higher than in F1 and crude protein. Similarly, a previous study revealed that the mycelium of *L. edodes* strain 18N44 exhibited a relative content of taste AAs in the following sequence: bitter > sweet > umami (Yu et al., 2023).

Table 4-4. Flavored AA content (mg/g protein) in crude protein, F1 and F2.

AA		Crude protein	F1	F2
Umami AAs	Content	81.5 ± 3.7	66.1 ± 5.0	73.3 ± 0.4

Sweet AAs	Content	129.0 ± 2.3	124.4 ± 3.2	130.1 ± 1.2
Bitterness AAs	Content	131.8 ± 4.1	161.3 ± 4.9	175.2 ± 5.5
Tasteless AAs	Content	2.25 ± 0.2	2.3 ± 0.0	2.4 ± 0.1

4.3.5 Immunostimulatory activities of purified protein fractions

PS are widely recognized as the chief bioactive component of edible and medicinal fungi and have been explored in many previous studies (Yin et al., 2021). One of the most notable bioactivities of the fungal PS is immunomodulatory activity as reported in the literature (Li et al., 2023; Janardhanan et al., 2024). Proteins represent another major class of biomacromolecules in edible fungi, some of which have also shown immunological function (Xu et al., 2023; Janardhanan et al., 2024), but have only been sparely documented. In a recent study from our group (Zhao et al., 2024), protein exhibited more potent immunostimulatory activity than PS fractions from *L. edodes* mushroom through TPP extraction. A reasonable explanation is that the protein molecules may contain bioactive peptides or AA sequences that can interact with the immune cells to regulate the immune responses (Ahmed et al., 2024). Motivated by the significant immunostimulatory activity observed in the crude protein, the present study was carried out to investigate the beneficial functions of proteins further. As shown in **Figure 4-6A**, the crude protein and the mixture M1 (PS + F1 + F2) exhibited slight inhibitory effects on the cell viability, while the F1, F2, and M2 (F1 + F2) had negligible effects on the RAW 264.7 cells at concentrations up to 4 μg/mL. The production of NO is a common response of immune cells, which contributes to the activation of the immune system and the defense against pathogenic invasions. As depicted in **Figure 4-6B**, the level of NO in RAW264.7 cell culture was significantly increased with the

treatment of all protein fractions crude protein, F1, and F2 and their mixtures M1 and M2. Crude protein and M1 had higher stimulatory activity than the two purified protein fractions F1 and F2 ($p < 0.05$); M2 and F2 had similar stimulatory activity and higher than PS ($p < 0.05$). Although the PS alone had a lower immunostimulatory, its mixture M1 with the two purified protein fractions showed higher activity, similar to crude protein, suggesting a synergistic effect of these PS and protein components in the crude protein into much higher immunostimulatory activity. An appropriate explanation is that PS may conjugate with proteins to form complexes, thereby improving stability and facilitating recognition by the immune cells. The carbohydrate components within these complexes can offer supplementary binding sites for receptors, such as Toll-like receptors (TLRs), which can specifically bind to the carbohydrate moieties of the complexes (Trinchieri & Sher, 2007). Meanwhile, proteins can also improve the specificity and effectiveness of immune activation by directly interacting with immune receptors such as TLR4, TLR2, cluster of differentiation (CD)14, CD91, and CD94 (Portnoy et al., 2016). As a result, the presence of both PS and protein components enables multi-target binding, leading to a more effective receptor activation and downstream signaling. It is well-known that PS-protein complexes of edible and medicinal fungi have notable immunostimulatory activities (Ooi & Liu, 2012; Zhang et al., 2014). It has also shown that the immunomodulation and some other biological activities of proteins were improved after binding with PS (Zhang et al., 2014). The missing α-helix structure in the two purified protein fractions might affect their immunological activity (Liu et al., 2020).

The phagocytic activity of macrophages is another useful indicator of immunomodulatory effects. As shown in **Figure 4-6C**, compared with the control group, all the protein fractions significantly enhanced the uptake of neutral red of RAW 264.7 cells, indicating their stimulating effects on macrophage phagocytosis or pinocytosis. Specifically, the pinocytic activity of the crude protein and M1 sample was also higher than that of the two purified fractions F1 and F2 as for the NO production. Similar to pinocytic activity, all protein fractions enhanced the production of ROS in the macrophage cells, with crude protein and M1 being the most active (**Figure 4-6D**). During phagocytosis, macrophage cells can generate ROS as defense elements and signal compounds in the immune pathways (Kohchi et al., 2009). Furthermore, 100 and 114 proteins were discovered for F1 and F2 fractions, respectively, through the LC-MS analysis and Mascot search. The details of these two fractions were provided in the supplementary Excel data file. With a Mascot score value above 70 ($p < 0.05$) and a number of significant matches, the relatively highly matched proteins in F1 mainly included heat shock protein 9, uncharacterized protein, 14-3-3 protein, heat shock protein HSS1and uncharacterized protein **(Table 4-5)**. The relatively highly matched proteins in F2 included heat shock protein 9, uncharacterized protein, heat shock protein HSS1, 14-3-3 protein, ubiquitin-like domain-containing protein, uncharacterized protein, lea domain-containing protein, lea domain protein and small heat shock protein **(Table 4-5)**. F2 showed a relatively strong correlation with heat shock protein HSS1 (a member of the heat shock protein 70 family) compared to F1, which has been reported to have immunostimulatory activity (Zininga et al., 2018). F2 was more likely to

contain a small heat shock protein compared to F1. Furthermore, it was discovered that small heat shock proteins also have immunomodulatory activity (Zininga et al., 2018). Therefore, F2 had slightly higher immunostimulatory activity than F1. Overall, the results confirmed the *in vitro* immunostimulatory activity of the protein fractions, which varied with the chemical composition and protein, and F2 had a slightly higher activity than F1, probably due to its higher protein content.

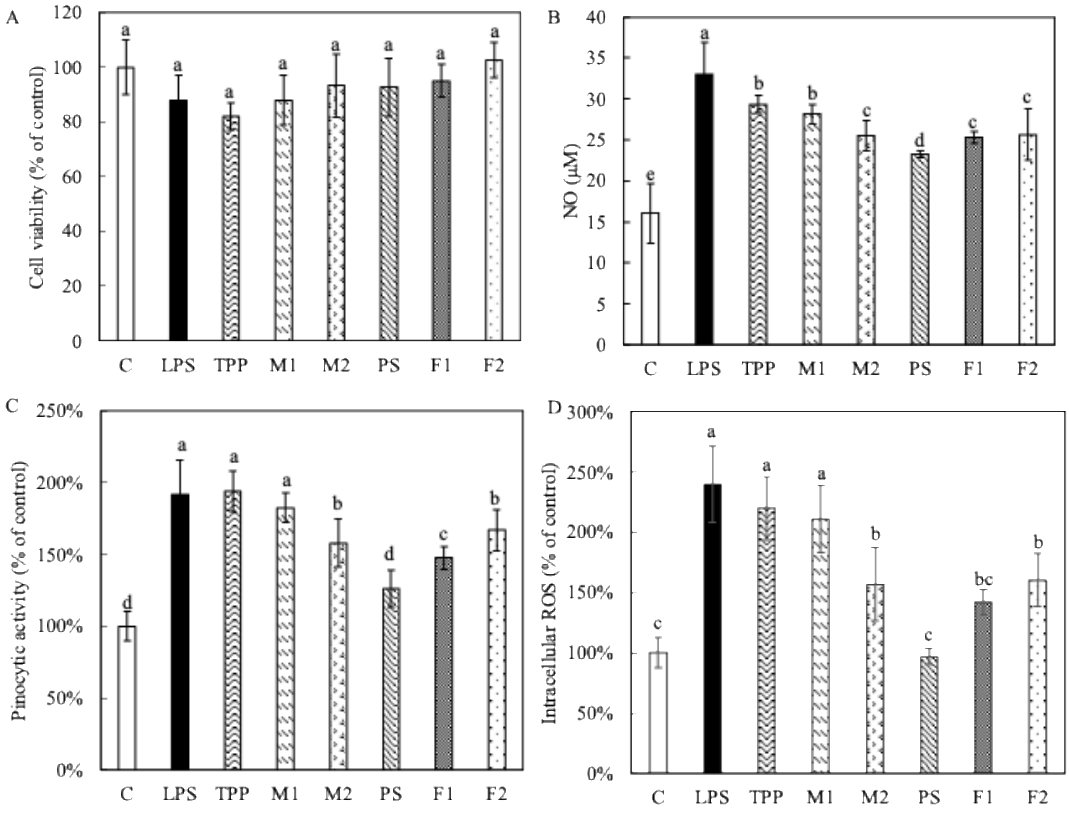

Figure 4-6. Immunostimulatory activities of crude protein, mixture, PS, and purified crude protein samples on RAW 264.7 cells. (A) Macrophage proliferation activity determined by MTS method; (B) NO level; (C) Phagocytic activity measured by neutral red uptake; (D) intracellular ROS generation. LPS (200 ng/mL) was used as a positive control. Different letters a, b…d indicate significant difference ($p < 0.05$); TPP: crude

protein; M1 (PS + F1 + F2 at 1:2:5) and M2 (F1 + F2 at 1:2.5), F1: F1 and F2: F2 at 4 μg/mL).

Table 4-5. LC-MS analysis identified that F1 and F2 mainly consisted of a specific type of protein.

Sample	Type of protein	Uniport	The primary type of protein	MW (Da)
F1	100 types	A0A1Q3E1W0	heat shock protein 9	9,120
		A0A1Q3E0W6	14-3-3 protein	35,999
		A0A1Q3EMH0	uncharacterized protein	34,967
		A0A1Q3EMJ0	heat shock protein HSS1	71,089
		A0A1Q3DVR1	uncharacterized protein	96,368
F2	114 types	A0A1Q3E1W0	heat shock protein 9	9,120
		A0A1Q3DVR1	uncharacterized protein	96,368
		A0A1Q3EMJ0	heat shock protein HSS1	71,089
		A0A1Q3E0W6	14-3-3 protein	35,999
		A0A0D0BMC9	Ubiquitin-like domain-containing protein	18,076
		A0A6A4HQ74	Uncharacterized protein	26,440
		A0A1Q3EMH2	Lea domain-containing protein	26,507
		A0A1Q3ECQ8	Lea domain protein	24,831
		A0A1Q3DVU7	Small heat shock protein	17,436

4.3.6 Analysis of the immunomodulatory pathway for two purified fractions

It is well established that the toll-like receptors (TLR) family is a crucial membrane receptor involved in macrophage activation, and TLR4 mediates the production of protein-induced cytokines such as TNF-α (Ru et al., 2021). As shown in **Figure 4-7,**

F1, and F2, as well as LPS (0.2 μg/mL), effectively activated the NF-κB pathway and released pro-inflammatory TNF-α. The method for improving the yield of protein fractions should be further investigated, along with exploring their wider applications. Immunofluorescence staining analysis was used to examine the expression of TLR4 in RAW264.7 macrophage cells, and the results (**Figure 4-8**) revealed a significant increase in the TLR4 expression by F1 and F2 compared to the control group. Another assay showed a significant expression in the macrophages stimulated by LPS and purified protein fractions (**Figure 4-8B**). Therefore, these results, together with the stimulated TNF-α (**Figure 4-7**), suggested that the TL-R4 receptor-mediated NF-κB pathway was involved in the induction of immune responses in the RAW264.7 macrophage cells by the protein fractions (F1 and F2) (Yusi et al., 2020).

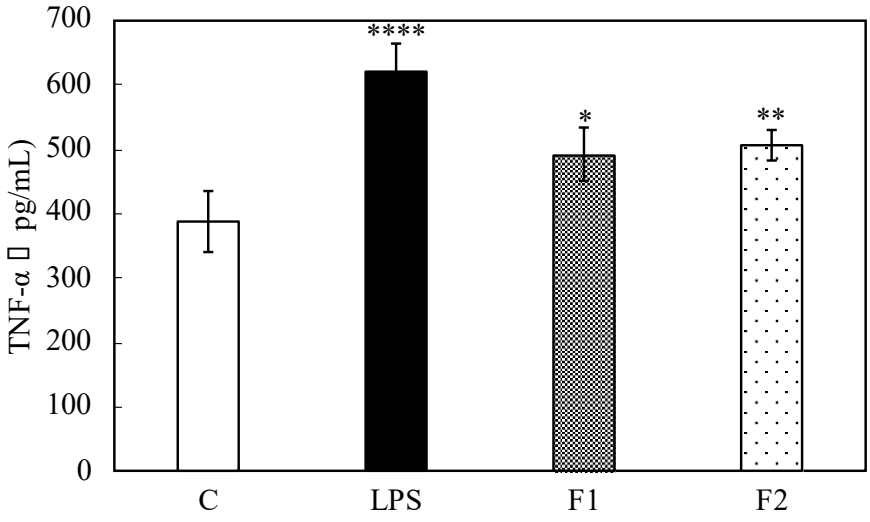

Figure 4-7. Effects of F1 and F2 immunostimulatory cytokine responses TNF-α on Raw246.7. *, **, and **** indicated statistically significant differences from the control group at $p < 0.05$, $p < 0.01$, and $p < 0.0001$, respectively.

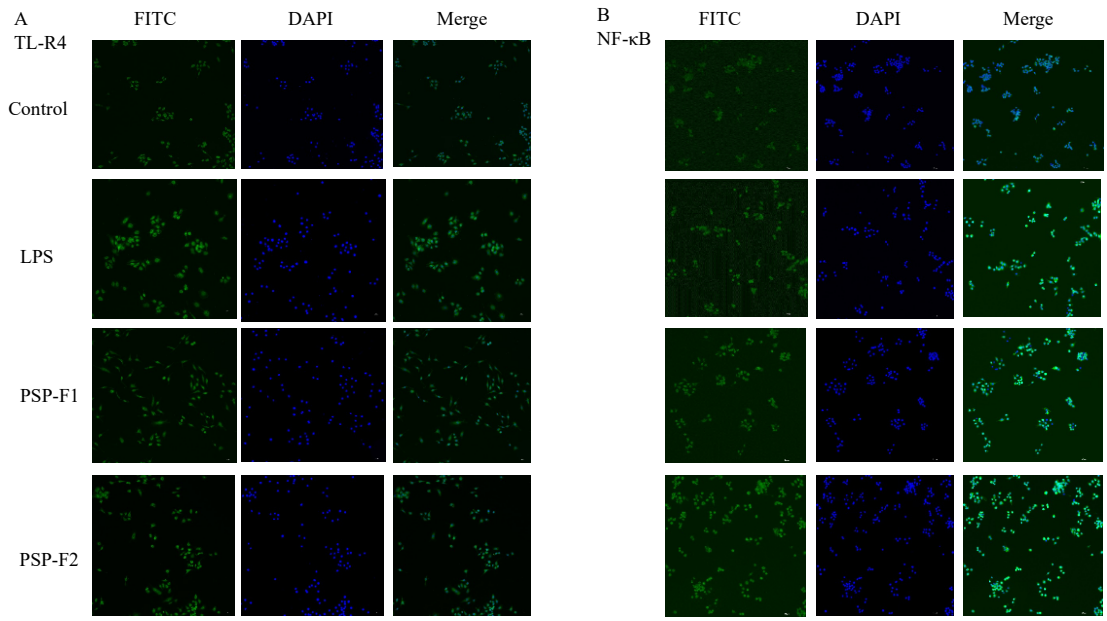

Figure 4-8. Immunofluorescence staining of RAW 264.7 macrophages grown for 24 h in standard culture medium in the presence of LPS, F1, and F2. The cells were stained with DAPI and Fluorescein (FITC) (green).

4.4 Conclusions

Through anion exchange chromatographic fractionation, two purified protein fractions, F1 and F2, were isolated from the crude protein fraction extracted from *L. edodes* mushroom. Both F1 and F2 exhibited β-sheet and random coil structures and contained essential and non-essential AAs. Compared with F1, F2 had a broader MW distribution from below 10 kDa to 95 kDa, and higher contents of total protein and EAAs, and also a higher immunostimulatory activity. In general, the purification process enriched not only the protein content but also the EAAs. However, the separated and protein purified fractions showed lower immunostimulatory activity than their combined mixture and the crude protein, which may suggest the synergistic action of different components to a higher activity. However, further research is necessary to

understand the PS-protein complex structure and its immunostimulatory relationship. The results from the present study show the valuable potential of protein fractions extracted from the *L. edodes* mushroom as a dietary supplement for AA nutrition and immunomodulatory function. Further research is still needed also worthwhile to develop and employ an effective purification strategy for the mushroom proteins, to attain homogeneous protein fractions with a high purity. To elucidate the specific protein classes responsible for the observed immunostimulatory activity, a targeted investigation will be conducted to identify the active immunomodulatory proteins. With these and the application of specialized techniques, we shall be able to determine their structures, and immunoactivity, and to elucidate more structure-activity relationships and mechanisms of action.

Chapter 5 Sequential Enzyme and Ultrasonic Extraction of *Lentinula edodes* Proteins leading to Enhanced Yield and Significant Immunoactivity

5.1 Introduction

Lentinula edodes is one of the more popular edible mushrooms and is especially favored in Asia for its appealing aroma, nutritional, and medicinal properties. Among the most abundant and beneficial components are polysaccharides (PS) and proteins (Wasser, 2004; Du et al., 2024). The PS derived from *L. edodes* have been extensively reported in the literature on their notable antitumor, and immunomodulatory effects and many other bioactivities (Wang et al., 2022). Proteins represent another important component of *L. edodes* are gaining attention for their nutritional and medicinal functions (Wong et al., 2010; Das & Prakash, 2022). The *L. edodes* proteins have shown abundant essential amino acids (EAA) (Yu et al., 2023) and multiple biological functions, such as immunomodulatory, antitumor and antioxidant activities (Ngai & Ng, 2003; Gao et al., 2023). With these beneficial functions, the *L. edodes* proteins are promising candidates for applications in the food industry as functional food ingredients. Their essential amino acid profile and high digestibility suggest that they could be used as a meat substitute (Ayimbila & Keawsompong, 2023). Additionally, they are useful for enriching plant-based foods and combating protein malnutrition in vegetarian and vegan diets (Contato & Conte-Junior, 2025). Mushroom proteins can also be utilized in nutraceutical products for improving human health (Ayimbila & Keawsompong, 2023). Our recent research has shown that the protein-rich fractions

from *L. edodes* have stronger immunostimulatory activity compared to the PS-rich extracts (Zhao et al., 2024) and also showed high nutritional value of AA (Zhao et al., 2024).

The extraction process is a crucial step for acquiring the desired proteins from mushrooms and other food and medicinal materials. Extraction with water is the most common method for extracting proteins from mushrooms (Zhao et al., 2021) or with aqueous solutions containing salt (NaCl) or surfactants (SDS, Triton100) for higher protein solubility (Chatterjee et al., 2012). Alkaline or acid extraction may be more effective for the proteins with high solubility in the respective pH (Du et al., 2018; Gerliani et al., 2019). After the extraction process, an organic solvent, typically ethanol or methanol, is usually used to precipitate and isolate the proteins from prolamin, urea, phenol, and other denaturing agents (Chatterjee et al., 2012; Capellini et al., 2017; Chen et al., 2019; Bose et al., 2019). However, the aforementioned extraction and separation methods are rather tedious and time-consuming, and environmentally unfriendly.

Enzyme-assisted extraction (EAE) and ultrasound-assisted extraction (UAE) are two of the most widely used methods for improving the water extraction of natural substances from diverse origins (Goktayoglu et al., 2023; Cannavacciuolo et al., 2024). It can be operated at a lower temperature than hot-water extraction, favorable for extraction of heat-sensitive components such as proteins. EAE and UAE have also been considered efficient in enhancing protein extraction from various mushrooms (Prandi et al. 2023; Ahmed et al. 2024; Ketemepi et al. 2024). It is well established that high-intensity ultrasound enhances water extraction mainly through acoustic cavitation in

water, which produces strong shear forces, causing cell wall disruption and promoting the release of cellular components (Wu, 2019). A recent study has shown that the use of UAE with high-intensity ultrasound at 50 °C significantly increased the protein extraction rate from *L. edodes* mushrooms (Wang et al., 2023). However, the strong shear forces generated by high-intensive ultrasound may also induce adverse effects on the integrity and quality of protein extracts (Higuera-Barraza et al., 2016). In contrast, EAE utilizes specific enzymes like cellulases to selectively degrade cellulose and other glycan components of the cell walls, keeping the proteins intact (Culter, 2008). The complex structure of the fungal cell wall mainly contains proteins, glucan, chitin, as well as cellulose (Rivillas-Acevedo & Soriano-García, 2006), necessitates enzymatic degradation for efficient extraction of intracellular bioactive components. Enzymes including cellulase, papain, and pectinase can help break down cell walls (Zhao et al., 2016), thereby aiding in the release of intracellular proteins. Previous studies have established the efficacy of cellulase-assisted extraction for mushroom protein recovery. For example, Zhang et al. (2015) showed enhanced yields from *Pleurotus eryngii* by EAE. Xu et al. (2020) further improved the protein extraction through a sequential UAE-EAE process, though the optimal sequence of the two extraction methods remains underexplored. On the other hand, while individual EAE and UAE methods have been separately to *L. edodes* (Wang et al., 2023; Prandi et al., 2023), their combined use remains to be explored. EAE using *B. licheniformis* protease or papain achieved 24% and 23% yields respectively for wood-grown *L. edodes*, albeit producing peptide fragments rather than intact proteins (Prandi et al., 2023). Comparative assessment of

protein extraction methods for *L. edodes* stems has shown notable yield variations, alkaline extraction (13.45%), cellulase-assisted EAE (18.57%), and UAE (22.44%) (Zhang, 2017). While UAE alone has shown high efficiency as a standalone method, these results suggest potential for further yield enhancement through combined extraction strategies.

