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**EVALUATION OF EGGSHELL MEMBRANE PROTEINS AS
ALTERNATIVE PROTEIN SOURCE**

MASTER THESIS

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ABSTRACT

EVALUATION OF EGG SHELL MEMBRANE PROTEINS AS ALTERNATIVE PROTEIN SOURCE

MSC THESIS

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BOLU ABANT İZZET BAYSAL UNIVERSITY

INSTITUTE OF GRADUATE STUDIES

DEPARTMENT OF FOOD ENGINEERING

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Eggs are the one of the foods with the highest waste capacity, and disposal of these wastes has become difficult due to bad odor and increased bacterial load. Even though eggshell and the three-layer eggshell membrane (ESM) inside the shell are very valuable in terms of protein and calcium concentrations; they cannot be utilized and valorized into new products. Studies with valorization of ESM are very limited because its separation from the shell is very hard and its solubilization is very limited. Thus, separation and solubilization of ESM from the shell, its characterization with functional and textural properties in addition to formulation of soft gel capsules with soluble ESM, determination of its microbial loads, and sensory properties were investigated. The optimal separation conditions at which membranes could be separated from the shells were determined using acetic acid, HCl, EDTA, and water. The highest protein levels of 83.36 ± 0.00 , 82.90 ± 0.00 , 81.75 ± 0.00 , 80.42 ± 0.00 , 80.38 ± 0.00 , 80.22 ± 1.45 , and $79.6 \pm 1.98\%$ were obtained from 100 mM EDTA at 1:20 ratio, water at 1:30 ratio, 50 mM EDTA at 1:20 ratio, 100 mM EDTA at 1:40 ratio, 1.25% acetic acid at 1:40 ratio, 1.25% acetic acid 1:30 ratio, and 0.185% HCl at 1:30 ratio, respectively by Kjeldahl method. Solubilization of the highest protein concentrations was performed by means of 0.1, 0.5 and 1% SDS and 0.1, 0.5 and 1% Triton X-100 (T) detergents at 0, 30, 60, 90, 120, and 1440 min in the Lowry analysis. The washing procedure was applied to remove lysis solution and to determine the effect of washing on protein content of the ESM samples. The samples treated with 100 mM EDTA at 1:20 ratio, 100 mM EDTA at 1:40 ratio, and water at 1:40 ratio samples having the highest soluble protein concentrations for further analyses. The samples had a high emulsion stability, low foaming activity index and no foaming abilities. The onset temperature of the samples ranged from 84.60 ± 0.01 °C to 101.22 ± 0.01 °C. Rheological analysis revealed high correlations with Bingham, Herschley-Bulkley, and Newtonian flow models. Moreover, the FT-IR spectra of the samples were similar to each other. 16 different types of aminoacids were identified in soluble ESM samples. The hardness and the springiness values were significantly different from each other ($p < 0.05$). Soft gels containing 1.45 mg/g ESM protein had lower sensory scores in terms of water 1:40 sample although had a microbiological stability of 32 weeks at +4°C. It was presented by this study that ESM considered as waste can be utilized as a protein supplement in the form of soft gel capsules.

KEYWORDS: Eggshell membrane; Solubilization; Eggshell membrane protein; Soft gel capsules.

ÖZET

YUMURTA KABUĞU ZARI PROTEİNLERİNİN ALTERNATİF PROTEİN KAYNAĞI OLARAK DEĞERLENDİRİLMESİ

YÜKSEK LİSANS TEZİ

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Günümüzde tarımsal atık yönetimi, gıda işletmecilerinin karşılaştığı en yaygın sorunlardan biridir. Tarım sektöründe oluşan atıkların artmasına paralel olarak kanatlı eti ve yumurta üretimi dünya çapında önemli ölçüde artmış ve halen en hızlı büyüyen tarım sektörlerinden biri olmaya devam etmektedir. Birleşmiş Milletler Gıda ve Tarım Örgütü'ne (FAO) göre, tavuk eti üretimi 2016 yılında dünya et üretiminin yaklaşık %36'sını oluşturmaktadır. 1961 ile 2016 yılları arasında tavuk eti endüstrisi üretim hacimlerini %13 artırmıştır. En büyük yumurta üreticilerinden biri olan Türkiye, dünya kanatlı yumurta üretiminde 10'uncu, Avrupa'da ise 3'üncü sırada yer almaktadır (1). 2018 yılı verilerine göre Türkiye'de 22.3 milyar ticari sofralık yumurta üretilmiştir (2). 2019 yılında yaklaşık 19.9 milyar (1.2 milyon ton) tavuk yumurtası üretimiyle tüm yılların en yüksek düzeyine ulaşıırken, 2020 yılında 1.127.934 ton üretim rapor edilmiştir (3). 2022 yılında ise yumurta üretimi 1.67 milyar adet olarak kaydedilmiştir (4). Çevre Koruma Ajansı, yumurta kabuğu atıklarını gıda endüstrisinin karşılaştığı en önemli 15. kirlilik sorunu olarak listelemiştir. Ayrıca, atıkların uygunsuz şekilde bertaraf edilmesi yalnızca insanın varlığını değil, aynı zamanda bitki ve hayvanlar gibi diğer ekosistem bileşenlerinin hayatlarını da riske atmaktadır. Yumurta kabukları atık olarak çevresel zorluklara yol açsa da geri dönüştürülmesinin karmaşık bir süreç olduğu kabul edilmektedir. Bu nedenle kabuk atıklarında membranın etkin bir şekilde ayrılmasına yönelik çalışmalar ve çözüm uygulamaları sınırlı kalmıştır (1). Yumurtanın iç kısmı çıkarıldıktan sonra yumurta kabuğu ve kabuğa yapışan iç zar hızla bozularak kötü bir koku yayar (6). Bu nedenle yumurta kabuğu genel olarak çabuk bozulan ve kötü kokulu atık olarak sınıflandırılır ve kullanılamaz. Bu da her yıl binlerce ton yumurta kabuğunun değerlendirilememesi ve atık olarak ayrılarak çevre kirliliğine yol açması anlamına gelmektedir. Bu nedenle yumurta kabuğu zarının değerli bir protein kaynağı olarak kullanılması çalışmanın temel amaçlarından biridir.

Yumurta kabuğu, gelişen embriyoya mekanik korumayı desteklemesi, kalsiyum başta olmak üzere birçok besin kaynağı sağlaması ve kontrollü gaz değişimi sağlaması açısından önemli bir yapıya sahiptir (7). Kabuk, bakteri, virüs ve diğer patojenlerin bulaşmasını önleyerek yumurtanın tüketiciye saf bir şekilde ulaşmasını sağlar. Sağlığa olumlu etkileri olan yumurta kabuğu güçlü bir kalsiyum takviyesi olduğu gibi aynı zamanda kalsiyum kaynağı olduğundan diş sağlığı alanında da kullanılabilir. Yapısındaki vitamin ve mineraller sayesinde cilt hücrelerini yenileyerek ciltte oluşan tahrişleri iyileştirir. Bunların dışında yapısında bulunan protein ve kalsiyum sayesinde kemik erimesini önler (6, 8).

Yumurta, insanın ihtiyaç duyduğu tüm besin maddelerini anne sütünden sonra sağlayan en zengin kaynaklarından biridir. Yumurtanın yenilebilir kısmının ve kalitesinin korunması, yumurta ağırlığının yaklaşık %10-12'sini oluşturan yumurta kabuğu tarafından sağlanmaktadır (2,8). Yumurta kabuğu tıp ve eczacılık alanında yardımcı maddelerin geliştirilmesi amacıyla gıda maddesi, gıda katkı maddesi ve kalsiyum takviyesinin yanı sıra tarımsal gübre bileşeni olarak da kullanılabilen zengin bir mineral kaynağıdır (8).

Yumurta kabuğu, kabuk ağırlığının ana bileşeni olan kalsiyum karbonat (%96), kalsiyum fosfat (%1) ve magnezyum karbonatın (%1) yanı sıra organik maddeler içeren protein lif ağından oluşur (9). Yumurta kabuğunun yapısında hava boşluğu oluşturacak şekilde bölündükleri bir nokta dışında iki iç zarı vardır. Membran yapısının yaklaşık %70-75'ini lizin, %10'unu kollajen, glikoproteinler, desmosin ve izodesmosin gibi proteinler oluşturur (9).

Yumurta, albümin (yumurta akı) ile çevrelenmiş merkezi bir sarıdan, yumurta kabuğu zarlarından, kalsifiye yumurta kabuğundan ve kütikülden oluşur (7, 10). Yumurta kabuğu katmanları -dıştan içe doğru- dış ve iç kabuk zarları, kütikül içeren gözenekleri, kristal (sınırlayıcı) katman, palisades ve memeli konisinden meydana gelmektedir (2). Kabuğun hemen altında yer alan kabuk altı zarlar, kabuk altı dış zarı (kabuk zarı), kabuk altı iç zarı (yumurta zarı) ve sınırlayıcı zar olmak üzere üçe ayrılmaktadır (9,11).

Meme tabakası birçok düzensiz konik yapıdan oluşur. Meme tabakası embriyonik gelişim sırasında embriyo için gerekli olan tek kalsiyum kaynağıdır (11).

Palisades tabakası süngerimsi tabaka olarak bilinir ve organik (%2-5) ve inorganik maddelerden oluşur. Palisades, yumurta kabuğunun en kalın (200 µm) tabakasıdır (10, 11).

Kütikül, yumurta kabuğunun dış yüzeyini kaplayan organik bir tabakadır ve mikroorganizmaların girişini önlemede kritik bir role sahiptir. Kütikül kalınlığı ile yumurta kabuğundaki bakteri yükü arasında yüksek bir ilişki bulunmuştur (11). Kütikül tabakası ayrıca kabuğun içindeki ve dışındaki nemi dengeleyen ve yumurtanın iç bileşenlerinin dehidrasyonunu önleyen bir bariyer görevi görmektedir (10, 11).

Yumurta zarının oluşumu için dış kabuk zarı gereklidir. Bu zar yumurta kabuğunun kireçlenmesi ve sertleşmesi için önemlidir (11, 15). Alt kabuk iç zarı, alt kabuk dış zarından bir hava boşluğu ile ayrılır. Dış zar kalınlığı 50-70 µm iken iç zar kalınlığı 15-26 µm arasındadır. İç membran liflerinin çapı (1-7 µm) dış membraninkinden (0.1 -3 µm) daha büyüktür ve her iki membranın lifleri iç içe geçmiş durumdadır (13, 16). Sınırlayıcı zar, yumurta beyazını çevreleyen en ince zardır ve yumurta kabuğu zarının en ince ve en lifli yapısını oluşturur (11, 16).

Yumurta kabuğu ile yumurta akı arasında ise yumurta kabuğu zarı bulunur (16, 17). Zarın yapısında I, V ve X tipi kollajenler, proteoglikanlar, glikoproteinler ve fibronektin bulunur. İç ve dış zarın her ikisi de protein liflerinden oluşan yarı geçirgen bir zar oluşturur. Bu nedenle ESM stabil ve suda çözünmeyen liflerden oluşan bir kafes yapısına sahiptir. ESM ayrıştırıldığında yan ürün olarak

değerlendirilen yumurta kabuğunun ağırlığı, yumurtanın yaklaşık %11'ini (60 g) temsil etmektedir (9).

ESM, yüksek çapraz bağlantılardan oluşan, çift katmanlı, açık pembe renkli bir membran olup yarı geçirgen bir yapıya sahiptir (16,18). ESM'nin %80-85'i organik, %15-20'si ise inorganik matriksten oluşur (11). Organik matris, esas olarak kolajen (tip I, V ve X) olan yapısal bileşenlerin %10'unu içerir. Kollajenin yaklaşık %70-75'i keratin, osteopontin, proteoglikan ve glikoproteinler gibi sekonder protein bileşenlerinden oluşur (15).

ESM ayrıca glikozaminoglikanlar (dermatan sülfat ve kondroitin sülfat gibi) ve sülfatlanmış glikoproteinler (heksozaminler) içerir (19). Diğer bileşenler ise hyaluronik asit (HA), ovalbümin, ovotransferrin, ovokaliksin-36, desmosin ve izodesmosin, lisil oksidaz, lizozim ve β -Nasetilglukozaminidazdır (8, 11, 15). Ayrıca dış ESM'nin ES ile bulunduğu uçlarda kalsiyum karbonat bulunur. Bu nedenle ESM doğal bir HA, kondroitin, glukozamin ve kollajen kaynağı olarak çok değerli bir yapıya sahiptir (15). ES'de iç ve dış kabuk altı membran matrisindeki protein sayısının 500'den fazla olduğu ve bu değer yumurtanın diğer kısımlarına göre çok daha yüksek olduğu belirtilmektedir (11). Kabuk altı zarlarında 62 proteinin olduğu rapor edilmiştir. Ancak ESM'de yalnızca 20'den az protein tanımlanabilmiştir ve bunların arasında hala tam olarak karakterize edilemeyen proteinler bulunmaktadır (18). Prolin, glutamik asit ve glisin membranlarda en çok bulunan bileşenlerdir (11). ESM yapısında sialik ve üronik asit gibi protein olmayan maddeler de bulunmaktadır (2, 14). Burada yer alan dış zar liflerinin çekirdekleri tip I kolajen içerirken, iç zar lifleri esas olarak tip I ve V kolajen içerir. Kolajen X'in her iki membranda da mevcut olduğu ve membranlardaki mineralizasyonun inhibisyonunu kolaylaştırdığı düşünülmektedir (11,13). Hem iç hem de dış ESM, esansiyel olanlar da dahil olmak üzere amino asitler açısından zengindir (20).

Yumurta yüzeyi steril değildir ve farklı floralarından çeşitli mikroorganizmalarla kirlenmiştir. Mikrobiyal floranın artışı önlemek, *Salmonella* spp.'yi ortadan kaldırmak ve kontaminasyonu önlemek için yumurta kabukları kullanılmadan önce yıkanmalı ve dezenfekte edilmelidir.

Genellikle ESM içeren yumurta kabuğu yumurtanın içi çıkarıldıktan sonra kullanılamaz. ESM, kabuğa sıkı bir şekilde bağlı olduğundan ESM'nin yumurta kabuklarından ayrılmasına yönelik çalışmalar yapılmaktadır. Bu çalışmalardan en kolay yumurta kabuğundan zarın elle çıkarılması yani elle soyulması işlemidir. Ayrılan membranlar 30 °C'deki fırında en az 30 dakika kurutulduktan sonra öğütülerek dondurucuda saklanır (21). Bu yöntemin etkili olduğu bildirilse de zahmetli ve zaman alıcıdır (22).

Bu nedenle yumurta kabuğu zarının kabuktan ayrılmasını sağlamak amacıyla farklı fiziksel, kimyasal ve mekanik yöntemler test edilmiştir. Nitekim, Amerika Birleşik Devletleri'nde ESM'yi yumurta kabuğundan düşük basınç altında mekanik aşınmayla ayıran ticari bir makine geliştirilmiştir (23). Levi New (24) tarafından icat edilen başka bir sistemde ise, hava akışı yoluyla yumurta kabuklarını toza ve zarları pullara dönüştürmek için pulver kurutucu kullanılmış ve kabuk ile membran tozlarını ayırmak için elek sallama sistemi kullanılmıştır. ES ve ESM'yi ayırt edebilen kimyasal olmayan bir diğer yöntem ise çözünmüş hava flotasyonunun

(DAF) kullanıldığı yöntemdir. Bu yöntemin prensibi yumurta kabuğu ile zarın özgül ağırlıklarının farklı olmasıdır. Yumurta kabuğu ve su belirli oranlarda 10 dakika karıştırıldıktan sonra hava-su karışımı enjekte edilerek bileşenler toplanmaktadır. Bu yöntemde DAF ayırma ünitesi kullanılarak ES'den iki saat içerisinde ESM'nin %96'sının ve ES'deki kalsiyum karbonatın %99'unun geri kazanıldığı belirlenmiştir (20, 21).

İç ve sınırlayıcı katmanlar mekanik olarak ayrılabilirken, dış membran ES'ye güçlü bir şekilde gömülmüştür ve membranı ayırmak için asidik işlem (EDTA veya HCl ile işlem gibi) gereklidir. Asidik bir ortamda ayırma iki şekilde gerçekleştirilebilir: ES'yi asit içerisine batırarak veya ES'nin ana bileşeni olan kalsiti çözerek. İlk işlem sonucunda asit içerisine daldırma ile ES tamamen çözünmese de ESM ve ES birbirinden ayrılabilir (13).

Membranları ES'den ayıran diğer bir işlem ise mikrodalga işlemidir. Bu prosesin temel prensibi ES ve ESM'nin ısıtılarak ve genişerek ayrılmasına dayanmaktadır. Membranların su içeriği kabuklara göre daha yüksektir ve kabuklara göre daha fazla elektromanyetik enerji absorblar ve ısınır. Genişleyen zar ile kabuk arasındaki yapışma kuvvetleri ısınma sonucu zayıfladığından, yumurta kabuğu zarının yumurta kabuğundan ayrılmasına yardımcı olur ve zar daha sonra elle kolaylıkla ayrılabilir (21, 24).

Bir başka yöntem olan ve ticari olarak kullanılan mekanik ayırma, yumurta kabuğu parçalarının sulu ortamda buharla ısıtılarak ayrılması esasına dayanır (21). Kabuk ile zar arasındaki bağlar asit uygulaması yardımıyla zayıflatılır ve daha sonra ayrılan zarlar elle soyulmaktadır.

Yumurta Kabuğu, Yumurta Kabuğu Membranı ve Çözünür Yumurta Kabuğu Membran Proteininin Kullanımı

İnsan beslenmesinde kalsiyum kaynaklarının yetersiz olduğu iyi bilinmektedir. Süt, yoğurt, peynir gibi kalsiyum kaynağı olduğu bilinen besinler, laktoz intoleransı olan kişiler tarafından rahatlıkla tüketilemez. Doktorların önerdiği takviyeler, fiyatlarının yüksek olması sebebiyle herkes tarafından satın alınamayabilir. Bu nedenle kalsiyum tüketimini arttırmak için kalsiyumca zengin gıdalar talep görmektedir. Bu tür gıdalar süt, kalsiyum fosfat ve kalsiyum karbonat gibi çeşitli kalsiyum kaynakları ile zenginleştirilebilir. Önceki bölümlerde de bahsedildiği gibi yumurta çok iyi bir protein ve mineral kaynağıdır. Ayrıca yumurta kabukları yaklaşık %38 kalsiyum içeriğiyle en büyük kalsiyum kaynaklarından biri olarak kabul edilmektedir (26). Yumurta kabuğunda bulunan bir kalsiyum türevi olan hidroksiapatit maddesi, kimyasal yapısı nedeniyle kemiklerde ve dişlerde bulunan mineral bileşenlere benzeyen bir maddedir. Özellikle insan yumuşak dokularıyla mükemmel uyumluluk sağlamaktadır. Bu nedenle ortopedi alanında ve diş implantlarında kullanılmaktadır. Bu malzemeden yapılan kompozit malzemeler kemik ve diş eksikliklerinin düzeltilmesinde kullanılabilir (27). ESM'nin çok iyi bir protein kaynağı olmasının yanı sıra yumurta kabuğu da çok iyi bir kalsiyum kaynağıdır (30). Kabuktaki kalsiyumun yaşlılarda osteoporotik kırıkların önlenmesinde etkili bir şekilde kullanılabileceği klinik olarak test edilmiştir (32).

1957'de (102) ESM'nin temel yapıları oluşturulmuştur. Yumurta kabuğunun içini kaplayan ince bir film olan ESM, esas olarak proteinden oluşmaktadır. Kimyasal olarak değiştirilmiş ESM, mekanik ve kimyasal özelliklerine ilişkin artan bilgi birikiminin bir sonucunda çok sayıda mühendislik alanında kullanılmaktadır. Pek çok farklı uygulamada, glikoz sensörü (104) ve TiO_2 'nin hiyerarşik sentezi (105) için uygulamalar olarak da dahil olmak üzere, ESM kullanılmıştır. SEP, hücre kültüründe kullanıldığında boyutunu ve şeklini ESM'ye göre daha kolay değiştirebilir. Ek olarak suda çözünmez, bu da biyo-uyumlu bir substrat olarak kullanılmasını kolaylaştırmaktadır. ESM'nin elektrikli cihazlarda uygulama potansiyeli, güneş pili (107), NiO kapasitör (108) ve Li-S pil (109) kullanımıyla ortaya konmuştur. Ayrıca doku mühendisliği ve kemik dokuda biyomateryal olarak kullanımı da araştırılmaktadır (34). Membranlar ayrıca ağır metalleri ve zararlı maddeleri absorbe etmek için emici olarak kullanılır. İçerdiği farklı kolajen türleri nedeniyle eklem ve cilt sağlığında kullanıldığı da rapor edilmiştir. ESM'de bulunan kolajen, dokuyu desteklemede ve hücrelerin yapılanmasında rol oynayan çok önemli bir proteindir. Bu nedenle kırışıklıkları önleyen, cildin kalınlığını ve elastikiyetini artıran birçok ürünün ana bileşeni olarak görev yapmaktadır (28). ESM proteinleri kolajen açısından zengin olduğundan kozmetik ürünlerin etkinliğini arttırmak için ana bileşen olarak kullanılabilirler. Bazı çalışmalarda ESM'den elde edilen proteinlerin diğer kaynaklardan elde edilenlere göre daha az alerjik olması nedeniyle kullanımının daha güvenli olduğu bildirilmiştir (27, 29).

Özellikle gıda israfının önlenmeye çalışıldığı bu dönemde uygun forma getirilmiş olan ESM insan tüketimine yönelik olarak gıda endüstrisinde protein kaynağı olarak kullanılabileceği gibi gıda aroması formunda da kullanılabilir (28, 29). Yumurta kabuğu membranı ve tozu sadece gıda endüstrisinde değil aynı zamanda kağıt endüstrisinde veya tarımda kireç yerine veya kalsiyum takviyesi olarak da kullanılabilir. Diğer kullanımlar arasında membran proteinlerinden biyolojik olarak parçalanabilen biyoplastiklerin üretimi yer almaktadır. Ancak kolajen deposu olan ESM proteinlerinin sindirilebilirliği ve net protein faydası halen araştırılan konular arasındadır. ESM'nin temel işlevi yumurta akı ve sarısına yabancı maddelerin ulaşmasını engellemektir. Dolayısıyla ESM bu haliyle vücuda alındığında sindirime uygun olmayabilir. Bu nedenle ESM'nin düşük molekül ağırlıklı protein ve peptidlere çözünebilirliği araştırılmaktadır (31).

Daha önce yapılan çalışmalarda yumurta kabuklarının insanlar ve hayvanlar için gıda takviyesi ve hayvan yemi olarak kullanılabileceği bildirilmiştir. Ayrıca kabuğun gözenekli yapısından dolayı biyodizel üretiminde heterojen katalizör olarak kullanılabileceği belirlenmiştir. Yumurta kabuğunda bulunan çeşitli fonksiyonel gruplar ve yüksek karbon içeriği, kabuğu atık su arıtımında ağır metallerin ve boyaların uzaklaştırılmasında adsorben olarak kullanılabilecek alternatif bir madde haline getirmektedir (30). Bisküvi formülasyonuna kalsiyum katkı maddesi olarak %3, 6 ve 9 oranlarında yumurta kabuğu tozu ilavesi, kalsiyumun doğrudan biyoyararlanım oranında sırasıyla %26.0, %35.4 ve %41.43 olacak şekilde önemli bir artışa neden olmuştur (33).

ESM proteininin (ESM-P) ve ESM hidrolizatının (ESM-H) farelerde sindirilebilirliği, karşılaştırma proteini olarak kazein kullanılarak araştırılmıştır (31). ESM-H proteinleri, ESM-P proteinlerinin alkalik proteaz ile sindirilmesi sonucu elde edilmiştir. ESM-P ve ESM-H'nin *in-vivo* sindirimi sırasıyla %80 ve

%95.1 olduğundan, ESM-H'nin sindirilebilirliğinin ESM-P'den önemli ölçüde daha yüksek olduğu sonucuna ulaşılmıştır. Ancak her iki proteinin de sindirilebilirliği kazeine göre daha düşük bulunmuştur (31).

Dişi destekleyen yapıların bozulmasından kaynaklanan periodontal hastalık, dünya nüfusunun %5-15'ini etkilemekte ve yetişkinlerde diş kaybına neden olmaktadır. Hasar görmüş periodontal doku ile kemik arasında yenileme amaçlı cerrahi yöntem, çok uzun ve yorucu bir süreç olmasına rağmen yaygın olarak uygulanmaktadır. Bu nedenle kollajen, sentetik polimerler ve poli-laktik asit (PLA) gibi doğal polimerleri içeren biyolojik olarak emilebilen membranların kullanımı giderek yaygınlaşmaktadır (34). Son yıllarda ESM'nin yanıklar için mükemmel biyoyumluluğa sahip olduğu rapor edilmiştir. Sulu 3-merkaptopropiyonik asit ve asetik asit kullanılarak ESM'den elde edilen çözünür ESM (SESM), tip I kollajene kıyasla zayıf mekanik yapı ve kırılabilir doku göstermiştir (33, 34). Önceki çalışmalar umut verici sonuçlar ortaya koysa da, ESM'nin farklı biyomateryallerle kombinasyonları sonucunda yeni membranların geliştirilmesine yönelik daha fazla çalışmaya ihtiyaç bulunmaktadır (34, 35).

Son çalışmalar, yüksek miktarda çapraz bağ nedeniyle çözünürlüğü çok sınırlı olmasına rağmen ESM'nin antioksidan potansiyeline işaret etmektedir. Bu amaçla ESM proteinleri çözünürlüğünü arttırmak amacıyla beş farklı proteaz ile hidrolize edilerek ve hidrolizatların antioksidatif aktiviteleri ve DNA hasarına karşı koruyucu etkileri kemoluminesans yöntemi kullanılarak araştırılmıştır. Sıvı yumurta kabuğu zar proteininden saflaştırılan peptidlerin kozmetikte antioksidan ve nutrasötik materyal olarak ya da gıda ürünlerinde katkı maddesi olarak kullanılabileceği sonucuna varılmıştır (37).

Resveratrol bileşiği, bitkilerde mantar enfeksiyonunu önlemek veya UV ışığına maruz kalmanın neden olduğu hasarı en aza indirmek için yaprak dokuları tarafından sentezlenen fenolik bir bileşiktir. Bu bileşiğin ısı ve ışık gibi çevresel koşullar altında stabilitesi sınırlıdır ve bu nedenle resveratrol gibi biyoaktif bileşikler kapsüllemek için proteinler kullanılmıştır. Resveratrol bileşiğinin stabilitesini korumak amacıyla elektrospreyleme tekniği ile trans-resveratrolün ESM proteinleri ve ipek fibroinden oluşan çekirdek/kabuk nanopartikülleri içerisine kapsüllemesi, trans-resveratrol stabilitesinin polifenol ve proteinler arasındaki etkileşimler tarafından sağlandığını ortaya çıkarmıştır. Kapsülleme parametrelerinin değiştirilmesiyle nanopartikül özelliklerinde değişiklik yapılması mümkün olmuştur. Ayrıca ESM proteinleri tarafından kapsüllenmiş resveratrolün UV ışığına karşı stabilitesi ve vücuda salındığında biyoyararlılığı artmaktadır (38). ESM'nin yara iyileştirme ve kozmetik gibi topikal uygulamalara yönelik çeşitli formülasyonlarda bileşen olarak kullanılması ile yetişkin hastalarda venöz bacak ülserlerinin tedavisinde kullanılması umut verici sonuçlar ortaya çıkarmıştır (37). Yaşlı kişilerde (70-100 yaş) inflamasyon semptomlarını azaltmak için günlük yumurta kabuğu zarı alımının (500 mg kapsül, 2/gün) etkinliği olumlu sonuçlar göstermiştir (5). Yüzdeki kılların yanı sıra cilt tonunu ve görünümünü iyileştirmek için hidrolize yumurta kabuğu zarının ağızdan alınması da çok umut verici sonuçlar vermiştir (39).

ESM, gıda ve kozmetik endüstrisinde diyet takviyesi, fonksiyonel içerik ve kişisel bakım ürünü olarak farklı uygulamalar için toz, kapsül ve hatta macun gibi çeşitli formülasyonlarda satılmaktadır. Ayrıca ESM şekerlemeler, içecekler ve sakız gibi

farklı gıda ürünlerinde de bulunabilmektedir (5). Sonuç olarak, ESM yaygın bir endüstriyel ve evsel atık olduğu için kullanım açısından pek çok faydası bulunmaktadır. Bunlardan bazıları şunlardır: (i) düşük maliyetli, elde edilmesi kolay ve kullanıma hazır; (ii) çevre dostu ve toksik değil; (iii) bol miktarda amino asitler ve doğal protein içerir, ESM'nin yüzeyinde çok sayıda fonksiyonel grup bulunur, bu da işlevselleştirme ve adsorpsiyon özelliğiyle sonuçlanır; (iv) çeşitli sentezlerle ve çözünme yoluyla kolaylıkla modifiye edilebilir. ESM ayrıca bu faydaları sayesinde kimya, malzeme, elektrik, çevre ve biyomedikal mühendisliği de dahil olmak üzere çeşitli mühendislik alanlarında kullanılmaktadır (17).

Yumurta Kabuğu ve Membranının Güvenlik Değerlendirmesi

ESM ve türevleri üzerinde yapılan *in vivo* testlerde, 100 µg dozunda insan kaynaklı epidermal keratinositlerin 20 saate kadar inkübasyonunu takiben hücre canlılığı testinde sitotoksik etkisinin olmadığı rapor edilmiştir (5). Ek olarak, 90 günlük tekrarlanan doz denemesinde, farelere oral yolla tek doz olarak uygulanan toz haline getirilmiş hidrolize ESM, herhangi bir patolojik toksisite belirtisi göstermemiştir (40). Ayrıca Avrupa Gıda Güvenliği Otoritesi (EFSA) Diyet Ürünleri, Beslenme ve Alerji Paneli, (AB) 2015/2283 Yönetmeliğine uygun olarak yumurta zarı hidrolizatının yeni bir gıda bileşeni olarak kullanımını onaylamıştır. Panel yetişkinler için gıda takviyesi olarak tüketilen hidrolize yumurta zarının 450 mg/gün dozunda güvenli olduğuna karar vermiştir (5). Yumurta kabuğu tüketiminin genotoksik olmadığı belirlenmiştir. Ancak yumurta ve türevleri bazı tüketiciler için alerjen olabilmektedir. Bu kapsamda üretilen gıda takviyelerinin etiketleri üzerinde uyarı bilgilerine yer verilmiştir (5). Hidrolize olmayan formuna kıyasla hidrolize ESM kullanımı, kollajen, antioksidanlar, HA ve antimikrobiyaller gibi aktif bileşenlerin biyoyararlanımının artması nedeniyle hidroliz sayesinde 2019 yılından bu yana daha fazla artış göstermiştir (5).

Daha önceki çalışmalarda ESM'nin kullanımına odaklanılmış olsa da farklı kimyasalların ESM'nin kabuktan ayrılma etkinliğini belirlemesi, ESM'nin çözündürülmesi ve yumuşak jel kapsüllerde olduğu gibi gıda takviyesi olarak kullanılması konusunda literatürde bilgi eksikliği bulunmaktadır. Yumurta kabukları en büyük atık kaynaklarından biri ve çevre sorunlarının nedeni olmasına rağmen, yumurta kabuğu zarı protein kaynağı olarak yeterince değerlendirilmemektedir. ESM'nin protein kaynağı olarak kullanılabilmesi için öncelikle kabuğundan ayrılması ve daha sonra çözündürülmesi gerekmektedir. Yumurta kabuğu ve ESM yakından bağlı olduğundan, öncelikle bir ayırma prosedürünün geliştirilmesi gerekir. ESM'de bulunan disülfid bağları insanların proteini sindirmesini engellemektedir; dolayısıyla ESM'nin sindirilebilmesi için bu bağların kırılması gerekir. Bu nedenle tezin amaçları 1) yumurta kabuğunu zarından ayırmaya yönelik bir proses geliştirmek ve optimize etmek, 2) protein kaynağı olarak kullanılacak ESM'yi çözündürerek fonksiyonel özelliklerini belirlemek, 3) çözündürülmüş ESM proteininden protein takviyesi olarak kullanılmak üzere yumuşak jel kapsüller geliştirmek ve 4) yumuşak jel kapsüllerin dokusal, mikrobiyolojik ve duysal özelliklerini belirlemektir. Yumurta kabuğundan elde edilen zarın sıfır atık kapsamında gıda atığı olarak geri dönüştürülmesi de teşvik edilmiştir.

