Food and Bioprocess Technology
https://doi.org/10.1007/s11947-025-04024-z

RESEARCH

Sequential Enzymatic and Ultrasonic Extraction of *Lentinula edodes* Mushroom Proteins Leading to Enhanced Yield and Significant Immunoactivity

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Received: 19 March 2025 / Accepted: 20 August 2025

Abstract

Lentinula edodes is a widely consumed edible fungus and a rich source of proteins with both nutritional and medicinal value. This study was to identify the most effective process for extraction of proteins from *L. edodes* mushroom by comparison of water extraction (WE), enzyme-assisted extraction (EAE), ultrasound-assisted extraction (UAE), and various combinations of EAE with UAE, EAE followed by UAE (EUE), UAE followed by EAE (UEE), and simultaneous EAE and UAE (SEUE). The two-step sequential scheme of EAE followed by UAE, designated as EUE, resulted in the highest protein yield compared to the UEE and SEUE extraction methods. The soluble protein yield by EUE (9.4%) was nearly three times that by UEE (3.6%) and around two times higher than by EAE (4.9%), respectively. Compared with other extraction methods, the protein fraction by EUE had the highest protein content (56.0%) and β-sheet content (55.8%) and exhibited the strongest in vitro immunostimulatory activity. Through statistically designed experiments and response surface methodology, EUE conditions were optimized as enzyme 0.28% (w/v), ultrasound amplitude 62%, and $(NH_4)_2SO_4$ saturation 69%, achieving 9.7% protein yield and 58.4% protein content. The distribution of protein molecular weights was below 10 kDa and between 25–75 kDa. The protein fraction contained nutritional amino acids and showed significant immunostimulatory activities in vitro. EUE has shown promising potential for efficient extraction of proteins from mushrooms in the food industry.

Keywords *Lentinula edodes* · Enzyme-assisted extraction · Ultrasound-assisted extraction · Proteins · Nutritional value · Immunoactivity

Introduction

Lentinula edodes is one of the most 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 (Du et al., 2024; Wasser, 2004). The PS derived from *L. edodes* have been extensively reported in the literature on their notable antitumor, immunomodulatory and other bioactivities (Wang et al., 2022). Proteins represent another important component of *L. edodes*, gaining attention for their nutritional and medicinal functions (Das & Prakash, 2021; Wong et al., 2010). The *L. edodes* proteins have shown abundant of essential amino acids (EAA) (Yu et al., 2023) and multiple biological functions, such as immunomodulatory, antitumor and antioxidant activities (Gao et al., 2023; Ngai & Ng, 2003). With these beneficial functions, *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., 2024a, 2024b) and also contained high nutritional value amino acids (Zhao et al., 2024a, 2024b).

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Published online: 17 September 2025

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 extracting 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 (Bose et al., 2019; Capellini et al., 2017; Chatterjee et al., 2012; Chen et al., 2019). However, the aforementioned extraction and separation methods are rather tedious and time-consuming, and environment 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 sources (Cannavacciuolo et al., 2024; Goktayoglu et al., 2023). 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 (Ahmed et al., 2024; Ketemepi et al., 2024; Prandi et al., 2023). 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-intensity 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, glucans, chitin, as well as cellulose (Rivillas-Acevedo & Soriano-García, 2006), necessitating enzymatic degradation for efficient extraction of intracellular bioactive components. Enzymes including cellulase, papain, and pectinase can help break cell walls (Zhao et al., 2016), facilitating the release of intracellular proteins. Previous studies have established the efficacy of cellulase-assisted extraction for mushroom protein recovery. For example, Zhang et al., (2015a, 2015b) 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 EAE and UAE methods have been applied separately to *L. edodes* (Prandi et al., 2023; Wang 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, these results suggest the 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. A systematic approach combining statistical experimental design and response surface methodology (RSM) was applied to develop an optimized procedure for combined extraction processes. Further efforts were made to elucidate the mechanistic interplay between enzymatic and physical disruption techniques, 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.

Materials and Methods

Chemical and Biochemical Reagents

The ammonium sulfate ($(NH_4)_2SO_4$) used was sourced from AnalaR Normapur, Vienna, Austria. Cellulase (50 U/mg) from Yuanye Company (Shanghai, China). Bovine serum albumin (BSA), penicillin, lipopolysaccharide (LPS), streptomycin, sulphanilamide, phosphoric acid, and anthrone were sourced from Sigma-Aldrich in St. Louis, MO, USA. Potassium persulphate and sodium nitrite were obtained from BDH in Poole, England. Fetal bovine serum and Dulbecco's modified Eagle medium were acquired from Thermo Fisher Scientific in Waltham, MA, in the United States. Sodium chloride (NaCl) was purchased from Macklin (Shanghai, China). All remaining chemicals were sourced from reliable vendors in analytical reagent (AR) grade or higher.

