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EFFECT OF ENZYMATIC HYDROLYSIS ON THE STRUCTURAL AND PROTEIN SOLUBILITY OF EGGSHELL MEMBRANE

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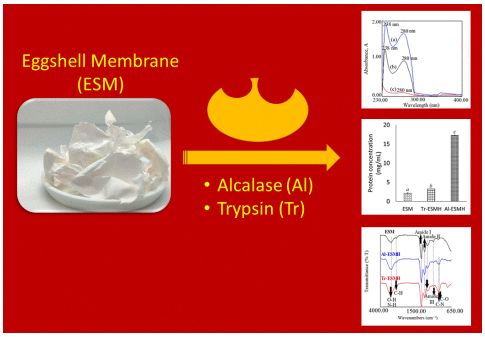
Graphical abstract



Abstract

Eggshell membrane (ESM) is considered food waste or a by-product of the egg processing industry. It has huge application potential in the engineering, nutraceutical, pharmaceutical, and cosmeceutical sectors. Recently, researchers have shown an increased interest in ESM hydrolysate (ESMH) from enzymatic hydrolysis in biomaterial applications. Nonetheless, there is lacking of information on the characteristics of ESMH. Thus, this study aims to characterise ESMH attained from enzymatic hydrolysis. The ESM was manually separated from the eggshell, and then subjected to enzymatic hydrolysis using two (2) different enzymes (alcalase (Al) and trypsin (Tr)). The alcalase-treated ESMH (Al-ESMH) and trypsin-treated ESMH (Tr-ESMH) were then characterised by using Ultra-violet Visible Spectroscopy (UV-Vis) and Fourier Transform Infrared Spectroscopy (FTIR). UV spectra displayed two intense absorbance peaks; 238 nm and 280 nm. The second peak at 280 nm was used to quantify the soluble protein in ESMHs. The results showed that the Al-ESMH had higher protein solubility (17.25±0.11 mg/mL) than the Tr-ESMH (3.28±0.11 mg/mL) and ESM (2.11±0.19 mg/mL). The FTIR spectra of ESMHs prove the presence of peptides for both treated ESM samples after enzymatic hydrolysis.

Keywords: Eggshell, eggshell membranes, hydrolysates, enzymatic hydrolysis, protein solubility

Abstrak

Membran kulit telur (ESM) dianggap sebagai sisa makanan atau hasil sampingan industri pemprosesan telur. Ia mempunyai potensi aplikasi yang besar dalam sektor kejuruteraan, nutraseutikal, farmaseutikal dan kosmeseutikal. Baru-baru ini, penyelidik telah menunjukkan minat yang semakin meningkat dalam ESM hidrolisat (ESMH) dari hidrolisis enzimatik dalam aplikasi biobahan. Namun begitu, terdapat kekurangan maklumat mengenai ciri-ciri ESMH. Oleh itu, kajian ini bertujuan untuk mencirikan ESMH yang

diperoleh daripada hidrolisis enzimatik. ESM diasingkan secara manual dari kulit telur, dan kemudian dihidrolisis secara enzimatik dengan menggunakan dua (2) enzim berbeza (alkalase (Al) dan tripsin (Tr)). ESMH yang dirawat alkalase (Al-ESMH) dan ESMH yang dirawat dengan tripsin (Tr-ESMH) kemudiannya dicirikan dengan menggunakan *Ultra-violet Visible Spectroscopy* (UV-Vis) dan *Fourier Transform Infrared Spectroscopy* (FTIR). Spektrum UV memaparkan dua puncak penyerapan yang ketara; 238 nm dan 280 nm. Puncak kedua pada 280 nm telah digunakan untuk mengukur protein larut dalam ESMH. Keputusan menunjukkan bahawa Al-ESMH mempunyai keterlarutan protein yang lebih tinggi (17.25±0.11 mg/mL) daripada Tr-ESMH (3.28±0.11 mg/mL) dan ESM (2.11±0.19 mg/mL). Spektrum FTIR ESMH membuktikan kehadiran peptida untuk kedua-dua sampel ESM yang dirawat selepas hidrolisis secara enzimatik.