Materyal ve Method

Bolu'daki çeşitli fırın işletmelerinden çiğ yumurta kabuğu temin edilmiştir. Toplanan yumurta kabuğu örnekleri su ile temizlenip yıkanarak 2 saat süreyle oda sıcaklığında kurutulmuştur. Yumurta kabukları kullanılıncaya kadar oda sıcaklığında muhafaza edilmiştir.

Yumurta kabukları yıkanıp kurutulduktan sonra yumurta kabuklarından zarların ayrılması işlemlerine geçilmiştir. Yumurta kabuklarından zarların ayrılması için EDTA 50, 100, 150 mM, %1.25, %2.5, ve %5 asetik asit ve %0.185, %0.37 ve %1.85 HCl ve saf su kullanılmıştır.

EDTA ile Yumurta Kabuğundan Membranın Ayrılması

Ayırma, sırasıyla 1:20, 1:30 ve 1:40 g/sıvı katı:sıvı oranlarıyla üç farklı EDTA (Sigma Aldrich, Steinheim, Almanya) konsantrasyonu (50 mM, 100 mM ve 150 mM) kullanılarak beherin içerisinde gerçekleştirilmiştir.

HCl ile Yumurta Kabuğundan Membranın Ayrılması

Balâz ve arkadaşlarının (43) çalışmalarından modifiye edilerek kullanılan yöntemde, HCl kullanarak zar yumurta kabuğundan ayrılmıştır. Ayırmada üç farklı HCl konsantrasyonu (%0.185, %0.37 ve %1.85) kullanılmıştır (Sigma Aldrich, Steinheim, Almanya). Yaklaşık 3.0 g yumurta kabuğu 1:20, 1:30 ve 1:40 g/mL oranlarında seyreltilerek HCl ile karıştırılmış ve her yarım saatte bir membranların ayrılma dereceleri izlenmiştir.

Asetik Asit ile Yumurta Kabuğundan Membranın Ayrılması

Tsai ve arkadaşlarıyla (9) birlikte Vladimir (23)'in uyguladıkları yöntemler modifiye edilerek zar yumurta kabuğundan ayrılmıştır. Ayırma, %1.25, %2.5 ve %5'lik üç farklı asetik asit konsantrasyonu (Sigma Aldrich, Steinheim, Almanya) ile gerçekleştirilmiştir. Yaklaşık 3 g yumurta kabuğu, seyreltilmiş asetik asit ile 1:20, 1:30 ve 1:40 g/mL oranlarında karıştırılarak her yarım saatte bir membranların ayrılma dereceleri izlenmiştir.

Asetik Asit ile Yumurta Kabuğundan Membranın Ayrılması

Son olarak damıtık su kullanılmıştır. Yaklaşık 3 g yumurta kabuğu 1:20, 1:30 ve 1:40 g/mL oranlarında distile su ile karıştırılarak her yarım saatte bir membranların ayrılma oranları izlenmiştir.

Yumurta kabuğu zarının yumurta kabuğundan elle soyulması sonrasında; EDTA, asetik asit, HCl ve suda bulanıklık, iletkenlik ve pH analizleri yapılmıştır. Bu aşamada yapılan bu analizlerle membranın kabuktan ayrılması sırasında çözelti içerisine protein ve diğer bileşenlerin geçişinin olup olmadığı kontrol edilmiştir. Bu analizlerin ardından ESM, oda sıcaklığında kurutulup toz haline getirilerek oda sıcaklığında desikatörde saklanmıştır. Ardından yumurta kabuğu zarı ile yapılacak analizlere geçilmiştir.

- ✓ Öncelikle ham protein oranının hesaplanması için Kjeldahl yöntemiyle protein tayini gerçekleştirilmiştir. Uluslararası AOAC (44) 990.03 yöntemine göre Kjeldahl yöntemiyle belirlenen numunelerin ham protein içeriği Düzce Üniversitesi Bilimsel ve Teknolojik Araştırma Uygulama ve

Araştırma Merkezi (DUBIT) (42) tarafından yapılmıştır. Ayırma çözeltisi ve konsantrasyonu, Kjeldahl analizlerinin sonuçlarına göre seçilmiştir. En yüksek protein içeriğine sahip olan 7 örnek seçilerek analizlere bu örneklerle devam edilmiştir.

- ✓ Yumurta kabuğu zarlarının fonksiyonel gruplarını belirlemek için yapılan FT-IR analizleri Düzce Üniversitesi Bilimsel ve Teknolojik Araştırma Uygulama ve Araştırma Merkezi (DUBIT) tarafından Shimadzu IR Prestige21 cihazı (Shimadzu, Tokyo Japonya) ile gerçekleştirilmiştir (13). Ölçüm modu olarak 4.0 eV çözünürlükte ve 1/cm 500-4500 aralıkta 20 taramayı temel alan % geçirgenlik değerleri kullanılmıştır (16).
- ✓ Numunelerin su tutma kapasitesi (WHC) ve yağ tutma kapasitesi (OHC) (g/g), 0.05 g ESM numuneleri ile 1.5 mL su/yağ kullanılarak gerçekleştirilmiştir. Karışımlar 30°C'de 30 dakika bekletilerek, 25°C'de 9000 rpm'de 20 dakika (NF 200 Model, Nüve, Ankara, Türkiye) santrifüj edilmiştir (45).
- ✓ Emülsiyon stabilite indeksi (ESI) ve emülsiyon aktivite indeksi (EAI) testlerinde, 0.25 g ESM tozu 2.5 mL yağ ile yüksek hızda tüp karıştırıcı ile karıştırıldıktan sonra içinden 50 µL numune alınmış ve SDS (7.5 mL, %1) çözeltisine aktarılmıştır. Karıştırıldıktan sonra 500 nm'de UV-görünür spektrofotometrede absorbans ölçülmüştür (46).
- ✓ ESM örneklerinin DSC analizi Düzce Üniversitesi Bilimsel ve Teknolojik Araştırma Uygulama ve Araştırma Merkezi'nde (DUBIT) yapılmıştır. ESM toz numuneleri (10 mg) hermetik olarak kapatılmış alüminyum numune kaplarına konarak diferansiyel taramalı kalorimetre (DSC) (Shimadzu TA-60 WS modeli, Tokyo, Japonya) cihazında analiz gerçekleştirilmiştir.

Yumurta kabuğu zarı çözünür olmadığından ham protein miktarı Kjeldahl yöntemi kullanılarak belirlenmiştir. En yüksek protein içeriğine sahip örnekler seçilerek ESM proteinleri çözünür hale getirilmiştir (18). Çözünür protein miktarı spektrofotometrik bir yöntem olan Lowry yöntemiyle belirlenmiştir. Örneklerde protein konsantrasyonunun belirlenmesinde iki farklı yöntemin kullanılmasının nedenlerini kısaca açıklamak gerekirse, öncelikle Kjeldahl analizi daha fazla örnek gerektirmekte ve bu analiz ile sadece protein konsantrasyonu değil aynı zamanda toplam azotlu bileşikler de belirlenmektedir. Ayrıca çözündürme aşamasından sonra elde edilen numune miktarı çok düşük (50 µL) olup Kjeldahl analizi için yeterli olmamıştır. Böylece numuneler, protein konsantrasyonunun belirlenmesi için Lowry yöntemiyle tekrar analiz edilmiştir. Lowry analizlerine göre en yüksek miktarda çözünür protein konsantrasyonunu içeren numuneler ileri testler için seçilmiştir. Yumurta kabuğu zarı proteini eldesinde ilk aşamada, yumurta kabuğu zarı toz haline getirilmiştir. Daha sonra, 0.1, 0.5 ve 1 g Triton X-100 (T) (Merck, Darmstadt, Almanya) ve sodyum dodesil sülfat (SDS) deterjanlarını içeren 100 mL 50 mM Tris-HCl tampon (lisis) (Merck, Darmstadt, Almanya) solüsyonları (Sigma Aldrich, Steinheim, Almanya) 0.1 mL 2-merkaptoetanol çözeltisi eklenerek hazırlanmıştır. Lisis çözelti (1000 µL), 100 mg yumurta kabuğu membran tozu ile karıştırılarak 3 dakika boyunca vortekslenmiştir. Karışım gece boyunca 4°C'de muhafaza edilmiştir. Ardından, ESM lizatı 1 dakika boyunca 10.000 rpm'de

santrifüjlenerek 400 µL süpernatant başka bir tüpe aktarılmıştır. Aktarılan süpernatanta 800 µL saf aseton eklenerek karışım ESM proteinlerinin çökmesi için gece boyunca 4°C'de muhafaza edilmiştir. Ardından karışım 10000 rpm'de 30 dakika boyunca santrifüjlenmiştir. Süpernatant uzaklaştırılarak protein tortusu, Lowry analizleri için 50 µL ultra saf su içerisinde yeniden süspansiyon edilmiştir (18). Yumurta kabuğu zarı proteinleri sıvılaştırıldıktan sonra sıvı protein üzerinde yapılacak olan analizler gerçekleştirilmiştir. Bunlardan ilki Lowry analizidir. Lowry protein tayini sonucunda protein oranlarına göre aynı Kjeldahl yönteminde olduğu gibi seçim yapılmıştır. 3 adet en yüksek protein oranına sahip örnek seçilerek, diğer analizlere bu örnekler üzerinden devam edilmiştir.

- ✓ Lowry analizi, Kjeldahl protein tayininden eleme yöntemiyle elde edilen 7 örnek için ayrı ayrı 50 µL protein izolatları üzerine Lowry kimyasalları eklenerek 0, 30, 60, 240, 720 ve 1440. dakikalarda spektrofotometrik olarak gerçekleştirilmiştir. BSA ile kalibrasyon eğrisi oluşturularak gerekli hesaplamalar yapılmıştır. Daha sonra en yüksek protein oranına sahip 3 örnek üzerinde su ve metanol ile yıkama suretiyle Lowry kimyasallarının uzaklaştırıldığı örnekler üzerinde de yıkamanın protein oranı üzerindeki etkisini belirlemek amacıyla Lowry protein tayini gerçekleştirilmiştir.
- ✓ ESM örneklerinde ana protein fraksiyonlarını (48) belirlemek için bazı modifikasyonlarla SDS-PAGE analizi Ankara Üniversitesi Ziraat Fakültesi Süt Teknolojisi Bölümü'nde (Ankara, Türkiye) gerçekleştirilmiştir (49).
- ✓ 1 mg çözünür yumurta zarı numunesi, 100 mL SDS (Sigma Aldrich, Steinheim, Almanya) numune tampon çözeltisi içerisinde çözülmüştür. Standart olarak Sigma marker (Sigma Aldrich), yağsız süt tozu ve peynir altı suyu tozu kullanılmıştır. Ayırma jelinin konsantrasyonu % 12.5 (%T) ve moleküler boyut ayırma aralığı 10000-100000 Mw olarak analiz gerçekleştirilmiştir. Çalışmada Protean II XL dikey jel ünitesi kullanılmıştır (Bio-Rad Laboratories Ltd., Watford, İngiltere). Numuneler yüklenmeden önce jeller, 200 V'de 40 dakika boyunca çalıştırılmıştır. Daha sonra jel içerisine 10 µL numune yüklenerek 200 V sabit voltajda çalışma gerçekleştirilmiştir. Çalışma tamamlandıktan sonra jeller fiksasyon solüsyonunda 60 dakika bekletildikten sonra boyanmıştır. Jeller, Agfa Fotolook v3.5 (Mortsel, Belçika) ile kontrol edilen Agfa Arcus 1200 tarayıcı (Mortsel, Belçika) kullanılarak taranmıştır.
- ✓ Köpürme kapasitesi ve stabilitesinin hesaplanması için 1 mL SEP alınmış ve 5 mL su ile karıştırılmıştır. Karışım maksimum hızda 5 dakika boyunca vortekslenmiştir. Numunelerin 1. ve 10. dakikadaki köpük hacimleri kaydedilerek gerekli hesaplamalar yapılmıştır (30). Ardından Hunter Lab Color Flex Spectrophotometer (Hunter Associates Laboratory Inc., Reston VA, USA) cihazıyla örneklerin L^* , a^* ve b^* değerleriyle birlikte ΔE , chroma ve Hue değerleri hesaplanmıştır.
- ✓ Reolojik ölçümler, Ankara Üniversitesi Ziraat Fakültesi Süt Teknolojisi Bölümü'nde (Ankara, Türkiye) Malvern Kinexus Lab Pro+ (Malvern Instruments Ltd., Malvern, İngiltere) dinamik reolojik ölçüm cihazı ile gerçekleştirilmiştir. Kayma gerilimi ölçümleri 0.01-100 Hz kayma hızı

aralığında ve 5°C'de yapılmıştır. Ölçümlerde 4° konik prob (Ø40 mm) kullanılmış olup, aralık 2 mm olacak şekilde ayarlanmıştır. Ölçüm sonrasında akış modeli cihaz yazılımı tarafından belirlenmiştir (29).

- ✓ Yumurta kabuğu zarının amino asit bileşimi, ikili pompa dağıtım sistemi, Agilent 1100 yüksek performanslı sıvı kromatografisi (Ramsey, MN, USA) ile TUBİTAK-MAM (Kocaeli, Türkiye) tarafından gerçekleştirilmiştir. Amino asit standartları, 95 µL 250 pmol/µL amino asit standart karışımının 5 µL 10 mM norvalin (Sigma Aldrich, Steinheim, Almanya) ile karıştırılmasıyla hazırlanmıştır. Kalibrasyon eğrisi 20, 50, 130, 250 veya 500 pmol/µL amino asit standart karışımının nihai konsantrasyonunu elde etmek için 1 nmol/µL amino asit standart çözeltisinin (Sigma Aldrich, Steinheim, Almanya) seyreltilmesiyle hazırlanmıştır. ESM ekstraktları (7-75 µg) cam test tüplerine aktarılmış ve 0.5 mM norvalin eklenmiştir. Düşük hızda santrifüjlenerek liyofilizatörde (115V ABD Modeli, Fisher Scientific, Waltham, MA, ABD) dondurularak kurutulmuştur. Numuneler, tabanında 0.5 mL HCl (Sigma Aldrich, Steinheim, Almanya) bulunan reaksiyon şişesine aktarılmıştır. Reaksiyon şişesi kapatılarak fırında (Mmert, Schwabach, Almanya) 110°C'de 18 saat önceden ısıtılmıştır. Reaksiyon şişesi oda sıcaklığına kadar soğutulmuştur. Kalıntılar 100 µL 0.1 N HCl içerisinde çözülerek HPLC cam ekleme şişelerine aktarılmış ve kuru toz halindeki ESM numunelerinin hidrolizasyonu gerçekleştirilmiştir. Hidrolize ESM numuneleri ve norvalin eklenmiş amino asit standart çözeltileri, o-fitalaldehit (OPA) ile otomatik örnekleyici tarafından analize hazırlanmıştır. Ardından her numuneden 2.5 µL alınarak 40 °C'de 338 nm'de çalıştırılan UV dedektörüyle (Agilent, Ramsey, MN, ABD) çalışan ve Zorbax Eclipse-AAA kolonu (5 µm x 150 x 4.6 mm) takılı olan HPLC cihazına enjekte edilmiştir. Mobil faz A, 40 mM NaH₂PO₄ (pH 7.8) (Sigma Aldrich, Steinheim, Almanya) ile oluşturulurken, mobil faz B asetonitril/metanol/su (45/45/10 h/h/v) (Sigma Aldrich, Steinheim, Almanya) ile oluşturulmuştur (30, 31).

SEP üzerinde yapılan analizler tamamlandıktan sonra yumuşak jel kapsül üretim aşamasına geçilmiştir. Lowry analizi sonucunda seçilen en yüksek protein konsantrasyonuna (çözünür ESM proteinleri) sahip 3 örnek, yumuşak jel kapsüllerde kullanılmıştır. Yumuşak jellerin formülasyonu için yaklaşık 200 mL su, 16 mL gliserin, 16 g sığır jelatini ve 10 g şeker kullanılmıştır. Öncelikle 200 mL suya 16 mL gliserin ve 10 g şeker ilave edilerek şeker tamamen eriyene kadar karıştırılmıştır. Kaynatılmadan önce pektin ve jelatin ilave edilerek karışım sürekli olarak karıştırılmıştır. Son olarak karışım kaynatıldığında 100 mM EDTA 1:20 ve 1:40 SEP ilave edilerek hazırlanacak olan kapsüllere gıdada kullanılabilir renklendirici (yeşil ve kırmızı renk, GLR Secret, Sıvı Gıda Boyası) ve aromalar (elma ve çilek, Aromsa) (3-4 damla) eklenirken; su 1:40 SEP örneklerinin ilave edileceği kapsüllere renklendirici ve aroma maddesi eklenmemiştir. Daha sonra karışım 45-50 °C'ye soğutulurken, karışıma 50 µL SEP ilave edilmiştir. Nihai karışım, her bir kapsülün yaklaşık 1 g olduğu tabletlere aktarılmıştır. Kalıp, yumuşak jel oluşumu tamamlanana kadar bir gece 4 °C'de bekletilmiştir. Her bir yumuşak kapsül 1.03 mg/g-1.45 mg/g ESM proteini içermektedir. Beslenme referans alımına (DRI) göre günlük protein alımı, vücut ağırlığının kilogramı başına 0.8 gram olarak önerilmektedir. Dolayısıyla eklenen ESM protein miktarı DRI değerleri göz önünde

bulundurulurak belirlenmiştir. Elde edilen kapsüller üzerinde tekstürel, mikrobiyolojik, duysal ve mikrobiyolojik analizler gerçekleştirilmiştir.

- ✓ Yumurta kabuğu zarından elde edilen kapsüllerde tekstür profil analizi (TA-XT PlusC Texture Analizörü, ABP, Ankara, Türkiye) için sertlik ve esneklik parametreleri incelenmiştir. Numunelerin esneklik ve sertlik değerleri çelik silindirik (P/35R) problu doku analiz cihazı ile yapılmıştır. İlk sıkıştırma eğrisinden belirlenen sertlik, numuneyi belirli bir seviyeye kadar deforme etmek için gereken kuvvet veya ilk sıkıştırma için gereken maksimum kuvvet olarak tanımlanmıştır. Esneklik, numunenin ikinci sıkıştırmadaki mesafesinin (Mesafe 2: M2) birinci sıkıştırma mesafesine (Mesafe 1:M1) (M2/M1) bölünmesiyle hesaplanmıştır. Esneklik, sıkıştırıldıktan sonra numune dokusunun yeniden şekillenme kapasitesini yansıtmakla birlikte numunenin orijinal şekline dönme yeteneğinin bir göstergesidir (55).
- ✓ Yumuşak jel kapsüllerin koku, renk, tatlılık ve ağızda kalan tat gibi duysal özellikleri, 15 eğitimli panelist tarafından 9 dereceli hedonik ölçekle ölçülmüştür. Numuneler rastgele 3 haneli rakamlarla panelistlere sunulurak numuneler arasında tuzsuz kraker tüketmeleri ve damaklarını temizlemek amacıyla numuneler arasında su içmeleri istenmiştir.
- ✓ Son olarak hava geçirmez cam şişelerde saklanan yumuşak jel kapsüller 4 °C’de muhafaza edilerek toplam mezofilik aerobik bakteri (TAMB), toplam maya ve küf (TMY) ve *Salmonella* spp. için 4. haftaya kadar haftada bir, 32. haftaya kadar ise 2 haftada bir numune alınarak mikrobiyolojik analizler gerçekleştirilmiştir (58). Sonuçlar log CFU/g olarak rapor edilmiştir.

Sonuçlar, Windows 17.0.0 sürümü için SPC yazılımı (Minitab®, Triola İstatistik Şirketi) kullanılarak analiz edilerek elde edilen veriler üzerinde tek yönlü varyans analizi (ANOVA) yapılmıştır. Karşılaştırmanın ortalama değerler açısından istatistiksel anlamlılığı Tukey HSD testi ile test edilmiştir.

Sonuçlar ve Tartışma

Numunelerin iletkenliğinin yumurta kabuğu:EDTA oranından ziyade artan EDTA konsantrasyonundan etkilendiği gözlenmiştir ($p < 0.05$). Numunelerin su ile iletkenliği, aynı yumurta kabuğu:çözelti oranında EDTA numunelerinkinden önemli ölçüde daha yüksek bulunmuştur ($p < 0.05$). Genel olarak numunelerin bulanıklığı EDTA konsantrasyonundan ve yumurta kabuğu:çözelti oranından etkilenmekle birlikte karışımların bulanıklığında belirgin bir eğilim gözlenmemiştir. Su ile hazırlanan numunelerin, konsantrasyon ve orana bakılmaksızın EDTA numunelerine göre önemli ölçüde daha yüksek iletkenliğe sahip olduğu gözlenmiştir. (Tablo 4.1). İletkenlik ölçümü suyun elektrik akımını taşıma yeteneğini göstermektedir. Fosfat, klorür, nitrat ve sülfat anyonları gibi negatif yüklü inorganik çözülmüş katılar veya sodyum, magnezyum, kalsiyum, demir ve alüminyum katyonları gibi pozitif yüklü iyonlar suyun iletkenliğini etkileyebilmektedir. Şeker, alkol, fenol ve yağ gibi elektrik iletkenliği düşük organik maddeler suda daha az iletkendir (114). EDTA çok düşük elektrik

iletkenliğine sahip olduğundan, EDTA ile hazırlanan tüm karışımlar daha düşük elektrik iletkenliği göstermiştir.

Numunelerin iletkenliğinin artan asetik asit konsantrasyonundan ve yumurta kabuğu:asetik asit oranından etkilendiği gözlenmiştir ($p < 0.05$). Numunelerin su ile iletkenliği, aynı yumurta kabuğu:çözelti oranında asetik asit numunelerinkinden önemli ölçüde daha yüksek bulunmuştur ($p < 0.05$). Genellikle numunelerin bulanıklığı asetik asit konsantrasyonundan ve yumurta kabuğu:çözelti oranından etkilenmiştir ancak karışımların bulanıklığındaki değişikliklerde belirgin bir eğilim gözlenmemiştir. Asetik asitli karışımların pH değeri yumurta kabuğu:asetik asit oranından fazla etkilenmezken, asetik asit konsantrasyonunun artmasından etkilenmiştir. Ayrıca su ile hazırlanan karışımların pH dereceleri aynı oranlarda asetik asit ile hazırlanan karışımlara göre daha yüksek bulunmuştur ($p < 0.05$). (Tablo 4.2).

Numunelerin iletkenliğinin hem HCl konsantrasyonunun hem de yumurta kabuğu:HCl oranının artmasıyla önemli ölçüde arttığı gözlenmiştir ($p < 0.05$). Numunelerin su ile iletkenliği ise yumurta kabuğu:çözelti oranının artmasıyla önemli ölçüde azalmıştır ve %1.85 HCl ile hazırlanan numunelerin iletkenliği ölçülememiştir. Genel olarak numunelerin bulanıklığı HCl konsantrasyonundan etkilenmiştir; 1:20 oranlı numunelerin bulanıklığı artan HCl konsantrasyonuyla birlikte artarken; ancak 1:30 ve 1:40 oranındaki numuneler, 1:20 oranındaki numunelere kıyasla %0.37 HCl ile en yüksek bulanıklığı göstermiştir. Su örneklerinin bulanıklığı, HCl örnekleriyle hazırlanan emsallerine göre önemli ölçüde daha düşük bulunmuştur ($p < 0.05$).

HCl içeren karışımların pH değeri, yumurta kabuğu:HCl oranından önemli ölçüde etkilenmiş ve hem yumurta kabuğu:çözelti oranı hem de artan HCl konsantrasyonu ile numunelerin pH'ı düştüğünden, HCl konsantrasyonunun arttığı gözlenmiştir. Su ile hazırlanan karışımların pH değeri, yumurta kabuğu:çözelti oranının artmasıyla önemli ölçüde değişmemiştir ($p < 0.05$) (Tablo 4.3). Tablo 4.1, 4.2 ve 4.3'te sunulan pH, iletkenlik ve bulanıklık ölçümüyle üç farklı oranda (1:20, 1:30 ve 1:40) farklı kimyasallarla (EDTA, HCl ve asetik asit) yapılan ölçümün amacı, sıvı kısma aktarılan katı konsantrasyonunun önemli olup olmadığını belirlemektir. Yapılan bu çalışma ile ESM'yi yumurta kabuğundan ayırmak için hangi kimyasalın hangi oranda daha iyi olduğu hakkında bilgi vermiştir. Ancak fiziksel özelliklerin ölçülmesiyle bir sonuca varılamadığından, daha yüksek protein konsantrasyonuyla en etkili ayırma yöntemini belirlemek amacıyla numunelerin ham protein içeriği Kjeldahl yöntemiyle belirlenmiştir.

Farklı yumurta kabuğu:sıvı oranları, EDTA, asetik asit, HCl ve suyun protein konsantrasyonu üzerinde farklı etkiler ortaya çıkarmıştır. Protein konsantrasyonlarına göre örnek sayısı 27'den 7'ye düşürülerek geri kalan çalışmalara bu 7 örnekle devam edilmiştir. Strelec ve arkadaşları (60) %5 HCl ile muamele edilen yumurta kabuğu zarında Kjeldahl analizi ile benzer protein oranının 97.03 ± 1.57 olduğunu bildirmiştir. Yumurta kabuğunu ve ESM'yi ayırmak için %5 EDTA çözeltisinin kullanılması, %88.2 protein konsantrasyonunu ortaya çıkarmıştır (42); bu sonucun, çalışmamızda 100 mM EDTA 1:20 (%83.36) ile muamele edilen numunelerin protein içeriğine yakın olduğu gözlenmiştir. Diğer örneklerin protein konsantrasyonu bu çalışmaya göre nispeten daha düşük olmuştur

ve bu durumun EDTA konsantrasyonu ve inkübasyon süresinden kaynaklanmış olabileceği tahmin edilmiştir. Tablo 4.4'teki sonuçlara bakıldığında suda tutulan zarların protein oranlarının birbirine yakın olduğu, HCl ile ayrılan zardaki protein oranının ise daha düşük olduğu görülmektedir. Torres-Mansilla ve Delgado-Mejía (61), yumurta kabuğu beyazının %5 HCl'ye maruz bırakılmasının ESM'deki hidrojen bağlarında denatürasyon ve kırılma gibi bazı kimyasal ve fiziksel değişikliklere neden olabileceğini belirtmiştir; bu değişiklikler muhtemelen asitle muamele edilmiş ESM'de su ile muamele edilmiş ESM'ye göre daha düşük protein içeriğinin belirlenmesine neden olmuştur (61). Genel olarak EDTA ile ekstrakte edilen örneklerin protein içeriği, asetik asit ve HCl örneklerinden daha yüksek protein konsantrasyonu sergilemiştir. Bu sonuçlar, protein olmayan organik maddenin ekstraksiyonundan ve/veya kısmi hidrolizinden kaynaklanmış olabileceğini göstermektedir (60). Genel olarak EDTA ile ekstrakte edilen örneklerin protein içeriği, asetik asit ve HCl örneklerinden daha yüksek protein konsantrasyonu sergilemiştir. Bu sonuçların protein olmayan nitrojenin ekstraksiyonundan ve/veya kısmi hidrolizinden kaynaklanabileceği düşünülmektedir (60). Kjeldahl analizi, serbest amino asit nükleotidleri, nükleik asitler, üre ve poliamidler de dahil olmak üzere 200'den fazla farklı bileşik içeren protein olmayan nitrojen fraksiyonunun %25'ini içerebilen toplam nitrojen içeriğini sağlamaktadır (113). Bu nedenle ESM örneklerinin toplam protein içeriğinin Tablo 4.4'te belirtilenden %25 daha az olduğu tahmin edilmektedir. Önceki adımdan en yüksek protein konsantrasyonlarına sahip numuneler, ESM proteinini çözündürmek için Triton X-100 (T) ve SDS ile %0.1, %0.5 ve %1.0 konsantrasyonlarda 1440 dakika tutma süresine kadar işlenmiştir.

EDTA, asetik asit ve HCl ön işlemlerinden en yüksek protein içeriğini ortaya çıkaran örnekler, T ve SDS ile ekstraksiyon işlemleri sonrasında uygulanan Lowry analizi sonucuna göre seçilmiştir. %1 SDS ile ekstrakte edilen 50 mM EDTA 1:20 örneği için en yüksek protein konsantrasyonu, Lowry analizindeki 240 dakikalık bekleme süresi sonunda 208.40 ± 0.00 mg/mL olarak bulunmuştur (Tablo 4.5). %1 SDS ile ekstrakte edilen 100 mM EDTA 1:20 örneği için ise en yüksek protein konsantrasyonu, Lowry analizindeki 60 dakikalık bekleme süresi sonunda 252.49 ± 0.19 mg/mL olarak bulunmuştur (Tablo 4.6). %1 SDS ile ekstrakte edilen 100 mM EDTA 1:40 örneği için en yüksek protein konsantrasyonu, Lowry analizindeki 60 dakikalık bekleme süresi sonunda 244.32 ± 0.12 mg/mL olarak bulunmuştur (Tablo 4.7). %0.5 SDS ile ekstrakte edilen %1.25 asetik asit 1:30 örneği için en yüksek protein konsantrasyonu, Lowry analizindeki 720 dakikalık bekleme süresi sonunda 168.79 ± 0.12 mg/mL olarak bulunmuştur (Tablo 4.8). %1 SDS ile ekstrakte edilen %1.25 asetik asit 1:40 örneği için en yüksek protein konsantrasyonu, Lowry analizindeki 240 dakikalık bekleme süresi sonunda 177.55 ± 0.12 mg/mL olarak bulunmuştur (Tablo 4.9). %1 SDS ile ekstrakte edilen %0.185 HCl 1:30 örneği için en yüksek protein konsantrasyonu, Lowry analizindeki 60 dakikalık bekleme süresi sonunda 165.39 ± 0.12 mg/mL olarak bulunmuştur (Tablo 4.10). %1 SDS ile ekstrakte edilen %100 su 1:40 örneği için en yüksek protein konsantrasyonu, Lowry analizindeki 240 dakikalık bekleme süresi sonunda 186.21 ± 0.00 mg/mL olarak bulunmuştur (Tablo 4.11).

En yüksek protein içeriğine sahip numuneler seçildikten sonra, lizis solüsyonlarında kullanılan deterjanlarla yapılan ekstraksiyon işleminin protein konsantrasyonuna etkisini gözlemlemek amacıyla numunelerin yıkama öncesi ve

sonrası protein konsantrasyonları yeniden belirlenmiştir. %1 SDS deterjanı ile ekstrakte edilen 100 mM EDTA 1:20 örnekleri üzerindeki yıkama işleminin etkisi incelendiğinde, Lowry analizindeki 720 dakikalık bekleme süresinin sonunda en yüksek protein oranı belirlenmiştir (Tablo 4.12). %1 SDS deterjanı ile ekstrakte edilen 100 mM EDTA 1:40 örnekleri üzerindeki yıkama işleminin etkisi incelendiğinde, Lowry analizindeki 1440 dakikalık bekleme süresinin sonunda en yüksek protein oranı belirlenmiştir (Tablo 4.13). %1 SDS deterjanı ile ekstrakte edilen su örnekleri üzerindeki yıkama işleminin etkisi incelendiğinde, Lowry analizindeki 1440 dakikalık bekleme süresinin sonunda en yüksek protein oranı belirlenmiştir (Tablo 4.14). Yıkama işlemi sonrasında numunelerin protein konsantrasyonunda yaklaşık %5-10 oranında artış gözlenmiştir. Yıkama işleminde kullanılan metanolün hidrojen bağlarını açarak protein konsantrasyonunu arttırması ve bu nedenle yıkanan numunelerin yıkanmamış numunelere göre daha yüksek protein içeriğine sahip olması mümkündür.