Based on the above background, we hypothesize that the proper combination of EAE and UAE may ultimately enhance the protein extraction through a biochemical mechanism (by enzyme) and a physical or mechanical mechanism (by ultrasound). This study aimed to identify and optimize the most efficient protein extraction scheme and conditions based on a combination of EAE and UAE. This investigation applied a systematic approach combining statistical experimental design and response surface methodology (RSM) to develop an optimized procedure for combined extraction processes. Further efforts were made to elucidate the mechanistic interplay between enzymatic and physical disruption techniques and characterize the structural and immunomodulatory properties of the extracted proteins. These findings lay the groundwork for scalable process optimization in the production of bioactive proteins.

5.2 Materials and methods

The majority of the chemical and biochemical reagents used in this study are described in section 2.1.

Molecular weight (MW) (SDS-PAGE analysis), Fourier transform infrared spectroscopy (FT-IR) spectra, and AA composition of the protein samples extracted using EUE were analyzed, as detailed in section 2.4. Furthermore, the nutritional

evaluation of the optimized EUE protein samples was carried out as described in section 2.4. The secondary structures of proteins extracted using WE, UAE, EAE, EUE, and optimized EUE methods were analyzed through Circular dichroism (CD) spectra, with the experimental method for CD detailed in section 2.4.

As previously reported, RAW 264.7 cell culture was used to evaluate the immunomodulatory activities of protein fractions. According to the previous study, the concentration of all protein fractions was set at 4 μg/mL, and LPS (200 ng/mL) was chosen as a positive control. Experiments including macrophage cell proliferation, nitric oxide (NO) generation, and phagocytic activity were initially conducted on protein extract samples from WE, EAE, UAE, and EUE to pinpoint the optimized protein exhibiting both high yield, and biological activities. The optimized EUE-extracted protein immunostimulatory activity was evaluated by cell viability, NO, phagocytic activity, and reactive oxygen species (ROS) analysis. The experiment procedure of cell viability, NO, phagocytic activity, and ROS analysis was described in section 2.5.

5.2.1 Protein extraction

The mushroom defatting procedure was carried out as described in Section 2.2. To identify the most effective extraction method, several methods were compared initially, including WE, UAE, EAE, and various combinations of EAE and UAE as described in detail below.

5.2.1.1 Water extraction (WE)

A preliminary experiment was conducted using the conventional water extraction (WE) method with alkaline and acid precipitation. Three grams of dry, defatted mushroom powder were dispersed in an aqueous solution at a 1:30 (w/v) ratio and macerated for 30 min. The pH was raised to 10 by adding 1 M NaOH (4-6 drops), and the mixture was agitated for 3 h at 50 °C. It was then centrifuged for 30 min at 4000 rpm and 4 °C. The supernatant was collected for protein precipitation by adjusting the pH to 4 with 1 M HCl (4-6 drops) and allowing it to stand overnight at 4 °C (selected based on its isoelectric point). The alkaline pH 10 maximizes protein solubility through enhanced ionization of functional groups, as demonstrated in previous studies, while avoiding excessive alkalinity (pH > 10) that could cause *L.edodes* protein degradation (Hu, 2019). The pH was then adjusted to 4 to facilitate protein precipitation, as this value approaches the isoelectric point of the target proteins. The subsequent procedure was followed in the optimized WE method described. Nevertheless, the protein yield obtained by this method remained low (3.5 ± 0.7%). As a result, the optimized WE method incorporating $(NH_4)_2SO_4$ and isoelectric precipitation was chosen. The WE procedure and conditions were selected based on the literature (Yang et al., 2023). The dried defatted mushroom powder (3 g) was dispersed into an aqueous solution of 1% (w/v) NaCl at 1:30 (w/v) and macerated for 30 min (in plastic centrifuge tubes). To increase the pH of the mixture to 10, 1 M NaOH was put into the samples. Then, the mixture was agitated for three h at 50 °C and centrifuged (30 min, 4000 rpm, 4 °C). The supernatant underwent protein precipitation by initially adjusting the pH to 4.0 using 1

M HCl. Subsequently, $(NH_4)_2SO_4$ was added to achieve 70% saturation (47.77 g/100 mL at 25 °C, as calculated with the ammonium sulfate calculator from EnCor Biotechnology Inc., Florida, USA). The mixture was then allowed to stand overnight at 4 °C. The liquid was centrifuged at 9000 rpm for 20 min, and the resulting pellet was gathered and dissolved in water. The liquid was centrifuged again (4000 rpm, 30 min, 4 °C) to attain a solid-free solution of crude protein. To remove salts, the protein solution was dialyzed in distilled water for 48 h by a 3.5 kDa molecular weight (MW) cutoff membrane. The crude protein fraction was then obtained by freeze-drying.

The protein yield (%) was represented by the mass percentage of freeze-dried soluble protein in the original mushroom powder. Lowry method was applied to the calculation of the total protein yield, and the Anthrone test, as outlined in an earlier study (Siu et al., 2016), was applied to measure the total sugar carbohydrate.

5.2.1.2 Ultrasound-assisted extraction (UAE)

The dried defatted mushroom powder (3 g) was pre-treated using the same method as outlined in the WE method, followed by UAE. UAE was performed using a probe-type ultrasonic processor (VCX 750, Sonics & Materials, Inc., USA) with a constant frequency of 20 kHz and a maximum power of 750 W, following established procedures with some adjustments (Cheung et al., 2012). In brief, a 13 mm-diameter ultrasonic horn was submerged into the liquid samples. Ice was employed during the UAE process to prevent overheating. The US amplitude was maintained at 60% (0.87 W/mL), and the ultrasonic process was conducted for 40 min. After UAE, the solid-liquid mixture was spun at 4000 rpm for thirty min. The collected liquid underwent

protein fraction isolation and partial purification, following the WE method as detailed in section 5.2.1.1 Water extraction (WE).

5.2.1.3 Enzyme-assisted extraction

Cellulase was chosen as the extracting enzyme for its well-known effectiveness in enhancing the extraction of mushrooms and other organisms (Fernandes, 2018). The enzyme-assisted extraction procedure was adapted from Xu et al. (2020). As for the EAE method, the dried defatted mushroom powder (3 g) was macerated for thirty min as well as the pH of the liquid was adjusted to 4.5 by adding 1 M HCl (the optimal pH for the enzyme). Cellulase (50 U/mg) was gradually introduced into the liquid at a dosage of 0.3% (enzyme-to-substrate mass ratio), and EAE was carried out at 45 °C for 1 h using a Shuxian HH-4 thermostatic water bath (Changzhou Jintan Chenyang electronic instrument factory, China) to ensure precise temperature control during enzymatic hydrolysis. The extraction process was stopped by briefly heating the sample solution at 100 °C for 10 min. Afterwards, the blend was cooled to ambient temperature. The solution pH was increased to 10 by 1 M NaOH. The sample was centrifuged for 30 min at 4000 rpm at 4 °C. The collected supernatant was utilized for protein isolation following the previously outlined procedure (5.2.1.1 Water extraction (WE)).

5.2.1.4 Combined EAE and UAE extraction

Three schemes of combined EAE and UAE extraction were initially tested including EUE (EAE followed by UAE), UEE (UAE followed by EAE), and SEUE (simultaneous use of EAE and UAE). **Table 5.1** shows the specific procedures and conditions for the combined extraction schemes. With all the extraction schemes, 3

grams of defatted mushroom powder were treated according to the procedure outlined for the WE method, and the supernatants obtained from these three schemes were applied for protein isolation and purification as for the WE method.

Table 5-1. Combined enzymatic and ultrasound-assisted extraction schemes and conditions.

Scheme	Procedure and conditions
EUE	Step 1: pH 4.5, cellulase 0.3% (enzyme-to-substrate mass ratio) and 45 °C for 1 h; Step 2: US amplitude: 60%, pH 10 for 40 min
UEE	Step 1: US amplitude: 60%, pH 10 for 40 min; Step 2: pH 4.5, cellulase 0.3% (enzyme-to-substrate mass ratio) and 45 °C for 1 h
SEUE	SEUE1: pH 4.5, cellulase 0.3% (enzyme-to-substrate mass ratio) under ultrasonication at 60% amplitude for 40 min; SEUE2: pH 10, cellulase 0.3% (w/v) under US amplitude at 60% for 40 min

5.2.2 Optimization of EUE extraction

Preliminary experiment results showed that the two-step sequential EUE method was the most effective in order to separate and extract the protein from the *L. edodes* (**Figure 5-1**). Therefore, the following extraction experiments were all conducted in two separate steps, enzyme using cellulase during the enzyme treatment. The major process factors, including enzyme content, enzyme treatment time, UAE treatment time, US amplitude percentage, and $(NH_4)_2SO_4$ saturation concentration, were evaluated for their effects on protein extraction yield. **Figure 5-2** presents a flowchart for the EUE extraction scheme, including all major experimental steps starting from the raw mushroom, including the major steps EAE, and UAE and the possible extraction mechanisms.

Cellulase enzyme (50 U/mg), within the concentration range of 0.2-1.0% (enzyme-

to-substrate mass ratio), was gradually introduced into the mushroom extract solution (pH 4.5) within a plastic centrifuge tube. The mixture was then extracted at 45°C for 30 to 120 min. The EAE was ceased by heating at 100°C (10 min). Once samples cooled, its pH was increased to 10 by 1 M NaOH. The treatment was further extended with UAE by adjusting the ultrasound amplitude to 20-100% (0.29-1.44 W/mL) for a duration of 20-60 min. After centrifuging for thirty min at 4000 rpm, the supernatant of samples was collected for protein precipitation by adding $(NH_4)_2SO_4$ to reach 20-100% saturation and allowing it to precipitate overnight at 4 °C. The following procedure was performed for partial purification and recovery of the protein fraction as for the WE method.

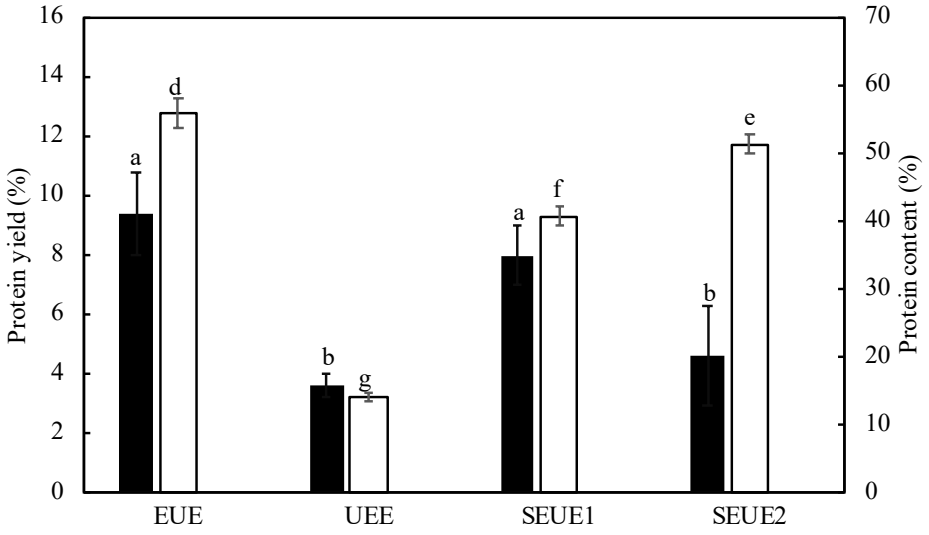

Figure 5-1. Comparison of protein yields, and protein content attained by different schemes of combined EAE and UAE. Data that lack the same letter indicates a significant difference.

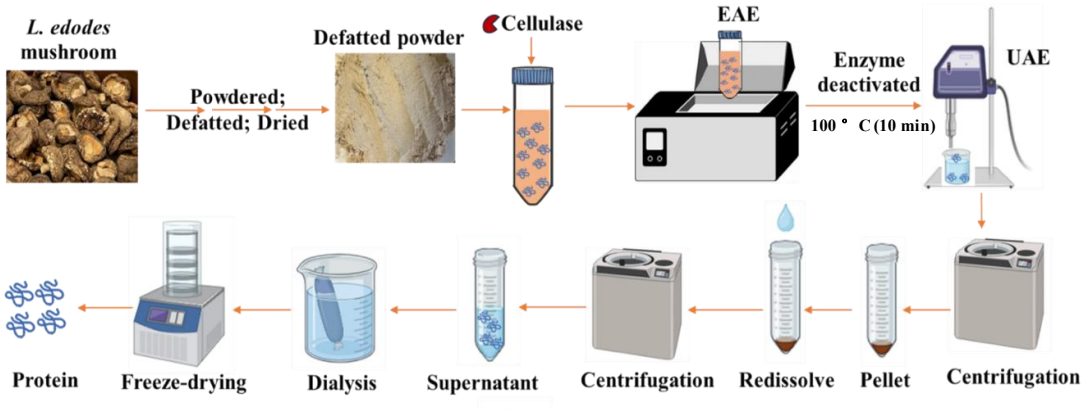

Figure 5-2. Process flow diagram for enzyme- and ultrasound-assisted extraction of proteins from mushrooms.

5.2.3 Statistical experiment design for EUE optimization by RSM

According to the results of the optimization of the EUE experiments mentioned above, a 3-central point definitive screening experimental design was selected. RSM was used to improve the protein extraction conditions utilizing a 3^3, three-factor, three-level factorial Box-Behnken design (BBD). This design created seven experimental runs, including 5 replicates at the central points and 12 factorial points. The choice of experimental factors and their respective levels was guided by the prior experiments (**Table 5-2**), with enzyme concentration (% w/v, X_1), ultrasound amplitude (US) (%, X_2), and $(NH_4)_2SO_4$ concentration (%, X_3) serving as the independent variables. The protein yield and content were chosen as the two response values. The response surface analysis was performed based on the Box-Behnken design (BBD) response surface design. The RSM was designed by the Design-Expert 11 software program (Stat-Ease, Inc., Minneapolis, USA). Analysis of Variance (ANOVA) was conducted to perform the statistical analysis, used to determine the optimal conditions for EUE extraction.

Table 5-2. Factors and levels used in the optimization of EUE.

Factor	Coded factor levels		
	-1	0	1
X_1 Enzyme amount (%)	0.1	0.3	0.5
X_2 US amplitude (%)	40	60	80
X_3 $(NH_4)_2SO_4$ saturation (%)	50	70	90

5.3 Results and discussion

5.3.1 Comparison of protein yield and content from various extraction methods

Table 5-3 is a summary of the crude protein yields by various extraction methods. When the UAE process was applied alone, the protein content was the lowest (41%) compared to the other methods. Compared to UAE, protein extraction using the WE extraction method with a NaCl solution and precipitation with $(NH_4)_2SO_4$ led to higher yields (6.5%) and content (47%) ($p < 0.05$). This may be caused by the ability of salt ions to inhibit electrostatic protein-protein interactions, consequently improving the extractability (Yang, et al., 2023). Cellulase is a highly effective biocatalyst for degrading carbohydrate fiber and cell walls, to facilitate protein extraction. In the single-step enzyme-assisted extraction EAE process, the protein yield was 4.9% and the protein content was 50% (**Table 5-3**). In comparison, the two-step EUE process resulted in a higher protein yield (9.4%) and content (56%).

Table 5-3. Protein yields, and contents by various separation methods.

	Protein yield (%)*	Total protein content (%)	Total sugar content (%)
WE	6.5 ± 1.1^{ab}	47.3 ± 1.0^{f}	43.1 ± 2.5^{l}
UAE	6.9 ± 1.5^{ab}	40.9 ± 3.8^{g}	51.4 ± 2.4^{k}

EAE	4.9 ± 1.3^{b}	50.2 ± 1.7^{ef}	45.8 ± 3.7^{kl}
EUE	9.4 ± 1.4^{a}	56.0 ± 2.2^{e}	38.7 ± 2.0^{l}

*. Equal to the mass of freeze-dried soluble protein divided by the mass of original mushroom powder; Different letters a, b…l indicating significant difference ($p < 0.05$).

Two alternative process schemes were compared with the EUE, UAE, followed by EAE (UEE) and simultaneous enzymatic and SEUE (concurrent application of EAE and UAE), both resulting in lower protein yield and content (**Figure 5-1**). The lower extraction efficiency of UEE than EUE suggests that the mechanical effect of ultrasound is more effective when the cell walls are partially disrupted by the enzyme cellulase, but less effective when the cell walls are intact. In the UEE method (enzyme extraction after ultrasound extraction), intact cell walls likely resist disruption due to their dense, unmodified structure. In contrast, the EUE method employs enzymatic pretreatment to weaken the cell wall, making it more susceptible to cavitation-induced fracture during ultrasound treatment (**Figure 5-3**). Specifically, cellulase breaks down cell walls by hydrolyzing β-1,4-glycosidic bonds in cellulose, degrading the crystalline cellulose network and hemicellulose matrix. This enzymatic action reduces cell wall rigidity and compromises its mechanical integrity (Nakazawa et al., 2024; Zhang et al., 2018). Enzymatic pretreatment fragments the cell wall structure, enhancing permeability and weakening its integrity (**Figure 5-3**), which reduces the energy required for ultrasound to breach the compromised cellulose-hemicellulose network, thereby maximizing physical disruption efficiency and facilitating the release of intracellular proteins for higher extraction yields. This concurs with previous findings

by Turker & Isleroglu (2024) that enzyme pretreatment followed by ultrasonication was effective for protein extraction. As for the SEUE scheme, the lower efficiency may be explained by the fact that the two require different optimal conditions for effective protein extraction. Consequently, the EUE scheme achieved a higher protein yield and content due to a greater synergy between the enzyme and ultrasound. Meanwhile, the EUE extraction method significantly enhances extraction efficiency and reduces extraction time, particularly when compared to the modified traditional WE method.

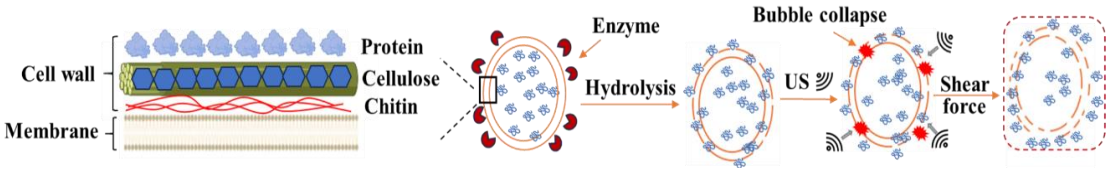

Figure 5-3. Possible mechanism of EUE protein extraction.

Additionally, the protein extracted by the EUE method exhibited stronger immunostimulatory activities than those by the other three methods (**Figure 5-4**). This is likely attributable to the higher protein content achieved through the EUE method compared to other extraction techniques, potentially offering more accessible epitopes for immune recognition (Zhao et al., 2024). Besides, the EUE method demonstrated the highest β-sheet content (55.8%) (**Table 5-4**), which contributes to creating a stable and rigid framework. This structural feature helps maintain the conformation of the epitope, thereby enhancing its accessibility for antibody binding (Rodrigues et al., 2019). Meanwhile, its high α-helix content (26.3%) contributes to overall structural stability without introducing excessive rigidity, potentially balancing stability with the flexibility necessary for optimal epitope exposure. Despite its low β-turn content (2.7%),

the high proportion of β-sheets compensates by preserving structural integrity and ensuring epitope accessibility. The enhanced stability of proteins extracted by the EUE method can be attributed to the synergistic effects of sequential cellulase hydrolysis and ultrasonication. Initially, cellulase selectively cleaves β-1,4-glycosidic bonds in the cell walls (Zhang et al., 2018; Nakazawa et al., 2024), effectively liberating proteins while minimizing structural disruption. This enzymatic pretreatment softens and partially degrades the cell wall, which not only facilitates protein release but also reduces the energy required for subsequent ultrasound treatment. Ultrasonication then generates cavitation effects that further disrupts the softened cell walls (Wu, 2019), accelerating the mass transfer of proteins from the intracellular matrix into the extraction medium (**Figure 5-3**). By softening the cell wall before ultrasound application, the sequential EUE approach avoids excessive mechanical stress and minimizes direct damage to protein structures, yielding the highest α-helix (26.3%) and β-sheet (55.8%) content, with minimum random coil (15.2%), thereby further preserving protein stability. In contrast, the random coil in EAE (29.5%) was almost two times higher than EUE (15.2%) ($p < 0.05$). This may be due to incomplete cell wall rupture during EAE, which can leave some proteins partially entrapped within the cellular matrix. During subsequent isolation steps, such as centrifugation, these trapped proteins are prone to forced aggregation (Challener, 2014), disrupting H-bonds and further increasing structural disorder. Furthermore, EAE has also been observed exhibiting the weakest activity, which can be attributed to its low β-turn content (7.4%) (**Table 5-4**). This deficiency may result in protein misfolding, reduced flexibility, and stability issues,

potentially hindering epitope exposure and effective binding to immune receptors (Marcelino & Gierasch, 2008). Although the WE method did not exhibit a high β-sheet structure (20.6%), it possessed a high random coil content (40.2%) (**Table 5-4**), which may enhance flexibility and improve epitope exposure, thereby facilitating recognition by immune cells (Fan et al., 2024). The higher random coil content observed in WE compared to other extraction methods may be due to the use of alkaline conditions, which promotes side-chain ionization and disrupt stabilizing interactions (Hadinoto et al., 2024). This disruption may facilitate the conversion of α-helix and β-sheets into random coils, thereby increasing the proportion of disordered conformation. Unlike EUE, UAE relies solely on cavitation to disrupt cells and proteins without enzymatic pretreatment. This can break hydrogen bonds, triggering the unfolding of α-helix into random coils (Sow & Du, 2024), consistent with our observed higher random coil content in UAE (33.3%) compared to EUE (15.2%). Meanwhile, UAE its β-turn content (14.3%) was higher than that of EAE (7.4%) ($p < 0.05$) (**Table 5-4**), which may aid in maintaining local conformational stability and facilitate the correct positioning of epitopes. In summary, the EUE method was chosen for optimization to achieve maximum protein yield because it can produce a high yield with substantial protein content and strong immunostimulatory activity.

