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Soluble Proteins From Conventional and Organic Eggshell Membranes With Different Proteomic Profiles Show Similar In Vitro Biofunctions

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Keywords: bone tissue regeneration | eggshell membrane | food waste utilization | proteomic analysis

ABSTRACT

The eggshell membrane (ESM), resembling the extracellular matrix (ECM), acts as a protective barrier against bacterial invasion and offers various biofunctions due to its porous structure and protein-rich composition, such as ovalbumin, ovotransferrin, collagen, soluble protein, and antimicrobial proteins. However, the structure of ESM primarily comprises disulfide bonds and heterochains, which poses a challenge for protein solubilization/extraction. Therefore, the method of dissolving and extracting bioactive protein components from ESM has significant potential value and importance for exploring the reuse of egg waste and environmental protection. In this study, soluble ESM proteins (SEPs) were extracted from conventional (industrial-fed) and organic (free-grounded) using an acidic 3-mercaptopropionic acid (3-MPA) extraction strategy. FTIR was employed to monitor the chemical changes in the ESM, while LC–MS/MS was used to conduct the proteomic analysis. The biocompatibility and effects of SEP cocktails on ECM synthesis were also investigated. The results indicated that the acidic 3-MPA strategy effectively altered the ESM chemical composition, thereby facilitating SEPs extraction. The SEPs from conventional and organic eggs have different protein profiles but with partial overlapping. SEPs from both sources showed similar desirable biosafety profiles and dose-dependent promotion of osteoblastic (ECM) component synthesis, suggesting that different egg sources may contribute to consistent core biological functions of protein products, they may also introduce different functional priorities.

Qianli Ma and Lya Paia contributed equally to this study as co-first authors.

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1 | Introduction

Sustainable development initiatives aim to meet the needs of future generations by adopting practices that promote material efficiency, including the reuse of processed materials and industrial waste [1]. The global egg industry generates substantial quantities of egg byproducts, primarily from food industries and household waste. Increasing egg consumption worldwide, reaching 93,000 million tons in 2020, and Norway's per capita consumption, which is 13.4 kg, highlight the significant need to reutilize egg waste [2–4].

Eggshell waste presents an opportunity to produce diverse materials, including inorganic components such as calcium carbonate ($CaCO_3$) and proteins [1, 5, 6]. The eggshell membrane (ESM), positioned between the eggshell and albumen [7], plays a vital role in initial eggshell mineralization and contributes to the mechanical properties of eggs. ESM is notably rich in fibers and proteins [8, 9], comprising 80%–85% organic matrix and 15%–20% inorganic matrix ($CaCO_3$) [10]. ESM contains collagen (Types I, V, and X) and smaller proteins (~70%) like keratin, glycoprotein, proteoglycan, and hyaluronic acid [2, 7, 11]. Consequently, egg waste was recognized as a valuable source of compounds applicable for various purposes, particularly in medical applications such as skin, bone, and osteochondral regeneration [12–17]. Eggshell and ESM mineral components, including amorphous calcium phosphate, have been investigated for their potential in bone tissue engineering [5, 9, 18]. Moreover, specific ESM components and ESM have shown promise in direct skin application for tissue regeneration, owing to their composition containing active substances conducive to skin regeneration [19].

The eggshell is a protective barrier and a dynamic entity capable of synthesizing unique proteins influenced by environmental factors such as diet, stress, and climate during its development in the hen's uterus [20]. Moreover, scientific findings have indicated the potential to extract these environmentally influenced proteins for medical applications [21, 22]. Filipiak-Florkiewicz et al. demonstrated the significant impact of hen nutrition on egg composition, with distinct differences observed between organic and conventional eggs. In their investigation, elevated levels of potassium and copper were quantified in organic eggs, whereas higher concentrations of magnesium and iron were detected in the yolk of conventional eggs. These findings imply an interconnected influence on the chemical composition of both the egg membrane and shell, indicating the potential impact of environmental factors [21, 22]. Numerous researchers have employed omics methodologies to discern the protein composition of eggs, particularly focusing on identifying and characterizing the primary proteins within the shell matrix [20, 23]. This area of inquiry continues to garner substantial interest, with ongoing efforts to elucidate protein and molecular constituents of the ESM [24–27]. For instance, Sun, Xu, and Yang delineated the ESM proteomic profiles of eggshells exhibiting disparate strengths, demonstrating a direct correlation between protein abundance and eggshell resilience [28]. Similarly, Rose-Martel, Du, and Hincke identified and evaluated the antimicrobial properties of 47 cuticle proteins extracted from eggshells [29, 30]. Mann and Mann identified 520 proteins pivotal in eggshell formation and vertebrate biomineralization, providing valuable insights into proteins present across various egg components [31]. Additionally, Xia et al. reported significant differences in the overall nutritional composition of conventional and organic eggs [32]. Therefore, it is reasonable to hypothesize that their ESMs exhibit different protein profiles, which may profoundly impact their biological functions and further affect ESM-related biomaterials' performance.

Most studies based on ESM proteomics include structural (insoluble) proteins and soluble proteins [33]. Considering our current research on bone regeneration, the extraction of soluble ESM proteins (SEPs) from ESM may be more conducive to combining them with other graft materials to achieve synergistic effects. This study aims to unlock the exciting potential of SEPs. Proteins were extracted from organic and conventional egg ESMs using a chemical process, followed by proteomics analysis and biocompatibility investigation. The acquisition of this information is poised to enhance our understanding of the impact and extent of different feeding conditions on the protein content of ESM waste [33, 34]. It is expected to provide theoretical guidance for selecting raw materials for the ESM waste utilization industry and obtaining high-value-added biomaterials with more precise biological properties to optimize tissue regeneration.