Kaweewong ve ark. (18) yumurta kabuğu atıklarını 10 dakika suda bekleterek zarlarından ayırmışlardır (18). Yumurta kabukları daha sonra sıvı nitrojenle işlenerek analizde kullanılmıştır. Çalışmada kullanılan membranlar, SDS ve Triton X-100 içeren lizis solüsyonları ile muamele edilerek çözünür hale getirilerek çözünen numuneler üzerinde protein konsantrasyonunu belirlemek için Lowry analizi yapılmıştır. Bu çalışma ile karşılaştırıldığında bu çalışmanın sonuçlarının belirgin bir şekilde daha yüksek olduğu görülmektedir. ESM'nin asitlerdeki protein konsantrasyonu suya göre daha yüksek olup, bunun farklı bekleme sürelerinden ve asitle soyulma işleminden kaynaklandığı düşünülmektedir. Kaweewong ve ark. (18) yaptıkları çalışmada bizim çalışmamızda da olduğu gibi SDS deterjanının Triton X-100 deterjandan daha etkili bir ajan olduğunu gözlemlemişlerdir. Her iki çalışmada da deterjan konsantrasyonunun %0.1'den %1.0'a çıkarılmasıyla ekstraksiyon yeteneğinin arttığı görülmüştür (18). Jain ve Anal'in (47) çalışmasında yumurta kabukları ve zarları saf su içerisinde tutularak ayrıştırılmıştır. Yaklaşık 36 saat süreyle saklanan protein hidrolizatlarında bulunan protein miktarı 177.3 mg/g olarak bulunmuştur. Cheng ve ark. (62) ise, *Pseudomonas aeruginosa* ME-4 suşundan yumurta kabuğu membranını parçalayan proteazın saflaştırılması ve karakterizasyonu ile ilgili bir çalışma gerçekleştirmişlerdir. Başka bir çalışmada Lowry yöntemiyle ölçülen protein konsantrasyonunu $140 \pm 3.4 \mu\text{g/U}$ olarak bildirilmiştir (62). Marimuthu ve ark. (63) membranları yumurta kabuğundan ayırmak için %20 NaOH, %5 metanol+%20 NaOH, %5 EDTA+%5 hekzan+%20 alkali gibi farklı işlemler uygulamışlardır. Bu işlemler sonucunda elde edilen protein oranları sırasıyla %91, %64.5 ve %65 olmuştur. Literatürde buna benzer bir çalışma bulmak zor olsa da SDS uygulanan numunelerin en yüksek verime sahip olduğu görülmektedir. Lizis solüsyonlarında kullanılan SDS ve Triton X-100 deterjanlarının yanı sıra maruz kalma süresinin de etkili bir parametre olduğu bulunmuştur. Yumurta kabuğu zarı proteinlerini çözünür hale getirmek zor olsa da en etkili yöntemin bu olduğuna karar verilmiştir.

Tez kapsamında yapılan çalışmalarda 1:20 oranındaki 100 mM EDTA'nın, 1:40 oranındaki 100 mM EDTA'nın ve 1:40 oranındaki suyun WHC ve OHC değerlerinin birbirinden önemli ölçüde farklı olmadığı bulunmuştur ($p > 0.05$) (Tablo 4.15). WHC, protein-su etkileşimlerini anlamak için kullanılan bir parametredir ve aynı zamanda bir molekülün, dış kuvvetlerin etkisi altında suyu tutma yeteneğini de gösterir (64). Yumurta kabuğu membranlarının WHC ve OHC

değerleri sırasıyla 1.90 ± 1.00 ile 2.90 ± 0.40 g/g ve 2.50 ± 1.00 ile 4.40 ± 0.40 g/g arasında değişmektedir (47). Bu sonuçlar mevcut çalışmada sunulan örneklerden daha düşük bulunmuştur. Püskürterek ve dondurarak kurutma yöntemiyle kurutulan yumurta beyazı proteininin WHC ve OHC'si sırasıyla 0.65 ± 0.06 'dan 1.37 ± 0.03 g/g'a ve 3.12 ± 0.07 'den 10.21 ± 0.13 mL/g'a kadar değişmiştir (65). Bu çalışma bizim çalışmamızla karşılaştırıldığında, Tablo 4.15'te su tutma kapasitesinin daha yüksek olduğu ve yağ tutma kapasiteleri birbirine çok yakın olduğu görülmektedir Yumuşak jel kapsülleri hazırlamak için 100 mM EDTA 1:20, 100 mM EDTA 1:40 ve en yüksek protein konsantrasyonuna sahip su numuneleri kullanılmıştır. Proteinin hem WHC'si hem de OHC'si ekstraksiyon için kullanılan kimyasallardan etkilenmiştir; çünkü bu kimyasalların proteinin denatürasyonuna neden olabileceği düşünülmektedir. Düşük WHC ve OHC değerlerinin, kullanılan EDTA kimyasalının yumurta kabuğu zarını kısmen tahrip etmesinden kaynaklanmış olabileceği bildirilmiştir. (44).

Numunelerin emülsiyon stabilite indeksi (ESI) birbirinden önemli ölçüde farklı bulunmuştur. 100 mM EDTA 1:40 örneklerinin ESI'si 77.27 ± 3.93 olarak ölçülürken; 100 mM EDTA 1:20 ve su 1:40 numunelerinin ESI'si sırasıyla 65.76 ± 1.57 ve 23.98 ± 1.76 olarak ölçülmüştür (Tablo 4.16). Numunelerin EAI'si de numuneler arasında önemli ölçüde farklılık göstermiş olup; 100 mM EDTA 1:20, 100 mM EDTA 1:40 ve su 1:40 numunelerinin EAI değerleri sırasıyla 0.0117 ± 0.0001 , 0.0016 ± 0.0000 ve 0.0021 ± 0.0000 m²/g olmuştur ($p < 0.05$) (Tablo 4.16). Protein molekülünün hidrofobiklik derecesine ve hidrasyona bağlı olan çözünürlüğün, proteinlerin en önemli fiziko-kimyasal ve fonksiyonel özelliklerinden biri olduğu bilinmektedir (66). Gıda ürünlerinde köpük, emülsiyon ve jel oluşumu gibi kullanım durumlarında proteinlerin çözünürlük oranlarının belli bir seviyenin üzerinde olması önemlidir (67). EAI, proteinin emülsiyon oluşturma kapasitesini ifade ederken, ESI, emülsiyon damlacıklarının dağılma, yağda yüzmeye ve belirli bir süre boyunca birikmeden dağılmış halde kalma yeteneğinin bir ölçüsüdür (68). Yumurta akı örneklerinin EAI'si 84.63 ± 1.81 ile 100.10 ± 1.86 arasında değişirken; ESI değeri ise 20.62 ± 1.17 ile 33.74 ± 2.43 arasında değişmiştir (39). Numunelerin hem EAI hem de ESI'si, proteinin türüne ve işleme yöntemine bağlı olarak farklılıklar göstermiştir.

Çözünür yumurta kabuğu proteini ekstraktı, uygulanan işleme bakılmaksızın hiçbir köpürme özelliği göstermemiştir. ESM'nin farklı konsantrasyonlardaki köpüklenme özellikleri, köpükleme kapasitesi ve köpük stabilitesi açısından sırasıyla 36.67 ± 2.89 ile 48.33 ± 2.89 ve 23.33 ± 3.33 ile 30.00 ± 2.89 arasında değişmektedir (69). Yumurta akının köpürme kapasitesi 31.00 ± 0.00 ; köpük stabilitesi ise 39.00 ± 0.00 olarak rapor edilmiştir (65). Bildirilen köpürme yetenekleri, proteinlerin kaynağına bağlı olarak büyük farklılıklar göstermektedir. 1:40 su örneklerinin toplam renk farkı ve chroması diğerlerinden önemli derecede farklı iken hue değerlerinde örnekler arasında istatistiksel olarak bir fark gözlenmemiştir ($p > 0.05$) (Tablo 4.17). Daha önce yapılan çalışmalarda iki farklı kuş türüne ait yumurta sarısının L^* , a^* ve b^* değerleri sırasıyla 73.4, -2.5 ve 25 olarak rapor edilmiştir (70). Çalışmamızda a^* değerleri daha düşük bulunurken, hem L^* hem de b^* değerleri yüksek bulunmuştur. Negatife yakın bir a^* değeri, yeşil tonun arttığı anlamına gelmektedir. Çalışmada yumurta sarısı ile çalıştıkları için a^* değerinin Tablo 4.17'de bulunan değerden düşük olması mantıklı bulunmuştur. Suyu ayıran membranların L^* ve a^* değerlerinin diğerlerinden farklı çıkmasının

nedeninin, EDTA kimyasalı nedeniyle EDTA ile ayrılan numunelerin yapısında meydana gelen değişikliklerden kaynaklı olabileceği tahmin edilmiştir. Kurutulmuş ve hidrolize edilmiş yumurta akı tozunun renk ölçümünde L^* değerinin 96-98 arasında olduğu ve bu çalışmadaki değerlerden oldukça yüksek olduğu görülmüştür (71). Bu çalışmada ESM protein ekstraktı ile karşılaştırıldığında, yumurta ürünlerinin farklı fraksiyonları, ham ürünlerin doğası ve/veya uygulanan işlemler nedeniyle önemli ölçüde farklı renk özellikleri sergilemiştir.

EDTA 1:40 ve su numunelerinin akış davranışlarının birbirine daha yakın bulunurken; EDTA 1:20 numunesi önemli ölçüde farklı akış davranışı göstermiştir. Şekil 4.1'de sunulduğu gibi, her numunenin kayma gerilimi, Newton tipi akışkan özellikleri sunan kayma hızındaki artışla birlikte yükselmiştir. 1:20 oranındaki EDTA, 1:40 oranındaki EDTA ve 1:40 oranındaki su örneklerinin modelleme çalışmaları, Bingham, Herschley-Bulkley ve Newton modelleriyle yüksek korelasyon ortaya koymuştur (Tablo 4.18). Yumurta sarısı ve çözünür ESM örneklerinin kayma hızının ve kayma geriliminin uygulanan kuvvetle orantılı olarak arttığı rapor edilmiştir (73). Çözünür yumurta kabuğu zarında olduğu gibi yumurta sarısında da kayma hızının artmasıyla birlikte viskozitenin azaldığı rapor edilmiştir (74). ESM protein ekstraktlarının reolojik analizleri literatürde bildirilmemiştir, bu nedenle bu çalışmanın sonuçları ESM protein ekstraktının reolojik özelliklerinin belirlenmesi için tek kaynak olarak karşımıza çıkmaktadır.

DSC termal analiz uygulamalarında yaygın olarak kullanılan bir tekniktir. Bu teknikte numune ile referans malzeme arasındaki enerji değişimi sıcaklığın bir fonksiyonu olarak belirlenebilir. Maddelerin ayırt edici özellikleri olan erime sıcaklığı ve ısı kapasitesi bu cihazla kolaylıkla ölçülebilmektedir (75). Çeşitli çapraz bağlı membranların çeşitli sıcaklıklardaki termal stabilitesi, dinamik taramalı kalorimetri yaklaşımı kullanılarak belirlenebilir (76). DSC, ESM'nin vücut sıcaklığında nasıl davrandığının yanı sıra membranın donma (0 °C), soğutma (2-4 °C) veya ortam koşullarında (23-25 °C) depolanırken bütünlüğünü koruma kapasitesini gösterebilir (77). Pik sıcaklık açısından en yüksek değer 100 mM EDTA numunelerinde 1:20 oranında tespit edilirken; en düşük pik sıcaklığı 100 mM EDTA örnekleri için 1:40 oranında tespit edilmiştir ($p < 0.05$). Numunelerin ısı değerleri 1:20 oranındaki 100 mM EDTA numuneleri için -1.70 ± 0.01 kJ/g ile 1:40 oranındaki su numuneleri için -1.47 ± 0.01 kJ/g arasında değişmektedir ve dolayısıyla örnekler ekzotermik sonuçlar göstermiştir (Tablo 4.19). ESM'nin farklı miktarlarda (%0, %20, %40 ve %60) su ile yapılan DSC analizi, endotermik piklerle birlikte 54.50-102.26 pik sıcaklık değerlerini ortaya çıkarmıştır. Örneklerin başlangıç sıcaklıkları 17.80-20.00 °C arasında değişmektedir. Ortaya çıkan sonuçlar, kolajenin termal denatürasyonu ile ilişkilendirilebilir. Kollajen denatürasyonunun, üç zinciri birbirine bağlayan hidrojen bağlarının kırılmasını ve kollajen üçlü sarmalının rastgele zincir konfigürasyonunda yeniden düzenlenmesini içeren bir süreç olduğu düşünülmektedir (29). Yumurta sarısının DSC analizinde pik sıcaklığı 83.3 °C, ısı değeri ise 1.29 J/g olarak kaydedilmiştir (78). Tablo 4.20'deki sonuçlarla karşılaştırıldığında yukarıdaki çalışmadaki sonucun düşük, ısı değerinin ise endotermik olduğu görülmüştür. Denatürasyon entalpisi, endotermik (hidrojen bağlarının parçalanması) ve ekzotermik değişikliklerin (hidrofobik etkileşimlerin veya protein toplanmasının bozulması) kombinasyonunun net değeridir. Bu nedenle ısı işlem sırasında protein yapısının kaybı, entalpideki azalmayla gösterilmiştir (78).

Genel olarak numunelerin FT-IR spektrumu birbirine benzer olup, birinci pik 3200-3500 cm^{-1} arasında, ikinci pik 2200-2400 cm^{-1} civarında ve üçüncü pik 1600-1700 cm^{-1} civarındadır. Numune 1 (1:20 oranında 100 mM EDTA) ve 2 (1:40 oranında 100 mM EDTA) birbirine benzer spektruma sahip olmasına rağmen, numune 2 1750 cm^{-1} civarında majör bir pik sergilemiştir. Ayrıca numune 3'ün (su) sırasıyla 1100-1250 ve 1300-1400 cm^{-1} civarında daha fazla pik verdiği gözlenmiştir (Şekil 4.2). Elle ayrılan yumurta kabuğu zarlarının özellikleri ve yumurta kabuğu mineralinin asitte çözünmesi, şekil ve yoğunluklarının yanı sıra emilim bantları açısından da farklılık göstermektedir. Çalışmada HCl örnekleri hariç tüm örneklerde amid III absorpsiyon bandı ve yumurta kabuğu mineraline ilişkin bant rapor edilmiştir (61). Bu bulgular ESM'nin karboksil, hidroksil ve amino grupları dahil olmak üzere çeşitli kimyasal gruplar içerdiğini göstermektedir (111). Bileşenlerin yapısı iç ve dış katmanlarda hemen hemen aynıdır (100). Daha önceki çalışmalarda ham ve HCl'den türetilmiş olan ESM örneklerinin FT-IR analizleri mevcut çalışmayla benzer veriler ortaya çıkarmıştır (60). Sonuçlara bakıldığında su kaynaklı ESM'lerin EDTA'lı ESM'lere göre daha fazla spektrum verdiği görülmüştür. Bunun temel nedeni, EDTA'nın kimyasal yapısından dolayı bazı proteinlerin inhibisyonu/denatizasyonu olarak yorumlanmaktadır. FT-IR spektroskopisi ile ESM üzerinde yapılan başka bir çalışmada ESM 3274 cm^{-1} (O-H ve N-H gerilmesi), 2929 cm^{-1} (C-H gerilmesi), 1643 cm^{-1} (amid C=O gerilmesi), 1536 cm^{-1} (amid N-H bükülmesi), 1451 cm^{-1} (CH_2 bandı), 1109 cm^{-1} (amin C-N germe) ve 661 cm^{-1} (C-S) noktalarında pikler ortaya çıkmıştır (110, 111, 112).

Örneklerin amino asit içerikleri incelendiğinde 1:20 oranındaki 100 mM EDTA, 1:40 oranındaki 100 mM EDTA ve 1:40 oranındaki su örneklerinde esansiyel aminoasitleri de içeren toplam 16 farklı amino asit tespit edilmiştir. Amino asitlerin konsantrasyonları örnekler arasında değişmektedir ve alanin, valin, lizin, izolisin, treonin, prolin, metiyonin, fenilalanin, histidin, tirozin konsantrasyonu, 1:40 oranında su örneklerinde en yüksek iken; glisin ve serin konsantrasyonları 100 mM EDTA 1:20 numunelerinde en yüksek bulunmuştur. Aspartik asit, glutamik asit ve lizin konsantrasyonu da 100 mM EDTA numunelerinde 1:40 oranında en yüksek bulunmuştur (Tablo 4.23). Buzağı (80), balık (37), domuz derisinden (81), yumurta kabuğu zarından (82), yumurta kabuğu zarı için fermente protein hidrolizatlarından (69) ve çözünür yumurta kabuğu zarından (83) elde edilen yumurta kabuğu zarı proteinlerinin amino asit bileşiminin karşılaştırılması Tablo 4.23'te gösterilmiştir. ESM'de hidroksiprolin, sistein, triptofan ve iminoasitler tespit edilmezken; buzağı, balık, domuz derisi, yumurta kabuğu zarı, yumurta kabuğu zarı için fermente protein hidrolizatları ve çözünür yumurta kabuğu zarında hidroksiprolin tespit edilmiştir. Ayrıca dana derisi, balık derisi, yumurta kabuğu ve SEP'teki sistein; fermente protein hidrolizatlarında triptofan ve fermente protein hidrolizatı ve SEP hariç tüm örneklerde iminoasitler bulunmuştur (Tablo 4.23).

Çözünür ESM proteinlerinden oluşan bir matris içindeki çözünür ve çözünmeyen proteinlerin SDS-elektroforetik modeli Şekil 4.6'da gösterilmiştir. Üç numunenin SDS-PAGE'inde farklılıklar gözlenmiştir. SDS-PAGE, 10 ile 66 kDa arasında yedi farklı göç bandını ortaya çıkarmıştır. Ovocleidin-17 olarak tanımlanan çözünmeyen fraksiyonun ana bantlarından ikisi olan 17 ve 23 kDa (Ovocleidin-23) bantları Mine ve arkadaşlarının çalışmasından belirlenmiştir (49). Yaklaşık olarak 14.3, 36, 45 ve 68.3 kDa olarak tespit edilen fraksiyonlarının sırasıyla lizozim, ovomukoid,

ovalbumin ve avidin olduğu belirlenmiştir (28, 58). Yumurta kabuğu matris proteinlerinden bazıları, yumurta beyazındaki ana proteinler olan lizozim (14.3 kDa) ve ovalbumin (45 kDa) tespit edilerek tanımlanmıştır. Ovocleidin-17 (17 kDa), yumurta kabuğuna özgü uterus proteini olarak tanımlanan ilk matriks proteinidir (49). Matriks proteinleri genel olarak asidik olmasına rağmen SDS ile ayrılan kabuk zarlarında da aynı proteinlere rastlanmıştır. Yumurta kabuğu matriksinden SDS ile protein ayrımı en etkili yöntem olmuştur. %1 SDS ile ayrılan yumurta kabuğu kütikülünde belirlenen protein fraksiyonlarının bu çalışmadaki fraksiyonlara benzer olduğu gözlenmiştir (12). Yumurta kabuğu proteininin SDS-PAGE analizi aynı zamanda 14 kDa lizozim proteinini de ortaya çıkarmıştır (85). EDTA ve asetik asit ile çözündürülmüş çeşitli kuş türlerinin yumurta kabuklarından elde edilen ESM'de 14.4 kDa'da lizozim, 17 kDa'da ovocleidin-17 ve 45 kDa'da ovalbumin proteinleri ortaya çıkmıştır (86).

1:20 oranında 100 mM EDTA, 1:40 oranında 100 mM EDTA ve 1:40 oranında su ekstraktlarıyla üretilen yumuşak jel kapsül, doku, duyusal ve mikrobiyolojik analizler açısından test edilmiştir. Numunelerin sertliği 1:40 oranındaki 100 mM EDTA numuneleri için 169.58 ± 24.40 g ve 1:40 oranındaki su için 388.60 ± 60.40 g arasında değişmektedir. Numunelerin esneme-gerilme özellikleri birbirinden önemli derecede farklı olduğu bulunmuştur ($p < 0.05$). 1:40 oranında su ile hazırlanan yumuşak jel kapsüllerin en yüksek sertliğe (388.60 ± 60.40) sahip olduğu bulunmuştur (Tablo 4.24). Numunelerin gerilme özelliği, $\%68.38 \pm 6.72$ ile $\%76.94 \pm 5.31$ arasında değişmektedir ($p < 0.05$) (Tablo 4.24). %2, %4 ve %6 oranında ESM tozu içeren ekmeklerin sertlik değeri, artan ESM konsantrasyonu ile birlikte artmış; gerilme değeri ise sabit kalmıştır. Artan sertlik değerinin daha yüksek kül içeriğiyle de ilişkili olabileceği belirtilmiştir (88). Yumuşak jel kapsüllerin dokusal özellikleri temel olarak formülasyon ve işleme teknolojilerinden etkilenir çünkü literatürde bildirilen yumuşak jeller farklı dokusal özelliklere sahiptir.

Çözünür ESM'yi protein takviyesi olarak kullanmak için yumuşak bir jel formülasyonu geliştirilmiştir. Üretimden sonra yumuşak jel kapsüller, 9 noktalı hedonik tanımlayıcı teste dayalı olarak duyusal analizlere tabi tutulmuştur. EDTA 1:20 ve 1:40 numuneleri ile hazırlanan yumuşak jel kapsüller yüksek duyusal puanlar alırken, su 1:40 numunesi ile hazırlanan yumuşak jel kapsüller duyusal panelden düşük puanlar almıştır. Renk, koku, tat ve ağızda kalan tat açısından su örneğiyle hazırlanan yumuşak jel kapsüllerin daha düşük duyusal puanlara sahip olmasının temel nedeninin jel hazırlama aşamasında EDTA örnekleri için hazırlanan jelle renk ve aroma maddeleri eklenirken; su örneği için hazırlanan jelle eklenmemesinin olabileceği düşünülmektedir.

Mikrobiyolojik analizler için 4°C'de saklanan numuneler için mikrobiyolojik çalışmalara 32 hafta boyunca devam edilmiştir. İlk 4 hafta örneklerde mikrobiyal üreme hızı düşük olduğundan, 4. haftadan sonra örnekler iki haftada bir analize tabi tutulmuştur. 1:20 oranındaki 100 mM EDTA numuneleri 28. haftadan sonra bozulmuş ve diğer numuneler ise 32. haftaya kadar mikrobiyolojik olarak analize tabi tutulmuştur. 1:20 oranında 100 mM EDTA, 1:40 oranında 100 mM EDTA ve 1:40 oranında sudan üretilen yumuşak jel kapsüllerin XLD plakalarında *Salmonella spp.* üremesi tespit edilmemiştir. Yumuşak jel kapsüllerin mikrobiyal sayısının çok düşük olması, bunların tüketilmesinin güvenli olduğu sonucunu ortaya çıkarmış ve

bu nedenle yumuşak jel kapsüllerle duyuşal analizler gerekleřtirilmiřtir. 1:20 rnekteki 100 mM EDTA'nın ortalama bařlangı TMAB sayısı 0.84 ± 1.20 log kob/g iken 28. haftanın sonunda 4.06 ± 0.02 log kob/g'ye ($p < 0.05$) ykselmiřtir. İlave olarak 1:40 oranında 100 mM EDTA ve 1:40 oranında su ile retilen yumuşak jel kapsln ortalama bařlangı TMY sayısı 0.00 ± 0.00 log kob/g iken bu sayı 3.79 ± 0.00 ve 3.61 ± 0.03 log kob/g 'ya ıkmıřtır. Devam eden 32 haftalık depolama sonunda sırasıyla (Tablo 4.25-4.26) TMAB ve TMY sayımlarındaki artıřta depolama sresi ve protein ekstraktının nemli olduėu belirlenmiřtir ($p < 0.05$).

1:20 oranında 100 mM EDTA ve 1:40 oranında 100 mM EDTA ile retilen yumuşak jel kapsller benzer duyuşal puanlara sahip olmuř ve bu iki numune arasında renk, řekil, koku, tat, doku ve aėızda kalan tat aısından anlamlı bir fark bulunmamıřtır. 1:40 oranında su ile retilen yumuşak jel kapsllerin, 1:20 oranında 100 mM EDTA ve 1:40 oranında 100 mM EDTA ile retilen yumuşak jel kapsllere gre nemli lde daha dřk duyuşal zelliklere sahip olduėu bulunmuřtur ($p < 0.05$) (Tablo 4.27, řekil 4.7). Jle tr řekerleme rnlerinin duyuşal ve dokusal zelliklerinin jelatin, řeker ve inlinin hem konsantrasyonundan hem de oranından etkilendiėi daha nceki alıřmalarla ortaya konmuřtur. Jelatinin artması sertliėin artmasına ve beėenin azalmasına neden olmuřtur. Ayrıca řeker konsantrasyonunun artması, beėeni dzeyinde de artıř saėlamıř ve aslında genel beėenin, inlin ve jelatin miktarı dřk, řeker miktarı yksek olduėunda en yksek seviyede olduėu gzlenmiřtir (92).

Yapılan denemeler sonucunda Kjeldahl ve Lowry analizleri sonrasında EDTA zeltisi ile ayrılan kabuklardan daha yksek protein verimi elde edilmesine raėmen; suyla ayrılan yumurta kabuėu zarlarından da yksek verimde protein elde edildiėi tespit edilmiřtir. Bu nedenle membran ayırmada suyun kullanılması kimyasal kullanımının minimize edilmesi aısından daha ok tercih edilebilir.

Alternatif proteinler, evresel srdrlebilirlik, hayvan refahı kaygıları ve saėlıkla ilgili hususlar dahil olmak zere eřitli nedenlerden dolayı ilgi ekmektedir. Ancak yumurta kabuėu zarı proteinlerine yapılan zel vurgu, daha yeni bilgiler gerektirebilir. Yumurta kabuėu zarı proteinlerine olan ihtiyaın artmasının bira nedeni olabilir:

- ✓ Beslenme ve saėlık yararları: Yumurta kabuėu zarı proteinleri, besin ierikleri ve potansiyel saėlık yararları nedeniyle tanınabilir ve bu da tketiciler talebinin artmasına yol aabilir.
- ✓ Srdrlebilir kaynak kullanımı: Et retimi gibi geleneksel protein kaynaklarının evresel etkileri konusunda artan bir farkındalık varsa, yumurta kabuėu zarından elde edilenler de dahil olmak zere alternatif proteinlere doėru bir geiř olabilir.
- ✓ Fonksiyonel gıda ve nutrastikler: Temel beslenmenin tesinde saėlık yararları sunan fonksiyonel gıdalara ve nutrastiklere olan ilgi, yumurta kabuėu zarı proteinleri gibi benzersiz protein kaynaklarına olan talebe katkıda bulunabilir.
- ✓ Arařtırma ve yenilik: Devam eden arařtırmalar ve teknolojik geliřmeler, yumurta kabuėu zarı proteinlerinden yararlanmaya ynelik yeni uygulamaları veya yntemleri ortaya ıkarabilir ve bu da talebi daha da artırabilir.

Dolayısıyla, yukarıda bahsedilen ESM'nin tüm yönlerini kapsayan bu çalışma, çevresel sürdürülebilirlik adına yumurta endüstrisinin ürettiği atıkların yeşil teknolojilerle azaltılması için ESM'nin kullanımına odaklanmıştır. Bu, atıklardan elde edilen esansiyel amino asitlerle alternatif protein kaynağı sağlamak için ESM'nin yumuşak jel kapsüllerde protein takviyesi olarak kullanılmasına odaklanan ilk rapordur. Bununla birlikte, alternatif bir protein kaynağı olarak etkinliğini belirlemek için ESM yumuşak jel kapsüllerinin biyoyararlanımına odaklanacak ileri çalışmalara ihtiyaç vardır.

ANAHTAR KELİMELEER: Yumurta kabuğu zarı; Çözünürlük; Yumurta kabuğu membran proteini; Yumuşak jel kapsüller.



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LIST OF ABBREVIATIONS AND SYMBOLS

μL	: Microliter
min	: Eggshell
BSA	: Bovine Serum Albumin
DAF	: Dissolved Air Flotation
DSC	: Differential Scanning Calorimetry
EDTA	: Ethylenediaminetetraacetic Acid
EAI	: Emulsion Activity Index
ES	: Eggshell
ESI	: Emulsion Stability Index
ESM	: Eggshell Membrane
ESM-H	: ESM Hydrolyzate
ESM-P	: Digestibility of ESM Protein
EFSA	: European Food Safety Authority
FC	: Foam Capacity
FS	: Foam Stability
FT-IR	: Fourier Transform and Infrared Spectroscopy
HA	: Hyaluronic Acid
HCA	: Hierarchical Cluster Analysis
HCl	: Hydrochloric Acid
HPLC	: High Pressure Liquid Chromatography
N	: Normality
OHC	: Oil Holding Capacity
PCA	: Plate Count Agar
PDA	: Potato Dextrose Agar
PLA	: Poly-lactic Acid
rpm	: Revolutions per Minute
SDS	: Sodium Dodecyl Sulfate
SDS-PAGE	: Sodium Dodecyl Sulfate Polyacrilamide Gel Electrophoresis
SEP	: Soluble Eggshell Membrane Protein
SESM	: Soluble ESM
T	: Triton X-100

TMAB : Total Mesophilic Aerobic Bacteria
TMY : Total Mold and Yeast
WHC : Water Holding Capacity
XLD : Xylose Lysine Deoxycholate



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1. INTRODUCTION

Today, agricultural waste management is one of the most common problems faced by food business operators. In parallel to increase in generated waste in agricultural industry, poultry meat and egg production has increased dramatically worldwide and remains one of the fastest growing agricultural sectors. According to the Food and Agriculture Organization of the United Nations (FAO), chicken meat production accounted for around 36% of world meat output in 2016. Between 1961 and 2016, the chicken meat industry increased output volumes by 13%. As one of the biggest egg producer, Türkiye is ranked at the 10th in the world poultry egg production and this rank is 3rd in Europe (1). Based on the data published 2018, 22.3 billion commercial table eggs were produced in Türkiye (2). While the highest level of all years was reached with the production of approximately 19.9 billion (1.2 million tons) chicken eggs in 2019, 1.127.934 tons of production were reported in 2020 (3). In 2022, egg production was recorded as 1.67 billion units (4).

The Environmental Protection Agency has listed eggshell waste as the 15th most significant pollution issue facing the food industry, if they are not disposed of as waste in certain areas properly (3). Improper waste disposal puts not only human existence but also the survival of other eco-system components like plants and animals at risk. While eggshells pose environmental challenges as waste, recycling them is acknowledged to be a complex process. For this reason, studies and solution practices on the effective separation of the membrane in bark wastes have been limited (1). Increase in egg production also brings the issue of the waste produced from the egg industry. Basically, the amount of the unwanted eggs and their shells from the used eggs are increasing every passing day creating environmental and public health problems (5). After the interior part of the egg is removed, the eggshells and the internal membrane attached to the shell deteriorate quickly and emit a bad odor (6). For this reason, eggshell is generally classified as a perishable and bad-smelling waste and cannot be used. This means that thousands of tons of eggshells cannot be utilized and are discarded as waste in every year, causing environmental pollution. Hence, a primary goal of this study is to use the eggshell membrane as a valuable source of protein.

2. AIM AND SCOPE OF THE STUDY

2.1 Eggshell and Eggshell Membrane Properties

The eggshell has an important structure in terms of supporting mechanical protection for the developing embryo, providing many nutritional sources, especially calcium, and a controlled gas exchange (7). The shell prevents the contamination of bacteria, viruses, and other pathogens. It assures that the egg reaches out the consumer in a purified form. Eggshell, which has positive effects on health, is a strong calcium supplement and also since it is one of the calcium source, the usage of it can be considered in the dental health field. Owing to the vitamins and minerals in its structure, it renews the skin cells and heals the irritation on the skin. Apart from these, it prevents bone resorption thanks to the protein and calcium in its structure (6, 8).

Egg is one of the best source of the all the nutrients that human needs after breast milk. Protection of the edible part of the egg and its quality is ensured by the eggshell (2) which constitutes approximately 10-12% of the egg weight (2, 8).

Eggshell is a rich resource of minerals that is able to utilized as a food ingredient, food supplement and calcium additive, as well as agricultural fertilizer component, in order to develop auxiliary substances in the field of medicine, dentistry, and pharmaceuticals (8).

The ingredients of the eggshell are consists of calcium carbonate as the main component (96%) of the shell weight, calcium phosphate (1%) and magnesium carbonate (1%), in addition to protein fiber network with organic materials (9). It has two internal membranes, apart from one point where they split to form an air space in the eggshell structure. Approximately 70-75% of the membrane structure is compromised of lysine, 10% collagen, and proteins such as desmosine and isodesmosine which are the composed of glycoproteins (8).

The egg consists of a central yolk surrounded by albumin (egg white), eggshell membranes, calcified eggshell, and cuticle (7, 10). Eggshell layers-from the inside to the outside- are the internal and external shell membranes, mammillary cone, palisades, crystal (limiting) layer, and cuticle containing pores (2). The subshell

membranes, located just below the shell, are divided into three as the subshell external membrane (shell membrane), the subshell internal membrane (egg membrane), and the limiting membrane (9, 11). The structure of the shell is shown in Fig. 2.1.

