RESEARCH ARTICLE

Methanol versus acetic acid extract effects on chicken eggshell membrane protein concentration, visualization and antioxidative potential

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Abstract:

Eggshell membrane (ESM) is a fibrous microstructure located between the eggshell's layers. ESM contains numerous protein constituents that serve its beneficial biological activities. Proteins such as ovalbumin, collagens, keratins, ovomucoid and ovocleidin-116 have been extracted from ESM. However, ESM is extremely insoluble in water and has poor stability. Solvents are often used in the extraction process; thus, this study aims to extract and compare the effect of methanol and acetic acid on proteins from chicken ESM. Findings from this study demonstrate differences in methanol compared to acetic acid in ESM protein extraction. ESM protein concentration from acetic acid (1.14 mg/ml) is slightly higher than methanol (1.01 mg/ml). The SDS-PAGE result shows both solvents can extract proteins from ESM with different bands intensity. Both solvents show moderate antioxidative activity compared to L-Ascorbic acid as a control in the FRAP assay. This study shows the potential of both solvents to extract and maintain the bioactivity of proteins from ESM. However, further purification and quantitative studies are needed to completely identify protein fragments isolated by both solvents to be used in future applications such as medicine, dentistry, and food industry.

Keywords: eggshell membrane, protein, antioxidant, ESM, SDS-PAGE, FRAP

1. INTRODUCTION

Eggs are the most largely consumed source of protein throughout the world. The hard part of eggshells is made mostly from calcium carbonate (Rath et al., 2016). However, eggshells have several layers, including the eggshell membrane (ESM). It contains over 500 protein constituents, such as collagen, ovalbumin, glycoproteins and many more (Kulshreshtha et al., 2022). These proteins are vital to the physiology of the egg itself and contributes to the safety for the maturation process of the embryo, including the safety from inflammatory process induced by free radicals such as reactive oxygen species (ROS). Fortunately, organisms have their own ways of fighting inflammation, one of which is the presence of proteins with antioxidative potential. These proteins neutralize free radicals, eventually halting cell damage and death (Xianhai Li et al., 2020). Once hydrolyzed, peptides from food and non-food sources possess a certain degree of bioactivity (Daliri et al., 2018). Research has shown that ESM proteins are of notably high quality, and their significance has been progressively recognized by the scientific community (Zhao et al., 2019). Prominent wound-healing properties of ESM has been well documented in literature (Xiaoyun Li et al., 2019; Wedekind et al., 2017).

The overabundance of eggshell waste often results in its disposal in landfills without pre-treatment to accelerate its decomposition (Amasuomo & Baird, 2016). In turn, this causes accumulation and, eventually, environmental spillage. Conversion of waste back into society is not something new by scientists and researchers (Shaari et al., 2018). As mentioned above, ESM contains several proteins with selected bioavailability that can be utilized and reused into various proteins, especially in the medical field. A study had successfully profiled the proteins present in ESM, however, there are differences in the extraction protein due to solvents used (Rath et al., 2016). In this research, the two most frequently utilized solvents for extractions, namely methanol and acetic acid, were employed. Methanol is a relatively popular extracting method and has even been applied in phytochemical extraction methods from plants. On the other hand, acetic acid is a gentle acid used in decalcifying the hard shell (Zhang et al., 2016). To evaluate the efficacy of the extraction methods, a preliminary protein quantification was carried out using Bradford's assay. After that, Sodium

Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was used to separate and visualize the protein. Finally, an antioxidant power assay utilizing Ferric Ions Reducing Antioxidant Power (FRAP) was used to evaluate its antioxidative potential.

2. MATERIALS AND METHODS

2.1 Materials

Empty chicken (*Gallus gallus domesticus*) eggshells waste was obtained from restaurants and households around Puncak Alam, Selangor. Chemicals used were sodium chloride (NaCl), ethylenediaminetetraacetic acid (EDTA), sodium hydroxide (NaOH), methanol, acetic acid, Bradford reagent, bovine serum albumin (BSA), acrylamide mix, sodium dodecyl sulfate (SDS), Tris base powder, tetramethyl ethylene diamine (TEMED), ammonium persulfate, bromophenol blue, glycerol, glycine, Coomassie blue powder, 170kDa protein ladder, Millon reagent, and ninhydrin.

