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BIOACTIVE POLYSACCHARIDE-PROTEIN
COMPLEXES ISOLATED FROM MYCELIAL
FERMENTATION OF MEDICINAL FUNGUS
CORDYCEPS CS-HK1

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PhD

The Hong Kong Polytechnic University

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Bioactive Polysaccharide-Protein Complexes
Isolated From Mycelial Fermentation Of
Medicinal Fungus *Cordyceps* Cs-HK1

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

August 2025

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Abstract

Inflammation, oxidative stress, and intestinal barrier dysfunction are closely interconnected in the development of many chronic disorders. Natural polysaccharide-protein (PSP) complexes from *Cordyceps sinensis* strain Cs-HK1 offer a sustainable source of multifunctional biomacromolecules with potential therapeutic and nutritional applications. This research systematically investigated two distinct types of PSPs: an extracellular PSP obtained from the liquid fermentation medium of Cs-HK1 (EPS-derived PSP), and an ultrasound-released PSP (US-PSP) derived from ultrasonic disruption of the mycelial fermentation broth, which releases intracellular PSP conjugates that combine with extracellular components.

The EPS-derived PSP was fractionated by stepwise ethanol precipitation. After the high-molecular-weight fraction was precipitated at 40% ethanol (v/v), additional ethanol was added to the supernatant to a final concentration of 80% (v/v) to recover the low-molecular-weight fraction (EPS-LM). EPS-LM had an average molecular weight of 6.58×10^5 Da and consisted of polysaccharide and protein components forming a typical polysaccharide-protein complex. In RAW264.7 macrophages, EPS-LM demonstrated potent anti-inflammatory and antioxidant activities by suppressing LPS-induced nitric oxide and reactive oxygen species production and inhibiting activation of the TLR4/MyD88/NF- κ B signaling pathway. Co-immunoprecipitation analysis further confirmed that EPS-LM disrupted the association between TLR4 and

MyD88, thereby suppressing downstream inflammatory signaling and cytokine production.

Further evaluation in Caco-2 epithelial cells revealed that EPS-LM effectively mitigated H₂O₂-induced oxidative stress, restored the activities of antioxidant enzymes (SOD, GSH-Px, CAT), and normalized NRF2-NQO1 redox signaling. It also reinforced epithelial barrier integrity, as evidenced by increased transepithelial electrical resistance, reduced fluorescein permeability, and upregulated expression of tight-junction proteins (Occludin, ZO-1, and Claudin-1). Activation of the ERK signaling pathway enhanced the stability of these junctions, indicating the potential of EPS-derived PSP as a functional ingredient for intestinal health.

To extend the functional applications of Cs-HK1-derived PSPs, the ultrasound-released PSP (US-PSP) was obtained through ultrasonic disruption of the mycelial fermentation broth, followed by enrichment using an alkali-solubilization and acid-precipitation method. The resulting US-PSP fraction was then utilized to develop a pH-induced, solvent-free quercetin-PSP complex as a natural carrier for poorly water-soluble flavonoids. The complex exhibited a uniform particle size, high encapsulation efficiency, and excellent thermal and ionic stability. Spectroscopic analyses confirmed that hydrogen bonding and hydrophobic interactions were the major driving forces for complex formation. Complexation with PSP significantly enhanced the antioxidant and anti-inflammatory activities of quercetin and increased its intestinal bioaccessibility to

approximately 70%, nearly five times that of free quercetin under simulated gastrointestinal conditions.

In conclusion, Cs-HK1-derived PSP complexes demonstrate dual bio-functional and delivery potential. The EPS-derived PSP functions as a potent anti-inflammatory, antioxidant, and barrier-protective agent, while the US-PSP serves as an effective biocompatible carrier that improves the stability, activity, and bioavailability of hydrophobic nutraceuticals such as quercetin. These findings provide fundamental and applied insights into the development of PSP-based functional foods, nutraceutical formulations, and health products targeting inflammation, oxidative stress, and intestinal health.

List of Publications

1. 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.
2. 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.
3. 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.
4. 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.
5. 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.
6. 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.
7. 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*, 18(3), 1–17.
8. 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- κ B pathway. *Nutrients*, 16(22), 3885.

9. Zhu, Y. Y., Wu, M. M. H., Zhao, Z. C., Gu, F. T., Huang, L. X., Kwok, K. W. H., & Wu, J. Y. (2025). An antioxidative exopolysaccharide-protein complex of *Cordyceps Cs-HK1* fungus and its epithelial barrier-protective effects in Caco-2 cell culture. *Antioxidants*, 14(12), 1501.

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Table 6-1 Chemical composition and molecular weight of Cs-HK1 PSP.

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List of Abbreviations and Symbols

Akt	Protein kinase B
EPS	Exopolysaccharides
HPGPC	High performance gel permeation chromatography
HPLC	High performance liquid chromatography
LPS	Lipopolysaccharides
MAPK	Mitogen-activated protein kinase
MS	Mass spectrometry
MW	Molecular weight
NF- κ B	Nuclear factor κ B (Nuclear factor kappa B)
NMR	Nuclear magnetic resonance
NO	Nitric oxide
PI3K	Phosphatidylinositol 3-kinase
SEM	Scanning electron microscopy
TLR2	Toll-like receptor 2
TLR4	Toll-like receptor 4

Chapter 1 Introduction

Inflammation, oxidative stress, and intestinal barrier dysfunction are interlinked drivers of many chronic diseases. Acute inflammation is indispensable for host defense and tissue repair, but when persistent or improperly resolved it sustains the production of proinflammatory cytokines and enzymes, fosters aberrant tissue remodeling and fibrosis, and contributes to pathologies such as inflammatory bowel disease, metabolic syndrome, cardiovascular disorders, neurodegeneration, and cancer (Ahmed, 2011). Oxidative stress, an imbalance between reactive oxygen/nitrogen species and endogenous antioxidant defenses, exacerbates this trajectory by inducing lipid peroxidation, protein carbonylation, and DNA damage, while activating redox-sensitive pathways that amplify inflammation and cell death (Birben et al., 2012). The intestinal epithelial barrier, maintained by tight junction proteins (e.g., Occludin, Claudins, ZO-1), is especially vulnerable: barrier disruption increases paracellular permeability, facilitates translocation of endotoxins and antigens, provokes mucosal inflammation and dysbiosis, and can propagate systemic inflammatory responses (Camilleri et al., 2012; Turner, 2009).

Cordyceps sinensis (*Ophiocordyceps sinensis*), a famous medicinal fungus, provides a tractable source of bioactive macromolecules through submerged mycelial fermentation. Compared with scarce and variable wild fruiting bodies, standardized mycelial fermentation, as exemplified by the Cs-HK1 strain, offers sustainable, scalable, and reproducible production of polysaccharide-protein (PSP) complexes. These

naturally occurring complexes feature mannose- and glucose-rich polysaccharide backbones that are covalently or physically associated with protein constituents. The integration of protein components introduces additional functional groups, such as amino, carboxyl, and hydroxyl moieties, that complement those already present in polysaccharide chains, enriching the physicochemical diversity of the complexes (Augustine et al., 2019; McClements, 2018; Yan et al., 2014).

Building on these structural features, PSP complexes have emerged as multifunctional bioactive macromolecules capable of modulating key physiological processes linked to inflammation, oxidative stress, and intestinal barrier dysfunction. Their structural diversity—including variations in monosaccharide composition, glycosidic linkage patterns, branching degree, molecular weight, and the nature of PSP associations—determines conformation, solubility, and the ability to engage with biological targets. Such structural complexity underlies a broad spectrum of biological activities: these complexes can regulate innate and adaptive immune responses, suppress pro-inflammatory signaling pathways, scavenge free radicals, activate endogenous antioxidant systems, and maintain epithelial integrity through modulation of tight junctions (J. H. Li et al., 2024; Xiao et al., 2020).

Despite the promising bioactivities of PSP complexes, the effective utilization of dietary or therapeutic polyphenols remains limited, because many flavonoids, such as quercetin, are poorly soluble in water and exhibit low stability and gastrointestinal bioavailability (Almeida et al., 2018). To address these challenges, covalently linked

PSP complexes can act as multifunctional macromolecules capable of forming noncovalent physical interactions with flavonoid polyphenols. The diverse binding domains of these complexes, including hydrophobic regions, hydrogen-bonding sites, and aromatic residues, facilitate stable physical associations with quercetin, improving its dispersion, colloidal stability, and resistance to environmental degradation. Such PSP complex-polyphenol interactions can enhance the apparent bioaccessibility of quercetin and reinforce the overall antioxidant and anti-inflammatory potential of the system (Ji et al., 2022; Pan et al., 2014; Shao et al., 2024).

In this study, submerged mycelial fermentation of Cs-HK1 was employed to obtain bioactive covalently linked PSP complexes with integrated structural and functional characteristics. These complexes are of particular interest because they combine the physicochemical and biological attributes of both polysaccharides and proteins within a single covalent framework, enabling the modulation of key processes related to inflammation, oxidative stress, and intestinal barrier integrity. Furthermore, the physical association of quercetin with the PSP complexes was designed to improve the physicochemical stability and intestinal bioaccessibility of this poorly water-soluble flavonoid, providing a model for synergistically functionalized biomacromolecules.

Chapter 2 Objectives and Significance

2.1 Objectives

This study aims to investigate the bioactive properties of polysaccharide-protein (PSP) complexes derived from *Cordyceps sinensis* strain Cs-HK1 mycelial fermentation and to develop PSP-based delivery systems for poorly water-soluble flavonoid polyphenols. Two distinct PSP samples were investigated: the extracellular PSP secreted into the medium (EPS-derived PSP), consisting of extracellular polysaccharide-protein complexes naturally secreted by fungal mycelia into the liquid culture medium, and the ultrasound-released PSP (US-PSP), obtained by ultrasonic disruption of the mycelial fermentation broth, which releases intracellular polysaccharide-protein conjugates that mix with the extracellular fraction to form a mixture containing both extracellular and intracellular components.

1.To prepare and fractionate both EPS-derived and ultrasound-released (US-PSP) complexes from Cs-HK1 mycelial fermentation, and to comprehensively characterize their molecular properties, including chemical composition, molecular-weight distribution, conformational features, and functional groups, to elucidate correlations between structural characteristics and potential bioactivities.

2.To evaluate the biological activities of the PSP fractions, particularly the anti-inflammatory, antioxidative, and epithelial-barrier-protective effects of the

EPS-derived PSP using relevant cellular models of inflammation, oxidative stress, and barrier dysfunction.

3.To further investigate the mechanisms responsible for the bioactivities of the EPS-derived PSP, focusing on key signaling pathways involved in inflammation, oxidative stress, and epithelial junction regulation, as well as potential interactions with gut-related processes.

4.To design and develop PSP-based delivery matrices using the US-PSP fraction for quercetin encapsulation, and to evaluate their encapsulation efficiency, physicochemical stability, gastrointestinal dispersibility, *in vitro* digestion behavior, and overall bioaccessibility.

2.2 Significance

Inflammation, oxidative stress, and intestinal barrier dysfunction are intricately interconnected processes that are central to the pathogenesis of numerous chronic disorders. Despite extensive mechanistic insights (Ahmed, 2011; Anderson et al., 2012; Birben et al., 2012), conventional therapeutic strategies such as antibiotics, corticosteroids, and low molecular weight antioxidants remain limited by adverse effects, drug resistance, and suboptimal long-term efficacy. These constraints highlight an urgent need for safer, food grade bioactive suitable for preventive or adjunctive management of inflammation associated diseases (Rao, 2025).

Natural PSP complexes, particularly those derived from medicinal fungi, are recognized as biological response modifiers (BRMs) with bioactivity functions (Wu, 2015). The Cs-HK1 strain, derived from *Cordyceps sinensis*, is noteworthy for its capacity to produce substantial amounts of PSP through mycelial fermentation. Characterizing these PSP fractions will elucidate how their structural attributes relate to antioxidant, anti-inflammatory, and barrier supporting functions, thereby providing mechanistic insights into the coupled axes of inflammation, redox imbalance, and epithelial dysfunction. The findings will advance understanding of food derived macromolecular networks that act at multiple biological levels to maintain intestinal and systemic homeostasis.

Furthermore, numerous flavonoid polyphenols such as quercetin exhibit potent in vitro antioxidant and anti-inflammatory activities but suffer from poor aqueous solubility, chemical instability, and low gastrointestinal bioavailability (C. Chen et al., 2022). Developing biocompatible PSP based delivery matrices offers a promising, food safe strategy to address these limitations by enhancing dispersibility, protecting against digestive degradation, and improving the bioaccessibility of hydrophobic polyphenols. The Cs-HK1PSP with flavonoid compounds exemplifies a synergistic approach that bridges the gap between naturally occurring bioactive and practical functional food or nutraceutical applications.

Collectively, this research provides a comprehensive platform for elucidating the compositional, functional, and mechanistic basis of Cs-HK1 PSP and for developing

PSP based carrier systems that potentiate flavonoid activity. The anticipated outcomes possess dual significance: scientifically, they contribute to a fundamental understanding of fungal macromolecular interactions underlying anti-inflammatory and antioxidative processes; industrially, they support the rational design and commercialization of evidence based functional foods and health products targeting the inflammation-oxidative stress-barrier dysfunction axis.

Chapter 3 Literature Review

3.1 Bioactive macromolecules in fungi

Medicinal and edible fungi constitute one of the most valuable natural resources for the discovery of novel bioactive substances. Rare species such as *Cordyceps* have been used for millennia in traditional Asian medicine and continue to serve as model organisms in modern biotechnology. These fungi exhibit a wide spectrum of pharmacological and health-promoting effects, including immunomodulation, enhancement of stress resistance, anti-inflammation, and metabolic regulation, which have been progressively validated by modern pharmacological studies (Hobbs, 2023). The structural and functional diversity underlying these effects primarily originates from the bioactive macromolecules that fungi synthesize during growth and development, notably polysaccharides, proteins, and a variety of PSP conjugates. These macromolecules have become a focal point of research at the crossroads of chemistry, immunology, and functional food science due to their high efficacy, low toxicity, and ecological sustainability (Li et al., 2020).

3.1.1 Polysaccharides as predominant functional components

Polysaccharides are natural carbohydrate polymers formed by monosaccharide residues linked through glycosidic bonds. In evolutionary terms, they play dual roles-serving as structural frameworks (e.g., chitin, β -glucans) and as energy reserves (e.g., glycogen, trehalose) (Kabir et al., 2022a; B. w. Xu et al., 2024). However, during the past few

decades, their function has expanded beyond these basic physiological roles. Fungal polysaccharides exhibit bio-regulatory and signaling activities that are rare among natural biomolecules, positioning them as a cornerstone of modern nutraceutical and immunopharmacological research (Mohammed et al., 2021).

The biological functions of fungal polysaccharides are intimately tied to their structural characteristics. β -D-glucans from *Ganoderma lucidum* and *Lentinus edodes*, mannose-rich polymers from *Cordyceps sinensis*, and heteropolysaccharides from *Tremella fuciformis* are prime examples of molecules whose activity depends on molecular weight, degree of branching, solubility, and spatial conformation (Khan et al., 2015). In many cases, β -1,3 and β -1,6 linkages form triple-helix structures that are recognized by pattern recognition receptors (PRRs) expressed on macrophages, dendritic cells, and neutrophils (Batbayar et al., 2012). Once engaged, these receptors, including Dectin-1 (dendritic cell-associated C-type lectin-1), complement receptor 3 (CR3, also known as CD11b/CD18), and Toll-like receptors 2 and 4 (TLR2 and TLR4), activate nuclear factor kappa-B (NF- κ B), mitogen-activated protein kinase (MAPK), and phosphatidylinositol-3-kinase/protein kinase B (PI3K/Akt) signaling cascades, leading to the production of cytokines and reactive oxygen species that are essential for innate and adaptive immune activation (Xiao et al., 2020).

Beyond immunostimulation, fungal polysaccharides exhibit context-dependent bidirectional regulation. Under inflammatory conditions, they suppress excessive activation of NF- κ B and nucleotide-binding oligomerization domain-like receptor

family pyrin domain-containing 3 (NLRP3) inflammasomes, reduce the release of proinflammatory mediators such as tumor necrosis factor-alpha (TNF- α) and interleukin-1 beta (IL-1 β), and induce M2 macrophage polarization, thereby promoting tissue repair and immune tolerance (Y.-Y. Zhu et al., 2024). These molecular processes translate into a broad physiological activity spectrum encompassing antioxidant, antiviral, anti-hyperglycemic, and hepatic-protective effects. Importantly, such multifaceted actions reflect the polysaccharides' ability to act on multiple targets and interconnected pathways, which is a distinctive advantage over single-target synthetic drugs (B. w. Xu et al., 2024). Collectively, the complexity of fungal polysaccharide architectures confers them a biological versatility that continues to inspire applications from immune adjuvants and anti-tumor co-therapeutics to prebiotic food additives aimed at modulating the gut microbiota and metabolic balance.

3.1.2 Fungal proteins as precision functional molecules

In parallel with polysaccharides, fungal proteins constitute another core class of bioactive macromolecules. Proteins are linear polymers of amino acids that fold into unique three-dimensional conformations, conferring them highly specific biological functions (Dhara & Nayak, 2022). Within fungi, many secreted or cell-associated proteins have evolved precise mechanisms to interact with host molecules, functioning as enzymes, ligands, or immunomodulators that fine-tune host pathways (Erjavec et al., 2012).

Among these, the fungal immunomodulatory proteins (FIPs) form the best-characterized group. *Ganoderma lucidum* produces FIP LZ-8, a ~13 kDa homodimeric protein rich in β -sheet structure. It binds T-cell receptors and mediates NF- κ B-dependent signaling, thereby adjusting Th1/Th2 cell responses, enhancing immune vigilance under suppression, or damping hyperactivation when inflammation is excessive (Liu et al., 2020). Such bidirectional immunoregulation presents clear advantages over non-specific immunostimulants.

Another widely studied category includes lectins, fungal proteins widely distributed in *Volvariella volvacea* and other species that participate in cell recognition, signaling, and immune modulation. Fungal lectins bind specifically to glycoconjugates on tumor cell surfaces, disturbing membrane signaling and triggering apoptosis or growth arrest (Glorani, 2019). Their glycan-selective recognition also contributes to anti-viral attachment inhibition, representing a bridge between innate defense and targeted therapy. Enzyme-based proteins, including copper- and manganese-containing oxidoreductases such as superoxide dismutase (SOD) and laccases, further contribute to antioxidative defense by scavenging free radicals and maintaining redox homeostasis (Erjavec et al., 2012). Moreover, several small peptides isolated from *G. lucidum* and *Cordyceps militaris* exhibit angiotensin-converting enzyme (ACE) inhibition, offering natural antihypertensive potential (Ahmed et al., 2024a). These emerging findings illustrate that fungal proteins are not only metabolic catalysts but also signaling molecules with therapeutic potential.

3.1.3 Polysaccharide-protein complexes and synergistic actions

Although both fungal polysaccharides and proteins exhibit unique bioactivities, their individual applications face inherent limitations (Dhara & Nayak, 2022; Erjavec et al., 2012). Polysaccharides, while potent immunomodulators and generally recognized as safe for consumption, often display structural heterogeneity, limited receptor specificity, and poor membrane permeability, which constrain their precision in regulating cellular responses (Kabir et al., 2022b). Conversely, fungal proteins possess high catalytic activity and molecular selectivity, making them effective in targeted biological processes; however, they are structurally unstable, being prone to denaturation or proteolytic degradation under adverse environmental conditions such as extreme pH or heat (Dhara & Nayak, 2022).

To address these drawbacks, the integration of polysaccharides and proteins into linked complexes effectively overcomes the limitations of each single component by combining their complementary strengths. The polysaccharide moiety forms a protective hydrophilic matrix that stabilizes proteins, enhances resistance to enzymatic digestion, and improves mucosal adhesion, whereas the protein fraction contributes to structural and immunological specificity that underlies receptor interactions and signaling activation (Ahmad et al., 2024; c Ooi & Liu, 2000; Fontes-Candia et al., 2025). This synergistic molecular architecture enables coordinated interaction with multiple targets, sustained biological responses, and optimized pharmacokinetic performance compared with either component alone (c Ooi & Liu, 2000).

In fungi, polysaccharides and proteins naturally coexist as covalently or noncovalently linked complexes, forming intrinsic macromolecular assemblies that give rise to enhanced and fine-tuned biological activities (Li et al., 2023; Q.-Z. Li et al., 2019). Such complexes integrate the broad-spectrum immune recognition of polysaccharides with the target specificity of proteins, generating multitarget and persistent signaling patterns. The polysaccharide framework further enhances protein stability and facilitates tissue adhesion, particularly within the intestinal mucosa. A representative example is polysaccharopeptide (PSK) from *Trametes versicolor*, which has been clinically approved as an adjuvant cancer therapy owing to its robust immunostimulatory and antitumor actions (Winder et al., 2021). Mechanistically, PSK activates macrophages, natural killer (NK) cells, and cytotoxic T lymphocytes, upregulates the secretion of interleukins and interferons, and concurrently suppresses immunosuppressive tumor microenvironments, yielding an integrated immunotherapeutic response.

Similarly, the extracellular polysaccharide-protein complex (EPS-P) isolated from *Cordyceps Cs-HK1* exhibits prominent anti-inflammatory and antioxidant activities through inhibition of NF- κ B activation, downregulation of proinflammatory cytokines (TNF- α , IL-1 β , and IL-6) (Li et al., 2020). Beyond these cases, other fungal PSP complexes, such as those derived from *Ganoderma lucidum*, *Lentinus edodes*, and *Flammulina velutipes*, have shown synergetic effects in immune enhancement, gut microbiota regulation, antioxidant defense, and metabolic modulation (Jun Hui Li et al.,

2024; Zhao, Gu, et al., 2024) . Collectively, these findings suggest that the conjugated configuration of PSPs not only stabilizes labile protein structures but also facilitates receptor cross-activation on immune and epithelial cells, leading to cooperative and sustained signal amplification. With their dual functional architecture and biocompatible nature, PSP complexes hold promise as next-generation multifunctional biomaterials for nutraceutical, pharmaceutical, and biomedical applications. They can serve as smart delivery vehicles that protect bioactive compounds, improve mucosal adhesion, and enable controlled immune or metabolic regulation.

Taken together, the interplay between fungal polysaccharides, proteins, and their conjugated complexes highlights the remarkable molecular versatility of fungi. These macromolecules not only function as protective and signaling agents within the fungal organism but also provide a diverse reservoir of bioactive candidates for modern medicine, nutrition, and functional food development. The representative molecules, their structural characteristics, cellular targets, and biological functions are summarized in Table 3-1.

Table 3-1 Functions and mechanisms of fungal polysaccharides, proteins, and polysaccharide-protein complexes.

Category	Sources	Structural features	Research models	Targets	Biological activities	References
Polysaccharide (β -glucans)	<i>Ganoderma lucidum</i> , <i>Lentinus edodes</i>	β -1,3/ β -1,6 linkages; triple-helix	In vitro macrophages; murine tumor models	Dectin-1, CR3, TLR-2/4	Immunomodulation, antitumor, antiviral	(Batbayar et al., 2012; Xiao et al., 2020)
Polysaccharide (mannose-rich)	<i>Cordyceps sinensis</i> mycelia	Mannose/glucose backbone, branched chains	PC12 oxidative model; animal stress model	ROS scavenging, NF- κ B inhibition	Antioxidant, neuroprotection	(Huang et al., 2013b; Leung et al., 2006)
Polysaccharide (heteropolysaccharide)	<i>Tremella fuciformis</i>	Mannose, fucose, xylose-rich	Clinical & food applications	Gut mucosal adhesion; PRRs	Moisturizing, hypoglycemic, gut barrier	(Khan et al., 2015; Wu, 2015)
Protein (FIPs)	FIP-LZ8 (<i>Ganoderma lucidum</i>)	~13 kDa small protein	T-lymphocyte assays; animal models	T-cell receptors; NF- κ B balance	Bidirectional immunoregulation	(Liu et al., 2020)
Protein (Lectins)	<i>Volvariella volvacea</i> , others	Glycan-binding domains	Tumor cell models	Glycans on tumor cell surface	Antitumor, apoptosis induction	(Glorani, 2019)
Enzymatic proteins	<i>Pleurotus</i> ; <i>Aspergillus</i> species	Cu/Mn-containing redox enzymes	Antioxidant, oxidative stress animals	ROS and O ₂ ^{-•} scavenging	Antioxidant; anti-aging; hepatoprotective	(Erjavec et al., 2012)

ACE-inhibitory peptides/proteins	<i>Ganoderma lucidum</i> ; <i>Cordyceps militaris</i>	Low-MW (< 5 kDa) hydrophobic peptides	Enzymatic hydrolysates; ACE activity assays	ACE active-site inhibition	Antihypertensive activity	(Ahmed et al., 2024a)
PSP complex (PSK)	<i>Trametes versicolor</i>	Covalently linked glycan–peptide (~100 kDa)	Cancer adjuvant therapy (Japan)	PRRs + T-cell signaling	Immunostimulatory, antitumor adjuvant	(Winder et al., 2021)
PSP complex (Cs-HK1 EPS)	<i>Cordyceps</i> Cs-HK1	Mixed glycan-protein	Cell/animal inflammation	NF-κB, cytokine balance	Anti-inflammatory, antioxidant	(Li et al., 2020)
Emerging complexes	<i>Hericium erinaceus</i> ; <i>Flammulina velutipes</i>	Glycoproteins (20–150 kDa); high carbohydrate ratio	Gut-microbiota or neuroprotection studies	PRRs; EGFR; STAT3 signaling	Neuroprotective; gut-immune modulation	(Contato & Conte-Junior, 2025)

3.2 Acquisition of fungal bioactive macromolecules

To further explore these bioactive macromolecules and elucidate their functions, reliable methods for acquisition, extraction, purification, and structural analysis are essential. The processes of extracting, purifying, and identifying polysaccharides from complex fungal materials constitute a systematic and technically demanding workflow. Typically, this workflow involves several stages: pretreatment, extraction, purification, and structural characterization.

3.2.1 Fungal polysaccharides

Fungal polysaccharides are among the most extensively studied bioactive macromolecules, exhibiting immunomodulatory, antioxidant, and anti-inflammatory properties (Guo et al., 2023). Their extraction forms a critical step that determines both yield and structural integrity.

Hot-water extraction remains the most classical and widely applied technique due to its safety and simplicity, though it often requires long processing times (Barbosa et al., 2020). To enhance efficiency, microwave-, ultrasound-, and enzyme-assisted extraction methods have been increasingly used to disrupt fungal cell walls and accelerate solute transfer, achieving higher yields and retaining biological activity (Cheong et al., 2016). Recent studies emphasize the integration of multiple extraction modes to balance process efficiency and molecular stability (Yang et al., 2022).

After extraction, fractional precipitation using stepwise ethanol addition (typically 30-90%) is commonly employed to separate polysaccharides according to molecular weight, while maintaining food-grade safety. To improve homogeneity and purity, anion-exchange and gel-filtration chromatography are often combined for higher resolution, yielding distinct neutral and acidic fractions (J. Yan et al., 2019). Despite extensive progress, large-scale production remains hindered by cost and the complexity of multi-step processes.

Compositional and structural analyses typically integrate colorimetric, chromatographic, and spectroscopic methods. The phenol-sulfuric acid assay is the standard for total carbohydrate content (DuBois et al., 1956), while high performance liquid chromatography (HPLC), ultra performance liquid chromatography mass spectrometry (UPLC-MS), and gas chromatography mass spectrometry (GC-MS) provide reproducible profiles of monosaccharide composition. High-performance gel-permeation chromatography coupled with light-scattering detection enables precise determination of molecular-weight distribution. Complementary structural elucidation through methylation analysis, nuclear magnetic resonance (NMR), and MS based fragmentation reveals glycosidic linkage patterns and conformational features such as triple helix structures (Galermo et al., 2018). Representative extraction, purification, and analytical methods for fungal polysaccharides, together with their main parameters, advantages, and limitations, are summarized in Table 3-2a.

Table 3-2a Extraction, purification, and analytical methods for fungal polysaccharides.

Step	Method	Principle & parameters	Yield / Application	Advantages / Limitations	References
Extraction	Hot water extraction	Boiling for several hours	~30% yield; <i>Pleurotus ostreatus</i>	Safe, classical / Low selectivity, time-consuming	(Barbosa et al., 2020)
Extraction	Microwave-assisted (MAE)	1300 W, 5 min	6.2% yield; <i>Cordyceps mycelia</i>	Fast, efficient / Possible degradation	(Cheong et al., 2016)
Purification	Stepwise ethanol precipitation	30-90% ethanol gradient	Fractionated EPS; <i>Hericium erinaceus</i>	Simple, food-grade / Low resolution	(Chou et al., 2019)
Purification	Ion-exchange (DEAE)	Separation by charge	Neutral vs acidic polysaccharides	High selectivity / Labor intensive	(J. Yan et al., 2019)
Purification	Gel filtration (Sephadex/Sepharose)	Size-exclusion separation	Homogeneous polysaccharides	Precise MW / Costly, not scalable	(Liu et al., 2015)
Analysis	HPGPC + MALLS/CAD	MW distribution profiling	2-200 kDa	Sensitive, accurate / Requires standards	(L. F. Li et al., 2022)
Analysis	Monosaccharide composition	Hydrolysis + GC-MS / PMP-HPLC	Glucose, mannose, galactose, etc.	High sensitivity / Hydrolysis variability	(Song et al., 2024)

3.2.2 Fungal proteins

Fungal proteins represent another major class of bioactive macromolecules exhibiting diverse enzymatic, structural, and therapeutic functions (Ahmed et al., 2024a; Rojas-Osnaya et al., 2024). Their extraction and purification are technically challenging because the rigid chitinous cell wall restricts release, and proteins themselves are highly sensitive to heat, shear stress, and extreme pH conditions.

The isolation of intracellular proteins requires effective disruption of the fungal cell wall composed mainly of chitin, glucans, mannans, and structural proteins. Physical approaches, such as liquid-nitrogen grinding, bead milling, ultrasonication, and high-pressure homogenization, are routinely applied at the laboratory or pilot scale (Gupta & Roy, 2021; Zin et al., 2025). These methods efficiently liberate intracellular proteins but may risk partial inactivation due to nonselective mechanical disruption. In contrast, extracellular proteins secreted into the culture broth, including hydrolases, antioxidant enzymes, and fungal immunomodulatory proteins, can be directly recovered by centrifugation and ultrafiltration or salting out, which better preserves native conformation but typically yields lower quantities (Rojas-Osnaya et al., 2024).

To enhance recovery and functional stability, green and solvent-based extraction strategies have gained prominence. Sub-critical water, super-critical CO₂, ultrasound- and microwave-assisted techniques, and emerging deep-eutectic solvent

systems enable efficient cell disruption and protein solubilization under mild conditions that minimize denaturation (Ahmed et al., 2024a; Pobiega et al., 2024). Buffer systems such as Tris-HCl remain widely used for their stabilizing effect on proteins and peptides (Choochan et al., 2022; Rojas-Osnaya et al., 2024), while solvent mixtures (acetone, ethanol, or isopropanol) are applied to recover hydrophobic or membrane-associated proteins.

Enzymatic extraction represents an alternative mild and selective approach, commonly employing proteolytic enzymes such as pepsin, trypsin, or alkaline protease. Process optimization through statistical techniques, including orthogonal design and response surface methodology, is increasingly adopted to maintain protein integrity while maximizing yield. Combined physical and enzymatic systems (e.g., ultrasound assisted enzymatic extraction) have been highlighted as green and efficient techniques for isolating active proteins and bioactive peptides (Ahmed et al., 2024a).

After crude extraction, precipitation and chromatographic purification steps are required to concentrate and refine protein products. Salting-out and pH-induced precipitation offer simple, economical solutions for large-scale concentration (Alvarez & Enriquez, 1988), whereas gel-filtration, hydrophobic-interaction, and affinity chromatography achieve higher resolution and purity, albeit with greater cost. Additionally, membrane-based separations, such as microfiltration and ultrafiltration, have proven effective for concentrating or desalting thermolabile proteins while avoiding thermal denaturation (Ahmed et al., 2024a).

Following extraction and purification, accurate quantification and characterization of the obtained proteins are essential to evaluate product quality, yield, and structural integrity. Total protein content is usually determined by Bradford, Lowry, or bicinchoninic acid (BCA) assays (Bradford, 1976; Lowry et al., 1951; Smith et al., 1985), while sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and two-dimensional electrophoresis (2-DE) are applied to assess purity and molecular-weight distribution. These electrophoretic fingerprints have been used to compare fungal strains and evaluate product quality (Li et al., 2018; Li et al., 2017). Furthermore, amino-acid composition analysis by automated HPLC analyzers provides quantitative insight into nutritional value and structural characteristics (J. Li et al., 2022). Representative extraction, purification, and analytical methods for fungal proteins, along with their main features and limitations, are summarized in Table 3-2b.

Table 3-2b Extraction, purification, and analytical methods for fungal proteins.

Step	Method	Principle & parameters	Yield / Application	Advantages / Limitations	References
Extraction	Physical disruption (LN ₂ grinding, bead milling)	Mechanical cell wall breakage	High recovery; intracellular proteins	Simple, effective / Limited scalability	(Zin et al., 2025)
Extraction	Ultrasound-assisted enzymatic extraction (UAEE)	25 °C, 100 W, 3.2 h + protease	45% protein, 16% peptide; <i>C. militaris</i>	Mild, preserves bioactivity / Costly, enzyme residues	(Ahmed et al., 2024b)
Purification	Salting-out (ammonium sulfate)	Precipitation by ionic strength, pH	Crude protein isolates	Inexpensive, scalable / Risk of denaturation	(Alvarez & Enriquez, 1988)
Analysis	SDS-PAGE / 2-DE	Electrophoretic separation	Protein purity, MW; QC in <i>C. sinensis</i>	High resolution / Labor intensive	(Li et al., 2017)
Analysis	Amino acid analyzer	Hydrolysis + HPLC quantification	Amino acid composition; nutritional analysis	Nutritional insight / Destructive method	(J. Li et al., 2022)

3.3 *Cordyceps sinensis* and its fermented mycelia

Among various medicinal fungi, *Cordyceps sinensis* has attracted considerable scientific and pharmacological interest owing to its unique entomopathogenic life cycle and diverse therapeutic activities (Zhang et al., 2012). The following sections summarize its biological features, cultivation strategies, and the structural and functional properties of its fermentation products.

3.3.1 Biological characteristics and traditional applications

Cordyceps sinensis commonly referred to as the caterpillar fungus, is an entomopathogenic complex formed through the parasitic infection of ghost moth larvae (Hepialidae) by the fungus in Figure 3-1. Following infection, the larval host is gradually mummified and colonized by fungal mycelia, from which a characteristic fruiting body emerges. *C. sinensis*, classified under the phylum Ascomycota, order Hypocreales, and family Clavicipitaceae, is regarded as the most extensively investigated species in the genus *Cordyceps*. Naturally, it is endemic to the high-altitude environments of the Tibetan Plateau, encompassing regions of Tibet, Qinghai, western Sichuan, and parts of Yunnan and India, where it has long been valued as a traditional Chinese medicine (Chen et al., 2013a).

Its unique life cycle involves the fungus infecting the larva and forming a sclerotium during winter, absorbing host nutrients, and then producing a fruiting body in summer—hence its name resembling both insect and plant. *Cordyceps* contains various bioactive

components, including polysaccharides, proteins, nucleosides, and peptides, conferring both nutritional and medicinal value. It has long been celebrated in traditional Chinese medicine as the “king of medicinal fungi” (Zhang et al., 2012). With rising demand for healthcare products and medicinal resources, the limited yield and high price of wild *Cordyceps* can no longer meet market needs, making artificial cultivation essential. Two main approaches are applied: solid-state cultivation using agricultural substrates, and liquid submerged fermentation that enables large-scale mycelial production within a short time (Yan & Wu, 2014).



Figure 3-1 *Cordyceps sinensis* fruiting body-caterpillar complexes: morphology and natural habitat (Paterson, 2008).

3.3.2 Artificial cultivation and fermentation technology

(Oh et al., 2007) reported that cultivation at 25 °C and mildly acidic initial pH 4-5, using sucrose (40 g/L) with yeast extract (6 g/L) and polypeptide (2 g/L) plus $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, supported vigorous growth and substantial extracellular biopolymer output. In a 5 L stirred-tank, maximal dry biomass (28 g/L) was obtained at 300 rpm, whereas extracellular biopolymer production peaked (2.5 g/L) at a milder agitation (150 rpm), underscoring shear/oxygen-dependent morphological control (looser, hairier pellets at higher aeration) as a lever for secretion. Size-exclusion profiling resolved three extracellular biopolymer fractions with distinct molecular weights (1831, 27, and 2.2 kDa) and compositions-mannose/galactose-rich polysaccharide moieties and protein moieties enriched in acidic/nonpolar amino acids (Asp, Glu, Gly, Val)-implicating protein-carbohydrate architecture in biofunction.

In larger-scale studies, the research group of Wu Jianyong at The Hong Kong Polytechnic University isolated the Cs-HK1 strain from wild *Cordyceps* collected in the Qinghai-Tibet Plateau. Fermentation in shake flasks or pilot-scale bioreactors yielded 20-25 g/L dry mycelia and 2-5 g/L extracellular polysaccharides (Yan & Wu, 2014). Kinetic studies revealed a strong correlation between extracellular polysaccharide synthesis and mycelial growth. During the exponential growth phase, polysaccharide content increased rapidly, whereas it plateaued during the stationary phase. Moreover, the chemical composition of extracellular polysaccharides varied with fermentation time: early-stage polysaccharides contained higher protein

proportions and lower sugar content, whereas later-stage polysaccharides exhibited increasing sugar content and decreasing protein ratios, suggesting a biosynthetic process in which a protein backbone is gradually extended by polysaccharide chains, consistent with the formation of polysaccharide-protein complexes (Leung et al., 2009).

3.3.3 Polysaccharides-protein complex from Cs-HK1 mycelial fermentation

Research on the PSP complexes derived from *Cordyceps sinensis* (Cs-HK1) has undergone systematic development since 2009, progressing from crude extracts to detailed structural characterization and mechanistic elucidation at the signaling level. In the early stage, (Leung et al., 2009) obtained crude extracellular polysaccharides (EPS) from Cs-HK1 culture supernatants by ethanol precipitation. The preparation contained approximately 65-70 % carbohydrate and 25 % protein, forming a typical β -D-glucan-based polysaccharide-protein complex with branched chains. It exhibited antioxidant activity (TEAC $\approx$ 35-40 $\mu\text{mol Trolox g}^{-1}$; FRAP $\approx$ 50 $\mu\text{mol Fe}^{2+} \text{g}^{-1}$), and the activity correlated positively with protein content, suggesting that the protein moieties contributed significantly to free-radical scavenging. Subsequently, (Wang et al., 2011) purified an acidic PSP fraction using DEAE-52 cellulose ion-exchange chromatography followed by Sephadex G-75 gel filtration. The fraction consisted of about 70 % carbohydrate and 30 % protein with a Man: Glc: Gal molar ratio of 4 : 11 : 1. Rich in carboxyl groups and negatively charged, it enhanced macrophage phagocytosis and nitric oxide production, indicating a dual role of the polysaccharide-protein complex in immunomodulation and antioxidation. (Huang et al., 2013b) further

fractionated the EPS by gradient ethanol precipitation to obtain three components (EPS-P1, P2, and P5) with increasing protein but decreasing carbohydrate content. Electron microscopy revealed spherical architectures composed of intertwining polysaccharide and protein chains; the higher the protein proportion, the stronger the antioxidant and cytoprotective effects, suggesting a synergistic relationship between the carbohydrate and protein components.

(J. Y. Wu et al., 2014) isolated a low-molecular-weight O-linked polysaccharide-protein complex (Cs-GP1) from the low-molecular fraction of EPS (EPS-2) using Superdex 75 gel filtration and DEAE-cellulose purification. Cs-GP1 contained about 30 % carbohydrate and 52 % protein, with a molecular weight of 6 kDa. The polysaccharide moiety, composed of glucose and mannose (3.2: 1), was covalently linked to a peptide backbone enriched in Asp, Glu, and Gly residues. FT-IR and NMR spectroscopy confirmed its O-linked structure. Cs-GP1 showed exceptionally high antioxidant capacity ($\text{TEAC} \approx 1180 \mu\text{mol Trolox g}^{-1}$; $\text{FRAP} \approx 610 \mu\text{mol Fe}^{2+} \text{ g}^{-1}$) and effectively protected PC12 neuronal cells against H_2O_2 -induced oxidative injury, revealing the potential of low-molecular polysaccharide-protein complexes in neuroprotective oxidative stress defense. (Cheung et al., 2015) employed an ultrasonic disruption-ethanol precipitation process to extract intracellular and extracellular PSP, obtaining a broad molecular-weight distribution (1-670 kDa) of PSP complexes (30-60 % carbohydrate, 10-70 % protein). The low-molecular species generated by ultrasonication exhibited greater solubility, higher bioavailability, and stronger

antioxidant and anti-apoptotic activities, effectively protecting PC12 cells from oxidative damage.

(Li et al., 2020) obtained a low-molecular EPS fraction (EPS-LM; 25.5 % carbohydrate, 20.1 % protein, major peak at 3.6×10^5 Da) through a two-step ethanol precipitation procedure and confirmed it as a stable PSP complex. EPS-LM significantly inhibited LPS-induced NF- κ B activation and suppressed the production of NO and IL-1 β ($IC_{50} < 5 \mu\text{g mL}^{-1}$), demonstrating prominent anti-inflammatory activity and representing a functional transition from antioxidative to signaling-level immunoregulatory mechanisms. Building on these findings, (Y. Y. Zhu et al., 2024) further confirmed that EPS-LM remained an active PSP complex and elucidated its molecular mechanism. Mechanistic studies showed that EPS-LM suppressed TLR4-MyD88 interaction and NF- κ B p65 nuclear translocation, thereby reducing the levels of NO, IL-1 β , and ROS. These results provide clear evidence that the complex exerts dual anti-inflammatory and antioxidant actions through modulation of the TLR4/MyD88/NF- κ B signaling pathway. The structural characteristics and representative bioactivities of these PSP complexes are summarized in Table 3-3.

Table 3-3 Structural features and biological activities of Cs-HK1 PSP complexes.

PSP source	Purification method	Sugar: protein	Molecular weight	Main structural features	Representative bioactivities
Crude EPS (Leung et al., 2009)	Ethanol precipitation of fermentation broth	Sugar $\approx$ 65-70%; Protein $\approx$ 25%	$\approx$ 5 kDa – > 200 kDa	β -D-glucan backbone with slight branching; protein cross-linking	Moderate antioxidant activity: activity increases with protein content.
Acidic PSP (Wang et al., 2011)	DEAE-52 anion exchange + Sephadex G-75 gel filtration	Sugar $\approx$ 70%; Protein $\approx$ 30%	$\approx$ 36 kDa	Acidic polysaccharide containing carboxyl groups; Man: Glc: Gal = 4: 11: 1	Enhances macrophage phagocytosis and NO production; immunomodulatory and antioxidant functions
EPS-P1, P2, P5 (Huang et al., 2013b)	Fractional ethanol precipitation (1/5 to 5 vol. ratio)	Sugar 30 -6%; Protein 28-64%	P1 $\approx$ 2.5×10^5 Da; P5 $\approx$ 16 kDa	Polysaccharide chains intertwined with proteins forming spherical complexes	Antioxidant and cytoprotective activities increase with protein ratio

Glycopeptide C s-GP1 (J.-Y. Wu et al., 2014)	Superdex75 gel filtration + DEAE cellulose chromatography	Sugar $\approx$ 30%; Protein $\approx$ 52%	6.0 kDa	Low-MW O-linked glycopeptide; Glc: Man $\approx$ 3.2: 1; peptide rich in Asp/Glu/Gly	Strong antioxidant and neuroprotective effects against H ₂ O ₂ -induced oxidative damage
Ultrasound-Extracted PSP (Cheung et al., 2015)	Ultrasonic cell disruption + ethanol precipitation	Sugar 30-60%; Protein 10-70% (adjustable)	1-670 kDa (broad)	Sonication enhances solubility and produces low-MW fractions	Enhanced antioxidant and anti-apoptotic activities; protects PC12 cells
EPS-LM (Low-MW Fraction) (Li et al., 2021b)	Two-step ethanol precipitation (second fraction).	Sugar 25.5%; Protein 20.1%	Main peak $\approx$ 3.6 $\times 10^5$ Da	Stable low-MW PSP Man: Glc: Gal = 7.76: 13.87: 1	Potent anti-inflammatory and antioxidant effects via TLR4/MyD88/NF- κ B pathway

3.4 Inflammation, oxidative stress, and barrier function

3.4.1 Overview of the inflammation-oxidative stress axis

Inflammation represents a fundamental biological defense mechanism activated by mechanical injury, microbial invasion, chemical irritation, or immune disturbance. The response involves a coordinated interplay of vascular, humoral, and cellular events, including vasodilation, increased permeability, leukocyte recruitment, and secretion of various inflammatory mediators that act synergistically to eliminate harmful stimuli and initiate tissue repair. Under tightly regulated conditions, this transient inflammation is indispensable for host protection and maintenance of internal homeostasis. However, when regulatory mechanisms fail, as in persistent infection, metabolic disturbance, or autoimmune activation, sterile chronic inflammation emerges. It is characterized by sustained cytokine release, oxidative burst, and progressive tissue breakdown. This pathological inflammation has been identified as an etiological factor in many disorders, including rheumatoid arthritis, inflammatory bowel disease (IBD), metabolic syndromes, cardiovascular disease, and cancer (Ahmed, 2011; Harvanová & Duranková, 2025; Rajendran et al., 2018).