Protein Extraction

The dried mushroom bodies were pulverized into a powder using an electric mill. Then, it was sifted via mesh sieves (850 µm) to varying mean particle sizes. Mushroom powder was then defatted using absolute ethanol (1:8 w/v) at room temperature (20–25 °C) under constant stirring for overnight (Zhao, et al., 2024a, 2024b) and dried at approximately 50 °C until the weight remained constant. 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.

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 three 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 ($3.5 \pm 0.7\%$). 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 determine the total protein yield, and the Anthrone test, as outlined in earlier study (Siu et al., 2016), was applied to measure the total carbohydrate.

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 30 min. The collected Liquid underwent protein fraction isolation and partial purification, following the WE method as detailed in Sect. 2.2.1 Water extraction (WE).

Enzyme-assisted Extraction (EAE)

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 30 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 heating the sample solution at 100 °C for 10 min. Afterwards, the mixture 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 procedure as described in 2.2.1 Water extraction (WE).

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 S1 shows the specific procedures and conditions for the combined extraction schemes. With all the extraction schemes, 3 g of defatted mushroom powder was 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.

Optimization of EUE Extraction

Preliminary experiment results showed that the two-step sequential EUE method was the most effective for extracting the proteins from the *L. edodes* mushroom (Figure S1). Therefore, the following extraction experiments were all conducted in two separate steps, using cellulase for 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 1 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 stopped by heating at 100 °C (10 min). After the sample was cooled, its pH was increased to 10 by 1 M NaOH. The treatment was further extracted with UAE by adjusting the ultrasound amplitude to 20–100% (0.29–1.44 W/mL) for 20–60 min. After centrifuging for thirty min at 4000 rpm, the supernatant 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.

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 1), with enzyme concentration (%, X_1), ultrasound amplitude (US) (%, X_2), and $(NH_4)_2SO_4$ concentration (%, X_3) serving as independent variables. The protein yield and content were chosen as the two response values. The response surface analysis was performed

Table 1 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

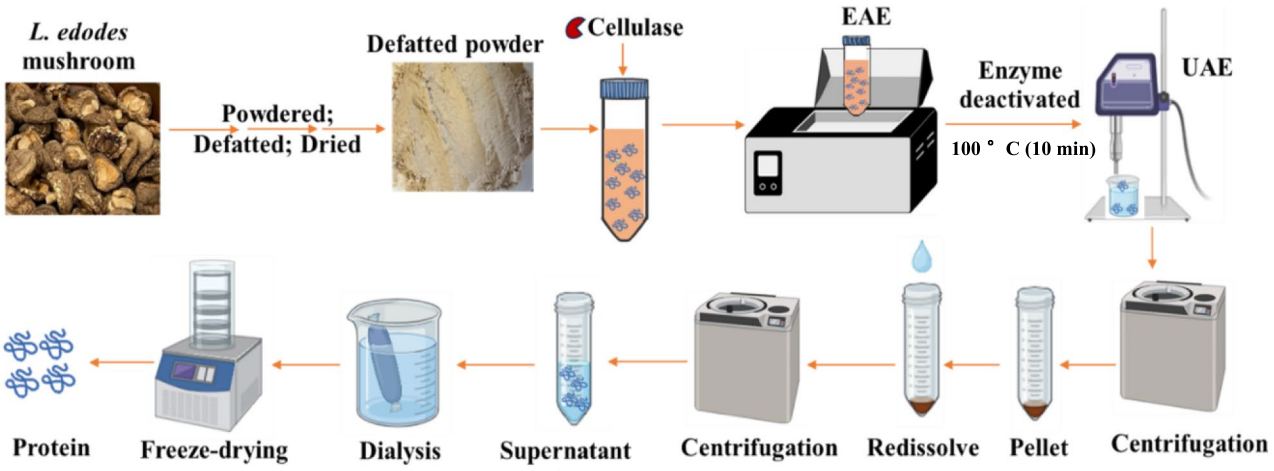

Fig. 1 Process flow diagram for enzyme- and ultrasound-assisted extraction of proteins from mushroom

based on the 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.

Analysis of Isolated Protein Fractions

Protein Secondary Structure Analysis

The secondary structures of the WE, UAE, EAE, EUE, and optimized EUE protein samples were analyzed using Circular Dichroism (CD) spectroscopy. The experiments were conducted at room temperature with a Jasco J-1500 CD spectropolarimeter from Japan. The identification of protein extract samples was performed as reported previously (Zhao et al., 2024a, 2024b).