2 | Materials and Methods

2.1 | Extraction of SEPs

ESM is collected from eggshells freshly discarded from the kitchen. The ESM was removed manually from the shell's interior and dried at room temperature (RT) for 24 h. Two types of eggs, conventional (industrial-fed, Nortura SA (Prior), Oslo, Norway) and organic (free-grounded, Solvinge AS, Orkanger, Norway), were used in this study. ESM protein extraction was performed as previously described [35, 36] with some modifications. Initially, the ESM was cut into small pieces and added to 1.25 M aqueous solution of 3-mercaptopropionic acid (3-MPA) in 10% (v/v) aqueous acetic acid solution. The suspension was stirred at 300 rpm for 24 h and kept for 5 h at 70°C, allowing the ESM to dissolve. Then, the suspension was cooled to RT and centrifuged (3600 g, 20 min, RT) to remove insoluble material. After that, the pH of the solution was adjusted to 5.0 using 5 M NaOH, at which point an albumen precipitate formed in the solution. When the pH of the protein solubilize was adjusted to 7, proteins precipitated because this pH is near the isoelectric point (pI) of many proteins, where they have no net electrical charge and reduced solubility. After centrifugation in distilled water (pH ~ 7), the precipitated proteins were collected and then resuspended. This white precipitate was collected by centrifugation at 5000 rpm. The white precipitate formed contains the eggshell proteins, which were dissolved in distilled water to form an aqueous solution of SEPs. The total protein concentration was measured using NanoDrop One Microvolume UV–Vis Spectrophotometers (ThermoFisher Scientific, USA). Triplicate extractions were performed, and the samples were further used for Fourier transform infrared (FTIR) and proteomic analysis.

2.2 | FTIR Spectroscopy

An FTIR Spectrum 400 (PerkinElmer, USA) was employed to investigate the FTIR spectra of ESM samples before and after

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3-MPA treatment for 24 h. All spectra were acquired between 400 and 4000 cm^{-1} by co-adding 64 scans with a resolution of 4 cm^{-1}.

2.3 | Proteomic Analysis

The SEPs of the conventional and organic ESMs were analyzed using LC–MS on a timsTOF Pro (Bruker Daltonik, Bremen, Germany), which was coupled online to a nanoElute nanoflow liquid chromatography system (Bruker Daltonik, Bremen, Germany) via a CaptiveSpray nanoelectrospray ion source. Three samples of each type of egg were quantified. All the raw files were converted to MASCOT generic files and searched against the Gallo Gallo Uniprot database using MASCOT (Version 2.7.0.1) with the following parameters: parent tolerance, 15 ppm; fragment tolerance, 0.03 Da; trypsin; max missed cleavage, 1; fixed modification, carbamidomethyl (+57 on C); variable modifications, +16 on M (oxidation), +42 on peptide N-terminal (acetyl).

The search results were imported to Scaffold (Version 5.3.0, Proteome Software Inc., Portland, OR) to validate MS/MS-based peptide and protein identifications. Peptide identifications were accepted if they could be established with greater than 95.0% probability by the Peptide Prophet algorithm with Scaffold delta-mass correction. Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least two unique peptides. The Protein Prophet algorithm calculates protein probabilities by integrating the probabilities of associated peptides. Setting a stringent protein probability threshold of greater than 99.0% and requiring multiple unique peptides per protein, the reliability of protein identification is enhanced, reducing the likelihood of false-positive results. By applying these high-confidence cutoff criteria, we aimed to ensure the accuracy and robustness of our proteomic analysis, allowing for reliable identification of proteins present in the soluble ESM extracts.

After Log_2FC transformation and centralization, the CNSknowall platform (https://cnsknowall.com/) was employed to analyze the protein abundance and generate hierarchical clustering information. Principal component analysis (PCA) was conducted by Prism 10.1.2 (GraphPad Software, USA). The expression of proteins in the Conventional group was set as a control. The *p* value data was transformed to an adjusted *p* value using the following equation: $p.\text{adj} = -1\,\text{Log}_{10}(p)$, and the volcano plot was generated. The ShinyGO software (Version 0.80) and String platform created a protein interaction network and GO biological enrichment plots.

2.4 | Cytotoxicity of SEPs

MC-3T3-E1 preosteoblastic cells (ATCC, USA) were cultured in α-MEM medium (α-MEM, A1049001, Gibco, USA) with 15% heat-inactivated fetal calf serum (FCS, 20170-106, Gibco, USA) at 37°C. Cells were passaged at 80% confluency. The experimental design was established according to previous studies, ISO standards, and guidance of the lactic dehydrogenase (LDH) testing kit (11644793001, Roche, USA) [37, 38]. MC-3T3-E1 cells were seeded in a 96-well plate at the concentration of 1000 cells/100 μL/well. After 24 h of cultivation, the media were replaced with fresh α-MEM containing SEPs at different concentrations (100 μg, 10 μg, 1 μg, 100 ng, 10 ng, 1 ng, and 0 ng (cell control, no SEPs)/mL). The cells were cultured with a background control (blank medium without cells) and corresponding positive controls (1% Triton X-100 added 0.5 h prior to supernatant collection). After 24 h of cultivation, the supernatants were collected, and LDH activity was measured by optical absorbance assay at a wavelength of 490 nm (ELx800, BioTek, USA). All absorbance values were presented as net values according to the following equation:

$$\text{Absorbance}_{\text{test.net}} = \text{Absorbance}_{\text{test}} - \text{Absorbance}_{\text{background}}$$

2.5 | Effects of SEPs on Cell Proliferation

MC-3T3-E1 cells were seeded in a 96-well plate at the concentration of 1000 cells/100 μL/well. After 24 h of cultivation, the media were replaced with fresh α-MEM containing SEPs at different concentrations (100 μg, 10 μg, 1 μg, 100 ng, 10 ng, 1 ng, and 0 ng (blank, no ESM proteins)/mL). After an additional 24 h of culturing, cells were washed with PBS and cultured in fresh α-MEM with 10% CCK8 reagent (ab228554, Abcam, USA) for an additional 2 h. The supernatants were collected, and the absorbance was measured at a wavelength of 450 nm.