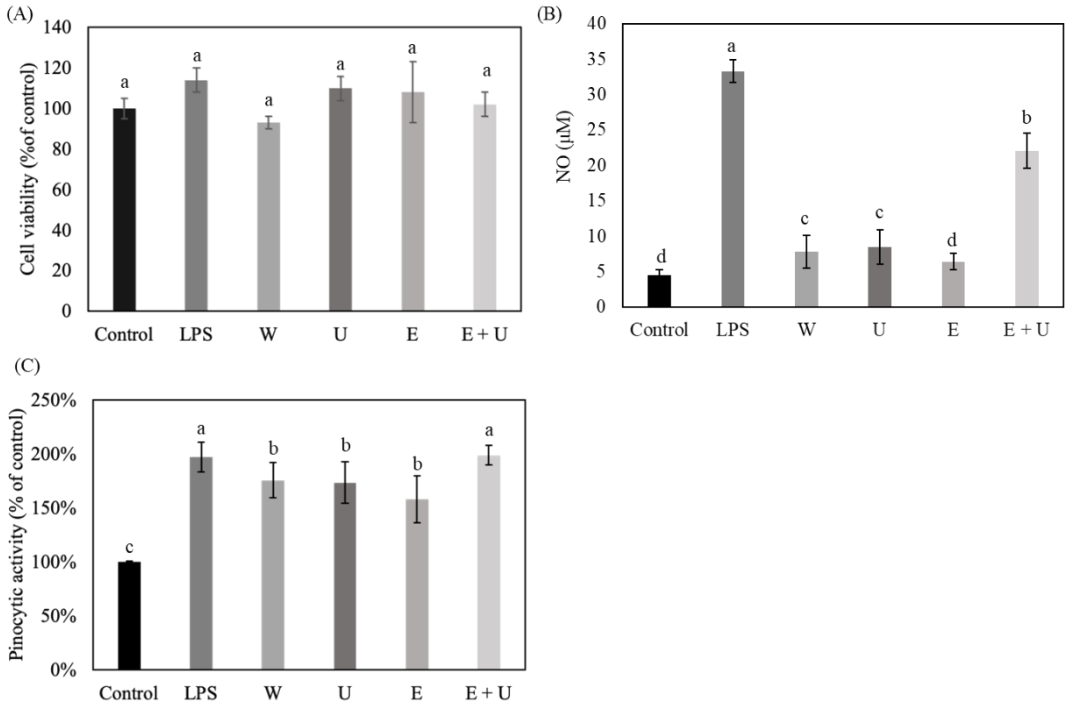

Figure 5-4. Immunoregulating activities of isolated fractions on the (A) cell viability, (B) NO release of macrophages, and (C) phagocytic activity measured by neutral red uptake. Different letters a, b…d indicate significant difference ($p < 0.05$).

Table 5-4. Protein secondary structures by different separation techniques.

	Protein secondary structure*			
	Random coil%	α-Helix%	β-Sheet%	β-Turn%
WE	40.2 ± 3.4^{a}	20.9 ± 1.7^{ef}	20.6 ± 1.6^{m}	18.3 ± 1.4^{o}
UAE	33.3 ± 0.3^{b}	13.9 ± 1.0^{g}	38.5 ± 4.0^{l}	14.3 ± 0.2^{p}
EAE	29.5 ± 3.3^{b}	19.9 ± 2.1^{f}	43.2 ± 1.9^{l}	7.4 ± 1.1^{q}
EUE	15.2 ± 1.3^{c}	26.3 ± 3.5^{e}	55.8 ± 3.9^{k}	2.7 ± 0.3^{r}

*. Different letters a, b…r indicate significant difference ($p < 0.05$).

5.3.2 Effects of EUE conditions on protein yield

Figure 5-5A demonstrates the impact of enzyme concentration ranging from 0.2 to 0.8% (enzyme-to-substrate mass ratio) on the protein yield. All other conditions held constant (EAE 60 min, UAE 40 min, US amplitude 60%, and $(NH_4)_2SO_4$ saturation 70%). As the amount of cellulase increased, both protein yield and content initially rose, then declined, and eventually stabilized. The lowest protein yield (6.0%) was achieved at 0.2%, which shows a significant difference compared to the yield at 0.3%. A 0.3% cellulase addition resulted in the highest protein yield (9.4%) and content (56.0%). At certain liquid-to-material ratios, protein extraction efficiency increases with higher cellulase levels. However, excessive enzyme concentrations beyond saturation may induce substrate limitation and trigger competitive inhibition among enzyme molecules, thereby suppressing catalytic efficiency (Zhang et al., 2015; Le et al., 2022). Moreover, as the amount of cellulase added increases, an excessive amount of enzyme results in over-hydrolysis, leading to an increase in total solids (Xu et al., 2020) and a rise in solution viscosity. This elevated viscosity could slow the diffusion of mushroom particles, thereby reducing their release into the solvent, and also restricts the movement of cellulase molecules, which limits their mobility and effectiveness. Consequently, this leads to a lower protein extraction rate. Therefore, 0.3% (enzyme-to-substrate mass ratio) was chosen as the central enzyme concentration for the RSM analysis.

Figure 5-5B indicates the effect of enzyme treatment time (30-120 min). Although there was insignificant ($p > 0.05$) between the various time periods, the protein output showed a rising and then declining pattern as treatment time rose, while the protein

content appeared a significant difference ($p < 0.05$). The protein extraction yield increased with time (30-60 min), reaching its maximum at 60 min, and then declining trend. With a longer treatment time, cellulase further hydrolyzes the broken cell walls of *L. edodes*, resulting in increased protein solubilization and extraction yield. However, the protein content decreased due probably to protein aggregation, which can negatively impact the extraction yield.

The protein yield was little impacted by changing the UAE time from 20 to 60 min, as seen in **Figure 5-5C** ($p > 0.05$), while it affected soluble on soluble protein content when the other conditions remained constant. As illustrated in **Figure 5-5C**, within the interval of 20 to 40 min of ultrasound treatment, the protein content (47%-56%) increases with the extension of ultrasound time, reaching its maximum (56%) at 40 min. With further increase in ultrasound treatment time, the protein content (56%-36%) starts to decrease, which may be protein denaturation and subsequently lowering the extraction yield.

The protein production varies with US amplitude, as shown in **Figure 5-5D**. At a US amplitude of 60%, the maximum protein content was observed at 56%. Protein content enhanced with the rise in US amplitude from 20%-60% but started to decline with a higher amplitude. Despite the decrease in protein extraction yield mentioned earlier, the crude protein yield continued to increase. This can be attributed to the release of other components such as PS, and polyphenols along with the protein as the US amplitude increased. These other components, although not affected by the destructive effects of ultrasound, could increase the crude protein yield. In order to

attain a high protein content, a central point of 60% US amplitude was chosen for the RSM experiments.

Figure 5-5E shows the effect of $(NH_4)_2SO_4$ saturation from 20% to 100% on protein extraction yield. With the rise of $(NH_4)_2SO_4$ amount, protein content and yield were both increased, probably attributed to the stronger salting-out effect. No significant difference was seen in the crude protein yield between 70% as well as 100% saturation of $(NH_4)_2SO_4$ ($p > 0.05$). A central point of 70% $(NH_4)_2SO_4$ saturation was selected for the RSM experiments.

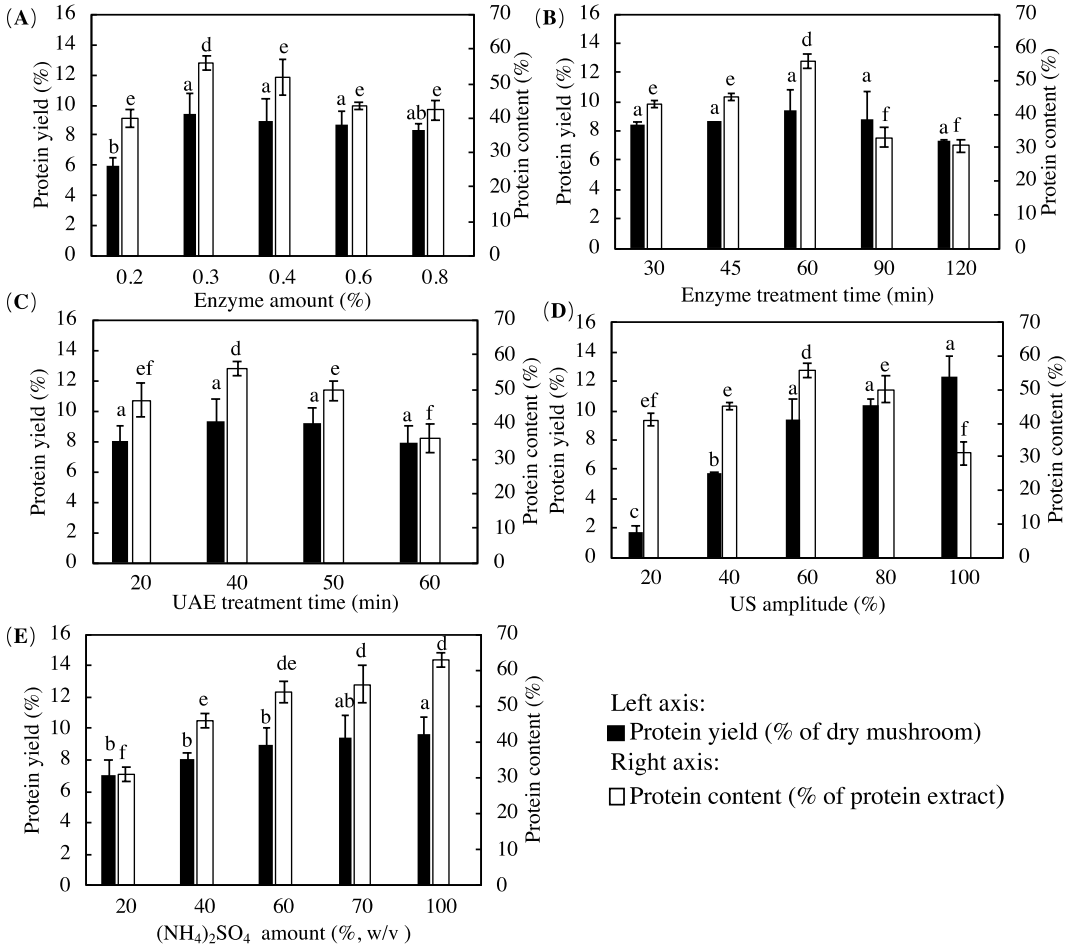

Figure 5-5. Effect of (A) cellulase addition amount, (B) enzyme treatment time, (C) UAE treatment time, (D) US amplitude, (E) $(NH_4)_2SO_4$ saturation concentration on

protein extraction yield and content of *L. edodes*. (Common conditions: enzyme concentration 0.3% (enzyme-to-substrate mass ratio), enzyme treatment time 60 min, ultrasound treatment time 40 min, US amplitude 60% and $(NH_4)_2SO_4$ 70% saturation; Different letters a, b…f indicate significant difference ($p < 0.05$).

5.3.3 EUE extraction conditions optimized using RSM

5.3.3.1 Model Fitting and Statistical Evaluation

RSM analysis was used to determine the optimal EUE protein extraction conditions from *L. edodes*. The effects of enzyme additional amount, US amplitude, and $(NH_4)_2SO_4$ saturation on the protein yield and content were tested using a BBD. The results of all 17 experimental runs are presented in **Table 5-5**. Regression analysis was performed by fitting a response surface model to all the responses, from which the multiple regression equation was derived. These equations represent the empirical correlation between the responses and the independent variables, as displayed:

$$Y_1 = -29.01 + 18.99X_1 + 0.47X_2 + 0.59X_3 + 0.20X_1X_2 - 0.01X_1X_3 - 0.003X_2X_3 - 53.24X_1^2 - 0.002X_2^3 - 0.003X_3^2 \quad (\mathbf{5.1})$$

$$Y_2 = -68.35 + 175.93X_1 - 0.10X_2 + 3.11X_3 - 0.02X_1X_2 - 0.90X_1X_3 - 0.01X_2X_3 - 207.06X_1^2 + 0.003X_2^2 - 0.02X_3^2 \quad (\mathbf{5.2})$$

In the above regression equations, Y_1 and Y_2 are the responses, representing protein yield and its content, respectively. X_1-X_3 denotes the actual values of the independent variables. The *F*-test was used to assess the significant impact of X_1-X_3 on the Y_1 and Y_2. US amplitude (X_2) and $(NH_4)_2SO_4$ saturation (X_3) were found to

significantly influence both protein yield and content ($p < 0.05$). In contrast, enzyme amount (X_1) as well as the interactive effects of US amplitude & $(NH_4)_2SO_4$ saturation ($X_2 : X_3$) significantly affected protein content ($p < 0.05$). However, enzyme amount (X_1) did not significantly affect protein yield. Besides, the model was determined to be significant for both responses (Y_1 and Y_2) (**Table 5-6**). The lack-of-fit test showed no significant difference, with *p*-values of 0.16 and 0.32 for model equations **5.1** and **5.2**, respectively. This demonstrates that the model is reliable for predicting protein yield and content. The adequacy of this model was confirmed via the coefficient of determination (R^2), with values of 0.944 (Y_1) and 0.924 for (Y_2), respectively. A higher R^2 value indicates a strong fit between the empirical models and the experimental data. Three-dimensional response surface plots were generated in order to explore the interaction effects of X_1-X_3 on Y_1 and Y_2 (**Figure 5-6**). One variable was kept constant while the other two were changed in these graphs. Multiple solutions were generated via this approach, with the most desirable solution, exhibiting a desirability value of 0.736, being presented in this study, which is close to 1. The contour plots (**Figure 5-7**) showed that the optimum conditions for the protein with high content and high yield by the EUE extraction were 0.28% (enzyme-to-substrate mass ratio) enzyme, 62% US amplitude and 69% $(NH_4)_2SO_4$ saturation, predicting a maximum protein yield of 9.7% and content of 57.1%. The optimal conditions were validated through a verification experiment. The verification experiment yielded a protein yield of 9.7 ± 1.6% and a content of 58.4 ± 1.3%, which closely matched the model-predicted value. The findings also indicated that the response models accurately represented the optimization targets.

In general, the accuracy was satisfactory, and the response surface models were effective for predicting responses.

Table 5-5. RSM experimental design (3^3 BBD) and the resulting protein yield and content outcomes.

Run	Independent variables			Response Y_1		Response Y_2	
	X_1	X_2	X_3	Experiment	Predicted	Experiment	Predicted
1	0.5	60	90	5.0 ± 0.5	5.1	39.3 ± 1.7	38.4
2	0.3	60	70	9.0 ± 1.4	9.5	55.4 ± 1.2	57.5
3	0.5	60	50	6.3 ± 0.5	6.6	43.6 ± 2.9	41.8
4	0.3	60	70	9.7 ±1.9	9.5	58.3 ± 2.9	57.5
5	0.3	40	90	5.9 ± 1.1	6.3	63.5 ± 0.8	62.9
6	0.1	60	50	7.3 ± 0.1	7.1	38.8 ± 1.3	39.8
7	0.1	80	70	7.3 ± 0.7	7.9	48.9 ± 0.9	47.4
8	0.1	40	70	6.1 ± 1.6	6.0	60.0 ± 1.9	58.7
9	0.3	80	90	7.5 ±1.7	7.3	45.6 ± 1.3	45.3
10	0.3	60	70	9.4 ± 1.3	9.5	57.2 ± 1.1	57.5
11	0.3	40	50	5.1 ± 0.7	5.3	52.5 ± 0.8	52.9
12	0.3	60	70	10.0 ± 1.9	9.5	60.3 ± 1.2	57.5
13	0.3	60	70	9.4 ± 1.3	9.5	56.4 ± 1.4	57.5
14	0.5	40	70	4.3 ± 0.5	3.8	52.2 ± 1.3	53.7
15	0.5	80	70	8.8 ± 0.4	8.9	40.7 ± 1.9	42.0
16	0.1	60	90	6.0 ± 0.02	5.7	48.9 ± 1.5	50.8
17	0.3	80	50	11.7 ± 0.5	11.3	47.0 ± 1.2	47.6

Table 5-6. ANOVA of fitted quadratic models of two responses.

Source	Y_1: protein yield (%)		Y_2: protein content (%)	
	F	*p*	*F*	*p*
Model	31.05	< 0.0001	22.63	0.0002
X_1-enzyme amount	2.55	0.1542	11.74	0.0110
X_2-US amplitude	99.06	< 0.0001	57.36	0.0001
X_3-$(NH_4)_2SO_4$ saturation	17.83	0.0039	6.39	0.0393
X_1X_2	10.49	0.0143	0.0080	0.9311
X_1X_3	0.0126	0.9139	11.39	0.0118
X_2X_3	25.72	0.0014	8.38	0.0231
X_1^2	77.77	< 0.0001	63.01	<0.001
X_2^2	9.39	0.0182	1.32	0.2876

X_3^2	26.12	0.0014	39.68	0.0004
Lack of Fit	2.95	0.1613	1.62	0.3192
	$R^2 = 0.976$	$R^2_{adj} = 0.944$	$R^2 = 0.967$	$R^2_{adj} = 0.924$
		C.V.% = 6.54		C.V.% = 4.19

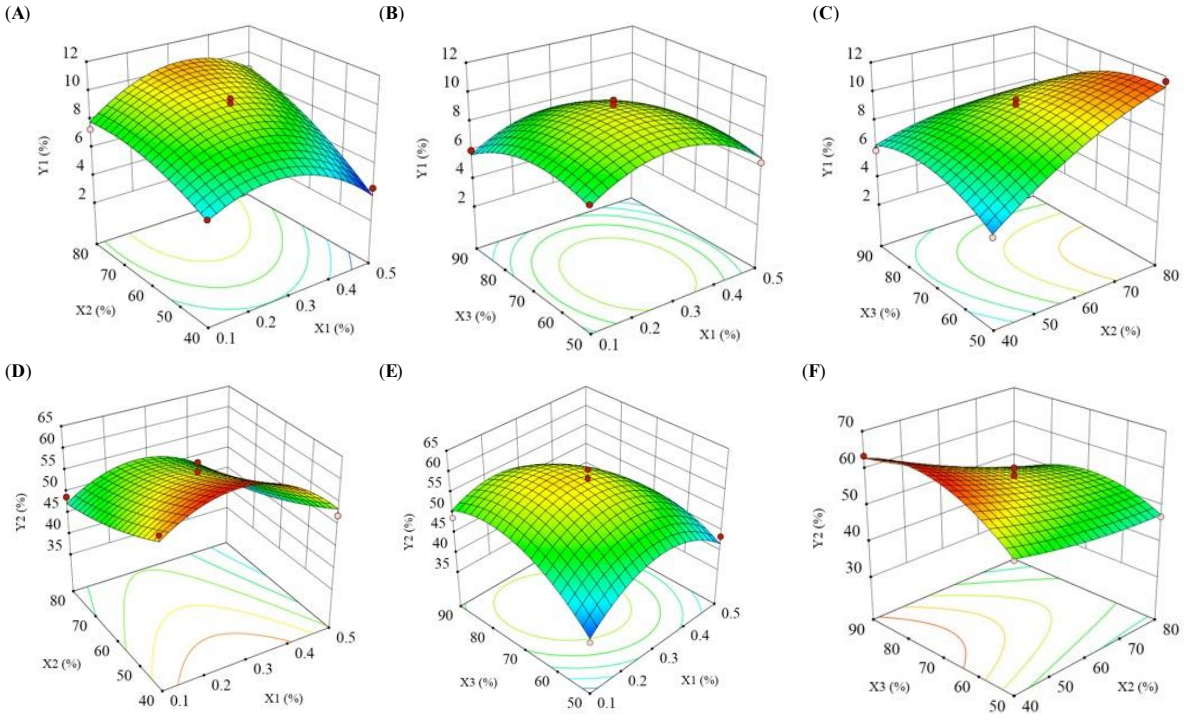

Figure 5-6. Response surface plots of Protein yield (Y_1) versus two experimental variables: (A) enzyme amount (X_1); and ultrasound amplitude (X_2); (B) enzyme amount (X_1) and the $(NH_4)_2SO_4$ saturation (X_3); (C) US amplitude power (X_2) and $(NH_4)_2SO_4$ saturation (X_3); Protein content (Y_2) versus two experimental variables: (D) enzyme amount (X_1); and US amplitude power (X_2); (E) enzyme amount (X_1) and the $(NH_4)_2SO_4$ saturation (X_3); (F) US amplitude power (X_2) and $(NH_4)_2SO_4$ saturation (X_3).