Kata kunci: Kulit telur, membran kulit telur, hidrolisat, hidrolisis enzimatik, keterlarutan protein

1.0 INTRODUCTION

Production of eggs worldwide has reached 5.8 x 10^{10} kg/year with approximately 167 of eggs consumption per person/year [1, 2]. It has been reported that the consumption of eggs in Malaysia has increased from 2.2 kg/ capita (1961) to 17.9 kg/ capita (2017). This consumption has superseded the Americans' consumption (15.6 kg/ capita) in the year 2017. In addition, China is not only the world's second-largest economy, but it has also ranked the highest in egg consumption (22.7 kg/ capita) in year the 2017 [3].

Subsequent to the trend of increasing egg consumption throughout the world, it has generated a huge amount of eggshell (ES) waste, and the egg industry is commonly abandoned and dumped the waste in landfills. Huge amount of ES waste is not favourable due to the limitation of handling at the landfill sites. In addition, eggshell membrane (ESM) is high in protein, it can easily attract rats and pests. Therefore, the disposal and management of the ES waste is costly [4, 5]. Currently, there is no estimated cost available for the ES waste management at the landfills in Malaysia, nevertheless, the cost of ES waste management for an egg processing factory in the United States of America is approximately around USD100,000 per annum [6].

ESM is part of the ES waste generated by the egg industry. This waste has huge application potential in engineering [7], nutraceutical [8, 9], pharmaceutical [10], and cosmeceutical [1] sectors. A vast number of ESM applications are due to the presence of cross-linked collagen, glycosaminoglycans (GAGs), egg white proteins, eggshell matrix proteins, uronic acid, and sialic acid [10, 11].

Besides the above-mentioned properties in the ESM, its FTIR characteristic has been reported but limited FTIR spectra is available for the enzymatically treated ESM [12-14]. In addition, a huge number of disulfide crosslinks in ESM structure have made it insoluble [15]. This non-solubilised nature of ESM has limited the UV-vis characterisation for the ESM [15-17].

Lately, there has been an increasing interest in ESM hydrolysate (ESMH) obtained through enzymatic hydrolysis [9, 10, 18], especially in the field of biomaterial application studies [19-21]. This is seen to be the solubilised ESM hydrolysate that can be reformed into bioactive materials according to desired shapes and sizes [15]. In addition to this, ESMH has a larger global market share with better biological activities than the ESM [9, 18].

Despite FTIR characterisation of ESM, so far, very little attention has been paid to the characteristic of ESM after enzymatic treatments including its protein solubility [10, 22]. Most of the reported characterisation of ESM after enzymatic hydrolysis was not carried out by using FTIR and UV-Vis [23, 24]. This suggests a need to further understand the chemical and molecular fingerprints of ESMH after enzymatic hydrolysis. Therefore, the purpose of this study is to characterise the enzymatically hydrolysed ESMHs by using UV-Vis and FTIR methods.

2.0 METHODOLOGY

2.1 Preparation of Eggshell Membrane (ESM)

The ESM was prepared according to the previously published protocol with slight modifications [22]. Eggs (NutriPlus) were purchased from a local supermarket (Giant) in Kuala Pilah, Negeri Sembilan, Malaysia. The eggshell (ES) was collected (after discarding the egg white and yolk) and washed thoroughly with tap water. The ES was dried in an oven (Shin Saeng Scientific Co. Ltd, South Korea) for 2 days at 37°C before weighing. The ES was placed in a beaker of simmering hot water (95 to 97°C) on a hot plate (HPS-7C Ceramic Hotplate Stirrer, USA) and heated for 15 minutes. Then, the ES was cooled by transferring them

into distilled water at 27°C (room temperature) after simmering. The ESM was carefully separated from the ES by manual peeling. The ESM was dried in the oven at 37°C for 2 days. The oven-dried ESM was ground into small particles using a grinder (Nima electric grinder, NM-8300, Japan). The fine powder of ESM was sieved through 0.25 mm sieve by using a siever (Endecotts Limited Sieves, London England). Then, the sieved ESM was recorded and stored in an airtight container separately until further use [22].