Composition, Molecular Weight, and FT-IR Spectroscopy Analysis

The MW of optimized EUE-extracted protein samples was examined using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis, following the literature procedure (Cheung et al., 2012). The optimized EUE protein samples were crushed into KBr pellets and subjected to Fourier transform-infrared (FT-IR) spectroscopy over the wavenumber range of 500–4000 cm^{-1} (Nexus 670 FT-IR spectrometer) (Thermo Nicolet Co., Cambridge, UK). The protein sample composition of AA was assessed by the description provided in a prior report. Before analysis using ultra-performance liquid chromatography-electrospray ionization triple quadrupole mass spectrometry (UPLC-ESI-TQMS) (Agilent Technologies, Santa Clara, CA, USA) coupled with a CORTECS™ UPLC C18 (1.6 µm, 2.1 × 150 mm column) as detailed by Gray et al. (2017) as well as Guba et al. (2022), protein samples underwent a 30 s treatment with nitrogen followed by hydrolysis with 12 M HCl at 110 ℃ for 24 h. Following this, the samples were diluted 60-fold to attain a final concentration of 0.1 M. The diluted samples were derivatized using the Waters Kairos AA kit (Waters, Milford, MA, USA).

Immunoactivity Assay in Vitro

As reported previously, RAW 264.7 cell culture was used to evaluate the immunomodulatory activities of protein fractions. (Zhao et al., 2024a, 2024b). 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.

Analysis of Cell Viability and Nitric Oxide (NO) Generation

RAW264.7 cell culture was prepared as reported previously (Zhao et al., 2024a, 2024b), and then seeded into 100 µL of 96-well plates (5×10^4 cells/mL). The cells in the logarithmic growth phase were treated with protein samples and LPS for 24 h. Cell viability was determined using MTS as previously described. The supernatants of the cell culture medium underwent treatment with Griess reagent to quantify NO. Absorbance readings were taken by utilizing a microplate reader at 540 nm. Subsequently, the absorbance measurement was calibrated against NO concentrations using sodium nitrite.

Neutral Red Uptake Analysis

The neutral red uptake method was applied to assess the phagocytic potential of cells. Cells were cultured and seeded as described in Sect. 2.6.1. After protein and LPS treatment for 24 h, the supernatant was removed, and the subsequent experiments were conducted as reported previously (Zhao et al., 2024a, 2024b).

ROS Production

The amount of reactive oxygen species (ROS) was assessed by the fluorescent probe 2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) from Sigma as described by Jayasinghe et al. (2023) with slight adjustments. In summary, each well of a 96-well black plate was seeded and incubated according to the procedure outlined in Sect. 2.6.1. After 24 h of cultivation, 100 µL of EUE-extracted protein (0.2 µg/mL) was further added to each well. Following a one-hour incubation at 37 ℃, cells were treated with DCFH-DA (10 µM) and further cultured for 23 h at 37 ℃. Fluorescence intensity was measured with an excitation wavelength of 485 nm and an emission wavelength of 527 nm.

Statistical Data Analysis

The results are presented as the mean ± standard deviation (SD) from three independent replicates. Statistical analyses were performed in Prism 9 using a student's T-test or one-way ANOVA followed by a Tukey post-hoc test. A p-value of less than 0.05 was considered statistically significant.

Results and Discussion

Comparison of Protein Yield and Content from Various Extraction Methods

Table 2 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 a higher content (47%) ($p < 0.05$). This may be attributed to 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 2). In comparison, the two-step EUE process resulted in a higher protein yield (9.4%) and content (56%).

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 S1). 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 (Fig. 2). Specifically, cellulase breaks the 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 disrupt the cell wall structure, enhancing permeability and release of intracellular cpmponents (Fig. 2), which reduces the energy required for ultrasound to break 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 and 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 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.

Additionally, the protein attained by the EUE method exhibited stronger immunostimulatory activities than those by the other three methods (Fig. 3). 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., 2024a, 2024b). Besides, the protein by EUE method exhibited the highest β-sheet content (55.8%) (Table 3), 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

Table 2 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 ($p < 0.05$)