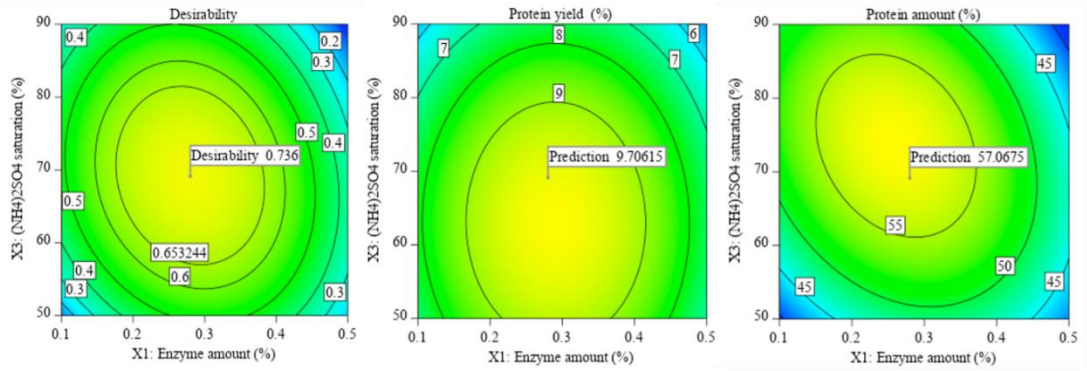

Figure 5-7. Contour plots with the desirable response goals to determine the optimum protein yield and content at an enzyme addition amount of 0.28%.

5.3.4 Structure characteristics of EUE-extracted proteins

In the CD spectrum (**Figure 5-8**), the protein structure was characterized by random coil 10.0 ± 1.4 %, α-helix 29.3 ± 2.6%, β-sheet 56.7 ± 6.2%, and β-turn 4.0 ± 0.6%. A previous study identified the β-sheet as the predominant structure in proteins extracted from the stipe of *L. edodes* mushroom, which is consistent with our findings (Hu Danhui, 2019). In **Figure 5-9**, the EUE-extracted protein under optimized conditions exhibits characteristic peaks of amide I (1638 cm^{-1}) and amide III (1242 cm^{-1}). Amide I and amide III bands result from the N-H and C-N stretching vibrations, as well as N-H bending vibrations. The absorption band at 3411 cm^{-1} is associated with the stretching vibration of O–H in the molecular structure, probably due to the presence of PS. This aligns with our findings, as the EUE-extracted protein can also acquire PS, as shown in **Table 5-3**. The anti-symmetric stretching vibration of alkanes (-C-H-) is responsible for the band at 2919 cm^{-1}. The absorption bands at 1547 cm^{-1} as well as 1080 cm^{-1} arise from the in-plane deformation vibration of NH_2 and the in-plane bending vibration of

NH, respectively, in the amide II region. From the SDS-PAGE analysis (**Figure 5-10**), it was observed that the optimized EUE protein primarily consisted of low-MW fragments, with the majority of protein bands appearing below 10 kDa. Additionally, distinct bands were detected at 25 kDa, 34 kDa, and a broad smear between 43-75 kDa, suggesting the presence of higher MW species, possibly due to incomplete degradation, or protein aggregation.

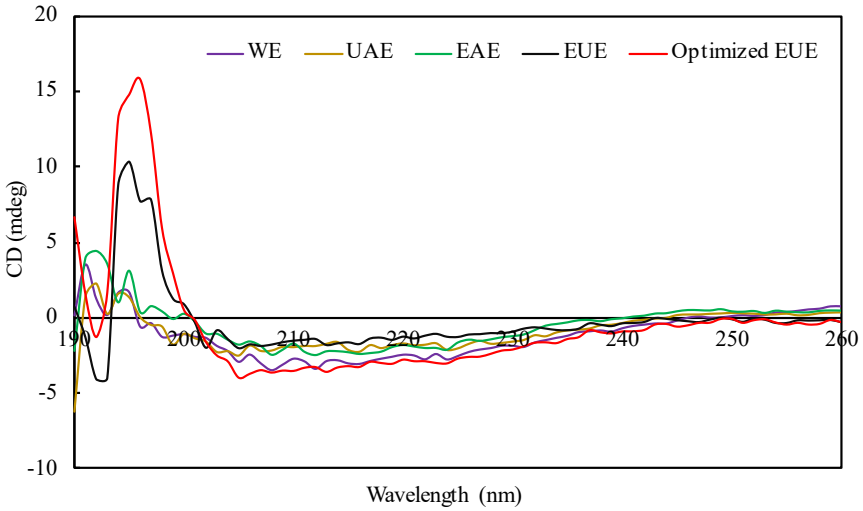

Figure 5-8. CD spectrum of isolated protein fraction from different extraction methods.

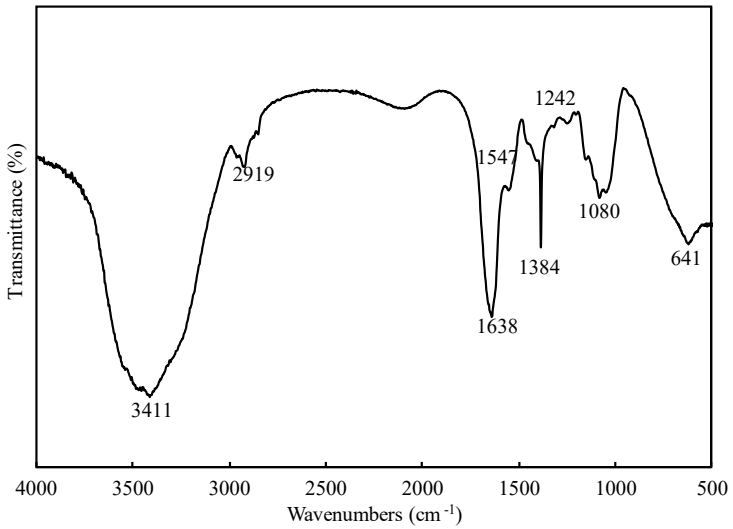

Figure 5-9. FT-IR spectrum of optimized EUE protein.

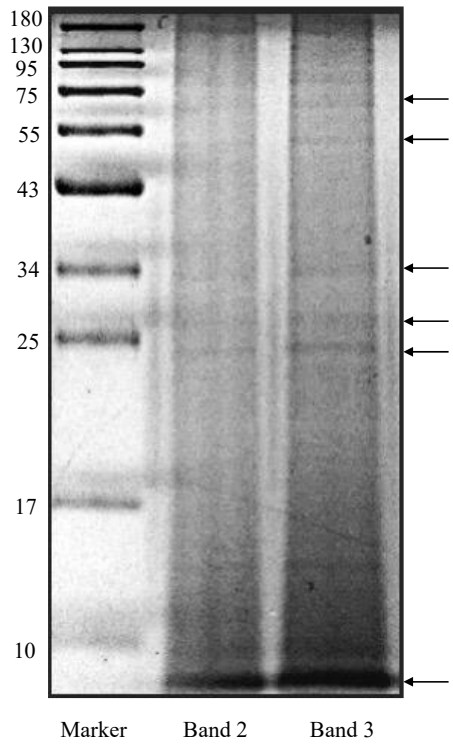

Figure 5-10. SDS-PAGE Analysis of Optimized EUE Protein (Bands 2 and 3).

5.3.5 Composition of proteins extracted through EUE

Table 5-7 presents the AA profiles of partially purified proteins extracted by EUE. As shown in **Table 5-7A**, the ratio of the sum of Glu and Asp to the sum of Lys, Arg, as well as His was 1.22. Thus, the EUE-extracted protein sample was acidic proteins. The protein nutritional value is primarily determined by the type, content, quantity, as well as composition of EAA. The protein extracted via EUE comprised EAA and NEAA with a total amount of 437.0 mg/g, which exceeded the amount of protein in other edible fungus powder such as *Pleurotus citrinopileatus* (oyster mushroom) and *Flammulina velutipes* (enoki mushroom) (Li et al, 2022). The content of EEA constitutes over 40% of the TAA content. EAA/NEAA value was close to 80%, in line with the recommendations proposed by the FAO/WHO ideal protein condition.

Furthermore, the value of isoleucine and valine exceeds the range required by FAO/WHO for adults (Wang et al., 2023), indicating that *L. edodes* can be used as a good nutritional protein source for adults.

As stated by the AA scoring model recommended by FAO/WHO and the AA profile of egg protein (**Table 5-8**), the amino acid score (AAS) as well as chemical score (CS) values for EUE protein are presented in **Table 5-7B**. When AAS was used as the standard, isoleucine content was the highest, 2.08 times of the standard. The first limiting AA was methionine and cysteine (Met + Cys), while Lys was the second limiting AA. The highest isoleucine content was 1.41 times the standard when CS was used as the standard. Met + Cys was also recognized as the first limiting AA. Therefore, the main limiting AA in the *L. edodes* protein was Met + Cys and lysine. Met + Cy was also identified as the first limiting AA based on the RC value. Lysine was the second limiting AA. This result aligns with a previous study that identified Met + Cys as the first limiting AA in the fungus *L. edodes*. It was also agreed with our previous findings, which showed that Met + Cys is lacking in *L. edodes*. The RC value of Leu was close to 1, indicating that the composition ratio of this AA in *L. edodes* protein was close to that in the model spectrum. Among detected AAs, Val showed the third lowest concentrations, while Ile and Thr demonstrated significantly higher abundance. Mushroom protein should be combined with other proteins to optimize its nutritional value based on the protein complementation theory. The nutritional quality of the protein improves as its SRC value increases. In this study, the SRC value of EUE-extracted protein was 69.8%, which was comparable to that of soy protein, milk

(72.60%), milk powder (67.31%), and walnuts (62.65%). The protein extracted from *L. edodes* mushroom using the EUE method possesses high nutritional value and offers potential for development and utilization.

Table 5-7. AAs in EUE-extracted protein samples.

(A) Amino acid composition and contents (mg/g crude protein)			
EAA	Content	NEAA	Content
Histidine (His)	9.8 ± 2.6	Alanine (Ala)	26.3 ± 1.6
Isoleucine (Ile)	33.3 ± 1.9	Asparagine (Arg)	2.3 ± 0.04
Leucine (Leu)	39.2 ± 2.6	Aspartic acid (Asp)	36.3 ± 2.7
Lysine (Lys)	23.2 ± 0.9	Arginine (Arg)	22.9 ± 1.5
Methionine (Met)	9.1 ± 1.0	Glutamic acid (Glu)	31.7 ± 2.2
Phenylalanine (Phe)	23.2 ± 1.5	Glycine (Gly)	23.0 ± 0.9
Threonine (Thr)	25.9 ± 1.4	Serine (Ser)	27.2 ± 2.0
Valine (Val)	25.0 ± 1.4	Tyrosine (Tyr)	17.6 ± 1.6
		Cysteine (Cys)	2.1 ± 0.3
		Glutamine (Glu)	38.4 ± 5.6
		Proline	20.5 ± 1.4

TAA	EAA	NEAA	EAA/TAA (%)	EAA/NEAA (%)
437.0 ± 33.1	188.7 ± 13.3	248.3 ± 19.8	43.2 ± 0.2	76.0 ± 0.7

(B) Amino acid and chemical scores of protein samples				
	AAS (%)	CS (%)	RAA	RC
Ile	83.3 ± 4.8^{a}	61.7 ± 3.5^{f}	0.83 ± 0.05^{k}	1.47 ± 0.01^{p}
Leu	56.0 ± 3.7^{c}	45.6 ± 3.0^{g}	0.56 ± 0.04^{m}	0.99 ± 0.00^{s}
Lys	42.2 ± 1.6^{d}	33.1 ± 1.3^{h}	0.42 ± 0.02^{n}	0.75 ± 0.02^{u}
Met + Cys	32.0 ± 1.7^{e}	19.6 ± 2.3^{i}	0.32 ± 0.02^{o}	0.56 ± 0.03^{v}
Phe + Tyr	68.0 ± 5.2^{b}	43.9 ± 3.3^{g}	0.68 ± 0.05^{l}	1.21 ± 0.01^{q}
Thr	64.8 ± 3.5^{bc}	55.1 ± 3.0^{f}	0.65 ± 0.03^{lm}	1.14 ± 0.01^{r}
Val	50.0 ± 2.8^{cd}	37.9 ± 2.1^{gh}	0.50 ± 0.03^{mn}	0.88 ± 0.01^{t}
SRC	69.8 ± 0.6%			

Note: AAS = content of a specific EAA in the protein sample divided by its content in the FAO/WHO model protein (FAO/WHO, 2007); CS = content of an EAA in a protein sample divided by its content in whole egg protein; RAA (AA ratio) = content of an AA in protein divided by its content in the FAO/WHO pattern spectrum; RC (ratio coefficient) = RAA value divided by the average RAA; SRC (score of ratio coefficient) = the standard deviation coefficient of RC (Zhao et al., 2024). Different letters a, b…v indicating significant difference ($p < 0.05$).

Table 5-8. FAO/WHO model protein and whole egg protein.

	FAO/WHO model score (mg/g)	Whole egg protein pattern score (mg/g)
Ile	40	54
Leu	70	86
Lys	55	70
Met + Cys	35	57
Phe + Tyr	60	93
Thr	40	47
Val	50	66

5.3.6 Immunomodulatory activities

At a concentration of 4 μg/mL, the EUE-extracted protein demonstrated no toxicity to RAW 264.7 cells (**Figure 5-11A**). NO is essential in various physiological functions, particularly in host defense. NO production serves as a reliable measure of the immunocompetence of RAW 264.7 cells. As displayed in **Figure 5-11B**, EUE-extracted protein NO production was more significant than the control group ($p < 0.05$). NO production level was around five times higher than that of the control group. Furthermore, phagocytosis is also one of the essential functions of macrophages. Neutral red phagocytosis was employed in this investigation as a marker of immune response activity, and the outcomes were consistent with the NO generation findings.

The protein group's neutral red uptake was almost 1.5 times more than the control group's ($p < 0.05$) (**Figure 5-11C &D**). Moreover, as defensive elements and signaling molecules in immunological pathways, ROS can be produced by macrophage cells during phagocytosis. As anticipated, the EUE protein fractions stimulated ROS production in macrophage cells similar to pinocytic activity (**Figure 5-11E**). Overall, the results confirmed that the EUE-extracted protein exhibited in *vitro* immunostimulatory activity.

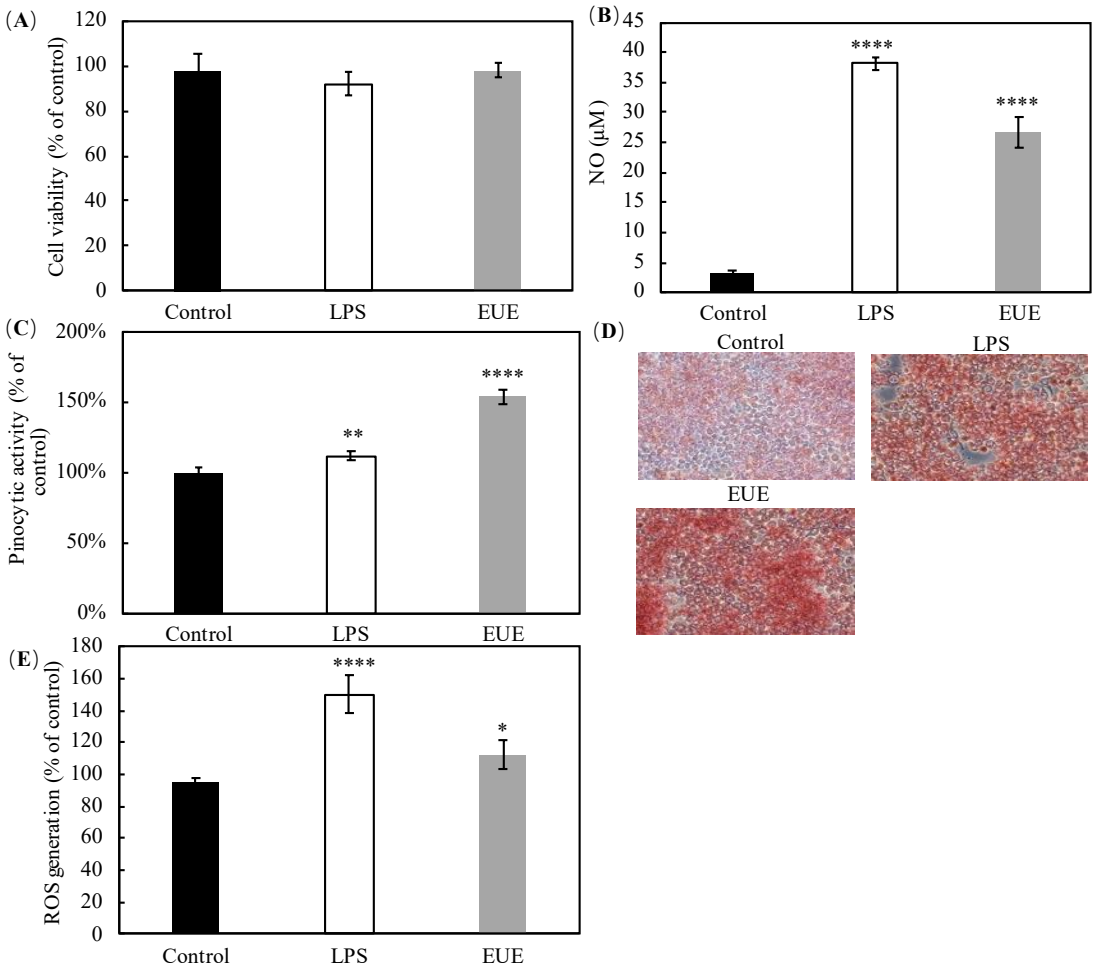

Figure 5-11. Immunoregulating activities of EUE-extracted protein on the (A) cell viability, (B) NO release of macrophages, (C, D) phagocytic activity measured by neutral red uptake, and (E) ROS expression. Data are expressed as the means ± SD (n

= 3). The error bar represents the standard deviation. *, ** and ****: statistically significant differences from the control group at $p < 0.05$ and $p < 0.01$, $p < 0.0001$ respectively.

5.4 Conclusions

This study demonstrated that the two-step, enzyme- followed by ultrasound-assisted extraction (EUE) was the most effective method for L. edodes mushroom protein extraction, achieving the highest protein yield (9.4%) and content (56%), as well as strong immunostimulatory activity, compared to one-step processes (WE, EAE, UAE). EUE also outperformed SEUE1 (yield: 8%, content: 40.7%), SEUE2 (yield: 4.6%, content: 51.4%), and UEE (yield: 3.6%, content: 14%). EUE reduced extraction time and cost, making it a more efficient and cost-effective approach than the traditional WE method. Key process factors for EUE included enzyme concentration, ultrasound power, and ammonium salt saturation. RSM optimization identified optimal conditions for maximum protein yield (9.7%) and content (58.4%): 0.28% enzyme-to-substrate mass ratio, 62% ultrasonic amplitude, and 69% $(NH_4)_2SO_4$ saturation. The extracted protein fraction showed high nutritional value and significant in vitro immunostimulatory activity. These findings provide a theoretical basis for using the EUE scheme in mushroom protein extraction, though further pilot-scale trials and economic analysis are needed to confirm industrial feasibility. Future research should focus on the immunostimulatory mechanisms of EUE-extracted proteins through molecular pathway analysis and characterization of key bioactive peptides and proteins. It is also important to further investigate protein structures and their interactions with PS

regarding immunostimulatory activities across different extraction methods. Additionally, more research is needed to clarify the molecular mechanisms behind the synergistic effects of enzyme and ultrasound treatments.

Chapter 6 General Conclusions and Future Studies

6.1 General conclusions

Overall, this research project involved a thorough experimental study of the molecular properties and immunostimulatory activities of crude and purified protein fractions from the *L. edodes* mushroom. Various extraction, isolation, and fractionation methods were employed. The results provide valuable insights and references for developing effective strategies and processes to extract, isolate, and fractionate proteins from *L. edodes* and potentially other natural sources. They also contribute to a better understanding of the relationship between molecular properties and bioactivity. The following findings and conclusions can be drawn from this project:

1. The three-phase partitioning (TPP) method efficiently isolates polysaccharides (PSs) and proteins from *L. edodes* mushroom, achieving superior yields and purity compared to traditional methods. Optimized extraction conditions, including ammonium sulfate ($(NH_4)_2SO_4$) concentration, *t*-butanol ratio, and temperature, maximized PS yield at 11.64% and protein yield at 2.06%.
2. The isolated PSs are primarily composed of D-Glc, D-Gal, and D-Man, while the protein fraction contains both essential and non-essential AAs and exhibits immunostimulatory activity.
3. Anion exchange chromatographic fractionation led to the isolation of two purified protein fractions, F1 and F2, from *L. edodes* mushrooms. Compared to F1, F2 exhibited a broader molecular weight (MW) range, higher protein content, and greater levels of essential amino acids (EAAs).

