Extraction of The Major Egg White Proteins- Optimization and Scale-Up

by

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ABSTRACT

Eggs are an excellent source of nutrients. Egg whites are used in food, cosmetic, and biotechnology industries for a plethora of physiological functions that they demonstrate due to their proteins. Although these proteins look promising, they can be utilised only after they have been isolated/extracted from the egg white. The objectives of this study were to develop a procedure for the extraction of the major egg white proteins, lysozyme (LSZ), ovomucin (OVM), ovotransferrin (OVT), and ovalbumin (OVA), and to simplify the developed method in order to execute a step wise scale-up from a lab scale (100 g egg white) to a pilot scale (20 kg egg white). The specific focus of this study was to improve the existing protocols in order to reduce the experimental setup requirement and thus reduce the cost of extraction. This was achieved by optimizing the extraction conditions for LSZ (cation exchange resin based separation), applying the traditional siphon filtration for the separation of resin particle, OVM, and OVT precipitates, and elimination of any heat treatments for OVA purification.

In case of LSZ, the extraction was achieved in a shorter time (180 min), with lesser resin dosage (2 g/100 g egg white) and without any pH buffers. For OVM, a modification of the 2-step method was performed and for the scale up study, siphon filtration was used. OVT was separated by the ammonium sulfate and citric acid salting out method and OVA was obtained as a supernatant at the end of the process and needed no further purification. The siphon filter used was designed indigenously in the lab using food grade transparent vinyl tubing, stainless steel mesh (100 mesh size for resin separation and 400 mesh size for precipitate separation), and waterproof silicone sealing tape to hold the filter in place with the tube opening and avoid any leakages. The proteins extracted in this study had comparable purities and yields than the previously reported methods.

For example, from 100 g egg white, an average yield of 58% for LSZ, 83% for OVM, 75% for OVT, and 99% for OVA were achieved, and the average purities were 92%, 85%, 69%, and 94%, respectively. The process developed in this study is easy to adapt and has a potential for scale-up to an industrial scale as was proven from our stepwise scale up batches of 1kg and 20kg which achieved similar average yields and purities *($p>0.05$)*.

PREFACE

This thesis contains original work by Manjot Singh and has been written as per the guidelines given by the Faculty of Graduate Studies and Research, University of Alberta. The concept of the research work in this thesis originated from my supervisor Dr. Jianping Wu.

This thesis contains five chapters: Chapter 1 provides a general introduction and the objectives of the thesis; Chapter 2 is a literature review that includes the information on egg white proteins, their respective properties and methods of extraction; Chapter 3 reports the optimization of extraction conditions for the major egg white proteins; Chapter 4 involves the stepwise scale-up of the developed extraction protocol using siphon filtration as a separation technique; Chapter 5 provides overall conclusions, significance of this improved extraction protocol for the food industry, limitations of the study and approaches to overcome them.

Dr. Jianping Wu greatly contributed to the experimental design, data interpretation, thesis preparation, and edits. I was responsible for literature search relevant for the above studies, designing and performing laboratory experiments, data collection and analysis, and drafting the manuscript and thesis.

DEDICATION

This thesis is dedicated to my dear parents: Kuldip Singh and Sukhpreet Kaur.

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The past two and a half years have been full of ups and downs, especially due to the pandemic! Everyday we faced new challenges, be it a sudden lab closure due to outbreaks or delay in the delivery of raw materials or unavailability of technical staff. However, even in these tough times, Dr. Jianping Wu kept motivating us in achieving our targets, one day at a time, for which I would like to sincerely thank him. I would also like to thank him for believing in me and giving me the opportunity to undertake research at the University of Alberta.