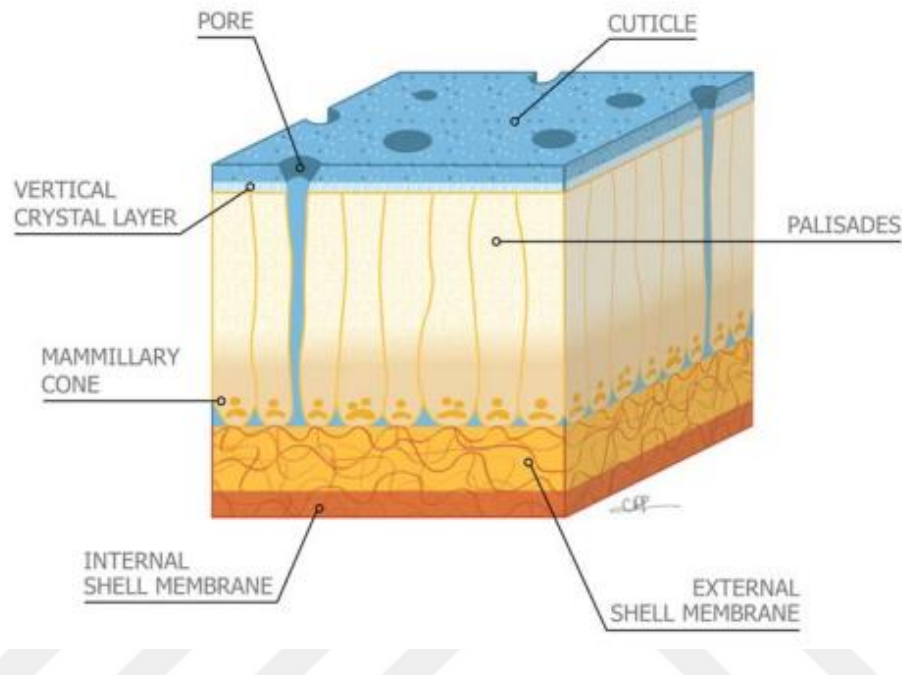


Figure 2.1. Conventional diagram of a hen's-eggshell to show the general structure (10).

The mammillary layer consists of many irregular conical structures. Each structure contains keratan sulfate together with an organic substance identified as mucopolysaccharide. These structures are found in areas where crystallization begins in the crust. The mammillary layer is the only source of calcium necessary for the embryo throughout embryonic development (11).

The palisades known as spongy layer consists of organic and inorganic substances. Organic materials constitute 2-5% of the palisades. The palisades is the thickest (200 μm) layer of the eggshell (10, 11).

Palisades layer ends up in the vertical crystal layer, which is the layer with the higher density crystalline structure. The external area of the palisades layer is a hard conformation made of large crystals and has organic layers that make it difficult for external influences to move through the crystal (10).

The cuticle is an organic layer that covers the external surface of the eggshell. The distribution of the cuticle is usually uneven. The cuticle has a critical role in preventing the entry of microorganisms. A high dependence was found between the cuticle thickness and the bacterial load in the eggshell (11). The cuticle layer also acts as a barrier that balances moisture in and out through the shell and avoids dehydration of the egg's internal components (10, 11).

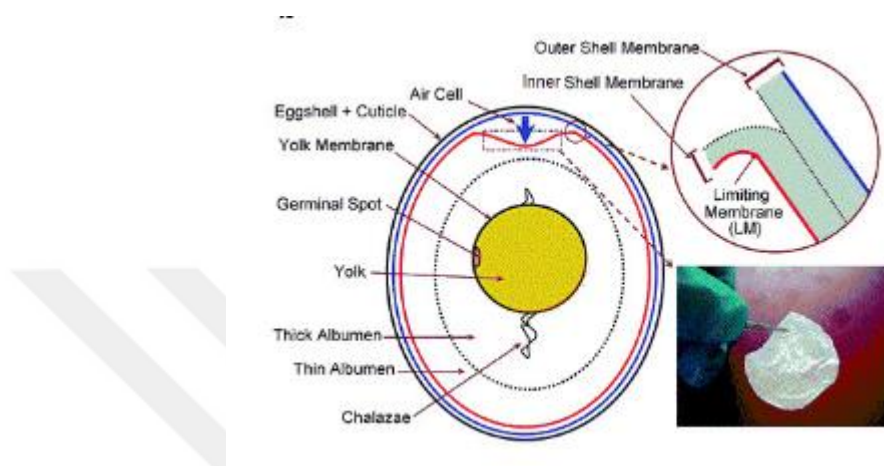


Figure 2.2. Structure of egg with the upper side- infrastructures of the eggshell membrane (ESM) and the lower side- featuring the photograph of the inner ESM (13, 14).

This internal structure has different morphological features from each other. The external crustal membrane is necessary for the formation of the egg membrane. This membrane is important for the calcification and hardening of the eggshell. It also prevents repulsive mineralization of egg white (11, 15). The subshell internal membrane is separated from the subshell external membrane by an air gap. The thickness of the external membrane is 50-70 μm , and the thickness of the internal membrane is between 15-26 μm . Diameter of internal membrane fibers (1-7 μm) is larger than that of the external membrane (0.1 -3 μm), and the fibers of both membranes are intertwined (13,16). In general, the fibers in the shell membranes are located in layers parallel to the egg surface. The limiting membrane is the thinnest membrane surrounding the egg white and forms the thinnest and fibrous dense structure of the eggshell membrane (11,16).

Eggshell membrane is located between the eggshell and egg white (16, 17). The membrane contains collagen types I, V, and X, proteoglycans, glycoproteins, and

fibronectin in its structure. The internal and external membrane both form a semi-permeable membrane composed of protein fibers. Therefore, ESM has a cage structure consisting of stable and water-insoluble fibers. The weight of the eggshell, which is considered as a by-product when the ESM is separated, represents approximately 11% of the egg (60 g) (9).

The ESM is composed of dual layers with a light pink hue, featuring a highly cross-linked structure and exhibiting a semi-permeable nature characterized by a collagenous organic matrix (16,18). It is a combination of an internal web of fine fibers and an external web of coarse fibers (15). ESM contains 80-85% organic and 15-20% inorganic matrix (11). The organic matrix consist of around 10% structural components which are made of collagen types I, V, and X. Of the collagen content, approximately 70-75% is made up of secondary protein components, such as keratin, osteopontin, proteoglycan, and glycoproteins (15).

The ESM also additionally includes ‘glycosaminoglycans like dermatan sulfate and chondroitin sulfate, along with sulfated glycoproteins (hexosamines) (19). Other components are hyaluronic acid (HA), ovalbumine, ovotransferrin, ovokalixsin-36, desmosine and isodesmosine, lysyl oxidase, lysozyme, and β -N-acetylglucosaminidase (8,11,15). In addition, the external ESM contains calcium carbonate at the ends where it meets the ES. Therefore, ESM has a very valuable structure as a natural source of HA, chondroitin, glucosamine, and collagen (15). It has been noted that the number of proteins in the internal and external subshell membrane matrix in ES is more than 500, and this value is much greater than in other parts of the egg (11). It has been reported that there are 62 proteins in the subshell membranes. However, only less than 20 proteins have been recognized in the ESM among which are still not fully characterized (18). ‘Proline, glutamic acid, and glycine’ are the most abundant components in the membranes (11). There are also non-protein substances such as sialic and uronic acid in the ESM structure (2, 14). The cores of the external membrane fibers contain type I collagen, while the internal membrane fibers mainly contain types I and V collagen. Collagen X presents in these two both membranes and is considered tahat it facilitate the constraint of mineralization in the membranes (11,13). Both inner and outer ESM are rich in amino acids including essentials (Table 2.1) (20).

Table 2.1. Percent amino acid composition of both the inner and outer eggshell membrane (20).

Amino acid	Inner ESM (%)	Outer ESM (%)
Proline	11.6	12.0
Glutamic acid	11.1	11.9
Glycine	11.1	0.6
Serine	9.2	9.2
Hydroxyproline	1.5	1.4
Aspartic acid	8.4	8.8
Valine	7.2	7.9
Threonine	6.9	6.9
Arginine	5.7	5.8
Leucine	5.6	4.8
Alanine	4.6	4.1
Histidine	4.1	4.3
Lysine	3.6	3.4
Isoleucine	3.3	3.4
Methionine	2.3	2.3
Tyrosine	2.2	1.7
Phenylalanine	1.6	1.5

2.2 Separation of the Eggshell Membrane from the Eggshell

Egg surface is not sterile, and in fact it is contaminated with microorganisms from different flora. To prevent growth of microbial flora and eliminate *Salmonella* spp. contamination, eggshells should be washed and disinfected with proper disinfection agents before use.

Generally, eggshell containing ESM is discarded and not used after the egg is removed. ESM is tightly bound to the shell, and thus, studies are performed to separate ESM from the eggshells. The easiest of these studies is manually removing the membrane from the eggshell, manual peeling. The separated membranes are dried in the oven at 30 °C for at least 30 min, then ground and kept in the freezer

(21). Even though this method is reported as effective, it is laborious and time-consuming (22).

A commercial machine was developed in the United States that separates the ESM from the eggshell by mechanical abrasion under low pressure. When the shells reach a certain humidity, separation occurs due to the different values their specific gravity (23). Another system invented by Levi New (24) used the pulver dryer to convert eggshells into powder and membranes into flakes through air flow. A sieve shaker system was used to separate the shell and membrane powders.

Another non-chemical method that can distinguish between ES and ESM is the method using dissolved air flotation (DAF). This method relies on the determination of the disparity in specific gravity between the eggshell and the membrane. After eggshell and water are mixed at certain proportions for 10 min, the air-water mixture was injected and the components were collected. It was determined that 96% of ESM and 99% of calcium carbonate in ES were recovered from ES within two hours by using the DAF separation unit (20, 21).

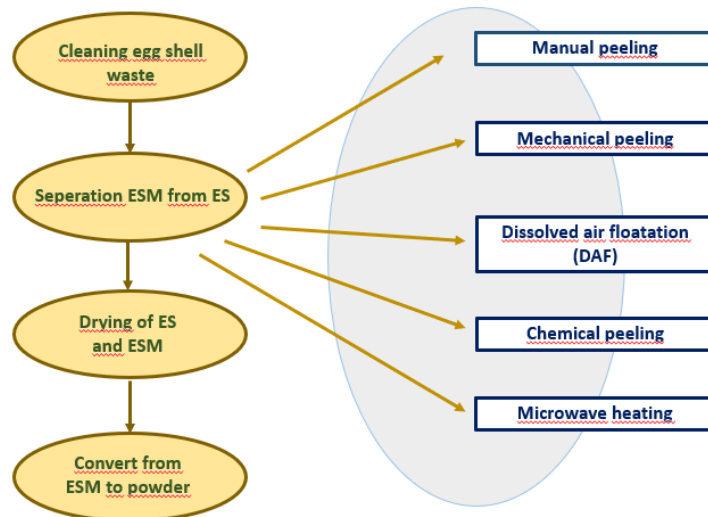


Figure 2.3. Different separation methods used to separate the eggshell and the membrane, modified by Aditya et al. (22).

While the internal and limiting layers can be separated mechanically, the external membrane is strongly embedded in ES and requires acidic treatment (such as treatment with EDTA or HCl) to separate it. Separation in an acidic environment can be achieved in two ways: by immersing ES in acid or by dissolving calcite, the

main ingredient of ES. As a result of the first process, immersion in acid, ESM and ES can be separated from each other, although ES is not completely dissolved (13).

Another process separates the membranes from ES is the microwave treatment. The basic principle of this process is based on the separation of ES and ESM by heating and expansion. Membranes exhibit elevated water content compared to shells, leading to increased absorption of electromagnetic energy and a higher temperature response. As the adhesive forces between the expanding membrane and the shell weaken as a result of heating, it helps the eggshell membrane to separate from the eggshell, and the membrane then would be able to separated by hand (21, 24).

Mechanical separation used commercially is based on the separation of eggshell pieces in an aqueous environment by heating them with steam (21). The forces between the shell and the membrane were weakened with the help of acids, and then the separated membranes were peeled off manually.

2.3 Utilization of Eggshell, Eggshell Membrane and Soluble Eggshell Membrane Protein

It is a widely recognized fact that there is a inadequate sources of calcium in the human diet. Foods known to be sources of calcium, such as milk, and yoghurt cannot be consumed easily by people with lactose intolerance. Supplements recommended by doctors may not be purchased by everyone due to their high prices. For this reason, the trend towards calcium-enriched foods is in demand to increase calcium consumption. Those foods can be enriched with many different calcium sources like milk, calcium phosphate, and calcium carbonate. As mentioned in previous sections. Eggs are a very rich in source of proteins and minerals. In addition, eggshells are considered the primary provider of calcium with approximately 38% calcium content (26). Moreover, the hydroxyapatite substance, a calcium derivative found in eggshells, is a substance which is similar to the mineral components which bones and teeth contains due to its chemical structure. It provides excellent compatibility especially with human soft tissues. Hence, it is utilized in the orthopedic field and dental implants. Composite materials made from this material can be used to correct bone and tooth deficiencies (27).

In 1957 (102) the basic structures of ESM were established. The ESM, a thin film that covers the inside of the eggshell, consists mainly of protein. In the past, studies have applied naked ESM to wound dressing (101), cell culture (102) and heavy metal adsorption (103). Chemically modified ESM was created and is utilized in numerous engineering domains as a result of increased knowledge of its mechanical and chemical characteristics. In many different applications, modified ESM has been employed, including as a glucose sensor (104) and as templates for the hierarchical synthesis of TiO_2 (105).

SEP, when utilized in cell culture, is more easily able to change its size and shape than ESM. Additionally, it is water insoluble, which makes it easier to employ as a biocompatible substrate. ESM is becoming more and more well-known as a future platform for electronics. A nanostructurally clustered PbSe semiconductor has been created via ESM (106). The potential of ESM for application in electrical devices was further demonstrated by its usage in a solar cell (107), a NiO capacitor (108), and a Li-S battery (109). Additionally, it has been investigated for use as a biomaterial in tissue engineering and bone replacement (34).

Membranes are also generally used as absorbents to absorb heavy metals and harmful substances. It has been reported that it is used in joint and skin health due to the different types of collagen that are contained. Collagen, a crucial protein present in ESM, plays a pivotal role in providing structural support to tissues and organizing cells. Consequently, it serves as a primary ingredient in various products designed to mitigate wrinkles and enhance skin thickness and elasticity (28). Since ESM proteins are rich in collagen, they can be used as the main ingredient to increase the effectiveness of cosmetic products. In some studies, it has been found that proteins obtained from ESM are safer to use because they have fewer allergic reactions compared to those obtained from other sources (27, 29).

ESM can also serve as a food flavoring agent in the food industry for human consumption, particularly when there is an effort to minimize food waste during this period (28, 29). Eggshell membrane and powder can find utility not only in the food industry but also as an alternative for lime or a calcium supplement in the paper industry or agriculture. Additional applications involve utilization of membrane proteins for the production of environmentally friendly bioplastics.

Furthermore, to ESM being a very good source of protein, eggshell is also a very good source of calcium (30). It has been clinically tested that the calcium in the bark can be used effectively to prevent osteoporotic fractures in elderly people (32). In addition, drying the shells and mixing them with plant and animal feed is a common practice adopted by the society (26, 28).

Nevertheless, the digestibility and overall high protein content of ESM proteins, which are collagen stores, are still among the issues being researched. The main function of ESM is to prevent foreign substances from reaching the egg white and yolk. Therefore, when ESM is taken into the body in this form, it may not be available for digestion. Thus, the solubilization of ESM and low molecular weight proteins and peptides are being searched (31).

It was reported in previous studies that eggshells can be utilized as food supplements and animal feed for humans and animals. Additionally, it has been determined that the shell can be used as a heterogeneous catalyst in biodiesel production due to its porous structure. The various functional groups and high carbon content found in eggshells make it an alternative substance that can be utilized as an absorbent in the removal of heavy metals and dyes in waste water treatment (30).

Addition of eggshell powder as a calcium additive into biscuit formulation at 3%, 6% and 9% ratio revealed that the addition of eggshell powder caused a considerable increase in direct bioavailability proportion of calcium as calcium bioavailability of biscuits containing 3%, 6%, and 9% ES was found to be 26.0%, 35.4%, and 41.43%, respectively (33).

The digestibility of ESM protein (ESM-P) and ESM hydrolyzate (ESM-H) in mice was investigated in reference to casein taken as the reference protein (31). ESM-H proteins were obtained by digesting ESM-P proteins with alkaline protease. The digestibility of ESM-H was found to be considerably greater than ESM-P as *in vivo* digestion of ESM-P and ESM-H were 80% and 95.1%, respectively. However, the digestibility of both proteins was lower than that of the casein (31).

Periodontal disease, caused by the deterioration of tooth-supporting structures, affects 5-15% of the World's population and causes tooth loss in adults. The surgical

method between the damaged periodontal tissue and the bone for renewal-even though it is very long and tiring process- is widely practiced. For this reason, the use of bioresorbable membranes containing natural polymers; for instance, collagen, synthetic polymers and poly-lactic acid (PLA) is becoming increasingly common. However, there is a need to search for natural biopolymers instead of synthetic biopolymers (34). In recent years, ESM has been reported to provide satisfactory results for burns or skin graft donor sites proving to have great biocompatibility. Soluble ESM (SESM) obtained from ESM using aqueous 3-mercaptopropionic acid and acetic acid showed weak mechanical structure and brittle texture compared to collagen type I (33, 34). Even though previous studies revealed promising results, more studies are in need to develop new membranes as a result of their combination with different biomaterials (34,35).

Following the discovery of SEP, the findings of SEP research began to improve. SEP, when utilized in cell culture, is more easily able to change its size and shape than ESM. Additionally, it is water insoluble, which makes it easier to employ as a biocompatible substrate (36). Recent reports indicate antioxidant potential of ESM even though its very limited solubility due to its high amount of cross-links. ESM proteins was hydrolyzed by five different proteases in order to increase its solubility, and the antioxidative activities and protective effects of the hydrolysates against DNA damage were examined using the chemoluminescence method. It was concluded that peptides purified from soluble eggshell membrane proteins protein can be used as antioxidants and nutraceutical materials in cosmetics or as additives in food products (37).

Resveratrol compound is a phenolic compound synthesized by leaf tissues to prevent fungal infection in plants or to minimize damage from UV light exposure. This compound has limited stability under environmental conditions such as heat and light, and thus, proteins have been used to encapsulate bioactive compounds such as resveratrol. Encapsulation of trans-resveratrol in core/shell nanoparticles consisting of ESM proteins and silk fibroin by electrospraying technique in order to maintain the stability of the resveratrol compound revealed that stability of trans-resveratrol is provided by the interactions between polyphenol and proteins. Changes in nanoparticle properties was possible by changing the encapsulation

parameters. Moreover, stability of encapsulated resveratrol by ESM proteins against UV light and its bioavailability increased when released into the body (38).

The utilization of ESM as a component in diverse formulations for topical applications; for instance, wound healing and cosmetics, as well as its application in treating venous leg ulcers in adult patients has shown encouraging outcomes (37). Effectiveness of daily eggshell membrane intake (500 mg capsule, 2/day) as a supplement to reduce inflammation symptoms in older people (70-100 years old) showed positive responses (5). Oral intake of hydrolyzed eggshell membrane to improve skin tone and appearance, as well as facial hair also resulted in very hopeful results (39).

ESM has been sold in various formulations such as powder, capsules and even paste for different applications such in food and cosmetics industry as dietary supplement, functional ingredient, and personal care product. Additionally, ESM can be found in different food products such as confectionery, beverages and chewing gum (5). In conclusion, since it is a common industrial and household waste, ESM has many benefits. Some of these are: (i) low cost, easy to obtain, and readily available for use; (ii) environmentally friendly and nontoxic; (iii) composed of many natural proteins, such as abundant amino acids, so many functional groups exist on the surface of ESM, resulting in functionalization and adsorption; (iv) easily modified through carbonization and dissolution, which complements its chemical weakness through various syntheses. ESM has been also used in a variety of engineering domains, such as chemical, materials, electrical, environmental, and biomedical engineering, due to these benefits (17).

2.4 Safety Evaluation of Eggshell and Membrane

In vivo tests on ESM and its variations were reported no cytotoxic effect at a dose of 100 µg on cell viability testing of human-derived epidermal keratinocytes following incubation for up to 20 hours (5). Furthermore, in a 90-day repeated dose trial, rats administered hydrolyzed ESM powder as a single oral gavage did not exhibit any notable pathological signs of toxicity (40). Moreover, the EFSA (European Food Safety Authority) Dietary Products, Nutrition and Allergy Panel has authorized the use of egg membrane hydrolyzate as a novel food in line with

Regulation (EU) 2015/2283. The panel decided that hydrolyzed egg membrane consumed as a food supplement for adults was safe at a dose of 450 mg/day (5). Consumption of eggshell has been determined to be non-genotoxic, but egg and its derivatives may be allergenic to some consumers. In this context, warning labels have been placed on the labels of food supplements produced (5). Compared to its non-hydrolyzed form, hydrolyzed ESM usage has shown more growth since 2019 by virtue of hydrolysis since the bioavailability of active ingredients; for instance, collagen, antioxidants, HA, and, antimicrobials are increased (5).

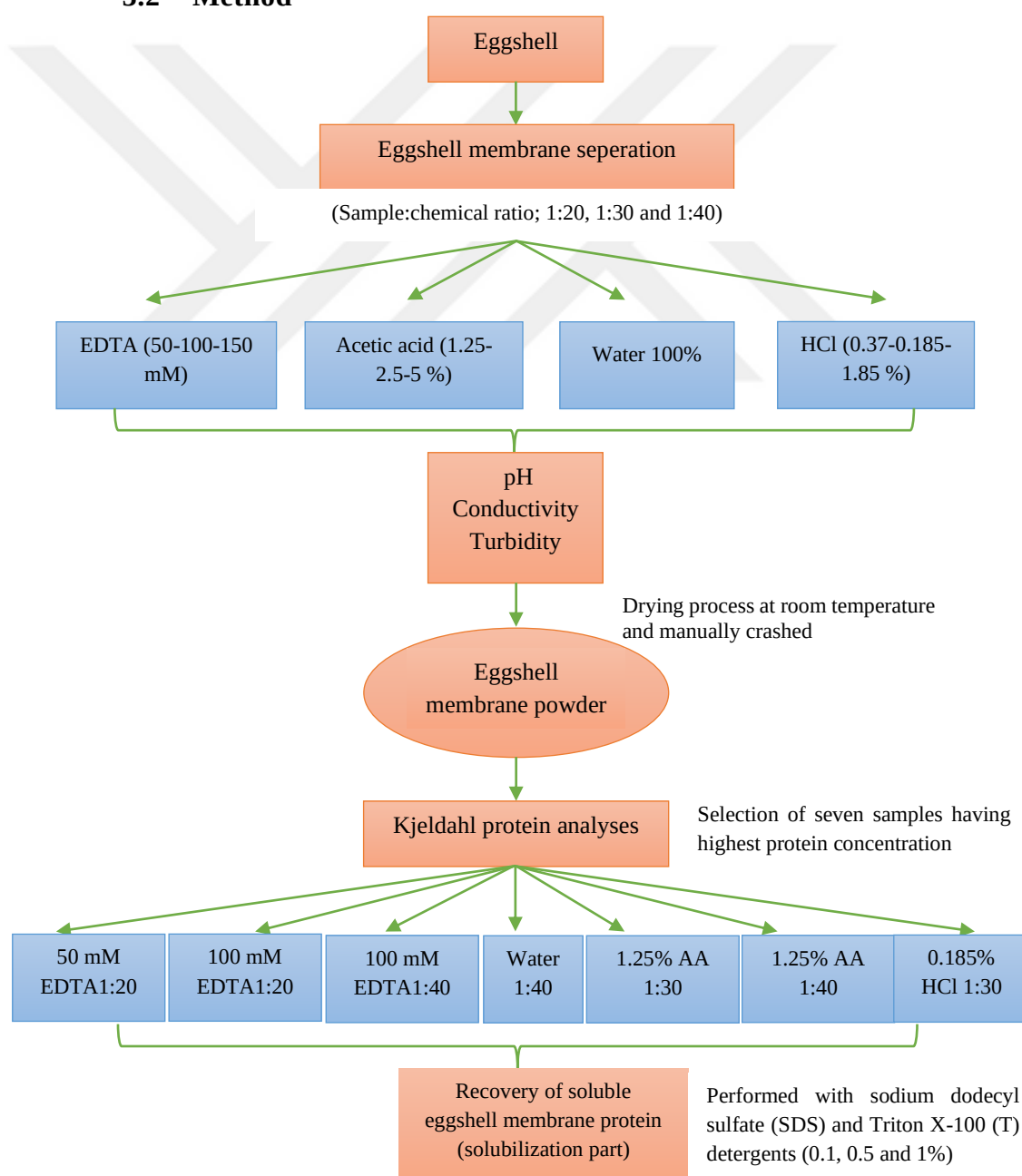
Even though previous studies focused on utilization of ESM, there is a lack of information in the literature regarding the determination of separation efficacy of different chemicals of ESM from the shell, solubilization of ESM, and its used as in soft gel capsules as food supplements. Although eggshells are one of the biggest source of the waste and a cause of environmental problems, eggshell membrane is underutilized as a source of protein. To be used as a source of dietary protein, ESM must first be separated from its shell then it needs to be solubilized. Since eggshell and ESM are closely bonded, a separation procedure must be developed first. Disulphur bonds, which are present in ESM, prevent humans from digesting the protein; therefore, in order for ESM to be digested, these bonds must be broken. Thus, the objectives of the thesis are 1) to develop and optimize a process to separate the eggshell from its membrane, 2) to solubilize the ESM to be used as protein source and determine its functional properties, 3) to develop soft gel capsules from the solubilized ESM protein as protein supplements, and 4) to determine textural, microbiological and sensory properties of soft gel capsules. Recycling the membrane obtained from eggshells as food waste within the scope of zero waste was also prompted.

3. MATERIALS AND METHODS

3.1 Material

Raw eggshell was collected from various bakery businesses in Bolu. The collected eggshell samples were cleaned and washed with water, and then dried for 2 h at 30°C. The eggshells were kept at room temperature until used. The whole process was summarized in Figure 3.1. All chemicals were purchased from Sigma (Sigma Aldrich, Steinheim, Germany).

3.2 Method



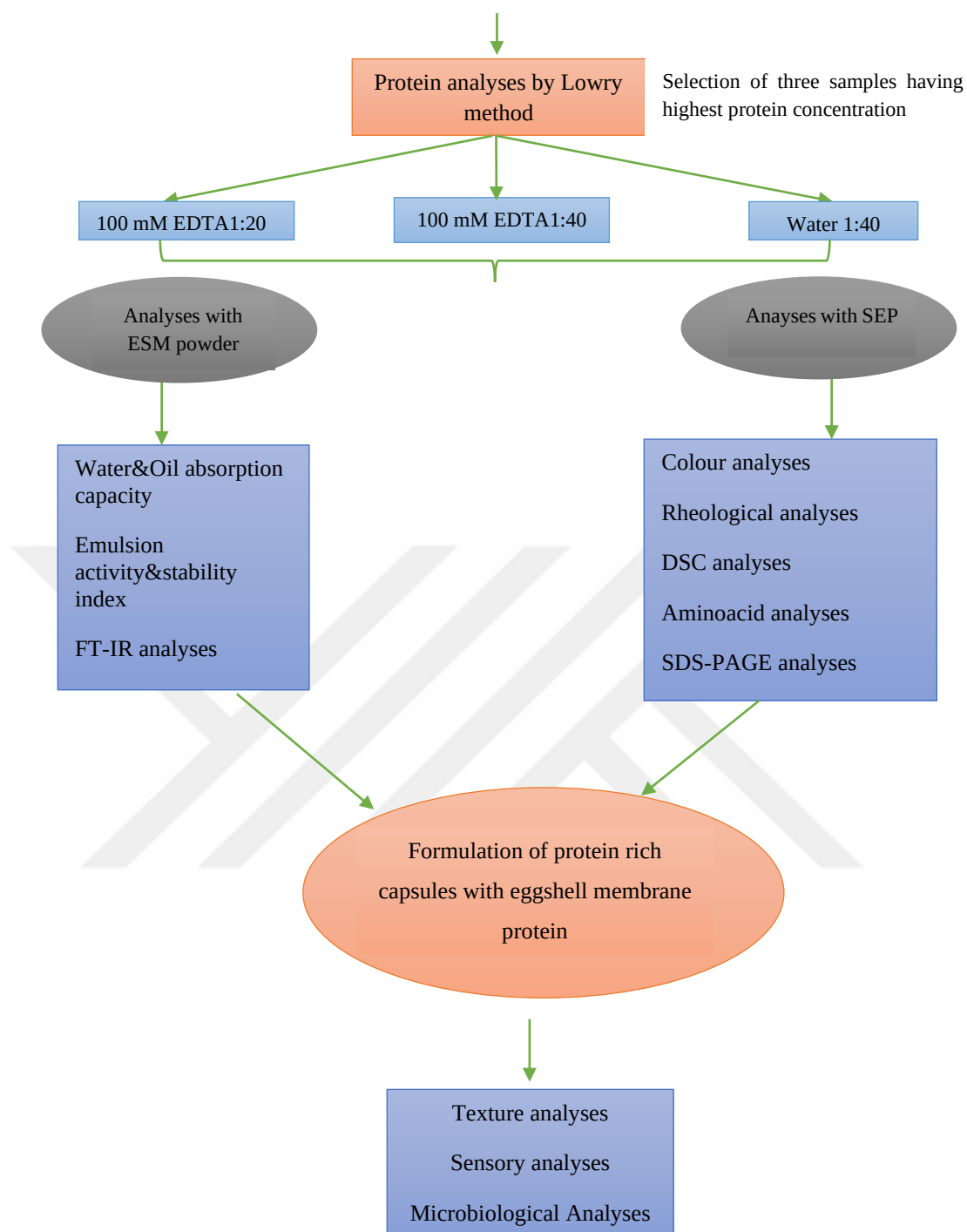


Figure 3.1. Flow chart of whole separation and solubilization process.

3.2.1 Cleaning and Storage of Eggshell Before the Analyses

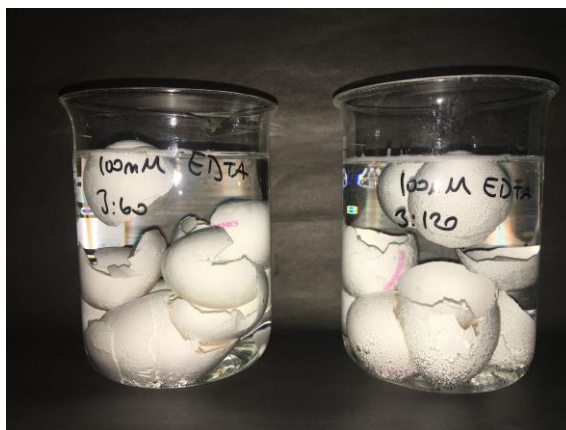
Leftover eggshells from the food industry tend to spoil rapidly, emitting an unpleasant odor since the shells are not clean and they carry some egg remains inside. Consequently, the collected eggshells underwent a washing process first soaking into warm water for 20 min and rinsing the eggshells with water twice. After that, cleaned eggshells were drained and left at room temperature for a day until all moisture was eliminated and completely dried. Following this, they were stored in a desiccator at 25°C until use.

3.2.2 Separation of Eggshell Membrane from the Eggshell

3.2.2.1 Eggshell Membrane Separation by EDTA

A method modified from previous works by Zhang et al. (41) and Ponkham et al. (42) was utilized to separate the eggshell from the membrane. The separation was accomplished by using three different EDTA (Sigma Aldrich, Steinheim, Germany) concentrations (50 mM, 100 mM, and 150 mM) with the solid:liquid (weight of eggshell:mL of solutions) ratios of 1:20, 1:30, and 1:40 g/mL, respectively (Picture 3.1). The rates of membrane separation were observed at every half hour. Whole separation processes were taken approximately one hour. Preparation of the mixtures were performed as follows;

- 50 mM EDTA Solution → 9.3 g of EDTA was weighed and stirred with 500 mL of pure water.
- 100 mM EDTA Solution → 18.6 g of EDTA was weighed and stirred with 500 mL of pure water.
- 150 mM EDTA Solution → 27.9 g of EDTA was weighed and stirred with 500 mL of pure water.



Picture 3.1. Separation process for eggshell membrane from eggshell with EDTA.

3.2.2.2 Eggshell Membrane Separation by HCl

A method modified from previous work by Baláz et al. (43) for separating the membrane from the eggshell using HCl was adopted. The separation was accomplished by three different concentrations of HCl (0.185%, 0.37%, and 1.85%) (Sigma Aldrich, Steinheim, Germany). Eggshell was mixed with diluted HCl at 1:20, 1:30, and 1:40 g/mL ratios, and the rates of membrane separation were observed at every half hour. The entire separation processes took about one hour.

- 0.185% HCl Solution → 1.5 mL of concentrated HCl (37%) was diluted with 298.5 mL of pure water.
- 0.37% HCl Solution → 3 mL of concentrated HCl (37%) was diluted with 297 mL of pure water.
- 1.85% HCl Solution → 15 mL of concentrated HCl (37%) was diluted with 275 mL with pure water.