4. Despite the increased protein content achieved through purification, the individual fractions showed lower immunostimulatory activity than the combined mixture, suggesting a potential synergistic effect between proteins and PSs.
5. The sequential enzyme-ultrasound-assisted extraction (EUE) method emerged as the optimal approach for *L. edodes* protein extraction, achieving superior outcomes through its two-step design. By integrating enzymatic pretreatment followed by ultrasound disruption, EUE outperformed single-step and alternative hybrid methods, delivering higher protein yields, enhanced structural preservation (e.g., β-sheet integrity), and greater immunostimulatory activity, highlighting the importance of sequential processing for synergistic extraction efficiency. The method effectiveness highlights its industrial potential.
6. The protein fractions extracted using the EUE method demonstrated high nutritional value and strong immunostimulatory activity, indicating their potential for use in dietary supplements.

6.2 Future studies

Our study provides valuable insights into the molecular characteristics and immunostimulatory activities of *L. edodes* proteins. Based on our findings and existing literature, we propose the following directions for future research.

1. Exploring ways to enhance the TPP method by integrating it with mechanical or non-mechanical techniques could further improve protein yield and purity, while also maximizing the immunostimulatory potential of the protein-rich fraction.

2. Additional research is needed to understand the synergistic interactions between polysaccharides and proteins that enhance immunostimulatory activity. This knowledge will help unlock the potential of protein fractions for use in dietary supplements, nutraceuticals, and functional foods. Additionally, there is a need to develop improved purification techniques to obtain highly pure and homogeneous protein fractions.
3. Evaluate the immunostimulatory effects of purified *L. edodes* protein in animal models, focusing on its chemical composition and molecular properties to confirm its pharmacological significance and functions.
4. Further research should include pilot-scale trials to assess the industrial feasibility and economic viability of this method. Additionally, applying the EUE process to other mushroom species and natural sources could broaden its applicability. By optimizing process parameters and scaling up operations, the EUE method has the potential to become a standard approach for protein extraction in various industrial settings.
5. Subsequent investigations should focus on detailed analyses of protein structures and their interactions with PS, particularly regarding immunostimulatory activities across different extraction methods. To enhance fundamental understanding and enable rational application, it is essential to elucidate the molecular mechanisms underlying the synergistic effects of enzyme and ultrasound interactions.

References

Abdoulaye, A., Noumavo, A. D. P., Dah-Nouvlessounon, D., Ohin, M. A. B., Bayraktar, H., Bade, F. T., et al. (2023). Purification of *Moringa oleifera* leaves protease by three-phase partitioning and investigation of its potential antibacterial activity. *American Journal of Plant Sciences*, *14*(01), 64–76. https://doi.org/10.4236/ajps.2023.141005

Ahmad, I., Arif, M., Xu, M., Zhang, J., Ding, Y., & Lyu, F. (2023). Therapeutic values and nutraceutical properties of shiitake mushroom (*Lentinula edodes*): A review. *Trends in Food Science & Technology*, *134*, 123–135. https://doi.org/10.1016/j.tifs.2023.03.007

Ahmad, M. I., Farooq, S., Alhamoud, Y., Li, C., & Zhang, H. (2022). A review on mycoprotein: History, nutritional composition, production methods, and health benefits. *Trends in Food Science and Technology*, *121*, 14–29. https://doi.org/10.1016/j.tifs.2022.01.027

Ahmed, T., Juhász, A., Bose, U., Shiferaw Terefe, N., & Colgrave, M. L. (2024). Research trends in production, separation, and identification of bioactive peptides from fungi – A critical review. *Journal of Functional Foods*, *119*. https://doi.org/10.1016/j.jff.2024.106343

Ahmed, T., Rana, M. R., Maisha, M. R., Sayem, A. S. M., Rahman, M., & Ara, R. (2022). Optimization of ultrasound-assisted extraction of phenolic content & antioxidant activity of hog plum (*Spondias pinnata L. f. kurz*) pulp by response surface methodology. *Heliyon*, *8*(10), e11109. https://doi.org/10.1016/j.heliyon.2022.e11109

Ahmed, T., Suzauddula, M., Akter, K., Hossen, M., & Islam, M. N. (2024). Green technology for fungal protein extraction—A Review. *Separations*, *11*(6), 186. https://doi.org/10.3390/separations11060186

Alfred, M. M. (2014). *Antioxidant, antiproliferative and immunomodulation activity of protein extracted from Pleurotus Eryngii (Dc.ex Fr.) Quel*. PhD diss., Nanjing Agricultural University.

Anjum, F. M., Ahmad, I., Butt, M. S., Sheikh, M. A., & Pasha, I. (2005). Amino acid composition of spring wheats and losses of lysine during chapati baking. *Journal of Food Composition and Analysis*, *18*(6), 523–532. https://doi.org/10.1016/j.jfca.2004.04.009

Araújo-Rodrigues, H., Sousa, A. S., Relvas, J. B., Tavaria, F. K., & Pintado, M. (2024). An overview on mushroom polysaccharides: Health-promoting properties, prebiotic and gut microbiota modulation effects and structure-function correlation. *Carbohydrate Polymers*, *333*. https://doi.org/10.1016/j.carbpol.2024.121978

Arruda, M. A. Z., Jesus, J. R., & Galazzi, R. M. (2019). Electrophoresis | Gel electrophoresis: Two-dimensional gel electrophoresis of proteins. *Encyclopedia of Analytical Science*. 2, 493-504. https://doi.org/10.1016/B978-0-12-409547-2.14480-4

Arya, A., & Rusevska, K. (2022). *Biology, cultivation and applications of mushrooms*. Singapore: Springer.

Ayimbila, F., & Keawsompong, S. (2023). Nutritional quality and biological application of mushroom protein as a novel protein alternative. *Current Nutrition*

Reports, *12*(2), 290–307. https://doi.org/10.1007/s13668-023-00468-x

Balavigneswaran, C. K., Sujin Jeba Kumar, T., Moses Packiaraj, R., Veeraraj, A., & Prakash, S. (2013). Anti-oxidant activity of polysaccharides extracted from *Isocrysis galbana* using RSM optimized conditions. *International Journal of Biological Macromolecules*, *60*, 100–108. https://doi.org/10.1016/j.ijbiomac.2013.05.014

Bisen, P. S., Baghel, R. K., Sanodiya, B. S., Thakur, G. S., & Prasad, G. B. K. S. (2010). *Lentinus edodes*: A macrofungus with pharmacological activities, *Current medicinal chemistry*, *17*(22), 2419-2430. https://doi.org/10.2174/092986710791698495

Bolje, A. (2021). Analytical techniques for structural characterization of proteins in solid pharmaceutical forms : An overview. *Pharmaceutics*, 13(4), 534. https://doi.org/10.3390/pharmaceutics13040534

Boraston, A. B., Bolam, D. N., Gilbert, H. J., & Davies, G. J. (2004). Carbohydrate-binding modules: Fine-tuning polysaccharide recognition. *Biochemical Journal*, *382*(3), 769–781. https://doi.org/10.1042/BJ20040892

Bose, U., Broadbent, J. A., Byrne, K., Hasan, S., Howitt, C. A., & Colgrave, M. L. (2019). Optimisation of protein extraction for in-depth profiling of the cereal grain proteome. *Journal of Proteomics*, *197*, 23–33. https://doi.org/10.1016/j.jprot.2019.02.009

Bubb, W. A. (2003). NMR spectroscopy in the study of carbohydrates: Characterizing the structural complexity. *Concepts in Magnetic Resonance Part A: Bridging Education and Research*, *19*(1), 1–19. https://doi.org/10.1002/cmr.a.10080

Cannavacciuolo, C., Pagliari, S., Celano, R., Campone, L., & Rastrelli, L. (2024). Critical analysis of green extraction techniques used for botanicals: Trends, priorities, and optimization strategies-A review. *TrAC - Trends in Analytical Chemistry*, *173*, 117627. https://doi.org/10.1016/j.trac.2024.117627

Capellini, M. C., Giacomini, V., Cuevas, M. S., & Rodrigues, C. E. C. (2017). Rice bran oil extraction using alcoholic solvents: Physicochemical characterization of oil and protein fraction functionality. *Industrial Crops and Products*, *104*, 133–143. https://doi.org/10.1016/j.indcrop.2017.04.017

Chan, L. G., Cohen, J. L., & De Moura Bell, J. M. L. N. (2018). Conversion of agricultural streams and food-Processing by-Products to value-added compounds using *Filamentous* fungi. *Annual Review of Food Science and Technology*, *9*, 503–523. https://doi.org/10.1146/annurev-food-030117-012626

Chan, M. Y. Y., Hoare, M., & Dunnill, P. (1986). The kinetics of protein precipitation by different reagents. *Biotechnology and Bioengineering*, *28*(3), 387–393. https://doi.org/10.1002/bit.260280312

Chang, S. T., & Miles, P. G. (2004). *Mushrooms: cultivation, nutritional value, medicinal effect, and environmental impact. CRC PRESS.*

Chatterjee, M., Gupta, S., Bhar, A., & Das, S. (2012). Optimization of an efficient protein extraction protocol compatible with two-dimensional electrophoresis and mass spectrometry from recalcitrant phenolic rich roots of Chickpea (*Cicer arietinum L.*). *International Journal of Proteomics*, *2012*(1), 536963.

https://doi.org/10.1155/2012/536963

Chen, H. Y., Linghu, C. L, Luo, Y., Wu, Y. Y., Zou, G., & Bao, D. P. (2021). Research progress on the preparation and application of edible mycoproteins. *Journal of Edible Fungi. 28*(6), 188–189.

Chen, Q. L., Li, Z. H., Chen, S. X. (2014). Analysis of amino acid composition and nutritional evaluation in five local edible fungus. *Food machinery*, *30*(6), 43–47.

Chen, R., Wang, X. J., Zhang, Y. Y., Xing, Y., Yang, L., Ni, H., & Li, H. H. (2019). Simultaneous extraction and separation of oil, proteins, and glucosinolates from *Moringa oleifera* seeds. *Food Chemistry*, *300*, 125162. https://doi.org/10.1016/j.foodchem.2019.125162

Chen, S., Liu, C., Huang, X., Hu, L., Huang, Y., Chen, H., et al. (2020). Comparison of immunomodulatory effects of three polysaccharide fractions from *Lentinula edodes* water extracts. *Journal of Functional Foods*, *66*, 103791. https://doi.org/10.1016/j.jff.2020.103791

Chen, X. Y., Liu, Y. Q., Yu, Z. L., Li, S. (2017). Analysis of extraction conditions and the functional properties of *Pleurotus eryngii* protein. *Food Science and Techonology*, (2012134), 235–240. https://doi.org/10.13684/j.cnki.spkj.2017.05.046

Chen, Y. F., Bao, D. P., Chen, H. Y., Feng, J., Luo, Y., Zhang, Q., Zou, G., Zhao, Y. (2021). Extraction and functional properties of protein from *Pleurotus mycelium* by liquid submerged fermentation. *Journal of Edible Fungi.*, *28*(3), 112–121.

Cheung, Y. C., Siu, K. C., Liu, Y. S., & Wu, J. Y. (2012). Molecular properties and

antioxidant activities of polysaccharide-protein complexes from selected mushrooms by ultrasound-assisted extraction. *Process Biochemistry*, *47*(5), 892–895. https://doi.org/10.1016/j.procbio.2012.02.004

Chew, K. W., Chia, S. R., Lee, S. Y., Zhu, L., & Show, P. L. (2019). Enhanced microalgal protein extraction and purification using sustainable microwave-assisted multiphase partitioning technique. *Chemical Engineering Journal*, *367*, 1–8. https://doi.org/10.1016/j.cej.2019.02.131

Citores, L., Ragucci, S., Russo, R., Gay, C. C., Chambery, A., Di Maro, A., et al. (2023). Structural and functional characterization of the cytotoxic protein ledodin, an atypical ribosome-inactivating protein from shiitake mushroom (*Lentinula edodes*). *Protein Science*, *32*(4), 1–17. https://doi.org/10.1002/pro.4621

Coimbra, C. G. de O., Lopes, C. E., & Calazans, G. M. T. (2010). Three-phase partitioning of hydrolyzed Levan. *Bioresource Technology*, *101*(12), 4725–4728. https://doi.org/10.1016/j.biortech.2010.01.091

Contato, A. G., & Conte-Junior, C. A. (2025). Mushrooms as meat substitute in plant-based diets. *European Food Research and Technology*. 1-14. https://doi.org/10.1007/s00217-025-04722-0

Coustets, M., Joubert-Durigneux, V., Hérault, J., Schoefs, B., Blanckaert, V., Garnier, J. P., & Teissié, J. (2015). Optimization of protein electroextraction from microalgae by a flow process. *Bioelectrochemistry*, *103*, 74–81. https://doi.org/10.1016/j.bioelechem.2014.08.022

Crevenna, A. H., Naredi-Rainer, N., Lamb, D. C., Wedlich-Söldner, R., & Dzubiella, J. (2012). Effects of hofmeister ions on the α-helical structure of proteins. *Biophysical Journal*, *102*(4), 907–915. https://doi.org/10.1016/j.bpj.2012.01.035

Cui, X. R., Wang, L., Shi, F. F., Pan, Y., Jia, H. L., ... & Song H. B. (2018). Extraction and antioxidant activity of *Stropharia rugosoannulata* protein. *Journal of Food Safety and Quality*, *9*(22). https://www.cabdirect.org/cabdirect/abstract/20193054042

Cutler, P. (Ed.). (2008). *Protein purification protocols (Vol. 244).* Springer Science & Business Media.

Das, S., & Prakash, B. (2022). *Edible mushrooms: nutritional composition and medicinal benefits for improvement in quality life. Research and technological advances in food science* (pp. 269-300). Academic Press.

de Falco, B., Incerti, G., Pepe, R., Amato, M., & Lanzotti, V. (2016). Metabolomic fingerprinting of romaneschi globe artichokes by NMR spectroscopy and multivariate data analysis. *Phytochemical Analysis*, *27*(5), 304–314. https://doi.org/10.1002/pca.2632

Deflores, L. P., Ganim, Z., Nicodemus, R. A., & Tokmakoff, A. (2009). Amide I′-II′ 2D IR spectroscopy provides enhanced protein secondary structural sensitivity. *Journal of the American Chemical Society*, *131*(9), 3385–3391. https://doi.org/10.1021/ja8094922

Dennison, C., & Lovrien, R. (1997). Three phase partitioning: Concentration and purification of proteins. *Protein Expression and Purification*, *11*(2), 149–161.

https://doi.org/10.1006/prep.1997.0779

Du, J., Xi, J., Chen, X., Sun, H., Zhong, L., Zhan, Q., & Zhao, L. (2024). Effects of different extraction methods on the release of non-volatile flavor components in shiitake mushroom (*Lentinus edodes*). *Journal of Food Composition and Analysis*, *128*, 106001. https://doi.org/10.1016/j.jfca.2024.106001

Du, M., Xie, J., Gong, B., Xu, X., Tang, W., Li, X., et al. (2018). Extraction, physicochemical characteristics and functional properties of Mung bean protein. *Food Hydrocolloids*, *76*, 131–140. https://doi.org/10.1016/j.foodhyd.2017.01.003

Dutta, R., Sarkar, U., & Mukherjee, A. (2015). Process optimization for the extraction of oil from *Crotalaria juncea* using three phase partitioning. *Industrial Crops and Products*, *71*, 89–96. https://doi.org/10.1016/j.indcrop.2015.03.024

Elizabeth, V. (1991). The roles of heat shock proteins in plants. *Annual review of plant physiology and plant molecular biology*, *42*(1), 579–620. https://doi.org/10.1146/annurev.pp.42.060191.003051

Fan, Y., He, Y., Li, Y., Yin, Z., Shi, J., Tian, T., et al. (2024). Design of a novel EmTSP-3 and EmTIP based multi-epitope vaccine against *Echinococcus multilocularis* infection. *Frontiers in Immunology*, *15*, 1425603. https://doi.org/10.3389/fimmu.2024.1425603

Fang, F. (2003). *Purification characteristics and bioactivity of antiviral protein from the Pleurotus eryngii and Agrocybe sp.* Master diss., Fujian Agriculture and Forestry University.

Fang, X., Chen, Z., Wu, W., Chen, H., Nie, S., & Gao, H. (2022). Effects of different protease treatment on protein degradation and flavor components of *Lentinus edodes*. *eFood*, *3*(6). https://doi.org/10.1002/efd2.41

FAO/WHO. (2007). Protein and amino acid requirements in human nutrition. *World Health Organization Technical Report Series*, *935*, 1–265. https://pubmed.ncbi.nlm.nih.gov/18330140/

Fernandes, P. (2018). *Enzymatic processing in the food industry.* Reference Module in Food Science. Elsevier.

Franca-Oliveira, G., Fornari, T., & Hernández-Ledesma, B. (2021). A review on the extraction and processing of natural source-derived proteins through eco-innovative approaches. *Processes*, *9*(9). https://doi.org/10.3390/pr9091626

Gagaoua, M., & Hafid, K. (2016). Three phase partitioning system, an emerging non-chromatographic tool for proteolytic enzymes recovery and purification. *Biosensors Journal*, *5*(1). https://doi.org/10.4172/2090-4967.1000134

Gaitán-Hernández, R., López-Peña, D., Esqueda, M., & Gutiérrez, A. (2019). Review of bioactive molecules production, biomass, and basidiomata of shiitake culinary-medicinal mushrooms, *Lentinus edodes* (*Agaricomycetes*). *International Journal of Medicinal Mushrooms*, *21*(9), 841–850. https://doi.org/ 10.1615/IntJMedMushrooms.2019031849

Gao, J., Li, X., Jia, S., Zeng, H., & Zheng, B. (2023). Structural characterization and antioxidant activity of a glycoprotein isolated from shiitake mushrooms. *Food*

Bioscience, *53*, 102608. https://doi.org/10.1016/j.fbio.2023.102608

Garg, R., & Thorat, B. N. (2014). Nattokinase purification by three phase partitioning and impact of *t*-butanol on freeze drying. *Separation and Purification Technology*, *131*, 19–26. https://doi.org/10.1016/j.seppur.2014.04.011

Gartshore, A., Kidd, M., & Joshi, L. T. (2021). Applications of microwave energy in medicine. *Biosensors*, *11*(4), 1–13. https://doi.org/10.3390/BIOS11040096

Gautério, G. V., da Silva, R. M., Karraz, F. C., Coelho, M. A. Z., Ribeiro, B. D., & Lemes, A. C. (2023). Cell disruption and permeabilization methods for obtaining yeast bioproducts. *Cleaner Chemical Engineering*, *6*, 100112. https://doi.org/10.1016/j.clce.2023.100112

Gerliani, N., Hammami, R., & Aïder, M. (2019). Assessment of the extractability of protein-carbohydrate concentrate from soybean meal under acidic and alkaline conditions. *Food Bioscience*, *28*, 116–124. https://doi.org/10.1016/j.fbio.2019.01.004

Gmoser, R., Fristedt, R., Larsson, K., Undeland, I., Taherzadeh, M. J., & Lennartsson, P. R. (2020). From stale bread and brewers spent grain to a new food source using edible filamentous fungi. *Bioengineered*, *11*(1), 582–598. https://doi.org/10.1080/21655979.2020.1768694

Goetz, H., Kuschel, M., Wulff, T., & Sauber, C. (2004). Comparison of selected analytical techniques for protein sizing, quantitation and molecular weight determination, *60*, 281–293. https://doi.org/10.1016/j.jbbm.2004.01.007

Goktayoglu, E., Oztop, M. H., & Ozcan, S. (2023). Proteomics approach to differentiate

protein extraction methods in sugar beet leaves. *Journal of Agricultural and Food Chemistry*, *71*(23), 9157–9163. https://doi.org/10.1021/acs.jafc.2c09190

Gong, P., Wang, S., Liu, M., Chen, F., Yang, W., Chang, X., et al. (2020). Extraction methods, chemical characterizations and biological activities of mushroom polysaccharides: A mini-review. *Carbohydrate Research*, *494*, 108037. https://doi.org/10.1016/j.carres.2020.108037

Gouseti, O., Larsen, M. E., Amin, A., Bakalis, S., Petersen, I. L., Lametsch, R., & Jensen, P. E. (2023). Applications of enzyme technology to enhance transition to plant proteins: A Review. *Foods*, *12*(13). https://doi.org/10.3390/foods12132518

Gray, N., Zia, R., King, A., Patel, V. C., Wendon, J., McPhail, M. J. W., et al. (2017). High-speed quantitative UPLC-MS analysis of multiple amines in human plasma and serum via precolumn derivatization with 6-Aminoquinolyl-N-hydroxysuccinimidyl carbamate: Application to acetaminophen-induced liver failure. *Analytical Chemistry*, *89*(4), 2478–2487. https://doi.org/10.1021/acs.analchem.6b04623

Guba, A., Bába, O., Tőzsér, J., Csősz, É., & Kalló, G. (2022). Fast and sensitive quantification of AccQ-Tag derivatized amino acids and biogenic amines by UHPLC-UV analysis from complex biological samples. *Metabolites*, *12*(3), 272. https://doi.org/10.3390/metabo12030272

Guo, L., Dai, H., Ma, J., Wang, J., Hua, Y., & Zhou, L. (2021). Isolation, structure characteristics and antioxidant activity of two water-soluble polysaccharides from *Lenzites betulina*. *BMC Chemistry*, *15*(1), 1–10. https://doi.org/10.1186/s13065-021-

00741-6

Guo, Q., Liu, L. X., & Sun, F. (2007). Advances in pharmacology of fungal immunomodulatory proteins. *Anhui Medicine*, *11*(5), 385–387.