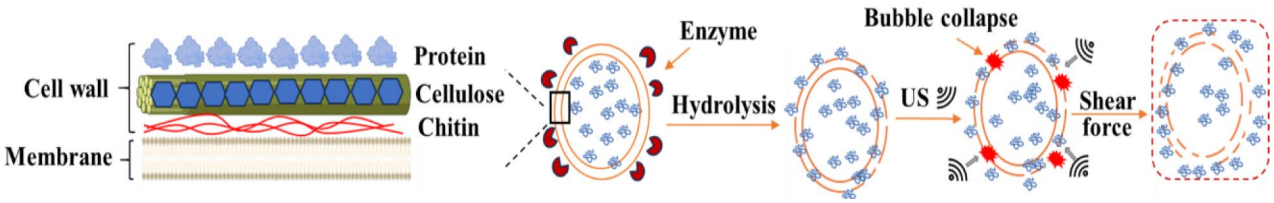

Fig. 2 Possible mechanism of EUE protein extraction

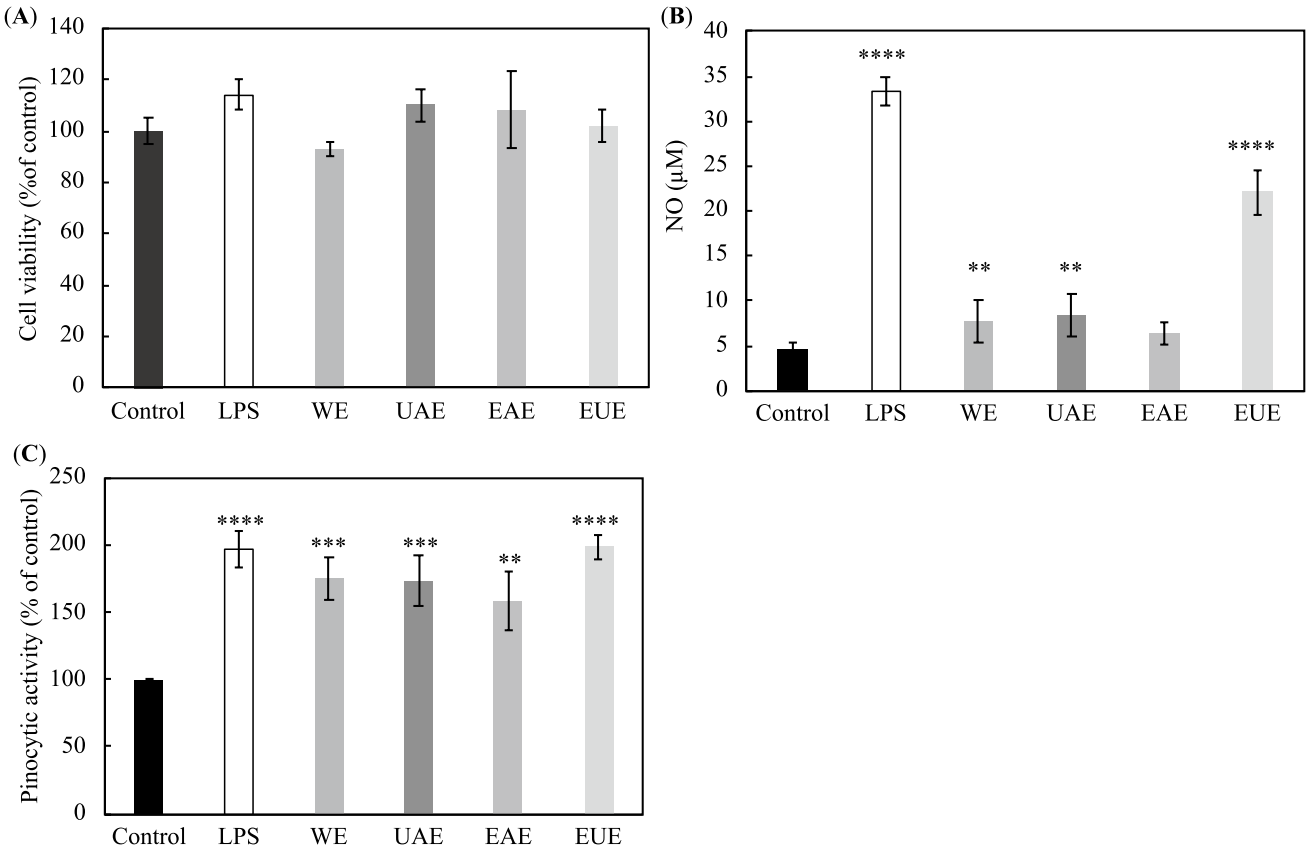

Fig. 3 Immunoregulating activities of isolated fractions on the (**A**) cell viability, **B** NO release of macrophages, and (**C**) phagocytic activity measured by neutral red uptake. **, *** and ****: statistically significant differences from the control group at $p < 0.01$ and $p < 0.001$, $p < 0.0001$ respectively

Table 3 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 indicating significant difference ($p < 0.05$)

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 (Nakazawa et al., 2024; Zhang et al., 2018), 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 (Fig. 2). 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 3). 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 3), 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 disrupt higher-order chain conformations and aggregates (Hadinoto et al., 2024). This disruption may facilitate the conversion of α-helices 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 the 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 33), which may help maintaining local conformational stability and facilitate the correct positioning of epitopes. In summary, the EUE method was chosen for following optimization to achieve maximum protein yield with substantial protein content and strong immunostimulatory activity.

Effects of EUE Conditions on Protein Yield

Fig. 4A shows the varation of cellulase enzyme concentration ranging from 0.2 to 0.8% (enzyme-to-substrate mass ratio) on the protein yield, while all other conditions were kept constant (EAE 60 min, UAE 40 min, US amplitude 60%, and $(NH_4)_2SO_4$ saturation 70%). As the cellulase concentration 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, much higher enzyme concentrations beyond saturation may induce substrate limitation and trigger competitive inhibition among enzyme molecules, thereby suppressing catalytic efficiency (Zhang et al., 2015a, 2015b; 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 restrict 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.

Fig. 4B indicates the effect of enzyme treatment time (30–120 min). Although there was insignificant ($p > 0.05$) between the various 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 rate 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 rate. However, the protein content decreased due probably to protein aggregation, which can negatively impact the extraction rate.