2.2 Eggshell Decalcification

Briefly, the eggshells were rinsed with distilled water to remove any remaining impurities, such as dirt, on their surface. Then, it was washed in 1 M NaCl and soaked for 2 hours with 100 mM EDTA at pH 8.5. The washed eggshells were left to dry at room temperature (25°C). After that, the dried eggshells were smashed quickly and stirred for 15 minutes at a solid/water ratio of 1:10 (Zhang et al., 2016). The lighter ESM floated on the water surface was collected on a sieve and separated for drying. Ten gram of ESM powder was solubilized in 200 ml NaOH. This is the "crude" ESM.

2.3 Methanol Fractionation

A method by Ahmed et al. (2017) was followed with minor modifications. Ten grams of ESM was mixed with 200 ml of 0.1% acetic acid acidified 70% methanol. The mixture was allowed to sit for 4 days at 25°C with continuous stirring. Then, it was centrifuged at 13000 rpm for 60 minutes. The precipitated ESM was taken and stored at 4℃ for other experimental protocols. This is the "methanol-extracted ESM".

2.4 Acetic Acid Extraction

The obtained ESM powder was mixed with 4M acetic acid with a ratio of 1:8 (w/v) and allowed to sit for 4 days at continuous stirring (Ponkham et al., 2011). The mixture was centrifuged at 15000 rpm for 30 minutes. The process was repeated three times. The precipitated compound was labelled as "acetic acid-extracted ESM".

2.5 Protein Quantification

A known molecular weight Bovine Serum Albumin (BSA) was used as a protein standard to generate a standard curve for determining an unknown protein sample (0.2-1.6 mg/ml). Absorbance values were plotted 595 nm (y-axis) versus their concentration in mg/ml (x-axis). The protein samples were mixed with the Bradford reagent in a 1:1 ratio and incubated at room temperature (20-25°C) for 5 minutes in a plastic disposable cuvette. Calorimetric optical reading was taken three times (triplicate) using a spectrophotometer at 595 nm wavelength.

2.6 Sodium Dodecyl Polyacrylamide Gel Electrophoresis (SDS-PAGE)

A standard procedure was followed to visualize the proteins extracted using both methods (Rath et al., 2016). Briefly, 12% polyacrylamide gels were used to separate the proteins and 4X Laemlli's loading buffer was loaded in the ratio of 1:3 to the protein sample. The mixtures were heated on a dry heating at 95°C for 5 minutes. Loading the wells were as follows:

1. 170 kDa protein marker was used as a protein standard.
2. The control, crude, and fractionate were loaded at varying concentrations of 4, 6, 8, and 10 µg.

A 120V constant current was applied to the gel for 60 minutes until the band's ends were observed at the bottom of the gel. The gels are submerged into Coomassie Brilliant Blue R-250 solution for 3-4 hours and de-stained overnight in acidified methanol until the separated protein bands could be seen clearly. GelDoc was used to visualize the gels and each protein band was analyzed using ImageJ software.

2.7 Ferric Ions Reducing Power (FRAP) Assay

A method by Singhal et al., (2011) was followed with minor modifications. Briefly, phosphate buffer and potassium ferricyanide were added to the protein samples. The mixture was incubated in a water bath with a temperature of 50°C for 20 minutes. Then, trichloroacetic acid (TCA) was added to the mixture. It was then subjected to centrifugation at 3000 rpm for 10 minutes. The supernatant was taken and mixed with freshly prepared ferric chloride and transferred into a plastic cuvette. A spectrophotometer was used to measure it's absorbance at 700 nm wavelength. Ascorbic acid (1000-50 µg/ml) was used as the standard to compare it activity.

3. RESULTS AND DISCUSSION

3.1. ESM Protein Potential

Spectrophotometric protein quantification analysis was used to quantify protein concentration using a Bradford assay

protocol. The colorimetric assay is based on the binding of protein molecules to coomassie dye stimulated by an acidic environment, resulting in a change of colour measured as absorbance. BSA was used to calculate the protein concentration of an unknown sample (Figure 1).