Hagel, L. (1998). Gel-filtration chromatography. *Current protocols in molecular biology, 44(1),* 10-9. https://doi.org/10.1002/0471142727.mb1009s44

He, Y. (2015). *Study on extraction and antioxidant activity of Morchella protein*. Master diss., Shanxi University.

Hibino, Y., Konishi, Y., Koike, J., Tabata, T., Ohashi, Y., & Sugano, N. (1994). Productions of interferon-γ and nitrite are induced in mouse splenic cells by a heteroglycan-protein fraction from culture medium of *Lentinus edodes* mycelia. *Immunopharmacology*, *28*(1), 77–85. https://doi.org/10.1016/0162-3109(94)90041-8

Higuera-Barraza, O. A., Del Toro-Sanchez, C. L., Ruiz-Cruz, S., & Márquez-Ríos, E. (2016). Effects of high-energy ultrasound on the functional properties of proteins. *Ultrasonics Sonochemistry*, *31*, 558–562. https://doi.org/10.1016/j.ultsonch.2016.02.007

Hobbs, C. (2000). Medicinal Value of *Lentinus edodes* (Berk.) Sing. (*Agaricomycetideae*). A Literature Review. *International Journal of Medicinal Mushrooms*, *2*(4), 16. https://doi.org/10.1615/intjmedmushr.v2.i4.90

Holtzer, M. E., & Holtzer, A. (1992). α-helix to random coil transitions: Determination of peptide concentration from the CD at the isodichroic point. *Biopolymers*, *32*(12), 1675–1677. https://doi.org/10.1002/bip.360321209

Hu, D. H. (2019). *Extraction and properties of the Lentinus edodes stem protein and the application of new nutritious bread's development*. Master diss., LiaoNing University.

Huang, L., Sun, F., Liang, C., He, Y. X., Bao, R., Liu, L., & Zhou, C. Z. (2009). Crystal structure of LZ-8 from the medicinal fungus *Ganoderma lucidium*. *Proteins: Structure, Function and Bioinformatics*, *75*(2), 524–527. https://doi.org/10.1002/prot.22346

Huang, X., Ai, C., Yao, H., Zhao, C., Xiang, C., Hong, T., & Xiao, J. (2022). Guideline for the extraction, isolation, purification, and structural characterization of polysaccharides from natural resources. *eFood*, *3*(6), 1–16. https://doi.org/10.1002/efd2.37

Huang, Y., Pu, D., Hao, Z., Liang, L., Zhao, J., Tang, Y., & Zhang, Y. (2022). Characterization of taste compounds and sensory evaluation of soup cooked with sheep tail fat and prickly ash. *Foods*, *11*(7), 896. https://doi.org/10.3390/foods11070896

Huang, Z., Tan, Q. L., Wu, T. X., Zhu, S. J., Yang, Z. T. (2016). Optimization of extraction technology of protein from *Grifola frondosa* mycelium by ultrasonic wave China Brewing, 5–8. https://doi.org/10.11882/j.issn.0254-5071.2016.07.033

Ichikawa, T., & Terada, H. (1979). Estimation of state and amount of phenylalanine residues in proteins by second derivative spectrophotometry. *Biochimica et Biophysica Acta (BBA)-Protein Structure*, *580*(1), 120–128.

Janardhanan, A., Govindan, S., Moorthy, A., Prashanth, K. V. H., Savitha Prashanth, M. R., & Ramani, P. (2024). An alkali-extracted polysaccharide from *Pleurotus eous*

and exploration of its antioxidant and immunomodulatory activities. *Journal of Food Measurement and Characterization*, *18*(4), 2489–2504. https://doi.org/10.1007/s11694-023-02318-4

Jayasinghe, A. M. K., Kirindage, K. G. I. S., Fernando, I. P. S., Kim, K. N., Oh, J. Y., & Ahn, G. (2023). The anti-Inflammatory effect of low molecular weight fucoidan from *Sargassum siliquastrum* in lipopolysaccharide-stimulated RAW 264.7 macrophages via inhibiting NF-κB/MAPK signaling pathways. *Marine Drugs*, *21*(6), 1–16. https://doi.org/10.3390/md21060347

Jeurink, P. V., Noguera, C. L., Savelkoul, H. F. J., & Wichers, H. J. (2008). Immunomodulatory capacity of fungal proteins on the cytokine production of human peripheral blood mononuclear cells. *International Immunopharmacology*, *8*(8), 1124–1133. https://doi.org/10.1016/j.intimp.2008.04.004

Ji, Y., Yang, X., Ji, Z., Zhu, L., Ma, N., Chen, D., et al. (2020). DFT-calculated IR spectrum amide I, II, and III band contributions of *N*-methylacetamide fine components. *ACS Omega*, *5*(15), 8572–8578. https://doi.org/10.1021/acsomega.9b04421

Jiskoot, W., & Crommelin, D. (Eds.). (2005). *Methods for structural analysis of protein pharmaceuticals.* Springer Science & Business Media.

Joint FAO/WHO ad hoc expert committee on energy, & protein requirements. (1973). *Energy and Protein Requirements: Report of a Joint FAO/WHO Ad Hoc Expert Committee, Rome*. Food and Agriculture Organization.

Jungbauer, A., & Hahn, R. (2009). *Ion-exchange chromatography*. *Methods in*

Enzymology (1st ed., Vol. 463). Elsevier Inc.

Kang, Z. L., Zhang, X. hua, Li, X., Song, Z. jun, Ma, H. jun, Lu, F., et al. (2021). The effects of sodium chloride on proteins aggregation, conformation and gel properties of pork myofibrillar protein running head: Relationship aggregation, conformation and gel properties. *Journal of Food Science and Technology*, *58*(6), 2258–2264. https://doi.org/10.1007/s13197-020-04736-4

Kayode, O. S., Siew, L. Q. C., Pillai, P., Haque, R., Rutkowski, K., & Caballero, M. R. (2020). Mushroom allergy: Case series. *Journal of Allergy and Clinical Immunology: In Practice*, *8*(1), 375–379. https://doi.org/10.1016/j.jaip.2019.08.048

Kayode, R. M. O., Olakulehin, T. F., Adedeji, B. S., Ahmed, O., Aliyu, T. H., & Badmos, A. H. A. (2015). Evaluation of amino acid and fatty acid profiles of commercially cultivated oyster mushroom (*Pleurotus sajor-caju*) grown on gmelina wood waste. *Nigerian Food Journal*, *33*(1), 18–21. https://doi.org/10.1016/j.nifoj.2015.04.001

Kelly, S. M., & Price, N. C. (2000). The use of circular dichroism in the investigation of protein structure and function. *Current protein and peptide science*, *1*(4), 349–384. https://doi.org/10.2174/1389203003381315

Ketemepi, H. K., Awang, M. A. Bin, Seelan, J. S. S., & Mohd Noor, N. Q. I. (2024). Extraction process and applications of mushroom-derived protein hydrolysate: A comprehensive review. *Future Foods*, *9*, 100359. https://doi.org/10.1016/j.fufo.2024.100359

Khotchai, W., Therdthai, N., & Ritthiruangdej, P. (2025). Effect of microwave-assisted extraction on quality and taste profiles of crude extracts from split gill mushroom. *Journal of Agriculture and Food Research*, *19*, 101588. https://doi.org/10.1016/j.jafr.2024.101588

Kilcoyne, M., Shah, M., Gerlach, J. Q., Bhavanandan, V., Nagaraj, V., Smith, A. D., et al. (2009). O-glycosylation of protein subpopulations in alcohol-extracted rice proteins. *Journal of Plant Physiology*, *166*(3), 219–232. https://doi.org/10.1016/j.jplph.2008.05.007

Ko, J. L., Lin, S. J., Hsu, C. I., Kao, C. L., & Lin, J. Y. (1997). Molecular cloning and expression of a fungal immunomodulatory protein, FIP-fve, from *Flammulina velutipes*. *Journal of the Formosan Medical Association= Taiwan yi zhi*, *96*(7), 517–524.

Ko, J. L., Hsu, C. I., Lin, R. H., Kao, C. L., & Lin, J. Y. (1995). A new fungal immunomodulatory protein, FIP-fve isolated from the edible mushroom, *Flammulina velutipes* and its complete amino acid sequence. *European Journal of Biochemistry*, *228*(2), 244–249. https://doi.org/10.1111/j.1432-1033.1995.tb20256.x

Kohchi, C., Inagawa, H., Nishizawa, T., & Soma, G. I. (2009). ROS and innate immunity. *Anticancer Research*, *29*(3), 817–822. https://ar.iiarjournals.org/content/29/3/817.long.

Kour, H., Kour, D., Kour, S., Singh, S., Hashmi, S. A. J., Yadav, A. N., ... & Ahluwalia, A. S. (2022). Bioactive compounds from mushrooms: An emerging bioresources of food and nutraceuticals. *Food Bioscience*, *102124*.

Kueltzo, L. A., Ersoy, B., Ralston, J. P., & Middaugh, C. R. (2003). Derivative absorbance spectroscopy and protein phase diagrams as tools for comprehensive protein characterization : A bGCSF case study, *Journal of Pharmaceutical Sciences*, *92*(9), 1805–1820. https://doi.org/10.1002/jps.10439

Kumar, M., Tomar, M., Potkule, J., Verma, R., Punia, S., Mahapatra, A., et al. (2021). Advances in the plant protein extraction: Mechanism and recommendations. *Food Hydrocolloids*, *115*, 106595. https://doi.org/10.1016/j.foodhyd.2021.106595

Kurahashi, A., Sato, M., Nishibori, K., & Fujimori, F. (2014). Heat shock protein 9 mRNA expression increases during fruiting body differentiation in *Grifola frondosa* and other edible mushrooms. *Mycoscience*, *55*(2), 98–102. https://doi.org/10.1016/j.myc.2013.06.001

Landi, N., Grundner, M., Ragucci, S., Pav, M., Pedone, P. V, Sep, K., & Maro, A. Di. (2022). Characterization and cytotoxic activity of ribotoxin-like proteins from the edible mushroom *Pleurotus eryngii*, *396*. https://doi.org/10.1016/j.foodchem.2022.133655

Leung, P. H., Zhao, S., Ho, K. P., & Wu, J. Y. (2009). Chemical properties and antioxidant activity of exopolysaccharides from mycelial culture of *Cordyceps sinensis* fungus Cs-HK1. *Food Chemistry*, *114*(4), 1251–1256. https://doi.org/10.1016/j.foodchem.2008.10.081

Li, B., Lu, F., Tian, Y., Zhao, S. (2010). Fractional purification and structural analysis of *Lentinus edodes* protein. *Natural Product Research and Development*, *2*, 257–260.

Li, B., Zhu, J., Cheng, J., Zhao, S., Tian, Y. (2009). Extraction and preparation methods of protein from *Lentinus edodes*. *Science and Technology of Food Industry*, *6*, 226–228. https://doi.org/10.13386/j.issn1002-0306.2009.06.099

Li, F., Wang, W., Wang, X., & Yu, J. (2014). Changes of structure and property of alkali soluble hydroxyethyl celluloses (HECs) and their regenerated films with the molar substitution. *Carbohydrate Polymers*, *114*, 206–212. https://doi.org/10.1016/j.carbpol.2014.08.015

Li, F., Wen, H. A., Zhang, Y. J., Min, A., & Liu, X. Z. (2011). Purification and characterization of a novel immunomodulatory protein from the medicinal mushroom *Trametes versicolor*, *54*(4), 379–385. https://doi.org/10.1007/s11427-011-4153-2

Li, J., Ma, J., Fan, S., Mi, S., & Zhang, Y. (2022). Comparison of the nutritional and taste characteristics of 5 edible fungus powders based on the composition of hydrolyzed amino acids and free amino acids. *Journal of Food Quality*, *2022(1),* 3618002. https://doi.org/10.1155/2022/3618002

Li, J. H., Zhu, Y. Y., Gu, F. T., & Wu, J. Y. (2023). Efficient isolation of immunostimulatory polysaccharides from *Lentinula edodes* by autoclaving-ultrasonication extraction and fractional precipitation. *International Journal of Biological Macromolecules*, *237*, 124216. https://doi.org/10.1016/j.ijbiomac.2023.124216

Li, L. Q., Song, A. X., Yin, J. Y., Siu, K. C., Wong, W. T., & Wu, J. Y. (2020). Anti-inflammation activity of exopolysaccharides produced by a medicinal fungus

Cordyceps sinensis Cs-HK1 in cell and animal models. *International Journal of Biological Macromolecules*, *149*, 1042–1050. https://doi.org/10.1016/j.ijbiomac.2020.02.022

Li, L., Sheng, J., Sun, J., He, X., Li, J., & Zheng, F. (2015). Review on nutritional value and comprehensive utilization of kumquat and present situation. *Shipin Gongye (Shanghai, China)*, *36*(9), 220–224.

Li, Q., Liu, J., Shang, X., Li, Y., Zhang, L., Li, Z., et al. (2021). Characterizing diversity based on the chemical and nutritional composition of shiitake culinary-medicinal mushroom *Lentinula edodes* (*Agaricomycetes*) commonly cultivated in China. *International journal of medicinal mushrooms*, *23*(8), 51–64. https://doi.org/10.1615/IntJMedMushrooms.2021039240

Li, Q. Z., Wang, X. F., & Zhou, X. W. (2011). Recent status and prospects of the fungal immunomodulatory protein family. *Critical Reviews in Biotechnology*, *31*(4), 365–375. https://doi.org/10.3109/07388551.2010.543967

Li, X., Zhang, Z., Wang, L., Zhao, H., Jia, Y., Ma, X., et al. (2023). Three-phase extraction of polysaccharide from *Stropharia rugosoannulata*: Process optimization, structural characterization and bioactivities. *Frontiers in Immunology*, *13*, 1–14. https://doi.org/10.3389/fimmu.2022.994706

Li, X. F. (2015). *Study on water-soluble protein and active peptide in mushroom stem*. Master diss., Shanxi University.

Li, X. M., Chen, K., Huang, Z. W., Shen Y. G., Lu, J. Q., U., Chen, H. Z., Liu, F. Y. et

al. (2020). Optimization of protein extraction process and its functional properties of white *Hypsizygus marmoreu*, *Food and Fermentation Industries*, *46(4)*, 239–246.

Li, Y., Kiani, H., Tiwari, B. K., & Halim, R. (2022). Unit operations applied to cell disruption of microalgae. *3rd generation biofuels, 225-248.*. https://doi.org/10.1016/B978-0-323-90971-6.00027-9

Li, Z., Hong, T., Shen, G., Gu, Y., Guo, Y., & Han, J. (2022). Amino acid profiles and nutritional evaluation of fresh sweet–waxy corn from three different regions of China. *Nutrients, 14(19),* 3887. https://doi.org/10.3390/nu14193887

Lin, W. H., Hung, C. H., Hsu, C. I., & Lin, J. Y. (1997). Dimerization of the *N*-terminal amphipathic α-helix domain of the fungal immunomodulatory protein from *Ganoderma tsugae* (Fip-gts) defined by a yeast two-hybrid system and site-directed mutagenesis. *Journal of Biological Chemistry*, *272*(32), 20044–20048. https://doi.org/10.1074/jbc.272.32.20044

Lindequist, U., Niedermeyer, T. H. J., & Jülich, W. D. (2005). The pharmacological potential of mushrooms. *Evidence-based Complementary and Alternative Medicine*, *2*(3), 285–299. https://doi.org/10.1093/ecam/neh107

Liu, Y. F., Chang, S. H., Sun, H. L., Chang, Y. C., Hsin, I. L., Lue, K. H., & Ko, J. L. (2012). IFN-γ Induction on carbohydrate binding module of fungal immunomodulatory protein in human peripheral mononuclear cells. *Journal of Agricultural and Food Chemistry*, *60*(19), 4914–4922.

Liu, D., Lebovka, N. I., & Vorobiev, E. (2013). Impact of electric pulse treatment on

selective extraction of intracellular compounds from *Saccharomyces cerevisiae* yeasts. *Food and Bioprocess Technology*, *6*(2), 576–584. https://doi.org/10.1007/s11947-011-0703-7

Liu, G. J., Zheng, H. H., Jiang, Y., Sang, H., Xue, J., Ji, G. H., Zhang, L. (2020). Study on the preparation technology of *Hericium erinaceus* mycelium protein. *Storage and processing*, *42*(2), 74–78. https://doi.org/10.1016/j.fochx.2021.100172

Liu, M., Li, J., & Kong, F. (1999). Purification, characterization and immunological function of a new polysaccharide-protein complex (LE) from cultured mycelia of *Lentinus edodes*. *Sheng wu hua xue yu sheng wu wu li xue bao Acta biochimica et biophysica Sinica*. *31(1)*, 46-50.

Liu Q., Li C., Geng Z. H., Z. Q. C. (2013). Optimization of prepartion of protein from *Pleurotus eryngii* via alkali extraction and acid precipitation. *Process technology*, *34*(10), 145–148.

Liu, Y. R., You, J. K., Deng, Y. Y., Wang, J., Yang, L. M., Chi, W. (2021). Research progress on the structure, carbohydrate specificity and immunoregulation of macrofungal Lectins. *Edible Fugni of China*, *40*(12), 1–9.

Liu, Y. R., Zhao, J., Zhao, Y., Zong, S., Tian, Y., Chen, S., et al. (2019). Therapeutic effects of lentinan on inflammatory bowel disease and colitis-associated cancer. *Journal of Cellular and Molecular Medicine*, *23*(2), 750–760. https://doi.org/10.1111/jcmm.13897

Liu, Y., Bastiaan-Net, S., & Wichers, H. J. (2020). Current understanding of the

structure and function of fungal immunomodulatory proteins. *Frontiers in Nutrition*, *7*(132), 1–17. https://doi.org/10.3389/fnut.2020.00132

Liu, Z. D., Yu, D., Li, L., Liu, X., Zhang, H., Sun, W., et al. (2019). Three-phase partitioning for the extraction and purification of polysaccharides from the immunomodulatory medicinal mushroom *Inonotus obliquus*. *Molecules*, *24*(3), 1–14. https://doi.org/10.3390/molecules24030403

Liu, Z. Q., Zheng, Z., Zhu, G., Luo, S., Zhang, D., Liu, F., & Shen, Y. (2021). Modification of the structural and functional properties of wheat gluten protein using a planetary ball mill. *Food Chemistry*, *363*, 130251. https://doi.org/10.1016/j.foodchem.2021.130251

Luan, H. Q. (2017). *Study on expression, purification and antitumor activity of Lentinula edodes latcripin-8 protein*. Master diss., Da Lian Medical University.

Luiking, Y. C., Engelen, M. P. K. J., & Deutz, N. E. P. (2010). Regulation of nitric oxide production in health and disease. *Current Opinion in Clinical Nutrition and Metabolic Care*, *13*(1), 97–104. https://doi.org/10.1097/MCO.0b013e328332f99d

Lupatini, A. L., de Oliveira Bispo, L., Colla, L. M., Costa, J. A. V., Canan, C., & Colla, E. (2017). Protein and carbohydrate extraction from *S. platensis* biomass by ultrasound and mechanical agitation. *Food Research International*, *99*, 1028–1035. https://doi.org/10.1016/j.foodres.2016.11.036

Ma, Z. Q., Zhang, B. S., Zhao, S. X., Zhang, Y. T. (2012). Optimization of ultrasonic-assisted extraction of protein from *Agricus bisporus* by response surface methodology.