The protein yield was Little influenced by changing the UAE time from 20 to 60 min, as seen in Fig. 4C ($p > 0.05$) while the soluble protein content was affected. As illustrated in Fig. 4C, within the interval of 20 to 40 min of ultrasound treatment, the protein content (47%−56%) increased with the extension of ultrasound time, reaching its maximum (56%) at 40 min. With a further increase in ultrasound treatment time, the protein content (56%−36%) starts to decrease, which may be protein denaturation and subsequently lowering the extraction rate.

Protein production varies with US amplitude, as shown in Fig. 4D. 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 rate 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 response surface methodology (RSM) experiments.

Figure 4E 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.

EUE Extraction Conditions Optimized Using RSM

Model Fitting and Statistical Evaluation

RSM analysis was used to determine the optimal EUE protein extraction conditions from *L. edodes*. The effects of

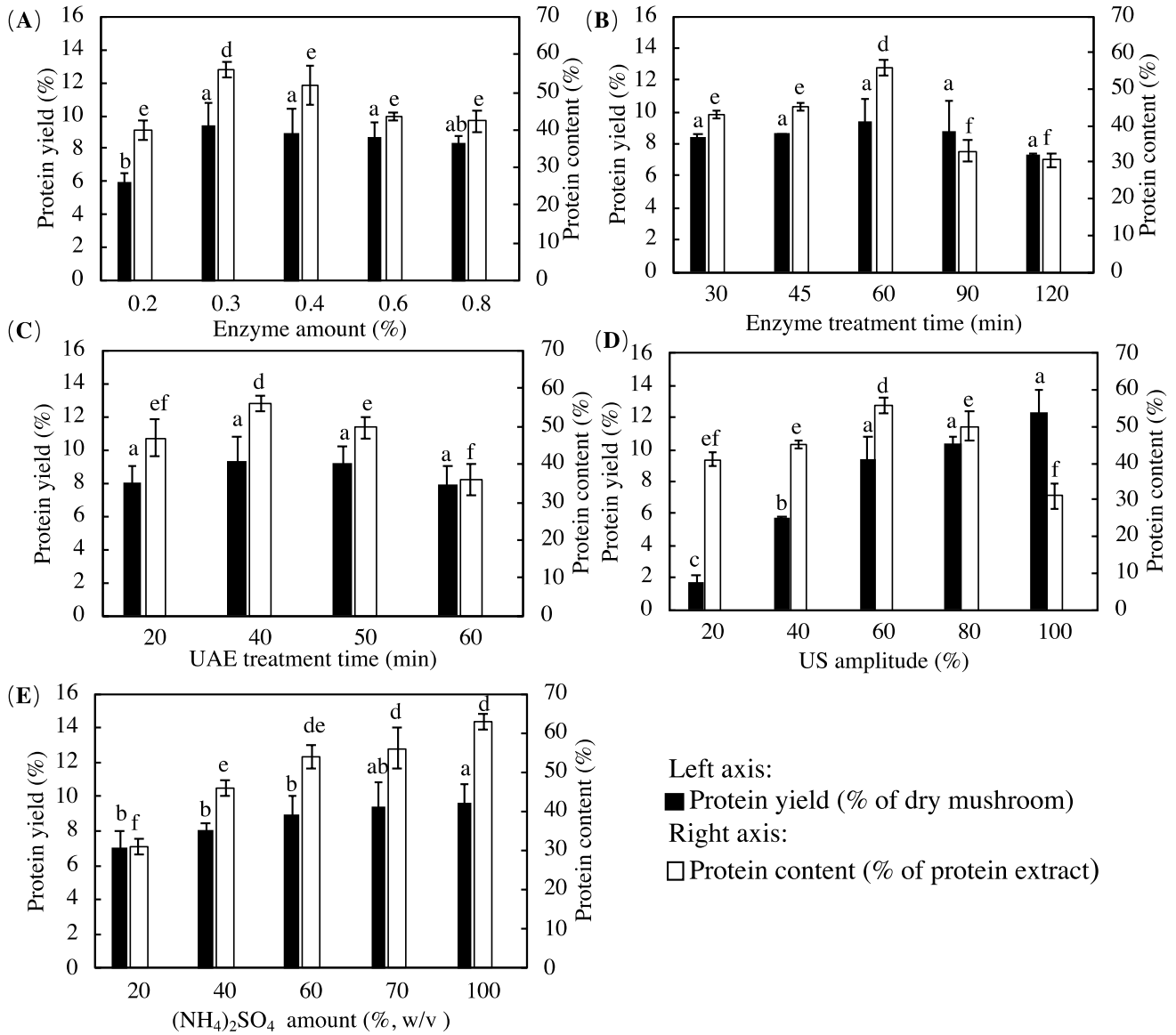

Fig. 4 Effect of (**A**) cellulase addition amount, (**B**) enzyme treatment time, (**C**) UAE treatment time, (**D**) US amplitude, (**E**) $(NH_4)_2SO_4$ saturation concentration on protein extraction yield and content of *L. edodes*. (Common conditions: enzyme concentration 0.3% (enzyme-to-substrate 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 indicating significant difference ($p < 0.05$)