3.2.2.3 Eggshell Membrane Separation by Acetic Acid

A method modified from previous works by Tsai et al. (9) and Vladamir (23) was slightly modified to separate the eggshell from the membrane. The separation was accomplished by three different acetic acid (Sigma Aldrich, Steinheim, Germany) concentrations of 1.25%, 2.5%, and 5%. Eggshell was mixed with diluted acetic acid at 1:20, 1:30, and 1:40 g/mL ratios, and the rates of membrane separation were observed at every half hour. The complete separation processes took approximately one hour.

- 1.25% acetic acid solution → 6.25 mL of glacial acetic acid was stirred with 293.75 mL of pure water.
- 2.5% acetic acid solution → 12.5 mL of glacial acetic acid was stirred with 287.5 mL of pure water.
- 5% acetic acid solution → 15 mL of glacial acetic acid was stirred with 275 mL of pure water.

3.2.2.4 Eggshell Membrane Separation by Water

Eggshell was mixed with pure water at 1:20, 1:30, and 1:40 g/mL ratios, and the separation rates of the rates of membrane separation were observed at every half hour (Picture 3.2). The separated eggshell membrane from the eggshell was illustrated in Picture 3.3.



Picture 3.2. Separation process for eggshell membrane from eggshell with water.



Picture 3.3. Separated eggshell membrane from eggshell.

3.2.3 Determination of the Properties of Eggshell Membrane Separation Mixtures

After the manual peeling of the eggshell membrane from the eggshell; turbidity, conductivity, and pH analysis were performed. These analyses were performed to determine if the most suitable separation method for ESM can be selected by measurement of pH, conductivity and turbidity. After these analyses, separated ESM samples were dried at 25°C for 24 hour, and processed into powder forms by home style grinding machine (Arçelik, Türkiye). Powder forms were stored at 25°C in a dessiccator until they were used.

3.2.3.1 Turbidity Analysis

The turbidity values of the samples treated with EDTA, HCl, acetic acid, and water were measured with a turbidimeter (MICRO TPI, Model 20008, HF Scientific Inc, Fort Myers, USA) at room temperature after solid particles were removed, and the results were recorded as NTU (nephelometric turbidity unit).

3.2.3.2 Conductivity Analysis

Electrical conductivity of the solutions containing eggshell and separation mixture was measured at 25°C after solid particles were removed using the Sension 5 model (HACH CO, USA) hand held conductivity meter, and the results were recorded as in $\mu\text{S}/\text{cm}$.

3.2.3.3 pH Analysis

The pH of the samples was measured at 25°C after solid particles were removed using Orion 420 A model pH meter (Inolab WTW, Germany).

3.2.4 Analysis of Eggshell Membranes

3.2.4.1 Determination of Crude Protein Content

The crude protein content of the samples assessed by standard Kjeldahl method based on the 990.03 method of AOAC International (44). Protein analysis were performed at the Düzce University Scientific and Technological Research Application and Research Center (DUBIT) (42). The total nitrogen content in the

raw materials was multiplied by the conversion factor (6.25) to assess the crude protein content of the samples. The results were determined by the formula below (Equations 1 and 2):

$$N \% (\text{Nitrogen}) = \frac{(V_1 - V_0) \times 0.014 \times 100}{M} \quad (\text{Equation 1})$$

$$\% \text{ Protein} = \% N \times F (6.25) \quad (\text{Equation 2})$$

V_1 = 0.1 N H_2SO_4 spent in titration for blank (mL)

V_0 = The amount of 0.1 N H_2SO_4 spent in the titration for the sample (mL)

M = g of sample

N = The normality of the H_2SO_4 solution used in the titration

F = Nitrogen factor

The most effective separation solutions along with its concentration were selected based on the results from Kjeldahl analyses. The samples revealed the greatest protein contents were chosen for further tests and the rest were eliminated.

3.2.4.2 FT-IR Analysis

FT-IR analyses were conducted to determine the functional groups of eggshell membrane powders (13). FT-IR analysis was performed by the Düzce University Scientific and Technological Research Application and Research Center (DUBIT) using the Shimadzu IR Prestige21 device (Shimadzu, Tokyo Japan). The % transmittance values based on 20 scans with a resolution of 4.0 eV and a 1/cm range of 500-4500 (16) were utilized as a measurement mode.

3.2.4.3 Water (WHC) and Oil Holding Capacity (OHC)

Water holding capacity (WHC) and oil holding capacity (OHC) (g/g) of the samples were performed using 1.5 mL water and oil with 0.05 g ESM powder samples, respectively. Then, the mixtures were incubated at 30°C for 30 min and centrifuged at 25°C for 20 min at 9000 rpm (NF 200 Model, Nüve, Ankara, Türkiye). The

sample weight and sediment weight were measured, and the calculations were performed as follows (Equation 3):

$$\text{WHO/OHC} = \left(\frac{m_2 - m_1}{m_1} \right) \quad (\text{Equation 3})$$

where m_1 represents the ESM mass and m_2 represents the sediment mass (45).

3.2.4.4 Emulsion Stability and Activity Index

Emulsion stability index (ESI) and emulsion activity index (EAI) were determined with 0.25 g ESM powder mixed with 2.5 mL oil. The mixture was vortexed at high speed for 1 min and then 50 μL of the sample was immersed into SDS (7.5 mL, 0.1%) solution. After vortexing the mixture, the absorbance at 500 nm was measured. The results were calculated by the following formulas 4-5:

$$\text{ESI (\%)} = \frac{A_{10} - A_0}{A_0} \times 100 \quad (\text{Equation 4})$$

$$\text{EAI (m}^2\text{/g)} = \frac{2 \times 2.303 \times n \times A_0}{C \times \phi \times 10000} \quad (\text{Equation 5})$$

where A_0 and A_{10} represent the absorbance values calculated at the time 0 and 10 min later, respectively, C represents the concentration of protein (g/mL), n represents the dilution factor, and ϕ is the oil volume fraction (v/v) of total emulsion volume (46).

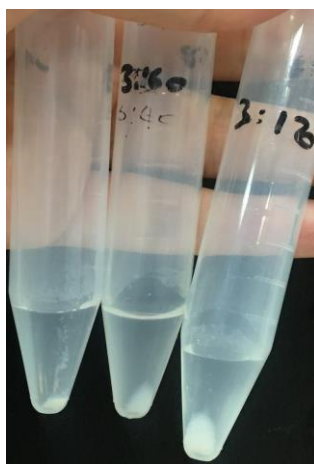
3.2.4.5 Differential Scanning Calorimetry Analysis

Differential scanning calorimeter (DSC) analysis in ESM samples with some modifications were performed in Düzce University DUBİT Research Center (Düzce, Türkiye). ESM powder samples (10 mg) were located in airtight aluminum pans and subjected to heating at a rate of 20 $^{\circ}\text{C}$ per min within a temperature range of 20-350 $^{\circ}\text{C}$ by (DSC) (Shimadzu TA-60 WS model, Tokyo, Japan).

3.2.5 Solubilization of Eggshell Membrane Protein and Determination of Protein Content

3.2.5.1 Soluble Protein Extraction from the Eggshell Membrane

Eggshell membranes were not soluble thus; the protein content was only determined by Kjeldahl analyses. Thus, solubilization of ESM proteins was conducted in order to measure the protein content by Lowry method. For this reason a method modified from previous works by Kaweewong et al. (18) was adopted to solubilize ESM. The first stage to get a soluble eggshell membrane protein was turning the eggshell membrane into powder. After that, 100 mL of 50 mM Tris-HCl buffer (lysis) (Merck, Darmstadt, Germany) solutions, containing 0.1, 0.5 and 1 g of Triton X-100 (T) (Merck, Darmstadt, Germany) and sodium dodecyl sulfate (SDS) (Sigma Aldrich, Steinheim, Germany) were prepared respectively, and then 0.1 mL of 2-mercaptoethanol was added, separately. This lysis solution (1000 μ L) was mixed with 100 mg of eggshell membrane powder, and vortexed for 3 min. The mixture was refrigerated at 4°C overnight. The ESM lysate was centrifuged at 10.000 rpm for 1 min, and 400 μ L of the supernatant was splitted out. The eliminated supernatant was combined with 800 μ L of acetone, and the mixture was refrigerated at 4°C overnight to induce the precipitation of the ESM proteins. Subsequently, the mixture was centrifuged at 10.000 rpm for 30 min to facilitate the separation of proteins by the acetone and settled down at the bottom. The supernatant above the sediment was disposed of, and the protein residue was resuspended in 50 μ L ultra-pure water for Lowry analyses (Picture 3.4).



Picture 3.4. SEP protein in bottom of the falcon tubes.

After Lowry analyses, in order to remove detergents used for solubilization, a two-step washing protocol was performed. Firstly, 100 μ L methanol followed by centrifugation at 10000 rpm for 5 minutes was performed. Then, the samples were washed with 100 μ L ultrapure water 2 times to remove methanol. The effectiveness of the washing procedure was measured by determination of protein concentration by Lowry method by comparing the protein concentration of the same samples before and after the washing (18).

3.2.5.2 Lowry Method

Protein content of the samples - after the solubilization process- was assessed by Lowry method, with bovine serum albumin (BSA) as a standard (Sigma Aldrich, Steinheim, German) used as a standard protein (17, 26).

The quantities and preparation steps of the chemicals used in the Lowry method are as follows:

- 0.1M NaOH $\rightarrow$ 0.4 g NaOH+100 mL distilled water
- %1 SDS in 0.1 M NaOH $\rightarrow$ 50 mg SDS + 5 mL 0.1 M NaOH
- Reagent A $\rightarrow$ 0.5 g of Na_2CO_3 was weighed. It was made up to 2% with 0.1 M 25 mL NaOH.
- Reagent B $\rightarrow$ 5 mg $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ g/mL

It was dissolved in 500 μ L pure water so as to gain a solution at 1% concentration.

- Reagent C $\rightarrow$ 10 mg of potassium sodium tartrate tetrahydrate was stirred with 0.5 mL of pure water.
- Reagent D $\rightarrow$ 500 μ L Reagent B + 500 μ L Reagent C
- Reagent E $\rightarrow$ 25 mL Reagent A + 1000 mL Reagent D

The standard preparation process and analysis is follows:

1. Stock solution was made with 0.001 g of BSA dissolved in 1 mL distilled water.
2. BSA stock solutions were made at the concentrations of 0, 20, 40, 60, 80, and 100 μ g/mL, and 0, 4, 8, 12, 16, and 20 μ L were taken from the

corresponding concentrations to eppendorf tubes and final concentration in each tube was adjusted to 200 μ L with 1% SDS in 0.1 M NaOH.

3. 150 μ L of 1% SDS/0.1 N NaOH and 50 μ L of ESM extract were transferred to six eppendorf tubes, respectively.
4. One mL of Reagent E was included in each of the six standard and six ESM extract tubes, and incubated for 5 min at room temperature.
5. 0.1 mL of Folin reagent was included in each of the tubes and incubated at 37°C for 30, 60, 240, 720, and 1440 min.
6. Blank was measured with 1% SDS at 660 nm.
7. The absorbance of the blue color was measured at 660 nm after 0, 30, 60, 240, 720, and 1440 min of incubation.
8. Protein content of the samples was calculated from the absorbance versus concentration equation 3. Calibration curve was illustrated in Figure 3.2.

Protein concentration of the samples before solubilization was assessed by Kjeldahl method but instead Lowry method was utilized to assess the protein content of the samples after solubilization. To explain the reasons for using two different protein assay methods, first, the Kjeldahl analysis requires more samples and not only protein concentration but also total nitrogenous compounds are determined by this analysis. In addition, the amount of sample obtained after the solubilization step was very low (50 μ L) and was not sufficient for Kjeldahl analysis. Thus, the samples were further analysed by Lowry method for the determination of protein concentration. The samples containing the highest amount of soluble protein concentration according to Lowry analyses were selected for further tests.

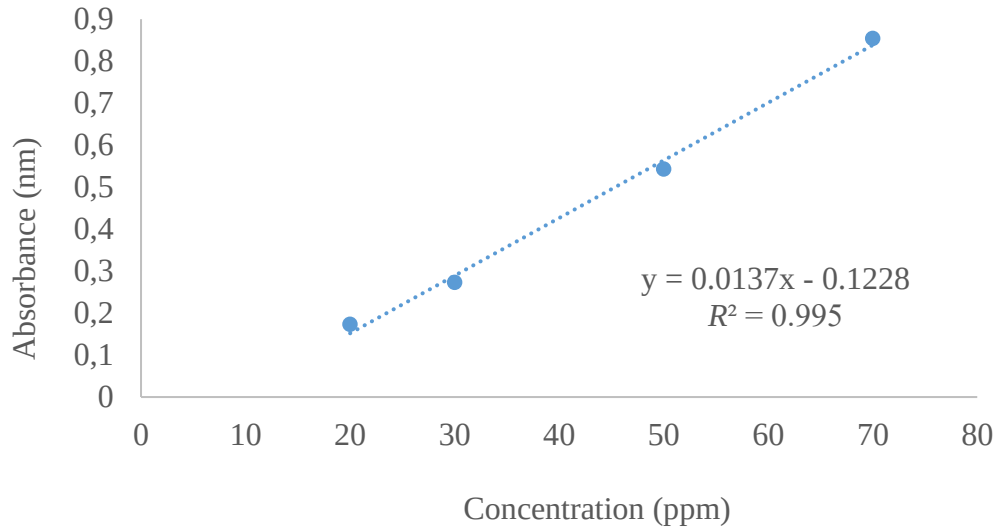


Figure 3.2. Calibration curve for Lowry analysis.

3.2.5.3 SDS-PAGE Analysis

SDS-PAGE gel electrophoresis analysis with some modifications was performed to identify the primary protein fractions (48) in ESM samples in Ankara University Faculty of Agriculture, Department of Dairy Technology (Ankara, Türkiye).

One mg of soluble egg membrane sample was dissolved in 100 mL of SDS (Sigma Aldrich, Steinheim, Germany) sample buffer solution. Sigma marker (Sigma Aldrich), skimmed milk powder, and whey powder were used as standards. The separation gel concentration was 12.5% (%T), and the molecular size separation range was 10.000-100.000 Mw.

The Protean II XL vertical gel unit was used in the study (Bio-Rad Laboratories Ltd., Watford, UK). The gels were pre-run for 40 min at 200 V before loading the samples. Then, 10 µL samples were loaded into the gel and the run was carried out with a constant voltage of 200 V. After the run was completed, the gels were soaked in fixation solution for 60 min, and then stained. The gels were scanned using Agfa Arcus 1200 scanner (Mortsel, Belgium) under the control of Agfa Fotolook v3.5 (Mortsel, Belgium).

3.2.6 Determination of the Functional Properties of Soluble Eggshell Membrane Protein

3.2.6.1 Foaming Capacity (FC) and Stability Index (FS)

Approximately one mL of SEP samples were homogenously mixed with 5 mL of pure water in a test tube, and then, vortexed at maximum speed for 5 min. Foam volumes of the samples at one and 10 min were recorded, and inserted into the formulas below (Equation 6-7) (50).

$$FC = \frac{V_{10}-V_0}{V_0} \times 100 \quad (\text{Equation 6})$$

$$FS = \frac{V_{10}}{V_0} \times 100 \quad (\text{Equation 7})$$

V_0 = Volume at 0 min

V_{10} = Volume after 10 min

3.2.6.2 Colour Measurements

Color L^* , a^* and b^* values were determined by Hunter Lab Color Flex Spectrophotometer (Hunter Associates Laboratory Inc., Reston VA, USA) by CIE Lab method. The L^* value, indicating luminosity, signifies the extent of lightness from black to white. In contrast, the a^* and b^* values denote the degree of redness to greenness and yellowness to blueness on scales ranging from red to green and yellow to blue, respectively. Moreover, total color difference (ΔE) value was determined from the measured color values by the formula below (Equation 8):

$$\Delta E = \sqrt{(\Delta L^2) + (\Delta a^2) + (\Delta b^2)} \quad (\text{Equation 8})$$

3.2.6.3 Rheological Analysis

A dynamic rheological measuring device was used to conduct rheological measurements (Malvern Kinexus Lab Pro+, Malvern Instruments Ltd., Malvern, UK) in Ankara University Faculty of Agriculture, Department of Dairy Technology (Ankara, Türkiye). Shear stress measurements were conducted at a shear rate range

of 0.01-100 Hz and at 5°C. A 4° conical probe (Ø40 mm) was used in the measurements, and the gap was adjusted to 2 mm. Measurements were started 30 s after the SEP samples were located on the measuring plate to equilibrate the samples. After this measurement, the flow model was determined by the device software.

3.2.6.4 Determination of Amino Acid Profile

Amino acid composition of the ESM samples was implemented by Agilent 1100 high performance liquid chromatograph (Ramsey, MN, USA) equipped with a binary pump delivery system, robotic auto sampler, column thermostat, and multi-wavelength detector (TUBITAK-MAM, Kocaeli, Turkiye).

Amino acid standards were prepared by mixing 95 µL of the 250 pmol/µL amino acid standard mixture with 5 µL of 10 mM norvaline (Sigma Aldrich, Steinheim, Germany). The calibration curve was generated by diluting the amino acid standard solution (Sigma Aldrich, Steinheim, Germany) with an initial concentration of 1 nmol/µL to achieve the final concentration of 20, 50, 130, 250, or 500 pmol/µL of amino acid standard mixture along with 0.5 mM norvaline.

Glass test tubes (50 × 6 mm) were immersed in a detergent solution for a minimum of 12 hours prior to the analyses. Subsequently, they underwent thorough rinsing with ultra-pure water multiple times to eliminate the detergent. The tubes were then located in a drying oven at 80°C overnight. Extracts from ESM (7–75 µg) were introduced into the glass test tubes and spiked with 0.5 mM norvaline. Following centrifugation at a low velocity, the samples were freeze-dried in a lyophilizer (115V US Model, Fisher Scientific, Waltham, MA, USA). The samples were placed into the reaction vial, which contains 0.5 mL of constant-boiling HCl (Sigma Aldrich, Steinheim, Germany) on the bottom. The vials were transferred into the oven (Mmert, Schwabach, Germany) at 110°C for 18 hours. After that, the vials were removed and they were cooled to room temperature. Once the temperature decreased to room temperature, the vials were turned on under an aspirated hood. The tubes were centrifuged, and then, dried in the lyophilizer to eliminate any remaining liquid traces, including condensed vapors. Dried samples were dissolved in 100 µL of 0.1 N HCl and they were transferred into HPLC glass vials.

Hydrolyzation (peptide bonds were broken under acidic conditions at high temperatures, resulting in free amino acid residue) of dry ESM samples were performed in TUBİTAK-MAM. Hydrolyzed ESM samples and the norvaline-spiked amino acid standard solutions were derivatized with o-phthalaldehyde (OPA) by autosampler, and 2.5 μ L of each sample was injected on a Zorbax Eclipse-AAA column (5 μ m x 150 x 4.6 mm) (Agilent, Ramsey, MN, USA) at 40 °C, with UV detector operated at 338 nm. Mobile phase A was formed by 40 mM NaH₂PO₄ (pH 7.8) (Sigma Aldrich, Steinheim, Germany), whereas the mobile phase B was formed by acetonitrile/methanol/water (45/45/10 v/v/v) (Sigma Aldrich, Steinheim, Germany). Mobile phase flow rate was adjusted to 2 mL/min with a gradient program that allowed for 1.9 min at 0 % B followed by a 16.3-min step that increased eluent B to 53% (30, 31).

3.2.7 Formulation and Characterization of Soft Gel Capsules Containing Eggshell Membrane Proteins

3.2.7.1 Formulation of Soft Gel Capsules with Eggshell Membrane Proteins

Three of samples with the highest protein concentration (soluble ESM proteins) selected as a result of Lowry analysis were used in the soft gel capsules. The formulation contains roughly 200 mL of water, 16 mL of glycerin, 16 g of beef gelatin, 5 g of pectin and 10 g sugar. First, 16 mL of glycerin and 10 g sugar were added to 200 mL of water and mixed until sugar was completely dissolved. Before it was boiled, pectin and gelatin were inserted and the mixture was constantly mixed. Then, when the mixture was boiled, food-grade colorants (the green and the red colour, GLR Secret, Liquid Food Coloring) and flavors (apple and strawberry, Aromsa, Türkiye) were added (3-4 drop) into gel mixtures for EDTA samples, whereas food-grade colorants and flavors were not added into gel mixtures for water sample. Finally, the mixtures were cooled to 45-50 °C, and 50 μ L of soluble ESM proteins was inserted, separately. The final mixture was transferred into the silicone molds where each molds contain 80 heart-shape holes of one g capacity. Thus, each capsule was approximately one g. The mold kept at refrigeration temperature (+4 °C) for one night until soft gel formation was completed. Each soft capsule contained 1.03 mg/g-1.45 mg/g of ESM protein (Picture 3.5). Dietary Reference

Intake (DRI) indicates the daily amount of protein to be taken as 0.8 grams per each kilogram of body weight.



Picture 3.5. Capsules containing SEP protein.

3.2.7.2 Characterization of the Soft Gel Capsules Prepared with Eggshell Membrane Proteins

3.2.7.2.1 Texture Profile Analysis of the Soft Gel Capsules

The hardness/firmness and springiness/elasticity parameters of the soft gel capsules were performed by the texture profile analysis (TA-XT PlusC Texture Analyzer, ABP, Ankara, Türkiye). The measurements were conducted with a steel cylindrical (P/35R) probe. The speed of the probe was 1 mm/s before the test and 1 mm/s at room temperature during the test. The distance between the probe and the sample was adjusted as 10 mm, and the dwell time was 60 s between the strains (32, 33). The 'hardness' determined from the initial compression curve was specified as the force required to deform the sample to a certain level or the maximum force required for initial compression. Elasticity was calculated by dividing the distance of the sample in the second compression (Distance 2: M2) by the first clamping distance (Distance 1:M1) ($M2/M1$). Elasticity reflects the remodeling capacity of the sample tissue after compression, but it is an indicator of the ability of the sample to return to its original shape (55).



Picture 3.6. Sof gel capsules under the TPA devices.

3.2.7.2.2 Microbiological Analysis of the Soft Gel Capsules

Soft gel capsules, kept in sealed glass bottles, were maintained at 4°C. Sampling occurred weekly up to the 4th week and every two weeks thereafter until the 32nd week for the assessment of total mesophilic aerobic bacteria (TMAB), total mold and yeast (TMY), and *Salmonella* spp. counts. Serial dilutions prepared with 0.1% peptone water at 1:9 ratio were surface plated for TMAB, TMY, and *Salmonella* spp. counts (58). The prepared dilutions were surface placed on Plate Count Agar (PCA, Fluka, Seelze, Germany) for TMAB, potato dextrose agar (Fluka, Seelze, Germany, PDA) for TMY, and XLD (Fluka, Seelze, Germany) agar for *Salmonella* spp. counts. Plates for TMAB and XLD were incubated at 35±2 °C for 24-48 hours, while the plates for TMY were incubated at 22±2 °C for 48-72 hours. Results were recorded as log CFU/g (56, 57).

3.2.7.2.3 Sensory Analysis of the Soft Gel Capsules

Sensory analysis of soft gel capsules was carried out simultaneously after the first week of microbiological studies, when it was determined that capsule consumption was safe in terms of microbial load. Sensory attributes of soft gel capsules such as “odor, color, sweetness, and aftertaste” were assessed using a 9-point hedonic scale sensory test by 15 trained panelists. The samples were randomly revealed to the

panelists with 3-digit numbers. They were instructed to consume unsalted crackers and drink water to cleanse their palates between the samples.

3.2.8 Statistical Analysis

Each experiment was performed in three replicates. Results were analyzed using the SPC software (Minitab®, Triola Statistics Company) for Windows version 17.0.0, and one-way analysis of variance (ANOVA) was conducted to assess the considerable differences among the samples. The statistical significance of the comparison for mean values was tested with the Tukey HSD test. The level of significance used for statistical testing was 95%.



4. RESULTS AND DISCUSSION

4.1 Utilization of EDTA, Acetic Acid, HCl and Water to Separate Eggshell Membrane from the Shell

The electrical conductivity values of the samples changed from 6.79 ± 0.00 mS/cm for 50 mM EDTA at 1:40 ratio to 184.66 ± 0.66 mS/cm with pure water at 1:20 ratio. The concentration of EDTA had a more pronounced impact on the conductivity of the samples compared to the eggshell:EDTA ratio ($p < 0.05$). Additionally, when considering the same eggshell:solution ratio, the conductivity of water samples was considerably greater than that of the EDTA samples ($p < 0.05$). Generally, the samples prepared with water had considerably greater conductivity than that of the EDTA samples regardless of the concentration and ratio (Table 4.1).

Conductivity measurement indicates the ability of water to carry an electrical current. Inorganic dissolved solids with a negative charge, such as phosphate, chloride, nitrate, and sulfate anions, or positively charged ions, such as “sodium, magnesium, calcium, iron, and aluminum cations” can impact the conductivity of water. When in water, organic substances with low electrical conductivity, such as sugar, alcohol, phenol, and oil, are less conductive (114). Since EDTA has very low electrical conductivity, all solutions with EDTA presented lower electrical conductivity.

The samples' turbidity values ranged from 0.40 ± 0.06 NTU for pure water samples at 1:30 ratio to 1.75 ± 0.6 NTU for 50 mM EDTA samples at 1:20 ratio. In general, turbidity of the samples was influenced by both the concentration of EDTA and eggshell:solution ratio, but no clear trend in turbidity among the mixtures was observed. Eggshell:water mixtures exhibited considerably lower turbidity than their counterparts prepared with EDTA ($p < 0.05$) (Table 4.1).

Table 4.1. Analyzing the characteristics of the EDTA solution used in the eggshell membrane separation process.

Concentration	Ratio	EDTA			Water
		50 mM	100 mM	150 mM	100 %
Conductivity (mS/cm)	1:20	6.83±0.00 ^{Cf}	10.81±0.01 ^{Ce}	14.95±0.01 ^{Bd}	184.66±0.66 ^{Aa}
	1:30	6.81±0.00 ^{Df}	10.96±0.01 ^{Ce}	14.96±0.01 ^{Bd}	65.50±1.21 ^{Ab}
	1:40	6.79±0.00 ^{Df}	10.92±0.00 ^{Ce}	14.92±0.01 ^{Bd}	61.03±0.11 ^{Ac}
Turbidity (NTU)	1:20	1.75±0.60 ^{Aa}	1.35±0.30 ^{Babc}	1.11±0.09 ^{Cbcd}	0.65±0.09 ^{Dde}
	1:30	0.63±0.05 ^{Ce}	0.91±0.06 ^{Bcde}	1.69±0.10 ^{Dab}	0.40±0.06 ^{De}
	1:40	0.56±0.07 ^{Cde}	1.44±0.09 ^{Aabc}	0.96±0.02 ^{Bcde}	0.57±0.02 ^{Cde}
pH	1:20	5.28±0.01 ^{Bc}	5.14±0.01 ^{Ccde}	5.08±0.00 ^{Dcde}	6.09±0.2 ^{Ab}
	1:30	5.17±0.01 ^{Bcd}	5.06±0.01 ^{Cde}	5.01±0.01 ^{Dde}	6.64±0.01 ^{Aa}
	1:40	5.15±0.00 ^{Bcde}	5.02±0.01 ^{Cde}	4.94±0.01 ^{De}	6.70±0.01 ^{Aa}

*Data in the similar column with distinct small letter and data in the similar row with distinct capital letters are considerably different ($p \leq 0.05$).

pH values changed from 4.94±0.01 with 150 mM EDTA at 1:40 ratio to 6.70±0.01 with pure water at 1:40 ratio. Overall, the pH of the mixtures containing EDTA declined with an increase in the eggshell:EDTA ratio from 1:20 to 1:40. Conversely, the mixtures prepared with water demonstrated greater pH levels compared to those at equivalent ratios prepared with EDTA (Table 4.1).

Table 4.2. Analyzing the characteristics of the acetic acid solution used in the eggshell membrane separation process.

Concentration	Ratio	Acetic acid			Water
		1.25 %	2.5 %	5 %	100 %
Conductivity (mS/cm)	1:20	3.83±0.01 ^{Cef}	3.05±0.00 ^{Dfg}	6.92±0.20 ^{Bd}	184.66±0.66 ^{Aa}
	1:30	2.26±0.01 ^{Dg}	2.70±0.00 ^{fgC}	4.31±0.00 ^{Be}	65.50±1.21 ^{Ab}
	1:40	2.32±0.00 ^{Cg}	2.25±0.00 ^{Cg}	3.73±0.01 ^{Bef}	61.03±0.11 ^{Ac}
Turbidity (NTU)	1:20	1.46±0.01 ^{Bb}	0.52±0.10 ^{Dcd}	4.29±0.08 ^{Aa}	0.65±0.09 ^{Ccd}
	1:30	0.45±0.01 ^{Bcd}	0.44±0.03 ^{Bcd}	1.56±0.03 ^{Ab}	0.40±0.06 ^{Cd}
	1:40	0.81±0.30 ^{Ac}	0.53±0.05 ^{Bcd}	0.57±0.00 ^{Bcd}	0.57±0.02 ^{Bcd}
pH	1:20	4.65±0.00 ^{Bcd}	4.69±0.01 ^{Bcd}	3.95±0.00 ^{Cf}	6.09±0.20 ^{Ab}
	1:30	4.71±0.00 ^{Bc}	4.48±0.00 ^{Cde}	3.74±0.00 ^{Dfg}	6.64±0.01 ^{Aa}
	1:40	4.69±0.00 ^{Bcd}	4.34±0.00 ^{Ce}	3.62±0.00 ^{Dg}	6.7±0.01 ^{Aa}

*Data in the similar column with distinct small letter and data in the similar row with distinct capital letters are considerably different ($p \leq 0.05$).

Conductivity of the samples varied between 2.25±0.00 mS/cm for 2.5 % acetic acid at 1:40 ratio to 184.66±0.66 mS/cm with pure water at 1:20 ratio. It was showed that conductivity of the samples was influenced by both raised concentration of acetic acid and eggshell:acetic acid ratio ($p < 0.05$). Except for the 1:30 ratio for 1.25 % acetic acid concentration, conductivity of the samples with 2.5% acetic acid were lower than that of the samples with 1.25% acetic acid concentration. Subsequently, an increase was observed when acetic acid concentration was increased to 5%. The conductivity values were treated with water were considerably greater than that of the acetic acid samples at the same eggshell:solution ratios ($p < 0.05$) (Table 4.2).

The samples' turbidity ranged from 0.40±0.06 NTU for 100% water samples at 1:30 ratio to 4.29±0.08 NTU for 5% acetic acid samples at 1:20 ratio. In general, both the concentration of acetic acid and eggshell:solution ratio influenced the turbidity of the samples; however, there was no obvious trend in turbidity values of the solutions (Table 4.2).

The maximum pH value was founded as 6.70 ± 0.01 for the samples prepared with water at 1:40 ratio, whereas the minimum value was founded as 3.62 ± 0.00 for the samples prepared with 5% acetic acid with 1:40 ratio. In general, pH value of the mixtures containing acetic acid was not significantly influenced by the eggshell:acetic acid ratio, but showed sensitivity to the elevated concentration of acetic acid. Moreover, pH of the solutions prepared with water was greater than that of the equivalent samples prepared with acetic acid at the same rates ($p < 0.05$) (Table 4.2).

Table 4.3. Analyzing the characteristics of the HCl solution used in the eggshell membrane separation process.

Concentration	Ratio	HCl			Water
		0.185 %	0.37 %	1.85 %	100 %
Conductivity (mS/cm)	1:20	22.70 ± 0.00^{Ci}	75.96 ± 0.3^{Bd}	ND	184.66 ± 0.66^{Aa}
	1:30	28.90 ± 0.00^{Ch}	91.76 ± 0.05^{Ac}	ND	65.50 ± 1.21^{Be}
	1:40	35.30 ± 0.00^{Cg}	97.63 ± 0.05^{Ab}	ND	61.03 ± 0.11^{Bf}
Turbidity (NTU)	1:20	1.01 ± 0.04^{Bc}	1.04 ± 0.02^{cB}	10.09 ± 0.20^{Aa}	0.65 ± 0.09^{Cde}
	1:30	0.16 ± 0.005^{Df}	0.76 ± 0.05^{Acd}	0.64 ± 0.05^{Bde}	0.40 ± 0.06^{Cef}
	1:40	0.57 ± 0.05^{Cde}	1.69 ± 0.10^{Ab}	0.73 ± 0.03^{Bcd}	0.57 ± 0.02^{Cde}
pH	1:20	1.77 ± 0.02^{Bc}	1.38 ± 0.00^{Cef}	0.93 ± 0.01^{Dg}	6.09 ± 0.2^{Ab}
	1:30	1.66 ± 0.01^{Bcd}	1.32 ± 0.00^{Cef}	0.84 ± 0.01^{Dg}	6.64 ± 0.01^{Aa}
	1:40	1.52 ± 0.00^{Bde}	1.28 ± 0.00^{Cf}	0.80 ± 0.01^{Dg}	6.70 ± 0.01^{Aa}

*Data in the similar column with distinct small letter and data in the similar row with distinct capital letters are considerably different ($p \leq 0.05$).