2.6 | Effects of SEPs on Extracellular Matrix (ECM) Synthesis

MC-3T3-E1 cells were seeded in a 24-well glass-bottom plate at 2×10^4 cells/mL/well for 3 days until preliminary confluency was formed. The culture media were then replaced with fresh α-MEM containing SEPs at different concentrations (100 μg, 10 μg, 1 μg, 100 ng, 10 ng, 1 ng, and 0 ng/mL) in the presence of 50 μg/mL ascorbic acid (A5960, Sigma-Aldrich, USA). After 21 days of culture, cells were fixed with 4% paraformaldehyde (PFA, P6148, Sigma-Aldrich, USA) for 20 min. After washing with PBS three times, samples were stained overnight in a 0.1 wt% Sirius Red (365548, Sigma-Aldrich, USA) in saturated picric acid solution (P6744, Sigma-Aldrich, USA). The samples were then washed with 0.1 M acetic acid (A6283, Sigma-Aldrich, USA), and microscope images were captured. For semiquantitative analysis, the stains were eluted in 500 μL elution solution (0.2 M NaOH/methanol 1:1, S5811 and 34860, Sigma-Aldrich, USA), and the absorbance was then measured at 490 nm.

2.7 | Statistics

Unless otherwise noted, experiments were repeated three times, with three to five replicates in each group. Other data were analyzed and plotted using Prism 10.1.2 and expressed as medians and quartiles. Significant differences between groups were identified using one- or two-way analysis of variance (ANOVA) followed by Tukey's post hoc test for parametric data or a Kruskal–Wallis rank-sum test followed by Dunn's multiple comparison test for nonparametric data (ranked test).

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Differences were considered statistically significant when $p < 0.05$.

3 | Results

3.1 | FTIR Inspection on Chemical Alteration of ESM After 3-MPA Treatment

The chemical profiles of ESM were obtained using FTIR spectroscopy. Figure 1 shows the vibration bands observed in ESM from organic eggs and conventional eggs before (Figure 1A,B) and after (Figure 1C,D) treatment with the 3-MPA solution. As shown in Figure 1A,B, the most prominent band at 3272 cm^{-1} corresponds to the stretching mode of O—H and N—H (Amide A), whereas the absorbance band at 2957 cm^{-1} (C—H stretching) is attributed to lipids. The lines at 1633, 1513, 1443, and 1239 cm^{-1} correspond to the amide C═O stretching (Amide I), C—N stretching/NH bending (Amide II), CH_2 scissoring (attributed to sulfates), and amine C—N stretching (Amide III), respectively. The absorption at 1076 cm^{-1} is attributed to polysaccharides, and at 620 cm^{-1} to C—S bonds [34, 39, 40]. These chemical species identify the components that constitute part of the protein, such as amides and polysaccharides. After

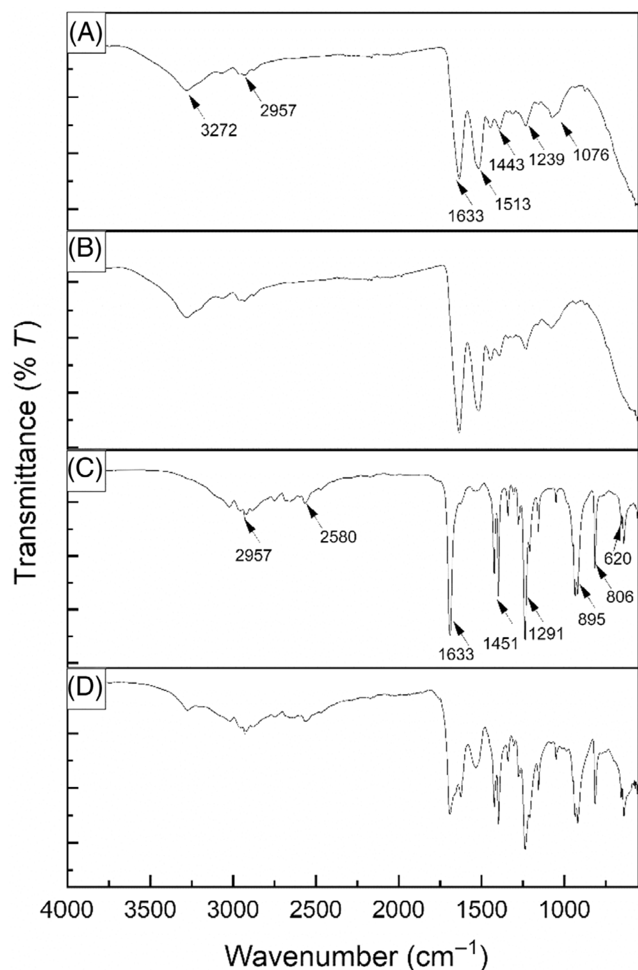

FIGURE 1 | FTIR spectra of ESM from organic eggs (A) and conventional eggs (B) before 3-MPA treatment. ESM from organic eggs (C) and conventional eggs (D) after 3-MPA treatment.

treatment with 3-MPA (Figure 1C,D), new absorption bands appear at 806, 1291, and 1451 cm^{-1} corresponding to the SO stretch and S═O stretch, while the band at 2580 cm^{-1} arises from the S—H stretching vibration, and the peak at 895 cm^{-1} corresponds to C═C bending [41]. These specific bands of disulfide bridges suggest that they were broken, making the proteins more soluble in solution.