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 4. 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:

$$Y_1 = -29.01 + 18.99X_1 + 0.47X_2 + 0.59X_3 + 0.20X_1X_2 - 0.01X_1X_3 - 0.003X_2X_3 - 53.24X_1^2 - 0.002X_2^3 - 0.003X_3^2 \quad (3.1)$$

$$Y_2 = -68.35 + 175.93X_1 - 0.10X_2 + 3.11X_3 - 0.02X_1X_2 - 0.90X_1X_3 - 0.01X_2X_3 - 207.06X_1^2 + 0.003X_2^2 - 0.02X_3^2 \quad (3.2)$$

Table 4 RSM experimental design (3^3 BBD) and the resulting protein yield and content outcomes

Run	Independent variables			Y_1: protein yield (%)		Y_2: protein content (%)	
	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

In the above equations, Y_1 and Y_2 represent the two responses, protein yield and protein 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). The lack-of-fit test showed no significant difference, with *p*-values of 0.16 and 0.32 for model Eqs. 3.1 and 3.2, 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 (Fig. 5). One variable was kept constant while the other two were changed in these graphs. Multiple

Table 5 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
	$R^2 = 0.976$	$R^2_{adj} = 0.944$ C.V.% = 6.54	$R^2 = 0.967$	$R^2_{adj} = 0.924$ C.V.% = 4.19

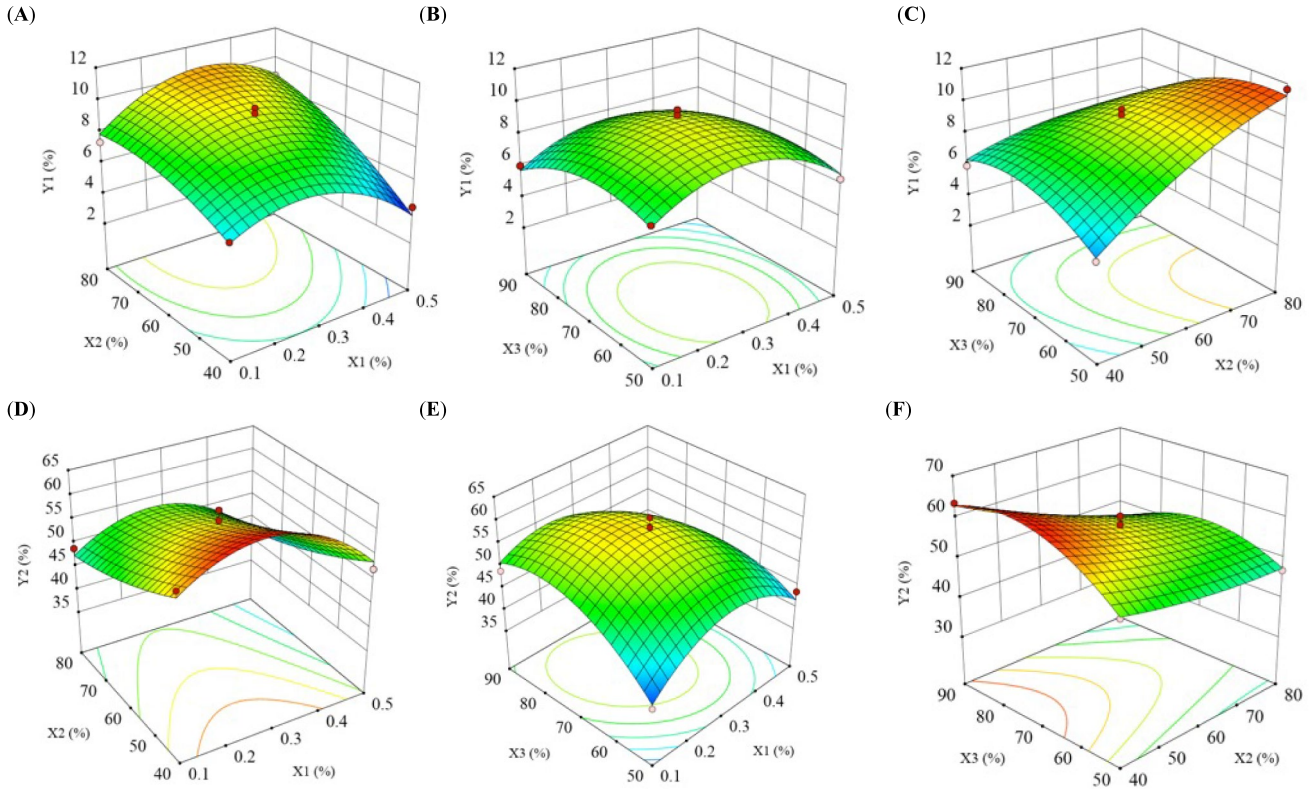

Fig. 5 Response surface plots of Protein yield (Y_1) versus two experimental variables: **A** enzyme amount (X_1); and ultrasound amplitude (X_2); **B** enzyme amount (X_1) and the $(NH_4)_2SO_4$ saturation (X_3); **C** US amplitude power (X_2) and $(NH_4)_2SO_4$ saturation (X_3); Protein content (Y_2)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