A defining molecular hallmark of chronic inflammation is its reciprocal relationship with oxidative stress. Activated immune cells, particularly macrophages and neutrophils, generate excessive reactive oxygen and nitrogen species (ROS/RNS) through NADPH oxidases, nitric oxide synthases, and the mitochondrial respiratory

chain. In turn, oxidative intermediates act as second messengers, stimulating pro-inflammatory enzymes and transcription factors such as NF- κ B. This close feedback loop, often called the inflammation-oxidative stress axis, allows inflammation to self-amplify and persist even without the initial triggering factor. Persistent ROS signaling disturbs cellular redox balance, propagates lipid peroxidation, and damages nucleic acids and proteins, thereby reinforcing cytokine overproduction and tissue dysfunction (Braunstein et al., 2025). The gastrointestinal epithelium, which is continuously exposed to microbial antigens and dietary oxidants, is particularly susceptible to this vicious cycle (Altanam et al., 2025).

3.4.2 Inflammatory cytokine network

Cytokines serve as molecular conductors orchestrating innate and adaptive immune responses. Among them, TNF- α acts as an early and potent initiator. Released by activated macrophages and monocytes, TNF- α rapidly induces the expression of endothelial adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), facilitating leukocyte rolling, attachment, and extravasation into inflamed tissues. At the same time, TNF- α stimulates neutrophils and macrophages to release proteases and additional cytokines, forming a cyclical amplification cascade (Opal & DePalo, 2000). IL-1 β complements TNF- α by inducing fever, stimulating prostaglandin synthesis, and promoting hepatic acute-phase responses. IL-6 bridges innate and adaptive immunity by promoting B-cell differentiation and antibody production while driving systemic metabolic changes.

Chemokines such as IL-8 (CXCL8) provide spatial guidance for neutrophil migration to inflammatory sites and play crucial roles in purulent reactions (Dash et al., 2025).

At the transcriptional level, the NF- κ B pathway governs cytokine regulation. Activation of TLR4 by microbial lipopolysaccharides or endogenous ligands initiates the MyD88-IRAK-TRAF6 signaling cascade, which then activates the inhibitor of kappa B (I κ B) kinase (IKK) complex. Degradation of inhibitor of I κ B frees NF- κ B to translocate to the nucleus, where it upregulates, genes encoding TNF- α , IL-1 β , IL-6, and cyclooxygenase-2 (COX-2), thereby amplifying inflammation (Akira & Takeda, 2004). Chronic or excessive NF- κ B activation transforms transient inflammation into a persistent pathological state, resulting in tissue injury, fibrosis, and even malignant transformation (Hayden & Ghosh, 2011). Anti-inflammatory cytokines such as IL-10 counterbalance this process by inhibiting NF- κ B translocation and suppressing pro-inflammatory gene transcription (Dinarello, 2000). The equilibrium between pro- and anti-inflammatory cytokines ultimately determines whether inflammation resolves or progresses toward chronic disease.

3.4.3 Oxidative damage and antioxidant defense

Under physiological conditions, ROS participate in essential signaling pathways such as cell differentiation, proliferation, and antimicrobial defense. However, when ROS production exceeds the neutralizing capacity of antioxidant systems, oxidative stress develops. Major enzymatic sources of ROS include NADPH oxidase (NOX) family

members, xanthine oxidase (XO), COX, and mitochondrial complexes I and III, all of which generate superoxide anions and hydrogen peroxide as metabolic byproducts (Bedard & Krause, 2007; Madamanchi & Runge, 2007). Superoxide radicals induce lipid peroxidation, oxidize protein thiols, and damage DNA bases, leading to cellular dysfunction, senescence, or apoptosis (Fujii et al., 2022).

Organisms maintain a multilevel antioxidant defense system to counteract such damage. Primary defenses comprise enzymatic scavengers such as superoxide dismutase (SOD), which convert superoxide into hydrogen peroxide. The latter is further detoxified by catalase (CAT) or glutathione peroxidases (GPx) in cooperation with reduced glutathione (GSH). Secondary defenses involve the thioredoxin (Trx) and peroxiredoxin (Prx) systems, which maintain protein thiol homeostasis and redox signaling integrity (Deneke, 2001; Sies, 2017).

Central to redox regulation is the Kelch-like ECH-associated protein 1 (Keap1)-nuclear factor erythroid 2-related factor 2 (Nrf2) signaling pathway, a cytoprotective transcriptional mechanism responsive to oxidative or electrophilic stress. Under basal conditions, Nrf2 is sequestered in the cytoplasm by Keap1 and targeted for proteasomal degradation via the Cullin 3 (Cul3)-based E3 ubiquitin ligase complex. When oxidative stress modifies cysteine residues in Keap1, this inhibition is lifted and Nrf2 translocates into the nucleus, where it binds antioxidant response elements (AREs). The resulting gene activation, including heme oxygenase-1 (HO-1) and NAD(P)H:quinone oxidoreductase 1 (NQO1), GPx, and CAT, restores glutathione reserves, maintains

NADPH recycling, and re-establishes redox balance (Kensler & Wakabayashi, 2010; Kobayashi et al., 2004).

A dynamic interplay exists between Nrf2 and NF- κ B pathways. ROS activate NF- κ B by inducing I κ B phosphorylation and degradation, which increases cytokine release. Meanwhile, persistent NF- κ B activation promotes NOX and COX expression, producing additional ROS and thereby perpetuating an inflammatory-oxidative cycle (Ozougwu, 2016). Conversely, Nrf2 activation mitigates this cycle by inducing antioxidant enzymes and inhibiting NF- κ B nuclear translocation, providing an intrinsic “off switch” that differentiates adaptive responses from pathological inflammation (Silvestro & Mazzon, 2023).

3.4.4 Barrier disruption and signaling crosstalk

The intestinal epithelium is constantly exposed to dietary antigens and microbial components. Its tight-junction (TJ) network, mainly composed of occludin, claudins, and zonula occludens-1 (ZO-1), functions as a selective barrier between the lumen and internal environment. When the integrity of this junctional network is compromised, endotoxins and pathogens can translocate across the epithelium, triggering mucosal immune activation (Oami et al., 2025).

Pro-inflammatory mediators such as TNF- α and IL-1 β reduce TJ expression and phosphorylate cytoskeletal regulators through the myosin light-chain kinase (MLCK) pathway. MLCK-driven phosphorylation of myosin light chain induces actomyosin

contraction, disrupts junctional scaffolds, and increases paracellular permeability (Al-Sadi et al., 2008; Shen et al., 2006). Oxidative stress further aggravates junctional remodeling by modifying junctional proteins and weakening cell adhesion. Simultaneously, excess ROS and cytokines promote epithelial apoptosis and suppress crypt stem cell proliferation, impairing mucosal repair (Wu et al., 2013).

The combined effect of NF- κ B-dependent cytokine production and redox-mediated stress forms a feed-forward loop that leads to barrier failure and generalized inflammation (John et al., 2011). Experimental studies consistently show that the sustained activation of the NF- κ B-MLCK axis contributes to “leaky-gut” pathology, characteristic of IBD, diabetes, and obesity (Neurath et al., 2025).

Protective signaling pathways counteract this damage. Nrf2 activation induces the expression of HO-1, NQO1, and glutathione-forming enzymes, reducing intracellular ROS levels and preserving TJ localization (Wen et al., 2019). In addition, MAPK pathways have dual functions: transient extracellular signal-regulated kinase 1/2 (ERK1/2) activation facilitates epithelial proliferation and migration during tissue restitution, promoting TJ reassembly; in contrast, persistent ERK activation under chronic inflammation works synergistically with NF- κ B to aggravate mucosal injury (Kuo et al., 2022).

Taken together, inflammation, oxidative stress, and epithelial barrier disruption represent interconnected pathological cascades that reinforce one another through

overlapping signaling networks. Persistent activation of the NF- κ B axis, redox imbalance, and cytoskeletal remodeling create a self-perpetuating loop that culminates in chronic mucosal injury and systemic inflammation (Rao, 2008).

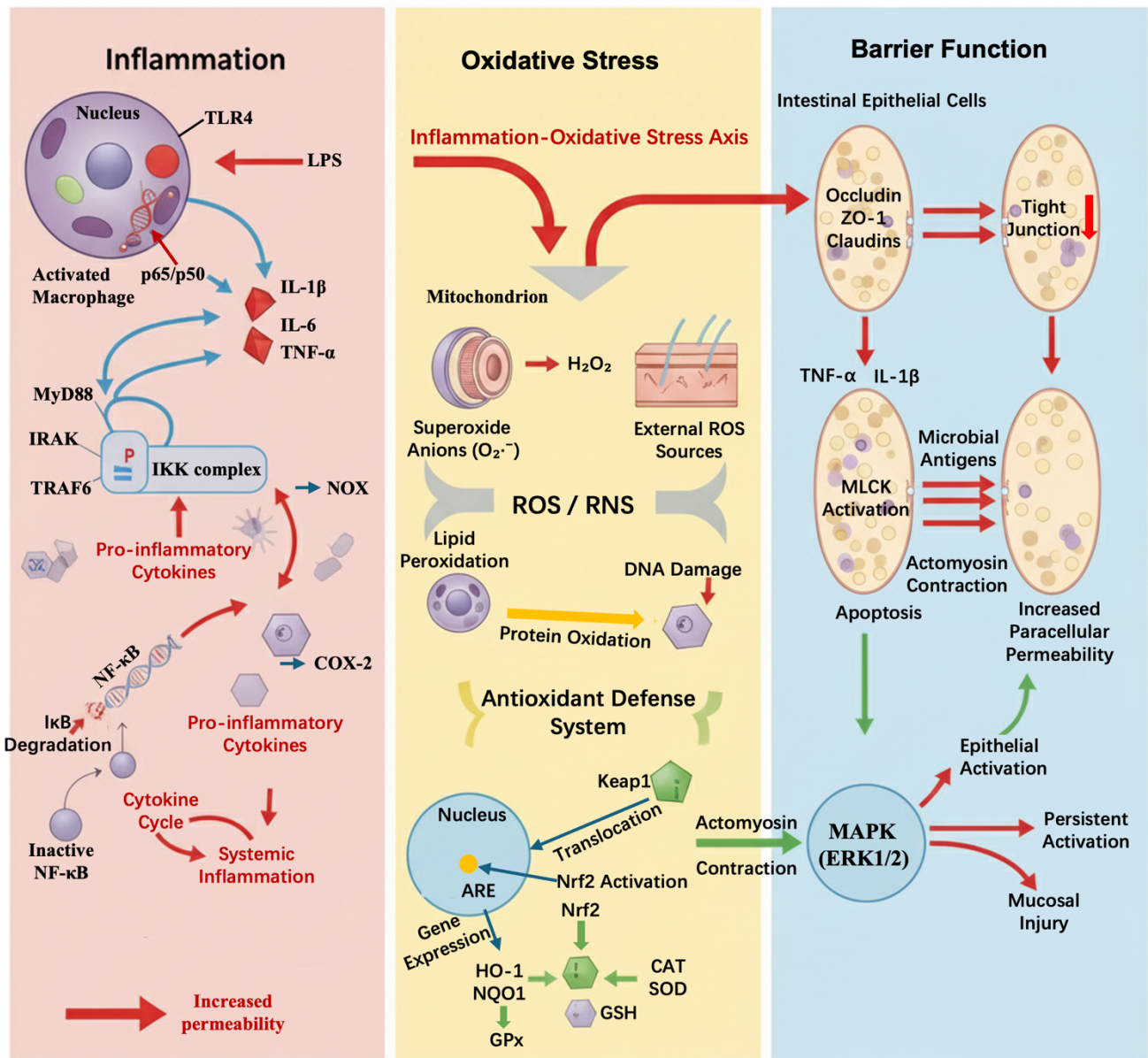


Figure 3-2 Schematic illustration showing the inflammation-oxidative stress-barrier dysfunction axis and the associated signaling pathways regulating epithelial permeability.

3.5 PSP complexes as bio carriers and protective agents

Biopolymers, especially natural polysaccharides and proteins have emerged as promising candidates for the design of biodegradable and biocompatible delivery systems. Their reactive functional groups, including hydroxyl, carboxyl, amino, and sulfhydryl groups, enable extensive intermolecular interactions through hydrogen bonding, electrostatic association, and hydrophobic attraction. These molecular features allow the fabrication of nanoparticles, hydrogels, films, and microcapsules with tunable surface charge, elasticity, and degradation behaviors that can be adapted for food, pharmaceutical, and biomedical applications (McClements, 2018).

Polyphenolic compounds, particularly flavonoids, possess potent antioxidant and anti-inflammatory activities; however, their poor aqueous solubility and chemical instability under variations in light, temperature, and pH often result in low bioavailability (Santos-Buelga et al., 2023). Encapsulation of these bioactive within PSP effectively protects them against oxidative degradation and improves compatibility with biological membranes, thus promoting controlled transport along the gastrointestinal tract (Liang et al., 2017).

The performance of carrier systems based on natural polymers largely depends on their structural configuration, degree of cross-linking, and interfacial interactions. Polysaccharides encapsulate small or chemically sensitive compounds primarily through hydrogen bonding and electrostatic forces. For example, chitosan, a cationic

polysaccharide, interacts with negatively charged polyphenols to form stable nanoparticles that protect loaded bioactive in acidic environments and enhance intestinal absorption through mucosal adhesion (Lawson, 2023). Proteins, owing to their amphiphilic nature, can encapsulate hydrophobic or aromatic bioactive via noncovalent interactions-for instance, casein and whey proteins effectively stabilize phenolic compounds and enable enzyme-responsive release during digestion (Dönmez et al., 2020; Viljanen et al., 2004).

When polysaccharides and proteins are combined, their complementary physicochemical characteristics result in hybrid systems with superior functional performance. Electrostatic attractions, hydrogen bonds, and hydrophobic interactions drive the self-assembly of these components into cohesive nanocomposites that exhibit improved thermal stability, oxidation resistance, and colloidal uniformity. Representative examples include chitosan-whey protein and alginate-gelatin nanocarriers, which show enhanced encapsulation efficiency, sustained release behavior, and increased stability in gastrointestinal environments (Eghbal & Choudhary, 2018). The integration of polysaccharide flexibility with protein structural versatility produces multifunctional and eco-friendly alternatives to synthetic nanocarriers. These synergistic interactions between polysaccharides and proteins lead to PSP hybrids with enhanced encapsulation efficiency, physiological stability, and tunable interfacial behavior, underscoring their crucial role as natural bio-carriers and protective agents (Oyervides-Guajardo et al., 2025).

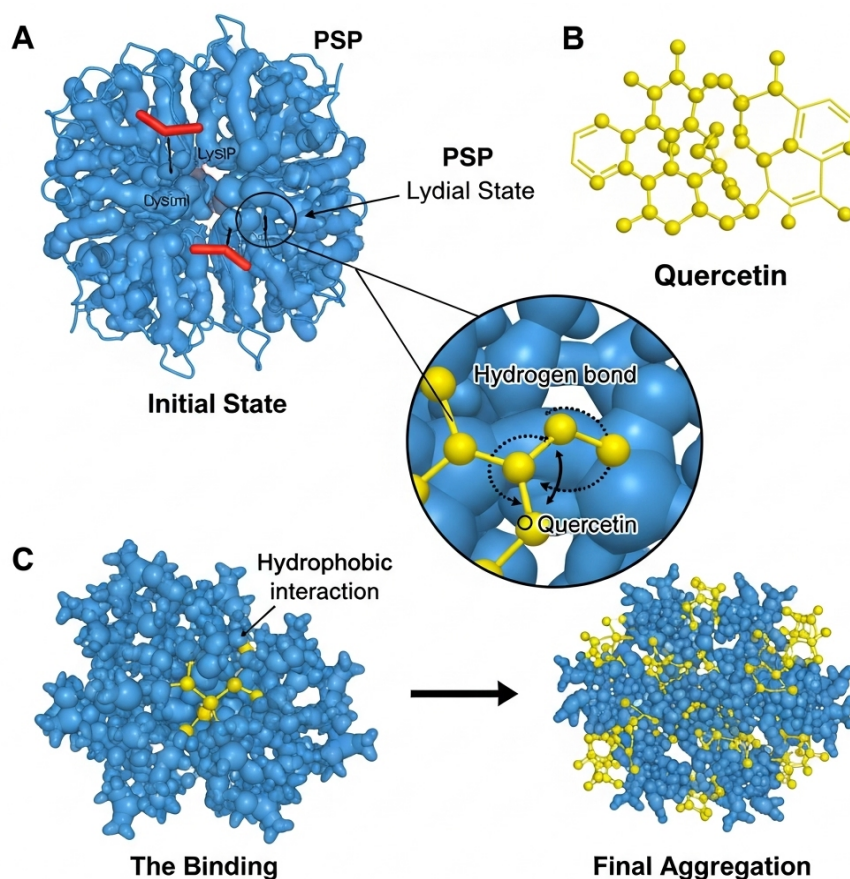


Figure 3-3 Schematic illustration of the noncovalent interaction and aggregation mechanism between PSP complexes and flavonoid polyphenols, exemplified by quercetin.

Building upon the structural and functional advantages of PSP hybrids described above, recent advances in biopolymer chemistry and biomedical engineering have significantly broadened their practical applications. By adjusting their molecular architecture and interfacial properties, these hybrid carriers can achieve precise control over the loading, protection, and release of bioactive compounds. Within the framework of personalized nutrition and multifunctional food design, such structural tunability allows the simultaneous and controlled delivery of multiple bioactive, thereby promoting synergistic physiological effects (Oyervides-Guajardo et al., 2025).

PSP complexes represent a new generation of natural biopolymeric delivery systems that combine adaptability, structural stability, and excellent biocompatibility.

Chapter 4 Anti-inflammatory effects of *Cordyceps* Cs-HK1 fungus EPS

4. 1 Introduction

Inflammation is a fundamental biological response in human body to protect against harmful stimuli, such as infections, injuries, and toxins. It plays a critical role in initiating defense mechanisms and facilitating tissue repair. Inflammation is beneficial and essential for recovery. However, when the inflammatory response becomes dysregulated, it can lead to pathological conditions. Chronic inflammation is implicated in the progression of numerous diseases, including autoimmune disorders (e.g., rheumatoid arthritis, lupus), cardiovascular diseases (e.g., atherosclerosis), neurodegenerative disorders (e.g., Alzheimer's disease), and various other chronic inflammatory conditions (Medzhitov, 2010; Nathan & Ding, 2010). Given the public health impact associated with these diseases, there is an urgent need for novel therapeutic strategies for effectively modulating the inflammatory responses and preventing or treating inflammation-related pathologies.

In the search for effective therapeutic options, natural products with anti-inflammatory properties have garnered considerable attention. Among these, *Cordyceps sinensis* (or *Ophiocordyceps sinensis*), generally known as the Chinese caterpillar fungus, has been widely recognized in traditional Chinese medicine (TCM) for its therapeutic benefits. Traditionally, *C. sinensis* was used to treat a variety of diseases, including respiratory disorders, chronic fatigue, and kidney dysfunction, with its primary functions in TCM being to "tonify the kidneys and lungs" and alleviate symptoms of chronic inflammation (Chen et al., 2013b). Recent studies have focused on the exopolysaccharides (EPS)

produced by the fermented mycelium of *C. sinensis* (Leung & Wu, 2007). The fermentation process has been optimized to produce high yields of bioactive EPS, which are secreted by the fungus as part of its extracellular matrix. One strain of *C. sinensis*, known as Cs-HK1, has been isolated and cultured in a controlled fermentation process to produce complex EPS. These EPS are further separated into two major fractions, EPS-HM and EPS-LM, based on differences in molecular weight and chemical composition (Li et al., 2020). Among these, EPS-LM, the lower molecular weight fraction, has demonstrated significantly higher anti-inflammatory activity. Specifically, EPS-LM has been shown to inhibit lipopolysaccharide (LPS)-induced pro-inflammatory responses in macrophage cell cultures. This is particularly important as LPS is commonly used to induce inflammation *in vitro*. EPS-LM has been shown to effectively suppress the activation of NF- κ B, a transcription factor that regulates the production of key pro-inflammatory mediators, including nitric oxide (NO), TNF- α , and interleukin-8 (IL-8) (Li et al., 2021a). Given the high bioavailability of EPS-LM and potent inhibitory effects on inflammation, it was selected for further investigation in the present study. This study aims to explore the molecular mechanisms by which EPS-LM, derived from fermented mycelium of *C. sinensis*, exerts its anti-inflammatory effects.

In addition to *C. sinensis*, polysaccharides from other medicinal fungi and plants have been demonstrated significant anti-inflammatory effects. For instance, polysaccharides from *Ganoderma lucidum* (GLP) and *Angelica sinensis* (ASP-Ag-AP) have been shown to modulate the TLR4/MyD88/NF- κ B signaling pathway, a key mediator of inflammatory responses. GLP has been demonstrated to protect against colitis and tumorigenesis by reducing the production of pro-inflammatory cytokines such as IL-1 β , inducible nitric oxide synthase (iNOS), and COX-2 (Guo et al., 2021). Furthermore,

GLP enhances gut barrier function and improves microbial dysbiosis, further contributing to its anti-inflammatory effects. Similarly, ASP-Ag-AP has demonstrated strong protective benefits in animal models of colitis, reducing inflammation and modulating gut microbiota and barrier function (Zou et al., 2023). These studies highlight the therapeutic potential of natural polysaccharides in managing inflammation by targeting key molecular pathways.

TLR4 acts as a transmembrane pattern-recognition receptor capable of responding to various pathogen-associated and damage-associated molecular patterns (Xiao et al., 2024; G. Xu et al., 2024). Upon activation, TLR4 engages myeloid differentiation factor 88 (MyD88), initiating a signaling cascade that leads to the activation of NF- κ B. This activation results in the upregulated transcription of genes encoding inflammatory cytokines. Furthermore, the TLR4/MyD88/NF- κ B signaling pathway is essential in facilitating the inflammatory responses induced by LPS, a constituent of the outer membrane of Gram-negative bacteria. Activation of this pathway results in the synthesis of pro-inflammatory mediators, including NO and reactive oxygen species (ROS). These mediators play a crucial role in the signaling pathways of the human immune response but may also cause adverse inflammatory effects and tissue damage if generated excessively (Kawai & Akira, 2010; Lu et al., 2008). The TLR4/MyD88/NF- κ B signaling pathway is a common therapeutic target for anti-inflammatory natural products, including natural polysaccharides (Wei et al., 2022; Zou et al., 2023).

This study aimed to investigate the anti-inflammatory effects of EPS-LM on LPS-stimulated macrophages through the TLR4/MyD88/NF- κ B signaling pathway. Based on the background above, we hypothesize that EPS-LM modulates this signaling

pathway, thereby exerting anti-inflammatory effects as shown in our previous studies (Li et al., 2021a). Experiments were performed in RAW 264.7 macrophage cell culture, which is one of the most popular in vitro models for immunomodulatory and anti-inflammatory studies.

4. 2 Materials and Methods

4.2.1 Preparation of different EPS fractions

The mycelial fermentation of Cs-HK1 was conducted in shake flasks to produce exopolysaccharides (EPS) with different molecular-weight ranges. Unless otherwise specified, all cultivations were carried out in the liquid medium reported previously (Leung et al., 2009; Li et al., 2020), the liquid medium contained 40 g/L glucose, 5 g/L peptone, 1 g/L KH_2PO_4 , 0.5 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 10 g/L yeast extract. For seed culture preparation, PDA-grown Cs-HK1 mycelia were inoculated into 250-mL Erlenmeyer flasks each containing 50 mL of the liquid medium (working volume 20% v/v). The flasks were incubated at 20 °C and 200 rpm for 7 days. For scale-up fermentation, 10 mL of the 7day seed culture (4% v/v) was transferred into 1L Erlenmeyer flasks each containing 250 mL of the same medium. The flasks were shaken at 200 rpm and 20 °C for another 7 days to obtain the main mycelial broth. At the end of fermentation, the culture broth was centrifuged at 12,000 rpm for 20 min at 4 °C to remove the mycelial biomass. The resulting supernatant was subjected to a two-step ethanol precipitation procedure (Li et al., 2021a), as illustrated in Figure 4-1. Absolute ethanol was slowly added to the supernatant with stirring to reach 40% (v/v),

followed by standing for 2 h at 25 °C and centrifugation at 9,000 rpm for 30 min. The precipitate obtained from this step was collected and, after re-dissolution in water and lyophilization, designated as the higher-molecular-weight EPS fraction (EPS-HM). The supernatant from the 40% ethanol step was then adjusted to 80% (v/v) ethanol, left to stand at 4 °C for 12 h, and centrifuged at 9,000 rpm for 30 min. The resulting precipitate was similarly re-dissolved and lyophilized to yield the lower-molecular-weight EPS fraction (EPS-LM).

Among the different fractions obtained from the Cs-HK1 culture, EPS-LM was selected for detailed characterization and bioactivity evaluation. In our preliminary screening, EPS-LM exhibited: (i) a relatively high protein content, suggesting the presence of polysaccharide-protein complexes, which are often associated with immunomodulatory activity; and (ii) a stronger stimulatory effect on RAW 264.7 cell viability and NO production at comparable concentrations than the other fractions. Therefore, EPS-LM was chosen as the representative extracellular PSP fraction for in-depth investigation in this thesis.

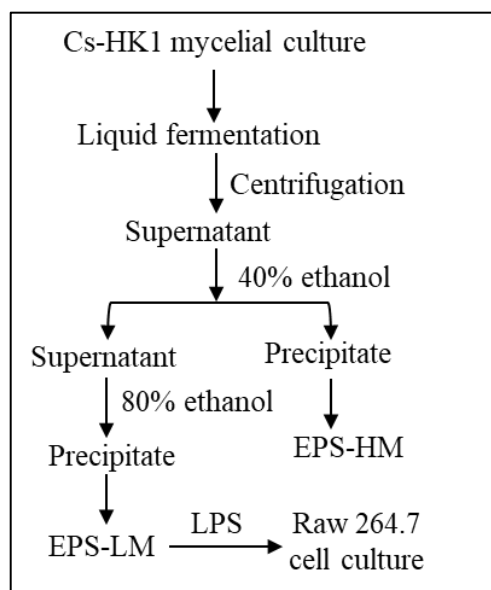


Figure 4-1 Major experimental steps for isolation of EPS-LM from Cs-HK1 mycelial fermentation and purification process.

4.2.2 Analysis of EPS composition and molecular weight

4.2.2.1 Total carbohydrate and protein content

The total carbohydrate and protein content of EPS-LM were determined by the Anthrone test and by Lowry method, respectively. As reported previously (Jun Hui Li et al., 2024; Zhao, Huang, et al., 2024), the EPS samples were hydrolyzed at 100°C with anthrone reagent in 80% H₂SO₄ and the absorbance of the sample solutions was measured at 620 nm, and converted to total carbohydrate by calibration with glucose as a reference standard. The protein content was quantified by measuring absorbance at 750 nm, using bovine serum albumin (BSA) as the reference standard.

4.2.2.2 monosaccharide composition

The monosaccharide composition of EPS-LM was analyzed by PMP-HPLC, following the previously published procedure (Li et al., 2021a). In brief, the EPS-LM sample was

hydrolyzed in 2 M TFA at 110°C for 4 hours, followed by vacuum evaporation to complete dryness. The remaining solid was redissolved in de-ionized water and mixed with equal volumes of 0.5 M PMP solution in methanol and 0.3 M NaOH solution, then incubated at 70°C for 30 minutes. The reaction was stopped by adding 0.3 M hydrochloric acid, then rinsed three times with chloroform, and the aqueous layer was collected for high-performance liquid chromatography (HPLC) analysis. An Agilent Zorbax Eclipse XDB-C18 column (150 mm × 4.6 mm) was used for the analysis on an Agilent 1100 instrument at a temperature of 25°C. The mobile phase comprised a potassium phosphate buffered saline solution (0.05 M, pH 6.9) with 15% acetonitrile (solution A) and 40% acetonitrile (solution B). Each sample was injected at 20 µL to the system detected with UV at 250 nm. The specific monosaccharide peaks were identified and quantified by calibration with monosaccharide standards (Sigma, St. Louis, Missouri, United States).

4.2.2.3 Analysis of the Molecular Weight

The MW of EPS-LM was measured using high-performance size-exclusion chromatography (HPSEC) with detection by refractive index (RI) and multi-angle laser light scattering (MALLS), as reported previously (Jun Hui Li et al., 2024). The instrumental system consisted of a Waters 1515 isocratic pump, two HPSEC columns (SB-806 HQ and SB-804 HQ, 7.8 × 300 mm, Shodex, Japan), a 2414 RI detector (Waters Co., Milford, MA, USA), and a MALLS detector (DAWN HELEOS II, Wyatt Technology, USA). An aliquot of 100 µL sample solution was injected into the system and eluted with water as the mobile phase at a flow rate of 0.5 mL/min. The average MW of the EPS-LM fractions was calculated using a refractive index increment (dn/dc)

value of 0.138 mL/g. Data collection and analysis were conducted using Astra software (version 6.1.7, Wyatt Technologies Co., Santa Barbara, CA, USA).

4.2.2.4 FT-IR Analysis

An infrared (IR) spectroscopic analysis was performed at room temperature using an Avatar 360 Fourier-transform infrared (FT-IR) spectrometer (Thermo Nicolet, Cambridge, UK). The analysis covered the spectral range of 4000-500 cm^{-1} with a resolution of 4 cm^{-1} . The solid EPS-LM was pulverized with potassium bromide (KBr) powder and pressed into a pellet for FT-IR analysis (Zhao, Huang, et al., 2024).

4.2.3 Assessment of EPS-LM bioactivities

4.2.3.1 RAW 264.7 macrophage cell culture

The in vitro anti-inflammatory activity assays were performed using LPS-stimulated RAW 264.7 macrophage cell cultures (Li et al., 2020). Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 $\mu\text{g/mL}$ streptomycin, all obtained from Gibco Biotechnology Co., Ltd. (Grand Island, NY, USA), was used as the base culture medium. All other agents related to cell culture, including trypsin, were also obtained from Gibco. The cell cultures were maintained at 37°C in a humidified incubator with 5% CO_2 .

4.2.3.2 Reagents and antibodies

Antibodies used included MyD88 (4283S, CST), NF- κB p65 (#8242, CST), interleukin-18 (IL-18) (A1115, ABclonal), TLR4 (Sc-293072, Santa Cruz), and β -actin (#4970, CST). Additionally, horseradish peroxidase (HRP)-conjugated goat anti-rabbit (A0208, Beyotime) and HRP-conjugated goat anti-mouse (A0216, Beyotime) were

utilized. LPS from *Escherichia coli* 0111: B4, along with reagents such as 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium (MTS), 2,7-Dichlorodihydrofluorescein diacetate (DCFH-DA), sulphanilamide, N-1-naphthyl ethylenediamine dihydrochloride, and phosphoric acid, were sourced from Sigma-Aldrich Co. Ltd. (St. Louis, MO, USA). Phenazine methosulfate (PMS) was obtained from Promega Corporation (Madison, WI, USA).

4.2.3.3 Cell viability assay

Cell viability was assessed using the MTS tetrazolium compound and the electron coupling reagent PMS, following the documented procedure (Barltrop et al., 1991). In metabolically active cells, dehydrogenases convert MTS into a water-soluble formazan, which can be quantified by measuring absorbance. RAW 264.7 cells in the logarithmic growth phase were seeded into 96-well plates (100 μ L/well) at a concentration of 2×10^5 cells/mL and incubated for 24 hours. The cells were then treated with EPS-LM at concentrations of 25, 50, 100, and 200 μ g/mL and incubated for another 24 hours. The absorbance of the resulting formazan was measured at 490 nm, with all operations conducted without exposure to light.

4.2.3.4 NO assay

The amount of NO formed was estimated from the accumulation of the stable NO metabolite, nitrite (NO_2^-), using the Griess assay. Cells were inoculated in 96-well plates at a density of 2×10^5 cells/mL, with culture conditions and treatments as described in Section 2.1. A reagent mixture of 1% (w/v) sulfanilamide, 0.1% (w/v) naphthyl ethylenediamine, and 2% (v/v) phosphoric acid was prepared. Samples were incubated with these reagents separately for 10 minutes at 25°C, and optical density

(OD) values were measured at 540 nm (Kleinbongard et al., 2002). The amount of nitrite was calculated from a NaNO₂ standard curve (0–100 µM).

4.2.3.5 Intracellular ROS assay (DCFH-DA probe)

Intracellular ROS levels were quantified using the method described by (Alia et al., 2005). DCFH-DA is a cell-permeant fluorogenic dye that measures hydroxyl, peroxy, and other ROS activities in cells. Cells were seeded in 96-well plates at a density of 2×10^5 cells/mL, following the culture conditions and treatments outlined in Section 2.1. DCFH-DA is deacetylated by cellular esterase into a non-fluorescent compound, which is subsequently oxidized by ROS into 2',7'-dichlorofluorescein (DCF). DCF fluorescence is detected with excitation at 485 nm and emission at 527 nm using a microplate reader (Molecular Devices SpectraMax Gemini X).

4.2.3.6 Western blotting analysis

The Western blot approach was used to identify the expression of inflammation-related proteins, as reported by Li et al., with minor adjustments (Li et al., 2021a). After treatment with LPS and EPS-LM, RAW 264.7 cells were collected and washed with cold phosphate-buffered saline (PBS, pH 7.4). Ice-cold cell lysis reagent (Catalogue #R0278, Sigma-Aldrich, Germany) was used to extract total cytoplasmic proteins, including those for IL-18, NF-κB p65, TLR4, and MyD88 detection. Actin served as an internal reference. The protein content in the supernatant was measured using the bicinchoninic acid (BCA) protein assay kit (Catalogue #23225, Thermo Fisher Scientific, Rockford, IL, USA). The lysate protein (20 µg per lane) was separated using 10% SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane. The membrane was blocked with 5% skimmed milk (w/v) and treated with specific primary antibodies overnight at 4°C, followed by the addition of secondary antibodies coupled

with horseradish peroxidase. The blots were then developed using enhanced chemiluminescence and visualized via autoradiography.

4.2.3.7 Immunofluorescence and confocal microscopy measurement

After treatment with LPS and EPS-LM for 24 hours, RAW 264.7 cells were fixed with 4% paraformaldehyde at room temperature for 10 minutes. The cells were then treated with 0.1% Triton X-100 for 5-10 minutes at room temperature, followed by three washes with PBS. To block nonspecific antibody binding, the cells were incubated with 1% BSA in PBS for 30 minutes. After blocking, the cells were incubated with diluted primary antibodies against MyD88, TLR4, and NF- κ B p65 at 4°C for 1 hour. The cells were washed 3-5 times with PBS for 5 minutes each to remove unbound primary antibodies. Next, they were incubated with diluted fluorescein isothiocyanate (FITC)-conjugated secondary antibodies at room temperature for 1 hour in the dark. The cells were washed three times with PBS for 5 minutes each to remove unbound secondary antibodies. For nuclear visualization, the cells were treated with DAPI (4',6-diamidino-2-phenylindole) for 5 minutes at room temperature and then washed 2-3 times with PBS. Images were visualized using confocal microscopy (Nikon Eclipse Ti2-E live-cell imaging system).

4.2.3.8 Co-immunoprecipitation (Co-IP) assay

Approximately 1.0×10^7 macrophage cells were seeded in each 6-cm dish and exposed to LPS and/or EPS-LM for 24 hours, followed by protein extraction as described above. Immunoprecipitation was performed with slight modifications to the previously reported method (Burckhardt et al., 2021). Protein A+G magnetic beads were prepared and incubated with antibodies against TLR4 and MyD88 or control immunoglobulin G (IgG), followed by washing and resuspension in Tris-buffered saline (TBS). The

protein samples were incubated with antibody-bound beads overnight at 4°C, washed, and eluted with SDS-PAGE sample buffer. The eluted proteins were separated by SDS-PAGE and transferred to a 0.45 µm PVDF membrane. Membranes were blocked, incubated with primary antibodies overnight, washed, and then incubated with secondary antibodies. Detection was performed using chemiluminescence, and images were captured and analyzed to assess the expression levels of TLR4 and MyD88.

4.2.4 Statistical data analysis

The data were analyzed using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA, USA) with one-way analysis of variance (ANOVA) for multiple comparisons, followed by Tukey's post hoc test. All numerical data are expressed as the mean ± standard deviation (SD) of at least three independent experiments. The significance level was set at $p < 0.05$.

4.3 Results

4.3.1 EPS-LM composition and effect on RAW 264.7 cell viability

As shown in Table 4-1, EPS-LM in this study had an average molecular weight of 6.58×10^5 Da, a sugar content of 18.7 wt%, and a relatively high protein level of 30.0 wt%. These values indicate that the present EPS-LM sample is a high-molecular-weight, polysaccharide–protein complex. In our previous study on EPS-LM from the same Cs-HK1 strain (Li et al., 2021a), a major molecular-weight peak of 3.60×10^5 Da was reported, together with a higher total carbohydrate content (25.5 wt%) and a lower protein content (20.1 wt%). Although the absolute values differ, both batches were produced using the same fermentation conditions and two-step ethanol precipitation workflow, and therefore represent the same EPS-LM fraction rather than different

products. The small variations in carbohydrate and protein levels are within the range expected for separate fermentation batches and are likely attributable to minor differences in manual handling and environmental conditions (e.g., humidity and temperature) during the multi-step preparation, which can slightly affect precipitation efficiency. In both studies, glucose and mannose were the main monosaccharide constituents of EPS-LM, with mannose being more abundant in the present batch. In addition, trace amounts of rhamnose and glucosamine were detected in the current EPS-LM sample, whereas these minor components were not reported previously. Overall, the main compositional profile and structural features of EPS-LM are consistent between the two batches, and the EPS-LM used in the present study can be regarded as equivalent to that examined by (Li et al., 2021a).

These batch-to-batch variations in sugar and protein contents are likely attributable to subtle differences in fermentation and downstream processing. For example, small fluctuations in inoculum age, dissolved oxygen, or effective cultivation time may have influenced the relative synthesis and secretion of polysaccharides versus extracellular glycoproteins. Variations in ethanol precipitation (e.g., mixing efficiency, standing time, or temperature control) and subsequent re-dissolution steps could also have altered the co-precipitation of protein-rich polysaccharide-protein complexes, thereby increased the measured protein proportion and slightly diluted the carbohydrate content in the current EPS-LM preparation. Nevertheless, the consistently high molecular weight and the dominance of glucose and mannose as major monosaccharides indicate that the core structural features of EPS-LM were preserved, supporting an overall stability of the bioprocess despite minor compositional differences between batches.

Table 4-1 Chemical composition and molecular weight of Cs-HK1 EPS-LM.

Original GPC results shown in Appendix Data **Figure A1**.

Sugar (wt%)	Protein (wt%)	Molecular weight (Da)		
18.71±2.27	30.00±0.05	6.58×10 ⁵ (±3.32%)		
Monosaccharide composition (molar ratio)				
Mannose	Glucose	Galactose	Rhamnose	Glucosamine
2.191	1.286	1	0.325	0.246

Figure 4-2 shows the FT-IR spectrum of EPS-LM, which reveals several characteristic peaks. The broad and intense peak around 3400 cm⁻¹ is attributed to the axial stretch of the -OH group in the glycol chain, indicating the presence of numerous hydroxyl groups commonly associated with polysaccharides. Additionally, the peak around 1390 cm⁻¹ is due to OH bending vibration, further confirming the abundance of hydroxyl groups in EPS-LM. A weak peak around 2911 cm⁻¹ corresponds to C-H stretching vibration, suggesting the presence of organic compounds with C-H groups. The peak at 1624 cm⁻¹ is characteristic of asymmetric and symmetric vibrations of the ring stretching of the carboxylate group, indicating the presence of carboxylate groups potentially related to the anti-inflammatory and antioxidant properties of EPS-LM. Absorption peaks in the region of 1000-1200 cm⁻¹ are associated with the stretching vibrations of C-O-H side groups and C-O-C glycosidic bond vibrations, which are indicative of glycosidic linkages and side groups typical of polysaccharide structures. The peak around 1059 cm⁻¹ is attributed to C-O stretching of pyranoside, particularly in glucose residues. The absence of an absorption peak around 1710-1740 cm⁻¹ confirms the absence of carboxylic acid groups in EPS-LM. The characteristics of EPS-LM observed in this

study are like those reported in previous research on EPS-LM produced by Cs-HK1. The FT-IR spectrum shows analogous peaks, suggesting that both studies describe EPS-LM with certain structural features that could potentially contribute to their comparable biological activities (Li et al., 2021a).

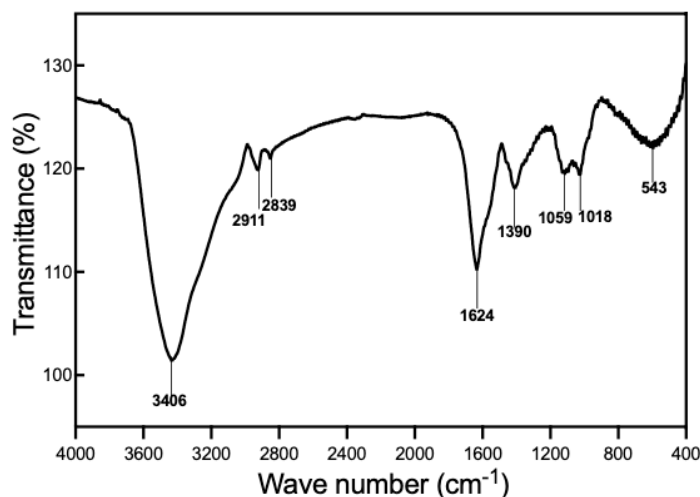


Figure 4-2 FT-IR spectrum of EPS-LM isolated from Cs-HK1 mycelial fermentation medium.

As shown in Figure 4-3, the OD values of the EPS-LM treated groups showed no significant difference from the control in the concentration range of 25-200 $\mu\text{g/mL}$. This indicates that EPS-LM is non-toxic to RAW 264.7 cells and promotes cell growth, which could be regarded as an immunostimulatory effect of the EPS-LM.

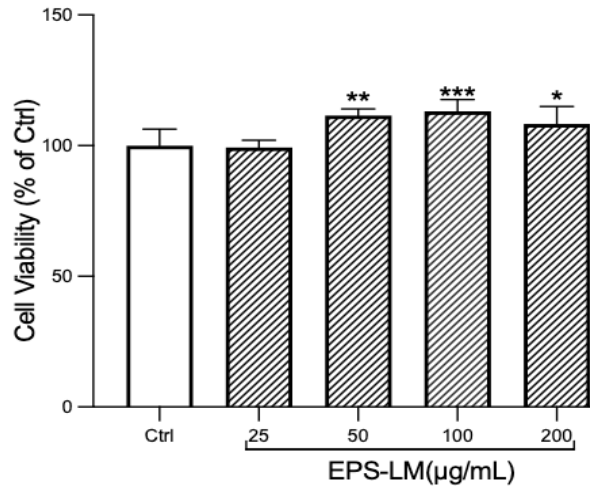


Figure 4-3 Effects of EPS-LM treatment on cell viability and proliferation in RAW 264.7 cells. (Data as mean $\pm$ SEM, $n = 6$. One-way ANOVA followed by Tukey's multiple comparison test: * $p < 0.1$, ** $p < 0.01$, *** $p < 0.001$ versus Ctrl).