3.2 | Proteomic Analysis of SEPs Extracted From Conventional and Organic ESMs

After FTIR analysis, the SEPs in conventional and organic samples were further quantified by LC–MS/MS, and the analytic data are shown in Figure 2. After data centralization, the relative protein expression profile was generated and plotted with individual samples in Figure 2A. The heatmap with clustering analysis (Figure 2A) clearly shows that conventional and organic ESM have different protein expression profiles. The PCA plot (Figure 2B) separated conventional and organic samples by Component 1 (PC1, *x*-axis). Compared with the clustered organic samples, conventional samples had greater differences (PC2, *y*-axis), indicating that conventional ESM's composition varies greatly from batch to batch, while organic SEPs were more consistent. Additionally, the proteins with the most different expression levels are depicted in the volcano plot (Figure 2C) in red (upregulated) and green color (downregulated). These protein-related mRNAs were further subjected to GO pathway enrichment to better understand their potential functions. The 50 detected proteins with the highest abundance were listed in Figure S1.

The detected proteins were divided into two categories (similar expression and different expression) based on whether there was a difference in expression between the conventional and organic SEPs. Protein interaction networks and GO biological process pathway enrichment were then conducted and are depicted in Figure 3. The similarly expressed proteins contributed consistent biofunctions to both conventional and organic SEPs, including response to corticosterone and acute-phase response, as shown in Figure 3A,B. On the other hand, differentially expressed proteins may lead to diverse biological characteristics between conventional and organic SEPs (Figure 3C,D). Although there were pronounced differences in protein profiles and biological functions between conventional and organic ESMs, there was still considerable overlap (Figures 2A and 3 and Table S1).

3.3 | Cytocompatibility and ECM Synthetic Functions of SEPs

The cytotoxicity of ESM proteins was tested at different concentrations. As shown in Figure 4, during the 24-h cultivation, neither conventional nor organic SEPs elicited LDH release at any of the tested concentrations, indicating that SEPs will not destroy the integrity of the cell membrane. However, the SEPs from different sources exhibited inconsistent effects on MC-3T3-E1 cell proliferation (Figure 5). Compared to conventional SEPs, organic SEPs started to facilitate cell growth when the concentration decreased from 100 to 10 μg/mL. This promotion gradually weakened as the protein concentration decreased

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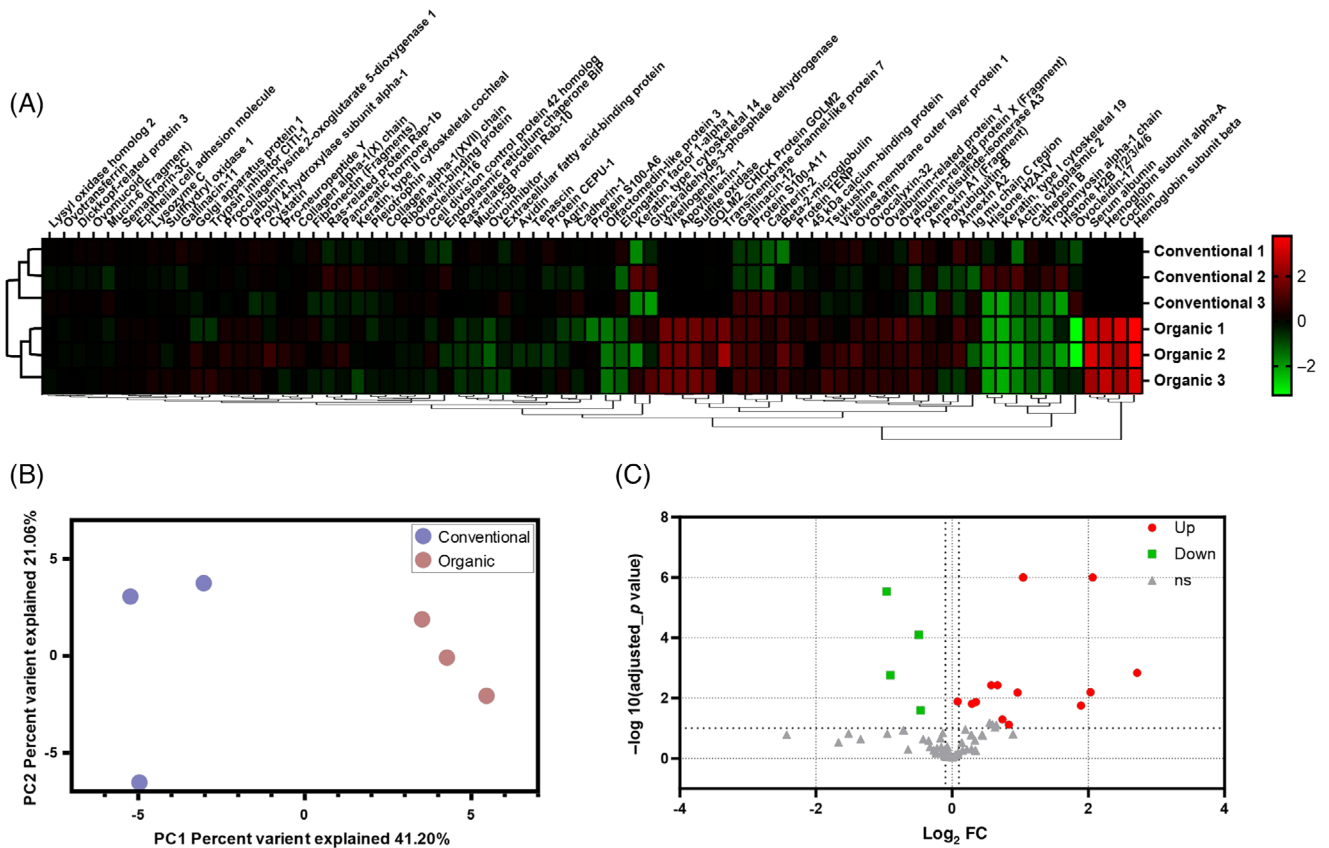

FIGURE 2 | Proteomic analysis of conventional and organic ESM proteins. (A) Clustering of the proteins that differ in expression between organic and conventional ESM proteins. The color bar indicates the Log_2 FC of individual protein versus conventional samples. (B) Principal component analysis (PCA) of conventional and organic ESM proteins across all independent samples. (C) Volcano plot of conventional and organic protein expression profiles. Proteins with differential expression levels are labeled red (upregulated) and green (downregulated) colors. $p < 0.05$, ($n = 6$).