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ABBREVIATIONS

ACS- American Chemical Society

ANOVA- Analysis of Variance

BCA- Bicinchoninic acid assay

BSA- Bovine serum albumin

CDAB- Cetyl dimethylammonium bromide

CMC- Carboxymethyl cellulose

CPUF- Carrier phase ultrafiltration

EW- Egg white

FPLC - Fast Protein Liquid Chromatography

HPLC - High Performance Liquid Chromatography

ID- Inner diameter

kDa - Kilo Dalton

LSZ- Lysozyme

MW - Molecular Weight

MWCO- Molecular Weight cutoff

NIH- National Institutes of Health

OD- Outer diameter

OVA- Ovalbumin

OVM- Ovomucin

OVT- Ovotransferrin

PEG- Polyethylene glycol

pI - Isoelectric Point

SDS-PAGE - Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

TFF- Tangential Flow Filtration

UF- Ultrafiltration

1. GENERAL INTRODUCTION AND THESIS OBJECTIVES

1.1. General Introduction

Chicken eggs are considered a perfect food due to their ability to sustain life. They have a large-scale consumption worldwide since egg proteins have a complete amino acid profile (Lesnierowski and Stangierski 2018). They also have several other applications. They may be used as a thickener or emulsifier in baking (Wu 2014). Structurally, the three main components of eggs are eggshell, egg white and yolk that make up around 10%, 28% and 60% (by weight) of the chicken egg respectively (Mine and Zhang 2013). Ovalbumin (OVA, 54%), ovotransferrin (OVT, 11%), ovomucin (OVM, 3.5%), and lysozyme (LSZ, 3.4%) are the major proteins of egg white, by weight; these proteins have many uses in foods, dietary supplements, vaccines, pharmaceuticals, antibacterial agents, and so on, after they are isolated from egg white (Wu 2019; Mann and Ndung'u 2020).

Ovalbumin, (OVA) was one of the first proteins to be extracted in its pure form from egg white by salting out, using large quantities of ammonium sulfate and acetic acid (Chick and Martin 1913). It is a phosphoglycoprotein comprising of 385 amino acid residues with a molecular weight of 45 kDa (Nisbet et al. 1981). OVA has been widely used for emulsification as well as for its nutritional properties (Mine et al. 1991). Many different methods are employed for the extraction of OVA like precipitation by salting out/solvent, at isoelectric pH, and liquid chromatography (Sorensen and Hoyrup 1915; Croguennec et al. 2000). These methods have been optimized over the years and the most common method is salting out using ammonium sulfate and citric acid (Abeyrathne et al. 2014). The precipitate (OVT) is separated out and the supernatant is heated to precipitate the impurities; the purified supernatant thus obtained is OVA (Abeyrathne et al. 2014). The yields for

small-scale (280g EW) and large-scale (3625g EW) OVA extractions were found to be 98% and 99% respectively, however, the purity reduced from 94% to 88% with scale up.

Ovotransferrin (OVT) from egg, just like lactoferrin from milk, is a member of the transferrin family of proteins (Superti et al. 2007). This egg white glycoprotein transfers ferric ions from the hen oviduct to the developing embryo. This protein is 40-70% homologous to mammalian (serum) transferrin and lactoferrin when comparing their domains (lobes) (Mazurier et al. 1983). OVT comprises of 686 amino acid residues and has a molecular weight of 76 kDa. It exists in 2 forms, namely Apo Ovotransferrin (iron free) and Holo Ovotransferrin (iron bound) where the Apo form is less stable and is easily destroyed by chemical and physical treatments as compared to the Holo form (Azari and Feeney 1958). The earliest method of OVT extraction from egg white suggests precipitation of OVA using saturated ammonium sulfate at pH 3 and then adding sodium chloride and ethanol to the supernatant to induce crystallization and recrystallization to obtain pure OVT (Warner and Weber 1951). Over the years, different methods of extraction have been proposed like ion exchange chromatography, ethanol precipitation, chemical precipitation and so on. Of these, ammonium sulfate citric acid precipitation is the most recent and widely used method for extraction of large quantities of OVT (Abeyrathne et al. 2014). In this method OVT is obtained as a precipitate and OVA as supernatant, as explained earlier for OVA.

Ovomucin (OVM) is responsible for the gel like properties of egg white and for maintaining the viscosity and structural integrity of egg albumen (Brooks and Hale 1959). OVM and its various peptides are used for their biological and nutraceutical properties. Depending on its form, OVM has varying molecular weight that ranges from 163 kDa (single monomer) to 23000 kDa (insoluble

OVM) (Lanni et al. 1949; Donovan et al. 1970; Tominatsu and Donovan 1972; Hayakawa and Sato 1976). Furthermore, OVM has two subunits: α, a mixture of α1 and α2, and β. The α subunit is carbohydrate poor and has around 15% dry matter whereas the β subunit is carbohydrate rich and has around 50% dry matter. The M.W of α subunit is around 200 kDa (α1=150kDa, α2=220kDa) whereas the M.W of β subunit varies between 500-700 kDa (Kato and Sato 1971; Itoh et al. 1987). Due to this, OVM's electrophoretic profile is unique as it shows 3 distinct bands in an SDS PAGE gel for the fractions α1, α2, and β (Itoh et al. 1987). The biggest challenge with OVM is to obtain large quantities of it with high purity as it tends to interact with lysozyme, ovalbumin as well as with ovotransferrin (Kato et al. 1976). However, some methods like the 2 step method by Omana & Wu, 2009 have proven to solve this issue to some extent as OVM with high purity (>90%) can be prepared. OVM extraction methods are broadly classified into 3 categories: chromatography, centrifugation, and precipitation. These methods have been modified or combined to achieve the desired purity and yields.