4.3.2 EPS-LM reduces ROS and inhibits oxidative stress induced by LPS

As shown in Figure 4-4, LPS treatment caused a dramatic increase in the ROS level (proportional to the fluorescence intensity) of macrophage cells compared with the control group. The addition of EPS-LM together with LPS, denoted as LPS+EPS-LM, significantly lowered the LPS-stimulated ROS production. The increase in EPS-LM concentration from 25 to 200 $\mu\text{g/mL}$ resulted in a more substantial suppression of ROS production. These results suggest that EPS-LM can effectively reduce the LPS-stimulated ROS production in RAW 264.7 cells.

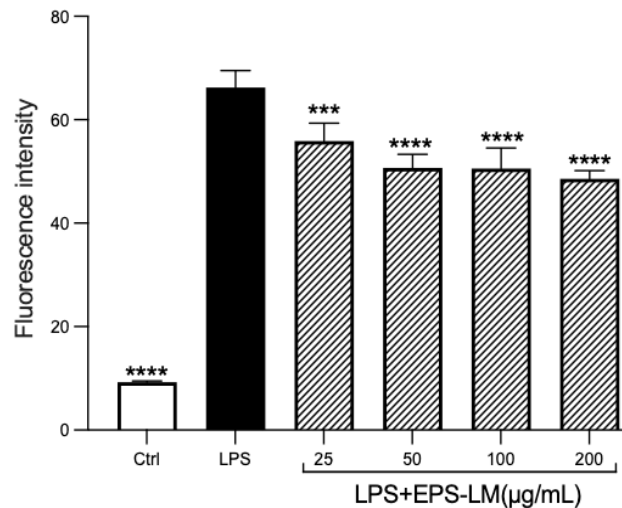
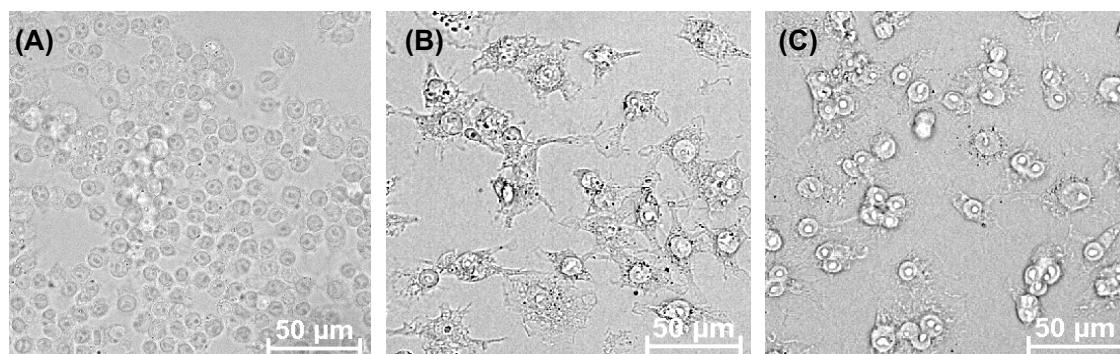


Figure 4-4 Effects of EPS-LM on LPS-induced ROS production in RAW 264.7 cells. (Data as mean $\pm$ SEM, n = 6. One-way ANOVA followed by Tukey's multiple comparison test: *** p < 0.001, **** p < 0.0001 versus LPS).

4.3.3 EPS-LM effects on LPS-induced morphological changes and NO production

LPS treatment of the macrophage cells also altered their morphology (**Figure 4-5 A-C**). While cells in the control group exhibited a uniform and oval shape, the LPS-treated cells showed obvious differentiation into a shuttle shape. This morphological change, combined with the results of the NO assay, indicates successful induction of an inflammatory response and establishment of the inflammation model. As shown in Figure 4-5D, EPS-LM significantly suppressed the LPS-induced production of NO. At the maximum concentration tested (200 μ g/mL), EPS-LM decreased the LPS-stimulated production of NO by about 40%. The inhibitory effects of EPS-LM on NO production followed a dose-dependent trend in the range of 25-200 μ g/mL.



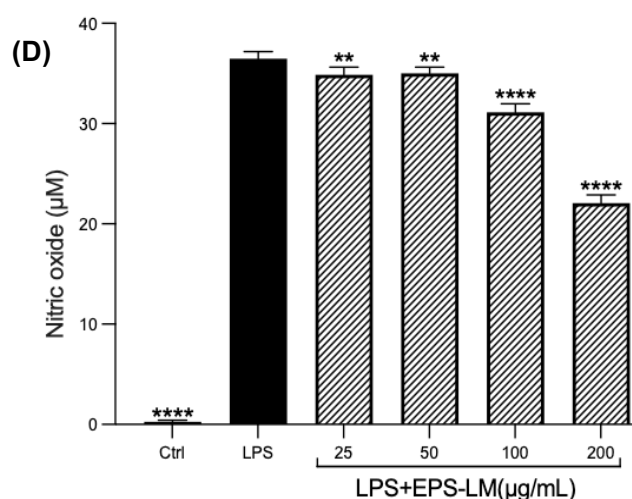


Figure 4-5 Effects of LPS and EPS-LM on RAW 264.7 cells: Morphology of (A) Control; (B) LPS-treated; (C) LPS + 200 µg/mL EPS-treated group; (D) NO production. (Data as mean $\pm$ SEM, n = 6; One-way ANOVA followed by Tukey's multiple comparison test: **p < 0.01, ****p < 0.0001 versus LPS).

4.3.4 EPS-LM inhibits the TLR4/MyD88/NF- κ B pathway to mitigate inflammation

In the present work, IL-18 was chosen as the primary cytokine marker rather than TNF- α and IL-1 β . This selection was based on the following considerations. First, previous studies on Cs-HK1 EPS-LM have already reported the induction of classical pro-inflammatory cytokines such as TNF- α and IL-1 β , whereas the role of IL-18 in polysaccharide-mediated macrophage activation has received comparatively less attention. Second, IL-18 serves as a key link between innate and adaptive immunity and is closely associated with inflammasome activation, which may provide additional insight into the immunomodulatory mechanisms of EPS-LM beyond the commonly studied cytokines. Therefore, IL-18 was measured as a representative cytokine to indicate TLR4/MyD88-dependent activation.

Figure 4-6 shows all the results from western blotting and immunofluorescence labeling assessment of the expression of critical proteins in the TLR4/MyD88/NF- κ B signaling pathway. The Western blotting results (Figure 4-6A) showed that LPS significantly increased the expression levels of IL-18, TLR4, NF- κ B p65, and MyD88 proteins in RAW 264.7 cells, validating its effectiveness in activating the TLR4/MyD88/NF- κ B pathway and eliciting an inflammatory response. Treatment of the cells with EPS-LM dramatically reduced the expression levels of these proteins in LPS-stimulated cells, indicating a substantial inhibitory effect on this pathway.

The immunofluorescence labeling assay provided further evidence for the changes in cellular localization and expression of these signaling molecules. The fluorescence intensity of TLR4 and MyD88 significantly increased after LPS exposure, indicating their heightened expression and involvement in the inflammatory process (Figure 4-6B and C). Additionally, LPS exposure prompted the nuclear translocation of NF- κ B p65, a crucial step for initiating the transcription of inflammatory genes (Figure 4-6D). Treatment with EPS-LM significantly decreased the fluorescence intensity of both TLR4 and MyD88 after LPS exposure, demonstrating its potent capacity to suppress these critical inflammation-inducing proteins (Figure 4-6E and F). This suggests that EPS-LM inhibited both the expression and potential activation of these key inflammatory mediators. Furthermore, EPS-LM treatment notably reduced the nuclear translocation of NF- κ B p65 (Figure 6G), indicating its role in obstructing the downstream signaling processes essential for initiating the transcription of pro-inflammatory genes. Therefore, these results provide support to the hypothesis that

EPS-LM suppressed the LPS-induced inflammatory responses in the macrophage cells by blocking the TLR4/MyD88/NF- κ B signaling pathway.

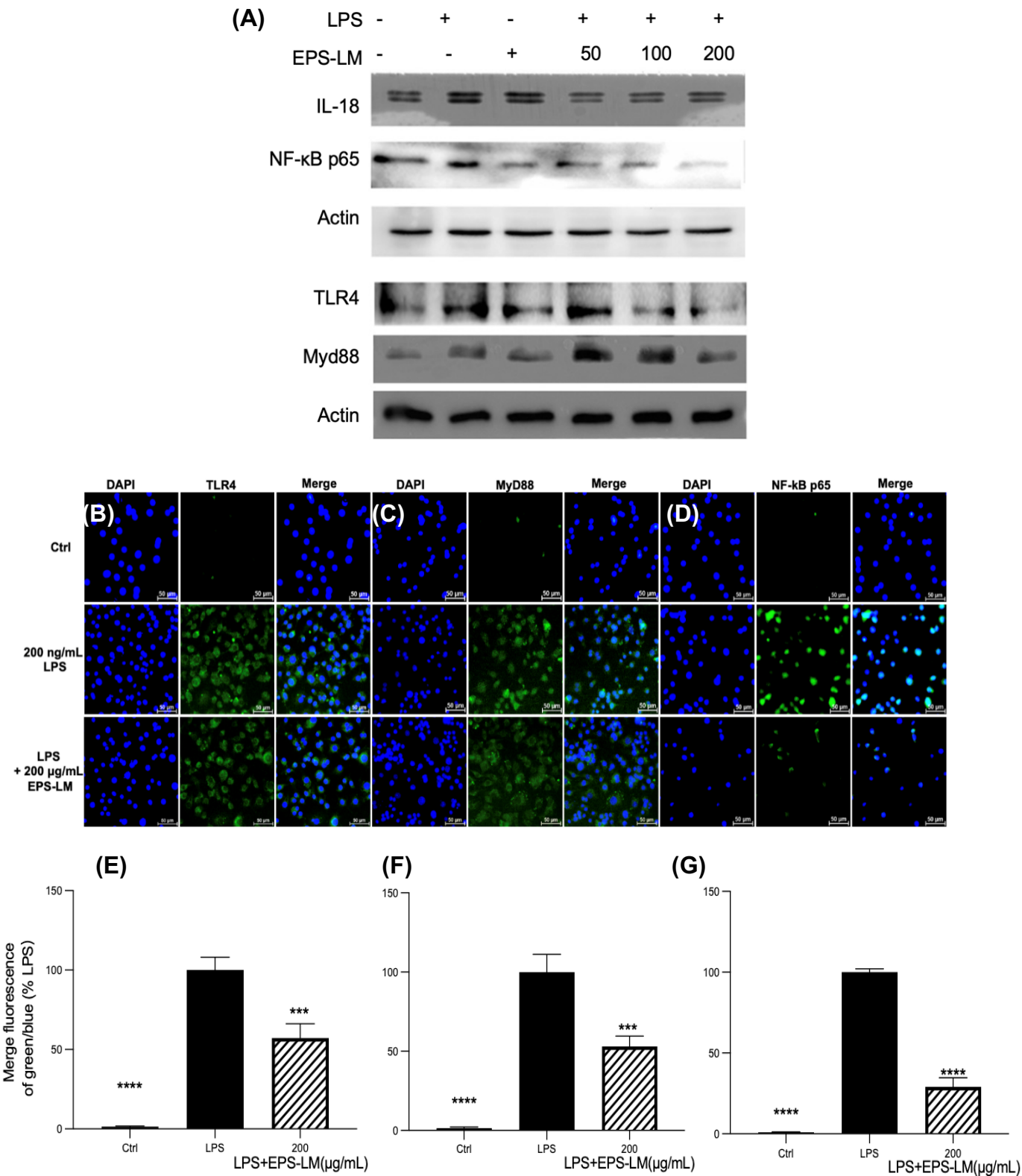


Figure 4-6 Western blotting and immunofluorescence analysis of the effect of EPS-LM on LPS-induced inflammatory protein expression in RAW 264.7 cells: (A) The representative bands of IL-18, NF- κ B p65, TLR4, and MyD88 were determined by Western blotting. Actin were used as internal control; (B) (C) (D) TLR4, MyD88, NF- κ B p65 expression levels in RAW 264.7 cells; (E) (F) (G) Statistical analysis of the ratio of green to blue fluorescence for images corresponding to TLR4, MyD88, and NF- κ B p65, respectively. (Data as mean $\pm$ SEM, n = 3. One-way ANOVA followed by Tukey's multiple comparison test: ***p < 0.001, ****p < 0.0001 versus LPS).

4.3.5 Effects of EPS-LM on the interaction between TLR4 and MyD88

The treatment effects on the interaction between TLR4 and MyD88 were detected via co-immunoprecipitation (Co-IP). As shown in **Figure 5-6**, 24 hours post-LPS stimulation, the contact between TLR4 and MyD88 increased considerably, suggesting that the TLR4/MyD88 signaling pathway had been successfully induced. However, EPS-LM significantly decreased the binding of TLR4 to MyD88, indicating that it efficiently disturbed their interaction. This result corroborated the above finding that EPS-LM suppressed the expression of TLR4 and MyD88 in LPS-stimulated RAW 264.7 cells, further proving the concept that EPS-LM exerts its anti-inflammatory effects by interfering with the TLR4/MyD88/NF- κ B signaling pathway.

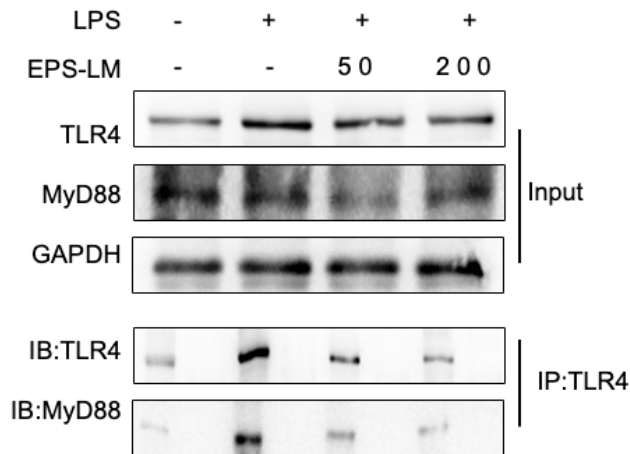


Figure 4-7 EPS-LM reduces the interaction between TLR4 and MyD88 in RAW 264.7 cells. RAW 264.7 cells were treated with LPS in the presence or absence of EPS-LM (50 and 200 $\mu\text{g/mL}$). Co-IP was performed to assess the interaction between TLR4 and MyD88. TLR4 and MyD88 were detected by immunoblotting (IB) in the input and TLR4-immunoprecipitated (IP: TLR4) samples. GAPDH was used as a loading control.

4.4 Discussion

The results demonstrated that EPS-LM significantly reduced NO and IL-18 levels in LPS-stimulated RAW 264.7 cells, indicating its potent *in vitro* anti-inflammatory activity. More specifically, EPS-LM inhibited the expression of TLR4, MyD88, and NF- κ B proteins, which are critical components of the TLR4/MyD88/NF- κ B signaling pathway, a key mediator of LPS-induced inflammatory responses. This downregulation reduces the generation of inflammatory mediators, suggesting a protective effect against LPS-induced cellular damage. By inhibiting the TLR4/MyD88/NF- κ B signaling pathway, EPS-LM substantially reduces inflammation (Zou et al., 2023). We further conducted experiments to investigate the interaction between TLR4 and MyD88. The Co-IP experiment was designed to elucidate the molecular interactions between TLR4 and MyD88 in the presence and absence of EPS-LM. After 24 hours of LPS stimulation, Co-IP analysis revealed a significant increase in the interaction between

TLR4 and MyD88, indicating successful activation of the TLR4/MyD88 signaling pathway. This interaction is a key step in the cascade that leads to the activation of NF- κ B and the subsequent production of pro-inflammatory cytokines (Akira & Takeda, 2004). Interestingly, in cells co-treated with EPS-LM and LPS, the Co-IP results showed a marked decrease in TLR4 binding to MyD88. This reduction suggests that EPS-LM effectively disrupts the critical connection between these two proteins, thereby impeding the signaling cascade at an early stage. By preventing the association of TLR4 with MyD88, EPS-LM inhibits the subsequent activation of NF- κ B p65. By inhibiting its activation, EPS-LM significantly reduces the production of inflammatory cytokines, thereby mitigating the inflammatory response (Liu et al., 2017).

The findings from our present study are consistent with earlier research on comparable polysaccharides, confirming their anti-inflammatory capabilities. Both EPS-HM and EPS-LM, along with total EPS, have been demonstrated to decrease LPS-induced activation of NF- κ B, a major transcription factor in the inflammatory response (Li et al., 2021a). Attenuating NF- κ B activation is critical because it induces the transcription of pro-inflammatory genes, including those encoding cytokines like TNF- α and interleukin-1 beta (IL-1 β), which are pivotal in immunological and inflammatory responses, particularly during infection and trauma. The NF- κ B signaling pathway stimulates cytokines, chemokines, and adhesion molecules, thereby controlling inflammation, immunity, and cell survival (Barnes & Larin, 1997; Hayden & Ghosh, 2011).

Our experiments, building on previous studies (Li et al., 2021a), further demonstrated that EPS-LM suppresses the expression of the downstream protein NF- κ B p65 by inhibiting the upstream pathway components TLR4 and MyD88 and their interactions

(Figure 4-8). By inhibiting the interaction between TLR4 and MyD88, EPS-LM blocks the activation of NF- κ B p65. NF- κ B p65 is a key subunit of the NF- κ B transcription factor complex, responsible for regulating the expression of multiple pro-inflammatory genes (S. Y. Zhang et al., 2024). By inhibiting the activation of NF- κ B, EPS-LM significantly reduces the production of pro-inflammatory cytokines, thereby alleviating inflammatory responses.

Oxidative stress is a key contributor to cellular damage and inflammation, often exacerbating the effects of inflammatory stimuli like LPS. Excessive ROS can lead to lipid peroxidation, protein oxidation, and DNA damage, which further amplify the inflammatory response and contribute to chronic inflammation and tissue damage (Shin et al., 2024; Y. Zhang et al., 2024). By suppressing the TLR4/MyD88/NF- κ B signaling pathway, EPS-LM not only reduces the production of pro-inflammatory cytokines but also lowers ROS levels, thereby providing a dual protective effect (Figure 8). The reduction of ROS by EPS-LM is particularly significant because oxidative stress and inflammation are closely linked processes. ROS can activate various signaling pathways, including NF- κ B, which in turn promotes the expression of pro-inflammatory genes. This creates a vicious cycle where inflammation leads to increased ROS production, and ROS further amplifies the inflammatory response (Abbas et al., 2024). By breaking this cycle through the inhibition of the TLR4/MyD88/NF- κ B pathway, EPS-LM effectively mitigates both oxidative stress and inflammation. Furthermore, the antioxidant properties of EPS-LM help maintain cellular redox balance. This is crucial for the proper functioning of cellular processes and the prevention of apoptosis (programmed cell death) triggered by oxidative damage. By preserving cellular integrity and function, EPS-LM supports the overall health and viability of cells exposed to inflammatory stimuli.

EPS-LM showed a unique and potentially superior ability to suppress both oxidative stress and inflammation compared to other well-known anti-inflammatory agents. For instance, nonsteroidal anti-inflammatory medicines (NSAIDs), such as ibuprofen and aspirin, primarily reduce inflammation by inhibiting cyclooxygenase (COX) enzymes, but their impact on ROS levels is limited (Vane & Botting, 1997). Corticosteroids, like dexamethasone, effectively suppress pro-inflammatory cytokine production by inhibiting the NF- κ B pathway, yet they do not directly target ROS, and long-term use can lead to side effects such as immunosuppression and metabolic disturbances (Barnes, 2006). In contrast, antioxidants like N-acetylcysteine (NAC) and vitamin E have been shown more effective in scavenging ROS but less in modulating the key inflammatory pathways like TLR4/MyD88/NF- κ B (Brigelius-Flohé & Davies, 2007; Zafarullah et al., 2003). EPS-LM was active not only in reducing ROS levels but also inhibiting the TLR4/MyD88/NF- κ B signaling pathway, thus breaking the vicious cycle where oxidative stress and inflammation exacerbate each other (Mittal et al., 2014).

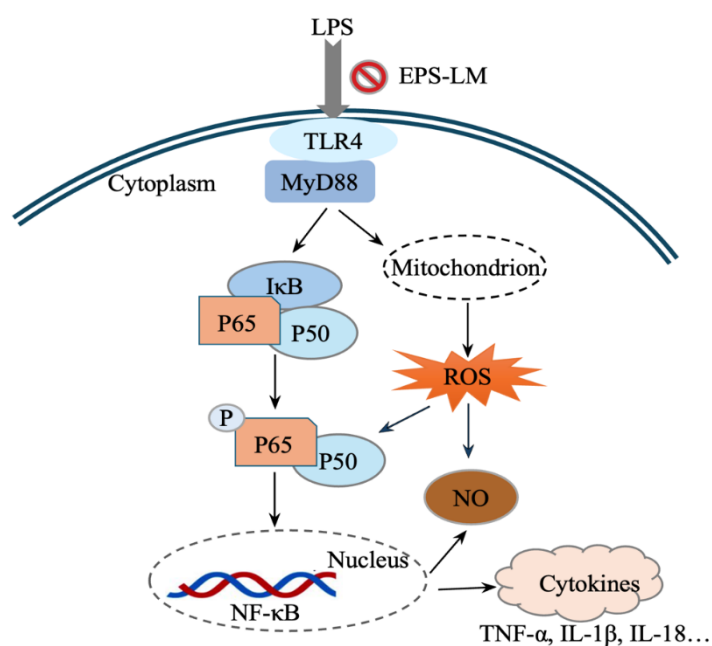


Figure 4-8 A hypothesized signal mechanism for the protective effects of EPS-LM against LPS-induced inflammatory injury and ROS via the TLR4/MyD88/NF- κ B

pathway: (1) LPS binding to TLR4 activates MyD88, leading to a signaling cascade that degrades I κ B, releasing and phosphorylating p50 and p65 subunits; (2) these subunits form an active NF- κ B complex that moves to the nucleus to transcribe pro-inflammatory cytokines like TNF- α and IL-1 β ; (3) LPS also induces ROS and NO production in mitochondria, contributing to inflammation and damage; (4) EPS-LM inhibits TLR4-MyD88 interaction, reducing ROS and NO generation and providing anti-inflammatory protection.

This dual action of anti-inflammation and anti-oxidation through the same signaling pathway highlights the multifaceted protective effects of EPS-LM. The molecular weight and composition of EPS-LM, as identified in this study, provide further insights into its anti-inflammatory potential. The high MW (6.58×10^5 Da) and significant protein content (30 wt%) suggest the presence of complex polysaccharide-protein formations known to enhance biological activity. Compared to previous findings (Li et al., 2021a), our study identified a higher protein content and a different monosaccharide composition, with a higher prevalence of mannose and the presence of rhamnose and glucosamine. These components interact with specific receptors on immune cells, such as Toll-like receptors. These interactions are critical for regulating immune responses and lowering inflammation. Larger polysaccharide molecules often have more complex and branched structures, enhancing their capacity to engage with immune cell receptors. Furthermore, polysaccharide-protein complexes add to this complexity by possibly providing numerous binding sites for receptor engagement and promoting a stronger anti-inflammatory response (Brown & Gordon, 2003; Li et al., 2020; Willment & Brown, 2008). The FT-IR spectrum analysis further confirms the structural complexity of EPS-LM, highlighting the presence of numerous functional groups associated with bioactivity. The similarities in FT-IR spectra between our study and previous research

on EPS-LM from Cs-HK1 underscore the stability and consistency of the fermentation process, while the minor differences observed, such as slight shifts in the FT-IR peaks corresponding to functional groups (e.g., carboxyl, hydroxyl, or glycosidic bonds), might account for variations in bioactivity, particularly in terms of antioxidant and anti-inflammatory effects.

Our findings demonstrate the potent anti-inflammatory and antioxidant properties of EPS-LM, primarily through its modulation of the TLR4/MyD88/NF- κ B signaling pathway. This dual action highlights the potential of EPS-LM as a holistic therapy for inflammatory conditions. The effectiveness of similar polysaccharides like ASP-Ag-AP and GLP in reducing colitis via the TLR4/MyD88/NF- κ B pathway suggests that EPS-LM, which also exerts anti-inflammatory effects through this pathway, may have comparable protective benefits against inflammatory conditions such as colitis. While our study focused on LPS-stimulated RAW 264.7 cells, the shared mechanism of action indicates that EPS-LM might be beneficial in managing various inflammatory diseases. We focused on the TLR4/MyD88-dependent signaling pathway for several reasons. First, numerous reports have demonstrated that fungal and mushroom-derived polysaccharides mainly activate macrophages through the TLR4/MyD88-NF- κ B axis, leading to the production of pro-inflammatory and immunoregulatory mediators. Second, our preliminary inhibitor experiments indicated that blocking MyD88 markedly attenuated the EPS-LM-induced activation of RAW 264.7 cells, suggesting that MyD88-dependent signaling plays a predominant role under the present experimental conditions. Third, given the scope and length constraints of this thesis, a detailed investigation of the MyD88-independent (TRIF-dependent) branch was not undertaken. Nonetheless, we acknowledge that MyD88-independent signaling

pathways may also contribute to the overall macrophage response to EPS-LM, and they warrant further investigation in future studies.

By modulating the TLR4/MyD88/NF- κ B pathway, EPS-LM reduces inflammation and alleviates oxidative damage, demonstrating its potential as a holistic therapy for inflammatory conditions. Future research should be focused on *in vivo* studies using animal models of inflammation, such as colitis or arthritis, to verify the bioavailability, efficacy, and safety of EPS-LM in complex physiological environments. Following successful animal studies, early-phase clinical trials are essential to assess the safety, tolerability, and efficacy of EPS-LM in humans, particularly in patients with inflammatory disorders such as inflammatory bowel disease (IBD) or rheumatoid arthritis. Moreover, combination therapy studies should investigate whether EPS-LM can enhance the effects of existing anti-inflammatory or antioxidant treatments without increasing side effects.

4.5 Conclusions

The present study has detected positively the specific activity of EPS-LM in inhibiting the TLR4/MyD88/NF- κ B signal pathway which is pivotal for the LPS-induced pro-inflammatory responses in the macrophage cells. EPS-LM has also been shown to suppress the LPS-induced ROS production in the macrophage cell culture. These altogether revealed the multifaceted anti-inflammatory effects of EPS-LM, making it a promising candidate for further research and development in the treatment of various inflammatory and oxidative stress-related conditions, offering a comprehensive approach to managing inflammation and protecting cells from damage. However, while this study highlights the potential of EPS-LM as a therapeutic agent, further *in vivo* and clinical research is crucial to fully establish its efficacy and safety in humans.

Chapter 5 An antioxidative exopolysaccharide-protein complex of *Cordyceps* Cs-HK1 fungus and its epithelial barrier-protective effects in Caco-2 cell culture

5.1 Introduction

The intestinal epithelial barrier represents the first line of host defense against luminal threats, preventing the translocation of pathogens and toxins into the internal circulation while selectively allowing nutrient and signal exchange between the host and environment (Bischoff et al., 2014). Tight junctions (TJs) are central to this barrier system, regulating paracellular permeability and protecting underlying tissues from luminal contents (Lu et al., 2013). Disruption of TJ integrity compromises epithelial protection, enabling the passage of luminal antigens and microbes. This breach provokes immune activation and chronic inflammation, which exacerbate gastrointestinal disorders including IBD, irritable bowel syndrome (IBS), and “leaky gut syndrome,” and can also contribute to systemic complications such as metabolic, autoimmune, and neurological diseases (Camilleri et al., 2012; Dunleavy et al., 2023).

Oxidative stress, largely driven by the overproduction of reactive oxygen species (ROS), plays a pivotal role in TJ disruption. Under physiological conditions, endogenous antioxidant systems maintain redox balance; however, during infection or inflammation, uncontrolled ROS can damage DNA, proteins, and lipids (Ngo & Duennwald, 2022; Patlevic et al., 2016). Excessive ROS specifically compromises TJ components such as

ZO-1, Claudins, and Occludin, leading to their redistribution or degradation, elevated paracellular permeability, and increased translocation of luminal substances. This barrier breakdown amplifies inflammation and oxidative stress, generating a self-perpetuating cycle that further deteriorates epithelial integrity (Kim & Breton, 2016; Petecchia et al., 2012).

Among TJ proteins, Claudin-1, Occludin, and ZO-1 play essential structural and regulatory roles. Claudin-1 forms selective paracellular barriers between epithelial cells, controlling ion and solute permeability, while Occludin contributes to junctional sealing and stabilizes interprotein connections with the cytoskeleton (Adil et al., 2021; Angelow et al., 2008). ZO-1, a multidomain cytoplasmic scaffolding protein, anchors transmembrane proteins such as Claudin-1 and Occludin to the actin cytoskeleton and mediates dynamic regulation of junctional complexes in response to signaling cues (Balda & Matter, 2008; Brunner et al., 2021). Together, these TJ proteins coordinate to sustain epithelial cohesion and preserve barrier functionality.

To counteract oxidative injury, cells activate the Kelch-like ECH-associated protein 1-nuclear factor erythroid 2-related factor 2 (KEAP1-NRF2) axis, a master regulatory pathway governing antioxidant and cytoprotective responses. Upon oxidant challenge (e.g., H_2O_2), critical cysteine residues on KEAP1 become modified, which disrupts the KEAP1-CUL3 complex and allows NRF2 to escape ubiquitin-mediated degradation. Accumulated NRF2 translocates into the nucleus and binds to antioxidant response elements (AREs), driving the transcription of detoxifying and phase II

enzymes such as NAD(P)H: quinone oxidoreductase 1 (NQO1), HO-1, and glutamate-cysteine ligase catalytic subunit (GCLC). Through these effectors, the NRF2 pathway restores cellular redox balance, enhances resilience against oxidative stress, and contributes to the maintenance of epithelial integrity (Ma, 2013; Suzuki & Yamamoto, 2017).

Natural polysaccharides-based complexes from food and medicinal sources can alleviate oxidative stress and preserve epithelial structure and function, thereby maintaining intestinal homeostasis (Huang et al., 2025). By reducing oxidative load, these biopolymers modulate NRF2-regulated genes and promote intracellular conditions favorable for TJ maintenance and barrier integrity (Huo et al., 2022; Yamamoto et al., 2018). *Cordyceps (Ophiocordyceps) sinensis*, a highly valued medicinal fungus in traditional Chinese medicine, exhibits multiple bioactivities including antioxidant and anti-inflammatory effects (Liu et al., 2022). Because natural *C. sinensis* is rare and costly, mycelial fermentation provides a sustainable and economical alternative. *Cordyceps sinensis* Cs-HK1, an anamorphic species identified by Wu's group, can produce mycelial biomass and exopolysaccharides (EPS) with notable biological functions (Leung et al., 2009). The EPS secreted during Cs-HK1 fermentation consist mainly of polysaccharide-protein complexes spanning a broad molecular weight range from several kDa to over 10,000 kDa (Huang et al., 2013a). Lower-molecular-weight fractions (EPS-LM) with relatively high protein content have demonstrated enhanced antioxidant and anti-inflammatory properties (Y.-Y. Zhu et al.,

2024). However, mechanistic evidence linking the antioxidant actions of EPS-LM to epithelial barrier preservation under oxidative stress remains limited, which this study aims to elucidate.

Based on this background and previous work, the present study investigated the protective effects of EPS-LM on intestinal epithelial barrier integrity under oxidative stress. The EPS-LM fraction was characterized for its chemical composition-including total carbohydrate and protein contents, as well as monosaccharide and amino acid profiles-and evaluated for antioxidant activities using chemical assays and cellular models measuring intracellular ROS and antioxidant enzyme activities. Furthermore, its protective impact on transepithelial electrical resistance (TEER) and TJ protein expression in H₂O₂ challenged Caco-2 monolayers was assessed to elucidate potential redox and signaling mechanisms underlying barrier restoration.

5.2 Materials and Methods

5.2.1 Chemical and biochemical agents

The Cs-HK1 mycelial fermentation, isolation, and preparation of EPS-LM were carried out as described in Section 4.2.1.

The Cs-HK1 EPS-LM used in Chapters 4 and 5 was obtained from different fermentation batches of the same strain under identical culture and two-step ethanol precipitation conditions. The two EPS-LM batches had very similar carbohydrate and protein contents and closely comparable monosaccharide compositions, with only small

differences in the measured values. Such minor variations are expected for separate batches and may arise from slight differences in manual handling and environmental factors (e.g., humidity and temperature) during the multi-step preparation, which can affect precipitation efficiency to a limited extent.

The FT-IR spectra of EPS-LM presented in Chapters 4 and 5 are also highly similar, all showing characteristic polysaccharide signals (a broad O-H band and strong C-O-C/C-O bands around 1,000-1,100 cm^{-1}) and typical protein-related bands (amide I band around 1,620 cm^{-1} and COO^- bands), indicating the same polysaccharide-protein functional-group pattern. The slight differences in band shape are within normal experimental variation and are more likely due to sample preparation than to genuine structural changes. Taking all compositional and spectroscopic data together, the main composition and structural features of EPS-LM are consistent between the two batches, and the EPS-LM used in Chapters 4 and 5 can be regarded as equivalent.

The analysis of EPS-LM composition, molecular weight, and FT-IR was performed using the same methods as described in Section 4.2.2.

5.2.1.1 HPGPC analysis of EPS-LM

The MW of EPS-LM was determined by high-performance gel permeation chromatography (HPGPC) as previously reported with slight modifications (Li et al., 2020). The system consisted of an Agilent 1260 Infinity HPLC with a G1362A refractive index detector (Beijing, China) and a Waters Ultrahydrogel Linear column

(7.8 mm × 300 mm; Waters, MA, USA). The detector and column were both maintained at 40 °C. The mobile phase consisted of 0.1 M NaNO₃, operated at a flow rate of 1 mL/min. Sample solutions were filtered through a 0.22 µm membrane filter prior to injection, and 20 µL of each sample was injected into the chromatographic system. Polyethylene glycol (PEG) standards (MW 4.30×10²-3.26×10⁵ Da) were used for calibration.

5.2.2 Spectroscopic and Structural Analysis of EPS-LM

5.2.2.1 Amino acid analysis

The total amino acid (TAA) composition of EPS-LM was determined using an automatic amino acid analyzer (LA8080, Hitachi, Japan) following a reported method (Hao et al., 2021) with minor modifications. A 100 mg sample was hydrolyzed in sealed glass tubes with 6 mol/L HCl at 110 °C for 23 hours. The hydrolysate was diluted to 10 mL with deionized water, filtered through two layers of filter paper, and 1.0 mL of the filtrate was transferred to a 50 mL beaker, where it was dried using an electric platen. The dried material was redissolved in 1.0 mL of 0.02 mol/L HCl for analysis. Amino acids were separated using cation exchange chromatography and quantified via ninhydrin post-column derivatization, with detection at 570 nm and 440 nm. The amino acid concentrations were calculated directly by the instrument, based on their absorption intensities in reference to the amino acid standards.

5.2.2.2 UV-Vis and fluorescence spectral analysis

The UV-Vis absorbance spectra of EPS-LM were recorded using a Varian Cary 4000 UV-Vis Spectrophotometer (Varian Co., USA). Samples were prepared at four concentrations, 1.0, 0.5, 0.25, and 0.125 mg/mL in deionized (DI) water, with DI water serving as a control. Absorbance was measured over a wavelength range of 200-800 nm using a 1 cm path length quartz cuvette at room temperature. The same sample concentrations were used for fluorescence analysis, and the fluorescence spectrum was recorded using a fluorescence spectrophotometer (Agilent G9800AA, Japan). The excitation wavelength was set to 280 nm, while the emission spectrum was scanned between 300 and 450 nm. Both the excitation and emission slit widths were adjusted to 5 nm to ensure optimal resolution and sensitivity.

5.2.2.3 Circular dichroism analysis

Circular dichroism (CD) was measured using a CD spectropolarimeter (Jasco J-1500, Japan) at room temperature with a 0.1 cm path length cuvette. The EPS-LM sample was prepared in PBS, and spectra were collected at a scan speed of 100 nm/min, with a digital integration time of 1 s and a bandwidth of 4 nm. For far-UV analysis, measurements were performed over the range of 260 to 190 nm with 1 nm step increments, and at least two scans were averaged. Secondary structural analysis was conducted using the JWSSE-513 program, which applies the classical least squares (CLS) method and incorporates the reference spectra (Zhao, Gu, et al., 2024).

5.2.3 In vitro antioxidant activity assays

5.2.3.1 DPPH radical-scavenging assay

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity of the samples was determined with slight modifications of reported method (Shen et al., 2022). EPS-LM samples were first dissolved in DI water to achieve concentrations ranging from 0.25 to 3 mg/mL. A working solution of DPPH radicals (0.2 mM, dissolved in ethanol) was freshly prepared, and 2 mL of the DPPH working solution was mixed with 2 mL of the diluted EPS-LM sample solution. The reaction mixtures were incubated in the dark at room temperature for 0 to 24 hours, with 30 µg/mL vitamin C (VC) included as a positive antioxidant reference. After the reaction, the absorbance of the supernatant was measured at 517 nm using a microplate reader (Thermo Scientific, Waltham, MA, USA). A mixture of the DPPH working solution and deionized water served as the blank. The DPPH radical scavenging activity (%) was represented by $(1 - A_{\text{sample}}/A_{\text{control}}) \times 100$, where A_{sample} and A_{control} are the absorbances of EPS-LM sample group and the control group, respectively.

5.2.3.2 ABTS radical-scavenging assay

The 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activity of the samples was determined with a reported method (You et al., 2010). EPS-LM samples were dissolved in deionized water to achieve concentrations ranging from 0.25 to 3 mg/mL. A working solution of ABTS was prepared by dissolving 0.007 g of

K₂S₂O₈ and 0.0406 g of ABTS in 10 mL of distilled water, followed by mixing the two solutions in equal volumes and allowing the mixture to react in the dark for 16 hours at room temperature to generate ABTS radicals. The resulting solution was then diluted 50 times with distilled water to create the ABTS working solution. To assess the scavenging activity, 3 mL of the ABTS working solution was mixed with 0.1 mL of the diluted EPS-LM sample. The reaction mixture was incubated at room temperature for 0-24 hours, with 30 µg/mL VC used as the positive control. The absorbance (A) of the supernatant was measured at 734 nm using a microplate reader. A mixture of 3 mL ABTS working solution and 0.1 mL deionized water was used as the blank. The ABTS radical scavenging activity (%) was represented by $(1 - A_{\text{sample}}/A_{\text{control}}) \times 100$.

5.2.4 Assessment of EPS-LM bioactivities

5.2.4.1 Caco-2 intestinal epithelial cell culture

The Caco-2 human colonic adenocarcinoma cell line and Eagle's Minimum Essential Medium (EMEM) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in EMEM supplemented with 20% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin in a humidified incubator at 37 °C with 5% CO₂. The culture medium was refreshed every two days until the cells reached 80% confluence. Cells were then digested with 0.25% trypsin at 37 °C for 3 minutes and seeded into 96, 24, 12, or 6 well culture plates for further experiments. All reagents for cell culture, including Hanks' Balanced Salt Solution

(HBSS), trypsin (0.25%), antibiotic-antimycotic solution (penicillin (100 U/mL) and streptomycin (100 µg/mL)), and FBS, were obtained from Gibco Biotechnology Co., Ltd. (Grand Island, NY, USA).

5.2.4.2 Reagents

3-(4,5-dimethylthiazol-2-yl) -5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS), Hydrogen peroxide (H₂O₂) and 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Phenazine methosulfate (PMS) was purchased from Promega Corporation (Madison, WI, USA). Fluorescein sodium was purchased from Shanghai Reagent (Shanghai, China). Glutathione peroxidase (GSH-Px), CAT, and SOD assay kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Transwell permeable supports (0.4 µm pore size) and cell culture plates were purchased from SPL Life Sciences Co., Ltd. (Gyeonggi-do, South Korea). The bicinchoninic acid (BCA) protein assay kit was purchased from Thermo Fisher Scientific (Rockford, IL, USA). Calcein acetoxymethyl ester/propidium iodide (Calcein-AM/PI) Live/Dead Cell Double Staining Kit was purchased from Servicebio (Wuhan, China). All other chemicals and reagents were of analytical grade.

5.2.4.3 Cell viability assay

The cell viability was assessed using the MTS assay (Zhao, Gu, et al., 2024). Caco-2 cells in the logarithmic growth phase were seeded into 96-well plates at a density of 1

$\times 10^5$ cells/mL (100 μ L/well) and incubated for 24 hours. To select the suitable concentration of H_2O_2 for the ROS-induced cell damage and the protective effect of EPS-LM, preliminary tests were performed by treating the cells at various concentrations of H_2O_2 (0-700 μ M) for 24 hours. The results showed (Appendix Figure A2) that cell viability started to decrease significantly when H_2O_2 concentrations exceeded 500 μ M and at 550 μ M, the cell viability reduced by 50% compared to the control. Therefore, 550 μ M H_2O_2 was selected to induce oxidative damage in this study. For the protective effect of EPS-LM against H_2O_2 -induced cytotoxicity, cells were simultaneously exposed to 550 μ M H_2O_2 and EPS-LM at concentrations of 50, 100, 200, and 400 μ g/mL for 24 hours. After the respective treatments, the culture medium in the 96-well plates was removed, and 45 μ L of MTS solution mixed with PMS was added to each well. The plates were incubated at 37 °C for 2 hours, and the optical density (OD) at 492 nm was measured using a microplate reader.

5.2.4.4 Cell viability staining assay

To further elucidate the protective effects of EPS-LM (50 and 200 μ g/mL) on Caco-2 cells following exposure to 550 μ M H_2O_2 , the cells were stained using the Calcein-AM/PI Double Stain Kit. The staining process involved incubating the cells with calcein AM and PI for 30 minutes, following the manufacturer's protocol. Fluorescent images were subsequently captured using an inverted fluorescence microscope (Ti2-E, Nikon, Japan).

5.2.4.5 Intracellular ROS assay

The Intracellular ROS assay (DCFH-DA probe) was performed as described in 4.2.3.5.

5.2.4.6 Measurement of antioxidant enzyme activities

Caco-2 cells were harvested and lysed with an ultrasonic cell disruptor while kept in cold water to maintain a low temperature and prevent protein degradation, and the resulting supernatants were collected for subsequent analysis. The protein concentration was determined using the BCA assay, following the protocol provided by the manufacturer. The changes in SOD, CAT, and GSH-Px activity were detected using the appropriate kits (Nanjing Jiancheng, Nanjing, China), with data normalized to total protein.

5.2.4.7 TEER measurement

The TEER assay was conducted to evaluate the TJ integrity of the Caco-2 monolayer (Marino et al., 2024). Caco-2 cells were seeded at a density of 1×10^5 cells/mL onto Transwell membranes (polyester, 12 mm diameter, 0.4 μ m pore size). A well without cells served as the blank control. The medium in the apical (AP) and basolateral (BL) chambers (0.2 mL and 0.6 mL, respectively) was replaced every 3 days. After 21 days of culture on Transwell inserts, the Caco-2 cells spontaneously differentiated into polarized monolayers. For protection studies, monolayers were allocated to the following groups for 24 h: (i) Control (no treatment); (ii) H₂O₂ Model (550 μ M H₂O₂ alone); and (iii) H₂O₂ + EPS-LM co-treatment (550 μ M H₂O₂ with EPS-LM at 50 or

200 µg/mL). Baseline TEER (TEER₀) was recorded immediately before initiating the 24 h treatment. TEER ($\Omega \cdot \text{cm}^2$) was measured once again immediately after completing the 24 h treatment (TEER_{24h}) using a Millicell Electrical Resistance System (ERS-2, Millipore-Sigma, Burlington, MA, USA) following blank correction, and calculated as $(R_m - R_i) \times A$, where R_m is the measured trans-epithelial resistance, R_i is the intrinsic resistance of cell-free medium, and A is the membrane surface area (cm^2). The change in resistance was defined as $\Delta\text{TEER} (\Omega \cdot \text{cm}^2) = \text{TEER}_{24h} - \text{TEER}_0$; negative values indicate a decrease from baseline.