2.2 Preparation of Alcalase Treated Eggshell Membrane Hydrolysates (Al-ESMH) and Trypsin Treated Eggshell Membrane Hydrolysates (Tr-ESMH)

The enzymatic hydrolysed ESMHs were prepared by using enzymes, namely alcalase (Al) (Merck) and trypsin (Tr) (Biobasic) according to the previously published protocol with slight modifications [22]. The dried ESM (5 %w/w) samples were heated at 50°C in distilled water for 15 min by using a water bath shaker (Wise Bath, WSB-30 WITEG, Germany). The samples were adjusted to the pH 8.0 at 55°C with 1 mol/L HCl (R&M Chemicals) or 1 mol/L NaOH (R&M Chemicals) before hydrolysing with 2 %(w/w) of Al and 2 %(w/w) of Tr separately for 7 hours at 120 rpm. The enzymatic reaction for both Al-treated eggshell membrane hydrolysates (Al-ESMH) and Tr-treated eggshell membrane hydrolysates (Tr-ESMH) were stopped by transferring both ESMH samples to a pre-heated water bath shaker at 95°C with 120 rpm for 10 min. The ESMH solutions were cooled down to 30°C before dividing into two portions. One portion was freeze-dried and another portion was centrifugated at 4500 g for 20 min (Centurion Scientific K2042, United Kingdom). After the centrifugation process, the supernatants were collected. The freeze-dried ESMHs and collected supernatants were stored at 4°C for future use [22]. In addition, the ESM soaked with distilled water at 55°C for 7 hours without the addition of any enzyme was used as a control sample.

2.3 Spectral Data and Protein Quantitation using Ultraviolet-visible Spectroscopy (UV-Vis)

The ESM was mixed with distilled water (5 %w/v) and the ESM mixture was processed according to the Al-ESMH preparation except the alcalase was replaced with distilled water. The ESM and ESMHs supernatant were then subjected to the ultraviolet-visible (UV-Vis) analysis. The ESM was used as a control to study the changes of the ESM after enzymatic treatments. The UV-Vis spectra were observed over the wavelength range from 190 to 400 nm with quartz cuvettes using a spectrophotometer (PG Instruments Ltd, T80+ UV/VIS Spectrometer, United Kingdom). The liquid samples were diluted in distilled water by 10 times, and the supernatant of the samples (Al-ESMH, Tr-ESMH, and ESM) were analysed. All spectra were recorded with measurement settings as follows: spectral bandwidth of 2.00 nm, an interval of 1.00 nm, slow speed of scanning, and 4 times smoothing [25].

Protein quantitation was also carried out by using UV–Vis absorption spectroscopy (PG Instruments Ltd, T80+ UV/VIS Spectrometer, United Kingdom). Quartz cuvette (1-cm-long) was used to load the samples and the absorbance at 280 nm was recorded for the quantification of protein concentration with five standards (0.4, 0.8, 1.2, 1.6, and 2.0 mg/mL). The standards were prepared from bovine serum albumin (BSA) lyophilised powder (Sigma-Aldrich) [26, 27]. Blank samples were prepared according to the preparation of Al-ESMH and Tr-ESMH without the addition of ESM. All the samples were diluted 10-fold with deionised water and vortexed. The diluted samples were analysed in triplicate.

2.4 Fourier Transform Infrared Spectroscopy (FTIR)

Infrared spectrum for the ESM and the freeze-dried ESMH samples (Al-ESMH and Tr-ESMH) were generated by Fourier Transform Infrared (FTIR) spectroscopy. The sample preparations were done by placing one (1) mg of the sample onto the small crystal area. The mode of the FTIR spectroscopy is Attenuated Total Reflectance (ATR); model Spectrum 100 (Perkin Elmer, United States). All spectra were recorded within a range from 650 to 4000 cm^{-1} at 4 cm^{-1} resolution in 32 scans with 4 times smoothing. The results were recorded in transmittance units [28].