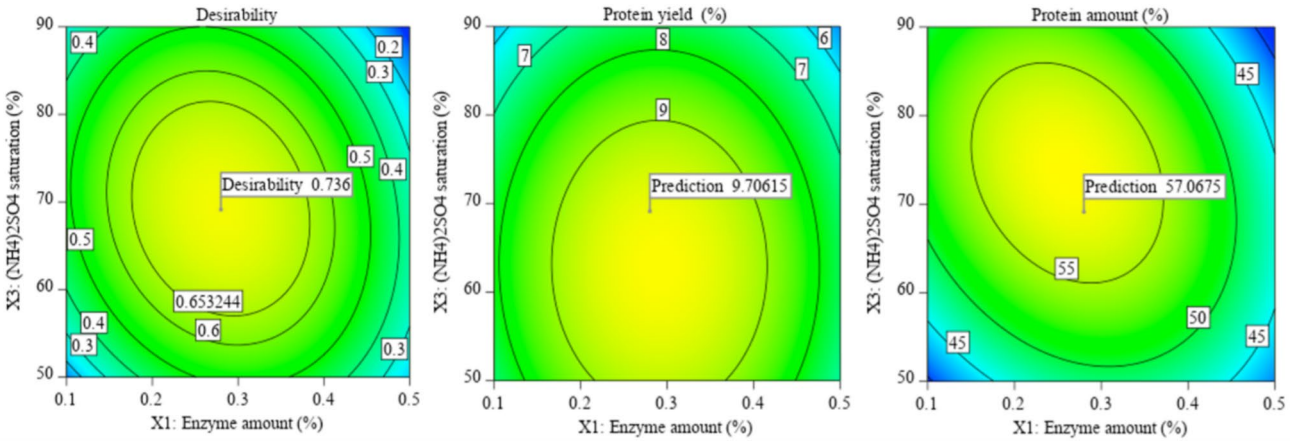

Fig. 6 Contour plots with the desirable response goals to determine the optimum protein yield and content at an enzyme additional amount of 0.28%

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 (Fig. 6) showed that the optimum conditions for the protein with high content and high yield by the EUE extraction were 0.28% (w/v) 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.

Structure characteristics of EUE-extracted proteins

In the CD spectrum (Figure S2), 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* mushrooms, which is consistent with our findings (Hu, 2019). In Figure S3, 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 2. 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 S4), 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.

Amino acid composition of proteins extracted through EUE

Table 6 presents the AA profiles of partially purified proteins extracted by EUE. As shown in Table 6A, 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 essential amino acids (EAAs). The protein extracted via EUE comprised EAAs and non-essential amino acids (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 EAA accounted for over 40% of the total amino acids (TAA) content. EAA/NEAA value was close to 80%, in line with the recommendations proposed by 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 model recommended by FAO/WHO and the AA profile of egg protein, the amino acid score (AAS) and chemical score (CS) values for EUE protein are presented in Table 6B. When AAS was used as the standard, isoleucine content was the highest, 2.08 times the standard. The first limiting AA was 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 (Table 6B). Mushroom proteins can 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, 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* mushrooms using the EUE method possesses high nutritional value and offers potential for development and utilization.

Immunomodulatory Activities

At a concentration of 4 μg/mL, the EUE-extracted protein demonstrated no toxicity to RAW 264.7 cells (Fig. 7A). NO is essential in various physiological functions, particularly in host defense. NO production serves as a reliable measure of

Table 6 Amino acids 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%			

AAS = content of a specific EAA in the protein sample divided by its content in the FAO/WHO model protein (Table S2) (FAO/WHO, 1973; FAO/WHO, 2007; CS = content of an EAA in protein sample divided by its content in whole egg protein (Table S2); 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., 2024a). Different letters a, b…v indicating significant difference ($p < 0.05$)

the immunocompetence of RAW 264.7 cells. As displayed in Fig. 7B, EUE-extracted protein's 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 ($p < 0.05$) (Fig. 7C&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 (Fig. 7E). Overall, the results confirmed that the EUE-extracted protein rich fraction exhibited in *vitro* immunostimulatory activity.