5.2.4.8 Cell permeability assay

The permeability assay using sodium fluorescein was adapted from previous studies (Chen et al., 2016; Masungi et al., 2004), with permeability reported as BL concentration (µg/mL) under sink conditions. After TEER evaluation, monolayers were gently washed three times with Hank's Balanced Salt Solution (HBSS, pH 7.4). During the final wash, cells were incubated at 37 °C for 30 min to allow equilibration. Then, 200 µL of sodium fluorescein solution (250 µg/mL) was added to the AP side, and 800 µL of HBSS was added to the BL side. BL samples (200 µL) were collected after 150 min. Absorbance was measured at 490 nm using a microplate reader, and BL concentrations (µg/mL) were determined from external calibration ($R^2 \geq 0.999$).

5.2.4.9 RT-qPCR analysis

Caco-2 cells were seeded into 12-well plates and incubated for 10 days to reach full differentiation. To evaluate the effects of H₂O₂-induced oxidative stress and the protective role of EPS-LM, the cells were treated with H₂O₂ and EPS-LM prior to RNA extraction. Total RNA was extracted using TRIzol Reagent (Invitrogen, USA) according to the supplier's protocol. The purity and concentration of RNA were measured using a NanoDrop 2000c Spectrophotometer (Thermo Scientific, USA). Primers specific to target genes were designed using Primer-BLAST, and real-time quantitative polymerase chain reaction (RT-qPCR) was performed with the Hifair III One-Step RT-qPCR SYBR Green Kit (Yeast, Shanghai, China). Reverse transcription (RT) was conducted at 42 °C for 10 min, followed by qPCR on an ABI QuantStudio 5 Real-time PCR system (Applied Biosystems) under the following conditions: 95 °C for 5 min, then 40 cycles of 95 °C for 10 s and 60 °C for 30 s. The relative gene expression levels were calculated using the $2^{-\Delta\Delta CT}$ method, with GAPDH as the reference gene (Chen et al., 2021). The specific primer sequences used in the experiment are listed in Table 5-1.

Table 5-1 Primer sequences of tight-junction genes used for RT-qPCR analysis.

Gene	Accession No.	Forward primer (5'-3')	Reverse primer (5'-3')
ZO-1	NM_003257.4	CTAAGGGAGCACATGGTGAAGGTAA	GTCGGGCAGAACTTGTATATGGTTT
Claudin-1	NM_021101.6	GAAGATGAGGATGGCTGTCATTGGG	GGTAAGAGGTTGTTTTTCGGGGAC

Occludin	NM_002538.3	AACTTCGCCTGTGGATGACTTCAG	TTTGACCTTCCTGCTCTTCCCTTTG
GAPDH	NM_002046.7	TCAAGAAGGTGGTGAAGCAGG	TCAAAGGTGGAGGAGTGGGT

5.2.4.10 Western blotting analysis

Western blotting was carried out as described in Section 4.2.3.6, with minor modifications for Caco-2 cells. Caco-2 cells were seeded in 6-well plates. For antioxidant-related protein analysis, cells were cultured for 24 h to reach confluence, while for TJ protein analysis, cells were allowed to differentiate for 10 days. After treatment with H₂O₂ and EPS-LM for 24 h. The primary antibodies used were rabbit anti-NQO1 (#T56710, Abmart), anti-ZO-1 (#GB111402, Servicebio), anti-Claudin-1 (#GB12032, Servicebio), anti-NRF2 (#ab62352, Abcam), and anti- β -actin (#GB15003, Servicebio), the latter serving as a loading control.

5.2.4.11 Immunofluorescence and confocal microscopy analysis

Immunofluorescence staining was performed according to the procedure described in Section 4.2.3.7, with minor modifications for Caco-2 cells. Briefly, Caco-2 cells were seeded onto collagen-coated coverslips in 12-well plates and cultured for 10 days until fully confluent and differentiated. After treatment with H₂O₂ and EPS-LM for 24 h, the cells were incubated with a primary antibody against Claudin-1, followed by an FITC-conjugated secondary antibody and DAPI counterstaining to evaluate Claudin-1 localization.

5.2.5 Statistical analysis

The data were analyzed in GraphPad Prism (GraphPad Software, La Jolla, CA, USA). One-way analysis of variance (ANOVA) was used for multiple comparisons, followed by Tukey's post hoc test. Data are presented as mean $\pm$ SEM from at least three independent experiments. P-value < 0.05 was considered statistically significant.

5.3 Results

5.3.1 Chemical composition and molecular weight of EPS-LM

EPS-LM contained 19.3% (w/w) total carbohydrate and 28.7% (w/w) protein (Table 5-2), consistent with previous reports (Y.-Y. Zhu et al., 2024), demonstrating the reproducibility of EPS-LM production from Cs-HK1 mycelial fermentation. HPGPC (Appendix Figure A3) revealed two predominant fractions with apparent molecular weights of 2.50×10^4 Da (41.9% area) and 1.70×10^3 Da (54.0% area) (Table 5-2). These size distributions were comparable to earlier findings on Cs-HK1 EPS fractions (Leung et al., 2009), which reported a broad MW range (5-200 kDa). High-molecular-weight polysaccharides have been proposed to protect the intestinal barrier by forming a hydrophilic gel-like layer that mimics intestinal mucus and may interact with membrane receptors to reinforce epithelial stability (Błaszczuk et al., 2015; Huo et al., 2022). By contrast, the stronger antioxidant activity associated with the low-MW fraction likely reflects its higher protein content, consistent with previous observations that protein-rich, low-MW EPS fractions exhibit greater antioxidant capacity (Leung et al., 2009; J.-Y. Wu et al., 2014).

Table 5-2 Chemical composition and structural characteristics of Cs-HK1 EPS-LM. Original GPC profiles are shown in Appendix Figure A3. Data are presented as mean $\pm$ SEM (n = 3).

Total carbohydrate (%)	Total protein (%)	MW (peak area, %)
19.29 $\pm$ 0.04	28.71 $\pm$ 0.16	2.50 $\times$ 10 ⁴ (41.93%)
		1.70 $\times$ 10 ³ (53.97%)
Protein structure (chain conformation forms)		
Random: 30.8 %	α -Helix: 19.3%	β -Sheet: 49.8%
Monosaccharide composition (molar ratio)		
Mannose: 2.127	Galactose: 1	Glucose: 0.4946 Galactosamine: 0.2688

5.3.2 Structural characteristics

As shown in Figure 5-1A, a distinct peak was observed in the near-UV region of the UV spectrum of EPS-LM, confirming the presence of protein components in the sample. UV–visible spectroscopy is a simple and direct method for detecting proteins, typically showing absorption between 250 and 310 nm (Brader, 2020). Fluorescence spectroscopy is a versatile and sensitive technique for high-throughput screening (Auer et al., 1998), particularly useful for rapid protein analysis (Brader, 2020). As shown in Figure 5-1B, the fluorescence spectrum of EPS-LM (excited at 280 nm) displayed emission peaks at 340-350 nm, attributable to aromatic amino acids such as tryptophan, tyrosine, and phenylalanine. EPS-LM exhibited the strongest fluorescence

intensity at 1 mg/mL, and the signal decreased proportionally with dilution, consistent with its concentration-dependent fluorescence behavior.

CD is an effective technique for elucidating the secondary structural features of macromolecules, including proteins, polysaccharides, and glycoproteins (Miles & Wallace, 2020; Wang et al., 2024). In aqueous solution, the sugar chains of glycoproteins undergo dynamic conformational changes such as helix formation, folding, turning, and random coiling, which enhance molecular asymmetry (Greenfield, 2006). As shown in Figure 5-1C and Table 5-2, the CD spectrum of EPS-LM exhibited a positive Cotton effect around 195 nm and a negative Cotton effect near 210 nm. Analysis of the CD data revealed that the secondary structure of EPS-LM was primarily composed of β -sheets (49.8 %), along with random coils (30.8 %) and α -helices (19.3 %). The predominance of β -sheets suggested a highly ordered and stable conformation, which may enhance molecular integrity and facilitate interactions with polysaccharides, contributing to protein stability and functionality (Lee et al., 2002; Wang et al., 2014; Zhou et al., 2017). The considerable proportion of random coils indicated structural flexibility, supporting dynamic interactions with polysaccharides and contributing to functional versatility (Chen et al., 2023).

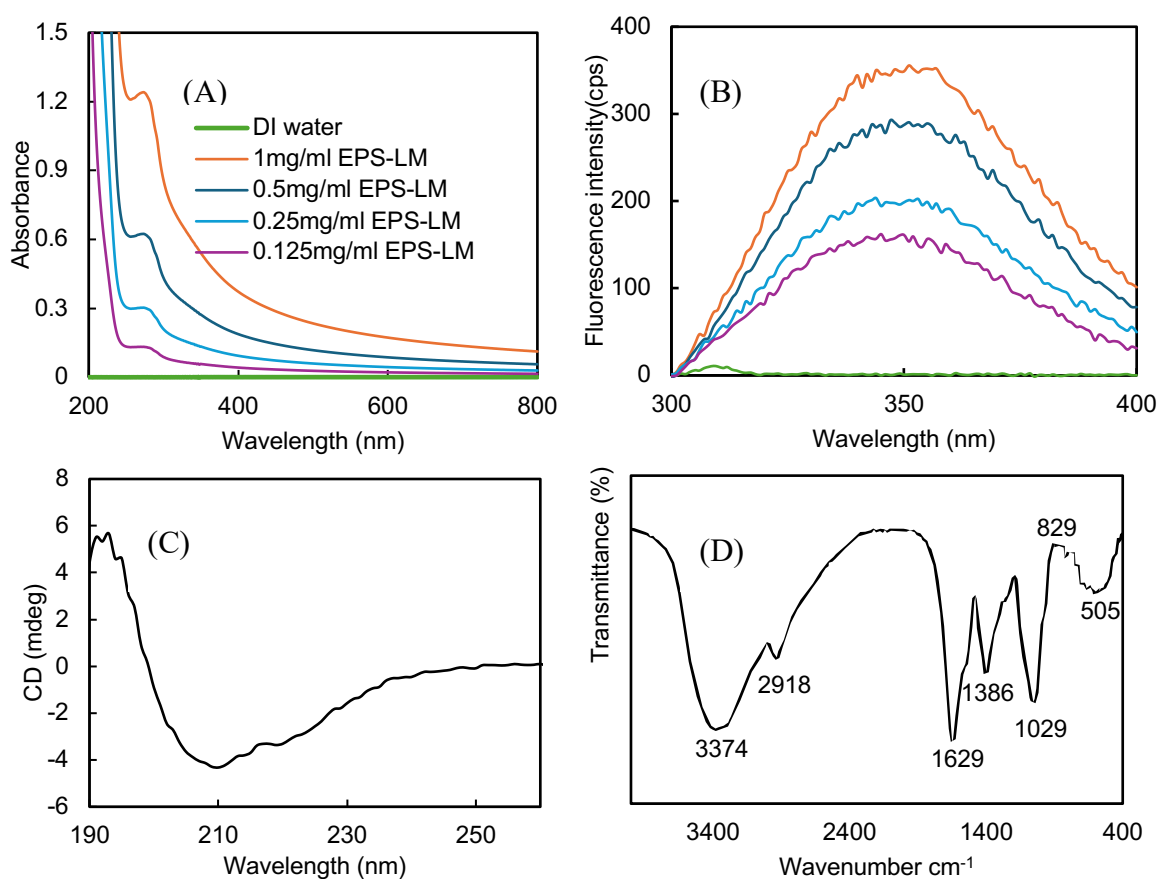


Figure 5-1 Spectroscopic characteristics of EPS-LM: (A) UV-Vis absorbance spectra at different concentrations; (B) fluorescence intensity (cps) at various concentrations; (C) circular dichroism (CD); (D) FT-IR spectrum.

The FT-IR spectrum (Figure 5-1D) provided further structural information on EPS-LM. The broad absorption band at 3374 cm^{-1} was attributed to O-H stretching vibrations, indicative of hydroxyl groups commonly present in polysaccharides and proteins. The peak at 2918 cm^{-1} corresponded to C-H stretching vibrations originating from aliphatic groups or C-H bonds within polysaccharide chains (Y.-Y. Zhu et al., 2024). The peaks at 1629 cm^{-1} and 1386 cm^{-1} were assigned to the amide I and amide II bands, respectively, confirming the presence of proteins. Specifically, the amide II band at

1386 cm^{-1} likely arose from N-H bending and C-N stretching vibrations, with potential shifts influenced by hydrogen bonding or interactions between proteins and polysaccharides (Zhao, Gu, et al., 2024). Additionally, the peak at 1029 cm^{-1} was associated with C-O-C and C-OH stretching vibrations, indicating the presence of polysaccharide moieties (Li et al., 2020).

The ^1H -NMR spectrum (Appendix Figure A4) displayed signals in the range of 4.4-5.5 ppm, characteristic of the anomeric protons in polysaccharides, confirming their presence (Yao et al., 2021). Signals between 3.0 and 4.4 ppm were attributed to the side-chain hydrogens of amino acids such as glutamine, glutamic acid, and alanine, while those between 2.5 and 3.0 ppm also originated from side-chain hydrogens, specifically from glutamine and arginine (de Falco et al., 2016; Nord et al., 2004; Ritota et al., 2010). Additionally, signals in the range of 1.2-1.5 ppm corresponded to the methyl groups of branched-chain amino acids, including isoleucine and valine, whereas signals between 0.8 and 1.0 ppm were characteristic of the methyl groups in aliphatic amino acids such as leucine and isoleucine (Bubb, 2003; Zhao, Gu, et al., 2024). These ^1H -NMR findings, together with the FT-IR results, further supported the presence of both protein and polysaccharide components in EPS-LM.

5.3.3 Monosaccharide and amino acid composition

As shown in Table 5-2, the monosaccharide composition of EPS-LM revealed mannose as the dominant sugar with a molar ratio of 2.127, followed by galactose (1),

glucose (0.4946), and galactosamine (0.2688). These findings indicated that EPS-LM is a mannose-rich heteropolysaccharide. The overall monosaccharide profile was consistent with previous reports on EPS derived from *C. sinensis* Cs-HK1 (Y.-Y. Zhu et al., 2024), which also contained mannose, galactose, and glucose as the main carbohydrate constituents. The amino acid composition of EPS-LM is presented in Table 5-3. The total amino acid content (TAA) was 210.4 mg/g, comprising 65.0 mg/g of essential amino acids (EAAs) and 145.4 mg/g of non-essential amino acids (NAAs). Aspartic acid (43.7 mg/g), glutamic acid (22.2 mg/g), and glycine (21.8 mg/g) were the predominant NAAs, whereas threonine (19.3 mg/g) and lysine (12.4 mg/g) were the most abundant EAAs. The amino-acid pattern of EPS-LM was comparable to that reported for the glycopeptide Cs-GP1 isolated from Cs-HK1 (J.-Y. Wu et al., 2014). These compositional data suggest that EPS-LM contains both polysaccharide and protein components, consistent with its characterization as a protein-polysaccharide complex.

Table 5-3 Amino acid composition and contents of EPS-LM (in mg/g). Data are presented as mean $\pm$ SEM (n = 3).

Essential Amino Acid (EAA)		Non-essential Amino Acid (NAA)	
Histidine	3.3 $\pm$ 0.1	Alanine	13.6 $\pm$ 0.1
Isoleucine	6.6 $\pm$ 0.1	Aspartic acid	43.7 $\pm$ 0.1
Leucine	5.7 $\pm$ 0.2	Arginine	8.4 $\pm$ 0.3

Lysine	12.4 ± 0.3	Glutamic acid	22.2 ± 0.1
Phenylalanine	7.0 ± 0.2	Glycine	21.8 ± 0.1
Threonine	19.3 ± 0.1	Serine	15.5 ± 0.1
Valine	10.7 ± 0.1	Tyrosine	3.7 ± 0.1
		Proline	16.5 ± 0.4
Total Amino Acids (TAA)		210.4 ± 1.1	
Essential Amino Acids (EAA)		65.0 ± 1.1	
Non-essential Amino Acids (NAA)		145.4 ± 0.6	

$$\text{TAA (mg/g)} = \text{MW/W} \times V_1/V_2 \times 10$$

Where MW = amino acid MW, W = 100 mg, V₁ = 50 mL, and V₂ = 20 µL.

5.3.4 Antioxidant activities

The antioxidant capacity of EPS-LM was assessed using ABTS and DPPH radical-scavenging assays (Figure 5-2A, B). In both systems, EPS-LM showed a clear concentration and time-dependent trend. At 3 mg/mL, EPS-LM showed a significant increase in scavenging activity within 24 h, which subsequently reached a plateau. Vitamin C (30 µg/mL) showed consistent antioxidant activity across all time points in both assays. As summarized in Table 5-4, the IC₅₀ values of EPS-LM decreased over time, indicating an overall enhancement of radical-scavenging capacity. Specifically, in the ABTS assay, the IC₅₀ declined from 0.689 mg/mL at 0 h to 0.305 mg/mL at 24 h, whereas in the DPPH assay it decreased from 1.023 mg/mL to 0.807 mg/mL over the

same period. These results clearly demonstrate that the antioxidant activity of EPS-LM increased with both concentration and incubation time.

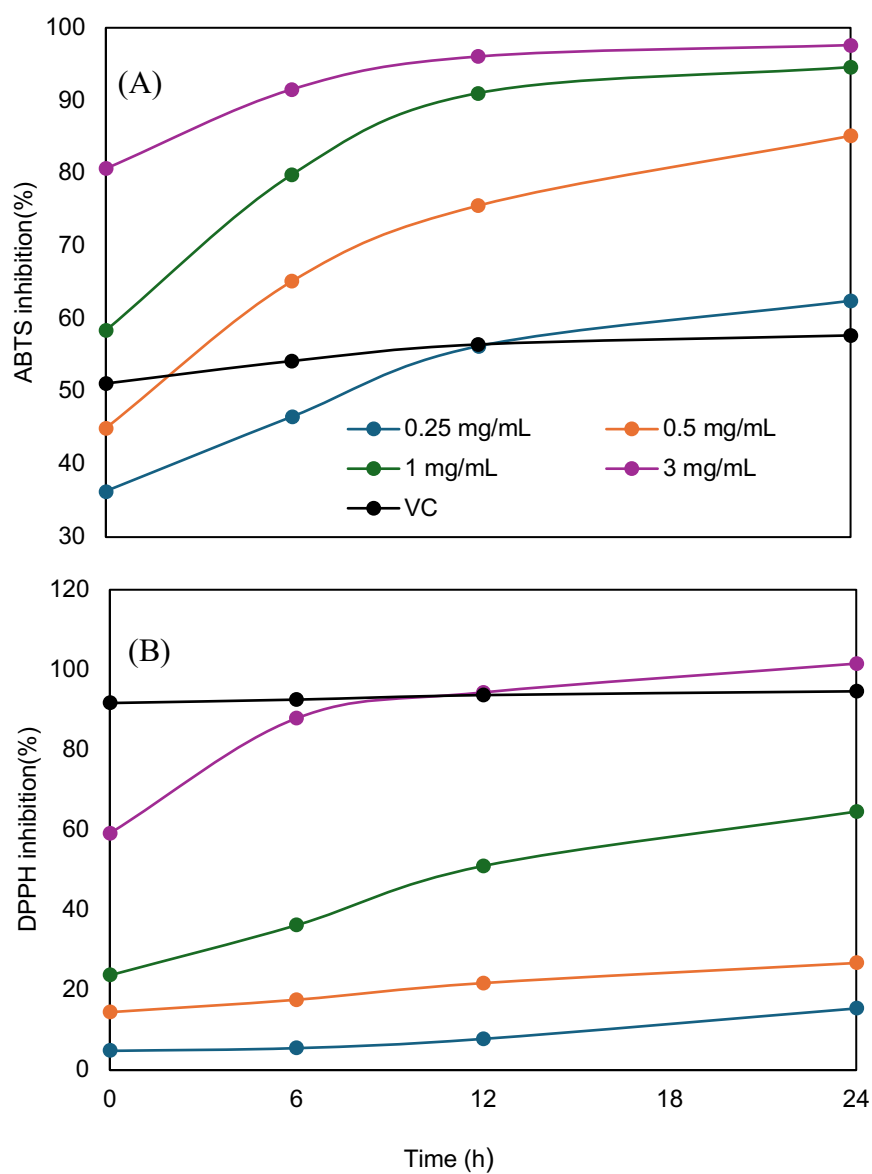


Figure 5-2 Radical-scavenging activity of EPS-LM at different concentrations (mg/mL) and incubation times: (A) ABTS• assay and (B) DPPH• assay, with vitamin C (30 µg/mL) as an antioxidant reference.

Table 5-4 IC₅₀ values (mg/mL) for the antioxidant activity of EPS-LM measured by ABTS and DPPH assays at different time points (0-24 h). Data are presented as mean $\pm$ SEM (n = 6).

Time (h)	IC ₅₀ (ABTS) (mg/mL) $\pm$ SEM	IC ₅₀ (DPPH) (mg/mL) $\pm$ SEM
0	0.689 $\pm$ 0.032	1.023 $\pm$ 0.036
6	0.296 $\pm$ 0.013	1.282 $\pm$ 0.032
12	0.34 $\pm$ 0.024	0.983 $\pm$ 0.055
24	0.305 $\pm$ 0.015	0.807 $\pm$ 0.030

5.3.5 Effects of EPS-LM on Caco-2 cell viability

Figure 5-3A shows that EPS-LM was non-toxic, with no significant change in Caco-2 cell viability across 0-400 μ g/mL. In Figure 5-3B, EPS-LM demonstrated a dose-dependent ability to preserve cell viability under H₂O₂ challenge, with the highest concentration (400 μ g/mL) almost restoring viability to control levels. This protective effect was consistently observed across a broader range of H₂O₂ concentrations (350-700 μ M), as shown in Appendix Figure A5. Figure 5-3C presents fluorescence microscopy images in which cells exposed to 550 μ M H₂O₂ showed intense red fluorescence (PI, dead cells) and reduced green fluorescence (Calcein-AM, viable cells). Treatment with 50 or 200 μ g/mL EPS-LM markedly decreased red fluorescence and increased green fluorescence in a dose-dependent manner, consistent with the MTS assay results.

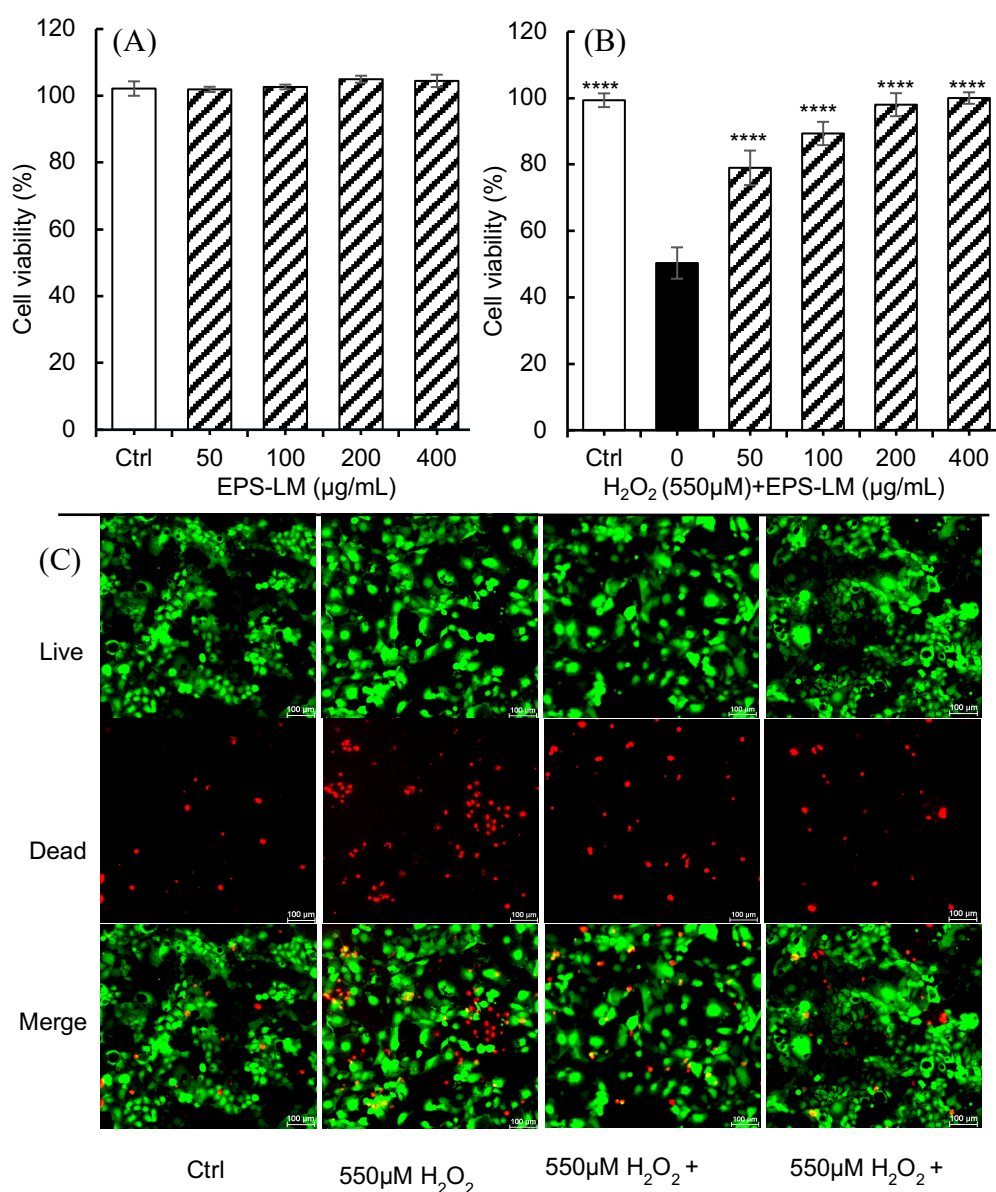


Figure 5-3 Impact of H₂O₂ and EPS-LM on Caco-2 cell viability: (A) treatment with EPS-LM alone at 0, 50, 100, 200, and 400 μg/mL; (B) treatment with 550 μM H₂O₂ plus EPS-LM at various concentrations; (C) confocal microscopy images of cells stained with calcein AM (green) for viable cells and propidium iodide (PI, red) for dead cells in control, 550 μM H₂O₂, and 550 μM H₂O₂ plus 50 or 200 μg/mL EPS-LM. Data are presented as mean ± SEM (n = 6). (****p < 0.0001 vs. H₂O₂).

5.3.6 Effects of EPS-LM on antioxidant responses in Caco-2 cells

5.3.6.1 EPS-LM reduces ROS levels and restores antioxidant enzyme activities

Exposure of Caco-2 cells to 550 μM H_2O_2 significantly increased intracellular ROS levels (Figure 5-4A), confirming the induction of oxidative stress. Treatment with EPS-LM reduced ROS accumulation in a concentration-dependent manner, with the maximal reduction observed at 200 $\mu\text{g/mL}$. The activities of key antioxidant enzymes were markedly affected by H_2O_2 exposure and subsequently restored by EPS-LM. As shown in Figure 5-4 B-D, treatment with 550 μM H_2O_2 significantly decreased the activities of SOD, CAT, and GSH-Px, whereas co-treatment with EPS-LM (50-200 $\mu\text{g/mL}$) dose-dependently recovered these enzyme activities. The highest restoration was observed at 200 $\mu\text{g/mL}$ EPS-LM, consistent with its effect on ROS reduction. These results indicate that EPS-LM effectively mitigated H_2O_2 -induced oxidative stress by decreasing intracellular ROS levels and promoting the recovery of antioxidant enzyme activities.

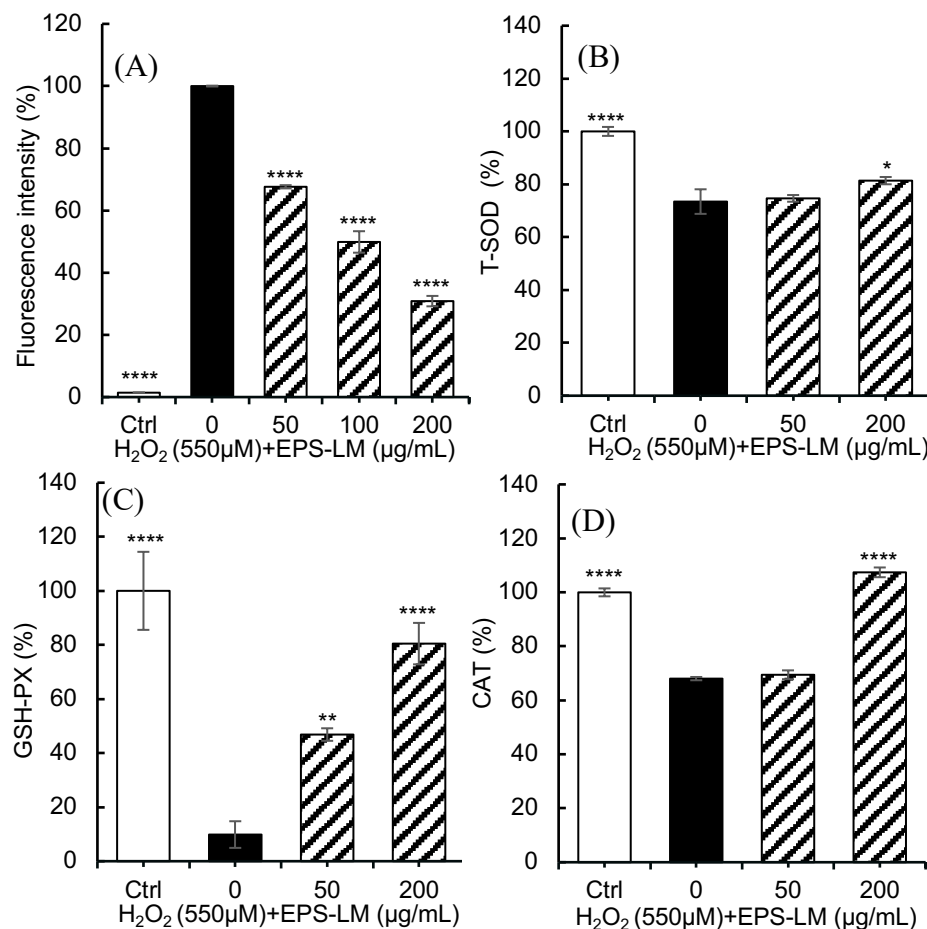


Figure 5-4 Effects of EPS-LM on intracellular ROS levels and antioxidant enzyme activities in Caco-2 cells exposed to H₂O₂. (A) ROS levels following exposure to H₂O₂ with or without EPS-LM treatment; (B) SOD; (C) GSH-Px; and (D) CAT. Data are presented as mean $\pm$ SEM (n = 6). *p < 0.05, **p < 0.01, ****p < 0.0001 vs. 550 μ M H₂O₂.

5.3.6.2 EPS-LM modulates NRF2-NQO1 signaling under oxidative stress

To assess oxidative burden and the involvement of the NRF2 pathway, NQO1 and total NRF2 protein levels were quantified by Western blotting and densitometry (Figure 5-5A-D). NQO1 limits ROS generation by catalyzing the two-electron reduction of quinones to hydroquinones, thereby preventing redox cycling and the formation of

semiquinone radicals and superoxide, whereas NRF2 coordinates antioxidant defenses by activating ARE-dependent genes under oxidative stress, as previously described (Ngo & Duennwald, 2022; Rashid et al., 2021). Exposure of Caco-2 cells to 550 μ M H₂O₂ significantly increased both NQO1 and total NRF2 levels compared with the control, indicating activation of the oxidative-stress response. Treatment with EPS-LM (200 μ g/mL) attenuated these H₂O₂-induced increases, resulting in NQO1 and NRF2 levels approaching those of the untreated control. Together with the results shown in Figure 4, these findings indicate that EPS-LM alleviated oxidative stress by lowering intracellular ROS, restoring antioxidant enzyme activities, and normalizing NRF2-NQO1 signaling, thereby contributing to redox balance in Caco-2 cells.

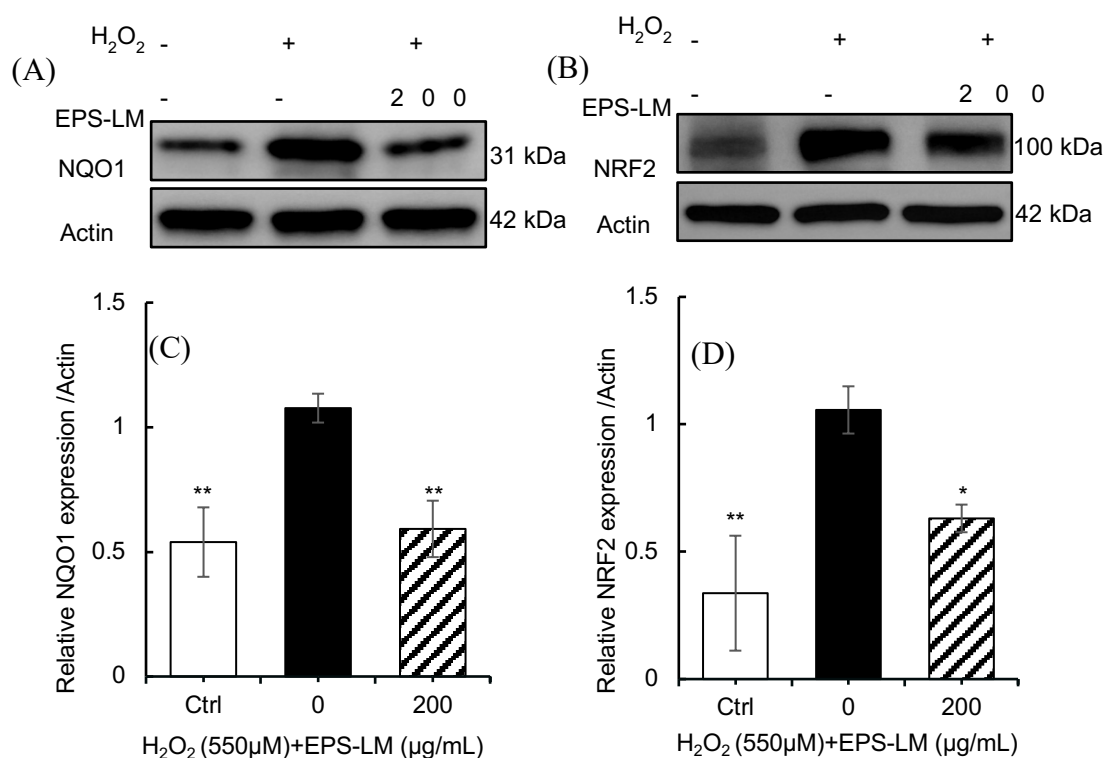


Figure 5-5 Effects of EPS-LM on NRF2 and NQO1 expression in Caco-2 cells under H₂O₂-induced oxidative stress. (A, B) Western blot analysis showing representative bands for NQO1 and NRF2 expression; (C, D) quantification of NQO1 and NRF2 expression levels. Data are presented as mean $\pm$ SEM (n = 3). *p < 0.05, p < 0.01 vs. 550 μ M H₂O₂.

5.3.7 Protective effects of EPS-LM on epithelial barrier function in Caco-2 cell culture

5.3.7.1 EPS-LM alleviates H₂O₂-induced epithelial barrier dysfunction

The integrity of the intestinal barrier was assessed using two complementary assays: TEER and sodium fluorescein permeability (Srinivasan et al., 2015). TEER reflects the electrical resistance across a cell monolayer and serves as an indicator of TJ integrity, whereas sodium fluorescein measures paracellular permeability, providing a sensitive evaluation of early barrier disruption (Szabo et al., 2008). Together, these methods provide a comprehensive assessment of intestinal barrier function by combining electrical resistance and fluorescence-based permeability measurements. As shown in Figure 5-6, exposure of Caco-2 cells to 550 μ M H₂O₂ for 24 h resulted in marked barrier dysfunction, evidenced by a 60 $\Omega \cdot \text{cm}^2$ decrease in TEER relative to baseline (TEER₀) and a corresponding increase in basolateral sodium fluorescein concentration to 2.58 μ g/mL. Co-treatment with 50 μ g/mL EPS-LM mitigated these changes, showing a smaller TEER decrease (-20 $\Omega \cdot \text{cm}^2$) and a lower basolateral

fluorescein level (2.36 $\mu\text{g/mL}$), indicating a protective trend at the lower dose. At 200 $\mu\text{g/mL}$, EPS-LM exerted a pronounced protective effect, with TEER values approaching or exceeding initial levels and fluorescein permeability significantly reduced to 1.90 $\mu\text{g/mL}$. These findings demonstrate that EPS-LM attenuated H_2O_2 -induced decreases in TEER and increases in paracellular permeability in a dose-dependent manner, supporting its role in maintaining tight-junction integrity and epithelial barrier function under oxidative stress.

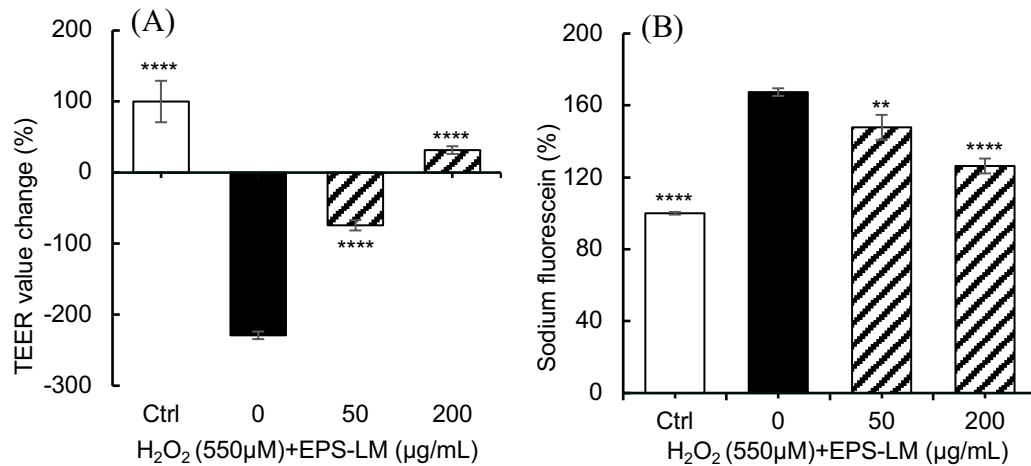
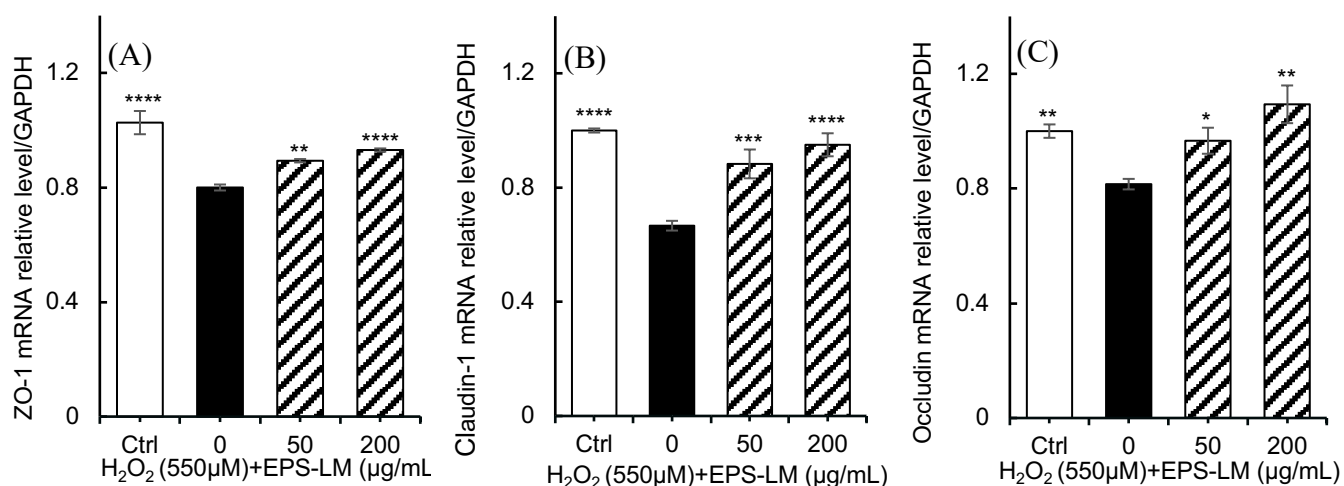


Figure 5-6 Effects of EPS-LM on H_2O_2 -induced changes in transepithelial electrical resistance (TEER) and sodium fluorescein permeability in Caco-2 cells. (A) TEER values after 24 h (% of control); (B) basolateral sodium fluorescein levels (% of control) at 150 min. Data are presented as mean $\pm$ SEM ($n = 6$). **** $p < 0.0001$, ** $p < 0.01$ vs. control.

5.3.7.2 EPS-LM restores tight-junction components under oxidative stress

As shown in Figure 5-7A-C, exposure of Caco-2 cells to 550 μM H_2O_2 significantly decreased the mRNA expression of the TJ genes ZO-1, Claudin-1, and Occludin,

indicating oxidative stress-induced disruption of barrier-related transcripts. Treatment with 50 $\mu\text{g/mL}$ EPS-LM partially restored the mRNA levels of these TJ components, and 200 $\mu\text{g/mL}$ EPS-LM further enhanced their expression, showing a clear dose-dependent improvement. Consistent with the transcriptional findings, Western blot analysis and densitometric quantification (Figure 7D-G) demonstrated that EPS-LM remarkably increased Claudin-1 and ZO-1 protein levels after H_2O_2 exposure, with the greatest recovery observed at 200 $\mu\text{g/mL}$. Immunofluorescence imaging of Claudin-1 (Figure 7H) further confirmed the protective effect of EPS-LM, showing restoration of continuous junctional localization along cell borders. Together, these results indicate that EPS-LM preserved tight-junction gene and protein expression in a dose-dependent manner, contributing to the maintenance of epithelial barrier integrity under oxidative stress.