Science and Technology of Food Industry, *33*, 229–233. https://doi.org/10.13386/j.issn1002-0306.2012.23.044

Mancheñ O, J. M., Tateno, H., Goldstein, I. J., Martínez-Ripoll, M., & Hermoso, J. A. (2005). Structural analysis of the *Laetiporus sulphureus* hemolytic pore-forming lectin in complex with sugars. *Journal of Biological Chemistry*, 280(17), 17251-17259. https://doi.org/10.1074/jbc.M413933200

Mantovani, M. S., Bellini, M. F., Angeli, J. P. F., Oliveira, R. J., Silva, A. F., & Ribeiro, L. R. (2008). Beta-Glucans in promoting health: prevention against mutation and cancer. *Mutation research*, *658*(3), 154–161. https://doi.org/10.1016/J.MRREV.2007.07.002

Manzi, P., Gambelli, L., Marconi, S., Vivanti, V., & Pizzoferrato, L. (1999). Nutrients in edible mushrooms: an inter-species comparative study. *Food Chemistry*, *65*(4), 477–482. https://doi.org/10.1016/S0308-8146(98)00212-X

Marcelino, A. M. C., & Gierasch, L. M. (2008). Roles of β-Turns in protein folding: from peptide models to protein engineering. *Biopolymers: Original Research on Biomolecules.*, *89*(5), 380–91. https://doi.org/10.1002/bip.20960

Miyazawa, M. (1998). Second derivative near infrared studies on the structural characterisation of proteins, *Journal of Near Infrared Spectroscopy*, 6(A), A253-A257. https://doi.org/10.1255/jnirs.204

Mohanta, T. K., Mishra, A. K., Khan, A., & Hashem, A. (2021). Virtual 2 - D map of the fungal proteome. *Scientific Reports*, 1–13. https://doi.org/10.1038/s41598-021-86201-6

Mohapatra, S., Bhakuni, P., Barman, S. R., & Nayak, B. (2024). RSM-CCD optimized hollow mesoporous silica nanospheres encapsulating sorafenib induce mitochondrial membrane potential-mediated apoptotic cell death in non-small cell lung cancer. *Microporous and Mesoporous Materials*, 113032. https://doi.org/10.1016/j.micromeso.2024.113032

Muñoz-Castiblanco, T., Mejía-Giraldo, J. C., & Puertas-Mejía, M. Á. (2022). *Lentinula edodes*, a novel source of polysaccharides with antioxidant power. *Antioxidants*, *11*(9). https://doi.org/10.3390/antiox11091770

Nakazawa, H., Okada, I., Ito, T., Ishigaki, Y., Kumagai, I., & Umetsu, M. (2024). Combinatorial optimization of the hybrid cellulase complex structure designed from modular libraries. *Scientific reports*, *14*(1), 22429. https://doi.org/10.1038/s41598-024-73541-2

Nehete, J. Y., Bhambar, R. S., Narkhede, M. R., & Gawali, S. R. (2013). Natural proteins : Sources, isolation, characterization and applications. *Pharmacognosy reviews*, *7*(14), 107. https://doi.org/10.4103/0973-7847.120508

Nemer, G., Louka, N., Vorobiev, E., Salameh, D., Nicaud, J. M., Maroun, R. G., & Koubaa, M. (2021). Mechanical cell disruption technologies for the extraction of dyes and pigments from microorganisms: A review. *Fermentation*, *7*(1), 1–17. https://doi.org/10.3390/fermentation7010036

Ngai, P. H. K., & Ng, T. B. (2003). Lentin, a novel and potent antifungal protein from shitake mushroom with inhibitory effects on activity of human immunodeficiency

virus-1 reverse transcriptase and proliferation of leukemia cells. *Life Sciences*, *73*(26), 3363–3374. https://doi.org/10.1016/j.lfs.2003.06.023

Niego, A. G., Rapior, S., Thongklang, N., Raspé, O., Jaidee, W., Lumyong, S., & Hyde, K. D. (2021). Macrofungi as a nutraceutical source: promising bioactive compounds and market value. *Journal of Fungi*, *7*(5). https://doi.org/10.3390/jof7050397

Nishimura, T., & Kato, H. (1988). Taste of free amino acids and peptides. *Food Reviews International*, *4*(2), 175–194. https://doi.org/10.1080/87559128809540828

Niu, R. (2010). Progress in research of protein separation and purification techniques. *Chemical Technology Market*, *4*, 16–18.

Nord, L. I., Vaag, P., & Duus, J. (2004). Quantification of organic and amino acids in beer by 1H NMR spectroscopy. *Analytical Chemistry*, *76*(16), 4790–4798. https://doi.org/10.1021/ac0496852

Novák, P., & Havlíček, V. (2016). Protein extraction and precipitation. *Proteomic Profiling and Analytical Chemistry: The Crossroads: Second Edition*, 52–62. https://doi.org/10.1016/B978-0-444-63688-1.00004-5

Ooi, V. E. C., & Liu, F. (2012). Immunomodulation and anti-cancer activity of polysaccharide-protein complexes. *Current Medicinal Chemistry*, *7*(7), 715–729. https://doi.org/10.2174/0929867003374705

Otero, M. A., Wagner, J. R., Vasallo, M. C., García, L., & Añón, M. C. (2000). Thermal behavior and hydration properties of yeast proteins from *Saccharomyces cerevisiae* and *Kluyveromyces fragilis*. *Food Chemistry*, *69*(2), 161–165.

https://doi.org/10.1016/S0308-8146(99)00245-9

Ou, C. C., Hsiao, Y. M., Wang, W. H., Ko, J. L., & Lin, M. Y. (2009). Stability of fungal immunomodulatory protein, FIP-gts and FIP-fve, in IFN-γ production. *Food and Agricultural Immunology*, *20*(4), 319–332. https://doi.org/10.1080/09540100903247688

Paaventhan, P., Joseph, J. S., Seow, S. V., Vaday, S., Robinson, H., Chua, K. Y., & Kolatkar, P. R. (2003). A 1.7 Å structure of Fve, a member of the new fungal immunomodulatory protein family. *Journal of Molecular Biology*, *332*(2), 461–470. https://doi.org/10.1016/S0022-2836(03)00923-9

Paisansak, S., Sangtanoo, P., Srimongkol, P., Saisavoey, T., Reamtong, O., Choowongkomon, K., & Karnchanatat, A. (2021). Angiotensin-I converting enzyme inhibitory peptide derived from the shiitake mushroom (*Lentinula edodes*). *Journal of Food Science and Technology*, *58*(1), 85–97. https://doi.org/10.1007/s13197-020-04517-z

Pakhale, S. V., & Bhagwat, S. S. (2016). Purification of serratiopeptidase from *Serratia marcescens* NRRL B 23112 using ultrasound assisted three phase partitioning. *Ultrasonics Sonochemistry*, *31*, 532–538. https://doi.org/10.1016/j.ultsonch.2016.01.037

Park, C., Cha, H. J., Lee, H., Kim, G. Y., & Choi, Y. H. (2021). The regulation of the TLR4/NF-κB and Nrf2/HO-1 signaling pathways is involved in the inhibition of lipopolysaccharide-induced inflammation and oxidative reactions by morroniside in

RAW 264.7 macrophages. *Archives of Biochemistry and Biophysics*, *706*, 108926. https://doi.org/10.1016/j.abb.2021.108926

Patil, P. D., & Yadav, G. D. (2018). Application of microwave assisted three phase partitioning method for purification of laccase from *Trametes hirsuta*. *Process Biochemistry*, *65*, 220–227. https://doi.org/10.1016/j.procbio.2017.10.006

Pérez Montes, A., Rangel-Vargas, E., Lorenzo, J. M., Romero, L., & Santos, E. M. (2021). Edible mushrooms as a novel trend in the development of healthier meat products. *Current Opinion in Food Science*, *37*, 118–124. https://doi.org/10.1016/j.cofs.2020.10.004

Phong, W. N., Show, P. L., Ling, T. C., Juan, J. C., Ng, E. P., & Chang, J. S. (2018). Mild cell disruption methods for bio-functional proteins recovery from microalgae-Recent developments and future perspectives. *Algal Research*, *31*, 506–516. https://doi.org/10.1016/j.algal.2017.04.005

Pobiega, K., Sękul, J., Pakulska, A., Latoszewska, M., Michońska, A., Korzeniowska, Z., et al. (2024). Fungal proteins: Sources, production and purification methods, industrial applications, and future perspectives. *Applied Sciences*, *14*(14), 6259. https://doi.org/10.3390/app14146259

Pohleven, J., Obermajer, N., Sabotič, J., Anžlovar, S., Sepčić, K., Kos, J., et al. (2009). Purification, characterization and cloning of a ricin B-like lectin from mushroom *Clitocybe nebularis* with antiproliferative activity against human leukemic T cells. *Biochimica et Biophysica Acta - General Subjects*, *1790*(3), 173–181.

https://doi.org/10.1016/j.bbagen.2008.11.006

Ponnusamy, C., Uddandrao, V. V. S., Pudhupalayam, S. P., Singaravel, S., Periyasamy, T., Ponnusamy, P., et al. (2022). *Lentinula Edodes* (edible Mushroom) as a Nutraceutical: A Review. *Biosciences Biotechnology Research Asia*, *19*(1), 1–11. https://doi.org/10.13005/bbra/2964

Portnoy, J. M., Williams, P. B., & Barnes, C. S. (2016). Innate immune responses to fungal allergens. *Current Allergy and Asthma Reports*, *16*(9), 1–6. https://doi.org/10.1007/s11882-016-0643-4

Prandi, B., Cigognini, I. M., Faccini, A., Zurlini, C., Rodríguez, Ó., & Tedeschi, T. (2023). Comparative study of different protein extraction eechnologies applied on mushrooms by-products. *Food and Bioprocess Technology*, *16*(7), 1570–1581. https://doi.org/10.1007/s11947-023-03015-2

Pushparajah, V., Fatima, A., Chong, C. H., Gambule, T. Z., Chan, C. J., Ng, S. T., et al. (2016). Characterisation of a new fungal immunomodulatory protein from tiger milk mushroom, *Lignosus rhinocerotis*. *Scientific Reports*, *6*, 1–12. https://doi.org/10.1038/srep30010

Quintana, F. J., & Cohen, I. R. (2011). The HSP60 immune system network. *Trends in Immunology*, *32*(2), 89–95. https://doi.org/10.1016/j.it.2010.11.001

Rahman, M. M., & Lamsal, B. P. (2021). Ultrasound-assisted extraction and modification of plant-based proteins: Impact on physicochemical, functional, and nutritional properties. *Comprehensive Reviews in Food Science and Food Safety*, *20*(2),

1457–1480. https://doi.org/10.1111/1541-4337.12709

Rajagopalan, A., & Sukumaran, B. O. (2018). Three phase partitioning to concentrate milk clotting proteases from *Wrightia tinctoria R. Br* and its characterization. *International Journal of Biological Macromolecules*, *118*, 279–288. https://doi.org/10.1016/j.ijbiomac.2018.06.042

Rajeeva, S., & Lele, S. S. (2011). Three-phase partitioning for concentration and purification of laccase produced by submerged cultures of *Ganoderma sp.* WR-1. *Biochemical Engineering Journal*, *54*(2), 103–110. https://doi.org/10.1016/j.bej.2011.02.006

Ramlal, A., & Samanta, A. (2022). In silico functional and phylogenetic analyses of fungal immunomodulatory proteins of some edible mushrooms. *AMB Express*, *12*(1). https://doi.org/10.1186/s13568-022-01503-w

Rappa, F., Farina, F., Zummo, G., David, S., Campanella, C., Carini, F., et al. (2012). HSP-Molecular chaperones in cancer biogenesis and tumor therapy: An overview. *Anticancer Research*, *32*(12), 5139–5150.

Rathore, H., Prasad, S., & Sharma, S. (2017). Mushroom nutraceuticals for improved nutrition and better human health: A review. *PharmaNutrition*, *5*(2), 35–46. https://doi.org/10.1016/j.phanu.2017.02.001

Reis, F. S., Martins, A., Vasconcelos, M. H., Morales, P., & Ferreira, I. C. F. R. (2017). Functional foods based on extracts or compounds derived from mushrooms. *Trends in Food Science and Technology*, *66*, 48–62. https://doi.org/10.1016/j.tifs.2017.05.010

Reis, R. Van, & Zydney, A. L. (2010). Protein ultrafiltration. *Encyclopedia of Industrial Biotechnology*, 1–20. https://doi.org/10.1002/9780470054581.eib515

Rezvani, V., Pourianfar, H. R., Mohammadnejad, S., Madjid Ansari, A., & Farahmand, L. (2020). Anticancer potentiality and mode of action of low-carbohydrate proteins and peptides from mushrooms. *Applied Microbiology and Biotechnology*, *104*(16), 6855–6871. https://doi.org/10.1007/s00253-020-10707-8

Ritota, M., Marini, F., Sequi, P., & Valentini, M. (2010). Metabolomic characterization of italian sweet pepper (*Capsicum annum L.*) by means of HRMAS-NMR spectroscopy and multivariate analysis. *Journal of Agricultural and Food Chemistry*, *58*(17), 9675–9684. https://doi.org/10.1021/jf1015957

Rivillas-Acevedo, L. A., & Soriano-García, M. (2006). Antimicrobial peptides from plants as mechanism of defense. *Actual Biol*, *28*(85), 155–169. https://doi.org/10.17533/udea.acbi.329395

Rodrigues, R. L., Menezes, G. D. L., Saivish, M. V., Costa, V. G. Da, Pereira, M., Moreli, M. L., & Silva, R. A. Da. (2019). Prediction of MAYV peptide antigens for immunodiagnostic tests by immunoinformatics and molecular dynamics simulations. *Scientific Reports*, *9*(1), 1–10. https://doi.org/10.1038/s41598-019-50008-3

Roncero-Ramos, I., & Delgado-Andrade, C. (2017). The beneficial role of edible mushrooms in human health. *Current Opinion in Food Science*, *14*, 122–128. https://doi.org/10.1016/j.cofs.2017.04.002

Rose, J. C. (1956). Electrophoresis. *Gp*, *14*(5), 101–102.

https://doi.org/10.1142/9781783266739_0003

Roy, I., & Gupta, M. N. (2002). Three-phase affinity partitioning of proteins. *Analytical Biochemistry*, *300*(1), 11–14. https://doi.org/10.1006/abio.2001.5367

Royer, C. A. (2006). Probing protein folding and conformational transitions with fluorescence. *Chemical reviews*, *106*(5), 1769–1784. ttps://doi.org/10.1021/cr0404390

Ru, Z., Xu, M., Zhu, G., Tu, Y., Jiang, Y., & Du, H. (2021). Ovotransferrin exerts bidirectional immunomodulatory activities via TLR4-mediated signal transduction pathways in RAW264.7 cells. *Food Science and Nutrition*, *9*(11), 6162–6175. https://doi.org/10.1002/fsn3.2569

Xu, D. Z. (2023). *Immune regulatory functions of biologically active proteins from Cordyceps Militaris*. Master diss., Nanjing University of Finance and Economics.

Scholtmeijer, K., van den Broek, L. A. M., Fischer, A. R. H., & van Peer, A. (2023). Potential protein production from lignocellulosic materials using edible mushroom forming fungi. *Journal of Agricultural and Food Chemistry*, *71*(11), 4450–4457. https://doi.org/10.1021/acs.jafc.2c08828

Schweiggert-Weisz, U., Eisner, P., Bader-Mittermaier, S., & Osen, R. (2020). Food proteins from plants and fungi. *Current Opinion in Food Science*, *32*, 156–162. https://doi.org/10.1016/j.cofs.2020.08.003

Scott, P. (2017). Global panel on agriculture and food systems for nutrition: food systems and diets: facing the challenges of the 21st century. *Food Security*, *9*(3), 653–654. https://doi.org/10.1007/s12571-017-0678-y

Seow, S. V., Kuo, I. C., Paaventhan, P., Kolatkar, P. R., & Chua, K. Y. (2003). Crystallization and preliminary X-ray crystallographic studies on the fungal immunomodulatory protein Fve from the golden needle mushroom (*Flammulina velutipes*). *Acta Crystallographica - Section D Biological Crystallography*, *59*(8), 1487–1489. https://doi.org/10.1107/S0907444903011879

Sharon, N., & Lis, H. (1989). Lectins as cell recognition molecules. *Science*, *246*(4927), 227–234. http://www.jstor.org/stable/1704348

Shen, L., Pang, S., Zhong, M., Sun, Y., Qayum, A., Liu, Y., et al. (2023). A comprehensive review of ultrasonic assisted extraction (UAE) for bioactive components: Principles, advantages, equipment, and combined technologies. *Ultrasonics Sonochemistry*, *101*, 106646. https://doi.org/10.1016/j.ultsonch.2023.106646

Sheng, K., Wang, C., Chen, B., Kang, M., Wang, M., Liu, K., & Wang, M. (2021). Recent advances in polysaccharides from *Lentinus edodes* (Berk.): Isolation, structures and bioactivities. *Food Chemistry*, *358*, 129883. https://doi.org/10.1016/j.foodchem.2021.129883

Shi, R., He, Y., Wang, Q., Cai, J., Gantumur, M. A., & Jiang, Z. (2024). Insight into the physicochemical characteristics, functionalities and digestion behavior of protein isolate derived from *Lactarius volemus* (*L. volemus*): Impacts of microwave-assisted extraction. *Food Chemistry*, *431*, 137070. https://doi.org/10.1016/j.foodchem.2023.137070

Shi, L. (2008). *Eucommia ulmoides protein purification, sequence analysis and antibacterial activity study*. Master diss., Guizhou University.

Sierra-Patev, S., Min, B., Naranjo-Ortiz, M., Looney, B., Konkel, Z., Slot, J. C., ... & Hibbett, D. (2023). A global phylogenomic analysis of the shiitake genus *Lentinula*. *Proceedings of the National Academy of Sciences*, *120*(10), 2017. https://doi.org/10.1073/pnas

Singh, B. R. (2000). Basic aspects of the technique and applications of infrared spectroscopy of peptides and proteins. *ACS Symposium Series*, *750*, 2–37. https://doi.org/10.1021/bk-2000-0750.ch001

Singh, M., Kamal, S., & Sharma, V. P. (2022). Species and region-wise mushroom production in leading mushroom producing countries - China, Japan, USA, Canada and India. *Mushroom Research*, *30*(2), 99. https://doi.org/10.36036/mr.30.2.2021.119394

Singh, R. S., Bhari, R., & Kaur, H. P. (2010). Mushroom lectins: current status and future perspectives. *Critical reviews in biotechnology*, *30*(2), 99–126. https://doi.org/10.3109/07388550903365048

Siu, K. C. (2015). *Isolation and structural characterization of mushroom polysaccharides in relationship with their antioxidant activities*. PhD diss. The Hong Kong Polytechnic University.

Siu, K. C., Chen, X., & Wu, J. Y. (2014). Constituents actually responsible for the antioxidant activities of crude polysaccharides isolated from mushrooms. *Journal of Functional Foods*, *11*(C), 548–556. https://doi.org/10.1016/j.jff.2014.08.012

Siu, K. C., Xu, L., Chen, X., & Wu, J. Y. (2016). Molecular properties and antioxidant activities of polysaccharides isolated from alkaline extract of wild *Armillaria ostoyae* mushrooms. *Carbohydrate Polymers*, *137*, 739–746. https://doi.org/10.1016/j.carbpol.2015.05.061

Sow, W. J. C., & Du, J. (2024). Effects of high-pressure homogenization and ultrasound on the composition, structure, and physicochemical properties of proteins extracted from *Nannochloropsis Oceania*. *Ultrasonics Sonochemistry*, 105, 106851. https://doi.org/10.1016/j.ultsonch.2024.106851

Stephan, A., Ahlborn, J., Zajul, M., & Zorn, H. (2018). Edible mushroom mycelia of *Pleurotus sapidus* as novel protein sources in a vegan boiled sausage analog system: functionality and sensory tests in comparison to commercial proteins and meat sausages. *European Food Research and Technology*, *244*(5), 913–924. https://doi.org/10.1007/s00217-017-3012-1

Stine, G. J., Strickland, W. N., & Barratt, R. W. (1964). Methods of protein extraction from *neurospora carassa*. *Canadian Journal of Microbiology*, *10*(1), 29–35. https://doi.org/10.1139/m64-005

Sun, J., Wang, W., & Yue, Q. (2016). Review on microwave-matter interaction fundamentals and efficient microwave-associated heating strategies. *Materials*, *9*(4). https://doi.org/10.3390/ma9040231

Sun, X., Sarteshnizi, R. A., & Udenigwe, C. C. (2022). Recent advances in protein–polyphenol interactions focusing on structural properties related to antioxidant

activities. *Current Opinion in Food Science*, *45*, 100840. https://doi.org/10.1016/j.cofs.2022.100840

Švajger, U., Pohleven, J., Kos, J., Štrukelj, B., & Jeras, M. (2011). CNL, a ricin B-like lectin from mushroom *Clitocybe nebularis*, induces maturation and activation of dendritic cells via the toll-like receptor 4 pathway. *Immunology*, *134*(4), 409–418. https://doi.org/10.1111/j.1365-2567.2011.03500.x

Tam, S., Khan, Z. I., Chen, J., Law, H. K., Yip, J. H., Yan, H., & Tam, T. (2023). Proteomic profiling of chemotherapy responses in FOLFOX-resistant colorectal cancer cells. *International journal of molecular sciences*, 24(12), 9899. https://doi.org/10.3390/ijms24129899

Tan, Z. J., Wang, C. Y., Yi, Y. J., Wang, H. Y., Zhou, W. L., Tan, S. Y., & Li, F. F. (2015). Three phase partitioning for simultaneous purification of aloe polysaccharide and protein using a single-step extraction. *Process Biochemistry*, *50*(3), 482–486. https://doi.org/10.1016/j.procbio.2015.01.004

Tanaka, S., Ko, K., Kino, K., Tsuchiya, K., Yamashita, A., Murasugi, A., et al. (1989). Complete amino acid sequence of an immunomodulatory protein, ling zhi-8 (LZ-8). *Journal of Biological Chemistry*, *264*(28), 16372–16377. https://doi.org/10.1016/s0021-9258(19)84715-4

Taskova, R. M., Zorn, H., Krings, U., Bouws, H., & Berger, R. G. (2006). A comparison of cell wall disruption techniques for the isolation of intracellular metabolites from *Pleurotus and Lepista sp*. *Zeitschrift für Naturforschung C*, *61*(5–6), 347–350.

https://doi.org/10.1515/znc-2006-5-608

Tie, W. F., Jia, J. Z., Guo, W. P. (2016). Advances of research on the structure and function of fungal immunomodulatory proteins. *Journal of Anhui Agri. Sci.*, *44*(35), 20–24. https://doi.org/10.13989/j.cnki.0517-6611.2016.35.007

Tiwari, S., Thakur, R., & Shankar, J. (2015). Role of heat-shock proteins in cellular function and in the biology of fungi. *Biotechnology Research International*, *2015*, 1–11. https://doi.org/10.1155/2015/132635

Trinchieri, G., & Sher, A. (2007). Cooperation of Toll-like receptor signals in innate immune defence. *Nature Reviews Immunology*, *7*(3), 179–190. https://doi.org/10.1038/nri2038

Tsan, M. F., & Gao, B. (2004). Heat shock protein and innate immunity. *Cellular & molecular immunology*, *1*(4), 274–279.