**ND: Not Determined.

The electrical conductivity values of the eggshell:HCl ratio changed from 22.7 ± 0.00 mS/cm for 0.185 % HCl at 1:20 ratio to 184.66 ± 0.66 mS/cm by pure water at 1:20 ratio. It was noticed that conductivities considerably raised by both increased concentration of HCl and eggshell:HCl ratio ($p < 0.05$). On the contrary, the samples conductivity with water were considerably decreased with an increased eggshell:solution ratio. Furthermore, conductivity of the samples with pure water were greater than that of the HCl samples at the same eggshell:solution ratio ($p <$

0.05). The electrical conductivities of the mixtures with 1.85% HCl could not be measured (Table 4.3).

The turbidity of the mixtures ranged from 0.16 ± 0.005 NTU for 0.185% HCl samples at 1:30 ratio to 10.09 ± 0.20 NTU for 1.85% HCl samples at 1:20 ratio. In general, the concentration of HCl influenced the samples turbidity as turbidity of the samples with 1:20 ratio increased with increased HCl concentration; however, the samples at 1:30 and 1:40 ratio presented the highest turbidity at 0.37% HCl than that of the samples at 1:20 ratio. Water samples illustrated considerably lower turbidity compared to their counterparts prepared with HCl samples ($p < 0.05$) (Table 4.3).

The maximum pH value was determined as 6.70 ± 0.01 for the samples prepared with pure water at 1:40 ratio, whereas the minimum pH of that was determined as 0.80 ± 0.01 for the samples prepared with 1.85% HCl at 1:40 ratio. The mixtures pH value with HCl was influenced considerably by the eggshell:HCl ratio, and raised concentration of HCl as pH of the samples decreased by both eggshell:solution ratio and increased HCl concentration. The mixtures pH prepared with water was not significantly changed by increased eggshell:solution ratio, and the pH of these samples were higher than the equivalents prepared with HCl at the same ratios ($p < 0.05$) (Table 4.3).

pH value of the mixtures prepared with water was greater than that of other solvents. While the highest value for the pH parameter was observed from the samples with water at 3:90 and 3:120 ratios, the lowest value was observed at 150 mM EDTA at 3:120 ratio. It was illustrated that there was no considerable distinction between the values of 50 mM EDTA at 3:120 ratio, 100 mM EDTA at 3:60 ratio and 50 mM EDTA at 3:60 ratio. Therefore, it can be said that solid:liquid ratio and concentration values were partially important on pH values. Proteins carry both positive and negative charges due to their structure. A large part of the electrical charge of a protein is figured out by the carboxyl and amino groups in the radical groups of the amino acids that make up the protein and the behavior of the environment. This affects both the electrical charge and the pH of the protein (59).

The samples by different chemicals (EDTA, HCl and acetic acid) at three different ratios (1:20, 1:30, and 1:40) with the measurement of pH, conductivity and turbidity

as showed in Tables 4.1, 4.2 and 4.3 were prepared to determine if the solids concentration transferred to liquid gave information about which chemicals at what ratio is the best to separate ESM from the eggshell. However, no conclusion was driven by the measurement of the physical properties, and thus, protein content of the samples was examined by Kjeldahl method so as to determine the most effective separation method with higher protein concentration.

4.2 Determination of Crude Protein Concentration by Kjeldahl Method

Protein concentration of the samples among EDTA, acetic acid, HCl, and water at different eggshell:liquid ratio revealed great variations depending on the chemicals used for separation and their ratio. The samples with the greatest crude protein concentrations of 83.36 ± 0.00 , $82.9 \pm 0.00\%$, 81.75 ± 0.00 , 80.42 ± 0.00 , 80.38 ± 0.00 , 80.22 ± 1.45 , and $79.6 \pm 1.98\%$ were obtained by 100 mM EDTA at 1:20 ratio, 100% water at 1:30 ratio, 50 mM EDTA at 1:20 ratio, 100 mM EDTA at 1:40 ratio, 1.25% acetic acid at 1:40 ratio, 1.25% acetic acid 1:30 ratio, and 0.185% HCl at 1:30 ratio, respectively ($p < 0.05$) (Table 4.4). Thus, the study continued with the seven samples presented higher protein concentration after narrowing down from the initial 27 pre-treatments based on their protein concentration.

Similar protein concentration of $97.03 \pm 1.57\%$ by Kjeldahl analysis in eggshell membrane treated with 5% HCl was reported by Strelec et al. (60). Use of 5% EDTA solution to separate the eggshell and ESM revealed 88.2% protein concentration (42) and this results was close the protein concentration of the samples treated with 100 mM EDTA 1:20 (83.36%) in the present study. Protein concentration of the other treatments were relatively lower than that of the present study, and this could be caused by both the concentration of EDTA and the incubation time.

Table 4.4. The impact of various separation solutions on the crude protein concentration (%) of the eggshell membrane.

	EDTA			Acetic Acid			HCl			Water
	50 mM	100 mM	150 mM	1.25 %	2.5 %	5 %	0.185 %	0.37 %	1.85 %	100 %
1:20	81.75±0.00 ^{Ca}	83.36±0.00 ^{Aa}	69.40±0.00 ^{Hc}	72.70±0.00 ^{Gb}	75.13±0.00 ^{Ea}	52.66±0.00 ^{Jc}	75.10±0.00 ^{Fb}	66.76±0.00 ^{Ic}	77.78±0.00 ^{Da}	
1:30	72.27±0.00 ^{Bb}	55.04±1.50 ^{Fc}	69.58±0.00 ^{Db}	80.22±1.45 ^{Aa}	71.85±0.00 ^{Cb}	67.86±4.76 ^{Ea}	79.60±1.98 ^{Aa}	73.50±2.13 ^{Ba}	71.42±0.00 ^{Db}	
1:40	58.36±0.00 ^{Ic}	80.38±0.00 ^{Bb}	76.82±0.00 ^{Ca}	80.42±0.00 ^{Aa}	68.81±0.00 ^{Gc}	60.82±0.00 ^{Hb}	71.82±0.00 ^{Dc}	71.31±0.00 ^{Eb}	69.28±0.00 ^{Fc}	82.90±0.00 ^A

*Data in the similar column with distinct small letter and data in the similar row with distinct capital letters are considerably different ($p \leq 0.05$).

Torres-Mansilla and Delgado-Mejía (61) stated that exposure of eggshell white to 5% HCl may cause some chemical and physical changes such as denaturation and breakage in hydrogen bonds in the ESM; these changes could possibly be the reason why lower protein content was determined in the acid-treated ESM than in the water-treated ESM (61). Generally, the samples protein content extracted by EDTA presented higher protein concentration compared to the acetic acid and HCl samples. These results could be stemmed from extraction and/or partial hydrolysis of non-protein nitrogen (60).

The eggshell membrane proteins contain many sulfhydryl groups and these sulfhydryl groups are not fragmented by the applications of EDTA, acetic acid, and HCl, and thus, the samples protein concentrations were first assessed using the Kjeldahl method and elimination of the samples with greatest protein concentrations were selected based on the results from Kjeldahl analysis. Kjeldahl analysis, on the other hand, provides total nitrogen content which may include 25% of non-protein nitrogen fraction which comprises more than 200 different compounds including free amino acids nucleotides, nucleic acids, urea, and polyamides (113). Thus, it can be estimated that total protein content of the ESM samples is 25% less than that of the indicated in Table 4.4.

Solubilization of ESM proteins would provide digestibility of the protein by breaking the sulfhydryl groups. Thus, solubilization of the ESM protein was studied as a next step in order to provide digestibility.

4.3 Solubilization of Eggshell Membrane Protein and Lowry Analysis

The highest protein concentrations of the samples from the previous step underwent treatments with Triton X-100 (T) and SDS at concentrations of 0.1%, 0.5%, and 1.0% for solubilization. After that, Lowry analysis was performed over a retention period of 0, 30, 60, 240, 720, and 1440 minutes to determine the protein content of the soluble ESM samples. Among the treatments, the samples treated with 1.0% SDS lysis solution for 240 min retention time in the Lowry analysis showed the greatest protein concentration of 208.40 ± 0.00 mg/mL ($p < 0.05$) (Table 4.5).

It was examined that the samples containing 0.1% T exhibited considerably greater protein concentration in comparison to the samples with 0.1% SDS at the same retention times ($p < 0.05$). However, the samples solubilized with 0.5% SDS and 1.0% SDS had considerably higher protein concentrations than that of their counterparts prepared with 0.5 % and 1.0 % T at the same retention times in the Lowry analysis ($p < 0.05$). Protein concentration of all samples showed a raise from 0 to 240 min retention time, and then showed a decline from 240 to 1440 min (Table 4.5).

When comparing detergents used for the solubilization of 100 mM EDTA (at a 1:20 ratio) samples (T and SDS at 0.1%, 0.5%, and 1.0%), the highest protein concentration value was observed in the sample solubilized with 1% SDS detergent, at 252.49 ± 0.19 mg/mL for 60 min retention time in the Lowry analysis. Samples solubilized with 0.1% T showed significantly higher protein concentration compared to the samples solubilized with SDS at 0.1% concentration for all retention times in Lowry analyses; while the samples solubilized with SDS at 0.5% and 1% concentrations had considerably greater protein concentration ($p < 0.05$) in comparison to the samples solubilized with T at 0.5% and 1.0% concentrations for all retention times. Generally, an increase in protein concentration was observed in all samples up to 240 minutes of retention time, followed by a decrease as the retention time increased from 240 to 1440 minutes ($p < 0.05$) (Table 4.6).

100 mM EDTA (1:40 ratio) samples solubilized with T and SDS at 0.1%, 0.5%, and 1.0% concentrations showed the greatest protein concentration of 244.32 ± 0.12 mg/mL on the samples 1% SDS at 60 min retention time in the Lowry analysis. Samples solubilized with SDS showed significantly greater protein concentration compared to the samples solubilized with T at the same concentrations of 0.1%, 0.5%, and 1.0% ($p < 0.05$). Protein concentration of the samples solubilized with T (0.1%) increased up to 240 min and then showed a decrease until 1440 min; whereas the samples solubilized with 0.5 % and 1.0 % T had an increased trend up to 60 and 720 min and then a decreased trend up to 1440 min. All the samples solubilized with SDS showed a raise up to 240 min. After that they showed a decrease up to 1440 min ($p < 0.05$) (Table 4.7).

Table 4.5. The protein concentration (mg/mL) of eggshell membrane samples solubilized with 50 mM EDTA at 1:20 ratio, using lysis solutions of triton X-100 (T) and sodium dodecyl sulfate (SDS) at varying concentrations and extended retention times of the Lowry analysis.

Sample		50 mM EDTA 1:20				
Lysis solution	0 min	30 min	60 min	240 min	720 min	1440 min
T (0.1 %)	121.20±0.12 ^{Fd}	143.88±0.00 ^{Dd}	169.47±0.32 ^{Bc}	175.02±0.12 ^{Ac}	150.69±0.12 ^{Cd}	133.66±0.19 ^{Ed}
T (0.5 %)	105.92±0.00 ^{Ee}	130.16±0.00 ^{Be}	118.96±0.25 ^{Df}	140.08±0.00 ^{Ae}	124.32±0.00 ^{Ce}	106.31±0.1 ^{Ee}
T (1.0 %)	121.59±0.12 ^{Fc}	155.17±0.12 ^{Bc}	148.84±0.00 ^{Dd}	163.05±0.12 ^{Ad}	154.39±0.00 ^{Cc}	141.74±0.1 ^{Ec}
SDS (0.1 %)	87.92±0.12 ^{Ff}	124.71±0.12 ^{Bf}	121.01±0.12 ^{Ce}	133.08±0.00 ^{Af}	112.64±0.00 ^{Df}	103.39±0.12 ^{Ef}
SDS (0.5 %)	125.00±0.12 ^{Fb}	164.32±0.38 ^{Db}	177.07±0.25 ^{Bb}	180.18±0.12 ^{Ab}	169.86±0.19 ^{Cb}	154.00±0.12 ^{Eb}
SDS (1.0 %)	166.17±0.12 ^{Ea}	201.30±0.12 ^{Ca}	205.58±0.12 ^{Ba}	208.40±0.00 ^{Aa}	191.57±0.12 ^{Da}	164.61±0.38 ^{Fa}

*Data in the similar column with distinct small letter and data in the similar row with distinct capital letters are considerably different ($p \leq 0.05$).

*The retention times in the table stand for the time period in the Lowry analysis.

Table 4.6. The protein concentration (mg/mL) of eggshell membrane samples solubilized with 100 mM EDTA at 1:20 ratio, using lysis solutions of triton X-100 (T) and sodium dodecyl sulfate (SDS) at varying concentrations and extended retention times of the Lowry analysis.

Sample	100 mM EDTA (1:20)					
Lysis solution	0 min	30 min	60 min	240 min	720 min	1440 min
T (0.1 %)	123.63±0.12 ^{Fc}	159.06±0.58 ^{Cc}	156.82±0.12 ^{Dd}	162.95±0.12 ^{Bc}	164.80±0.12 ^{Ac}	132.78±0.19 ^{Ed}
T (0.5 %)	114.10±0.00 ^{Ff}	136.77±0.12 ^{Ce}	138.04±0.00 ^{Be}	145.05±0.00 ^{Ae}	129.86±0.38 ^{Df}	125.48±0.19 ^{Ee}
T (1.0 %)	119.55±0.25 ^{Ed}	116.82±0.12 ^{Ff}	136.97±0.12 ^{Ce}	153.12±0.12 ^{Ad}	151.37±0.12 ^{Be}	125.48±0.19 ^{De}
SDS (0.1 %)	116.43±0.19 ^{Fe}	146.80±0.19 ^{Dd}	162.08±0.12 ^{Bc}	153.22±0.00 ^{Cd}	163.34±0.12 ^{Ad}	134.05±0.12 ^{Ec}
SDS (0.5 %)	160.72±0.12 ^{Fb}	179.21±2.84 ^{Db}	176.00±0.19 ^{Eb}	198.87±0.12 ^{Ab}	189.62±0.12 ^{Bb}	180.96±0.58 ^{Cb}
SDS (1.0 %)	211.62±0.19 ^{Fa}	236.82±0.32 ^{Da}	252.49±0.19 ^{Aa}	247.82±0.00 ^{Ba}	239.74±0.12 ^{Ca}	222.61±0.12 ^{Ea}

*Data in the similar column with distinct small letter and data in the similar row with distinct capital letters are considerably different ($p \leq 0.05$).

*The retention times in the table stand for the time period in the Lowry analysis.

Table 4.7. The protein concentration (mg/mL) of eggshell membrane samples solubilized with 100 mM EDTA at 1:40 ratio, using lysis solutions of triton X-100 (T) and sodium dodecyl sulfate (SDS) at varying concentrations and extended retention times of the Lowry analysis.

Sample	100 mM EDTA (1:40)					
Lysis solution	0 min	30 min	60 min	240 min	720 min	1440 min
T (0.1 %)	130.16±0.19 ^{Dd}	135.80±0.12 ^{Ce}	136.00±0.00 ^{Cf}	142.03±0.10 ^{Ae}	136.87±0.00 ^{Bf}	118.18±0.19 ^{Ef}
T (0.5 %)	108.26±0.19 ^{Ef}	131.23±0.45 ^{Cf}	143.20±0.12 ^{Ae}	131.03±0.00 ^{Cf}	135.41±0.19 ^{Be}	127.33±0.32 ^{De}
T (1.0 %)	121.69±0.00 ^{Fe}	137.65±0.12 ^{Dd}	149.33±0.12 ^{Cd}	153.81±0.19 ^{Bd}	157.31±0.19 ^{Ad}	133.17±0.32 ^{Ed}
SDS (0.1 %)	140.86±0.12 ^{Ec}	156.24±0.12 ^{Dc}	174.15±0.12 ^{Ac}	172.69±0.12 ^{Bc}	160.81±0.00 ^{Cc}	138.14±0.12 ^{Fc}
SDS (0.5 %)	178.91±0.00 ^{Fb}	226.99±0.12 ^{Aa}	223.68±0.12 ^{Bb}	220.18±0.12 ^{Cb}	208.60±0.12 ^{Db}	203.15±0.00 ^{Eb}
SDS (1.0 %)	188.40±0.12 ^{Fa}	221.54±0.12 ^{Db}	244.32±0.12 ^{Aa}	227.38±0.00 ^{Ba}	224.46±0.00 ^{Ca}	208.55±0.12 ^{Ea}

*Data in the similar column with distinct small letter and data in the similar row with distinct capital letters are considerably different ($p \leq 0.05$).

*The retention times in the table stand for the time period in the Lowry analysis.

The 1.25% acetic acid samples at 1:30 ratio solubilized with T and SDS at 0.1%, 0.5%, and 1.0% concentrations showed the maximum protein content of 168.79 ± 0.12 mg/mL on the samples solubilized with 0.5% SDS at 720 min in the Lowry analysis. In general, the samples solubilized with T had lower protein concentration compared to the samples solubilized with SDS with the exception of the samples treated by both 0.1% and 0.5% T at 0 min. An increase in protein concentration was detected in the samples solubilized with T up to 240 min, and then it decreased when retention time increased to 1440 min. The samples solubilized with 0.1% SDS showed an increase in protein concentration up to 720 min, whereas the samples treated with 0.5% and 1.0% SDS illustrated a raise in protein concentration up to 720 min, and then, presented a decrease by retention time up to 1440 min ($p < 0.05$) (Table 4.8).

The 1.25% acetic acid samples at 1:40 ratio solubilized with T and SDS lysis solutions showed the maximum protein concentration of 177.55 ± 0.12 mg/mL in the samples solubilized with 1.0% SDS at 240 min in the Lowry analysis. Overall, the samples solubilized with SDS exhibited a greater protein concentration than those solubilized with T at equivalent concentrations ($p < 0.05$). An increase in protein concentration up to 60 min and a decline in protein concentration up to 1440 min were observed in the samples treated with 0.1% T. The samples treated with both 0.5% and 1.0% T showed an increase up to 720 min and then a decrease was observed up to 1440 min. Protein concentration of the samples solubilized with SDS increased up to 240 min and then it was decreased with raised retention time up to 1440 min ($p < 0.05$) (Table 4.9).

Table 4.8. The protein concentration (mg/mL) of eggshell membrane samples solubilized with 1.25% acetic acid at 1:30 ratio, using lysis solutions of triton X-100 (T) and sodium dodecyl sulfate (SDS) at varying concentrations and extended retention times of the Lowry analysis.

Sample	1.25% Acetic acid (1:30)					
Lysis solution	0 min	30 min	60 min	240 min	720 min	1440 min
T (0.1 %)	34.55±0.03 ^{Ce}	42.49±0.03 ^{ABe}	44.43±0.00 ^{Ae}	44.43±2.96 ^{Af}	38.59±0.00 ^{BCf}	36.67±0.06 ^{Cf}
T (0.5 %)	39.25±0.00 ^{Fd}	46.14±0.03 ^{Ed}	50.47±0.03 ^{Cc}	58.08±0.00 ^{Be}	49.59±0.03 ^{De}	61.78±0.08 ^{Ae}
T (1.0 %)	42.71±0.03 ^{Ec}	61.25±0.03 ^{Dc}	43.05±0.04 ^{Ef}	126.17±0.12 ^{Ac}	110.50±0.45 ^{Bc}	96.68±0.32 ^{Cc}
SDS (0.1 %)	32.44±0.03 ^{Ff}	42.24±0.00 ^{Ef}	48.01±0.00 ^{Dd}	85.09±0.12 ^{Ad}	74.78±0.12 ^{Cd}	76.24±0.12 ^{Bd}
SDS (0.5 %)	134.63±0.12 ^{Eb}	143.78±0.12 ^{Da}	110.59±0.00 ^{Fb}	167.43±0.12 ^{Ba}	168.79±0.12 ^{Aa}	146.60±0.12 ^{Ca}
SDS (1.0 %)	140.47±0.12 ^{Da}	137.94±0.12 ^{Eb}	140.37±0.00 ^{Da}	154.58±0.12 ^{Bb}	157.99±0.12 ^{Ab}	145.05±0.19 ^{Cb}

*Data in the similar column with distinct small letter and data in the similar row with distinct capital letters are considerably different ($p \leq 0.05$).

*The retention times in the table stand for the time period in the Lowry analysis.

Table 4.9. The protein concentration (mg/mL) of eggshell membrane samples solubilized with 1.25% acetic acid at 1:40 ratio, using lysis solutions of triton X-100 (T) and sodium dodecyl sulfate (SDS) at varying concentrations and extended retention times of the Lowry analysis.

Sample	1.25% Acetic acid (1:40)					
Lysis solution	0 min	30 min	60 min	240 min	720 min	1440 min
T (0.1 %)	32.75±0.00 ^{Ef}	38.20±0.03 ^{Df}	43.61±0.03 ^{Af}	40.66±0.08 ^{Bf}	39.35±0.03 ^{Ce}	40.73±0.03 ^{Bf}
T (0.5 %)	45.97±0.04 ^{Fd}	51.00±0.00 ^{Ed}	56.45±0.03 ^{Ad}	55.16±0.00 ^{Bd}	52.88±0.03 ^{Dd}	54.29±0.04 ^{Cd}
T (1.0 %)	42.10±0.00 ^{Ff}	48.93±0.03 ^{De}	51.08±0.00 ^{Ce}	52.78±0.03 ^{Be}	52.88±0.03 ^{Ad}	48.11±0.03 ^{Ee}
SDS (0.1 %)	51.59±0.00 ^{Fce}	66.23±0.03 ^{Ec}	105.34±0.19 ^{Cc}	116.72±0.00 ^{Ac}	106.80±0.00 ^{Bc}	84.32±0.19 ^{Dc}
SDS (0.5 %)	131.03±0.19 ^{Eb}	171.71±0.12 ^{Aa}	142.90±0.12 ^{Db}	146.51±0.00 ^{Bb}	144.17±0.00 ^{Cb}	127.04±0.12 ^{Fb}
SDS (1.0 %)	149.52±0.12 ^{Fa}	166.65±0.30 ^{Cb}	168.40±0.19 ^{Ba}	177.55±0.12 ^{Aa}	161.69±0.38 ^{Da}	153.22±0.19 ^{Ea}

*Data in the similar column with distinct small letter and data in the similar row with distinct capital letters are considerably different ($p \leq 0.05$).

*The retention times in the table stand for the time period in the Lowry analysis.

The 0.185% HCl (1:30) samples solubilized with T and SDS lysis solutions showed the greatest protein concentration of 165.39 ± 0.12 mg/mL for the samples solubilized with 0.5% SDS at 60 min in the Lowry analysis. Except for the samples solubilized with 0.1% T for 30 min, the other samples solubilized with 0.1% T had the greater protein concentration in comparison to the samples solubilized with SDS 0.1% for the other retention times ($p < 0.05$). The samples treated with 1.0% T, on the other hand, presented lower protein concentration than that of their counterparts treated with 1.0% SDS for all retention times ($p < 0.05$). The samples treated with 1.0% T had the lower protein concentration compared to the samples treated with 1.0% SDS at both 0 and 30 min. However, same samples illustrated a greater protein concentration for 60, 240, 720, and 1440 min of retention time in the Lowry analysis ($p < 0.05$) (Table 4.10).

The pure water (1:40) samples solubilized with T and SDS lysis solutions at 0.1%, 0.5%, and 1.0% concentrations showed the greatest protein concentration of 186.21 ± 0.00 mg/mL for the samples solubilized with 1.0% SDS at 240 min in the Lowry analysis. The samples solubilized with 0.1% T exhibited higher protein concentrations at 60, 240, 720, and 1440 min of the Lowry analysis than that of the samples solubilized with 0.1% SDS; whereas the samples solubilized with 0.5% and 1% T had lower protein concentration in comparison to their counterparts treated with 0.5% and 1% SDS for all retention times ($p < 0.05$) (Table 4.11).

Table 4.10. The protein concentration (mg/mL) of eggshell membrane samples solubilized with 0.185% HCl at 1:30 ratio, using lysis solutions of triton X-100 (T) and sodium dodecyl sulfate (SDS) at varying concentrations and extended retention times of the Lowry analysis.

Sample	0.185% HCl (1:30)					
Lysis solution	0 min	30 min	60 min	240 min	720 min	1440 min
T (0.1 %)	100.37±0.00 ^{Fd}	117.11±0.12 ^{De}	132.78±0.00 ^{Cd}	138.04±0.19 ^{Ad}	136.29±0.00 ^{Bc}	116.43±0.19 ^{Ed}
T (0.5 %)	95.80±0.12 ^{Ee}	106.99±0.12 ^{Cf}	120.03±0.12 ^{Bf}	127.24±0.19 ^{Af}	120.13±0.12 ^{Be}	100.37±0.38 ^{Df}
T (1.0 %)	113.51±1.42 ^{Fc}	135.51±0.12 ^{Ed}	150.69±0.32 ^{Cb}	156.63±0.12 ^{Aa}	155.95±0.12 ^{Ba}	143.88±0.00 ^{Da}
SDS (0.1 %)	98.43±0.12 ^{Ff}	140.08±0.00 ^{Ac}	123.15±0.19 ^{De}	128.21±0.12 ^{Be}	124.22±0.12 ^{Cd}	103.39±0.12 ^{Ee}
SDS (0.5 %)	121.20±0.12 ^{Cb}	148.94±0.12 ^{ABa}	165.39±0.12 ^{Aa}	146.21±0.19 ^{Bb}	153.32±12.13 ^{ABa}	124.51±0.12 ^{Cc}
SDS (1.0 %)	125.19±0.00 ^{Ea}	140.37±0.19 ^{Cb}	134.54±0.00 ^{Dc}	142.13±0.19 ^{Bc}	150.59±0.00 ^{Ab}	140.86±0.12 ^{Cb}

*Data in the similar column with distinct small letter and data in the similar row with distinct capital letters are considerably different ($p \leq 0.05$).

*The retention times in the table stand for the time period in the Lowry analysis.

Table 4.11. The protein concentration (mg/mL) of eggshell membrane samples solubilized with 100% water at 1:40 ratio, using lysis solutions of triton X-100 (T) and sodium dodecyl sulfate (SDS) at varying concentrations and extended retention times of the Lowry analysis.

Sample	100% Water (1:40)					
Lysis solution	0 min	30 min	60 min	240 min	720 min	1440 min
T (0.1 %)	96.38±0.12 ^{De}	88.89±0.12 ^{Ef}	97.07±0.12 ^{Ce}	118.09±0.12 ^{Ae}	104.56±0.12 ^{Be}	86.65±0.19 ^{Fe}
T (0.5 %)	106.21±0.00 ^{Fc}	131.32±0.19 ^{Cc}	137.55±0.12 ^{Ac}	134.92±0.12 ^{Bc}	126.75±0.12 ^{Dc}	116.14±0.00 ^{Ec}
T (1.0 %)	106.02±0.12 ^{Dd}	104.07±0.12 ^{Ed}	122.95±0.12 ^{Bd}	130.16±0.00 ^{Ad}	114.88±0.12 ^{Cd}	94.92±0.12 ^{Fd}
SDS (0.1 %)	160.42±0.32 ^{Ea}	93.37±0.00 ^{Be}	92.88±0.12 ^{Bf}	96.00±0.00 ^{Af}	86.75±0.12 ^{Cf}	81.98±0.19 ^{Df}
SDS (0.5 %)	130.25±0.12 ^{Eb}	136.87±0.00 ^{Db}	149.13±0.00 ^{Bb}	154.78±0.12 ^{Ab}	144.75±0.00 ^{Cb}	122.66±0.12 ^{Fb}
SDS (1.0 %)	160.42±0.12 ^{Fa}	169.18±0.12 ^{Da}	183.88±0.12 ^{Ba}	186.21±0.00 ^{Aa}	180.47±0.12 ^{Ca}	162.95±0.12 ^{Ea}

*Data in the similar column with distinct small letter and data in the similar row with distinct capital letters are considerably different ($p \leq 0.05$).

*The retention times in the table stand for the time period in the Lowry analysis.

Solubilization of ESM protein was performed by the application of SDS and T and these chemicals should be removed before ESM protein to be is used as protein additive. Thus, washing procedure was implemented to eliminate the lysis solutions utilized for protein solubilization. The solutions exhibiting the maximum protein concentrations, namely 100 mM EDTA at a 1:20 ratio, 100 mM EDTA at a 1:40 ratio, and 100% water at a 1:40 ratio solubilized with 1% SDS, were chosen for further analyses. In order to remove lysis solution from ESM protein extract, a washing step with methanol followed by water was undertaken. Then, the variations in the protein concentration between the washed and unwashed samples were analyzed to determine the effect of the washing procedure on protein concentration. When comparing the unwashed and washed samples of 100 mM EDTA at 1:40 ratio treated with 1% SDS, it was observed that the washed samples at 720 minutes of retention time in the Lowry analysis exhibited the greatest protein concentration of 269.81 ± 0.84 mg/mL ($p < 0.05$). The unwashed samples at 0, 30, and 60 min had higher protein concentration; whereas the washed samples at 240, 720, and 1440 min had higher protein concentration compared to their counterparts ($p < 0.05$) (Table 4.12).

The greatest protein concentration of 313.43 ± 0.36 mg/mL was obtained in the washed 100 mM EDTA at 1:40 ratio samples solubilized by 1% SDS at 1440 min. Generally, washed samples had higher protein concentration in comparison to the unwashed counterparts (Table 4.13).

The maximum protein concentration reached 222.90 ± 0.33 mg/mL for the samples solubilized with 1% SDS (pure water at a 1:40 ratio) following the washing procedure at 1440 minutes. While the unwashed samples at 0, 30, and 60 min showed greater protein concentration compared to then washed samples, the washed samples at 240, 720, and 1440 min revealed higher protein concentration than the unwashed samples ($p < 0.05$) (Table 4.14).

Owing to eggshell membrane solubilization and washing procedure, the washed samples of 100 mM EDTA at 1:20 ratio at 720 min (269.81 ± 0.84 mg/mL), 100 mM EDTA at 1:40 ratio at 1440 min (313.43 ± 0.36 mg/mL), and water at 1:40 ratio at 1440 min (222.90 ± 0.33 mg/ml) (prepared with 1% SDS) with the greatest protein concentrations were chosen for further analyses.

Table 4.12. Assessing the impact of the washing process on protein concentration after protein extraction (mg/mL) with 1% sodium dodecyl sulfate (SDS) on the samples treated with 100 mM EDTA at 1:20 ratio.

Sample	1% SDS (100 mM EDTA 1:20)					
	0 min	30 min	60 min	240 min	720 min	1440 min
Lysis solution						
Washed	185.82±0.16 ^{Kb}	214.24±0.29 ^{Hb}	200.81±0.29 ^{Jb}	251.91±0.87 ^{Ba}	269.81±0.84 ^{Aa}	238.28±0.73 ^{Ea}
Unwashed	211.62±0.19 ^{Ia}	236.82±0.32 ^{Fa}	252.49±0.19 ^{Ba}	247.82±0.00 ^{Cb}	239.74±0.12 ^{Db}	222.61±0.12 ^{Gb}

*Data in the similar column with distinct small letter and data in the similar row with distinct capital letters are considerably different ($p \leq 0.05$).

*The retention times in the table stand for the time period in the Lowry analysis.

Table 4.13. Assessing the impact of the washing process on protein concentration after protein extraction (mg/mL) with 1% sodium dodecyl sulfate (SDS) on the samples treated with 100 mM EDTA at 1:40 ratio.

Sample	1% SDS (100 mM EDTA 1:40)					
	0 min	30 min	60 min	240 min	720 min	1440 min
Lysis solution						
Washed	238.87±0.16 ^{Ga}	281.59±0.60 ^{Ca}	271.96±0.44 ^{Ea}	305.82±0.16 ^{Ba}	277.90±0.21 ^{Da}	313.43±0.36 ^{Aa}
Unwashed	188.40±0.12 ^{Lb}	221.54±0.12 ^{Jb}	244.32±0.12 ^{Fb}	227.38±0.00 ^{Hb}	224.46±0.00 ^{Ib}	208.55±0.12 ^{Kb}

*Data in the similar column with distinct small letter and data in the similar row with distinct capital letters are considerably different ($p \leq 0.05$).

*The retention times in the table stand for the time period in the Lowry analysis.

Table 4.14. Assessing the impact of the washing process on protein concentration after protein extraction (mg/mL) with 1% sodium dodecyl sulfate (SDS) on the samples treated with 100% water at 1:40 ratio.