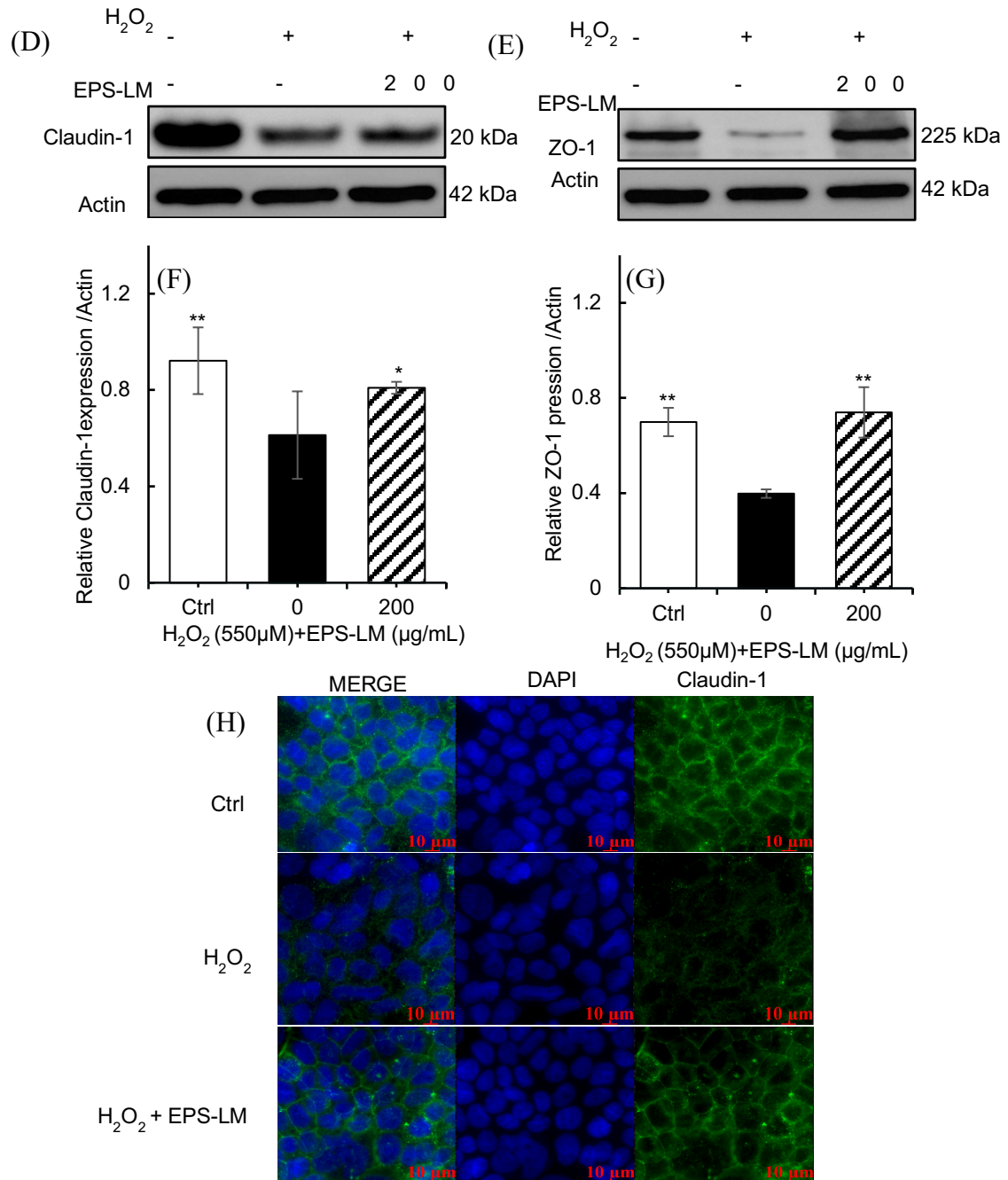


Figure 5-7 Effects of EPS-LM on tight-junction proteins in Caco-2 cells under H_2O_2 -induced oxidative stress. (A-C) mRNA expression levels of tight-junction proteins ZO-1, Claudin-1, and Occludin in Caco-2 cells; (D, E) protein expression of Claudin-1 and ZO-1 determined by Western blotting; (F, G) quantification of Claudin-1 and ZO-1 protein levels normalized to β -actin; (H) immunofluorescence staining of

Claudin-1 in Caco-2 cells. Data are presented as mean $\pm$ SEM (n = 3). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 vs. 550 μ M H₂O₂.

5.4 Discussion

Polysaccharides rich in mannose are widely recognized for their pronounced antioxidant activities. Mannose residues enhance radical-scavenging and reducing capacity (Lo et al., 2011; Xiang et al., 2012) and stimulate antioxidant enzyme expression (Cor et al., 2018). Furthermore, mannose plays a role in stabilizing polysaccharide structures, such as in xanthan, where the terminal mannose reinforces the double helix (Wu et al., 2019). The coexistence of galactose and glucose in EPS-LM may further modulate its biological effects. Galactose has been linked to macrophage activation and immunostimulation (R. Chen et al., 2022), whereas glucose residues confer metabolic flexibility and redox buffering capacity (Nimgampalle et al., 2021). Thus, the heterogeneous monosaccharide composition of EPS-LM may provide complementary biochemical roles that enhance both structural integrity and functional antioxidant potential.

In addition to its sugar composition, the amino acid profile of EPS-LM may also contribute to its observed bioactivity. The overall amino acid composition of EPS-LM shows a noticeable resemblance to that reported for Cs-GP1, a bioactive glycopeptide isolated from Cs-HK1. Cs-GP1 is rich in glutamic acid, aspartic acid, and glycine and has demonstrated significant antioxidant and cytoprotective properties, effectively

mitigating oxidative damage and preserving cell viability under stress conditions (J.-Y. Wu et al., 2014). In addition, lysine and threonine in EPS-LM may provide complementary physiological functions, as lysine is involved in collagen formation and mineral absorption, whereas threonine supports mucin synthesis, which is essential for intestinal surface protection (Aggarwal & Bains, 2022; Mao et al., 2011). Together, these observations suggest that the coexistence of polysaccharide and amino acid components within EPS-LM acts synergistically to enhance its antioxidant and intestinal barrier-protective capacities, providing the molecular foundation for the potent antioxidant and cytoprotective effects observed in subsequent experiments.

Building on these compositional insights, the antioxidant assays revealed a time-dependent scavenging behavior of EPS-LM, suggesting that effective neutralization of free radicals requiring prolonged molecular interactions, likely involving gradual formation of hydrogen-bonded networks between reactive groups. Such kinetic behavior is consistent with observations in polysaccharide-based eutectogels, where radical scavenging increases over time as hydrogen-bond donors reorganize into more reactive configurations (Marullo et al., 2023).

Excessive accumulation of ROS induces oxidative damage and cell death, contributing to a variety of pathological processes (Schieber & Chandel, 2014). Consistent with the observed decreases in intracellular ROS levels and the restoration of antioxidant enzyme activities shown in Figure 4, EPS-LM effectively mitigated H₂O₂-induced oxidative stress through restoration of key antioxidant enzymes. SOD catalyzes the

conversion of superoxide anions into hydrogen peroxide and molecular oxygen, thereby preventing superoxide accumulation and initiating ROS detoxification (Pisoschi & Pop, 2015). CAT subsequently decomposes hydrogen peroxide into water and oxygen (Heck et al., 2010), while GSH-Px reduces hydrogen peroxide and lipid hydroperoxides, protecting membrane lipids and preserving cellular integrity (Birben et al., 2012). The significant recovery of these enzymatic antioxidant activities in EPS-LM-treated cells suggests that EPS-LM supports the enzymatic defense network responsible for ROS clearance.

In addition to direct enzymatic restoration, EPS-LM treatment rebalanced the NRF2-NQO1 signaling axis, which serves as a key cellular regulator of oxidative defenses. NQO1 limits ROS generation by catalyzing the two-electron reduction of quinones to hydroquinones, thereby preventing redox cycling and the formation of semiquinone radicals and superoxide. In parallel, NRF2 coordinates antioxidant defenses by activating ARE-dependent genes upon KEAP1 cysteine modification under oxidative stress (Ngo & Duennwald, 2022; Rashid et al., 2021). NRF2 activation promotes the transcription of downstream antioxidant and phase II detoxification enzymes, including NQO1 (Shaw & Chattopadhyay, 2020), thereby reinforcing the intracellular antioxidant system. Together, these findings imply a dual protective mechanism of EPS-LM involving direct ROS scavenging and activation of endogenous antioxidant pathways. Collectively, these effects highlight EPS-LM as a potent redox-modulating

polysaccharide that maintains oxidative homeostasis and protects cells from H₂O₂-induced injury.

Consistent with its antioxidant potential observed in Caco-2 cells, EPS-LM substantially preserved intestinal epithelial barrier integrity under oxidative challenge. Both TEER and sodium-fluorescein permeability assays demonstrated that EPS-LM mitigated H₂O₂-induced barrier dysfunction, indicating reinforcement of TJ function. Importantly, this protection likely arises from redox-mediated stabilization of the actin cytoskeleton. Oxidative stress is well known to trigger TJ disruption via activation of MLCK/MLC and RhoA/ROCK pathways, which increase cytoskeletal tension and cause separation of junctional complexes (Brunner et al., 2021; Wang et al., 2023). By suppressing ROS accumulation and maintaining antioxidant enzyme activities, EPS-LM may prevent these stress-activated contractile events, preserving junctional continuity. At the molecular level, EPS-LM upregulated Claudin-1, Occludin, and ZO-1 expression and promoted their continuous localization at cell–cell contacts, which are core events required for TJ sealing (Adil et al., 2021; Angelow et al., 2008). The tight correspondence between functional improvement (increased TEER, decreased flux) and molecular stabilization indicates a redox–junctional coupling mechanism, wherein antioxidant regulation directly supports epithelial architecture and paracellular selectivity.

Integrating these findings, a mechanistic model is proposed (Figure 5-8). Under oxidative stress, excessive ROS disturb redox balance and impair TJ assembly, leading

to increased epithelial permeability. EPS-LM first alleviates this oxidative burden by scavenging ROS and restoring endogenous defense systems, including SOD, CAT, and GSH-Px. These effects subsequently normalize the KEAP1-NRF2-NQO1 signaling pathway, facilitating NRF2 nuclear translocation and re-establishing transcriptional control of antioxidant defenses, thereby maintaining cellular redox homeostasis. The resulting decrease in oxidative tension diminishes cytoskeletal contraction, thereby preventing TJ mislocalization and disassembly. This sequential process translates biochemical redox correction into measurable structural and functional recovery, reflected by elevated TEER, restored TJ protein networks, and reduced paracellular flux. Comparable protective effects between enhanced antioxidant capacity and TJ reinforcement have been documented for other polysaccharides and polyphenolic compounds, illustrating the generality of the mechanism proposed here. For instance, In line with this model, polysaccharide interventions in DSS-injured Caco-2 monolayers alleviated oxidative stress and improved barrier endpoints (TEER, paracellular permeability, TJ protein expression) (He et al., 2025). Similarly, in vivo polyphenol treatment increased Occludin expression and corrected small-intestinal barrier dysfunction in HFD-fed rats (Wang et al., 2023). Together, these findings demonstrate that EPS-LM, like other antioxidant bioactive, translates redox protection into restored TJ architecture and maintained epithelial barrier integrity.

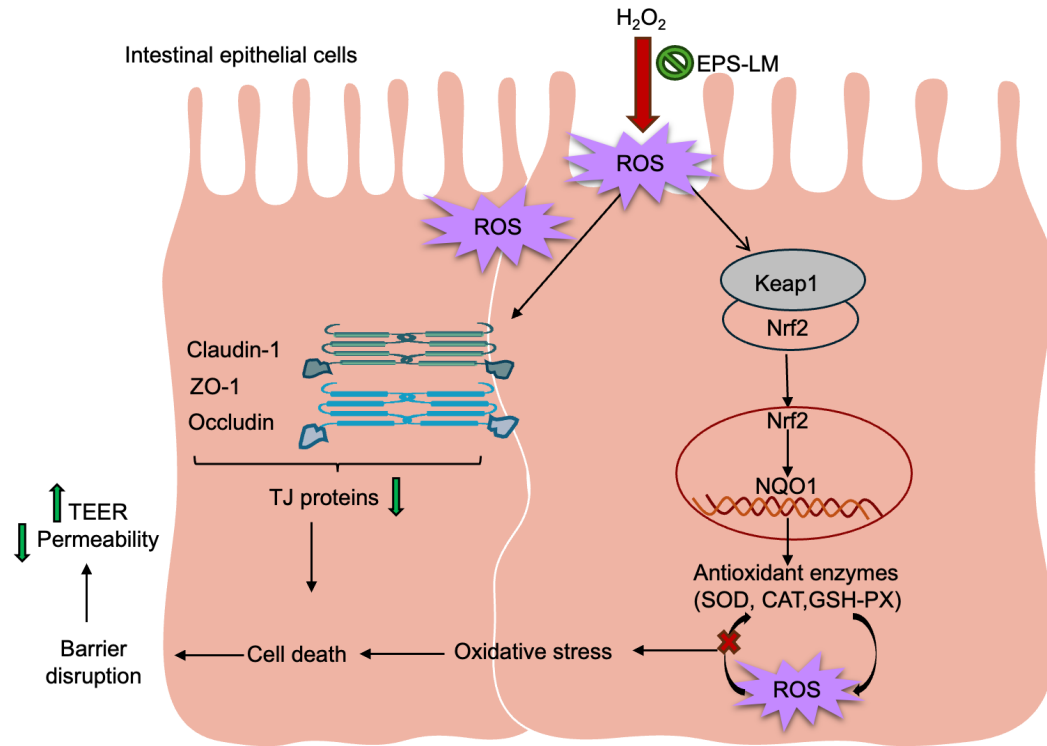


Figure 5-8 Hypothesized mechanism and signaling network for the protective effects of EPS-LM against oxidative-stress-induced epithelial barrier dysfunction in Caco-2 cell culture: (1) Exposure to H₂O₂ induces excessive ROS generation, damaging intestinal epithelial cells, reducing TEER, and disrupting TJ proteins (Claudin-1, ZO-1, Occludin), thereby impairing barrier integrity; (2) Accumulated ROS increase oxidative stress, leading to cellular injury and barrier dysfunction; (3) ROS promote NRF2 dissociation from KEAP1, enabling NRF2 nuclear translocation and transcriptional activation of antioxidant genes such as NQO1; (4) EPS-LM enhances SOD, CAT, and GSH-Px expression, facilitates ROS clearance, alleviates oxidative damage, and stabilizes TJ proteins, thereby protecting epithelial barrier function.

5.5 Conclusions

In summary, EPS-LM serves as a dual-function biomolecule linking antioxidant capacity and barrier protection. Rather than acting solely as a radical scavenger, EPS-LM functions as a redox modulator orchestrating barrier resilience by integrating chemical, enzymatic, and structural defenses within epithelial cells. This multifunctional profile highlights EPS-LM as a promising nutraceutical candidate for mitigating oxidative-stress-related intestinal disorders characterized by oxidative-inflammatory and barrier-compromised conditions. Further *in vivo* validation and gut-microbiota interaction studies are warranted to clarify its mechanistic interplay and translational potential under complex physiological conditions.

Chapter 6 Synthesis, characterization and enhanced bioavailability, and bioactivities of quercetin complex with polysaccharide-bond protein fraction of Cs-HK1

6.1 Introduction

Quercetin is a widely consumed flavonoid present in vegetables, fruits, and traditional Chinese medicines. It exerts diverse health-promoting effects, including cardiovascular protection, antioxidant activity, and obesity management, making it a valuable candidate for functional nutritional supplements (Azeem et al., 2023). However, its practical applications are limited by poor water solubility, low stability during food processing and digestion, and limited bioavailability. Therefore, improving quercetin absorption and efficacy through advanced delivery strategies is essential to fully realize its potential (Kandemir et al., 2022).

To overcome these limitations, various protein-based colloidal carriers have been explored to improve the dispersion, stability, and bioavailability of quercetin and other flavonoids. Proteins such as zein, soybean protein isolate, and pecan protein possess tunable amphiphilicity and excellent biocompatibility, enabling them to encapsulate hydrophobic compounds and enhance their performance in aqueous systems (Huang et al., 2021; H. Li et al., 2019; Wang et al., 2020). However, these single-protein systems often exhibit limited structural stability and are susceptible to environmental stresses such as pH, ionic strength, and temperature changes, which can cause aggregation or

phase separation. Such instability restricts their practical use in complex food formulations and gastrointestinal conditions.

To enhance structural robustness and environmental tolerance, hybrid protein-polysaccharide (PSP) conjugates have been developed as multifunctional delivery matrices. The introduction of polysaccharides can modulate surface charge, improve water affinity, and provide steric hindrance, thereby reinforcing the colloidal and interfacial integrity of protein-based assemblies. Nevertheless, most PSP systems reported to date are formed through physical complexation stabilized by non-covalent interactions (e.g., hydrogen bonding or electrostatic attraction). These weakly associated structures remain sensitive to processing conditions and exhibit limited reproducibility and long-term stability (Wei & Huang, 2019).

In contrast to physically mixed PSP systems, naturally occurring PSP conjugates possess intrinsic covalent architectures that confer stronger molecular cohesion and greater environmental stability. They are biosynthesized in certain fungi, bacteria, and algae, where proteins and polysaccharides become covalently linked during metabolic or secretory processes to form stable macromolecular complexes with synergistic bioactivities (J. Y. Wu et al., 2014). Among these natural sources, the *Cordyceps* Cs-HK1 strain, an anamorphic isolate of *C. sinensis*, has been recognized as a promising source of naturally covalent PSP conjugates. This fungus produces PSP complexes, and previous studies have identified glycopeptide components in which the protein backbone is covalently linked to glucose- and mannose-rich polysaccharide

chains (Leung et al., 2009; J. Y. Wu et al., 2014). Such findings indicate that covalent PSP linkages naturally exist within the Cs-HK1 polysaccharide fraction, supporting its potential to form stable PSP frameworks with high molecular stability and pronounced antioxidant activity (Y. Y. Zhu et al., 2024).

Building upon these natural advantages, the development of green, low-cost, and food-grade delivery systems has become a critical focus. Traditional preparation methods often rely on organic solvents such as dimethyl sulfoxide (DMSO) or ethanol, which raise toxicity and cost concerns (Ji et al., 2022; Modrzynski et al., 2019), while other techniques like nanogel formation require complex, non-scalable procedures (Ding et al., 2023). The pH-induced co-assembly approach provides a more sustainable alternative; under alkaline conditions, proteins dissociate and quercetin deprotonates, enabling spontaneous co-assembly into stable complexes upon neutralization (Liu, Sun, Zhang, et al., 2022).

Accordingly, the present study employs this solvent-free, pH-driven co-assembly strategy to construct quercetin-PSP complexes derived from the Cs-HK1PSP fraction. The resulting complexes were systematically characterized in terms of loading efficiency, physicochemical stability, antioxidant capacity, and intestinal delivery performance, providing a robust foundation for future functional-food and nutraceutical applications.

6.2 Materials and methods

6.2.1 Materials

Quercetin (CAS number 117-39-5, purity of 97 %) was purchased from Shanghai Macklin Biochemical Technology Co., Ltd. (Shanghai, China). ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) was obtained from Roche (Mannheim, Germany). Pepsin (3000 U/g), trypsin (250 U/g) and bile salts were provided by Solarbio (Beijing, China). The murine macrophage cell line RAW264.7 were originally from ATCC. Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), trypsin and other agents for cell culture were obtained from Gibco Biotechnology Co., ltd. (Grand Island, NY, USA). Lipopolysaccharide (LPS) and MTS assay kits were obtained from Sigma-Aldrich Co. Ltd. (St. Louis, MO, USA). All other chemicals and reagents used in this study were of analytical grade.

6.2.2 Cs-HK1 fermentation and ultrasound treatment

The Cs-HK1 mycelial fermentation was carried out as described in Section 4.2.1. After incubation, the mycelial fermentation broth (containing fungal mycelia and extracellular polysaccharides) of Cs-HK1 was collected from the flasks and subjected to ultrasonic treatment to extract intracellular and extracellular polysaccharide-protein complexes (PSPs) from this viscous broth. The broth was first diluted to 66 % of its original concentration to reduce viscosity and enhance cavitation efficiency. Ultrasonic extraction was performed using a VCX 750 ultrasonic processor (Sonics & Materials Inc., USA) operating at 20 kHz with a 13 mm probe horn inserted

approximately 2 cm into 50 mL of the broth in a 100 mL centrifuge tube. The sample tube was placed in an ice bath to prevent overheating and maintain the temperature at 30 ± 5 °C during sonication. The high-intensity ultrasound generated strong cavitation and shear forces that disrupted the fungal mycelia, thereby markedly reducing broth viscosity and promoting the release of PSP conjugates into the liquid phase (Cheung et al., 2015). The resulting PSP fraction thus contained both extracellular and cell-associated PSP complexes that were released during sonication.

6.2.3 US-PSP extraction and recovery from Cs-HK1 fermentation broth

For the construction of the PSP-based drug delivery system, the ultrasonicate-derived PSP (US-PSP) from Cs-HK1 mycelial biomass was selected instead of EPS-LM. US-PSP showed better physicochemical properties for nanoparticle fabrication, including higher solubility, more homogeneous molecular size distribution and a higher protein content, which facilitated self-assembly and drug loading. In contrast, EPS-LM, as an extracellular ethanol-precipitated fraction, contained more heterogeneous high-molecular-weight aggregates that were less suitable for forming stable nanoparticles under the current preparation conditions. Therefore, US-PSP was chosen as the PSP component for the drug delivery system in Chapter 6.

US-PSP from the Cs-HK1 mycelial fermentation broth were extracted under optimized conditions based on previously established methods (Sun et al., 2017). The selection of pH played a critical role in optimizing both protein extraction and precipitation.

Alkaline extraction at higher pH levels enhanced protein yield but introduced challenges such as extract darkening, increased viscosity, and reduced centrifugation efficiency. Taking these factors into account, pH 9.5 was identified as the optimal condition for protein extraction. To extract US-PSP, the fermentation broth underwent ultrasonic treatment and was then adjusted to pH 9.5 using 0.1 M NaOH or HCl. The solution was shaken at 200 rpm at room temperature for 30 minutes to facilitate protein solubilization under alkaline conditions. The mixture was subsequently centrifuged at 4000×g for 20 minutes to separate the protein-rich supernatant. Acid precipitation was performed on the supernatant at various pH levels (3.5-6) to determine the isoelectric point that yielded maximum protein recovery.

6.2.4 Analysis of chemical composition and molecular weight

The total carbohydrate content performed using the same methods as described in Section 4.2.2.

The protein content of US-PSP was determined using the Kjeldahl method with a Pro-Nitro M Kjeldahl Steam Distillation Unit (JP Selecta, Barcelona, Spain), following a previous study (Sampaio et al., 2020). For the protein analysis, 500 mg of freeze-dried PSP powder was digested with 25 mL of concentrated sulfuric acid and a catalyst tablet (6.25% $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and potassium sulfate) in a digestion box at sequential temperatures of 125°C, 270°C, and 400°C to ensure the complete conversion of nitrogen into ammonium sulfate. After cooling, the digested sample was distilled with

3 × 25 mL of 40% NaOH, and the released ammonia was collected in 50 mL of 4% boric acid solution containing bromocresol green and methyl red indicators. The borate formed was titrated with standardized HCl, and the nitrogen content was calculated to determine the protein content using a nitrogen-to-protein conversion factor of 6.25.

The molecular weight distribution of US-PSP samples was analyzed using SDS-PAGE according to the method of (Laemmli, 1970), with slight modifications. Samples were mixed with 5× reducing loading buffer, heated at 95 °C for 5 min, and loaded together with a protein molecular weight marker onto a gel containing 5% stacking gel and 12% separating gel. Electrophoresis was run at 75 V until the samples entered the separating gel, followed by 120 V until completion. Afterward, the gel was stained with Coomassie Brilliant Blue G-250 and destained until the background was clear. Protein bands were visualized using an Azure C600 imaging system (Azure Biosystems), and their apparent molecular weights were estimated by comparison with the marker.

6.2.5 Analysis of protein composition

The total amino acid (TAA) composition of PSP performed using the same methods as described in Section 5.2.2.1.

6.2.6 Preparation of quercetin-PSP complex

For the preparation of the quercetin-PSP complex, the procedure based on (Liu et al., 2025) with minor modifications. US-PSP (2 g) was dispersed in 200 mL of deionized water, the pH was adjusted to 7, and the solution was allowed to hydrate overnight at

4 °C. A 50 mL portion was then mixed with quercetin at 8.33, 12.5, 25, or 50 mg, corresponding to mass ratios of 1:60, 1:40, 1:20, and 1:10 (Quercetin:PSP), respectively. The pH of each mixture was raised to 12 with 1 M NaOH and stirred at 400 rpm for 30 min to facilitate quercetin incorporation, with additional NaOH added as needed to maintain alkalinity. The pH was subsequently lowered to 7 using 1 M HCl, followed by an additional 30 min of stirring. Fresh samples were analyzed immediately, while the remaining portions were freeze-dried for storage.

6.2.7 Characterization of US-PSP (particle size, PDI, and zeta potential)

Determination of US-PSP particle size, polydispersity index (PDI), and zeta potential was carried out with a Zetasizer Nano-ZS ZEN3600 (Malvern Instruments, UK). Particle size and PDI were analyzed by dynamic light scattering (DLS), while zeta potential was evaluated using electrophoretic light scattering (ELS). Freshly prepared PSP dispersions were diluted 100-fold with deionized water to minimize multiple scattering, and all measurements were conducted at 25 °C. The procedure was based on (Liu et al., 2024) with appropriate adjustments.

6.2.8 Evaluation of quercetin encapsulation in US-PSP systems

Encapsulation efficiency (EE) and loading capacity (LC) of quercetin in US-PSP complexes were assessed following (X. Yan et al., 2019) with slight modifications. Fresh samples were centrifuged at 10,000 rpm for 15 min using an Avanti JXN-26 centrifuge (Beckman Coulter, USA) to remove non-encapsulated quercetin. The pellet

fraction was re-dissolved in ethanol, appropriately diluted, and analyzed by UV-Vis spectrophotometry (Cary 60, Agilent, USA) at 374 nm. Quercetin content was quantified against a standard calibration curve (0-10 µg/mL, $y = 0.0665X - 0.0037$, $R^2 = 0.9999$). EE and LC values were then derived according to the standard formulas:

$$EE (\%) = \left(1 - \frac{\text{unencapsulated quercetin content (mg)}}{\text{total quercetin content (mg)}}\right) \times 100$$

$$LC (\%) = \frac{\text{mass of encapsulated quercetin (mg)}}{\text{mass of quercetin and PSP (mg)}} \times 100$$

6.2.9 Surface hydrophobicity analysis of quercetin-PSP complexes

Surface hydrophobicity was determined using 1-anilino-8-naphthalenesulfonic acid (ANS) as a fluorescence probe following the procedure of (Liu, Sun, Cheng, et al., 2022) with minor modifications. Fluorescence spectra were recorded on a Cary Eclipse spectrometer (Agilent, USA) at an excitation wavelength of 370 nm, with emission scanned from 400 to 700 nm and both slit widths set to 5 nm. Prior to measurement, PSP-quercetin samples were diluted 20-fold with deionized water, mixed with 20 µL of ANS solution (8 mM, pH 7), and incubated for 20 min without light exposure. The maximum fluorescence intensity was taken as an indicator of the surface hydrophobicity of the complexes.

6.2.10 Fluorescence spectral characterization of quercetin-PSP complexes

Intrinsic fluorescence spectra of PSP-quercetin samples were recorded using a Cary Eclipse spectrofluorometer (Agilent, USA) to assess structural changes upon complex

formation. Excitation was conducted at 280 nm, and emission spectra were collected between 300 and 450 nm with both slit widths fixed at 5 nm. Prior to measurement, freshly prepared samples were diluted 50-fold with deionized water.

6.2.11 Circular dichroism (CD) spectroscopy analysis

Circular dichroism (CD) was performed using the same method as described in Section 5.2.2.3.

6.2.12 Fourier transform infrared (FT-IR) spectroscopy analysis

FT-IR was performed using the same method as described in Section 4.2.2.

6.2.13 SEM observation of PSP-quercetin complexes

For morphological analysis, PSP-quercetin complexes at a mass ratio of 1:20 were examined by scanning electron microscopy using a Tescan Vega 3 LMU system (TESCAN, Brno, Czech Republic). Powder samples were mounted on aluminum stubs with conductive adhesive and sputter-coated with a thin layer of gold under vacuum to enhance conductivity. Surface features were subsequently visualized at appropriate accelerating voltages.

6.2.14 Stability assessment of PSP-quercetin complexes

The stability of PSP-quercetin complexes (quercetin-to-protein mass ratio of 1:20) was evaluated under thermal and ionic stress conditions. For thermal stability, samples were incubated at 60, 70, 80, and 90 °C for 30 min, followed by cooling to ambient

temperature in an ice bath. Ionic strength effects were assessed by adjusting NaCl concentrations to 100-500 mM. After each treatment, hydrodynamic size and PDI were measured using a Zetasizer Nano-ZS ZEN3600 (Malvern Instruments, UK), and the changes in complex stability were compared across conditions.

6.2.15 ABTS assay for antioxidant activity of US-PSP complexes

The ABTS assay was carried out according to the method described in Section 5.2.3.2.

6.2.16. Assessment of bioactivities of the PSP Complex

The cell lines and culture conditions used in this chapter were maintained according to the methods described in Section 4.2.3. Cell viability, nitric oxide (NO) production, and reactive oxygen species (ROS) assays were performed as previously described in Section 4.2.3.

6.2.16.1 Analysis of cytokines by ELISA

RAW 264.7 cells were seeded into 96-well plates at a density of 2×10^5 cells/well and cultured for 24 h. The cells were then treated with LPS, US-PSP, quercetin, or the PSP-quercetin complex, followed by an additional 24 h incubation to induce the release of pro-inflammatory cytokines IL-1 β and TNF- α . Culture supernatants were collected and centrifuged to remove debris, and cytokine concentrations were quantified using ELISA kits (eBioscience™, Thermo Fisher Scientific, USA) according to the manufacturer's instructions.

6.2.17 Simulated gastrointestinal digestion of quercetin-PSP complexes

In vitro digestion of the quercetin-PSP complex was carried out according to (Liu et al., 2025) with modifications. Quercetin (50 mg) was dissolved in 50 mL deionized water and combined with 50 mL of US-PSP solution to match the quercetin concentration in the 1:20 (w/w) Quercetin:PSP complex. To simulate gastric digestion, 5 mL of this mixture was added to 45 mL of 0.1 M HCl, shaken at 37 °C (100 rpm) for 10 min, and then treated with pepsin (10 mg) after adjusting the pH to 2.0. After 60 min, bile salts (250 mg) were introduced, the pH set to 7.0, and the sample incubated for 10 min before adding trypsin (20 mg) for 120 min of intestinal digestion. Aliquots were withdrawn from each stage for particle size, zeta potential, and PDI measurements. Following intestinal digestion, quercetin, the physical mixture with US-PSP, and the PSP-quercetin complex were centrifuged at 10,000 rpm for 15 min to collect the mixed micelle fraction. Ethanol-diluted samples (before centrifugation and from the micelle fraction) were analyzed at 374 nm by UV-Vis spectrophotometry, and quercetin concentration was calculated using the calibration curve ($y = 0.0665X - 0.0037$, $R^2 = 0.9999$). Bioaccessibility was expressed as the percentage of quercetin incorporated into mixed micelles.

$$\text{Bioaccessibility (\%)} = \frac{C_M}{C_D} \times 100$$

where C_M and C_D are the concentrations of quercetin in the mixed micelle phase and intestinal digestion phase, respectively.

6.2.18 Statistical analysis

Experiments were conducted in triplicate, and the results were expressed as mean $\pm$ standard deviation (SD). Statistical analysis was performed using one-way ANOVA in SPSS 22.0 software, and significant differences between groups were determined by Duncan's multiple range test ($p < 0.05$).

6.3 Results and discussion

6.3.1 Optimal pH for isoelectric precipitation of Cs-HK1 US-PSP

The yield of US-PSP obtained from Cs-HK1 fermentation broth varied with pH during isoelectric precipitation (Figure 6-1). The highest recoveries were achieved at pH 4.0 and 5.5, in line with the principle that proteins precipitate most readily near their isoelectric points, where net surface charge approaches zero and solubility is minimized (Qing et al., 2022). Among the tested conditions, pH 4.0 produced the maximum US-PSP yield (2.265 g/L) and was therefore selected as the optimal precipitation condition. These results highlight that pH 4.0 not only ensures higher yield and recovery efficiency but also demonstrates the feasibility of applying isoelectric precipitation at scale for US-PSP extraction from Cs-HK1 mycelial broth.

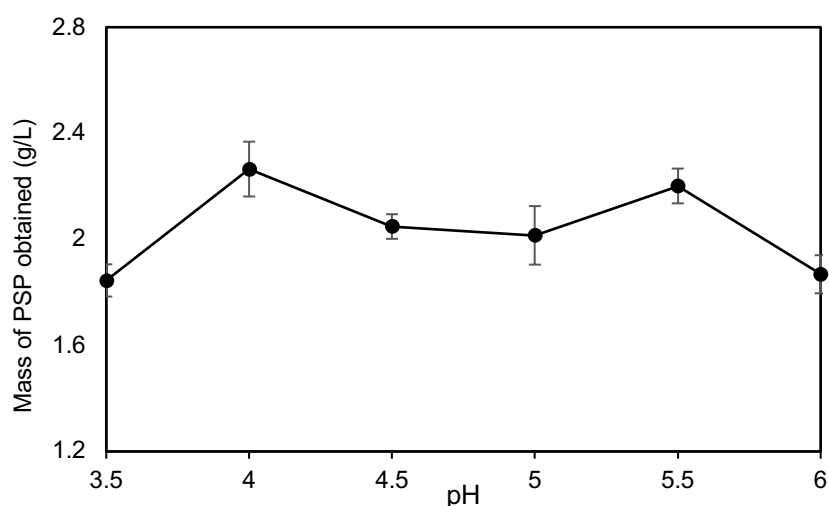


Figure 6-1 Effect of pH on the mass of US-PSP obtained (g/L) from Cs-HK1 fermentation broth.

6.3.2 Chemical Composition of Cs-HK1 US-PSP

The chemical composition and molecular weight distribution of Cs-HK1 US-PSP are presented in Table 6-1. Protein was identified as the major component (53.16%), whereas total carbohydrate accounted for 12.8%, reflecting the presence of polysaccharides that may influence solubility and bioactivity. SDS-PAGE analysis (Appendix Figure A6) showed protein bands mainly at 40 kDa and lower, together with additional fractions at 66 kDa and 135 kDa, indicating a heterogeneous protein profile with potential functional diversity.

Table 6-1 Chemical composition and molecular weight of Cs-HK1 US-PSP.

Protein (wt%)	Total carbohydrate (wt%)	Mainly, MW of US-PSP
---------------	--------------------------	----------------------

analyzed by SDS-PAGE		
53.16 ± 0.31	12.80 ± 0.91	40 kDa and below, 66 kDa,135 kDa

The amino acid composition of US-PSP is summarized in Table 6-2, providing insight into its potential interactions with quercetin in complex formation. The total amino acid (TAA) content was 222.73 mg/g, comprising 88.50 mg/g essential amino acids (EAAs, 40% of TAA) and 134.23 mg/g non-essential amino acids (NAAs, 60% of TAA). Among the EAAs, valine (16.04 mg/g), leucine (15.12 mg/g), and lysine (14.89 mg/g) were the most abundant, whereas methionine (3.36 mg/g) was least represented. For NAAs, glutamic acid (26.24 mg/g) and aspartic acid (24.32 mg/g) predominated, followed by proline (18.66 mg/g) and alanine (17.19 mg/g), while cysteine showed the lowest level (1.47 mg/g). This amino acid profile is consistent with previous reports that emphasize the role of protein composition in stabilizing bioactive compound-protein complexes (J. Y. Wu et al., 2014; Zhao, Gu, et al., 2024). Collectively, these findings suggest that Cs-HK1 US-PSP possesses a favorable amino acid composition for efficient quercetin loading, thereby supporting the stability and functionality of the resulting complex.

Table 6-2 Amino acids in protein of US-PSP (mg/g).

Essential Amino Acid (EAA)	Non-essential Amino Acid (NAA)
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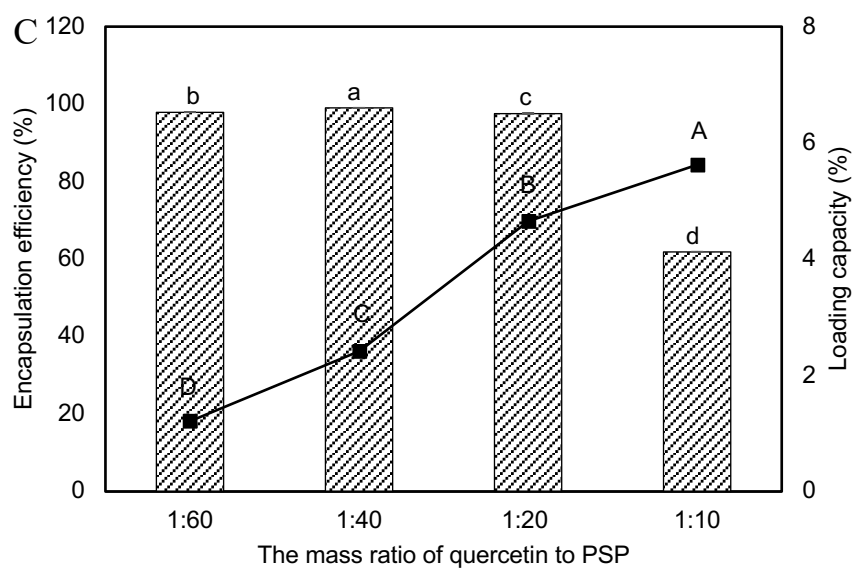
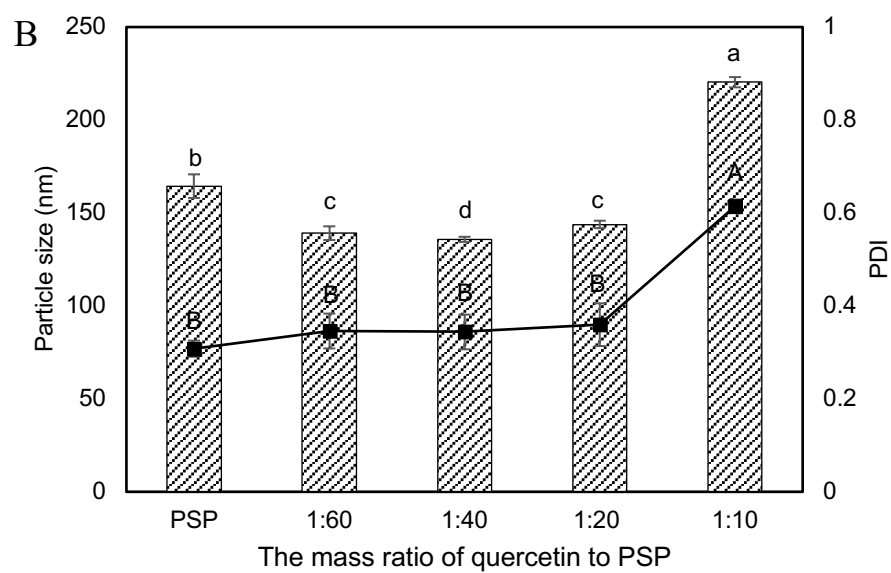
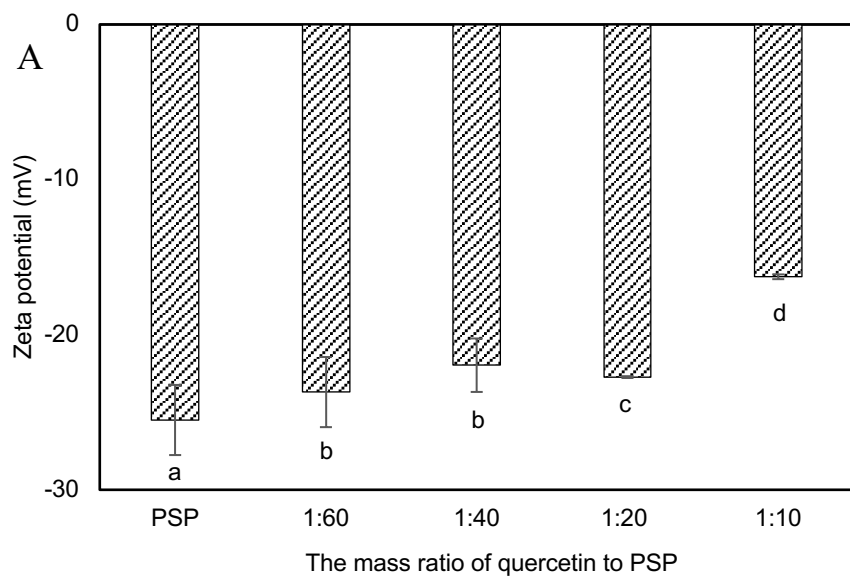
Histidine	7.70 ± 0.29	Alanine	17.19 ± 0.09
Isoleucine	10.10 ± 0.66	Aspartic acid	24.32 ± 0.16
Leucine	15.12 ± 0.17	Arginine	15.67 ± 0.45
Lysine	14.89 ± 0.13	Glutamic acid	26.24 ± 0.29
Phenylalanine	9.91 ± 0.45	Glycine	12.27 ± 0.05
Threonine	11.38 ± 0.13	Serine	11.08 ± 0.07
Valine	16.04 ± 0.14	Tyrosine	7.03 ± 0.47
Methionine	3.36 ± 0.13	Proline	18.66 ± 2.06
		Cysteine	1.47 ± 0.04
Total Amino Acids (TAA)		222.73 ± 4.58	
Essential Amino Acids (EAA)		88.50 ± 2.52	
Non-essential Amino Acids (NAA)		134.23 ± 3.84	

6.3.3 Quercetin loading characteristics

6.3.3.1 Characteristics of PSP-quercetin complexes

Pure US-PSP exhibited a zeta potential of -25 mV (Figure 6-2A). With increasing quercetin content, the zeta potential shifted progressively toward less negative values. A significant change was first observed at a mass ratio of 1:60 ($p < 0.05$), with further increases at 1:40, 1:20, and 1:10, indicating a continuous trend toward higher potentials

as quercetin loading increased. This progressive shift is likely attributable to quercetin-induced conformational rearrangements in US-PSP that expose polar groups, thereby enhancing electrostatic repulsion and improving colloidal stability (Xie et al., 2024). Particle size analysis (Figure 6-2B) showed that US-PSP alone had an average diameter >160 nm. Incorporation of quercetin at ratios $\leq 1:20$ reduced particle size markedly, consistent with protein-polyphenol interactions that promote a more compact structure, a phenomenon also reported for whey protein (Liu et al., 2025). In contrast, at a 1:10 ratio, mean size exceeded 200 nm, indicating aggregation induced by excess quercetin. The PDI followed the same pattern: ratios $< 1:20$ yielded narrow distributions (PDI < 0.4), whereas higher ratios produced broader distributions (PDI > 0.4). Collectively, these findings suggest that moderate quercetin loading favors compact and homogeneous PSP-quercetin complexes, while excessive incorporation promotes particle enlargement and structural heterogeneity.



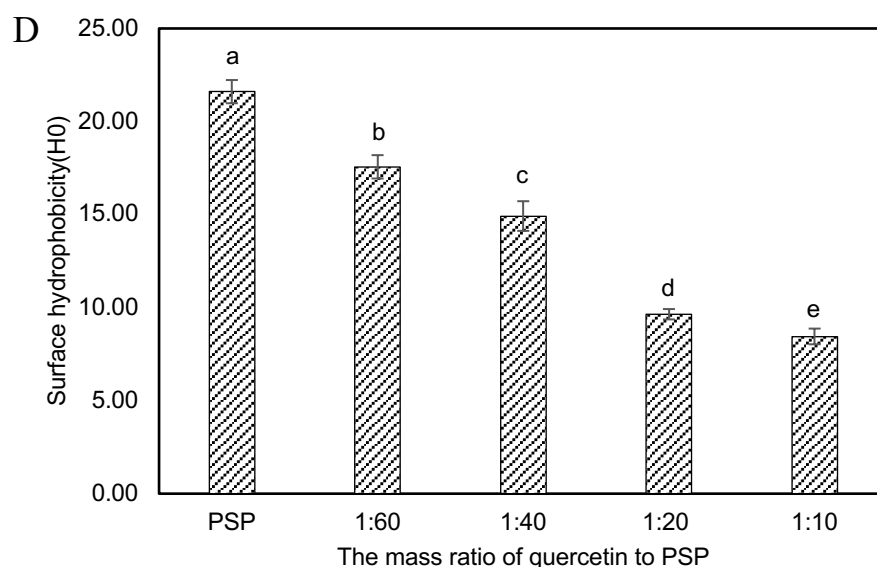


Figure 6-2 Effects of the quercetin-to-PSP mass ratio on the particle size and PDI (A), zeta potential (B), encapsulation efficiency and loading capacity of quercetin (C), and surface hydrophobicity (D).