3.0 RESULTS AND DISCUSSION

3.1 Yield of Eggshell Waste

In this study, eggshell membrane (ESM) was obtained from eggshell (ES). The ES was washed, dried, and weight before being treated with hot water. The hot water treatment eased the manual peeling process of ESM from the ES. Table 1 shows the yields of ES and ESM. The yield of the ES per egg is 11.04 %(w/w) and the yield of the ESM per egg is merely 0.47 %(w/w). Since the ES is considered an agricultural waste and is usually discarded [4], if a recovery process of the ESM could be done from the ES, it is expected that the recovery of the ESM is approximately 4 %(w/w) (Table 1).

Table 1 Yields of eggshell (ES) and eggshell membrane (ESM)

Eggs (*n*=10)	
Weight per egg (g) (mean±SD)	50.71±1.13
Weight of ES per egg (g) (mean±SD)	5.60±0.53
Weight of ESM (g) (mean±SD)	0.24±0.07
Yield of ES per egg (g) %(w/w)	11.04
Yield of ESM per ES (g) %(w/w)	4.29
Yield of ESM per egg (g) %(w/w)	0.47

The yield of ESM reported in the current study (4 %) is within the range (6 %±3) of ESM recovery study from ES waste through acetic acid and foam separator system [14].

3.2 Ultraviolet-Visible Spectroscopy (UV-Vis) Characterisation and Protein Quantitation

The absorbance of the enzymatically treated ESM was studied as a function of wavelength via UV-Vis spectroscopy in the current study. The spectra result for the Al-ESMH, Tr-ESMH, and ESM solutions obtained from the UV-Vis analysis can be compared in Figure 1.

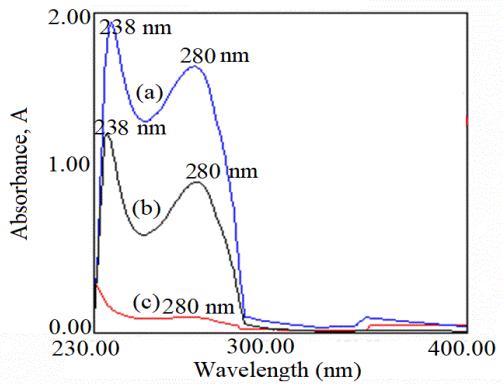


Figure 1 The UV-Vis spectra of (a) Al-ESMH, (b) Tr-ESMH and (c) ESM non-freeze-dried supernatant

There are significant peak shifts at 238 and 280 nm. It can be seen from the spectra that the ESM absorbance increased after the hydrolysis process with the Al-ESMH having the highest absorbance peak at wavelength 238 nm. Trypsin hydrolysis has also intensified the absorbance of this peak. Nevertheless, very little was found in the literature on the peak of 238 nm for the ESMH. Several attempts were done to figure out the possible substance at which the absorbance occurs. It was done by looking into the UV-Vis spectra of several other primary compounds present in the ESM and ESMH such as collagen, dermatan sulfate, hyaluronic acid, desmosine, and sialic acid [11, 29], however, no such absorbance was reported in the region around 238 nm.

A further study on this peak was carried out by investigating alcalase and trypsin subjected to the preparations of the Al-ESMH and Tr-ESMH without ESM. UV-Vis spectra (Figure 2) reveal that the unknown 238 nm absorbance peaks for the ESM after the enzymatic hydrolysis treatments in Figure 1 may most likely cause by the compounds present in alcalase and trypsin enzymes.

UV-Vis spectrum peak at 280 nm is expected and usually used to quantitate soluble protein concentration. Since the ESM is not well-dissolved in water, the UV-Vis spectrum peak for the ESM at 280 nm is not obvious. However, the peaks were clearly observed for the enzymatically treated ESM samples after blank subtraction, namely Al-ESMH and Tr-ESMH solutions as shown in Figure 1. This indicates the enzymatic hydrolysis enhances the solubility of the ESM with the existence of soluble protein with aromatic rings amino acids such as tyrosine (Tyr) and tryptophan (Trp), the solubilised ESM is useful for further application in engineering, nutraceutical, pharmaceutical, and cosmeceutical industries. These two aromatic amino acids, Tyr and Trp are usually located at the hydrophobic centre of the protein structure, the detection of these amino acids indicates that the peptide's microenvironment was weakened during enzymatic hydrolysis [30]. Thus, quantitation of soluble protein is usually done by using a spectrophotometer to measure the absorbance at 280 nm [31].