Sample	1% SDS (100% Water 1:40)					
	0 min	30 min	60 min	240 min	720 min	1440 min
Lysis solution						
Washed	142.42±0.00 ^{Kb}	162.18±0.60 ^{lb}	165.39±0.58 ^{Hb}	194.97±0.58 ^{Ca}	207.04±0.44 ^{Ba}	222.90±0.33 ^{Aa}
Unwashed	160.42±0.12 ^{Ja}	169.18±0.12 ^{Ga}	183.88±0.12 ^{Ea}	186.21±0.00 ^{Db}	180.47±0.12 ^{Fb}	162.95±0.12 ^{lb}

*Data in the similar column with distinct small letter and data in the similar row with distinct capital letters are considerably different ($p \leq 0.05$).

*The retention times in the table stand for the time period in the Lowry analysis.

The samples containing the highest protein content from EDTA, acetic acid, and HCl pretreatments were selected after based on the treatments of T and SDS after the Lowry analysis. The greatest protein content of 208.40 ± 0.00 mg/mL at 240 min was obtained in the membrane samples separated by 50 mM EDTA at 1:20 ratio and then solubilized with 1% SDS (Table 4.5). The second greatest protein content of 252.49 ± 0.19 mg/mL at 60 min was observed in the membrane samples separated by 100 mM EDTA at 1:20 ratio and then solubilized with 1% SDS (Table 4.6). The third greatest protein content of 244.32 ± 0.12 mg/mL at 60 min was obtained in the membrane samples separated by 100 mM EDTA at 1:40 ratio and then treated with 1% SDS (Table 4.7).

The greatest protein ratio of 168.79 ± 0.12 mg/mL at 720 min was obtained in the membrane samples separated by 1.25% acetic acid at 1:30 ratio and then solubilized with 0.5% SDS (Table 4.8). The second greatest protein ratio of 177.55 ± 0.12 mg/mL at 240 min was obtained in the membrane samples separated by 1.25% acetic acid at 1:40 ratio and then solubilized with 1% SDS (Table 4.9). The third highest protein ratio of 165.39 ± 0.12 mg/mL at 60 min was obtained in the membrane samples separated by 0.185% HCl at 1:30 ratio and then solubilized with 0.5% SDS (Table 4.10). The greatest protein concentration of 186.21 ± 0.00 mg/mL at 240 min was obtained in the membrane samples separated by water at the ratio of 1:40 then solubilized with 1% SDS (Table 4.11).

After the samples with the greatest protein content were selected, the protein concentrations of the samples before and after washing were re-determined to observe the effect of the lysis solutions on the protein concentration. When the impact of the washing process on the 100 mM EDTA samples at ratio of 1:20 solubilized with 1% SDS was examined, the greatest protein ratio was assessed at the end of 720 min in the washed samples (Table 4.12). Moreover, when the impact of the washing process on the 100 mM EDTA samples at ratio of 1:40 solubilized with 1% SDS was examined, the greatest protein ratio was assessed at the end of 1440 min in the washed samples (Table 4.13). In the samples that water was used, the greatest protein ratio was assessed after 1440 min in washed samples (Table 4.14). Approximately 5-10% increase in the protein concentration of the samples were observed after washing procedure. It is likely that methanol utilized in washing procedure, because a raise in the protein concentration by opening their hydrogen

bonds further, and for that reason washed samples had higher protein content than that of their unwashed counterparts (94, 95).

Kaweewong et al. (18) separated the eggshell wastes from their membranes by soaking them in water for 10 min (18). The eggshells were then treated with liquid nitrogen and utilized in the analysis. The membranes utilized in the study were made soluble by treating them with lysis solutions containing SDS and Triton X-100, and Lowry analysis to determine protein concentration was performed on the solubilized samples. The protein concentrations reported for the samples prepared with 0.1%, 0.5% and 1% SDS were 0.56 ± 0.05 , 0.81 ± 0.21 , and 1.36 ± 0.42 $\mu\text{g}/\mu\text{L}$, respectively. Protein concentration found for soluble proteins obtained with Triton X-100 detergent were 0.50 ± 0.02 , 0.51 ± 0.16 , and 1.04 ± 0.18 $\mu\text{g}/\mu\text{L}$, respectively. Compare to this study, it is observed that the results of this study are clearly higher. The protein concentrations of the ESM in acids were higher than that of the water. This variance was due to the different waiting time, and the peeling process with acid. Kaweewong et al. (18) observed in their study that SDS detergent is a more effective agent than Triton X-100 detergent, as it is true for our study. Extraction ability raised with increasing detergent concentration from 0.1% to 1.0% in both studies (18).

In the study of Jain and Anal (47), eggshells and membranes were separated by keeping them in pure water. The amount of protein was 177.3 mg/g on protein hydrolysates kept for 36 hours. In another study, Cheng et al. (62) performed a study about purification and characterization of an eggshell membrane decomposing by protease from *Pseudomonas aeruginosa* strain ME-4. They reported the protein concentration of 140 ± 3.4 $\mu\text{g}/\text{U}$ measured by the Lowry method (62). Marimuthu et al. (63) applied different treatments such as “20% NaOH, 5% methanol+20% NaOH, and 5% EDTA+5% hexane+20% alkali” to separate the membranes from the eggshell. The protein ratios obtained from these treatments were 91%, 64.5%, and 65%, respectively. It is observed that the samples solubilized with SDS had the greatest efficiency. In addition to the Triton X-100 and SDS detergents, the exposure time was also found to be an effective parameter. Although it is difficult to solubilize eggshell membrane proteins, it was decided that solubilization with SDS 1% was the most efficient method. The samples of 100 mM EDTA 1:20, 100

mM EDTA 1:40, and pure water with the highest protein concentrations were used to prepare the soft gel capsules.

4.4 Characterization and Functional Properties of the Solubilized Eggshell Membrane and SEP Proteins

4.4.1 Water and Oil Holding Capacities of the Eggshell Membrane

Water holding capacity of the 100 mM EDTA samples at 1:20 ratio, 100 mM EDTA samples at 1:40 ratio, and water samples at 1:40 ratio were not considerably different from each other ($p > 0.05$). The OHC of the samples also demonstrated no significant difference ($p > 0.05$) (Table 4.15).

Table 4.15. Assessing the water holding capacity and oil holding capacity of eggshell membrane powder.

Samples	Water holding capacity (g/g)	Oil holding capacity (g/g)
100 mM EDTA 1:20	12.54±2.17 ^a	11.52±1.18 ^a
100 mM EDTA 1:40	11.70±1.78 ^a	11.01±1.23 ^a
Water 1:40	13.00±0.77 ^a	10.60±2.09 ^a

*Data in the similar column with distinct small letter are considerably different ($p \leq 0.05$).

WHC is a parameter used to understand protein-water interactions, it also illustrates the ability of a molecule to retain water under the influence of external force (64). WHC and OHC of eggshell membranes in previous studies varied from 1.90±1.00 to 2.90±0.40 g/g and from 2.50±1.00 to 4.40±0.40%, respectively (47); and these results were lower than that of the samples illustrated in the current study. Both WHC and OHC of the protein are affected by the chemicals used for extraction since these chemicals can cause denaturation of the protein. In fact, it is reported that the lower WHC and OHC were stemmed from the partial destruction of eggshell membrane by the EDTA chemical used (44).

The WHC and OHC of the egg white protein dried both by spray and freeze drying ranged from 0.65±0.06 to 1.37±0.03 g/g and from 3.12±0.07 to 10.21±0.13 mL/g, respectively (65). When the results of egg white and our results were compared, it

was illustrated that both WHC and OHC of the ESM protein were higher as it is presented in Table 4.15.

4.4.2 Emulsion Stability Index and Emulsion Activity Index of the Eggshell Membrane

The emulsion stability index (ESI) exhibited significant variations among the samples. While ESI of the 100 mM EDTA 1:40 samples were measured as $77.27 \pm 3.93\%$; ESI of the 100 mM EDTA 1:20 and water 1:40 samples were $65.76 \pm 1.57\%$ and $23.98 \pm 1.76\%$, respectively (Table 4.16). EAI of the samples were also considerably differed among the samples. EAI of the 100 mM EDTA 1:20, 100 mM EDTA 1:40, and water 1:40 samples were 0.0117 ± 0.0001 , 0.0016 ± 0.0000 , and $0.0021 \pm 0.0000 \text{ m}^2/\text{g}$, consequently ($p < 0.05$) (Table 4.16).

Table 4.16. Assessing the emulsion capacities of eggshell membrane powder resulting from various applications.

Samples	Emulsion stability index (%)	Emulsion activity index (m^2/g)
100 mM EDTA 1:20	65.76 ± 1.57^b	0.0117 ± 0.0001^a
100 mM EDTA 1:40	77.27 ± 3.93^a	0.0016 ± 0.0000^c
Water 1:40	23.98 ± 1.76^c	0.0021 ± 0.0000^b

*Data in the similar column with distinct small letter are considerably different ($p \leq 0.05$).

Solubility depending on the degree of hydrophobicity of protein molecule and hydration is one of the most crucial physico-chemical and functional properties of the proteins (66). To obtain a desired level of gel and emulsion formations, proteins should have high solubility (67). While EAI refers to the emulsion-forming capacity of the protein, ESI stands for the ability of emulsion droplets to remain dispersed, float in oil, and remain dispersed without accumulation over a period of time (68). In a similar study, the EAI and ESI of 37.10 ± 1.50 and 19.10 ± 1.50 were reported for the ESM samples treated with ultrasonication (47). EAI of egg white samples ranged from 84.63 ± 1.81 to 100.10 ± 1.86 ; whereas ESI of the egg white samples changed from 20.62 ± 1.17 to 33.74 ± 2.43 (39). ESI and EAI of the egg white samples were 23 and 32, respectively (65). Both EAI and ESI of

the samples showed variations depending on the type of the protein and processing method.

EAI and ESI of the proteins vary greatly depending on the molecular structure. EAI of pH-treated small aggregates (2.50 m²/g) was found significantly greater than that of large aggregates (2.17 m²/g) of NaOH–HCl/gluconolactone. Similar to EAI, myofibrillar protein aggregates exhibited considerably greater ESI in comparison with aggregate samples because of their relatively extended conformations and exposed hydrophobic groups. In comparison to large aggregates (66.60%), the ESI value of small aggregates (93.67%) was much greater (46).

4.4.3 Foam Stability of the Eggshell Membrane Protein Extracts

Soluble ESM protein extracts presented no foaming properties regardless of the treatment. However, previous studies reported some degree of foaming ability. For example, Jain and Anal (2016) performed foaming capacity analysis on ESM protein hydrolysates obtained through ultrasonication and alcalase enzyme (47). Foaming capacity and foam stability of the ESM proteins were reported as 38.30±2.90 and 25.00±1.30 in that study, respectively (47). Foaming properties of ESM at different concentrations changed from 36.67±2.89 to 48.33±2.89 and from 23.33±3.33 to 30.00±2.89 for foaming capacity and foam stability, respectively (69). Foaming capacity of the egg white was 31.00±0.00; while the foam stability was reported as 39±0.00 (65). Reported foaming abilities illustrated high variations relying on the source and the concentration of the proteins in addition to the process applied for extraction.

4.4.4 Colour Analyses of the Eggshell Membrane Protein Extracts

Color parameters of L^* , a^* , and b^* values for the 100 mM EDTA at 1:20 ratio, 100 mM EDTA at 1:40 ratio, and pure water samples were very close to each other. Total color difference (ΔE) and Chroma of the water samples; however, were considerably different from the others, and no considerable variation was detected among the samples for hue values ($p > 0.05$) (Table 4.17).

Table 4.17. Overall colour variation of the soluble protein extracts.

Samples	L^*	a^*	b^*	ΔE	Chroma	Hue
EDTA 1:20	80.39±0.00 ^b	-0.92±0.01 ^b	2.72±0.02 ^a	80.43±0.00 ^b	2.87±0.02 ^a	-1.24±0.00 ^a
EDTA 1:40	80.40±0.00 ^b	-0.90±0.00 ^b	2.71±0.02 ^a	80.45±0.00 ^b	2.85±0.02 ^a	-1.24±0.00 ^a
Water 1:40	80.63±0.01 ^a	0.88±0.01 ^a	2.63±0.03 ^b	80.67±0.01 ^a	2.77±0.03 ^b	-1.24±0.00 ^a

*Data in the similar column with distinct small letter are considerably different ($p \leq 0.05$).

L^* , a^* and b^* values of the egg yolk from two different bird species were reported as 73.4, -2.5, and 25, respectively (70). While a^* values were found to be lower, both L^* and b^* values were recorded to be higher in our study. a^* value closer to negative means an increase in green hue. Since they worked with egg yolk in the study, it was logical that the a^* value was lower comparison to the value showed in Table 4.17. The reason why the L^* and a^* values of the ESM separated by water were different from the samples prepared with EDTA can be explained by the variation in the chemicals used for extraction as EDTA samples were not considerably different from each other, on the other hand, both were considerably different from the samples prepared with water. Likewise, since the b^* value denotes yellow color, a high b^* value with a large margin was acceptable. Color measurement of the dried and hydrolyzed egg white powder revealed L^* value between 96-98 which is considerably greater than the values in the present study (71). Color values of only hydrolyzed egg white powder samples resulted in L^* values between 85-97 with total color difference between 0-15% (45). Compare to ESM protein extract in the present study, different fraction of egg products presented significantly different color properties because of the nature of the raw products and/or applied processes.

4.4.5 Rheological Analysis

It was observed that flow behavior of the 100 mM EDTA 1:40 and water samples were closer to each other; while 100 mM EDTA 1:20 sample illustrated considerably different flow behavior. As showed in Fig. 4.1, the shear stress of each sample raised with an increase in shear rate presenting Newtonian type fluid properties.

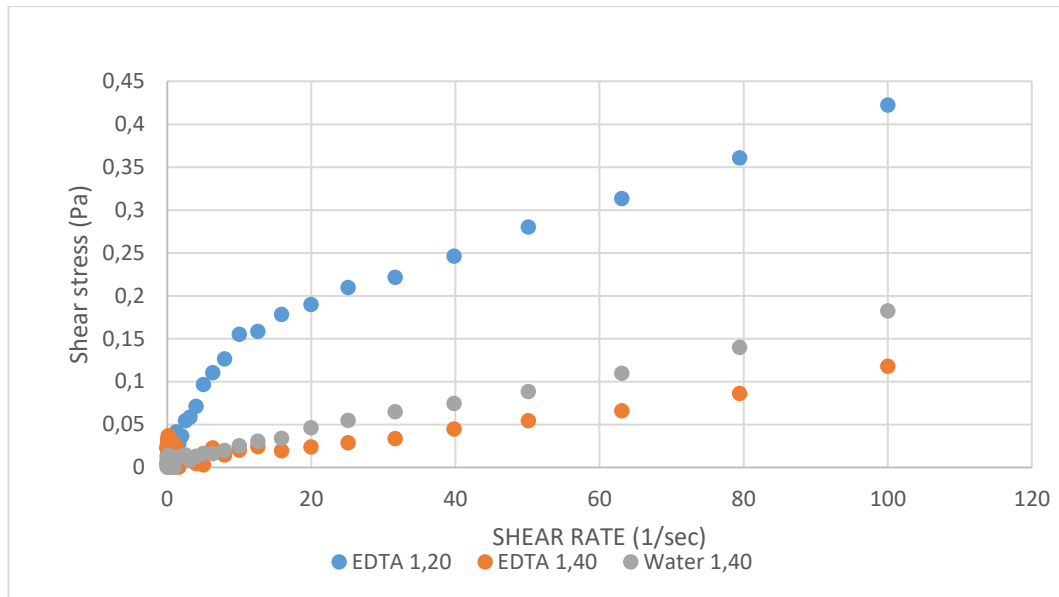


Figure 4.1. Shear rate versus shear stress of soluble eggshell membrane protein.

Modeling studies conducted with samples with EDTA at 1:20 ratio, EDTA at 1:40 ratio, and pure water at 1:40 ratio presented a strong correlation with Bingham, Herschley-Bulkley, and Newtonian models (Table 4.18). Correlation coefficients of the models were very close to each other, thus rheological properties of soluble ESM proteins can be explained by the three models. It was reported that shear rate and shear stress of the egg yolk and soluble ESM samples increased proportionally with applied force (73).

Decrease in viscosity with an increase in shear rate was reported in egg yolk as in soluble eggshell membrane (74). Rheological analyses of the ESM protein extracts were not reported in the literature, thus the results from the present study are the only source for the assessments of the rheological characteristics of ESM protein extract.

Table 4.18. Establishing correlations with Bingham, Herschley-Bulkley, and Newtonian models.

Bingham Model					
Samples	Yield Stress (Pa)	K1	n	Chi Square	Correlation Coefficient
EDTA 1:20	0.04	0.00	-	0.04	0.9538
EDTA 1:40	0.02	0.00	-	0.01	0.8719
Water 1:40	0.01	0.00	-	0.00	0.9934
Herschel-Bulkley Model					
EDTA 1:20	0.0122	0.03	05504	0.01	0.9927
EDTA 1:40	0.01729	0.00	1.583	0.00	0.8985
Water 1:40	0.00665	0.00	0.9846	0.00	0.9934
Newtonian Model					
EDTA 1:20	0.00796	-	-	0.3098	0.9538
EDTA 1:40	0.00214	-	-	0.03953	0.8719
Water 1:40	0.00229	-	-	0.00794	0.9934

4.3.5. Differential Scanning Calorimetry Analysis of the Eggshell Membrane Protein Extracts

The DSC analyses revealed that the samples exhibited varying peak temperatures, with the highest value observed for 100 mM EDTA samples at a 1:20 ratio and the lowest for 100 mM EDTA samples at a 1:40 ratio ($p < 0.05$). Additionally, the pure water extracts displayed the greatest onset temperature of 101.22 ± 0.01 °C, while the 100 mM EDTA samples at a 1:20 ratio exhibited the minimum onset temperature of 84.60 ± 62.40 °C. The heat values changed from -1.70 ± 0.01 kJ/g for 100 mM EDTA samples at a 1:20 ratio to -1.47 ± 0.01 kJ/g for water samples at a 1:40 ratio. All samples exhibited exothermic peaks (Table 4.19).

DSC is a widely used technique in thermal analysis applications. In this technique, the energy change between the sample and reference material can be assessed as a function of temperature. Melting temperature and heat capacity, which are the distinguishing features of substances, can be easily measured with this device (75).

Moreover, the thermal stability of various cross-linked membranes at various temperatures can be established using the DSC approach (76). When used in biomedical applications like wound dressing, DSC can show how ESM behaves at body temperature likewise the membrane's capacity to keep its integrity while being stored at freezing (0 °C), refrigeration (2-4 °C), or ambient temperatures (23-25 °C) (77).

Table 4.19. Analyzing the results of differential scanning calorimetry for soluble protein extracts.

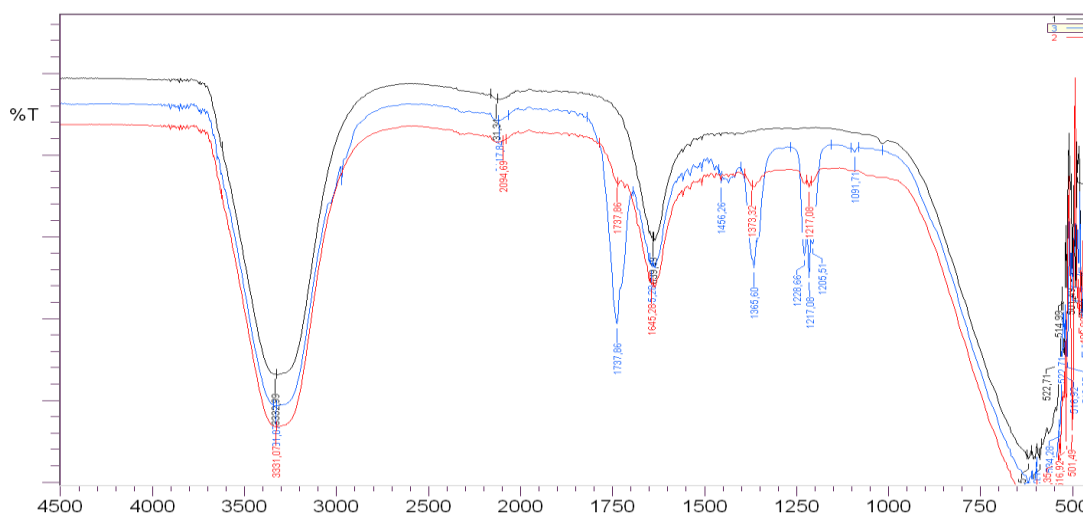
Samples	Peak temperature (°C)	Onset temperature (°C)	Heat (kJ/g)
100 mM EDTA 1:20	137.49±0.03 ^a	84.60±0.01 ^a	-1.70±0.01 ^c
100 mM EDTA 1:40	100.46±0.01 ^c	88.14±0.01 ^a	-1.51±0.01 ^b
Water 1:40	113.84±0.01 ^b	101.22±0.01 ^a	-1.47±0.01 ^a

*Data in the similar column with distinct small letter are considerably different ($p \leq 0.05$).

DSC analysis conducted on the ESM with varying water content (0%, 20%, 40%, and 60%) exhibited peak temperature values ranging from 54.50 to 102.26 °C, accompanied by endothermic peaks. The onset temperatures of the samples diversified within the range of 17.80 to 20.00 °C. The observed peaks are indicative of the thermal denaturation of collagen. The process of collagen denaturation is believed to involve the disruption of hydrogen bonds connecting the three chains, leading to the rearrangement of the collagen triple helix into a random chain configuration (29).

DSC analysis of egg yolk showed the peak temperature of 83.3 °C, whereas the heat value was recorded as 1.29 J/g (78). In comparison to the results in Table 4.20, it was showed that the peak value in the above study was low and the heat value was endothermic. The enthalpy of denaturation represented the net result of the combination of endothermic processes (breakdown of hydrogen bonds) and exothermic changes (disruption of hydrophobic interactions or protein aggregation). Consequently, the decrease in residual enthalpy indicated the loss of protein structure during heat treatment (78).

4.4.6 Fourier Transform and Infrared Spectroscopy Spectrum of the Eggshell Membrane Protein Extracts



(Black line: 100 mM EDTA at 1:20 ratio, Red line: 100 mM EDTA at 1:40 ratio Blue line: Water at 1:40 ratio.)

Figure 4.2. Fourier transform and infrared spectroscopy spectrum of eggshell membrane protein extracts.

In general, FT-IR spectrum of the samples were similar to each other revealing a first peak between $3200\text{--}3500\text{ cm}^{-1}$, second peak around $2200\text{--}2400\text{ cm}^{-1}$, and third peak around $1600\text{--}1700\text{ cm}^{-1}$. While sample 1 (100 mM EDTA at 1:20 ratio) and 2 (100 mM EDTA at 1:40 ratio) had similar spectrum to each other, sample 2 showed a major peak around 1750 cm^{-1} . Furthermore, sample 3 (water) had more distinct peak at around $1100\text{--}1250$ and $1300\text{--}1400\text{ cm}^{-1}$, respectively (Figure 4.2).

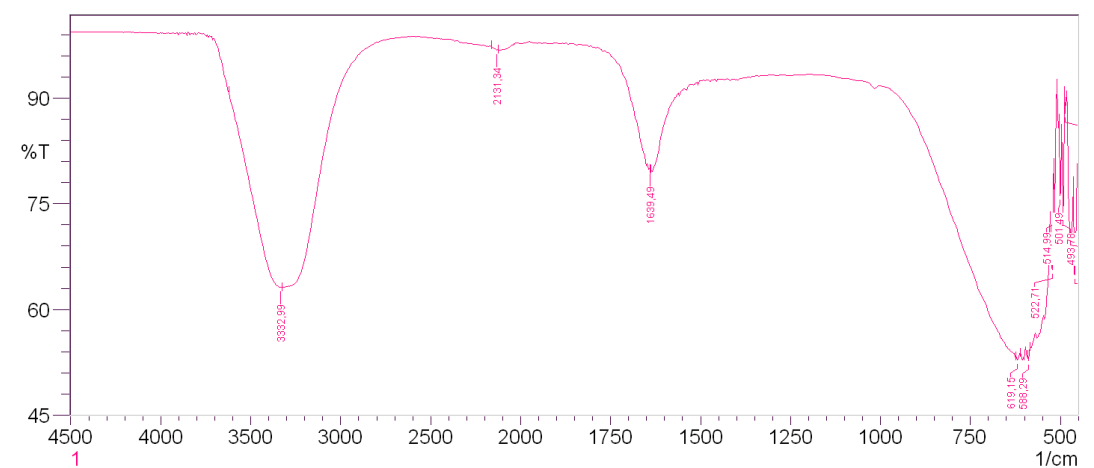


Figure 4.3. FTIR spectra of SEP in 100 mM EDTA at 1:20 ratio.

Table 4.20. IR spectra for concentration of 100 mM EDTA (1:20).

Frequency (cm ⁻¹)	Functional Group
3332.99 (s)	Amide-NH stretch
2131.34 (v)	C≡C stretch
1639.49 (m)	Amide I: C-O stretch
522.71 (w)	C-Br

w: weak, s: strong, m: medium, v: variable

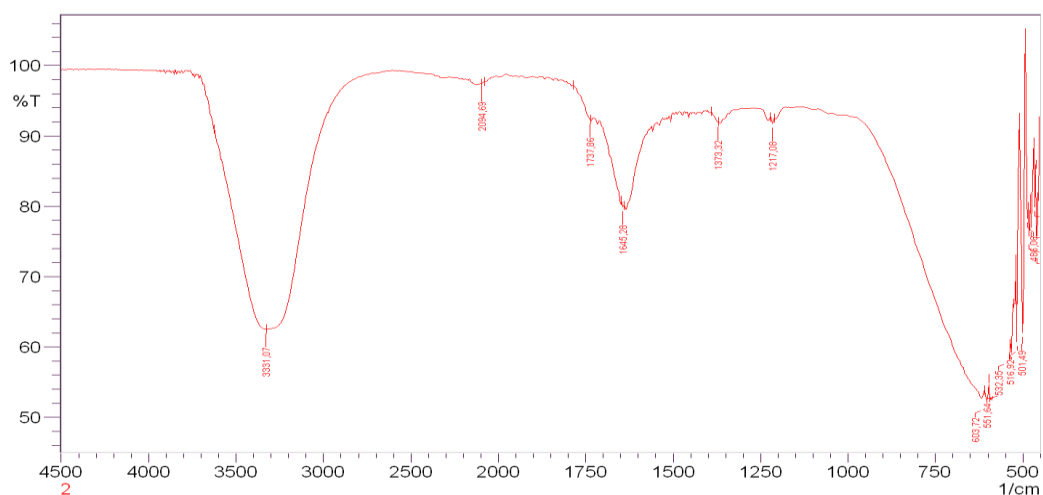


Figure 4.4. FTIR spectra of SEP in 100 mM EDTA at 1:40 ratio.

Table 4.21. IR spectra for concentration of 100 mM EDTA (1:40).

Frequency (cm ⁻¹)	Functional Group
3331.07 (s)	Amide-NH stretch
2094.69 (v)	C≡C stretch
1737.86 (w)	C=O ketone
1645.28 (s)	Amide I: C-O stretch
1373.32 (m)	C-F
1217.08 (m)	C-F
501.49 (w)	C-Br

*w: weak, s: strong, m: medium, v: variable

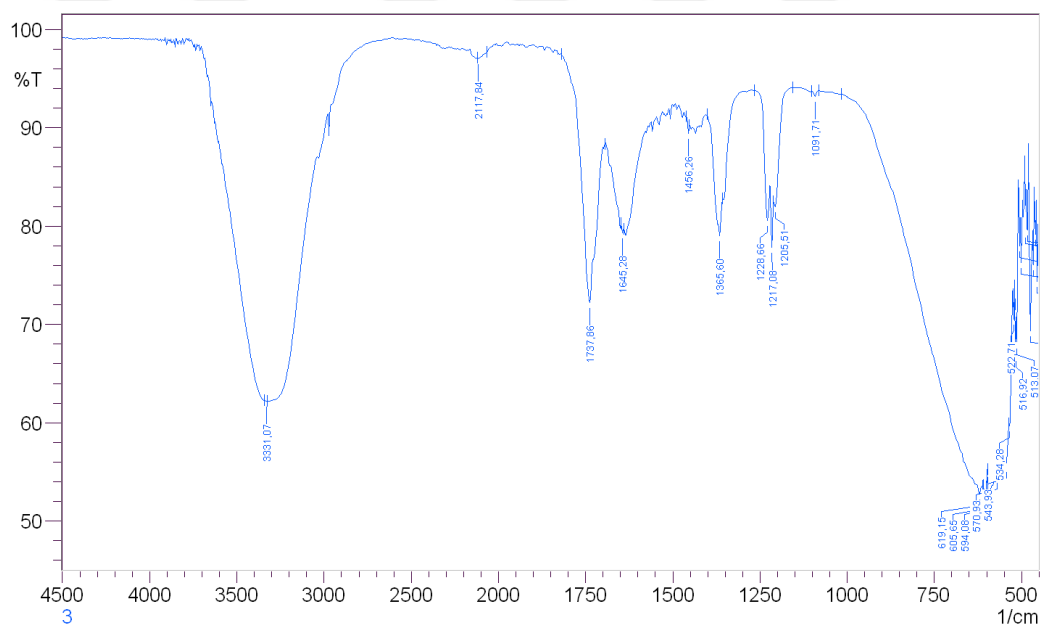


Figure 4.5. FTIR spectra of SEP in water at 1:40 ratio.

Table 4.22. IR spectra for concentration of water at 1:40 ratio.

Frequency (cm ⁻¹)	Functional Group
3331.07 (s)	Amide-NH stretch
2117.84 (v)	C≡C stretch
1737.86 (s)	C=O ketone
1645.28 (s)	Amide I: C-O stretch
1456.26 (m)	Amide II: C- N stretch
1365.60 (m)	C-F
1228.66 (w)	C-F
1217.08 (w)	C-F
1205.51 (w)	C-F
1091.71 (w)	C-O-C stretch
522.71 (w)	C-Br
516.92 (w)	C-Br

*w: weak, s: strong, m: medium, v: variable

FT-IR analysis for eggshell membranes separated with 100 mM EDTA 1:20 and 1:40 ratios and pure water samples at 1:40 ratio was shown in Figure 4.2. Absorption peaks at different frequencies (cm⁻¹) were illustrated in Tables 4.20-4.22. Different considerable peak intensities for 100 mM EDTA at both 1:20 and 1:40 ratios and water samples at 1:40 ratio was revealed at 3332.99, 3331.07, and 3331.07 cm⁻¹, respectively with the possibility of OH stretch bands.

In the study of Amirsadeghi et al. (79) FT-IR spectrum of egg membrane concentrate was obtained by soluble egg membrane by “20% NaOH, soluble egg membrane by 5% methanol+5% NaOH” and chemically refined EM by “5% EDTA+5% hexane+20% NaOH” revealed more peaks for each sample compared to the peaks summarized in Tables 4.21-4.23. However, it was illustrated that almost all of the wavelengths obtained in Tables 4.20-4.22 and Figures 4.2-4.4 were similar to the previous studies (79).

The characteristics of the eggshell membranes obtained through manual separation and acid resolution of the eggshell mineral varied in terms of shape and density, and absorption bands. The amide III absorption band and the band related to eggshell mineral were reported for all samples in the study (61). FT-IR analyses of

both the untreated and HCl-treated ESM samples exhibited similar peaks in the current study with additional peaks (60).

The band centered at 3332 cm^{-1} had N-H and O-H stretch vibrations in proteins and polysaccharides, the bands at $2094\text{--}2131\text{ cm}^{-1}$ have a C-C stretch vibration in lipids, and the bands at $1639\text{--}1645\text{ cm}^{-1}$ are due to amide bonds found only in proteins, including collagen fibers. It can be assigned to C-O and C-N stretching vibrations, as well as to amide bonds found in N-acetyl glucosamine and N-containing glycosaminoglycans (60). These findings suggest that ESM contains a variety of chemical groups, including carboxyl, hydroxyl, and amino groups (105) components' variance is about the same in the inner and outer layers (100).

A study performed with ESM revealed peaks in ESM- 3274 cm^{-1} (O-H and N-H stretching), 2929 cm^{-1} (C-H stretching), 1643 cm^{-1} (amide C=O stretching), 1536 cm^{-1} (amide N-H bending), 1451 cm^{-1} (CH_2 scissoring), 1109 cm^{-1} (amine C-N stretching), and 661 cm^{-1} (C-S) by FT-IR spectroscopy (110, 111, 112).

When we examined our results, it was observed that water-derived ESMs give more spectrum than that of the EDTA-derived ones. The main reason for this can be interpreted as EDTA inhibition/denaturing of some proteins due to its chemical structure.