6.3.3.2 EE and LC

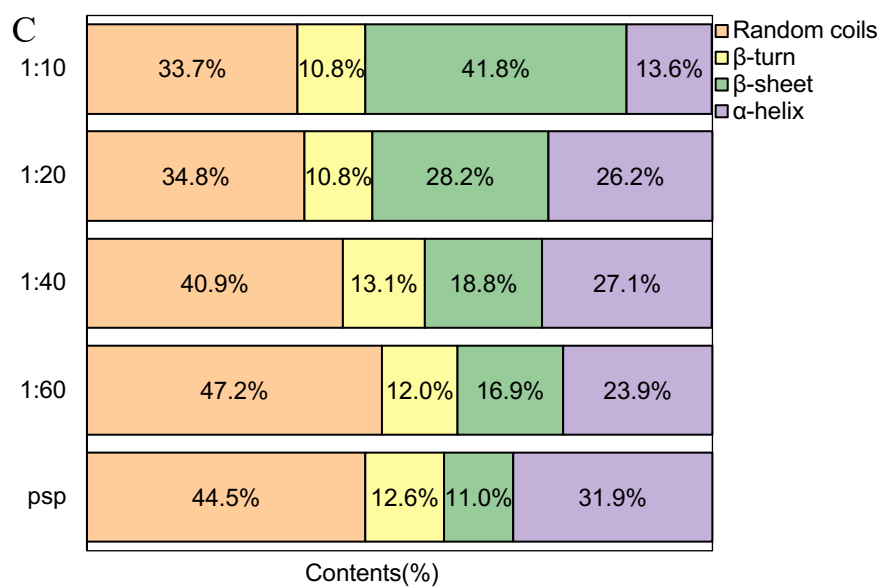
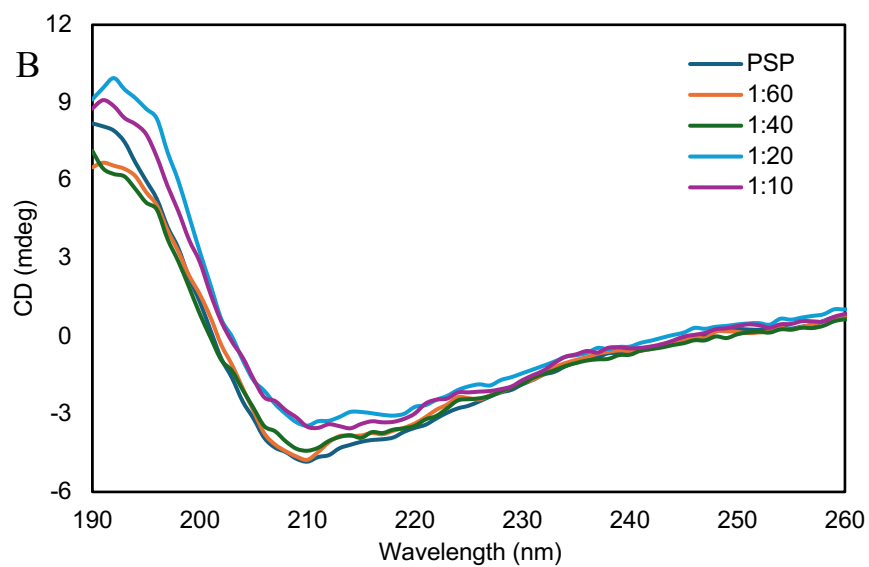
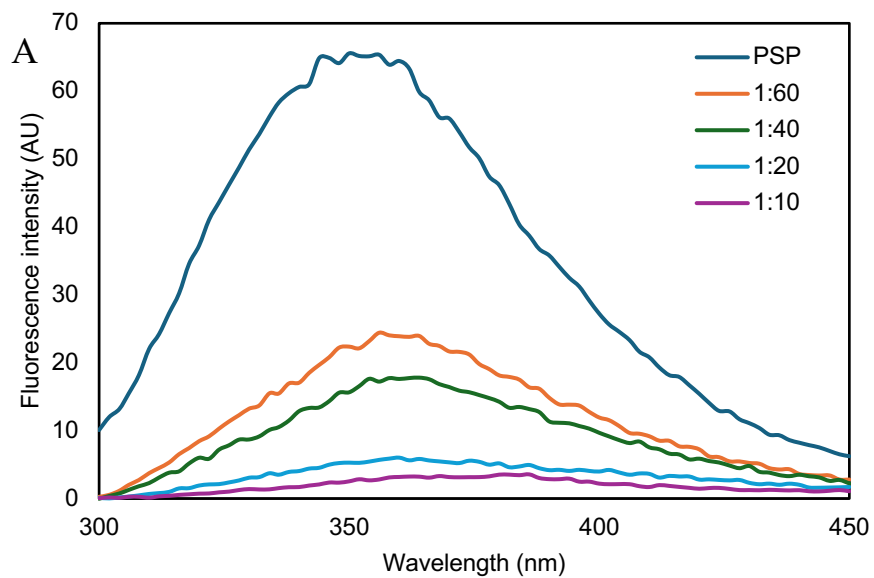
EE analysis showed that US-PSP effectively accommodated quercetin across a broad range of mass ratios (Figure 6-2C). At most ratios, EE exceeded 97%, with the maximum value of 99.01% at 1:40. High efficiencies were also maintained at 1:60 (97.95%) and 1:20 (97.58%), confirming the robustness of the pH-driven encapsulation approach. In contrast, at 1:10, EE fell sharply to 61.85%, suggesting saturation of PSP binding sites and the presence of unencapsulated quercetin (Moon et al., 2021). A similar pattern has been observed in whey protein systems, where moderate ratios ensured efficient encapsulation but quercetin excess markedly reduced EE (Liu et al., 2025). Unlike EE, the LC of US-PSP increased steadily with rising quercetin content

($p < 0.05$). LC values rose from 1.21% at 1:60 to 2.41% at 1:40, and further to 4.65% at 1:20. The maximum LC of 5.62% was obtained at 1:10, highlighting the notable ability of US-PSP to accommodate high quercetin amounts despite the drop in EE. Overall, these results indicate that US-PSP is an efficient carrier, achieving high encapsulation efficiency under most conditions while also providing strong loading capacity at elevated quercetin ratios.

Surface hydrophobicity analysis revealed a distinct response of US-PSP upon quercetin incorporation (Figure 6-2D). Native US-PSP showed the highest hydrophobicity value, whereas the successive addition of quercetin led to a marked decrease. Moderate reductions appeared at 1:60 and 1:40, with a more pronounced decline at 1:20 and 1:10, the latter showing the lowest value. This decline can be attributed to quercetin interactions with aromatic and other hydrophobic residues on US-PSP, which restrict ANS binding and reduce apparent surface hydrophobicity (Cheng et al., 2023). Integrating these results with other indicators (EE, LC, particle size, and zeta potential; Figure 6-2), the 1:20 ratio appeared optimal. Under this condition, PSP-quercetin complexes maintained high encapsulation efficiency (97.58%) and considerable loading capacity (4.65%), while exhibiting decreased surface hydrophobicity, smaller and more uniform particle size, and improved electrostatic stability. Taken together, these findings indicate that US-PSP provides favorable interfacial properties and functions as a stable and effective carrier for quercetin.

6.3.3.3 Fluorescence spectroscopy analysis

The intrinsic fluorescence of US-PSP originates mainly from tryptophan residues, which exhibit a characteristic emission maximum at 352 nm (Figure 6-3A). Upon quercetin addition, the overall fluorescence intensity decreased progressively, whereas the spectral profile and peak position remained essentially unchanged. This pronounced quenching indicates direct interactions of quercetin with the tertiary structure of US-PSP, where binding alters the microenvironment of tryptophan residues and reduces their emissive capacity (Kristo et al., 2012). The dose-dependent decline in fluorescence intensity mirrors the increasing association between quercetin and US-PSP with higher ratios, consistent with the enhancement of loading capacity (Figure 6-2C). In parallel, the suppression of intrinsic fluorescence aligns with the observed decrease in surface hydrophobicity (Figure 6-2D), suggesting that quercetin masks hydrophobic amino acid side chains and thereby promotes colloidal stability. Similar quenching behaviors of tryptophan fluorescence have been reported in other polyphenol-protein systems, highlighting the generality of this interaction mechanism (Liu et al., 2025).



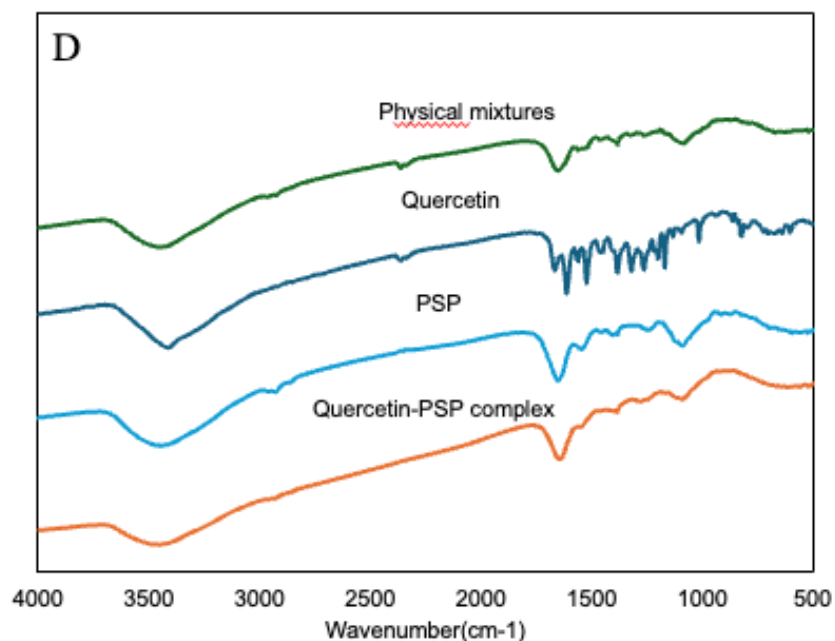


Figure 6-3 Effects of the quercetin-to-PSP mass ratio on the fluorescence spectra (A), FT-IR spectra (B) of PSP, quercetin, physical mixtures, and complex, CD spectra (C), and secondary structure content (D) of the complex. The mass ratio of quercetin to protein was 1:20 in the physical mixtures and the complex used in FT-IR spectra analysis.

6.3.3.4 Circular dichroism (CD) spectroscopy analysis

CD spectra revealed the conformational responses of US-PSP to quercetin binding (Figure 6-3C-D). Native PSP showed characteristic α -helical signatures with negative bands at 208 and 222 nm, together with a weaker band near 215 nm associated with β -sheet structures. Deconvolution indicated a predominantly disordered conformation, consisting of random coils (44.5%), α -helix (31.9%), β -turn (12.6%), and β -sheet (11.0%). Quercetin addition induced concentration-dependent reorganizations. At moderate loadings (1:60 and 1:40), α -helix content increased to 23.9% and 27.1%, accompanied by modest rises in β -sheet content (16.9% and 18.8%). More pronounced

changes occurred at higher loadings (1:20 and 1:10), where random coil content decreased to 34.8% and 33.7%, while β -sheet content expanded markedly to 28.2% and 41.8%. β -turn proportions fluctuated only slightly, remaining near 11%. Overall, these data suggest that quercetin binding drives US-PSP toward greater secondary structural order, characterized by a coil-to-sheet transition, in agreement with previous observations for protein-polyphenol systems (Xu et al., 2019).

6.3.3.5 FT-IR spectroscopy analysis

The FTIR spectrum of the PSP-quercetin complex displayed clear shifts compared with the individual components, indicating noncovalent interactions (Figure 6E). The broad O-H stretching band became more intense and shifted downward by 5-15 cm^{-1} , a characteristic sign of strengthened hydrogen bonding. The amide I and II bands of PSP, initially at 1658 and 1548 cm^{-1} , shifted to 1649 and 1542 cm^{-1} , suggesting participation of quercetin phenolic hydroxyl and carbonyl groups in intermolecular interactions. Importantly, no new band appeared in the ester region (1735-1745 cm^{-1}), confirming the absence of covalent linkages. For comparison, quercetin alone exhibited O-H stretching near 3350 cm^{-1} , a C=O band overlapping the aromatic region at 1656 cm^{-1} , and C-O vibrations at 1075-1035 cm^{-1} . US-PSP showed a broad hydroxyl absorption around 3350 cm^{-1} , weak aliphatic C-H absorptions (2924, 2852 cm^{-1}), carbohydrate bands at 1150-1030 cm^{-1} , protein amide I and II peaks (1654, 1544 cm^{-1}), and carboxylate absorptions (1602, 1415 cm^{-1}). In a simple physical mixture, superposition

of both spectra produced only minor broadening near 1650 and 1545 cm^{-1} . These spectral features demonstrate that hydrogen bonding and other noncovalent forces are the primary drivers of PSP-quercetin assembly, resulting in a stable composite without covalent linkages. Similar hydrogen-bond-dominated interactions have been documented in other polyphenol-protein systems (Liu et al., 2025), emphasizing the central role of noncovalent networks in stabilizing bioactive-biopolymer complexes.

6.3.3.6 Proposed binding mechanism

At neutral pH, proteins generally maintain a compact conformation with only limited hydrophobic pockets and polar groups exposed, whereas quercetin exhibits poor dispersion due to its low aqueous solubility (C. Chen et al., 2022). Raising the pH to 12 induces partial unfolding of the protein moieties within the US-PSP conjugates through electrostatic repulsion, thereby exposing additional hydrophobic domains and polar residues. Simultaneously, deprotonation of quercetin hydroxyl groups markedly enhances its solubility and dispersion (Jurasekova et al., 2009). Upon subsequent neutralization, the protein components undergo partial refolding and aggregation, during which quercetin molecules can be trapped within the newly formed hydrophobic cavities. Importantly, the polysaccharide chains covalently linked to the US-PSP backbone remain solvated and flexible during this process, forming a hydrophilic outer layer that improves water affinity and sterically hinders excessive protein-protein aggregation. These polysaccharide moieties also provide abundant hydroxyl and

carboxyl groups that are capable of hydrogen bonding with quercetin, thereby reinforcing the overall structural integrity of the assemblies. Consequently, a cooperative binding mode can be proposed in which hydrophobic interactions between the protein core and the aromatic rings of quercetin are complemented by hydrogen bonding between polysaccharide hydroxyl groups and quercetin's phenolic hydroxyls. As illustrated in Figure 6-4, this integrated core-shell architecture explains the high stability and encapsulation efficiency of the pH-driven Cs-HK1 PSP-quercetin complexes, where the protein domains act as primary hydrophobic binding sites and the polysaccharide chains provide secondary hydrogen-bonding interactions and colloidal stabilization.

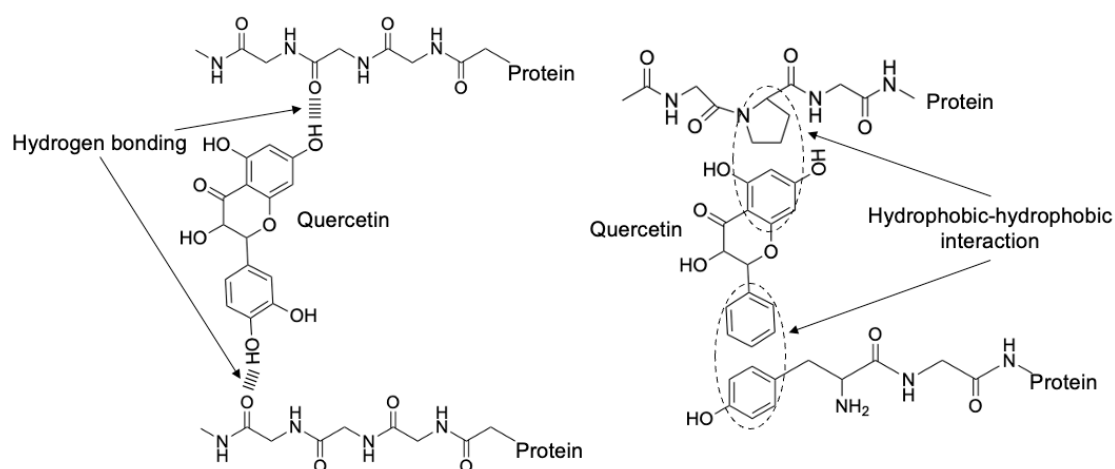


Figure 6-4 Schematic illustration of protein-quercetin interactions via hydrogen bonding and hydrophobic-hydrophobic interactions.

6.3.3.7 SEM analysis

As shown in Fig. 5, US-PSP alone displayed a loose, sponge-like three-dimensional framework with smooth walls and interconnected pores (Figure 6-5B). In contrast, free

quercetin appeared as elongated rods or needle-shaped crystals, consistent with its strong propensity to crystallize (Figure 6-5A). Upon complexation, the porous PSP network was partially filled and reinforced by quercetin, producing a more compact and heterogeneous microstructure (Figure 6-5C). This transformation suggests that quercetin molecules were not simply deposited on the surface but also accommodated within the internal cavities of US-PSP, thereby forming a denser matrix. Such rearrangements are most likely driven by multiple noncovalent interactions, particularly hydrophobic association and hydrogen bonding, which together contribute to the stabilization of the PSP-quercetin complex.

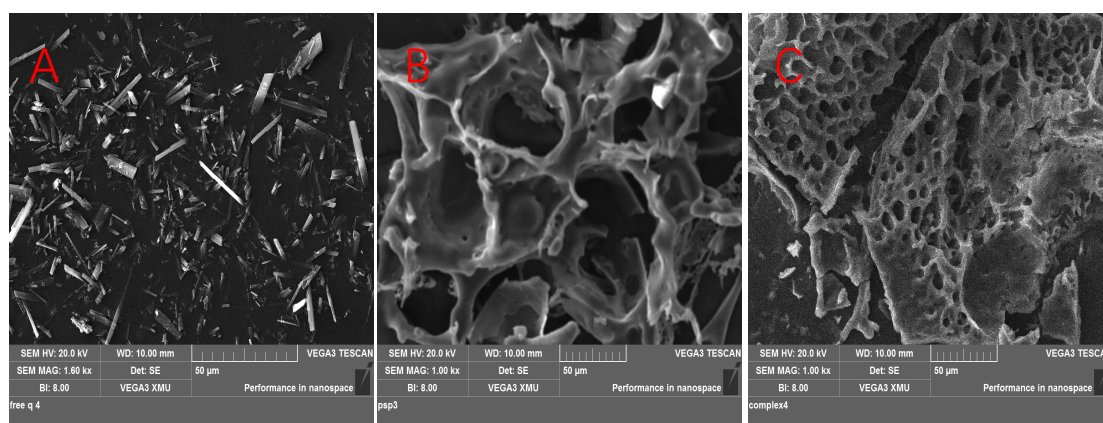
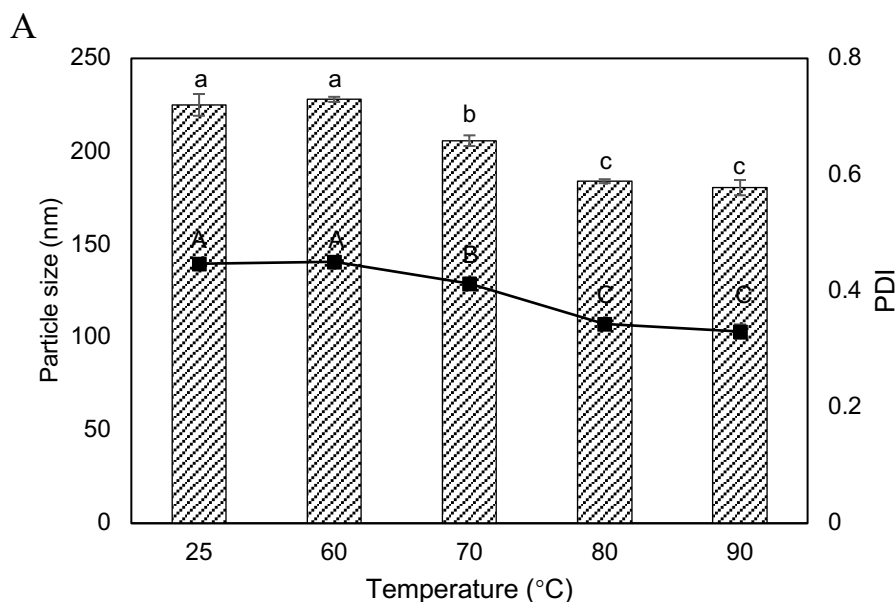


Figure 6-5 SEM images of quercetin (A), US-PSP (B), and the quercetin-PSP (C). The mass ratio of quercetin to protein was 1:20 in the complex.

6.3.4 Stability analysis

The quercetin-PSP complex exhibited notable stability under both thermal and ionic conditions (**Figure 6-6**). As the temperature increased from room temperature to 90 °C, the average particle diameter decreased from 225 nm to 180 nm. Meanwhile, the

dispersion became more uniform, with the PDI dropping below 0.4 at 90 °C. This response likely results from the porous and flexible structure of US-PSP, which accommodates thermal motion and restricts large-scale aggregation. Thus, the complex not only maintained stability during heating but also displayed improved homogeneity. Under ionic stress, increasing NaCl concentration produced gradual particle growth, from approximately 260 nm in salt-free solution to about 390 nm at 400 mM (Figure 6-B). This enlargement suggests that sodium ions weaken electrostatic repulsion, leading to partial aggregation. Meanwhile, the polydispersity index remained nearly constant (0.48-0.50), indicating that the particle distribution did not broaden substantially. These results indicate that elevated ionic strength primarily reduces stability by increasing particle size, rather than by introducing significant heterogeneity.



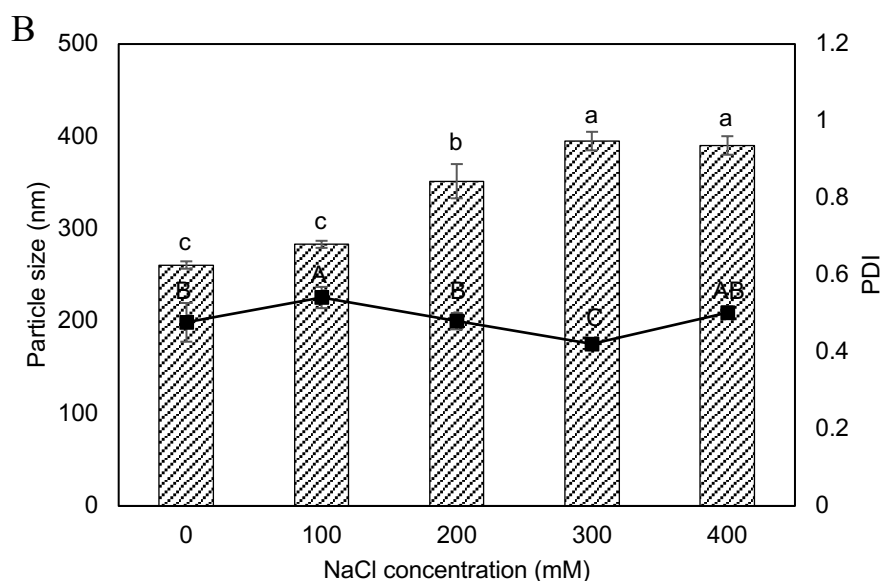


Figure 6-6 Effects of NaCl concentration on the particle size and PDI (A) of the quercetin-PSP complex. Effects of temperature on the particle size and PDI (B) of the quercetin-PSP complex. The mass ratio of quercetin to protein was 1:20 in the complex.

6.3.5 Antioxidant activity analysis

Figure 6-7 shows the antioxidant performance of the quercetin-PSP complex. Free quercetin displayed only modest ABTS• scavenging activity, consistent with earlier reports that poor water solubility limits the radical reactivity of lipophilic polyphenols (Yi et al., 2016). Encapsulation in PSP markedly enhanced its antioxidant capacity. At 25 µg/mL, the quercetin-PSP complex achieved a scavenging rate of about 51%, compared with 35% for free quercetin. At 50 µg/mL, the complex reached nearly 86% activity, which was more than 20 percentage points higher than that of free quercetin (62%). Comparable improvements have been reported for other hydrophilic carriers that increase the dispersibility and reactivity of lipophilic polyphenols (Luo et al., 2024).

This enhancement is most likely attributable to the high loading capacity of US-PSP, which improves quercetin's aqueous dispersion and facilitates radical contact, consistent with observations for protein-polysaccharide-based delivery systems (Liu, Sun, Cui, et al., 2022).

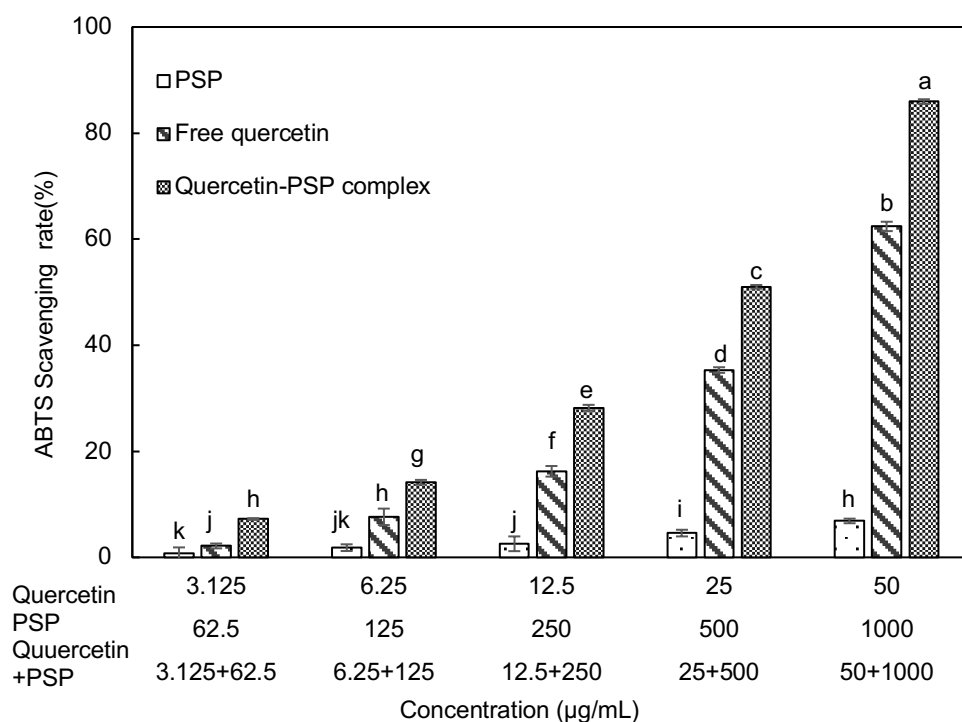


Figure 6-7 ABTS• scavenging activity of free quercetin, PSP and quercetin-PSP complexes at different concentrations.

6.3.6 In vitro anti-inflammatory activity

6.3.6.1 In vitro cytotoxicity assay

Figure 6-8 shows the cytotoxic effects of free quercetin, US-PSP, and their complexes on RAW264.7 cells. For both free quercetin and the quercetin-PSP complex, cell viability remained above 90% within the concentration range of 1.25-15 μg/mL,

showing no evident cytotoxicity compared to the control. Similarly, US-PSP alone exhibited no significant cytotoxic effect even at concentrations up to 300 µg/mL. For both free quercetin and the quercetin-PSP complex, cell viability remained above 90% within the concentration range of 1.25-15 µg/mL, showing no evident cytotoxicity compared to the control. Similarly, US-PSP alone exhibited no significant cytotoxic effect even at concentrations up to 300 µg/mL.

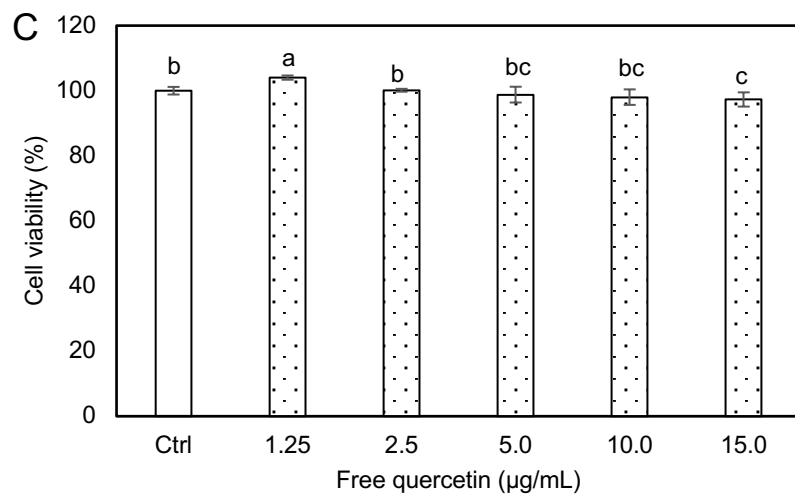
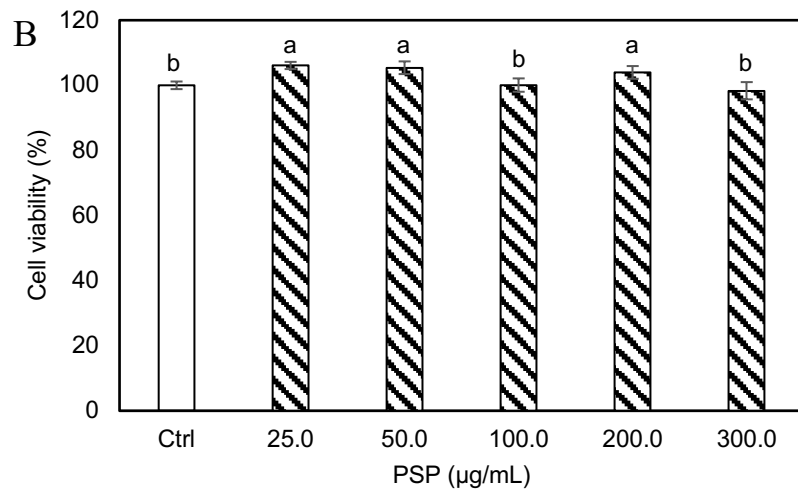
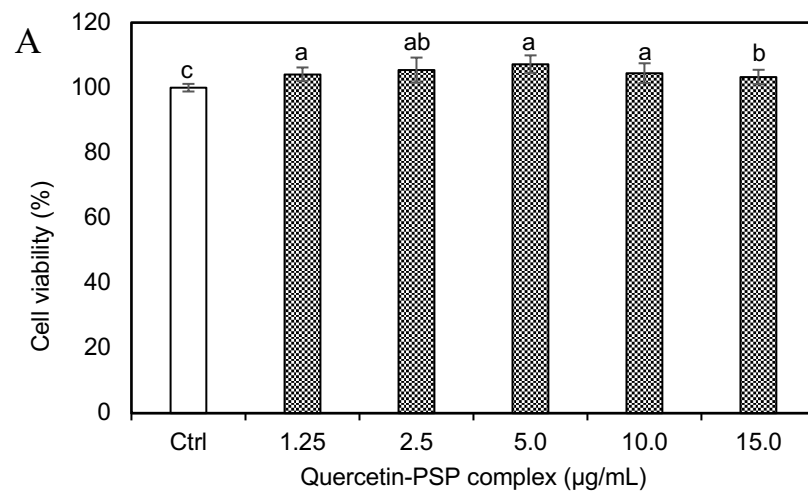
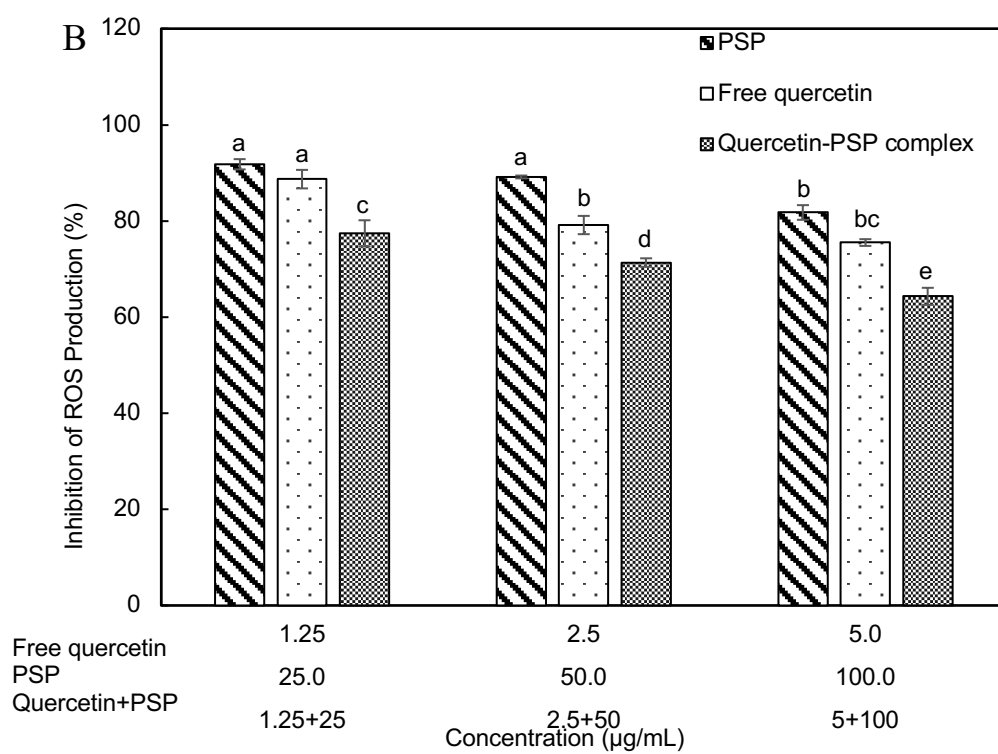
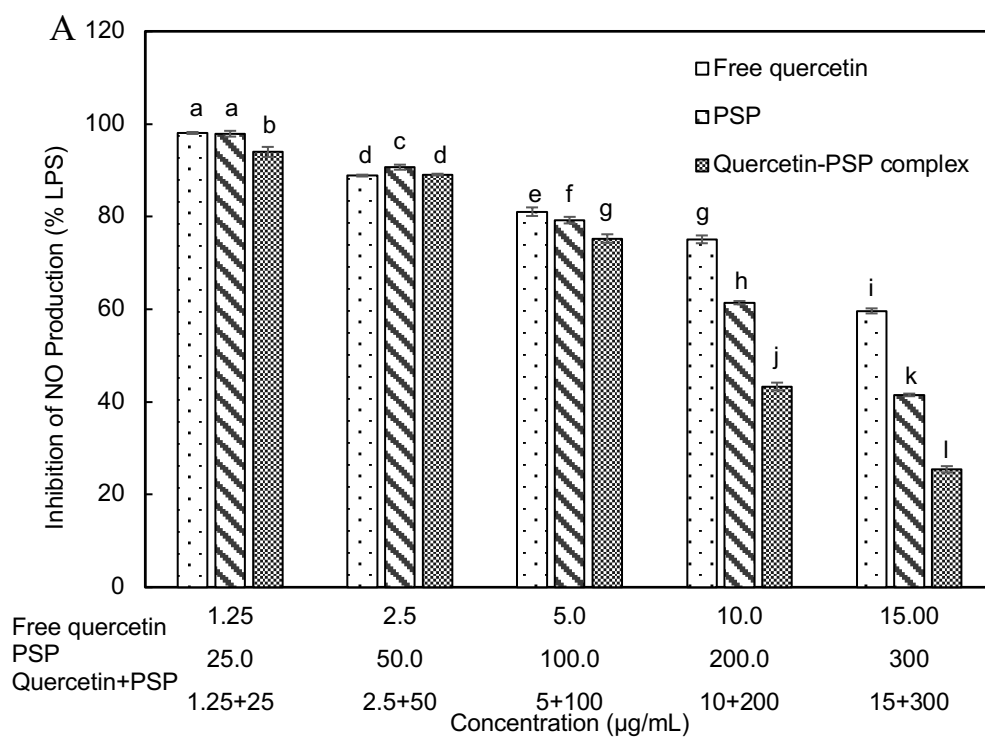


Figure 6-8 The cytotoxic effects of free, US-PSP and encapsulated quercetin on RAW264.7 cells. (A) free quercetin (B) Quercetin-PSP complex (C) US-PSP. The mass ratio of quercetin to protein was 1:20 in the complex.

6.3.6.2 Anti-inflammatory effects of quercetin and US-PSP on RAW264.7 cells

As shown in Figure 6-9, LPS stimulation markedly increased the production of NO, ROS, and pro-inflammatory cytokines in RAW264.7 cells. For NO release (Figure 9A), free quercetin at low concentrations showed little effect, whereas PSP alone elicited modest suppression. Among the treatments, the quercetin-PSP complex consistently produced the strongest inhibition at all tested concentrations, reflecting enhanced protective activity. A comparable trend was observed for ROS generation (Figure 6-9B), where the complex displayed more pronounced suppression than either component alone. Regarding cytokine secretion (Figure 6-9C-D), all treatments reduced LPS-induced IL-1 β and TNF- α relative to the model group, but the quercetin-PSP complex (10 + 200 μ g/mL) achieved the greatest reduction, outperforming quercetin (10 μ g/mL) or US-PSP (200 μ g/mL) administered separately. Together, these findings indicate that PSP-quercetin complexation potentiates the anti-inflammatory efficacy of quercetin, leading to more effective attenuation of macrophage-mediated inflammatory responses.



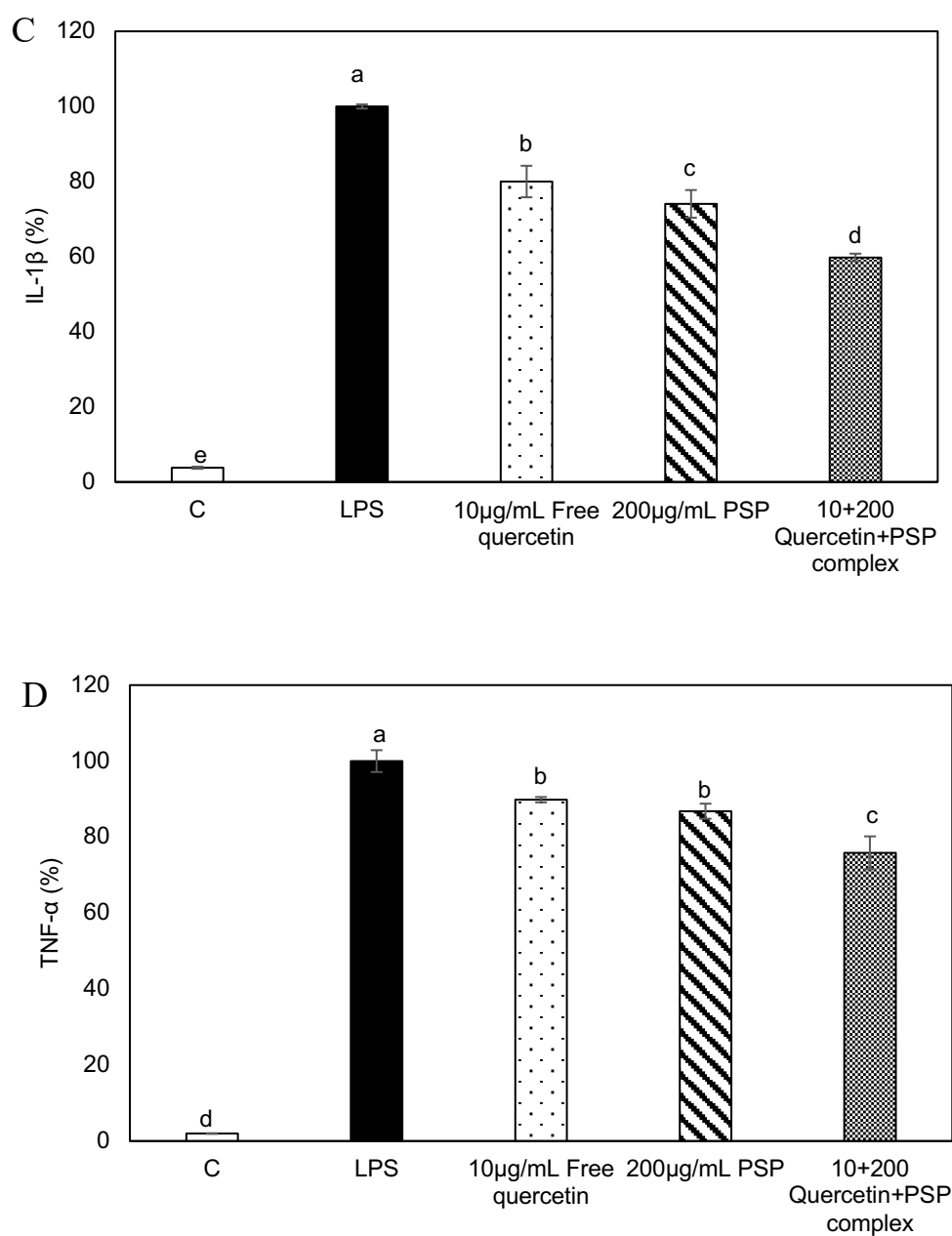


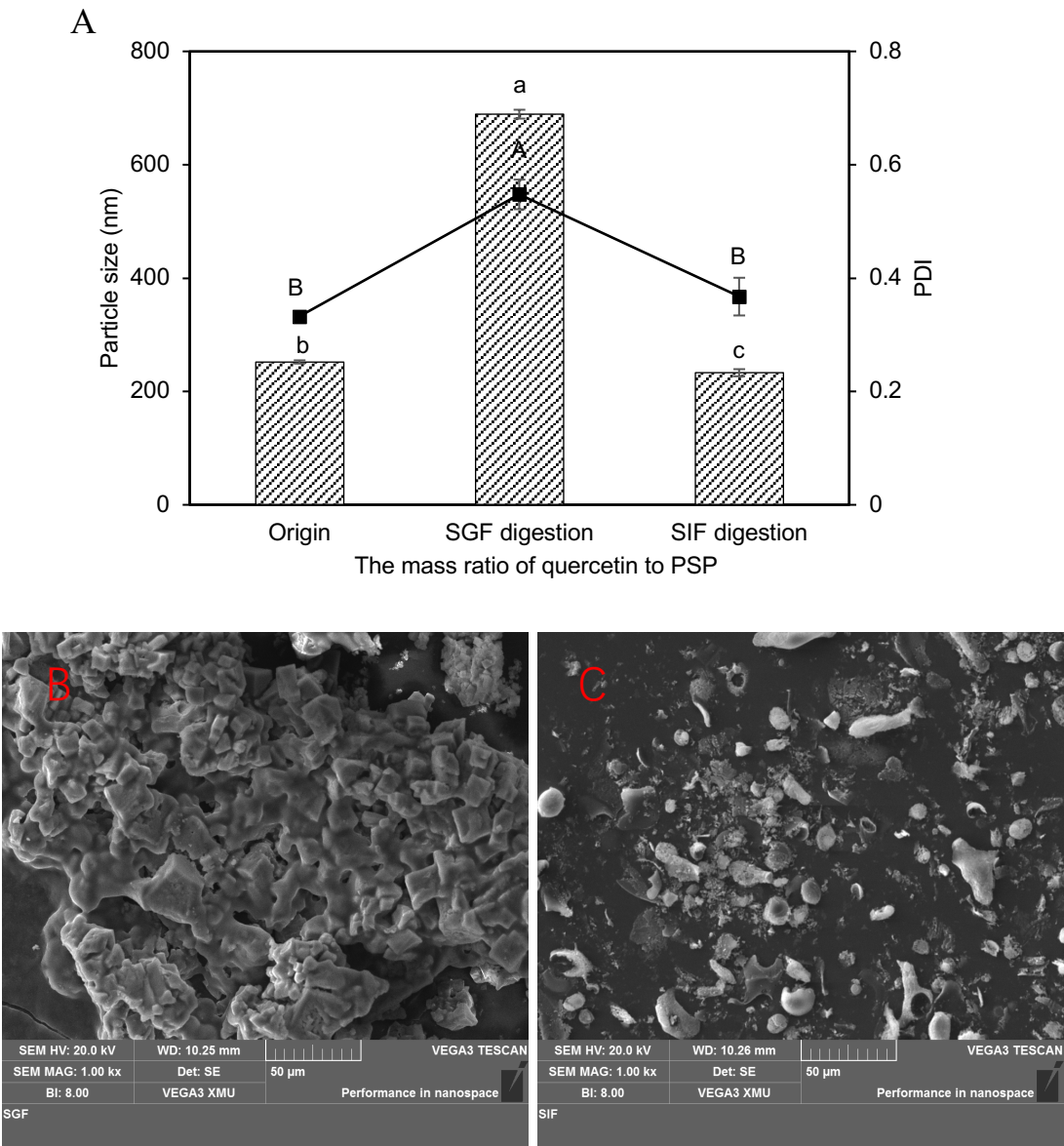
Figure 6-9 The inhibition of NO, ROS, TNF- α , and IL-1 β production by free quercetin, US-PSP, and quercetin-PSP complexes on RAW264.7 cells. (A) NO production, (B) ROS production, (C) TNF- α production, (D) IL-1 β production. The mass ratio of quercetin to protein in the quercetin-PSP complex was 1:20.