(Figure 5B). Although the difference between conventional and organic SEPs was not pronounced at concentrations ranging from 100 µg/mL to 10 ng/mL, organic SEPs promoted cell proliferation at 1 ng/mL (Figure 5C).

In addition to cell proliferation, ECM synthesis better reflects the macroscopic effect of proteins on host osteogenesis. To our surprise, few cells survived in the presence of high concentrations of ESM proteins (100 µg/mL), and this adverse effect gradually attenuated as the protein concentration decreased (Figure 6A). More importantly, SEPs significantly increased extracellular collagen synthesis within a specific concentration range (from 10 ng/mL to 1 µg/mL, Figure 6B,C). Both conventional and organic SEPs exhibited consistent dose-dependent effects on collagen synthesis (Figure 6D).

4 | Discussion

Innovative methods for converting waste into valuable biomaterials significantly reduce landfill waste and promote sustainability [42]. For instance, technologies have been developed to transform food waste into biodegradable plastics and biowastes [43]. Additionally, pyrolysis and engineered microorganisms can convert biomass and industrial by-products into renewable products, energy, and biodegradable materials, thereby supporting a circular economy and minimizing environmental impact [44, 45]. Using ESM for protein extraction offers significant environmental benefits compared to traditional waste management strategies. Conventional methods of managing eggshell waste, such as landfilling and incineration, contribute to environmental pollution through greenhouse gas emissions and leachate production [42]. Therefore, valorizing ESM for protein extraction mitigates these environmental impacts and promotes a circular economy [45]. This sustainable strategy aligns with global efforts to minimize waste and promote resource efficiency, demonstrating a practical application of waste-to-resource technologies that can be replicated across various industries.

In the context of tissue regeneration, ESM proteins have emerged as promising biomaterials because of their unique composition and bioactive properties. The ESM is a complex network of collagenous proteins and glycoproteins that plays a crucial role in forming and protecting avian eggs. Recent research has highlighted the potential of these proteins in various biomedical applications, particularly in tissue engineering and regenerative medicine [46, 47]. One of the key advantages of SEPs is their ability to promote cell adhesion and proliferation. Studies have shown that ESM contains collagen Types I, V, and X, along with other proteins, such as osteopontin and sialoprotein, which are known to support cell attachment and growth [46, 47]. These proteins provide a natural ECM-like environment that facilitates the adhesion and proliferation of various cell types, including

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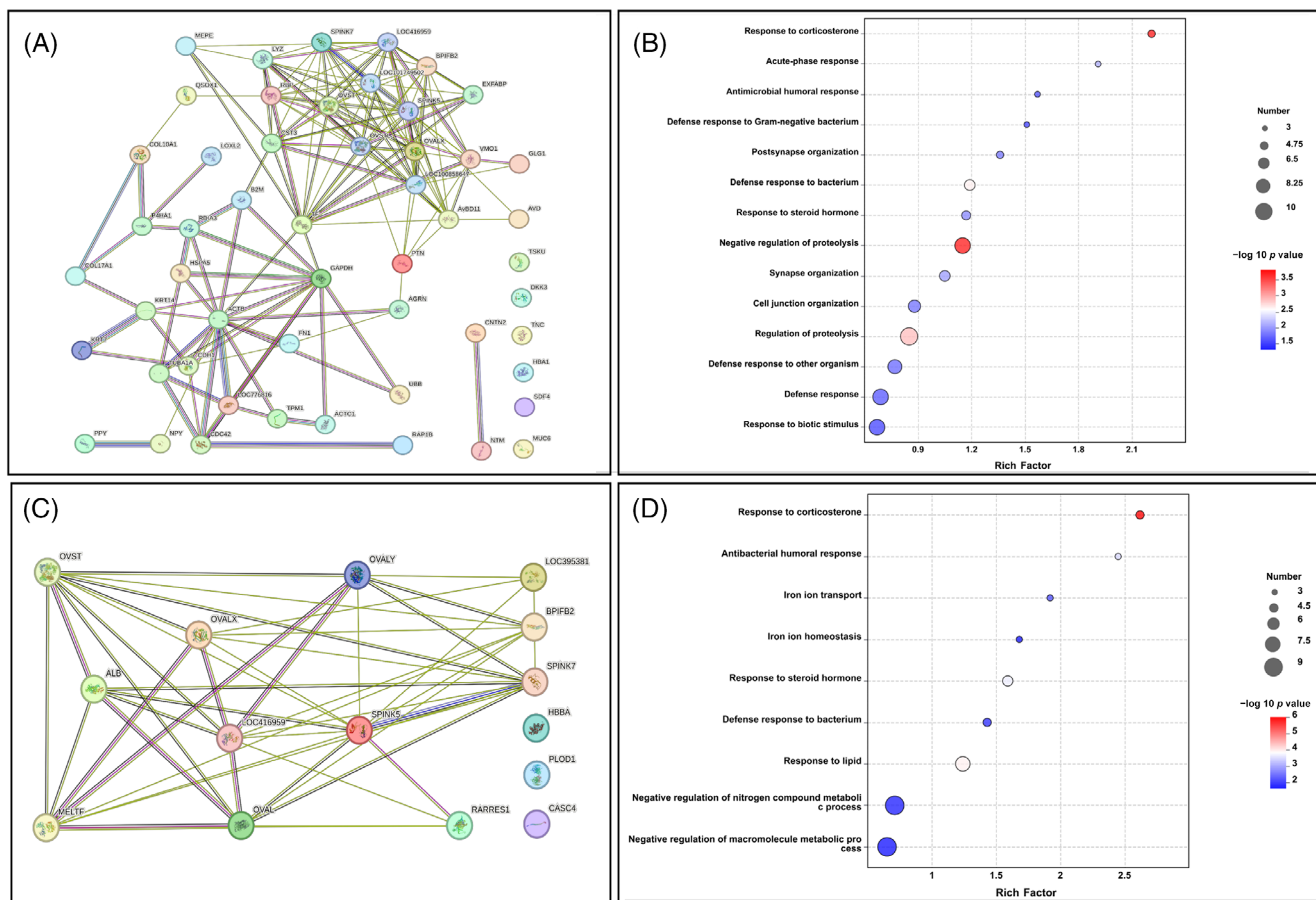