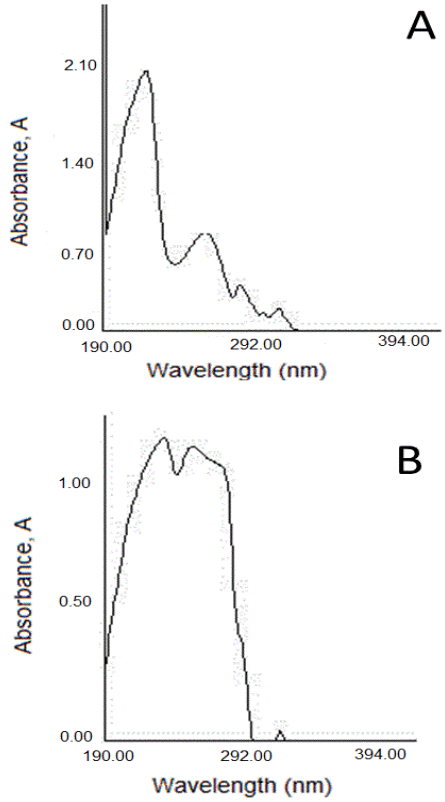


Figure 2 UV-Vis spectra of (A) alcalase and (B) trypsin subjected to the preparations of the Al-ESMH and Tr-ESMH without ESM

By plotting of the absorbance at 280 nm versus bovine serum albumin (BSA) concentration (Figure 2) in this study, a calibration curve was derived for quantifying the soluble protein concentration of the ESM, Al-ESMH, and Tr-ESMH. Figure 3 shows the standard curve for protein quantitation (R^2=0.9948) with different BSA concentrations ranging from 0.0 to 2.0 mg/mL.

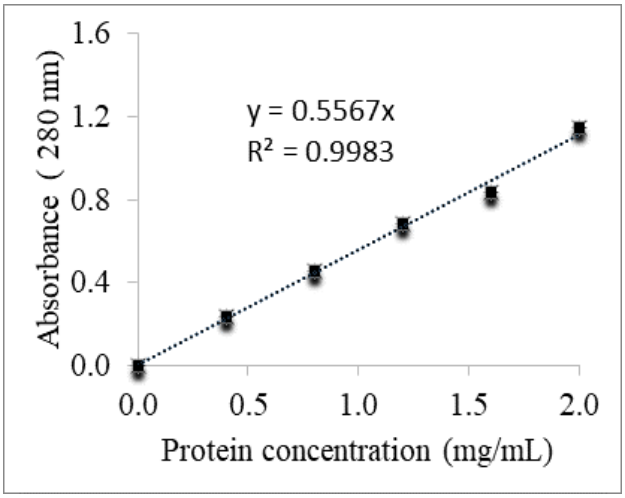


Figure 3 Standard curve for protein quantitation by using different BSA concentrations (0.0, 0.4, 0.8, 1.2, 1.6 and 2.0 mg/mL) at 280 nm

The bar chart below shows the soluble protein concentration of the ESM, Al-ESMH, and Tr-ESMH supernatant samples (*n*=3) after 7 after hours of respective treatment at 50°C with 120 rpm agitation in a water bath. The protein concentrations reported for the Al-ESMH, Tr-ESMH, and ESM solutions were after blank subtraction with the respective enzymes. The Al-ESMH has a greater soluble protein concentration (17.25±0.11 mg/mL) in comparison to the Tr-ESMH (3.28±0.11 mg/mL) and ESM (2.11±0.19 mg/mL) as shown in Figure 4.