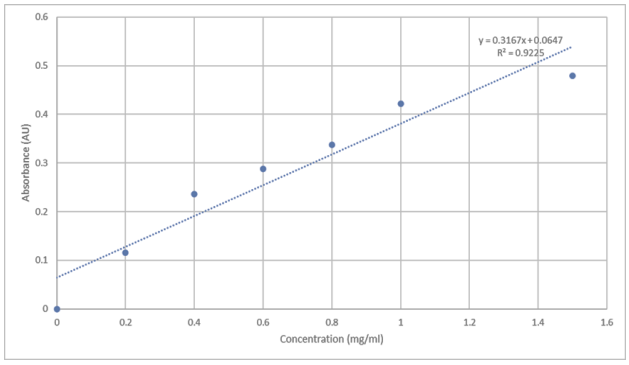

Figure 1. Graph of absorbance at 595 nm against BSA standard protein concentration (mg/ml)

Table 1. Protein concentration from different samples based on absorbance reading at 595 nm.

Sample	ΔAbsorbance (mean ± S.E.M.; 595nm)	Protein Concentration (mg/ml)
Eggshell crude	0.17333 ± 0.001202	0.34
Methanol-extracted eggshell	0.18167 ± 0.003712	0.37
Acetic acid- extracted eggshell	0.19733 ± 0.002167	0.42
ESM crude	0.18700 ± 0.006928	0.39
Methanol-extracted ESM	0.38300 ± 0.000577	1.01
Acetic acid-extracted ESM	0.42544 ± 0.000242	1.14

Data are pooled for all triplicate reading and summarized as mean ± S.E.M.

Table 1 shows that both solvents protocol yielded higher protein concentrations compared to the crude ESM with acetic acid-extracted ESM being the highest with 1.14 mg/ml of protein. This result suggests that both solvents are effective for ESM proteins extraction. This finding also matches previous study which found that both methanolic and acetic acid extracts had good efficiency to detect the presence of functional protein molecules in ESM (Kumer, 2017). The principle of methanolic extraction was the solubilization which had promoted ESM solubility through chemical degradation of peptide bonds and the associated cross-linkages (Ahmed et al., 2017). On the other hand, Husnaeni et al. (2019) suggested acetic acid reacts and hydrolysed the primary structure of the proteins. Swelling affect by pH value was one of the elements that can diminish the ability of an internal molecular structure to bind and promote protein unfolding by disrupting non-covalent bonding. The difference in molecular extraction approaches is taught to be the reason why acetic acid yielded higher protein concentration than methanol which is a much harsher solvent.

It is also notable that crude eggshell contains the least amount of extracted protein (0.34 mg/ml). This shows that proteins are more abundant in ESM than eggshells. The composition of eggshell consists primarily of calcium carbonate ($CaCO_3$) which is 95% and only 3.5% is an organic matrix including glycoproteins, proteins, and proteoglycans (Cordeiro & Hincke, 2011). In contrast, ESM contains 69.2% protein, 2.7% fat, 1.5% moisture, and 27.2% ash (King`ori, 2011). Furthermore, it is also high in proline, glutamic acid, glycine, and cysteine (Santana et al., 2016).

3.2. ESM Protein Expression

SDS-PAGE is a semi-qualitative technique used to separate and visualize protein mixture in any biological samples based on their size. The relative mobility of charged molecules influences protein separation in the sample. Because there is minimal resistance during electrophoresis, relatively small molecules move faster, leaving the larger molecules at the top of the gel. In this study, the electrophoretic profiles of the crude, methanol-extracted, and acetic acid-extracted ESM proteins were visualized at varying concentrations such as 4, 6, 8, and 10 µg. A 15 to 110 kD protein ladder was used as a reference for the molecular weight (MW) of separated proteins.

Both gels showed no protein bands in crude ESM sample (Figure 2 and 3). This indicates no protein was able to be extracted using the decalcification step. In contrast, methanol and acetic acid ESM extracts revealed proteins with prominent band intensity. The relative intensity of produced bands is used to estimate protein abundance. Methanol-extracted ESM showed higher protein abundance visualized by dense bands compared to acetic acid-extracted ESM. A much smaller protein at ~16.6 kDa was detected in the methanol-extracted ESM. In contrast, acetic acid did not manage to extract the smaller proteins. Both stained gels showed various protein bands at different molecular weights that indicates lower purity of protein samples.