FIGURE 3 | Protein interaction and pathway enrichment of different SEPs. Protein interaction network (A) and GO biological process pathway enrichment (B) of similarly expressed proteins in conventional and organic ESM. Protein interaction network (C) and GO biological process pathway enrichment (D) of differentially expressed proteins in conventional and organic ESM ($n = 6$).

mesenchymal stem cells and osteoblasts, making ESM an attractive scaffold material for tissue regenerative applications.

ESM proteins have also demonstrated potent osteoinductive properties, which can stimulate the differentiation of mesenchymal stem cells into osteoblasts and promote mineralized tissue formation [18]. This osteogenic potential is attributed to the presence of specific proteins and growth factors within the ESM, such as transforming growth factor-β (TGF-β) and bone morphogenetic proteins (BMPs) [46]. In addition, the anti-inflammatory and antioxidant properties of ESM proteins also contribute to their regenerative potential [48, 49]. These properties may help create a more favorable tissue repair and regeneration. Given that the bioactivities of ESM are based on its functional proteins, the extraction of ESM proteins has become a focal point of research. Nevertheless, the solubility of ESM is limited due to the presence of numerous cross-links between cystine (e.g., keratin), hydroxylysinonorleicine, and desmosines [50]. Although preparing egg membrane freeze-dried powder is currently the simplest and most easily industrialized method, this strategy is still in its early stages, with SEPs and ESM scaffold components remaining combined [16]. Only by destroying the disulfide cross-links in the ESM macrostructure can the solubility of ESM be effectively improved. A modified 3-MPA acidic extraction, employed in this study, was considered an ideal strategy, and the obtained SEPs had an amino acid composition similar to that of natural ESM. However, due to the cleavage of disulfide bonds during the solubilization process, the dimer cystine easily converted to cysteine [35, 36, 51]. In this study, several characteristic vibration peaks (at 806, 1291, 1451, and 2580 cm^{-1}, corresponding to the S—O/S═O stretch) appeared in the FTIR spectrum of ESM after 3-MPA treatment, suggesting that the integrity of these bonds was broken. This strategy was practical in preparing a considerable amount of SEP mixture from ESM waste (5.95–6.51 mg/mL/egg, conventional and organic eggs, respectively). Considering that collagenous components may be degraded or lost during acidic treatment, the abundance of nonstructural bioactive proteins in SEPs should be promoted, as confirmed by proteomic analysis (Table S1). While our study primarily focused on the soluble proteins extracted from ESM after 3-MPA treatment, it is vital to acknowledge the potential utility of the undissolved ESM debris. This residue likely contains essential collagenous components, which are key structural proteins in biomaterial applications [52, 53]. These proteins could be pivotal in designing collagen-rich graft materials by providing spatial scaffolding that facilitates tissue ingrowth and vascularization [46, 54]. However, due to their insolubility, achieving an even distribution in cell culture media remains a challenge. Incorporating these insoluble proteins into hydrogels could offer a viable solution, particularly for in vivo studies where they could enhance the structural integrity and functionality of the biomaterial. Future research should explore the integration of these collagen-rich residues to expand the potential applications of ESM-derived biomaterials.

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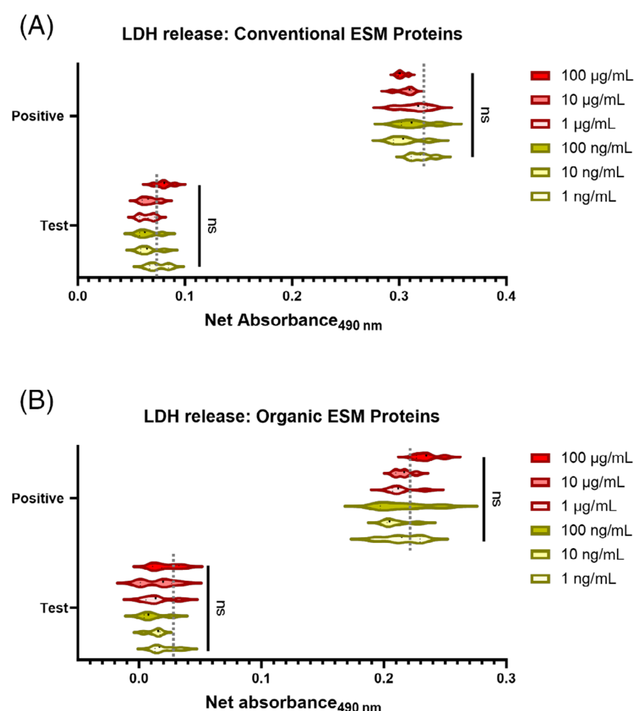

FIGURE 4 | LDH release of MC-3T3-E1 cells in the presence of conventional and organic SEPs for 24 h. (A) LDH release in the presence of conventional SEPs at different concentrations. (B) LDH release in the presence of organic SEPs at different concentrations. Left panel: Absorbance, LDH release during natural cultivation; right panel: absorbance, LDH release during positive cultivation (in the presence of 1% Triton X-100). ns, no significance ($p > 0.05$). Dotted lines, average absorbance of cell control without ESM proteins. Kruskal–Wallis rank-sum test and Dunn's multiple comparisons ($n = 5$).