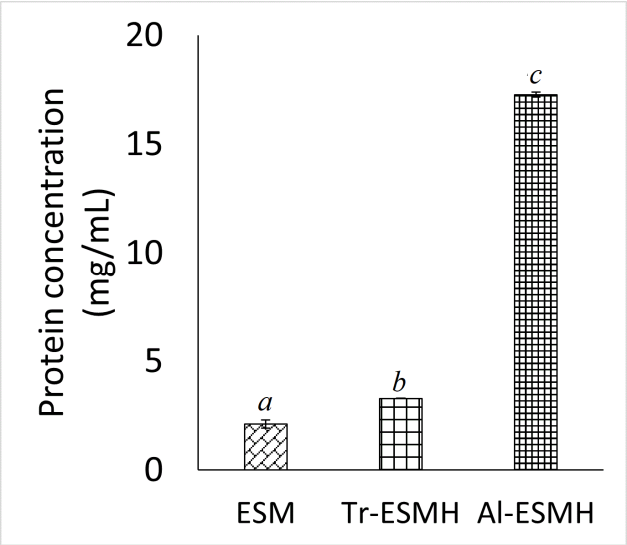


Figure 4 Protein concentration for the ESM, Al-ESMH and Tr-ESMH

The current finding on soluble protein quantitation study for the ESM broadly supports the work of other ESM studies. There are getting more reports on the use of alcalase in ESM preparation due to its ability in solubilising the non-soluble ESM [32-34]. Additionally, the ultrasonic coupled with alcalase enzyme hydrolysis treatment for the ESM has also demonstrated the alcalase enzyme ability in increasing protein solubility and degree of hydrolysis [22].

From the bar graphs in Figure 4, the ESM has significantly lower ($P \leq 0.05$) soluble protein concentration than the two ESMHs. In general, ESM is not soluble in water [10, 35], however, a minute amount of protein was quantitated in the ESM supernatant in this study. A possible explanation for this might be likely due to the long mild heat treatment (50°C) process of the ESM. The heat treatment may start denaturing the collagen fibre in the ESM caused by the multistep thermal decomposition [36]. Since the initial separation of the ESM from the ES obtained in this study was not processed with hydrochloric acid, sodium hydroxide, and ethylenediaminetetraacetic acid, the collagen present in the ESM is likely to remain. The collagen was then solubilised during the long hour of mild heat treatment, thereafter detected as a soluble protein in ESM supernatant [35].

Even though a quick protein quantitation performed in this study is usually carried out using 280 nm wavelength [31], this account must be approached with some caution because the absorbance at this wavelength may indicate not only proteins but free amino acids and small peptides, too [37]. Therefore, FTIR was performed for the freeze-dried ESMHs sample to study the structural changes of the ESM after the enzymatic treatments.

3.3 FTIR Characterisation

Figure 5 presents the FTIR spectra of the ESM, Al-ESMH, and Tr-ESMH. The data of each characteristic band is tabulated in Table 2. The FTIR spectrum of ESM reveals hydroxyl (O-H), carbonyl (C=O) stretching of the amide I at 1642.61 cm-1, and N-H bending vibration of amide II at 1535.14 cm-1. This finding is in accordance with the previous characterisation studies for ESM [12-14].

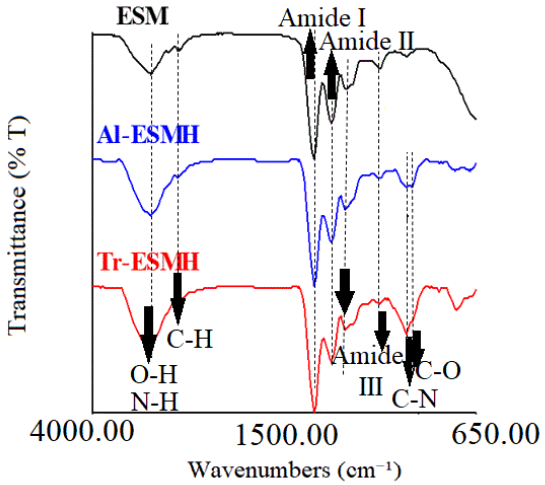


Figure 5 FTIR spectrum for the ESM, Al-ESMH and Tr-ESMH

As can be seen from Figure 5 and Table 2, the characteristic bands of O-H, amide I, and amide II for the Al-ESMH were retained, this indicates the alcalase enzyme hydrolysis did not alter the significant characteristic of the ESM. In the present work, the band widths of the Al-ESMH in the 3200-3400cm-1 region were bigger and more intense, it is apparent that stronger hydrogen bonding in the Al-ESMH could be induced by the alcalase enzyme.