6.3.7 In vitro digestive characteristics analysis

6.3.7.1 Digestive stability analysis

To further evaluate the gastrointestinal fate of the quercetin-PSP complex, a simulated digestion model was employed. In simulated gastric fluid (SGF), the particle size increased significantly from 252.1 nm to 689.2 nm ($p < 0.05$), accompanied by a higher PDI (Figure 6-10A). This enlargement suggests that the complexes tended to aggregate under acidic conditions. Similar gastric aggregation was reported for whey protein nanoparticles encapsulating citrus peel extracts (Hu et al., 2019). Upon transfer to simulated intestinal fluid (SIF), the mean particle size decreased sharply to 233 nm ($p < 0.05$). Trypsin-mediated hydrolysis promoted the breakdown of these aggregates, yielding smaller and more dispersed particles. This trend is consistent with observations for zein-alginate nanogels, which disassembled in SIF following initial aggregation under SGF (Ding et al., 2023). Comparable results have also been observed for soy isoflavones-whey protein nanoparticles, which remained relatively stable in gastric fluid but subsequently disintegrated in the intestine to enhance bioaccessibility (Liu, Sun, Zhang, et al., 2022). Functionally, this “transient aggregation” during gastric transit may limit premature quercetin release, while disassembly during the intestinal phase promotes efficient release and absorption. As shown in Figure 6-10B, SEM imaging confirmed that after SGF digestion the complexes aggregated into irregular block-like structures, reflecting protein matrix instability under acidic conditions. Subsequently, after SIF digestion the aggregates decomposed into smaller, more discrete particles (Figure 6-10C), consistent with the reduction in particle size and PDI.

This morphological transition provides evidence that pepsin-induced hydrolysis and acidic pH triggered aggregation in the gastric phase, whereas trypsin digestion facilitated disintegration in the intestinal phase.



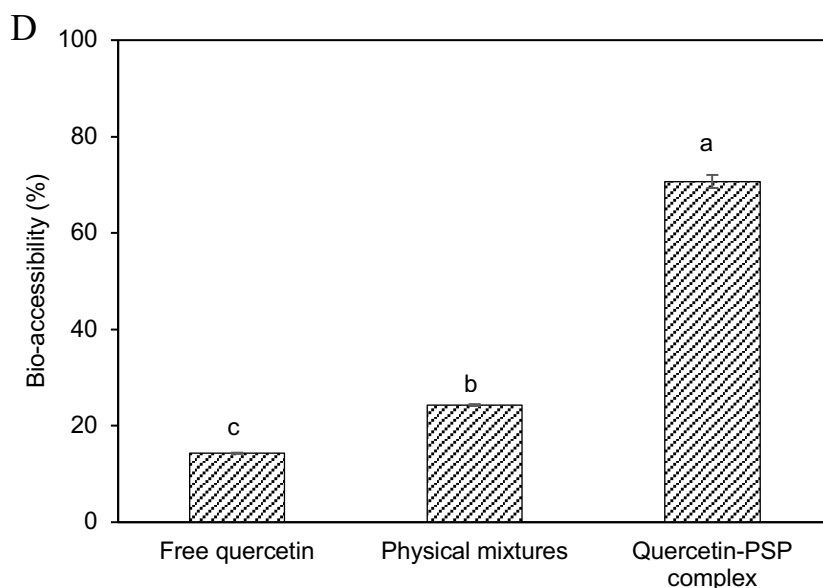


Figure 6-10 Particle size and PDI of quercetin-PSP complex at different digestion stages (A). SEM images of the complex after digestion in SGF (A) and SIF (B). Bioaccessibility of quercetin after simulated gastrointestinal digestion (D). The mass ratio of quercetin to protein was 1:20 in the physical mixtures and the complex.

6.3.7.2 Quercetin bioaccessibility analysis

Figure 6-10D summarizes the intestinal bioaccessibility of quercetin in different formulations. Free quercetin exhibited the lowest value (14.3%), which was attributed to its crystalline state and poor water dispersibility, leading to limited transfer into the micellar phase. When physically blended with US-PSP, the bioaccessibility increased to 24.3%, suggesting partial interactions among quercetin, proteins, digestive enzymes, and bile salts during digestion. Importantly, encapsulation within the quercetin-PSP complex further elevated the bioaccessibility to 70.7%, which is almost five times that of free quercetin and nearly three times that of the physical mixture. This value was consistent with that of a quercetin-whey protein complex prepared by a similar

pH-induced co-assembly method (62.7%), which was reported to be almost five times higher than free quercetin and nearly twice that of the physical mixture (Liu et al., 2025). The enhanced stability and bioaccessibility observed for quercetin encapsulated in zein/soluble soybean polysaccharide nanoparticles further highlight the protective role of protein-polysaccharide co-assemblies in shielding hydrophobic polyphenols during gastrointestinal transit and promoting efficient intestinal uptake. Comparable results were also reported for whey protein nanofibril-stabilized zein nanoparticles, which preserved structural integrity under gastric conditions and facilitated controlled release and antioxidant activity in the intestine (H. Li et al., 2019; Liu et al., 2021). Together, these findings emphasize the general utility of proteinaceous carriers in improving intestinal absorption of hydrophobic polyphenols.

6.4 Conclusion

This study demonstrated that a green pH-induced co-assembly strategy enabled the formation of quercetin-PSP complexes with high encapsulation efficiency, structural stability, and improved functional properties. The complexes exhibited enhanced antioxidant and anti-inflammatory activities and displayed favorable gastrointestinal behavior, characterized by transient aggregation in gastric fluid followed by disassembly in intestinal fluid, which markedly increased quercetin bioaccessibility. Collectively, these results identify Cs HK1-derived US-PSP as an effective and

sustainable carrier for improving the intestinal delivery and functional efficacy of quercetin in food and nutraceutical applications.

Chapter 7 General Conclusions and Future Studies

7.1 General conclusions

This series of studies comprehensively investigated the compositional characteristics, biological functions, and application potential of Cs-HK1-derived polysaccharide-protein complexes. Through systematic chemical, structural, and bio-functional analyses, the research revealed how these PSP complexes modulate inflammatory, oxidative, and epithelial-barrier-related processes and demonstrated their potential as natural carrier platforms for enhancing the bioavailability of flavonoid polyphenols such as quercetin.

1. Physicochemical characteristics

Mycelial fermentation of Cs-HK1 provided reproducible yields of bioactive PSP complexes with integrated polysaccharide and protein components. Comprehensive compositional analyses-covering monosaccharide and amino acid profiling, FT-IR spectroscopy, circular dichroism, fluorescence, and gel-permeation chromatography-confirmed that the PSP macromolecules consisted mainly of mannose-, glucose-, and galactose-rich polysaccharide chains covalently or physically linked to protein moieties enriched in aspartic acid, glutamic acid, and glycine. These components formed β -sheet-dominant secondary structures that endowed the complexes with both structural rigidity and conformational flexibility.

2. Anti-inflammatory effects in macrophage models

The low-molecular-weight exopolysaccharide-protein fraction (EPS-LM), isolated from the liquid medium of Cs-HK1 mycelial fermentation, exhibited anti-inflammatory activity in LPS-stimulated RAW 264.7 macrophages. EPS-LM significantly decreased NO and cytokine production and suppressed the TLR4/MyD88/NF- κ B signaling cascade, as demonstrated by Western blotting, immunofluorescence, and co-immunoprecipitation analyses. It effectively impaired the interaction between TLR4 and MyD88, thereby preventing NF- κ B p65 nuclear translocation and downstream cytokine transcription. Besides its anti-inflammatory role, EPS-LM reduced ROS accumulation, revealing a concurrent redox-modulatory function.

3. Antioxidative and epithelial-barrier protection mechanisms

Further investigations in Caco-2 intestinal epithelial cells demonstrated that EPS-LM strongly protected against H₂O₂-induced oxidative injury and barrier dysfunction. It significantly reduced cellular ROS levels, restored the activities of key antioxidant enzymes (SOD, CAT, GSH-Px), and normalized NRF2-NQO1 pathway activation, collectively re-establishing intracellular redox balance. Functionally, EPS-LM prevented the decline in TEER, reduced paracellular permeability, and restored the expression and junctional localization of tight junction proteins (Claudin-1, Occludin, and ZO-1). These effects demonstrate that EPS-LM maintains epithelial integrity by coupling antioxidative defense with structural reinforcement of junctional complexes. Mechanistically, the results substantiate a redox-dependent protective model in which

EPS-LM mediates ROS suppression and antioxidant enzyme recovery, subsequently stabilizing cytoskeletal and tight junction networks to preserve barrier function.

4. Functionalization and delivery enhancement with quercetin

The PSP fraction ultrasonically extracted from the Cs-HK1 mycelial fermentation broth, which contained both extracellular and intracellular polysaccharide-protein complexes, was further exploited as a natural, food-grade carrier for quercetin via a solvent-free, pH-induced co-assembly process. The resulting PSP-quercetin complexes displayed high encapsulation efficiency (> 97%), improved particle homogeneity and colloidal stability, and strong non-covalent binding interactions through hydrogen bonding and hydrophobic association. After complexation with PSP, quercetin exhibited markedly enhanced antioxidant and anti-inflammatory activities, and *in vitro* gastrointestinal simulations showed that the complexes remained stable under acidic gastric conditions but disassembled efficiently in the intestinal phase, resulting in a five-fold improvement in bioaccessibility compared with free quercetin. These findings confirm that PSPs from *Cordyceps sinensis* (Cs-HK1) provide a versatile and biocompatible matrix for stabilizing hydrophobic nutraceuticals while enhancing their release behavior and bioavailability.

In this thesis, two distinct polysaccharide-protein complexes derived from the Cs-HK1 culture were investigated, namely EPS-LM and US-PSP. EPS-LM is an extracellular polysaccharide-protein fraction obtained from the fermentation broth by two-step

ethanol precipitation, whereas US-PSP is an intracellular/mycelial fraction released by ultrasonication of the Cs-HK1.

EPS-LM tends to have a higher average molecular weight and a higher polysaccharide content, while US-PSP is relatively enriched in protein. These differences may underlie their distinct physicochemical behaviors and suitability for different applications.

Functionally, EPS-LM primarily acts as an anti-inflammatory agent that activates macrophages via the TLR4/MyD88-NF- κ B pathway and enhances the production of inflammatory mediators. In contrast, US-PSP was exploited as a structural component for constructing a drug delivery system, where its amphiphilic polysaccharide-protein nature and favorable solubility profile facilitated nanoparticle formation and drug loading. Thus, while both are Cs-HK1-derived PSPs, EPS-LM and US-PSP represent complementary functional entities: one serving mainly as a bioactive anti-inflammatory, and the other as a biomaterial platform for therapeutic delivery.

7.2 Future studies

Future studies on *Cordyceps sinensis* (Cs-HK1) polysaccharide-protein complexes (PSPs) should focus on expanding fundamental knowledge, verifying biological efficacy under physiological conditions, and promoting industrial application.

1. *In vivo* efficacy and safety evaluation

Comprehensive animal and clinical studies are needed to verify the anti-inflammatory, antioxidant, and barrier-protective effects of PSPs within whole-body systems. Pharmacokinetic and metabolic analyses should clarify their absorption, distribution, and biotransformation *in vivo*, while systematic safety assessments, including toxicity, allergenicity, and long-term health effects, are essential to ensure consumer and regulatory confidence.

2. Gut health and host-microbiota interaction

Future research should explore how PSPs modulate gut microbiota, strengthen mucosal barriers, and influence host immune and metabolic homeostasis. Elucidating the links between microbial metabolites, intestinal signaling pathways, and overall physiological outcomes will help clarify how PSPs contribute to gut-systemic health regulation.

3. Structure-function correlation and multifunctional delivery development

More advanced analytical and computational tools, such as multidimensional spectroscopy, molecular simulation, and imaging techniques, should be employed to

reveal the relationship between PSP molecular architecture and biological activity. These insights can guide the rational design of PSP-based carrier systems for delivering various nutraceutical and therapeutic compounds with improved stability and bioavailability.

4. Industrial scale-up and functional-food innovation

Large-scale fermentation, green extraction, and purification processes require optimization to guarantee yield, quality, and cost-effectiveness. Applied research should focus on developing PSP-based foods, beverages, and nutraceutical formulations that combine safety, sensory quality, and storage stability, ultimately establishing PSPs as reliable natural ingredients for health-oriented industries.

Reference

- Abbas, Z., Tong, Y. C., Zhang, J., Wang, J. Y., Guo, H. N., Cheng, Q., Marhaba, Zhou, Y. C., Ahmad, B., Wei, X. B., Si, D. Y., & Zhang, R. J. (2024). Enhancing the antioxidant and anti-inflammatory potentials of mulberry-derived postbiotics through submerged fermentation with *B. subtilis* H4 and *B. amyloliquefaciens* LFB112. *Food Bioscience*, 60, Article 104252. <https://doi.org/10.1016/j.fbio.2024.104252>
- Adil, M. S., Narayanan, S. P., & Somanath, P. R. (2021). Cell-cell junctions: structure and regulation in physiology and pathology. *Tissue Barriers*, 9(1), 1848212. <https://doi.org/10.1080/21688370.2020.1848212>
- Aggarwal, R., & Bains, K. (2022). Protein, lysine and vitamin D: critical role in muscle and bone health. *Crit Rev Food Sci Nutr*, 62(9), 2548-2559. <https://doi.org/10.1080/10408398.2020.1855101>
- Ahmad, K., Meng, Y., Fan, C., Din, A. S. U., Jia, Q., Ashraf, A., Zhang, Y., & Hou, H. (2024). Collagen/gelatin and polysaccharide complexes enhance gastric retention and mucoadhesive properties. *International Journal of Biological Macromolecules*, 266, 131034. <https://doi.org/https://doi.org/10.1016/j.ijbiomac.2024.131034>
- Ahmed, A. U. (2011). An overview of inflammation: mechanism and consequences. *Frontiers in Biology*, 6(4), 274-281.
- Ahmed, T., Suzauddula, M., Akter, K., Hossen, M., & Islam, M. N. (2024a). Green technology for fungal protein extraction—A review. *Separations*, 11(6), 186.
- Ahmed, T., Suzauddula, M., Akter, K., Hossen, M., & Islam, M. N. (2024b). Green Technology for Fungal Protein Extraction—A Review. *Separations*, 11(6). <https://doi.org/10.3390/separations11060186>
- Akira, S., & Takeda, K. (2004). Toll-like receptor signalling. *Nature Reviews Immunology*, 4(7), 499-511. <https://doi.org/10.1038/nri1391>
- Al-Sadi, R., Ye, D., Dokladny, K., & Ma, T. Y. (2008). Mechanism of IL-1 β -induced increase in intestinal epithelial tight junction permeability. *The Journal of Immunology*, 180(8), 5653-5661.
- Alia, M., Ramos, S., Mateos, R., Bravo, L., & Goya, L. (2005). Response of the antioxidant defense system to tert-butyl hydroperoxide and hydrogen peroxide in a human hepatoma cell line (HepG2). *J Biochem Mol Toxicol*, 19(2), 119-128. <https://doi.org/10.1002/jbt.20061>

- Altanam, S. Y., Darwish, N., & Bakillah, A. (2025). Exploring the Interplay of Antioxidants, Inflammation, and Oxidative Stress: Mechanisms, Therapeutic Potential, and Clinical Implications. *Diseases*, 13(9), 309.
- Alvarez, R., & Enriquez, A. (1988). Nucleic acid reduction in yeast. *Applied microbiology and biotechnology*, 29(2), 208-210.
- Anderson, R., Dalziel, J., Gopal, P., Bassett, S., Ellis, A., & Roy, N. (2012). The role of intestinal barrier function in early life in the development of colitis. In *Colitis*. Intechopen.
- Angelow, S., Ahlstrom, R., & Yu, A. S. (2008). Biology of claudins. *Am J Physiol Renal Physiol*, 295(4), F867-876. <https://doi.org/10.1152/ajprenal.90264.2008>
- Auer, M., Moore, K. J., Meyer-Almes, F. J., Guenther, R., Pope, A. J., & Stoeckli, K. A. (1998). Fluorescence correlation spectroscopy: lead discovery by miniaturized HTS. *Drug Discovery Today*, 3(10), 457-465. [https://doi.org/10.1016/S1359-6446\(98\)01240-9](https://doi.org/10.1016/S1359-6446(98)01240-9)
- Augustine, C. A., Panoth, D., & Paravannoor, A. (2019). High Performance Li-ion battery Anodes based on Si Nano core in an LATP Matrix with better Electrolyte Compatibility and Temperature Resistance. *Chemistryselect*, 4(24), 7090-7095. <https://doi.org/10.1002/slct.201901208>
- Azeem, M., Hanif, M., Mahmood, K., Ameer, N., Chughtai, F. R. S., & Abid, U. (2023). An insight into anticancer, antioxidant, antimicrobial, antidiabetic and anti-inflammatory effects of quercetin: a review. *Polym Bull (Berl)*, 80(1), 241-262. <https://doi.org/10.1007/s00289-022-04091-8>
- Balda, M. S., & Matter, K. (2008). Tight junctions at a glance. *J Cell Sci*, 121(Pt 22), 3677-3682. <https://doi.org/10.1242/jcs.023887>
- Barbosa, J. R., Freitas, M. M. S., Oliveira, L. C., Martins, L. H. S., Almada-Vilhena, A. O., Oliveira, R. M., Pieczarka, J. C., Brasil, D. d. S. B., & Junior, R. N. C. (2020). Obtaining extracts rich in antioxidant polysaccharides from the edible mushroom *Pleurotus ostreatus* using binary system with hot water and supercritical CO₂. *Food Chemistry*, 330, 127173.
- Barltrop, J. A., Owen, T. C., Cory, A. H., & Cory, J. G. (1991). 5-(3-CARBOXYMETHOXYPHENYL)-2-(4,5-DIMETHYLTHIAZOLYL)-3-(4-SULFOPHENYL)TE TRAZOLIUM, INNER SALT (MTS) AND RELATED ANALOGS OF 3-(4,5-DIMETHYLTHIAZOLYL)-2,5-DIPHENYLTETRAZOLIUM BROMIDE (MTT) REDUCING TO PURPLE WATER-SOLUBLE FORMAZANS AS CELL-VIABILITY INDICATORS. *Bioorganic & Medicinal Chemistry Letters*, 1(11), 611-&. [https://doi.org/10.1016/s0960-894x\(01\)81162-8](https://doi.org/10.1016/s0960-894x(01)81162-8)

- Barnes, P. J. (2006). How corticosteroids control inflammation: Quintiles prize lecture 2005. *British Journal of Pharmacology*, 148(3), 245-254.
<https://doi.org/10.1038/sj.bjp.0706736>
- Barnes, P. J., & Larin, M. (1997). Mechanisms of disease - Nuclear factor-kappa b - A pivotal transcription factor in chronic inflammatory diseases. *New England Journal of Medicine*, 336(15), 1066-1071.
<https://doi.org/10.1056/nejm199704103361506>
- Batbayar, S., Lee, D. H., & Kim, H. W. (2012). Immunomodulation of Fungal β -Glucan in Host Defense Signaling by Dectin-1. *Biomol Ther (Seoul)*, 20(5), 433-445.
<https://doi.org/10.4062/biomolther.2012.20.5.433>
- Bedard, K., & Krause, K.-H. (2007). The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. *Physiological reviews*, 87(1), 245-313.
- Birben, E., Sahiner, U. M., Sackesen, C., Erzurum, S., & Kalayci, O. (2012). Oxidative stress and antioxidant defense. *World allergy organization journal*, 5, 9-19.
- Bischoff, S. C., Barbara, G., Buurman, W., Ockhuizen, T., Schulzke, J.-D., Serino, M., Tilg, H., Watson, A., & Wells, J. M. (2014). Intestinal permeability—a new target for disease prevention and therapy. *BMC gastroenterology*, 14, 1-25.
- Błaszczak, K., Wilczak, J., Harasym, J., Gudej, S., Suchecka, D., Królikowski, T., Lange, E., & Gromadzka-Ostrowska, J. (2015). Impact of low and high molecular weight oat beta-glucan on oxidative stress and antioxidant defense in spleen of rats with LPS induced enteritis. *Food Hydrocolloids*, 51, 272-280.
<https://doi.org/10.1016/j.foodhyd.2015.05.025>
- Brader, M. L. (2020). Chapter 5 - UV-absorbance, fluorescence and FT-IR spectroscopy in biopharmaceutical development. In D. J. Houde & S. A. Berkowitz (Eds.), *Biophysical Characterization of Proteins in Developing Biopharmaceuticals (Second Edition)* (pp. 97-121). Elsevier.
<https://doi.org/https://doi.org/10.1016/B978-0-444-64173-1.00005-6>
- Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, 72(1-2), 248-254.
- Braunstein, I., Motohashi, H., Dallenga, T., Schaible, U. E., & Benhar, M. (2025). Redox signaling in innate immunity and inflammation: focus on macrophages and neutrophils. *Free Radical Biology and Medicine*, 237, 427-454.
<https://doi.org/https://doi.org/10.1016/j.freeradbiomed.2025.06.006>
- Brigelius-Flohé, R., & Davies, K. J. A. (2007). Is vitamin E an antioxidant, a regulator of signal transduction and gene expression, or a 'junk' food?: Comments on the

- two accompanying papers:: "Molecular mechanism of α -tocopherol action" by A. Azzi and "Vitamin E, antioxidant and nothing more" by M. Traber and J. Atkinson. *Free Radical Biology and Medicine*, 43(1), 2-3. <https://doi.org/10.1016/j.freeradbiomed.2007.05.016>
- Brown, G. D., & Gordon, S. (2003). Fungal β -glucans and mammalian immunity. *Immunity*, 19(3), 311-315. [https://doi.org/10.1016/s1074-7613\(03\)00233-4](https://doi.org/10.1016/s1074-7613(03)00233-4)
- Brunner, J., Ragupathy, S., & Borchard, G. (2021). Target specific tight junction modulators. *Adv Drug Deliv Rev*, 171, 266-288. <https://doi.org/10.1016/j.addr.2021.02.008>
- Bubb, W. A. (2003). NMR spectroscopy in the study of carbohydrates: Characterizing the structural complexity. *Concepts in Magnetic Resonance Part A*, 19A(1), 1-19. <https://doi.org/10.1002/cmr.a.10080>
- Burckhardt, C. J., Minna, J. D., & Danuser, G. (2021). Co-immunoprecipitation and semi-quantitative immunoblotting for the analysis of protein-protein interactions. *Star Protocols*, 2(3), Article 100644. <https://doi.org/10.1016/j.xpro.2021.100644>
- c Ooi, V. E., & Liu, F. (2000). Immunomodulation and anti-cancer activity of polysaccharide-protein complexes. *Current medicinal chemistry*, 7(7), 715-729.
- Camilleri, M., Lasch, K., & Zhou, W. (2012). Irritable bowel syndrome: methods, mechanisms, and pathophysiology. The confluence of increased permeability, inflammation, and pain in irritable bowel syndrome. *Am J Physiol Gastrointest Liver Physiol*, 303(7), G775-785. <https://doi.org/10.1152/ajpgi.00155.2012>
- Chen, C., Zhong, Q., & Chen, Z. (2022). Improved aqueous solubility, bioaccessibility and cellular uptake of quercetin following pH-driven encapsulation in whey protein isolate. *International Journal of Food Science & Technology*, 57(5), 2747-2755. <https://doi.org/10.1111/ijfs.15579>
- Chen, L., Lu, X., Liang, X., Hong, D., Guan, Z., Guan, Y., & Zhu, W. (2016). Mechanistic studies of the transport of peimine in the Caco-2 cell model. *Acta Pharm Sin B*, 6(2), 125-131. <https://doi.org/10.1016/j.apsb.2016.01.006>
- Chen, P. X., Wang, S., Nie, S., & Marcone, M. (2013a). Properties of Cordyceps Sinensis: A review. *J Funct Foods*, 5(2), 550-569. <https://doi.org/10.1016/j.jff.2013.01.034>
- Chen, P. X., Wang, S. A., Nie, S. P., & Marcone, M. (2013b). Properties of Cordyceps Sinensis: A review. *Journal of Functional Foods*, 5(2), 550-569. <https://doi.org/10.1016/j.jff.2013.01.034>

- Chen, R., Xu, J., Wu, W., Wen, Y., Lu, S., El-Seedi, H. R., & Zhao, C. (2022). Structure-immunomodulatory activity relationships of dietary polysaccharides. *Curr Res Food Sci*, 5, 1330-1341. <https://doi.org/10.1016/j.crfs.2022.08.016>
- Chen, Y., Miao, J., & Lou, G. (2021). Knockdown of circ-FURIN suppresses the proliferation and induces apoptosis of granular cells in polycystic ovary syndrome via miR-195-5p/BCL2 axis. *J Ovarian Res*, 14(1), 156. <https://doi.org/10.1186/s13048-021-00891-0>
- Chen, Y., Yao, M., Yang, T., Fang, Y., Xiang, D., & Zhang, W. (2023). Changes in structure and emulsifying properties of coconut globulin after the atmospheric pressure cold plasma treatment. *Food Hydrocolloids*, 136. <https://doi.org/10.1016/j.foodhyd.2022.108289>
- Cheng, J., Dudu, O. E., Zhang, J., Wang, Y., Meng, L., Wei, W., Li, X., & Yan, T. (2023). Impact of binding interaction modes between whey protein concentrate and quercetin on protein structural and functional characteristics. *Food Hydrocolloids*, 142. <https://doi.org/10.1016/j.foodhyd.2023.108787>
- Cheong, K. L., Wang, L. Y., Wu, D. T., Hu, D. J., Zhao, J., & Li, S. P. (2016). Microwave-Assisted Extraction, Chemical Structures, and Chain Conformation of Polysaccharides from a Novel Cordyceps Sinensis Fungus UM01. *J Food Sci*, 81(9), C2167-2174. <https://doi.org/10.1111/1750-3841.13407>
- Cheung, Y. C., Liu, X. X., Wang, W. Q., & Wu, J. Y. (2015). Ultrasonic disruption of fungal mycelia for efficient recovery of polysaccharide-protein complexes from viscous fermentation broth of a medicinal fungus. *Ultrason Sonochem*, 22, 243-248. <https://doi.org/10.1016/j.ultsonch.2014.05.006>
- Choochan, M. A., Jabeen, R., & Bibi, N. (2022). Extraction and quantification of antimicrobial peptides from medicinal plants through TrisNaCl and PBS buffer. *International Journal of Natural Medicine and Health Sciences*, 1(3).
- Chou, C.-H., Sung, T.-J., Hu, Y.-N., Lu, H.-Y., Yang, L.-C., Cheng, K.-C., Lai, P.-S., & Hsieh, C.-W. (2019). Chemical analysis, moisture-preserving, and antioxidant activities of polysaccharides from *Pholiota nameko* by fractional precipitation. *International Journal of Biological Macromolecules*, 131, 1021-1031.
- Contato, A. G., & Conte-Junior, C. A. (2025). Lion's Mane Mushroom (*Herichium erinaceus*): A Neuroprotective Fungus with Antioxidant, Anti-Inflammatory, and Antimicrobial Potential—A Narrative Review. *Nutrients*, 17(8), 1307.
- Cor, D., Knez, Z., & Knez Hrnčić, M. (2018). Antitumour, Antimicrobial, Antioxidant and Antiacetylcholinesterase Effect of *Ganoderma lucidum* Terpenoids and Polysaccharides: A Review. *Molecules*, 23(3). <https://doi.org/10.3390/molecules23030649>

- Dash, U. C., Nayak, V., Navani, H. S., Samal, R. R., Agrawal, P., Singh, A. K., Majhi, S., Mogare, D. G., Duttaroy, A. K., & Jena, A. B. (2025). Understanding the molecular bridges between the drugs and immune cell. *Pharmacology & Therapeutics*, 267, 108805. <https://doi.org/https://doi.org/10.1016/j.pharmthera.2025.108805>
- 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. *Phytochem Anal*, 27(5), 304-314. <https://doi.org/10.1002/pca.2632>
- Deneke, S. M. (2001). Thiol-based antioxidants. *Current topics in cellular regulation*, 36, 151-180.
- Dhara, A. K., & Nayak, A. K. (2022). Biological macromolecules: sources, properties, and functions. In *Biological macromolecules* (pp. 3-22). Elsevier.
- Dinarello, C. A. (2000). Proinflammatory cytokines. *Chest*, 118(2), 503-508.
- Ding, R., Zhang, M., Zhu, Q., Qu, Y., Jia, X., & Yin, L. (2023). Curcumin loaded Zein-alginate nanogels with "core-shell" structure: formation, characterization and simulated digestion. *Int J Biol Macromol*, 251, 126201. <https://doi.org/10.1016/j.ijbiomac.2023.126201>
- Dönmez, Ö., Mogol, B. A., Gökmen, V., Tang, N., Andersen, M. L., & Chatterton, D. E. (2020). Modulation of gastrointestinal digestion of β -lactoglobulin and micellar casein following binding by (-)-epigallocatechin-3-gallate (EGCG) and green tea flavanols. *Food & function*, 11(7), 6038-6053.
- DuBois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*, 28(3), 350-356.
- Dunleavy, K. A., Raffals, L. E., & Camilleri, M. (2023). Intestinal Barrier Dysfunction in Inflammatory Bowel Disease: Underpinning Pathogenesis and Therapeutics. *Dig Dis Sci*, 68(12), 4306-4320. <https://doi.org/10.1007/s10620-023-08122-w>
- Eghbal, N., & Choudhary, R. (2018). Complex coacervation: Encapsulation and controlled release of active agents in food systems. *Lwt*, 90, 254-264.
- Erjavec, J., Kos, J., Ravnikar, M., Dreo, T., & Sabotič, J. (2012). Proteins of higher fungi—from forest to application. *Trends in biotechnology*, 30(5), 259-273.
- Fontes-Candia, C., Díaz-Piñero, L., Vega-Gómez, L. M., Molina-Gilarranz, I., & Martínez-Sanz, M. (2025). Relevance of Protein-Polysaccharide Interactions on Nutritional Quality and Gastrointestinal Digestion of Protein-Based Foods. *J Agric Food Chem*, 73(9), 4998-5004. <https://doi.org/10.1021/acs.jafc.4c11949>

- Fujii, J., Homma, T., & Osaki, T. (2022). Superoxide Radicals in the Execution of Cell Death. *Antioxidants (Basel)*, 11(3). <https://doi.org/10.3390/antiox11030501>
- Galermo, A. G., Nandita, E., Barboza, M., Amicucci, M. J., Vo, T.-T. T., & Lebrilla, C. B. (2018). Liquid chromatography–tandem mass spectrometry approach for determining glycosidic linkages. *Analytical Chemistry*, 90(21), 13073-13080.
- Glorani, G. (2019). Structural studies of a Tremella fuciformis mushroom lectin: a novel protein with antitumoral properties.
- Greenfield, N. J. (2006). Using circular dichroism spectra to estimate protein secondary structure. *Nature Protocols*, 1(6), 2876-2890. <https://doi.org/10.1038/nprot.2006.202>
- Guo, C., Guo, D., Fang, L., Sang, T., Wu, J., Guo, C., Wang, Y., Wang, Y., Chen, C., Chen, J., Chen, R., & Wang, X. (2021). Ganoderma lucidum polysaccharide modulates gut microbiota and immune cell function to inhibit inflammation and tumorigenesis in colon. *Carbohydr Polym*, 267, 118231. <https://doi.org/10.1016/j.carbpol.2021.118231>
- Guo, Q., Liang, S., Xiao, Z., & Ge, C. (2023). Research progress on extraction technology and biological activity of polysaccharides from Edible Fungi: A review. *Food Reviews International*, 39(8), 4909-4940.
- Gupta, M. N., & Roy, I. (2021). *Three phase partitioning: applications in separation and purification of biological molecules and natural products*. Elsevier.
- Hao, X., Yang, W., Zhu, Q., Zhang, G., Zhang, X., Liu, L., Li, X., Hussain, M., Ni, C., & Jiang, X. (2021). Proteolysis and ACE-inhibitory peptide profile of Cheddar cheese: Effect of digestion treatment and different probiotics. *Lwt*, 145. <https://doi.org/10.1016/j.lwt.2021.111295>
- Harvanová, G., & Duranková, S. (2025). Inflammatory process: Factors inducing inflammation, forms and manifestations of inflammation, immunological significance of the inflammatory reaction. *Alergologia Polska-Polish Journal of Allergology*, 12(1), 54-61.
- Hayden, M. S., & Ghosh, S. (2011). NF- κ B in immunobiology. *Cell Research*, 21(2), 223-244. <https://doi.org/10.1038/cr.2011.13>
- He, F., Lu, H., Chen, X., Zhao, J., Ou, X., & Xie, J. (2025). Mesona chinensis Benth polysaccharide improves intestinal barrier function in dextran sodium sulfate-induced human Caco-2 cells and its molecular mechanisms. *Food Science and Human Wellness*.
- Heck, D. E., Shakarjian, M., Kim, H. D., Laskin, J. D., & Vetrano, A. M. (2010). Mechanisms of oxidant generation by catalase. *Ann N Y Acad Sci*, 1203, 120-125. <https://doi.org/10.1111/j.1749-6632.2010.05603.x>

- Hobbs, C. (2023). The health and clinical benefits of medicinal fungi. In *Biochemical engineering and biotechnology of medicinal mushrooms* (pp. 285-356). Springer.
- Hu, Y., Kou, G., Chen, Q., Li, Y., & Zhou, Z. (2019). Protection and delivery of mandarin (*Citrus reticulata* Blanco) peel extracts by encapsulation of whey protein concentrate nanoparticles. *Lwt*, 99, 24-33. <https://doi.org/10.1016/j.lwt.2018.09.044>
- Huang, M., Wang, Y., Ahmad, M., Ying, R., Wang, Y., & Tan, C. (2021). Fabrication of pickering high internal phase emulsions stabilized by pecan protein/xanthan gum for enhanced stability and bioaccessibility of quercetin. *Food Chem*, 357, 129732. <https://doi.org/10.1016/j.foodchem.2021.129732>
- Huang, Q.-L., Siu, K.-C., Wang, W.-Q., Cheung, Y.-C., & Wu, J.-Y. (2013a). Fractionation, characterization and antioxidant activity of exopolysaccharides from fermentation broth of a *Cordyceps sinensis* fungus. *Process Biochem.*, 48(2), 380-386. <https://doi.org/10.1016/j.procbio.2013.01.001>
- Huang, Q.-L., Siu, K.-C., Wang, W.-Q., Cheung, Y.-C., & Wu, J.-Y. (2013b). Fractionation, characterization and antioxidant activity of exopolysaccharides from fermentation broth of a *Cordyceps sinensis* fungus. *Process Biochemistry*, 48(2), 380-386. <https://doi.org/https://doi.org/10.1016/j.procbio.2013.01.001>
- Huang, Y., Guo, H., Liu, Y., Jin, W., Palanisamy, C. P., Pei, J., Oz, F., & Abd El-Aty, A. M. (2025). Effects of Natural Polysaccharides on the Gut Microbiota Related to Human Metabolic Health. *Mol Nutr Food Res*, 69(11), e202400792. <https://doi.org/10.1002/mnfr.202400792>
- Huo, J., Wu, Z., Sun, W., Wang, Z., Wu, J., Huang, M., Wang, B., & Sun, B. (2022). Protective Effects of Natural Polysaccharides on Intestinal Barrier Injury: A Review. *J Agric Food Chem*, 70(3), 711-735. <https://doi.org/10.1021/acs.jafc.1c05966>
- Ji, W., Yang, F., & Yang, M. (2022). Effect of change in pH, heat and ultrasound pre-treatments on binding interactions between quercetin and whey protein concentrate. *Food Chem*, 384, 132508. <https://doi.org/10.1016/j.foodchem.2022.132508>
- John, L. J., Fromm, M., & Schulzke, J.-D. (2011). Epithelial barriers in intestinal inflammation. *Antioxidants & redox signaling*, 15(5), 1255-1270.
- Jurasekova, Z., Torreggiani, A., Tamba, M., Sanchez-Cortes, S., & Garcia-Ramos, J. V. (2009). Raman and surface-enhanced Raman scattering (SERS) investigation of the quercetin interaction with metals: Evidence of structural changing processes in aqueous solution and on metal nanoparticles. *Journal of Molecular Structure*, 918(1-3), 129-137. <https://doi.org/10.1016/j.molstruc.2008.07.025>

- Kabir, S. F., Rahman, A., Yeasmin, F., Sultana, S., Masud, R. A., Kanak, N. A., & Haque, P. (2022a). Chapter One - Occurrence, distribution, and structure of natural polysaccharides. In M. Naeem, T. Aftab, & M. M. A. Khan (Eds.), *Radiation-Processed Polysaccharides* (pp. 1-27). Academic Press. <https://doi.org/https://doi.org/10.1016/B978-0-323-85672-0.00005-2>
- Kabir, S. F., Rahman, A., Yeasmin, F., Sultana, S., Masud, R. A., Kanak, N. A., & Haque, P. (2022b). Occurrence, distribution, and structure of natural polysaccharides. In *Radiation-Processed Polysaccharides* (pp. 1-27). <https://doi.org/10.1016/b978-0-323-85672-0.00005-2>
- Kandemir, K., Tomas, M., McClements, D. J., & Capanoglu, E. (2022). Recent advances on the improvement of quercetin bioavailability. *Trends in Food Science & Technology*, 119, 192-200. <https://doi.org/10.1016/j.tifs.2021.11.032>
- Kawai, T., & Akira, S. (2010). The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat Immunol*, 11(5), 373-384. <https://doi.org/10.1038/ni.1863>
- Kensler, T. W., & Wakabayashi, N. (2010). Nrf2: friend or foe for chemoprevention? *Carcinogenesis*, 31(1), 90-99.
- Khan, M. S., Zhang, X., You, L., Fu, X., & Abbasi, A. M. (2015). Structure and bioactivities of fungal polysaccharides. *Polysaccharides*, 1851-1866.
- Kim, B., & Breton, S. (2016). The MAPK/ERK-Signaling Pathway Regulates the Expression and Distribution of Tight Junction Proteins in the Mouse Proximal Epididymis. *Biol Reprod*, 94(1), 22. <https://doi.org/10.1095/biolreprod.115.134965>
- Kleinbongard, P., Rassaf, T., Dejam, A., Kerber, S., & Kelm, M. (2002). Griess method for nitrite measurement of aqueous and protein-containing samples. *Nitric Oxide, Pt D*, 359, 158-168.
- Kobayashi, A., Kang, M.-I., Okawa, H., Ohtsuji, M., Zenke, Y., Chiba, T., Igarashi, K., & Yamamoto, M. (2004). Oxidative stress sensor Keap1 functions as an adaptor for Cul3-based E3 ligase to regulate proteasomal degradation of Nrf2. *Molecular and cellular biology*, 24(16), 7130-7139.
- Kristo, E., Hazizaj, A., & Corredig, M. (2012). Structural changes imposed on whey proteins by UV irradiation in a continuous UV light reactor. *J Agric Food Chem*, 60(24), 6204-6209. <https://doi.org/10.1021/jf300278k>
- Kuo, W. T., Odenwald, M. A., Turner, J. R., & Zuo, L. (2022). Tight junction proteins occludin and ZO-1 as regulators of epithelial proliferation and survival. *Annals of the New York Academy of Sciences*, 1514(1), 21-33.

- Laemmli, U. K. (1970). Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. *Nature*, 227(5259), 680-685. <https://doi.org/10.1038/227680a0>
- Lawson, M. K. (2023). Improvement of therapeutic value of quercetin with chitosan nanoparticle delivery systems and potential applications. *International Journal of Molecular Sciences*, 24(4), 3293.
- Lee, H. J., Choi, C., & Lee, S. J. (2002). Membrane-bound alpha-synuclein has a high aggregation propensity and the ability to seed the aggregation of the cytosolic form. *J Biol Chem*, 277(1), 671-678. <https://doi.org/10.1074/jbc.M107045200>
- Leung, P., Zhang, Q., & Wu, J. (2006). Mycelium cultivation, chemical composition and antitumour activity of a *Tolypocladium* sp. fungus isolated from wild *Cordyceps sinensis*. *Journal of applied microbiology*, 101(2), 275-283.
- Leung, P. H., & Wu, J. Y. (2007). Effects of ammonium feeding on the production of bioactive metabolites (cordycepin and exopolysaccharides) in mycelial culture of a *Cordyceps sinensis* fungus. *J Appl Microbiol*, 103(5), 1942-1949. <https://doi.org/10.1111/j.1365-2672.2007.03451.x>
- 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, C.-H., Zuo, H.-L., Chen, C., Hu, Y.-J., Qian, Z.-M., Li, W.-J., Xia, Z.-N., & Yang, F.-Q. (2018). SDS-PAGE and 2-DE protein profiles of *Ganoderma lucidum* from different origins. *Pakistan journal of pharmaceutical sciences*, 31(2).
- Li, C.-H., Zuo, H.-L., Zhang, Q., Wang, F.-Q., Hu, Y.-J., Qian, Z.-M., Li, W.-J., Xia, Z.-N., & Yang, F.-Q. (2017). Analysis of soluble proteins in natural *Cordyceps sinensis* from different producing areas by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and two-dimensional electrophoresis. *Pharmacognosy research*, 9(1), 34.
- Li, H., Wang, D., Liu, C., Zhu, J., Fan, M., Sun, X., Wang, T., Xu, Y., & Cao, Y. (2019). Fabrication of stable zein nanoparticles coated with soluble soybean polysaccharide for encapsulation of quercetin. *Food Hydrocolloids*, 87, 342-351. <https://doi.org/10.1016/j.foodhyd.2018.08.002>
- 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.

- Li, J. H., Gu, F. T., Yang, Y., Zhao, Z. C., Huang, L. X., Zhu, Y. Y., Chen, S., & 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. *Carbohydr Polym*, 343, 122478. <https://doi.org/10.1016/j.carbpol.2024.122478>
- Li, J. H., Gu, F. T., Yang, Y., Zhao, Z. C., Huang, L. X., Zhu, Y. Y., Chen, S., & 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. <https://doi.org/10.1016/j.carbpol.2024.122478>
- Li, L. F., Zhang, Q. W., & Han, Q. B. (2022). Recent advances in qualitative and quantitative analysis of polysaccharides in natural medicines: A critical review. *J Pharm Biomed Anal*, 220, 115016. <https://doi.org/10.1016/j.jpba.2022.115016>
- Li, L. Q., Song, A. X., Wong, W. T., & Wu, J. Y. (2021a). Isolation and Assessment of a Highly-Active Anti-Inflammatory Exopolysaccharide from Mycelial Fermentation of a Medicinal Fungus Cs-HK1. *Int J Mol Sci*, 22(5). <https://doi.org/10.3390/ijms22052450>
- Li, L. Q., Song, A. X., Wong, W. T., & Wu, J. Y. (2021b). Modification and enhanced anti-inflammatory activity by Bifidobacterial fermentation of an exopolysaccharide from a medicinal fungus Cs-HK1. *Int J Biol Macromol*, 188, 586-594. <https://doi.org/10.1016/j.ijbiomac.2021.08.084>
- 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. *Int J Biol Macromol*, 149, 1042-1050. <https://doi.org/10.1016/j.ijbiomac.2020.02.022>
- Li, M., Huang, X., Wen, J., Chen, S., Wu, X., Ma, W., Cui, S. W., Xie, M., & Nie, S. (2023). Innate immune receptors co-recognition of polysaccharides initiates multi-pathway synergistic immune response. *Carbohydrate Polymers*, 305, 120533.
- Li, Q.-Z., Zheng, Y.-Z., & Zhou, X.-W. (2019). Fungal immunomodulatory proteins: characteristic, potential antitumor activities and their molecular mechanisms. *Drug Discovery Today*, 24(1), 307-314.
- Liang, J., Yan, H., Puligundla, P., Gao, X., Zhou, Y., & Wan, X. (2017). Applications of chitosan nanoparticles to enhance absorption and bioavailability of tea polyphenols: A review. *Food Hydrocolloids*, 69, 286-292.
- Liu, Q., Chen, A., Hong, P., Zhou, C., Li, X., & Xie, M. (2024). pH-induced interface protein structure changes to adjust the stability of tilapia protein isolate

- emulsion prepared by high-pressure homogenization. *Food Chem X*, 24, 101841. <https://doi.org/10.1016/j.fochx.2024.101841>
- Liu, Q., Cheng, J., Sun, X., & Guo, M. (2021). Preparation, characterization, and antioxidant activity of zein nanoparticles stabilized by whey protein nanofibrils. *Int J Biol Macromol*, 167, 862-870. <https://doi.org/10.1016/j.ijbiomac.2020.11.043>
- Liu, Q., Liu, P., & Ban, Q. (2025). Green development strategy for efficient quercetin-loaded whey protein complex: Focus on quercetin loading characteristics, component interactions, stability, antioxidant, and in vitro digestive properties. *Food Chem*, 472, 142939. <https://doi.org/10.1016/j.foodchem.2025.142939>
- Liu, Q., Sun, Y., Cheng, J., & Guo, M. (2022). Development of whey protein nanoparticles as carriers to deliver soy isoflavones. *Lwt*, 155. <https://doi.org/10.1016/j.lwt.2021.112953>
- Liu, Q., Sun, Y., Cui, Q., Cheng, J., Killpartrik, A., Kemp, A. H., & Guo, M. (2022). Characterization, antioxidant capacity, and bioaccessibility of Coenzyme Q10 loaded whey protein nanoparticles. *Lwt*, 160. <https://doi.org/10.1016/j.lwt.2022.113258>
- Liu, Q., Sun, Y., Zhang, J., Zhang, M., Cheng, J., & Guo, M. (2022). Physicochemical and in vitro digestion properties of soy isoflavones loaded whey protein nanoparticles using a pH-driven method. *Innovative Food Science & Emerging Technologies*, 82. <https://doi.org/10.1016/j.ifset.2022.103209>
- Liu, T., Zhang, L. Y., Joo, D., & Sun, S. C. (2017). NF- κ B signaling in inflammation. *Signal Transduction and Targeted Therapy*, 2, Article e17023. <https://doi.org/10.1038/sigtrans.2017.23>
- Liu, X., Wang, L., Zhang, C., Wang, H., Zhang, X., & Li, Y. (2015). Structure characterization and antitumor activity of a polysaccharide from the alkaline extract of king oyster mushroom. *Carbohydrate Polymers*, 118, 101-106.
- 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.
- Lo, T. C.-T., Chang, C. A., Chiu, K.-H., Tsay, P.-K., & Jen, J.-F. (2011). Correlation evaluation of antioxidant properties on the monosaccharide components and glycosyl linkages of polysaccharide with different measuring methods. *Carbohydrate Polymers*, 86(1), 320-327. <https://doi.org/10.1016/j.carbpol.2011.04.056>
- Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *J Biol Chem*, 193(1), 265-275.