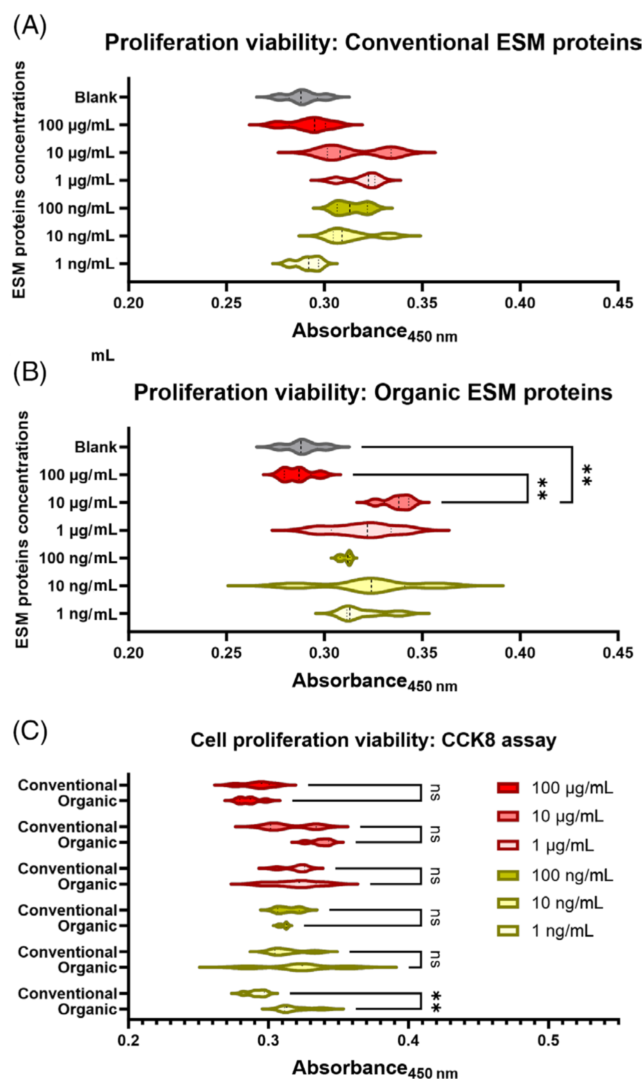

FIGURE 5 | Proliferation viability of MC-3T3-E1 cells in the presence of conventional and organic SEPs for 24 h. (A) Cell proliferation in the presence of conventional SEPs at different concentrations measured by CCK8 assay. (B) Cell proliferation in the presence of organic SEPs at different concentrations measured by CCK8 assay. (C) Comparison of cell proliferation in the presence of conventional/organic SEPs at specific concentrations. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$; ns, no significance ($p > 0.05$). Kruskal–Wallis rank-sum test and Dunn's multiple comparisons ($n = 5$).

Regarding different housing and feeding environments, organic eggs were considered "healthier" than conventional eggs. Proteomic analysis revealed that conventional and organic SEPs had different protein profiles but with partial overlapping in the proteins identified. This evidence indicates that while the core biological functions of SEP are consistent across different egg sources, there may be variations in functional priorities based on the specific proteins present. The differences in expression profiles could be attributed to the varying conditions under which conventional and organic eggs are produced.

Both conventional and organic SEPs demonstrated desirable biosafety profiles, indicating their suitability for biomedical applications. The biocompatibility of SEPs was confirmed through various assays, which showed that these proteins do not elicit adverse responses on cell integrity and proliferation (Figures 4 and 5). This consistent safety profile across different egg sources underscores the potential of SEP as reliable biomaterials for tissue engineering and regenerative medicine. Interestingly, although both SEPs exhibited excellent biocompatibility over a wide concentration range in short-term cultivation (< 24 h), SEP in relatively high concentration resulted in impaired extracellular collagen deposition and cell detachment (Figure 6). One possible explanation is that some proteins in SEPs may have growth-inhibitory effects or toxicity on mammalian cells at high concentrations during long-term cultivation. However, when SEPs were within a certain concentration range, such inhibition could reverse to promotion. This dose-dependent enhancement of ECM components may be attributed to the bioactive proteins containing the arginine–glycine–aspartic acid (RGD) motif [55]. Integrins on cell membranes recognize RGD peptides and mediates intracellular Ca^{2+} release, thereby promoting host osteogenesis [49, 56]. For the application of SEPs in tissue engineering, further optimization of protein concentration and biofunctions is essential.

In our previous work, amorphous calcium phosphate bone graft granules were prepared from eggshell waste, which presented excellent osteoinductivity and ideal biosafety [5]. For