Turker, I., & Isleroglu, H. (2024). Ultrasound and enzymatic treatments to improve protein extraction from cress seeds, and the characterization of protein isolates. *Food Bioscience*, *60*, 104443. https://doi.org/10.1016/j.fbio.2024.104443

Valverde, M., Hernández, T., & Paredes, O. (2015). Edible mushrooms: improving human health and promoting quality life. *International journal of microbiology*, *2015*, 1–14. https://doi.org/10.1155/2015/376387

Van Noort, J. M., Bsibsi, M., Nacken, P., Gerritsen, W. H., & Amor, S. (2012). The link between small heat shock proteins and the immune system. *International Journal of Biochemistry and Cell Biology*, *44*(10), 1670–1679.

https://doi.org/10.1016/j.biocel.2011.12.010

Vetal, M. D., & Rathod, V. K. (2015). Three phase partitioning a novel technique for purification of peroxidase from orange peels (*Citrus sinenses*). *Food and Bioproducts Processing*, *94*, 284–289. https://doi.org/10.1016/j.fbp.2014.03.007

Walport, L. J., Mackay, J. P., Low, J. K. K., & Matthews, J. M. (2021). The characterization of protein interactions – what, how and how much? *Chemical Society Reviews.*, (Section 3), 12292–12307. https://doi.org/10.1039/d1cs00548k

Wan Rosli, W. I., & Solihah, M. A. (2014). Nutritive qualities of patties prepared with mixture of meat and oyster mushroom. *International Food Research Journal*, *21*(5), 2001–2006.

Wang, Z. B., Chen, B. B., Luo, L., & Yan, J. K. (2016). Fractionation, physicochemical characteristics and biological activities of polysaccharides from *Pueraria lobata* roots. *Journal of the Taiwan Institute of Chemical Engineers*, *67*, 54–60. https://doi.org/10.1016/j.jtice.2016.07.029

Wang, H., Geng, H., Chen, J., Wang, X., Li, D., Wang, T., et al. (2020). Three phase partitioning for simultaneous extraction of oil, protein and polysaccharide from rice bran. *Innovative Food Science and Emerging Technologies*, *65*, 102447. https://doi.org/10.1016/j.ifset.2020.102447

Wang, J. Z., Fan, M. (2001). *Handbook of Protein Technology*. Beinjing : Science Press.

Wang, M. L., Hou, Y. Y., Chiu, Y. S., & Chen, Y. H. (2013). Immunomodulatory activities of Gelidium amansii gel extracts on murine RAW 264.7 macrophages.

Journal of Food and Drug Analysis, *21*(4), 397–403. https://doi.org/10.1016/j.jfda.2013.09.002

Wang, R., Yun, J., Wu, S., Bi, Y., & Zhao, F. (2022). Optimisation and characterisation of novel angiotensin-converting enzyme inhibitory peptides prepared by double enzymatic hydrolysis from *Agaricus bisporus Scraps*. *Foods*, *11*(3). https://doi.org/10.3390/foods11030394

Wang, W., Tan, J., Nima, L., Sang, Y., Cai, X., & Xue, H. (2022). Polysaccharides from fungi: A review on their extraction, purification, structural features, and biological activities. *Food Chemistry: X*, *15*, 100414. https://doi.org/10.1016/j.fochx.2022.100414

Wang, X. M., Zhang, J., Wu, L. H., Zhao, Y. L., Li, T., Li, J. Q., et al. (2014). A mini-review of chemical composition and nutritional value of edible wild-grown mushroom from China. *Food Chemistry*, *151*, 279–285. https://doi.org/10.1016/j.foodchem.2013.11.062

Wang, Y. T., Niu, H. M., Ma, Y., Yuan, G. X. (2024). Isolation, purification, fractionation, and hepatoprotective activity of polygonatum polysaccharides. *Molecules.*, *29*(5), 1038. https://doi.org/10.3390/molecules29051038.

Wang, Y. Y., Ma, H., Yan, J. K., Wang, K. Di, Yang, Y., Wang, W. H., & Zhang, H. N. (2019). Three-phase partitioning system with dimethyl carbonate as organic phase for partitioning of exopolysaccharides from *Phellinus baumii*. *International Journal of Biological Macromolecules*, *131*, 941–948.

https://doi.org/10.1016/j.ijbiomac.2019.03.149

Wang, Y. Y., Qiu, W. Y., Wang, Z. Bin, Ma, H. Le, & Yan, J. K. (2017). Extraction and characterization of anti-oxidative polysaccharide-protein complexes from *Corbicula fluminea* through three-phase partitioning. *RSC Advances*, *7*(18), 11067–11075. https://doi.org/10.1039/c7ra00117g

Wang, Z. H. , Fan, X., Yao, F., Yin, C., Shi, D., Gao, H., & Shen, W. (2023). Optimization of extraction, functional characteristic, and amino acid evaluation of *Lentinula edodes* protein. *Modern Food Science and Technology*, *39*(6), 186–194. https://doi.org/10.13982/j.mfst.1673-9078.2023.6.0627

Wasser, S. P. (2004). Shiitake (*Lentinus edodes*). *Encyclopedia of Dietary Supplements*, 653–664. https://doi.org/10.1081/E-EDS-120024880

Wong, J. H., Ng, T. B., Cheung, R. C. F., Ye, X. J., Wang, H. X., Lam, S. K., et al. (2010). Proteins with antifungal properties and other medicinal applications from plants and mushrooms. *Applied Microbiology and Biotechnology*, *87*(4), 1221–1235. https://doi.org/10.1007/s00253-010-2690-4

Wu, J. (2019). Power ultrasound for extraction and modification of polysaccharides from medicinal fungi. *In Advances in food processing technology (pp. 101-124). Singapore: Springer Singapore*. https://doi.org/10.1007/978-981-13-6451-8

Wu, J. Q., Wu, Z. L. (2015). Study on extraction technology of mushroom polypeptide. *Light Industry Science and Technology*, *9*, 21–23.

Wu, M. Y., Hsu, M. F., Huang, C. S., Fu, H. Y., Huang, C. T., & Yang, C. S. (2007).

A 2.0 Å structure of GMI , a member of the fungal immunomodulatory protein family from *Ganoderma microsporum*. *Protein crystallography*, *2*, 132.

Xiang, D., Ma, Y., Yao, J., Zhao, M., Li, H., Marchioni, E., & Zhou, L. (2025). Discriminate shiitake mushroom (*Lentinus edodes*) from different origins by lipidomics based on UHPLC-QE HR-AM/MS/MS. *Journal of Food Composition and Analysis*, *137*(PA), 106957. https://doi.org/10.1016/j.jfca.2024.106957

Xiao, L. I., Chang, W., Yan, Z. I., Tong, C., Zhi, H. A. O., & Wen, Y. U. (2015). Study on the extraction technology and molecular composition of water − soluble protein from *lentinus edodes* stems. *Food Industry Technology*, *36*(09), 343–349.

Xiao, D. Z., Zongman, Z., Ding, Z., & Tao, W. (2017). Advance in the research of proteins technology separation by pressure-driven membrane. *Guangdong Chemical Industry*, 21–24.

Xu, X. Y., Cui W.Y., Huang, Z. T., Gong, Z. Q., Wang, W. L., Wang, Y. S. (2020). Optimization of protein preparation of *Pleurotus eryngii* by ultrasonic assisted cellulase. *Food Research And Development*, *41*, 108–114.

Xu, J., Xu, D., Hu, Q., Ma, N., Pei, F., Su, A., & Ma, G. (2023). Immune regulatory functions of biologically active proteins from edible fungi. *Frontiers in Immunology*, *13*, 1–22. https://doi.org/10.3389/fimmu.2022.1034545

Xu, L., Guo, S., Li, Y., Guo, W., Guo, X., & Hong, S. (2023). Ultrasound-assisted enzymatic extraction and bioactivity analysis of polypeptides from *Cordyceps militaris*. *Journal of Chemistry*, *2023*. https://doi.org/10.1155/2023/1233867

Xu, X., Yan, H., Chen, J., & Zhang, X. (2011). Bioactive proteins from mushrooms. *Biotechnology Advances*, *29*(6), 667–674. https://doi.org/10.1016/j.biotechadv.2011.05.003

Xu, X., Yan, H., Tang, J., Chen, J., & Zhang, X. (2014). Polysaccharides in *Lentinus edodes*: Isolation, structure, immunomodulating activity and future prospective. *Critical Reviews in Food Science and Nutrition*, *54*(4), 474–487. https://doi.org/10.1080/10408398.2011.587616

Xu, X., Yan, H., & Zhang, X. (2012). Structure and immuno-stimulating activities of a new heteropolysaccharide from *lentinula edodes*. *Journal of Agricultural and Food Chemistry*, *60*(46), 11560–11566. https://doi.org/10.1021/jf304364c

Xuan, L., Hum, C. X., Qi, S. (2014). Effect of low-temperature water extraction on the extraction efficiency of protein and polysaccharides from mushroom. *China Condiment*, *39*(11), 23–26.

Xu, Z. D., Shen, J. Q., Cui, Q. Q., Zhong, J. G., Sun, C. S. (2022). Improvement of substrate and analysis of nutritional components in *Cordyceps Chanhua*. *Advances in Microbiology*, *11*(2), 102–111. https://doi.org/10.12677/amb.2022.112012

Xue, D., & Farid, M. M. (2015). Pulsed electric field extraction of valuable compounds from white button mushroom (*Agaricus bisporus*). *Innovative Food Science and Emerging Technologies*, *29*, 178–186. https://doi.org/10.1016/j.ifset.2015.03.012

Yan, J. K. (2021). Three phase partitioning-based strategies for highly efficient separation of bioactive polysaccharides from natural resources. *Three Phase*

Partitioning, 223–242. https://doi.org/10.1016/b978-0-12-824418-0.00012-6

Yan, J. M. (2018). *Comparative studies on structures of the polysaccharides from ten edible mushrooms*. PhD diss., Northeast Normal University.

Yan, J. K., Wang, Y. Y., Qiu, W. Y., Ma, H., Wang, Z. Bin, & Wu, J. Y. (2018). Three-phase partitioning as an elegant and versatile platform applied to nonchromatographic bioseparation processes. *Critical Reviews in Food Science and Nutrition*, *58*(14), 2416–2431. https://doi.org/10.1080/10408398.2017.1327418

Yang, M., Qian, Z., Zhan, Q., Zhong, L., Hu, Q., & Zhao, L. Y. (2023). Application of definitive screening design to optimization of the protein extraction and functional properties of proteins in *Auricularia auricula*. *Journal of the Science of Food and Agriculture*, *103*(3), 1226–1236. https://doi.org/10.1002/jsfa.12217

Yang, M. Y., Tarun, B., Hari, P. D., Li, L., & Luo, Z. (2019). Trends of utilizing mushroom polysaccharides (MPs) as potent nutraceutical components in food and medicine: A comprehensive review. *Trends in Food Science and Technology*, *92*, 94–110. https://doi.org/10.1016/j.tifs.2019.08.009

Yang, N., Bai J. (2017). Research progress on protein separation and purification technology. *World Latest Medicne Information*, (101), 2–3.

Yao, H. Y. Y., Wang, J. Q., Yin, J. Y., Nie, S. P., & Xie, M. Y. (2021). A review of NMR analysis in polysaccharide structure and conformation: Progress, challenge and perspective. *Food Research International*, *143*, 110290. https://doi.org/10.1016/j.foodres.2021.110290

Yasar, S., Tosun, R., & Sonmez, Z. (2020). Fungal fermentation inducing improved nutritional qualities associated with altered secondary protein structure of soybean meal determined by FTIR spectroscopy. *Measurement: Journal of the International Measurement Confederation*, *161*, 107895. https://doi.org/10.1016/j.measurement.2020.107895

Yin, Z., Liang, Z., Li, C., Wang, J., Ma, C., & Kang, W. (2021). Immunomodulatory effects of polysaccharides from edible fungus: a review. *Food Science and Human Wellness*, *10*(4), 393–400. https://doi.org/10.1016/j.fshw.2021.04.001

Young, R., & Pellett, P. L. (2018). Plant proteins in relation to human and amino acid nutrition1 ' 2 whereas. *American Journal of Clinical Nutrition*, *59*, 1203–1212.

Yu, C. X., Zhang, Y. R., Ren, Y. F., Zhao, Y., Song, X. X., Yang, H. L., & Chen, M. J. (2023). Composition and contents of fatty acids and amino acids in the mycelia of *Lentinula edodes*. *Food Science and Nutrition*, *11*(7), 4038–4046. https://doi.org/10.1002/fsn3.3392

Yu, J. F., Feng Y. L., Fang Y., Ma M., Guo X. (2021). Research progress of macrofungal lectin. *Edible and medicinal mushrooms*, *29*(6), 488–495.

Yu, M. (2017). Study on the technology of slow-freezing-traditional water extraction to extract mushroom protein. *Modern agriculture*, *7*(456), 29–30.

Yuan, G., Wang, Y., Niu, H., Ma, Y., & Song, J. (2024). Isolation, purification, and physicochemical characterization of *Polygonatum* polysaccharide and its protective effect against CCl4-induced liver injury via Nrf2 and NF-κB signaling pathways.

International Journal of Biological Macromolecules, *261*. https://doi.org/10.1016/j.ijbiomac.2024.129863

Zeng, B., Nilsson, K., Teixeira, P. G., & Bergenståhl, B. (2023). Study of mycoprotein extraction methods and its functional properties. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, *659*, 0–7. https://doi.org/10.1016/j.colsurfa.2022.130800

Zhang, F., Sun, Z., Li, X., Kong, B., Sun, F., Cao, C., et al. (2023). Ultrasound-assisted alkaline extraction of protein from *Tenebrio molitor larvae*: Extraction kinetics, physiochemical, and functional traits. *Ultrasonics Sonochemistry*, *95*. https://doi.org/10.1016/j.ultsonch.2023.106379

Zhang, L., Zhou, L. Y., Song, H. B., Li, P., Li, Y. R., Cao, Z. Z., Wei, M., Li, S. R. (2015). Optimization of enzymatic extraction of protein from root of *Flammulina velutipes* by response surface methodology. *Food Research And Development*, *36*(4), 40–45. https://doi.org/10.3969/j.issn.1005-6521.2015.04.011

Zhang, L. (2017). *Preparation and antioxidant study of polypeptides from mushroom stems*. Master diss., Nothwest University.

Zhang, M., Cui, S. W., Cheung, P. C. K., & Wang, Q. (2007). Antitumor polysaccharides from mushrooms: a review on their isolation process, structural characteristics and antitumor activity. *Trends in Food Science and Technology*, *18*(1), 4–19. https://doi.org/10.1016/j.tifs.2006.07.013

Zhang, M. T., Yang, W. J., Pei, F., Zhao, L. Y., An, X. X., Ma, N., Cheng, W., Hu, Q.

H. (2015). Optimization of enzymatic hydrolysis for preparing *Pleurotus eryngii* protein by response surface methodology and nutritional evaluation. *Food Science*, *36*(13), 125–129. https://doi.org/10.7506/spkx1002-6630-201513024

Zhang, N. (2019). *Study on preparation, separation and biological activity of Lentinus edodes stalk polypeptide*. Master diss., Northwest University.

Zhang, Q. W., Lin, L. G., & Ye, W. C. (2018). Techniques for extraction and isolation of natural products: A comprehensive review. *Chinese Medicine (United Kingdom)*, *13*(1), 1–26. https://doi.org/10.1186/s13020-018-0177-x

Zhang, X., Li, Y., Cheng, J., Liu, G., Qi, C., Zhou, W., & Zhang, Y. (2014). Immune activities comparison of polysaccharide and polysaccharide-protein complex from *Lycium barbarum L*. *International Journal of Biological Macromolecules*, *65*, 441–445. https://doi.org/10.1016/j.ijbiomac.2014.01.020

Zhang, Y. Y., Li, S., Wang, X., Zhang, L., & Cheung, P. C. K. (2011). Advances in lentinan: Isolation, structure, chain conformation and bioactivities. *Food Hydrocolloids*, *25*(2), 196–206. https://doi.org/10.1016/j.foodhyd.2010.02.001

Zhang, Y. R., Wang, D., Chen, Y., Liu, T., Zhang, S., Fan, H., et al. (2021). Healthy function and high valued utilization of edible fungi. *Food Science and Human Wellness*, *10*(4), 408–420. https://doi.org/10.1016/j.fshw.2021.04.003

Zhao, J., Song, Y., Liu, P. H., Li, Q. H., & Liao, X. J. (2021). Advances in the utilization of plant-based alternative protein. *Science and Technology of Food Industry*, *42*(18). https://doi.org/10.13386/j.issn1002-0306.2021070082

Zhao, S., Gao, Q., Rong, C., Wang, S., Zhao, Z., Liu, Y., & Xu, J. (2020). Immunomodulatory effects of edible and medicinal mushrooms and their bioactive immunoregulatory products. *Journal of Fungi*, *6*(4), 1–37. https://doi.org/10.3390/jof6040269

Zhao, W., Duan, M., Zhang, X., & Tan, T. (2018). A mild extraction and separation procedure of polysaccharide, lipid, chlorophyll and protein from *Chlorella spp*. *Renewable Energy*, *118*, 701–708. https://doi.org/10.1016/j.renene.2017.11.046

Zhao, X., Zhang, B., Luo, Z., Yuan, Y., Zhao, Z., & Liu, M. (2023). Composition analysis and nutritional value evaluation of amino acids in the fruit of 161 *Jujube Cultivars*. *Plants*, *12*(9), 1–12. https://doi.org/10.3390/plants12091744

Zhao, Y. M., Wang, J., Wu, Z. G., Yang, J. M., Li, W., & Shen, L. X. (2016). Extraction, purification and anti-proliferative activities of polysaccharides from *Lentinus edodes*. *International Journal of Biological Macromolecules*, *93*, 136–144. https://doi.org/10.1016/j.ijbiomac.2016.05.100

Zhao, Z. C., Gu, F. T., Li, J. H., Zhu, Y. Y., Huang, L. X., & Wu, J. Y. (2024). Fractionation, characterization, and assessment of nutritional and immunostimulatory protein-rich polysaccharide-protein complexes isolated from *Lentinula edodes* mushroom. *International Journal of Biological Macromolecules*, *280*, 136082. https://doi.org/10.1016/j.ijbiomac.2024.136082

Zhao, Z. C., Huang, L. X., Dong, X. L., & Wu, J. Y. (2024). Evaluation of three-phase partitioning for efficient and simultaneous isolation of immunomodulatory

polysaccharides and proteins from *Lentinula Edodes* mushroom. *Food and Bioprocess Technology*, *17*(8), 2277–2291. https://doi.org/10.1007/s11947-023-03262-3

Zheng, X. L., Li, L. C., Teng, J. W., Wei, B. Y., Huang, Li, Xia, N., Tan, L. D. (2019). Optimization of extraction and functional properties of *Agaricus bisporus* Protein. *Science and Technology of Food Industry*, *40(16)*, 126-132.

Zhu, L. C., Luo, H., Zeng, B., Yin, S. W. (2013). Preliminary study on isolation and purification of antifungal protein from *Sagittaria Sagittifolia*. *Journal of Jinggangshan University (Natural Science)*, *34*(4), 34-36. https://doi.org/10.3969/j.issn.1674

Zhu, S. T., & Wu, K. (1988). Nutritional evaluation of protein. *ACTA Nutrimenta Sinica*, *10*(2), 187–190.

Zininga, T., Ramatsui, L., & Shonhai, A. (2018). Heat shock proteins as immunomodulants. *Molecules*, *23*(11), 2846. https://doi.org/10.3390/molecules23112846

Zong, Y. (2022). *Isolation and identification of protein-polysaccharide complex from Shiitake mushroom and its application in pickering emulsion*. Master diss., Jiangnan University.