4.4.7 Amino Acid Concentration of the Eggshell Membrane Protein Extracts

The samples of 100 mM EDTA at a 1:20 ratio, 100 mM EDTA at a 1:40 ratio, and water at a 1:40 ratio revealed the presence of sixteen individual amino acids, including essential ones. These amino acids concentrations varied across the samples. “Alanine, valine, lysine, isolysine, threonine, proline, methionine, phenylalanine, histidine, tyrosine” exhibited considerably higher concentrations in the samples prepared with water and 100 mM EDTA samples at 1:40 ratio compared to other samples. Meanwhile, the glycine and serine concentrations were considerably greater in 100 mM EDTA samples at 1:20 ratio, and the concentration of “aspartic acid, glutamic acid, and lysine” were the greatest in 100 mM EDTA samples at 1:40 ratio, respectively (Table 4.23).

Comparison of the amino acid composition of eggshell membrane proteins obtained from calf (80), fish (37), pork skin (81), eggshell membrane (82), fermented protein hydrolysates for eggshell membrane (69) and soluble eggshell membrane protein (83) were shown in Table 4.23. Concentration of proline changing from $10.50 \pm 1.00\%$ to $11.60 \pm 1.00\%$ was the highest in three samples. In addition, glutamic acid (6.40 ± 1.00 - $9.30 \pm 1.00\%$), threonine (6.90 ± 1.00 - $8.10 \pm 1.00\%$), valine (6.70 ± 1.00 - $7.90 \pm 1.00\%$), and lysine (6.60 ± 1.00 - $7.10 \pm 1.00\%$) were the amino acids with higher concentrations followed after proline. Hydroxyproline, cysteine, tryptophan, and iminoacids were not found in the ESM; however, hydroxyproline was found in calf, fish, pork skin, eggshell membrane, fermented protein hydrolysates for eggshell membrane, and soluble eggshell membrane; cysteine in calf skin, fish skin, eggshell, and SEP; tryptophan in fermented protein hydrolysates, and iminoacids in all samples except for fermented protein hydrolysate and SEP (Table 4.23). This study agrees with previous works that both the concentration and variation of amino acids differs in different protein sources.

Table 4.23. Analyzing the types and concentrations of amino acids in eggshell membrane protein extracts in comparison to calf skin (80), fish skin (37), pork skin (81), eggshell membrane (82), fermented protein hydrolysates for eggshell membrane (69), and soluble eggshell membrane protein (83).

Amino acids (g/100 g)	100 mM EDTA 1:20	100 mM EDTA 1:40	Water 1:40	Calf skin (%)	Fish skin (%)	Pork skin (%)	Eggshell (%)	Fermented protein hydrolysates (%)	SEP (%)
Alanine	2.60±1.00 ^b	2.50±1.00 ^c	2.90±1.00 ^a	7.80	13.50	11.20	10.30	2.30	3.80
Glycine	6.40±1.00 ^a	6.20±1.00 ^b	6.20±1.00 ^b	24.10	35.30	33.00	31.80	-	10.70
Valine	7.00±1.00 ^b	6.70±1.00 ^c	7.90±1.00 ^a	2.80	2.10	2.60	2.00	3.30	7.40
Leucine	4.30±1.00 ^b	4.10±1.00 ^c	4.70±1.00 ^a	3.30	1.70	2.40	2.40	2.80	4.30
Isoleucine	3.50±1.00 ^b	3.20±1.00 ^c	3.90±1.00 ^a	1.70	0.90	1.00	1.30	2.10	3.20
Threonine	7.90±1.00 ^b	6.90±1.00 ^c	8.10±1.00 ^a	2.00	2.40	1.80	2.30	-	6.20
Serine	6.10±1.00 ^a	5.90±1.00 ^b	5.90±1.00 ^b	3.50	3.30	3.50	3.40	3.40	7.00
Proline	11.10±1.00 ^b	10.50±1.00 ^c	11.60±1.00 ^a	11.70	10.80	13.20	11.20	5.10	13.00
Arginine	4.20±1.00 ^c	5.50±1.00 ^a	4.60±1.00 ^b	7.30	5.20	4.90	6.20	4.00	5.30
Aspartic acid	4.40±1.00 ^b	4.90±1.00 ^a	2.70±1.00 ^c	6.80	4.60	4.60	5.50	5.70	8.10
Methionine	3.30±1.00 ^b	3.00±1.00 ^c	4.00±1.00 ^a	-	1.20	4.00	0.90	2.30	3.20
Glutamic acid	9.30±1.00 ^b	9.40±1.00 ^a	6.40±1.00 ^c	10.90	6.90	7.20	9.80	8.40	11.40
Phenylalanine	1.70±1.00 ^b	1.60±1.00 ^c	1.90±1.00 ^a	2.40	1.30	1.40	0.80	1.60	1.70
Lysine	6.70±1.00 ^b	7.10±1.00 ^a	6.60±1.00 ^c	4.00	2.80	2.70	2.10	-	3.70
Histidine	4.40±1.00 ^b	4.20±1.00 ^c	4.50±1.00 ^a	1.10	0.60	4.00	0.70	2.40	3.50
Tyrosine	1.90±1.00 ^b	1.70±1.00 ^c	2.10±1.00 ^a	6.00	0.40	3.00	0.60	1.10	1.20
Hydroxyproline	-	-	-	9.90	6.80	9.10	8.30	0.60	1.00
Cysteine	-	-	-	1.00	0.20	-	0.40	-	4.80
Tryptophan	-	-	-	-	-	-	-	0.90	-
Iminoacids	-	-	-	20.80	27.40	22.30	19.50	-	-

*Data in the similar row with distinct small letter are considerably different ($p \leq 0.05$).

4.4.8 SDS-PAGE Analysis of the Eggshell Membrane Protein Extracts

SDS-electrophoretic model of soluble and insoluble proteins in a matrix of soluble ESM proteins is shown in Figure 4.6. Differences were observed in the SDS-PAGE of three samples. SDS-PAGE showed seven different migration bands ranging from 10 to 66 kDa. Within the insoluble fraction, two prominent bands at 17 and 23 kDa (recognized as Ovocleidin-23), were identified as Ovocleidin-17, in accordance with the findings of Mine et al (49). The 14.3, 36, 45 and 68.3 kDa fractions were found to be “lysozyme, ovomucoid, ovalbumin, and avidin”, respectively (28, 58). Identification of eggshell matrix proteins involved the detection of key proteins found in egg white, lysozyme (14.3 kDa) and ovalbumin (45 kDa). Ovocleidin-17 (17 kDa) emerged as the initial matrix protein recognized as an eggshell-specific uterine protein (49). Although the matrix proteins are generally acidic, the same proteins were detected in the shell membranes separated by SDS.

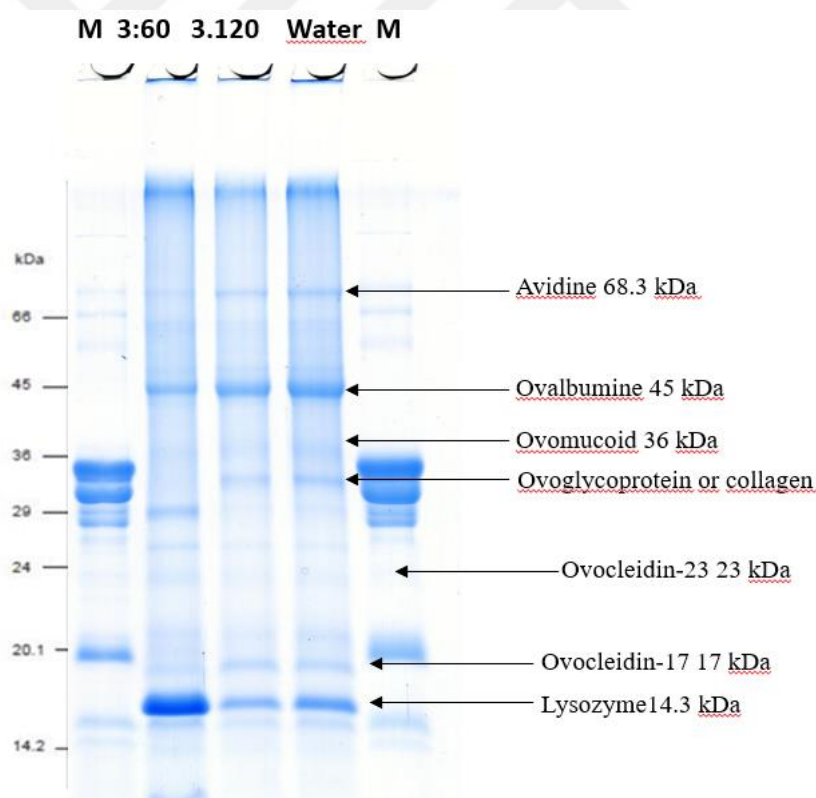


Figure 4.6 SDS-PAGE of ESM bounds protein.

Zhang et al. (41) conducted a study on eggshell matrix proteins extracted by EDTA, phosphate buffer, and acetic acid. It was noted that the most efficient method to

differentiate the protein bands by SDS-PAGE were phosphate buffer followed by acetic acid. Conversely, less protein bands were obtained with EDTA. Protein separation by SDS from eggshell matrix was the most efficient method. Protein fractions determined in eggshell cuticle separated with 1% SDS were similar to that of the fractions in the present study (12). SDS-PAGE analysis of eggshell protein also revealed 14 kDa lysozyme protein (85). ESM obtained from eggshells of various bird species solubilized with EDTA and acetic acid revealed lysozyme at 14.4 kDa, ovocleidin-17 at 17 kDa, and ovalbumin proteins at 45 kDa (86).

4.5 Production and Properties of the Soft Gel Capsules Produced from the Eggshell Membrane Protein Extract

A soft gel capsules were formulated with 100 mM EDTA at 1:20 ratio, 100 mM EDTA at 1:40 ratio, and water at 1:40 ratio extracts were tested for texture, sensory, and microbiological analyses.

4.5.1 Texture Profile Analysis of the Soft Gel Capsules

Hardness/firmness of the samples changed from 169.58 ± 24.40 g for 100 mM EDTA samples at 1:40 ratio to 388.60 ± 60.40 g for water at 1:40 ratio, whereas the elasticity/springiness among the samples illustrated considerable differences ($p < 0.05$), and the soft gel capsules prepared with water at 1:40 ratio showed the greatest firmness of $388.60 \pm 60.40\%$ (Table 4.24). Springiness of the samples changed from 68.38 ± 6.72 % for the 100 mM EDTA at 1:40 ratio samples to 76.94 ± 5.31 % for the pure water at 1:40 ratio samples ($p < 0.05$) (Table 4.24).

Table 4.24. Assessing of texture profile analysis on protein gel capsules.

Samples	Firmness (g)	Springiness (%)
100 mM EDTA 1:20	243.00 ± 21.08^b	73.57 ± 3.6^b
100 mM EDTA 1:40	169.58 ± 24.40^c	68.38 ± 6.72^{bc}
Water 1:40	388.60 ± 60.40^a	76.94 ± 5.3^a

*Data in the similar column with distinct small letter are considerably different ($p \leq 0.05$).

Previous studies subjected to ESM samples containing 1.5% NaCl showed that addition of NaCl influences hardness, and samples containing 1.5% NaCl had lower

hardness compared to the samples without ESM (87). Hardness value of the breads containing 2%, 4%, and 6% ESM powder increased with increased ESM concentration; whereas springiness value remained constant. It was mentioned that the rise in hardness value might also be linked to an elevated ash content (88). Rahman et al. (89) prepared three different gels made from bovine and porcine for TPA analyzes and observed textural differences among the samples was due to the type of gelatin (porcine, bovine and fish gelatine) used to make the gel (89). Pratiwi et al. (90) obtained gummy candies containing pineapple peel in four different ratios. Consequently, the TPA analysis performed on the gummy candies (90), it was noticed that the firmness values of the gummy candies were higher than the samples presented in Table 4.24. Textural properties of the soft gel capsules are mainly affected by the formulation and processing technology is because the soft gels reported in the literature had the different textural properties.

4.5.2 Microbiological Analysis of the Soft Gel Capsules

Microbiological analysis was assessed for 32 weeks period for the samples kept at 4°C. Due to the initially slow microbial growth rate within the first 4 weeks, subsequent analyses were conducted every 2 weeks. The 100 mM EDTA samples at 1:20 ratio showed signs of deterioration after the 28th week, while the stability of other samples was maintained until the end of 32nd weeks.

The soft gel capsules made with 100 mM EDTA at 1:20 ratio, 100 mM EDTA at 1:40 ratio, and pure water at 1:40 ratio presented no *Salmonella* spp. colonies in XLD plates. The initial TMAB counts of the soft gel capsules made with 100 mM EDTA at 1:20 ratio, 100 mM EDTA at 1:40 ratio, and pure water at 1:40 ratio, at the beginning of the microbiological analysis, were 0.89 ± 1.20 , 0.00 ± 0.00 , and 0.84 ± 1.20 log CFU/g, respectively (Table 4.25). TMY counts of the samples in the same order were determined as 0.84 ± 1.20 , 0.00 ± 0.00 , and 0.00 ± 0.00 log CFU/g, consequently (Table 4.26). The mean initial TMY count of the 100 mM EDTA at 1:20 samples (0.84 ± 1.20 log CFU/g) raised to 4.06 ± 0.02 log CFU/g ($p < 0.05$) at the end of the 28th week. The soft gel capsules made with 100 mM EDTA at 1:40 ratio and water at 1:40 ratio had the mean initial TMY count of 0.00 ± 0.00 log CFU/g, and this number raised to 3.79 ± 0.00 and 3.61 ± 0.03 log CFU/g at the end of

the 32-week storage, respectively (Table 4.25-4.26). The duration of storage significantly influenced the escalation in both TMAB and TMY counts ($p < 0.05$).

Table 4.25. Counting the TMAB in soft gel capsules stored at 4°C.

Total mesophilic aerobic bacteria count (log CFU/g)			
Weeks	100 mM EDTA 1:20	100 mM EDTA 1:40	Water 1:40
0	0.89±1.20 ^{Abc}	0.00±0.00 ^{Bc}	0.84±1.20 ^{AcD}
1	0.00±0.00 ^{Ac}	0.00±0.00 ^{Ac}	0.00±0.00 ^{Ad}
2	0.00±0.00 ^{Ac}	0.00±0.00 ^{Ac}	0.00±0.00 ^{Ad}
3	0.00±0.00 ^{Ac}	0.00±0.00 ^{Ac}	0.00±0.00 ^{Ad}
4	1.15±1.63 ^{Abc}	1.37±1.94 ^{Abc}	1.15±1.63 ^{Abcd}
6	1.15±1.63 ^{Abc}	0.00±0.00 ^{Ac}	0.00±0.00 ^{Ad}
8	2.30±0.00 ^{Aabc}	0.00±0.00 ^{Ac}	0.00±0.00 ^{Ad}
10	0.15±0.21 ^{Ac}	0.00±0.00 ^{Ac}	0.00±0.00 ^{Ad}
12	2.00±0.00 ^{Aabc}	2.00±0.00 ^{Aabc}	2.00±0.00 ^{Aabcd}
14	2.38±0.55 ^{Aabc}	2.38±0.55 ^{Aab}	2.45±0.63 ^{Aabc}
16	3.11±0.47 ^{Aab}	2.80±0.70 ^{Aab}	2.57±0.81 ^{Aabc}
18	3.33±0.54 ^{Aab}	3.36±0.40 ^{Aab}	2.62±0.88 ^{Aabc}
20	3.86±0.00 ^{Aa}	3.38±0.03 ^{Ba}	3.38±0.06 ^{Bab}
22	3.94±0.00 ^{Aa}	3.59±0.09 ^{Ba}	3.48±0.03 ^{Ba}
24	3.99±0.00 ^{Aa}	3.69±0.07 ^{Ba}	3.61±0.04 ^{Ba}
26	4.05±0.02 ^{Aa}	3.74±0.07 ^{Ba}	3.67±0.03 ^{Ba}
28	3.96±0.03 ^{Aa}	3.60±0.12 ^{Ba}	3.62±0.03 ^{Ba}
30	-	3.92±0.04 ^{Aa}	3.89±0.01 ^{Aa}
32	-	3.99±0.01 ^{Aa}	3.91±0.02 ^{Ba}

*Data in the similar column with distinct small letter and data in the similar row with distinct capital letter are considerably different ($p \leq 0.05$).

Table 4.26. Counting the TMY in soft gel capsules stored at 4°C.

Total mold and yeast count (log CFU/g)			
Weeks	100 mM EDTA 1:20	100 mM EDTA 1:40	Water 1:40
0	0.84±1.20 ^{Ac}	0.00±0.00 ^{Ac}	0.00±0.00 ^{Ad}
1	0.84±1.20 ^{Ac}	0.84±1.20 ^{Abc}	1.00±1.41 ^{Ac}
2	0.00±0.00 ^{Ad}	0.00±0.00 ^{Ac}	0.00±0.00 ^{Ad}
3	0.84±1.20 ^{Ac}	0.84±1.20 ^{Abc}	0.00±0.00 ^{Ad}
4	0.00±0.00 ^{Ad}	1.00±1.41 ^{Abc}	1.24±1.75 ^{Abcd}
6	0.00±0.00 ^{Ad}	0.00±0.00 ^{Ac}	0.00±0.00 ^{Ad}
8	2.15±0.21 ^{Aabcd}	0.00±0.00 ^{Bc}	0.00±0.00 ^{Bd}
10	2.00±0.00 ^{Abcd}	0.00±0.00 ^{Bc}	0.00±0.00 ^{Bd}
12	2.30±0.00 ^{Aabc}	2.30±0.00 ^{Aab}	2.30±0.00 ^{Aabcd}
14	3.16±0.02 ^{Aab}	3.16±0.02 ^{Aa}	2.52±0.73 ^{Babc}
16	3.58±0.57 ^{Aab}	3.28±0.01 ^{Aa}	2.52±0.73 ^{Aabc}
18	3.75±0.41 ^{Aab}	3.21±0.18 ^{Aa}	2.90±0.59 ^{Babc}
20	4.03±0.06 ^{Aab}	3.50±0.05 ^{Ba}	3.18±0.09 ^{Cabc}
22	4.10±0.13 ^{Aab}	3.59±0.00 ^{Ba}	3.30±0.07 ^{Cabc}
24	4.17±0.15 ^{Aa}	3.70±0.03 ^{Ba}	3.51±0.09 ^{Cab}
26	4.18±0.14 ^{Aa}	3.75±0.01 ^{Ba}	3.61±0.03 ^{Ca}
28	4.06±0.02 ^{Aab}	3.75±0.01 ^{Ba}	3.61±0.00 ^{Ca}
30	-	3.75±0.01 ^{Aa}	3.63±0.02 ^{Ba}
32	-	3.79±0.00 ^{Aa}	3.61±0.03 ^{Ba}

*Data in the similar column with distinct small letter and data in the similar row with distinct capital letter are considerably different ($p \leq 0.05$).

Vergara et al. (91) prepared gel candy in two distinct formulations utilizing red pitanga fruit in their study. When the storage period completed, both formulations displayed comparable stability and remained appropriate for consumption over the 120-day storage period. The samples demonstrated microbiological stability for 90 days at 25 °C with no observed growth of *Salmonella* spp. Pratiwi et al. (90) reported that TMY counts in sugars of pineapple peel extract ranged from 0.08×10^1 to 0.31×10^1 CFU/g. A decrease in yeast and mold counts was observed through the microbiological analysis due to organic acids in the pineapple peel.

4.5.3 Sensory Analyses of the Soft Gel Capsules

Soft gel capsules prepared with 100 mM EDTA at 1:20 ratio and 100 mM EDTA at 1:40 ratio exhibited comparable sensory scores with no considerable differences noticed between these two samples in terms of color, shape, odor, taste, texture, and aftertaste. However, the sensory properties of the soft gel capsules prepared with pure water at 1:40 ratio were considerably lower sensory characteristics compared to the soft gel capsules made with 100 mM EDTA at 1:20 ratio and 100 mM EDTA at 1:40 ratio ($p < 0.05$). The main reason why soft gel capsules prepared with water sample had lower sensory scores in terms of color, odor, taste and aftertaste; it may be food-grade colorants and flavors were not added to the gel prepared for the water sample during the gel preparation stage (Table 4.27, Figure 4.7).

Table 4.27. Assessing the sensory characteristics of the soft gel capsules.

	100 mM EDTA 1:20	100 mM EDTA 1:40	Water 1:40
Color	8.63±0.92 ^a	8.63±0.92 ^a	5.50±1.93 ^b
Shape	8.38±1.30 ^a	8.38±1.30 ^a	7.88±1.64 ^a
Odor	8.75±1.39 ^a	8.63±1.39 ^a	3.88±1.64 ^b
Taste	7.63±1.41 ^a	7.88±1.41 ^a	4.25±1.67 ^b
Texture	8.50±1.31 ^a	8.50±1.31 ^a	7.50±2.51 ^a
Aftertaste	8.25±1.28 ^a	8.13±1.28 ^a	4.75±1.28 ^b

*Data in the same column with different small letter are considerably different ($p \leq 0.05$).

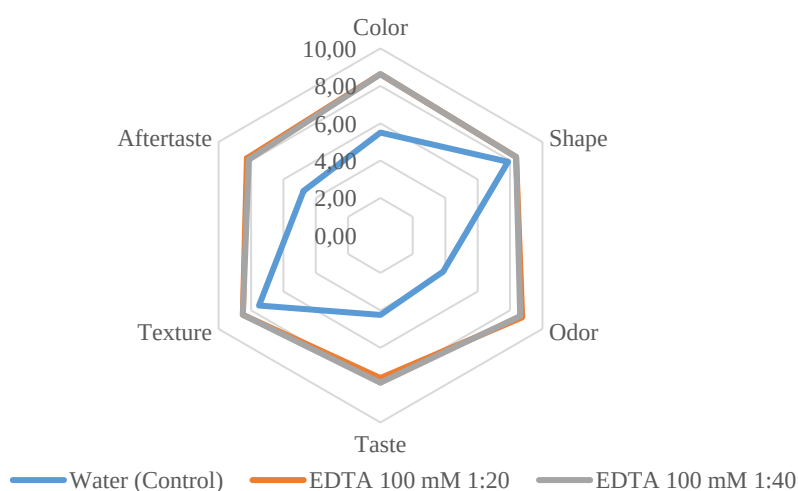


Figure 4.7. Sensory properties of soft gel capsule

It was revealed by the previous studies that sensory and textural properties of the jelly type confectionary products are influenced by both the concentration and the ratio of gelatin, sugar and inulin. Increase in gelatin increased causes raise in hardness and decline in liking. Moreover, raise in sugar concentration provided an increase in liking level, and in fact, overall liking was highest when the amount of inulin and gelatin was low and the amount of sugar was high (92).



5. CONCLUSIONS AND RECOMMENDATIONS

Egg production has raised by almost 150% in the last 30 years, with Asia seeing the biggest growth, with production quadrupling (96). Both domestically and industrially, these enormous quantities of eggs are used to produce and process food as a source of nourishment. Increased egg production will result in a larger volume of eggshell trash, which is often thrown away and dumped in landfills.

However, disposal of egg wastes has become difficult over time due to bad odor and increased bacterial load. Like other wastes, food wastes pose not only odor and inhibition problems to our environment, but also overheating and negatively affecting global warming. Various studies and projects have started to be carried out to make food waste recyclable within the scope of zero waste. Even though eggshell with membrane because of high calcium concentration is used in milk tablets as supplement, formulation of lactose free milk to be used by lactose intolerant individuals, bone graft substitute as nano-sheets to reduce surface roughness in dentistry to impair the oral cavity, adsorbent to remove lead, cadmium, and copper, chromium separation from water, heavy metal removal from acid mine drainage. Moreover, eggshell is also utilized in biodiesel production, cement formulations, biosorbent and/or adsorbent in dye industry. Rigid polyurethane foam composite and low-density polyethylene development are also realized with eggshell. The most common use of egg shell include as utilization of animal feed or fertilizer for plant (26). These valorization attempts use the whole shell without separation the membrane from the shell is because it is simpler and do not require additional processing step. The part rich with protein which is the ESM, on the other hand, is not fully valorized if it is used with the shell.

The sluggish progress in the waste management area made this issue even worse. Food waste can be utilized as a raw material to make a variety of goods with additional value because it is a storehouse of different nutrients. Eggshells are among the most common food wastes, and the fact that they are a precious commodity adds to the difficulty of this problem (97). It is imperative to turn garbage into commodities that are useful for sustainable development in this era of ever-increasing attempts to turn waste into wealth. The goal should be to recycle,

reuse, and channel waste materials while focusing our efforts on producing high-value items in order to do that. It is a good idea to look at how well eggshell, such a valuable food, waste may be turned into usable goods (99).

In addition to the high nutritional value of egg white and yolk, the eggshell and the 3-layer eggshell membrane inside the shell are also very valuable in terms of the protein and calcium they contain. The eggshell membrane is a thin fibrous structure located between the shell and the egg white. The structure of the membrane contains I, V and X type collagens, proteoglycans, fibronectin, and glycoproteins. Both the inner and outer membrane forms a semi-permeable membrane consisting of protein fibers. Therefore, ESM is insoluble in water and has a semi-permeable structure. ESM contains up to 10% of structural components, mainly collagen (types I, V and X). Approximately 70-75% of collagen consists of secondary protein components such as keratin, osteopontin, proteoglycans and glycoproteins (15).

ESM also contains glycosaminoglycans and sulfated glycoproteins. Other components are HA, ovotransferrin, ovalbumin, ovocalixin-36, desmosin and isodesmosin, lysozyme, lysyl oxidase, and β -N- acetylglucosaminidase. Additionally, there is calcium carbonate at the ends where the outer membrane combines the shell. For this reason, ESM has a very valuable structure as a natural source of collagen, glucosamine, chondroitin, and HA.

Eggshells are considered the primary source of calcium with approximately 38% calcium content. In addition, eggshell is a substance that resembles the mineral components found in bones and teeth due to its chemical structure. The use of ESM is limited by its tight binding to the shell and its separation due to its solubility. The solubility of a protein is a crucial characteristic for its utilization as protein supplements in the food industry. The eggshell membrane is rich in disulfide bonds both between and within protein molecules, featuring cross-linkages of lysine derivatives and heterochain chains making the membrane very difficult to dissolve (93). Thus, the present study was designed to determine the effective procedure to separate ESM from the ES and solubilization of ESM protein in addition to characterization of soluble ESM proteins by means of its protein concentration, amino acid content and protein fractions, functional, textural and thermal

properties. Moreover, utilization of ESM in soft gel capsules with determination of sensory properties, and microbial load was performed.

In order to determine the most efficient method to separate ESM from the shell, different treatments including ultrasonication, crushing the eggshell and then separating ESM from the shell by separation in the air stream, manual separation and different chemicals in various concentrations were tested. After careful considerations, chemical separation method including acetic acid, HCl and EDTA was used to separate the membranes in eggshells. Acetic acid, HCl, EDTA, and water were prepared in 3 different ratios (1:20, 1:30 and 1:40) to determine the most efficient separation method revealing 27 different trials. Turbidity, conductivity, and pH values of the eggshell samples in the above mentioned chemicals were measured. The samples with the greatest crude protein concentrations of $83.36 \pm 0.00\%$, $82.90 \pm 0.00\%$, $81.75 \pm 0.00\%$, $80.42 \pm 0.00\%$, $80.38 \pm 0.00\%$, $80.22 \pm 1.45\%$, and $79.6 \pm 1.98\%$ obtained from the samples prepared with 100 mM EDTA at 1:20 ratio, water at 1:30 ratio, 50 mM EDTA at 1:20 ratio, 100 mM EDTA at 1:40 ratio, 1.25% acetic acid at 1:40 ratio, 1.25% acetic acid 1:30 ratio, and 0.185% HCl at 1:30 ratio, respectively were selected and the rest were eliminated for further studies. Selected ESM samples with higher protein concentrations were solubilized by using 0.1%, 0.5% and 1% SDS and 0.1%, 0.5% and 1% Triton X-100 (T) detergents and their protein concentrations were assessed spectrophotometrically using the Lowry method at 0, 30, 60, 90, 120, and 1440 min. The washing procedure was applied to remove lysis solution and effect of washing on protein content of the ESM samples was re-measured. As a results of the protein analyses, the 100 mM EDTA at 1:20 ratio, 100 mM EDTA at 1:40 ratio, and water at 1:40 ratio samples revealing the greatest soluble protein concentrations were selected for further analyses. These selected samples were analyzed to determine their functional, textural, and thermal properties in addition to their protein fractions and amino acid contents.

All three soluble ESM samples showed moderate WHC and OHC allowing them to use in food formulations. Although the samples had no foaming ability, they exhibited quite good EAI ability. Thermal characteristics of the samples indicated that the water extracts exhibited the greatest onset temperature of 101.22 ± 0.01 °C; while the 100 mM EDTA samples at 1:20 ratio showed the minimum onset

temperature value of 84.60 ± 62.40 °C. Heat values varied across the samples ranging from -1.70 ± 0.01 kJ/g for 100 mM EDTA samples at 1:20 ratio to -1.47 ± 0.01 kJ/g for water samples with distinct exothermic peaks.

Rheological analyses of the samples revealed a high correlation with Bingham, Herschley-Bulkley, and Newtonian models. All samples showed Newtonian type flow as their shear stress increased in parallel to increased shear rate.

Color characteristics of the soluble ESM proteins showed variations in terms of L^* , a^* and b^* values. It was revealed that color properties were influenced by the treatments applied for solubilization as the samples treated with EDTA were significantly different from the samples prepared with water.

FT-IR spectrums of the samples were similar to each other having the first peak between $3200\text{--}3500$ cm^{-1} , the second peak around $2200\text{--}2400$ cm^{-1} , and the third peak around $1600\text{--}1700$ cm^{-1} . Generally, FT-IR spectrum of the samples were similar to each other but their intensities changed among the samples.

Amino acid analyses revealed that the solubilized ESM proteins contains 16 distinct amino acids including some essential amino acids. Major proteins of avidin, ovalbumin, ovomucoid, ovoglycoprotein, ovocleidin, and lysozyme were determined by SDS-PAGE analysis; whereas arginine, alanine, valine, methionine, glycine, threonine, leucine, isoleucine, proline, serine, aspartic acid, phenylalanine, lysine, glutamic acid, histidine, and tyrosine amino acids were determined by HPLC analysis in soluble ESM samples.

In order to utilize soluble ESM as protein supplements, a soft gel formulation was developed. All soft gel capsules contained 1.45 mg/g of ESM protein. After production, soft gel capsules were subjected to sensory analyses based on 9-point hedonic descriptive test. While the soft gel capsules prepared with EDTA 1:20 and 1:40 samples received high sensory scores, the soft gel capsules prepared with water 1:40 sample received low scores from the sensory panel.

In order to determine the microbiological stability of the soft gel capsules, they were kept at $+4^\circ\text{C}$ for 32 weeks, and the TMY and TMAB counts of the gel capsules were less than 4.0 log CFU/g after 8-month of storage at refrigeration temperature.

The microbiological stability of the soft gel capsules was equal or longer than that of the similar capsules sold in markets.

Textural properties of the soft gel capsules were measured by means of both firmness and springiness. Firmness of the capsule samples changed from 169.58 ± 24.40 to 388.60 ± 60.40 whereas springiness of the samples was considerably different from the each other and changed from $68.38 \pm 6.72\%$ to $76.94 \pm 5.31\%$.

This study demonstrates an alternative method to separate ESM from the eggshell, provide solubilization of the ESM so that it can be used as protein supplements, and utilization of the soluble ESM as a protein supplement in the form of soft gel capsules. This is the first and only report involving soluble ESM protein as protein supplement. However, further studies are needed to determine the bioavailability and bioaccessibility of soft gel capsules to better understand the ESM utilization.

Alternative proteins have been gaining attention for various reasons, including environmental sustainability, animal welfare concerns, and health considerations. However, the specific emphasis on eggshell membrane proteins might require more recent information. An increased need for eggshell membrane proteins, it may be due to several factors:

- Nutritional and health benefits: Eggshell membrane proteins may be recognized for their nutritional content and potential health benefits, which could lead to increased consumer demand.

- Sustainable sourcing: If there is a growing awareness of the environmental impact of traditional protein sources, such as meat production, there could be a shift towards alternative proteins, including those derived from eggshell membrane.

- Functional food and nutraceutical trends: The interest in functional foods and nutraceuticals, which offer health benefits beyond basic nutrition, could contribute to the demand for unique protein sources like eggshell membrane proteins.

- Research and innovation: Ongoing research and technological advancements may uncover new applications or methods for utilizing eggshell membrane proteins, further driving their demand.

Thus, this study covering all the aspects of ESM abovementioned focused on the utilization of ESM to reduce waste produced by egg industry with green technologies for the sake of environmental sustainability. This is the first report focusing on utilization of ESM into soft gel capsules as protein supplements to provide alternative protein source with essential amino acids driven from the waste. However, further studies need to focus on bioavailability of the ESM soft gel capsules to determine their effectiveness as an alternative protein source.



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