This is as expected due to the absence of a purifications step and abundance of proteins present in ESM including lysozyme, polyubiquitin-B ubiquitin, ovocalyxin-36 precursor, and structural proteins such as keratins. These proteins play specific roles in the physiology of the egg itself. For instance, ubiquitin is a 76-amino-acid protein that aids in

the breakdown of a variety of cytosolic, nuclear, and ER (endoplasmic reticulum) proteins (Alves-Rodrigues et al., 1998). The ovocalyxin-36 precursor is involved in hyaluronate lyase activity while keratin, type 1 cytoskeletal is a structural constituent of the cytoskeleton in ESM (Kaweewong et al., 2013). Extensive research showed that antimicrobial proteins of ESM, such as lysozyme, defend the embryo of the egg until it produces its immunoglobulins and are also effective against viruses and some toxins (Liu et al., 2013). Concurrently, this study could not really point out all the proteins present in ESM compared to more advanced extraction and purification methods described in other literatures. It is also important to note that ESM can easily lose quality due to storage, gaseous environment, humidity, and temperature (Qiu et al., 2012).

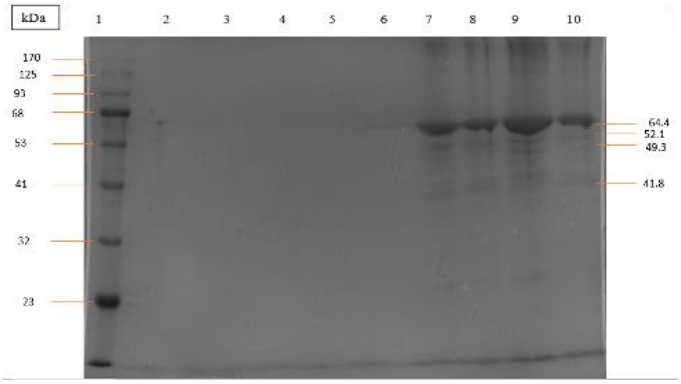

Figure 2. SDS-PAGE of the eggshell matrix protein extracted by methanol. Lane 1, protein marker; Lane 2, control (methanol); Lane 3, 4µg of crude ESM; Lane 4, 6µg of crude ESM; Lane 5, 8µg of crude ESM; Lane 6, 10µg of crude ESM; Lane 7, 4µg of methanol-extracted ESM; Lane 8, 6µg of methanol-extracted ESM, Lane 9, 8µg of methanol-extracted ESM; Lane 10, 10µg of methanol-extracted ESM.

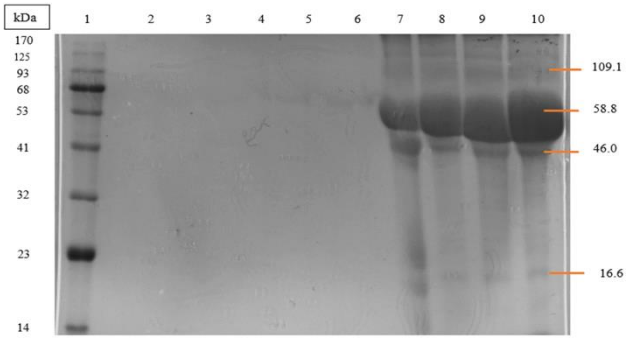

Figure 3. SDS-PAGE of the eggshell matrix protein extracted by acetic acid. Lane 1, protein marker; Lane 2, acetic acid as control; Lane 3, ESM crude 4 µg; Lane 4, ESM crude 6 µg; Lane 5, ESM crude 8 µg; Lane 6, ESM crude 10 µg; Lane 7, acetic acid extracted ESM 4 µg; Lane 8, acetic acid extracted ESM 6 µg; Lane 9, acetic acid extracted ESM 8 µg; Lane 10 acetic acid extracted ESM 10 µg.

3.3. ESM Proteins Antioxidative Potentials

Reactive oxygen species (ROS) such as superoxide anions produced by physiological metabolisms will react with proteins, DNA, and other molecules in the body. In turn, a level of oxidative damage is expected to cells and tissue (Chakrabarti et al., 2014). Accumulation of ROS and constant cellular damage will eventually cause mutations resulting in chronic disease manifestations such as cancer. Antioxidative proteins and peptides have been shown to neutralize these conditions (Shi et al., 2014). ESM is one of the greatly researched materials that possess antioxidative properties used in the field of tissue engineering.