- Lu, Y. C., Yeh, W. C., & Ohashi, P. S. (2008). LPS/TLR4 signal transduction pathway. *Cytokine*, 42(2), 145-151. <https://doi.org/10.1016/j.cyto.2008.01.006>
- Lu, Z., Ding, L., Lu, Q., & Chen, Y. H. (2013). Claudins in intestines: Distribution and functional significance in health and diseases. *Tissue Barriers*, 1(3), e24978. <https://doi.org/10.4161/tisb.24978>
- Luo, A., Xie, M., Li, X., Zhou, C., & Liu, Q. (2024). Preparation, characterization, stability, and antioxidant of quercetin-loaded hyaluronic acid complexes via pH-induced co-assembly. *Food Bioscience*, 62. <https://doi.org/10.1016/j.fbio.2024.105399>
- Ma, Q. (2013). Role of nrf2 in oxidative stress and toxicity. *Annu Rev Pharmacol Toxicol*, 53, 401-426. <https://doi.org/10.1146/annurev-pharmtox-011112-140320>
- Madamanchi, N. R., & Runge, M. S. (2007). Mitochondrial dysfunction in atherosclerosis. *Circulation research*, 100(4), 460-473.
- Mao, X., Zeng, X., Qiao, S., Wu, G., & Li, D. (2011). Specific roles of threonine in intestinal mucosal integrity and barrier function. *Front Biosci*, 3(4), 1192-1200.
- Marino, M., Rendine, M., Venturi, S., Porrini, M., Gardana, C., Klimis-Zacas, D., Riso, P., & Del Bo, C. (2024). Red raspberry (*Rubus idaeus*) preserves intestinal barrier integrity and reduces oxidative stress in Caco-2 cells exposed to a proinflammatory stimulus. *Food Funct*, 15(13), 6943-6954. <https://doi.org/10.1039/d4fo01050g>
- Marullo, S., Petta, F., Infurna, G., Dintcheva, N. T., & D'Anna, F. (2023). Polysaccharide-based supramolecular bicomponent eutectogels as sustainable antioxidant materials. *Green Chemistry*, 25(11), 4513-4527.
- Masungi, C., Borremans, C., Willems, B., Mensch, J., Van Dijck, A., Augustijns, P., Brewster, M. E., & Noppe, M. (2004). Usefulness of a novel Caco-2 cell perfusion system. I. In vitro prediction of the absorption potential of passively diffused compounds. *J Pharm Sci*, 93(10), 2507-2521. <https://doi.org/10.1002/jps.20149>
- McClements, D. J. (2018). Delivery by design (DbD): A standardized approach to the development of efficacious nanoparticle-and microparticle-based delivery systems. *Comprehensive Reviews in Food Science and Food Safety*, 17(1), 200-219.
- Medzhitov, R. (2010). Inflammation 2010: new adventures of an old flame. *Cell*, 140(6), 771-776. <https://doi.org/10.1016/j.cell.2010.03.006>
- Miles, A. J., & Wallace, B. A. (2020). Biopharmaceutical applications of protein characterisation by circular dichroism spectroscopy. In *Biophysical*

- Characterization of Proteins in Developing Biopharmaceuticals* (pp. 123-152). <https://doi.org/10.1016/b978-0-444-64173-1.00006-8>
- Mittal, M., Siddiqui, M. R., Tran, K., Reddy, S. P., & Malik, A. B. (2014). Reactive Oxygen Species in Inflammation and Tissue Injury. *Antioxidants & redox signaling*, 20(7), 1126-1167. <https://doi.org/10.1089/ars.2012.5149>
- Modrzynski, J. J., Christensen, J. H., & Brandt, K. K. (2019). Evaluation of dimethyl sulfoxide (DMSO) as a co-solvent for toxicity testing of hydrophobic organic compounds. *Ecotoxicology*, 28(9), 1136-1141. <https://doi.org/10.1007/s10646-019-02107-0>
- Mohammed, A. S. A., Naveed, M., & Jost, N. (2021). Polysaccharides; Classification, Chemical Properties, and Future Perspective Applications in Fields of Pharmacology and Biological Medicine (A Review of Current Applications and Upcoming Potentialities). *Journal of Polymers and the Environment*, 29(8), 2359-2371. <https://doi.org/10.1007/s10924-021-02052-2>
- Moon, H., Lertpatipanpong, P., Hong, Y., Kim, C.-T., & Baek, S. J. (2021). Nano-encapsulated quercetin by soluble soybean polysaccharide/chitosan enhances anti-cancer, anti-inflammation, and anti-oxidant activities. *Journal of Functional Foods*, 87. <https://doi.org/10.1016/j.jff.2021.104756>
- Nathan, C., & Ding, A. (2010). Nonresolving inflammation. *Cell*, 140(6), 871-882. <https://doi.org/10.1016/j.cell.2010.02.029>
- Neurath, M. F., Artis, D., & Becker, C. (2025). The intestinal barrier: a pivotal role in health, inflammation, and cancer. *The Lancet Gastroenterology & Hepatology*, 10(6), 573-592. [https://doi.org/https://doi.org/10.1016/S2468-1253\(24\)00390-X](https://doi.org/https://doi.org/10.1016/S2468-1253(24)00390-X)
- Ngo, V., & Duennwald, M. L. (2022). Nrf2 and Oxidative Stress: A General Overview of Mechanisms and Implications in Human Disease. *Antioxidants*, 11(12). <https://doi.org/10.3390/antiox11122345>
- Nimgampalle, M., Chakravarthy, H., & Devanathan, V. (2021). Chapter 8 - Glucose metabolism in the brain: An update. In B. Viswanath (Ed.), *Recent Developments in Applied Microbiology and Biochemistry* (pp. 77-88). Academic Press. <https://doi.org/https://doi.org/10.1016/B978-0-12-821406-0.00008-4>
- Nord, L. I., Vaag, P., & Duus, J. Ø. (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>
- Oami, T., Shimazui, T., Yumoto, T., Otani, S., Hayashi, Y., & Coopersmith, C. M. (2025). Gut integrity in intensive care: alterations in host permeability and the

- microbiome as potential therapeutic targets. *J Intensive Care*, 13(1), 16.
<https://doi.org/10.1186/s40560-025-00786-y>
- Oh, J.-Y., Cho, E.-J., Nam, S.-H., Choi, J.-W., & Yun, J.-W. (2007). Production of polysaccharide–peptide complexes by submerged mycelial culture of an entomopathogenic fungus *Cordyceps sphecocephala*. *Process Biochemistry*, 42(3), 352-362. <https://doi.org/10.1016/j.procbio.2006.09.005>
- Opal, S. M., & DePalo, V. A. (2000). Anti-inflammatory cytokines. *Chest*, 117(4), 1162-1172.
- Oyervides-Guajardo, V. G., Claudio-Rizo, J. A., Cabrera-Munguia, D. A., Cano-Salazar, L. F., León-Campos, M. I., & Tamayo-Ordoñez, M. C. (2025). Protein–Polysaccharide Hydrogels: A Review on Smart Materials for the Future of Biotechnology. *Polymers for Advanced Technologies*, 36(8).
<https://doi.org/10.1002/pat.70295>
- Ozougwu, J. C. (2016). The role of reactive oxygen species and antioxidants in oxidative stress. *International Journal of Research*, 1(8), 1-8.
- Pan, K., Luo, Y., Gan, Y., Baek, S. J., & Zhong, Q. (2014). pH-driven encapsulation of curcumin in self-assembled casein nanoparticles for enhanced dispersibility and bioactivity. *Soft Matter*, 10(35), 6820-6830.
<https://doi.org/10.1039/c4sm00239c>
- Paterson, R. R. (2008). Cordyceps: a traditional Chinese medicine and another fungal therapeutic biofactory? *Phytochemistry*, 69(7), 1469-1495.
<https://doi.org/10.1016/j.phytochem.2008.01.027>
- Patlevic, P., Vaskova, J., Svorc, P., Jr., Vasko, L., & Svorc, P. (2016). Reactive oxygen species and antioxidant defense in human gastrointestinal diseases. *Integr Med Res*, 5(4), 250-258. <https://doi.org/10.1016/j.imr.2016.07.004>
- Petecchia, L., Sabatini, F., Usai, C., Caci, E., Varesio, L., & Rossi, G. A. (2012). Cytokines induce tight junction disassembly in airway cells via an EGFR-dependent MAPK/ERK1/2-pathway. *Lab Invest*, 92(8), 1140-1148.
<https://doi.org/10.1038/labinvest.2012.67>
- Pisoschi, A. M., & Pop, A. (2015). The role of antioxidants in the chemistry of oxidative stress: A review. *Eur J Med Chem*, 97, 55-74.
<https://doi.org/10.1016/j.ejmech.2015.04.040>
- Pobiega, K., Sękul, J., Pakulska, A., Latoszevska, M., Michońska, A., Korzeniowska, Z., Macherzyńska, Z., Pląder, M., Duda, W., Szafraniuk, J., Kufel, A., Dominiak, Ł., Lis, Z., Kłusek, E., Kozicka, E., Wierzbička, A., Trusińska, M., Rybak, K., Kot, A. M., & Nowacka, M. (2024). Fungal Proteins: Sources,

- Production and Purification Methods, Industrial Applications, and Future Perspectives. *Applied Sciences*, 14(14). <https://doi.org/10.3390/app14146259>
- Qing, R., Hao, S., Smorodina, E., Jin, D., Zalevsky, A., & Zhang, S. (2022). Protein Design: From the Aspect of Water Solubility and Stability. *Chem Rev*, 122(18), 14085-14179. <https://doi.org/10.1021/acs.chemrev.1c00757>
- Rajendran, P., Chen, Y. F., Chen, Y. F., Chung, L. C., Tamilselvi, S., Shen, C. Y., Day, C. H., Chen, R. J., Viswanadha, V. P., & Kuo, W. W. (2018). The multifaceted link between inflammation and human diseases. *Journal of cellular physiology*, 233(9), 6458-6471.
- Rao, P. P. (2025). Natural products and nutraceuticals in the management of chronic inflammatory diseases: efficacy, mechanisms, and comparative insights. *Inflammopharmacology*, 1-21.
- Rao, R. (2008). Oxidative stress-induced disruption of epithelial and endothelial tight junctions. *Frontiers in bioscience: a journal and virtual library*, 13, 7210.
- Rashid, M. H., Babu, D., & Siraki, A. G. (2021). Interactions of the antioxidant enzymes NAD(P)H: Quinone oxidoreductase 1 (NQO1) and NRH: Quinone oxidoreductase 2 (NQO2) with pharmacological agents, endogenous biochemicals and environmental contaminants. *Chem Biol Interact*, 345, 109574. <https://doi.org/10.1016/j.cbi.2021.109574>
- 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. *J Agric Food Chem*, 58(17), 9675-9684. <https://doi.org/10.1021/jf1015957>
- Rojas-Osnaya, J., Quintana-Quirino, M., Espinosa-Valencia, A., Bravo, A. L., & Nájera, H. (2024). Hydrophobins: multitask proteins. *Frontiers in Physics*, 12, 1393340.
- Sampaio, S. L., Fernandes, A., Pereira, C., Calhelha, R. C., Sokovic, M., Santos-Buelga, C., Barros, L., & Ferreira, I. (2020). Nutritional value, physicochemical characterization and bioactive properties of the Brazilian quinoa BRS Piabiru. *Food Funct*, 11(4), 2969-2977. <https://doi.org/10.1039/d0fo00055h>
- Santos-Buelga, C., González-Paramás, A. M., & González-Manzano, S. (2023). Phenolic acids and derivatives: description, sources, properties, and applications. In *Natural Secondary Metabolites: From Nature, Through Science, to Industry* (pp. 37-72). Springer.
- Schieber, M., & Chandel, N. S. (2014). ROS function in redox signaling and oxidative stress. *Curr Biol*, 24(10), R453-462. <https://doi.org/10.1016/j.cub.2014.03.034>
- Shao, F., Zhang, Y., Wan, X., Duan, Y., Cai, M., & Zhang, H. (2024). Improving the properties of whey protein isolate-zein nanogels with novel acidifiers: Re-

- dispersity, stability and quercetin bioavailability. *Int J Biol Macromol*, 266(Pt 2), 131284. <https://doi.org/10.1016/j.ijbiomac.2024.131284>
- Shaw, P., & Chattopadhyay, A. (2020). Nrf2-ARE signaling in cellular protection: Mechanism of action and the regulatory mechanisms. *J Cell Physiol*, 235(4), 3119-3130. <https://doi.org/10.1002/jcp.29219>
- Shen, L., Black, E. D., Witkowski, E. D., Lencer, W. I., Guerriero, V., Schneeberger, E. E., & Turner, J. R. (2006). Myosin light chain phosphorylation regulates barrier function by remodeling tight junction structure. *Journal of cell science*, 119(10), 2095-2106.
- Shen, X., Xue, S., Tan, Y., Zhong, W., Liang, X., & Wang, J. (2022). Binding of Licochalcone A to Whey Protein Enhancing Its Antioxidant Activity and Maintaining Its Antibacterial Activity. *J Agric Food Chem*, 70(50), 15917-15927. <https://doi.org/10.1021/acs.jafc.2c06125>
- Shin, H. Y., Kim, Y. S., Ha, E. J., Koo, J. P., Jeong, W. B., Joung, M. Y., Shin, K. S., & Yu, K. W. (2024). Anti-inflammatory action and associated intracellular signaling of Centella asiatica extract on lipopolysaccharide-stimulated RAW 264.7 macrophage. *Food Bioscience*, 61, Article 104614. <https://doi.org/10.1016/j.fbio.2024.104614>
- Sies, H. (2017). Hydrogen peroxide as a central redox signaling molecule in physiological oxidative stress: Oxidative eustress. *Redox biology*, 11, 613-619.
- Silvestro, S., & Mazzon, E. (2023). Nrf2 Activation: Involvement in Central Nervous System Traumatic Injuries. A Promising Therapeutic Target of Natural Compounds. *International Journal of Molecular Sciences*, 24(1), 199.
- Smith, P. e., Krohn, R. I., Hermanson, G., Mallia, A., Gartner, F., Provenzano, M., Fujimoto, E., Goeke, N., Olson, B., & Klenk, D. (1985). Measurement of protein using bicinchoninic acid. *Analytical Biochemistry*, 150(1), 76-85.
- Song, J., Lee, H., & Lee, H. (2024). Extensive analysis of monosaccharide diversity in fermented soybean paste using LC-MS/MS. *Journal of Food Composition and Analysis*, 130, 106149.
- Srinivasan, B., Kolli, A. R., Esch, M. B., Abaci, H. E., Shuler, M. L., & Hickman, J. J. (2015). TEER measurement techniques for in vitro barrier model systems. *J Lab Autom*, 20(2), 107-126. <https://doi.org/10.1177/2211068214561025>
- Sun, L.-H., Lv, S.-W., & He, L.-Y. (2017). Comparison of Different Physical Technique-Assisted Alkali Methods for the Extraction of Rice Bran Protein and its Characterizations. *International Journal of Food Engineering*, 13(10). <https://doi.org/10.1515/ijfe-2017-0070>

- Suzuki, T., & Yamamoto, M. (2017). Stress-sensing mechanisms and the physiological roles of the Keap1-Nrf2 system during cellular stress. *J Biol Chem*, 292(41), 16817-16824. <https://doi.org/10.1074/jbc.R117.800169>
- Szabo, A., Vollmar, B., Boros, M., & Menger, M. D. (2008). In vivo fluorescence microscopic imaging for dynamic quantitative assessment of intestinal mucosa permeability in mice. *J Surg Res*, 145(2), 179-185. <https://doi.org/10.1016/j.jss.2006.05.045>
- Turner, J. R. (2009). Intestinal mucosal barrier function in health and disease. *Nature Reviews Immunology*, 9(11), 799-809.
- Vane, J. R., & Botting, R. M. (1997). Mechanism of action of anti-inflammatory drugs. In H. Sinzinger, B. Samuelsson, J. R. Vane, R. Paoletti, P. Ramwell, & P. Y. K. Wong (Eds.), *Recent Advances in Prostaglandin, Thromboxane, and Leukotriene Research* (Vol. 433, pp. 131-138).
- Viljanen, K., Kylli, P., Kivikari, R., & Heinonen, M. (2004). Inhibition of protein and lipid oxidation in liposomes by berry phenolics. *Journal of Agricultural and Food Chemistry*, 52(24), 7419-7424.
- Wang, Q., Wei, H., Deng, C., Xie, C., Huang, M., & Zheng, F. (2020). Improving Stability and Accessibility of Quercetin in Olive Oil-in-Soy Protein Isolate/Pectin Stabilized O/W Emulsion. *Foods*, 9(2). <https://doi.org/10.3390/foods9020123>
- Wang, Y., Chen, R., Bai, C., Bai, H., Yang, C., Tian, L., Pang, J., Pang, Z., Li, D., & Wu, W. (2024). Screening of sweet potatoes varieties, physicochemical and biological activity studies of glycoproteins of selected varieties. *Food Bioscience*, 61. <https://doi.org/10.1016/j.fbio.2024.104927>
- Wang, Y., Gao, N., Nieto-Veloza, A., Zhou, L., Sun, X., Si, X., Tian, J., Lin, Y., Jiao, X., & Li, B. (2023). Lonicera caerulea polyphenols inhibit fat absorption by regulating Nrf2-ARE pathway mediated epithelial barrier dysfunction and special microbiota. *Food Science and Human Wellness*, 12(4), 1309-1322. <https://doi.org/10.1016/j.fshw.2022.10.013>
- Wang, Z., Li, Y., Jiang, L., Qi, B., & Zhou, L. (2014). Relationship between Secondary Structure and Surface Hydrophobicity of Soybean Protein Isolate Subjected to Heat Treatment. *Journal of Chemistry*, 2014, 1-10. <https://doi.org/10.1155/2014/475389>
- Wang, Z.-M., Peng, X., Lee, K.-L. D., Tang, J. C.-o., Cheung, P. C.-K., & Wu, J.-Y. (2011). Structural characterisation and immunomodulatory property of an acidic polysaccharide from mycelial culture of Cordyceps sinensis fungus Cs-HK1. *Food Chemistry*, 125(2), 637-643. <https://doi.org/10.1016/j.foodchem.2010.09.052>

- Wei, X., Sun, W., Zhu, P., Ou, G., Zhang, S., Li, Y., Hu, J., Qu, X., Zhong, Y., Yu, W., You, Z., Wang, Y., & Wu, Y. (2022). Refined polysaccharide from *Dendrobium devonianum* resists H1N1 influenza viral infection in mice by activating immunity through the TLR4/MyD88/NF-kappaB pathway. *Front Immunol*, 13, 999945. <https://doi.org/10.3389/fimmu.2022.999945>
- Wei, Z., & Huang, Q. (2019). Assembly of Protein-Polysaccharide Complexes for Delivery of Bioactive Ingredients: A Perspective Paper. *J Agric Food Chem*, 67(5), 1344-1352. <https://doi.org/10.1021/acs.jafc.8b06063>
- Willment, J. A., & Brown, G. D. (2008). C-type lectin receptors in antifungal immunity. *Trends Microbiol*, 16(1), 27-32. <https://doi.org/10.1016/j.tim.2007.10.012>
- Winder, M., Bulska-Będkowska, W., & Chudek, J. (2021). The use of *Herichium erinaceus* and *Trametes versicolor* extracts in supportive treatment in oncology. *Acta Pharmaceutica*, 71(1), 1-16.
- Wu, J.-Y. (2015). Polysaccharide-protein complexes from edible fungi and applications. In *Polysaccharides* (pp. 927-937). Springer.
- Wu, J.-Y., Chen, X., & Siu, K.-C. (2014). Isolation and structure characterization of an antioxidative glycopeptide from mycelial culture broth of a medicinal fungus. *International Journal of Molecular Sciences*, 15(10), 17318-17332.
- Wu, J. Y., Chen, X., & Siu, K. C. (2014). Isolation and structure characterization of an antioxidative glycopeptide from mycelial culture broth of a medicinal fungus. *Int J Mol Sci*, 15(10), 17318-17332. <https://doi.org/10.3390/ijms151017318>
- Wu, M., Qu, J., Tian, X., Zhao, X., Shen, Y., Shi, Z., Chen, P., Li, G., & Ma, T. (2019). Tailor-made polysaccharides containing uniformly distributed repeating units based on the xanthan gum skeleton. *Int J Biol Macromol*, 131, 646-653. <https://doi.org/10.1016/j.ijbiomac.2019.03.130>
- Wu, Y., Zhang, X., Kang, X., Li, N., Wang, R., Hu, T., Xiang, M., Wang, X., Yuan, W., Chen, A., Meng, D., & Chen, S. (2013). Oxidative stress inhibits adhesion and transendothelial migration, and induces apoptosis and senescence of induced pluripotent stem cells. *Clin Exp Pharmacol Physiol*, 40(9), 626-634. <https://doi.org/10.1111/1440-1681.12141>
- Xiang, Y., Xu, X., & Li, J. (2012). Chemical properties and antioxidant activity of exopolysaccharides fractions from mycelial culture of *Inonotus obliquus* in a ground corn stover medium. *Food Chem*, 134(4), 1899-1905. <https://doi.org/10.1016/j.foodchem.2012.03.121>
- Xiao, S., Li, W. W., Zhang, P., Zhang, G. W., Lin, H. W., & Hu, X. (2024). Mechanisms of food-derived bioactive compounds inhibiting TLR4 activation and regulating TLR4-mediated inflammation: A comprehensive review and future directions.

- Xiao, Z., Zhou, W., & Zhang, Y. (2020). Fungal polysaccharides. *Advances in pharmacology*, 87, 277-299.
- Xie, M., Zhou, C., Li, X., Ma, H., Liu, Q., & Hong, P. (2024). Preparation and characterization of tilapia protein isolate - Hyaluronic acid complexes using a pH-driven method for improving the stability of tilapia protein isolate emulsion. *Food Chem*, 445, 138703. <https://doi.org/10.1016/j.foodchem.2024.138703>
- Xu, B. w., Li, S. s., Ding, W. l., Zhang, C., Rehman, M. U., Tareen, M. F., Wang, L., & Huang, S. c. (2024). From structure to function: A comprehensive overview of polysaccharide roles and applications. *Food Frontiers*, 6(1), 15-39. <https://doi.org/10.1002/fft2.490>
- Xu, G., Yu, Z. P., & Zhao, W. Z. (2024). Immunomodulation effects of isochlorogenic acid a from apple on RAW264.7 cells via modulation of TLR2 and TLR4 target proteins. *Food Bioscience*, 58, Article 103773. <https://doi.org/10.1016/j.fbio.2024.103773>
- Xu, H., Lu, Y., Zhang, T., Liu, K., Liu, L., He, Z., Xu, B., & Wu, X. (2019). Characterization of binding interactions of anthraquinones and bovine beta-lactoglobulin. *Food Chem*, 281, 28-35. <https://doi.org/10.1016/j.foodchem.2018.12.077>
- Yamamoto, M., Kensler, T. W., & Motohashi, H. (2018). The KEAP1-NRF2 System: a Thiol-Based Sensor-Effector Apparatus for Maintaining Redox Homeostasis. *Physiol Rev*, 98(3), 1169-1203. <https://doi.org/10.1152/physrev.00023.2017>
- Yan, J., Zhu, L., Qu, Y., Qu, X., Mu, M., Zhang, M., Muneer, G., Zhou, Y., & Sun, L. (2019). Analyses of active antioxidant polysaccharides from four edible mushrooms. *International Journal of Biological Macromolecules*, 123, 945-956.
- Yan, J.-K., & Wu, J.-Y. (2014). Submerged Fermentation of Medicinal Fungus *Cordyceps sinensis* for Production of Biologically Active Mycelial Biomass and Exopolysaccharides. In K.-Y. Paek, H. N. Murthy, & J.-J. Zhong (Eds.), *Production of Biomass and Bioactive Compounds Using Bioreactor Technology* (pp. 93-120). Springer Netherlands. https://doi.org/10.1007/978-94-017-9223-3_5
- Yan, J. K., Wang, W. Q., & Wu, J. Y. (2014). Recent advances in *Cordyceps sinensis* polysaccharides: Mycelial fermentation, isolation, structure, and bioactivities: A review. *J Funct Foods*, 6, 33-47. <https://doi.org/10.1016/j.jff.2013.11.024>
- Yan, X., Zhang, X., McClements, D. J., Zou, L., Liu, X., & Liu, F. (2019). Co-encapsulation of Epigallocatechin Gallate (EGCG) and Curcumin by Two

- Proteins-Based Nanoparticles: Role of EGCG. *J Agric Food Chem*, 67(48), 13228-13236. <https://doi.org/10.1021/acs.jafc.9b04415>
- Yang, Y., Liang, M., Ouyang, D., Tong, H., Wu, M., & Su, L. (2022). Research Progress on the Protective Effect of Brown Algae-Derived Polysaccharides on Metabolic Diseases and Intestinal Barrier Injury. *Int J Mol Sci*, 23(18). <https://doi.org/10.3390/ijms231810784>
- Yao, H. 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 Res Int*, 143, 110290. <https://doi.org/10.1016/j.foodres.2021.110290>
- Yi, J., Fan, Y., Zhang, Y., Wen, Z., Zhao, L., & Lu, Y. (2016). Glycosylated α -lactalbumin-based nanocomplex for curcumin: Physicochemical stability and DPPH-scavenging activity. *Food Hydrocolloids*, 61, 369-377. <https://doi.org/10.1016/j.foodhyd.2016.05.036>
- You, S.-J., Udenigwe, C. C., Aluko, R. E., & Wu, J. (2010). Multifunctional peptides from egg white lysozyme. *Food Research International*, 43(3), 848-855. <https://doi.org/10.1016/j.foodres.2009.12.004>
- Zafarullah, M., Li, W. Q., Sylvester, J., & Ahmad, M. (2003). Molecular mechanisms of *N*-acetylcysteine actions. *Cellular and Molecular Life Sciences*, 60(1), 6-20. <https://doi.org/10.1007/s000180300001>
- Zhang, S. Y., Mao, C. W., Liu, R. W., Zeng, X. A., & Lin, S. Y. (2024). Pulsed electric field (PEF) activates immune activity in RAW 264.7 macrophages by altering pine nut peptide-TLR4 binding sites. *Food Bioscience*, 58, Article 103779. <https://doi.org/10.1016/j.fbio.2024.103779>
- Zhang, Y., Li, E., Wang, C., Li, Y., & Liu, X. (2012). Ophiocordyceps sinensis, the flagship fungus of China: terminology, life strategy and ecology. *Mycology*, 3(1), 2-10.
- Zhang, Y., Zhang, C. C., Luo, M. H., Yang, S. H., Wang, Y. Z., Xu, S., & Xu, Q. R. (2024). Musa basjoo Sieb polysaccharide improves inflammation in RAW264.7 cells and zebrafish colitis. *Food Bioscience*, 61, Article 104471. <https://doi.org/10.1016/j.fbio.2024.104471>
- 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. *Int J Biol Macromol*, 280(Pt 4), 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>
- Zhou, L., Wu, F., Zhang, X., & Wang, Z. (2017). Structural and functional properties of Maillard reaction products of protein isolate (mung bean, *Vigna radiata* (L.) with dextran. *International Journal of Food Properties*, 1-13. <https://doi.org/10.1080/10942912.2017.1338727>
- 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- κ B Pathway. *Nutrients*, 16(22).
- 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-kappaB Pathway. *Nutrients*, 16(22). <https://doi.org/10.3390/nu16223885>
- Zin, M. T., Kaewkod, T., Chaipoot, S., Kanthakat, G., Chen, Y.-Y., Cheirsilp, B., & Srinuanpan, S. (2025). Ultrasound-Assisted Deep Eutectic Solvent Extraction of Antioxidant and Anti-Colorectal Cancer Proteins from *Spirulina* Biomass: Process Intensification, Characterization, and Bioactivity Evaluation. *Antioxidants*, 14(3), 365.
- Zou, Y. F., Li, C. Y., Fu, Y. P., JiZe, X. P., Zhao, Y. Z., Peng, X., Wang, J. Y., Yin, Z. Q., Li, Y. P., Song, X., Li, L. X., Zhao, X. H., Feng, B., Huang, C., Ye, G., Tang, H. Q., Chen, J., Li, R., Chen, X. F., & Tian, M. L. (2023). Angelica sinensis aboveground part polysaccharide and its metabolite 5-MT ameliorate colitis via modulating gut microbiota and TLR4/MyD88/NF-kappaB pathway. *Int J Biol Macromol*, 242(Pt 1), 124689. <https://doi.org/10.1016/j.ijbiomac.2023.124689>

Appendix

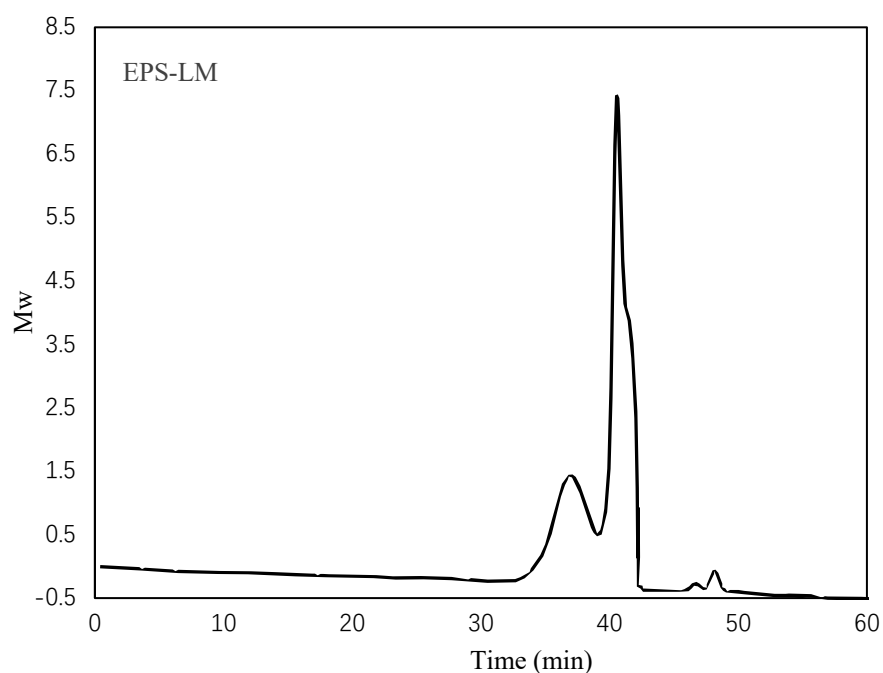


Figure A1 High-performance size-exclusion chromatography (HPSEC) profiles of EPS-LM.

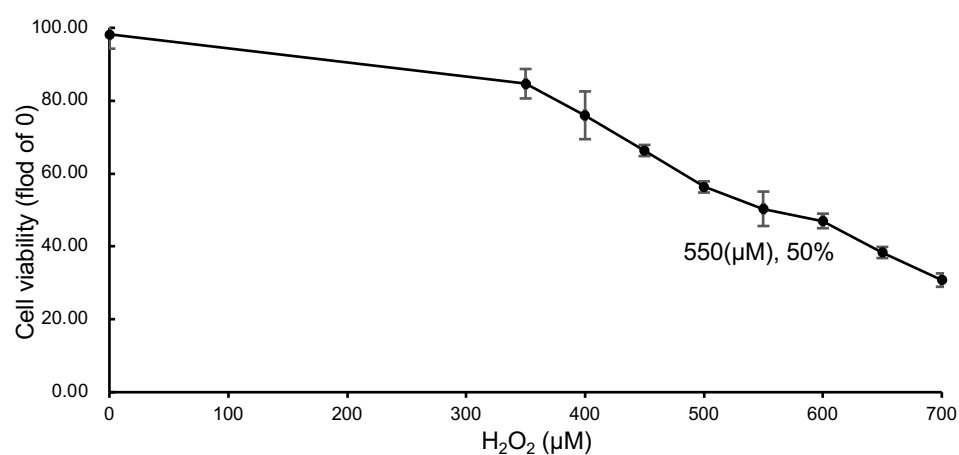


Figure A2 Preliminary dose-response analysis of H₂O₂-induced reduction in Caco-2 cell viability within the concentration range of 0–700 μM. Data are presented as mean ± SEM (n = 6).

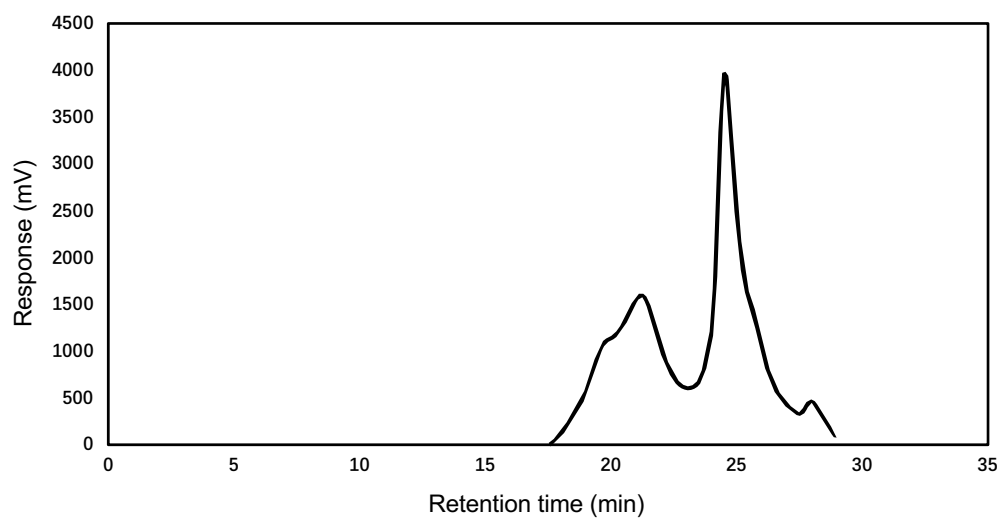


Figure A3 High-performance gel permeation chromatography (HPGPC) profile of EPS-LM.

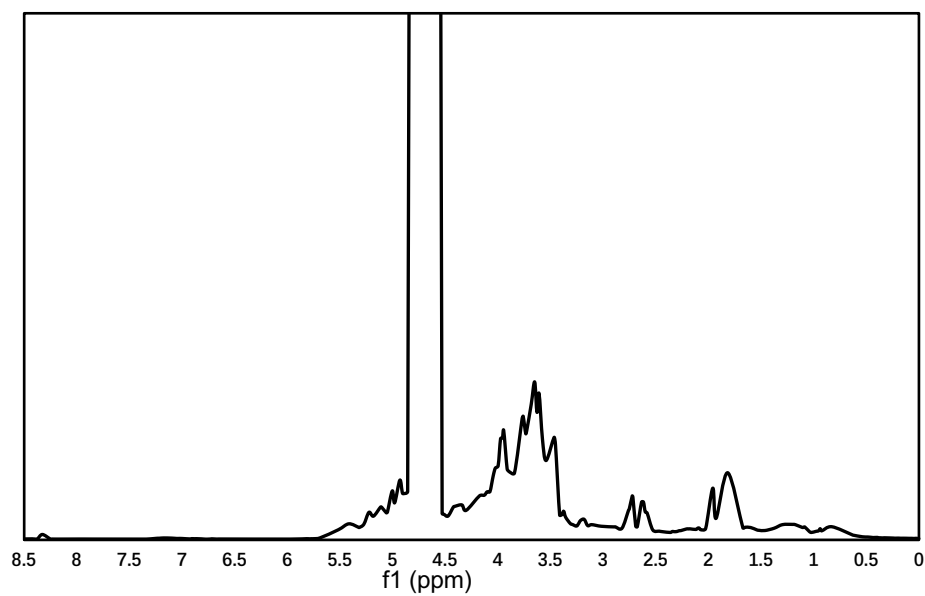
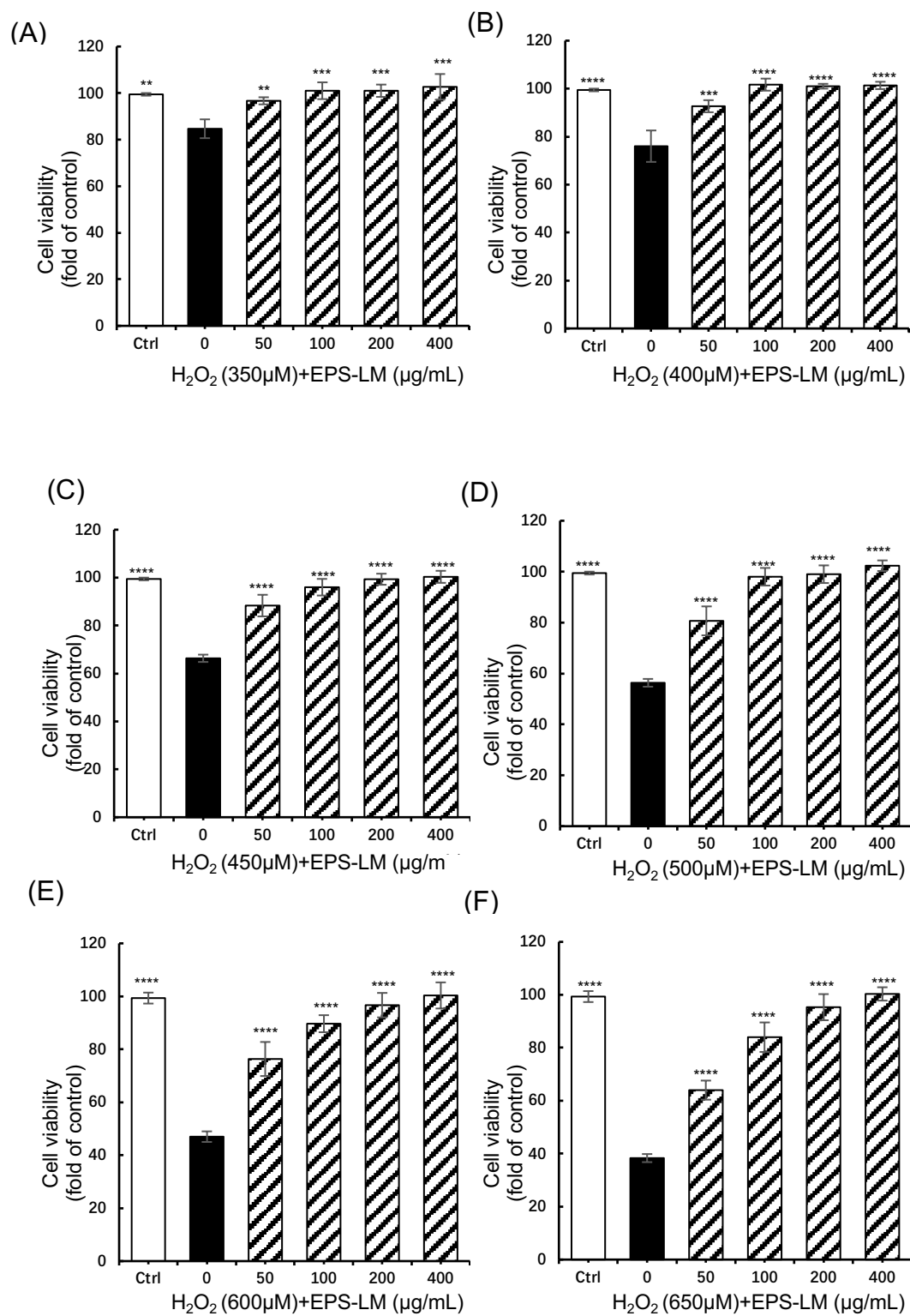


Figure A4 ^1H NMR spectrum of EPS-LM.



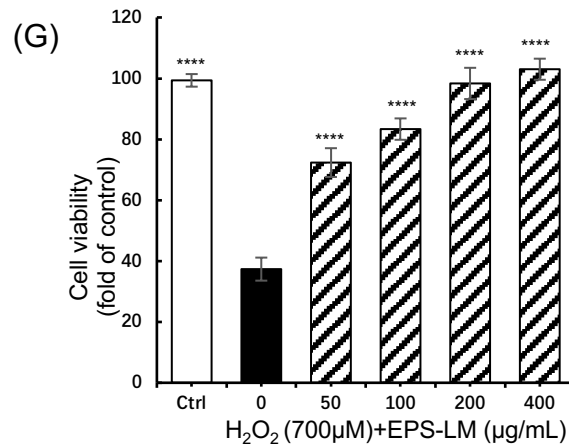


Figure A5 Protective effect of EPS-LM on Caco-2 cell viability under H₂O₂-induced oxidative stress at concentrations ranging from 350 μM to 700 μM (E–G). Data are presented as mean ± SEM (n = 6). (**p < 0.01, ***p < 0.001, ****p < 0.0001 vs. H₂O₂).

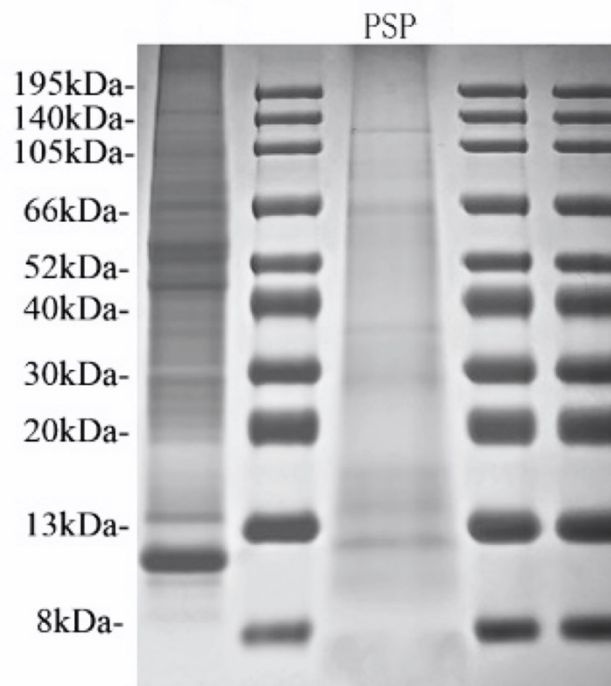


Figure A6 SDS-PAGE analysis of PSP extracted from Cs-HK1 mycelial fermentation, showing the molecular weight distribution of major protein fractions.