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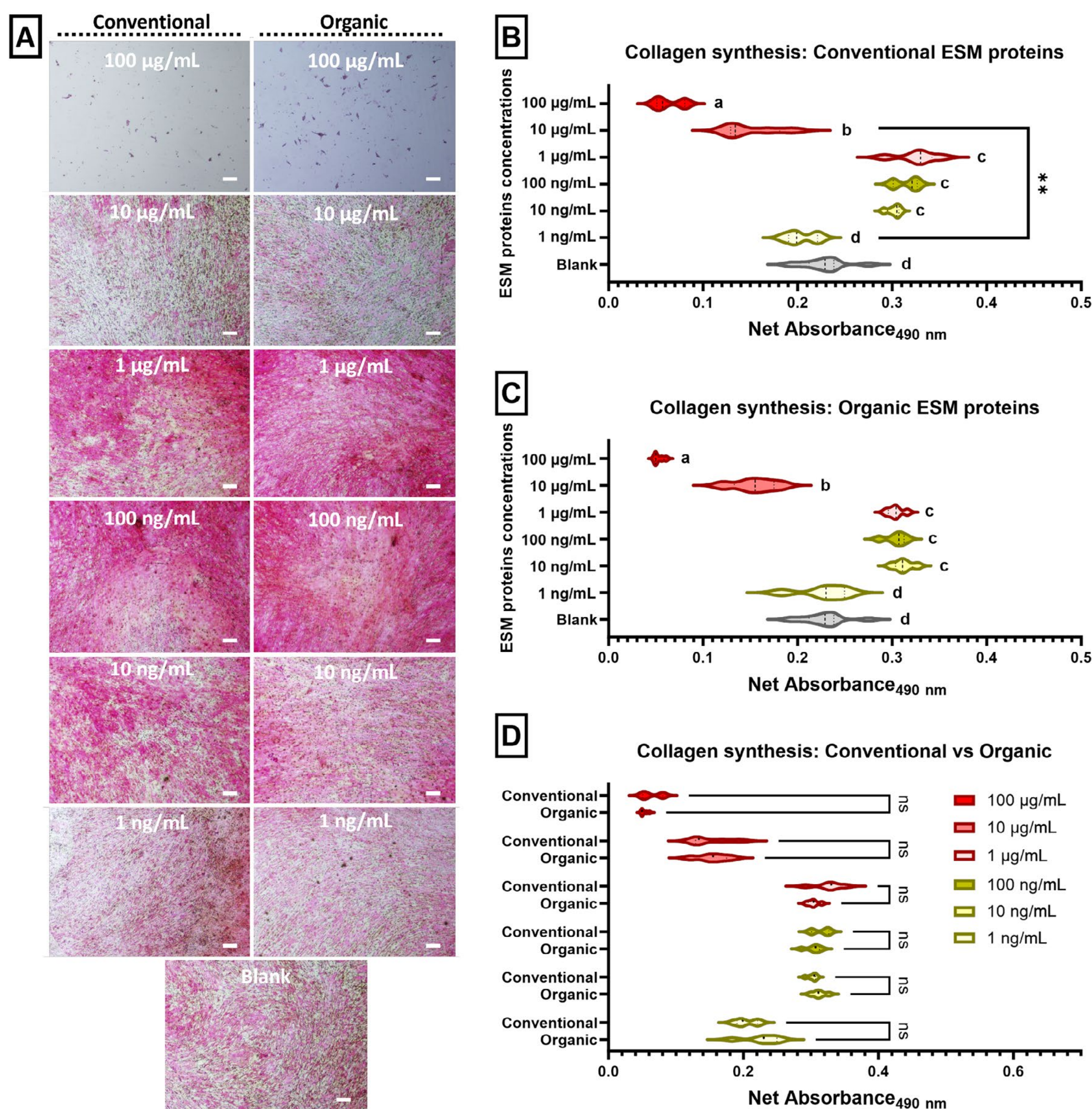

FIGURE 6 | Collagen synthesis of MC-3T3-E1 cells in the presence of conventional and organic SEPs for 21 days. (A) Sirius red staining for collagen synthesis from MC-3T3-E1 cells. (B) Semiquantitative measurement of collagen synthesis by elution assay in the presence of conventional ESM proteins. (C) Semiquantitative measurement of collagen synthesis by elution assay in the presence of organic ESM proteins. (D) Paired comparison of collagen synthesis in the presence of conventional and organic ESM proteins. Significance differences are indicated between labels a, b, c, and d ($p < 0.0001$); specific significance is indicated additionally. $^{**}p < 0.01$. ns, no significance, ($n = 6$). Scale bar = 100 μm.

better handling properties and spatial stability, we developed a novel cold-sintering technology to prepare a porous ACP scaffold with interconnected porosity (pore size: 300–550 μm, Figure S1). With an SEP coating layer, this bio-modified ACP scaffold was expected to have more desirable osteogenic activities, and the in vivo implantation study is ongoing. Utilizing the inorganic and organic components of eggshells is promising in establishing a comprehensive strategy for converting eggshell waste into multidimensional bone graft products. Moreover, because SEPs were used in cocktail form in this study, only their proteomic components and overall bioactivity were evaluated. Detailed work is still needed to clarify the biofunctions of individual proteins. For instance, ovotransferrin with high abundance in SEPs can significantly attenuate RANKL-mediated osteoclastogenesis and associated bone resorption while exerting antimicrobial, antiviral, and antioxidant properties [57, 58]. Therefore, developing an industrial technology for single protein extraction from ESM is also urgently needed. As for the application of SEPs cocktails or single ESM protein products, careful decisions should be made based on clinical evaluation and the protocol of personalized medicine. In addition, only organic and conventional eggs sold in Nordic countries were included in this study. It is necessary to broaden the research samples to more countries and regions to discover the universal laws of ESM protein composition and breeding conditions.

5 | Conclusions

The acidic 3-MPA strategy effectively alters the chemical composition of the ESM, thereby facilitating SEP extraction. The

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SEPs from conventional and organic eggs have different proteomic profiles but with partial overlapping. Both organic and conventional SEPs exhibited similar desirable biosafety and similar dose-dependent effects on osteoblastic ECM synthesis, suggesting that while different egg sources may contribute to the consistent core biological functions of protein products, they may also introduce different functional priorities. This discovery highlights the potential of transforming eggshell waste into high-value bioproducts. Although SEPs in organic and conventional eggs exhibit similar biological core functions, further research should include eggs sold in more countries and regions need to be clarify the impact of feeding conditions on ESM protein composition over a larger geographical scale.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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