In this study, FRAP assay was used to evaluate the antioxidant potential in methanol-extracted and acetic acid-extracted ESM (Figure 4 and 5). Its principle is based on the ability of a compound to donate an electron to free radicals to convert them to stable components which is referred to as reducing power (Jain and Anal, 2017). A known standard which is L-ascorbic acid was used to compare its antioxidative capacity. The effect of solvents on extracted ESM sample is shown in Figure 4. A dose-dependent response to its reducing power was obtained in which the highest reducing power (10%) of both methanol and acetic acid extracted ESM was at the concentration of 50 mg/ml. It can also be seen that L- ascorbic acid surpassed both extracts in every concentration used.

Based on the result, both ESM extracts showed moderate reduction of power activity against ferric ions. The notable feature of ESM as an antioxidative agent is validated. The findings from this study suggested that ESM extract could be a new functional supplement that acts as a beneficial antioxidant against oxidative stress (Shi et al., 2014).

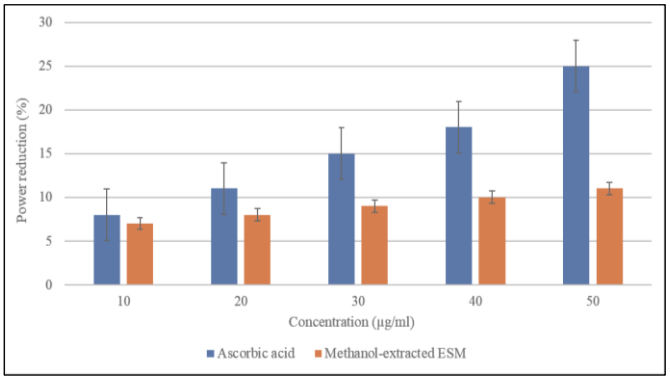

Figure 4. FRAP assay of the ESM samples extracted by methanol as compared to L-ascorbic acid as standard.

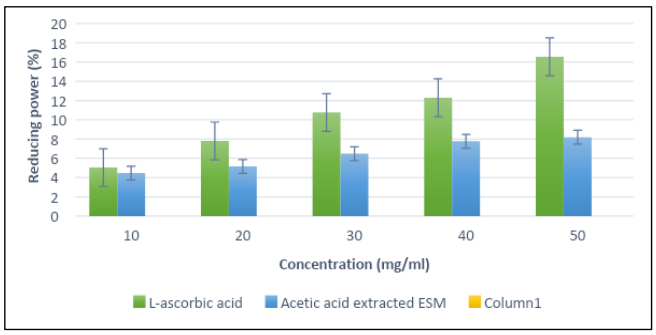

Figure 5. FRAP assay of the ESM samples extracted by acetic acid as compared to L-ascorbic acid as standard.

ROS generation typically reduces antioxidant enzymes, upregulates the MAPK signaling pathway and its associated cytokines production. When administered orally, ESM exhibits antioxidative potential by upregulating the antioxidant enzyme SOD1 and downregulating the MAPK signaling pathway and the production of proinflammatory cytokines IL-1, IL-6, and TNF-α (Sim et al., 2023).

4. CONCLUSION

ESM is a good source for a variety of biological proteins and can be used as an antioxidant. In conclusion, methanol and acetic acid are both versatile as an extraction platform solution that can extract a variety of proteins from ESM. These methods could also be applied to other eggshells or any other biological samples. From the extracted protein sample, a comprehensive study and manipulation could be done to further investigate its potential to be used as an agent of different bioactivity properties. An efficient and rapid method for solubilizing and extracting protein with a high yield was established. From this study, the ability to separate ESM from the eggshell itself successfully plays a vital role in the extraction of beneficial proteins with bioactivities. Both ESM extracts in this study show moderate antioxidative potential that could be contributed by the presence of abundant proteins found. Studies have shown hatched ESM proteins hydrolyzed into peptides reduced the level of inflammatory proteins such as ROS, matrix metalloprotease 3 (MMP3), MMP13, and IL-6 in a hydrogen peroxide induced human chondrocytes (Zhu et al., 2022). However, advanced purification techniques are needed to purify and isolate specific protein of interest to be studied and manipulated into beneficial products.

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