A similar finding was observed from the work reported on the study of egg white hydrolysate, and the hydrolysate was derived from egg white subjected to alcalase enzymatic treatment [30]. The Al-ESMH spectrum exhibits a significant absorption band of amide III at 1240. 74 cm^{-1} which is associated with C-N stretching. Another characteristic band for the hydrolysate was found at 1080.17 cm-1 which is related to the skeletal vibration of C-O. Both significant bands are attributed to the peptide bonds which is consistent with the purpose of ESM enzymatic hydrolysis in producing smaller biopeptides [23, 24]. The Al-ESMH FTIR characteristic findings from this study make several contributions to the current literature since alcalase enzyme is frequently used for ESM hydrolysate preparation [38]

Table 2 Characteristic bands and functional groups in the ESM, Al-ESMH and Tr-ESMH

Functional Group	ESM (cm^{-1})	Al-ESMH (cm^{-1})	Tr-ESMH (cm^{-1})
O-H	3296.17	3283.08	3304.47
C-H	2964.28 & 2932.53	2956.34	2912.69
Amide I (C=O)	1642.61	1642.40	1643.85
Amide II (C=O)	1535.14	1536.12	1540.08
Amide III (C-N)	*	1240.74	1236.78
C-O	*	1080.17	*

*Not detected

The spectrum of the Tr-ESMH included all the characteristic bands of the ESM and Al-ESMH. In the spectrum, higher intensity of the amide III band was observed which might be due to the specificity of trypsin hydrolysates to the ESM. This may lead to a stronger absorption band at amide III bonds. However, the skeletal vibration of C-O was found absent. This observation may be due to the Maillard reaction during the hydrolysis that causes the disappearance of some absorbance bands. The same phenomenon has been reported in a previous study where absorption bands at amide I are absent after the hydrolysis of the egg white by the trypsin due to the Maillard reaction [30].

Several spectra characterisations of the ESM based on the Fourier Transform Infrared (FTIR) have been reported. In general, the FTIR spectrum of the ESM can be divided into two regions, the first one is between 3750 cm^{-1} and 2500 cm^{-1}, and the other one below 1700 cm^{-1}. There is significant appearance of amide and amine bands observed in the ESM infrared spectrum due to the ESM glycoprotein fibers [12, 13, 39]. Another study shows that the FTIR spectra of ESM exhibits the characteristic infrared absorption bands at 3316 cm^{-1} (C=O), 1652 cm^{-1} (–CONH–), 1427 cm^{-1} (C–N), 875 cm^{-1} (N–H), and 710 cm^{-1} (C–C), this indicates the presence of peptide bonds in the ESM [39].

Even though peptides can be prepared through condensation reactions, however, the process is subject to the massive usage of organic solvents and toxic chemicals [40]. In the present FTIR characterisation for the ESMHs, it shows that the soluble protein quantified is very likely peptides and amino acids that have been successfully attained through the enzymatic hydrolysis. Similar ESM enzymatic hydrolysis has been reported earlier, however, the researchers characterised the ESMH via electrophoresis. In their finding, they reported the hydrolysis of chicken ES membrane with alcalase has successfully produced peptides. The electrophoretic profile showed the existence of small molecular-sized peptides in the ESMH samples [22].

4.0 CONCLUSION

The purpose of the current study was to characterise the enzymatically hydrolysed ESM by using UV-Vis and FTIR. The enzymatic treated ESM showed higher protein solubility with a soluble protein concentration of 17.25±0.11 mg/mL. Based on the FTIR analysis, the presence of peptides and amino acids was likely detected in both treated ESM samples after enzymatic hydrolysis. Further work will be conducted in order to determine the calcium binding capacity of these ESMHs and its role in calcium bioavailability.

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