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 was also outperformed shown more efficient than simultaneous enzyme and ultrasonic extraction or two-step ultrasonic followed by enzymatic extraction. EUE may save extraction time and process cost, making it a more efficient and cost-effective strategy than the traditional WE process. 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,

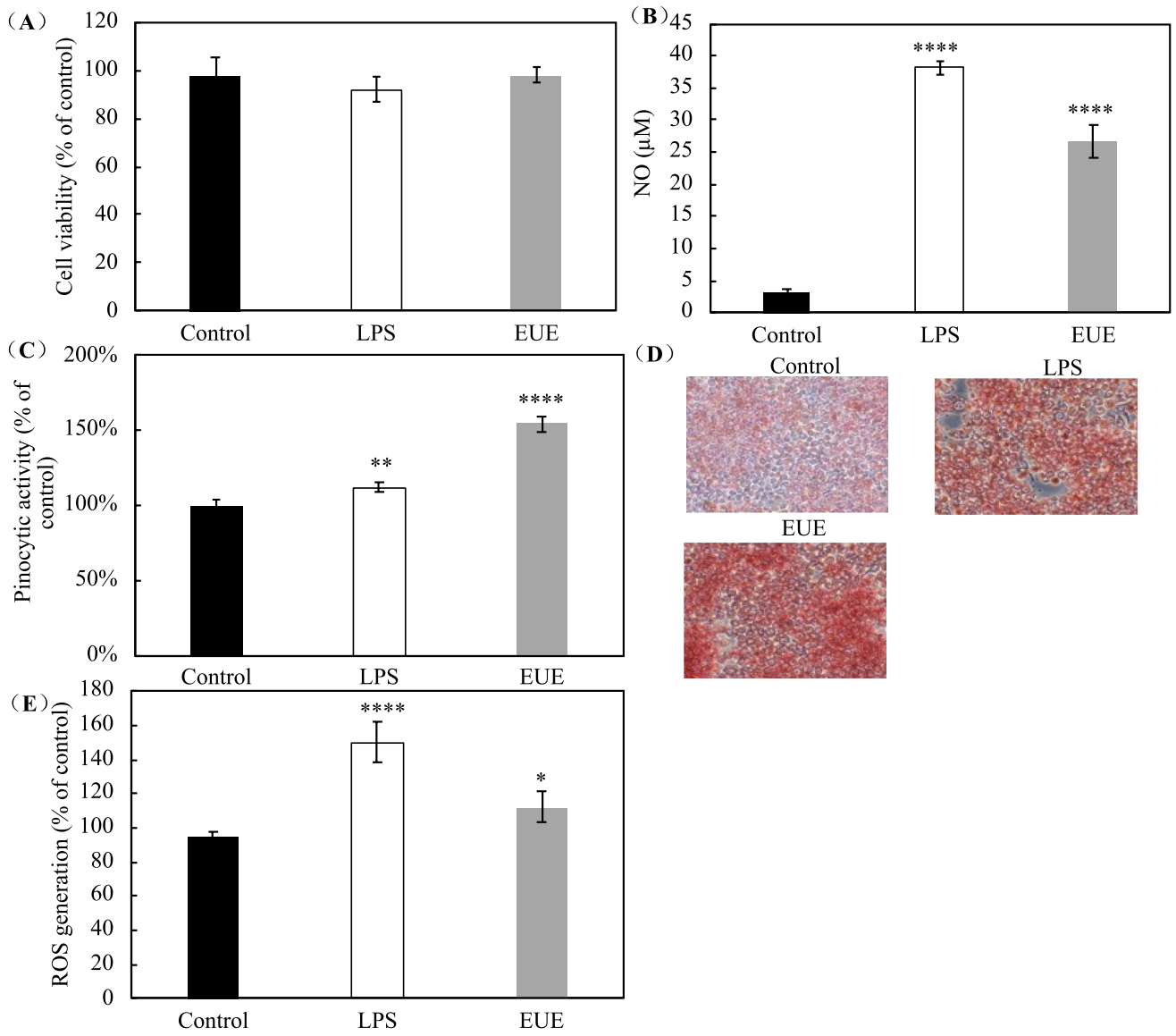

Fig. 7 Immunoregulating activities of EUE-extracted protein on the (**A**) cell viability, **B** NO release of macrophages, **C**, **D** phagocytic activity measured by neutral red uptake, and (**E**) ROS expression. Data are expressed as the means ± SD ($n = 3$). The error bar represents the standard deviation. *, ** and ****: statistically significant differences from the control group at $p < 0.05$ and $p < 0.01$, $p < 0.0001$ respectively

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

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11947-025-04024-z.

Acknowledgements No.

Author Contributions Zi Chen ZHAO: Methodology, Investigation, Visualization, Writing - original draft. Yan Yu ZHU: Methodology. Fang Ting GU: Methodology. Lin Xi Huang: Methodology. Xuwei LIU: Methodology. Jian Yong Wu: Conceptualization, Project administration, Supervision, Writing - review & editing.

Funding Open access funding provided by The Hong Kong Polytechnic University. Funding was provided by The Hong Kong Polytechnic University (Ri-Food Project CD59).

Data Availability No datasets were generated or analysed during the current study.

Declarations

Competing interests The authors declare no competing interests.

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