The name lysozyme means 'lysis' and 'enzyme' (Hartsell 1947). Lysozymes are a class of enzymes that are widespread in different areas of life, including bacteria, phages, and plants, as well as in organs, tissues, and excrement of vertebrates (Van Herreweghe and Michiels 2012). Depending on the source, they may have different molecular weights, however, all of them have the same property of instant lysis of the bacterial cell wall by the action of the enzyme muramidase. Egg white contains lysozyme (LSZ) with a molecular weight of 14.2 kDa. It is one of the lowest molecular weight egg white proteins with an isoelectric point in the range of 10.5-11 (Alderton et al. 1945). It is due to these properties that LSZ is easier to extract as compared to other egg white protein and can be scaled up easily with high purity via ion exchange chromatography using a

suitable resin (Abeyrathne et al. 2013a). Abeyrathne et al. (2014) demonstrated the extraction of LSZ with the resin Amberlite™ FPC3500. The yield of the isolated LSZ was more than 82% and its purity was more than 90%. This resin proved to be more convenient for handling on an industrial scale than the previously used CMC resin (Abeyrathne et al. 2013b). Various other methods of extraction have also been devised like crystallization and precipitation, direct membrane filtration, affinity chromatography, gel filtration, and ultrafiltration (Leśnierowski and Cegielska-Radziejewska 2012) but their large scale application is limited.

Most of the methods currently employed for the extraction of these proteins have several drawbacks which limits their scalability and ease of operation. This is mainly either due to the use of materials like organic solvents or due to the need for expensive and bulky equipment like chromatography columns, ultracentrifuge units and so on. There are also some processing parameters in the existing methods that are either not optimized or are not necessary in the process. For example, in case of LSZ extraction by Amberlite FPC 3500 resin, a long contact time (24 hrs i.e., overnight) for the resin with egg white solution, a low mixing temperature (4°C), and a high quantity of resin were used (Abeyrathne et al. 2014). All of these increase the cost of operation. Also, the use of centrifuge in case of OVM extraction limits the scalability of the process as it requires a high centrifugal speed of 10000 RPM which is difficult to be achieved on an industrial scale (Sleigh et al. 1973; Kato et al. 1975; Omana and Wu 2009).

This demands for a novel, economical, and robust scheme of separation of the egg white protein precipitates from the supernatant. Vacuum filtration can be one of the plausible solutions but has high operating cost due to pump and maintenance of the filter membranes. Another possible

solution which looks promising but needs to be explored further is siphon filtration as it could help overcome the shortcomings of the earlier methods discussed above. In many African villages where there is no power supply, a tool called 'ceramic siphon filter' is used to remove dirt, bacteria, protozoa and viruses from the water (Barnes et al. 2009). A siphon is any tube (generally 'U' shaped) which causes a liquid to flow upward, above the surface of a reservoir, with no pump, but powered by the fall of the liquid as it flows down the tube under the pull of gravity, then discharging at a level lower than the surface of the reservoir from which it came. Adding a filter of desired porosity at the inlet of the tube makes it a siphon filter. This filter can be modified according to the particles that need to be separated. This method of separation has never been attempted before with protein precipitates.

1.2. Objectives

Many of the published articles/literatures claim the easy scalability of the process but no evidence is available to support these claims. Some articles have scaled up to 3 kg egg white (Abeyrathne et al. 2014) which can be considered small scale, generally 1-5 kg, but not pilot/bulk scale (generally 10 kg and above) (Metz and Data 2015). Hence, we hypothesized that by optimizing the current methods and by employing siphon filtration as a separation technique, we can achieve a pilot level scale up within a laboratory setup, thereby proving the ease, efficiency, and cost effectiveness of our separation scheme and scale-up.

The overall objectives of this study were:

1. To establish a scheme for sequential extraction of egg white proteins namely, Lysozyme, Ovomucin, Ovotransferrin, and Ovalbumin
2. To scale up the extraction

The specific objectives of this thesis research were:

1. To develop a process for the extraction of egg white proteins, that is economical, sustainable, and produces comparable results to the existing methods. This would involve the optimization of certain steps in the current methods using 100 g egg white.
2. To scale up the operation in a step wise manner to ensure that the designed process gives similar yields and purity to the small-scale results, considering factors like the new separation technique 'siphon filtration' and the associated setup requirements for it. The aim was to keep the cost of setup as low as possible thereby reducing the cost of extraction. The extraction process and setup were to be designed in such a way that they are easy to adapt on industrial large-scale operations for which extractions with 1 kg and 20 kg EW were to be conducted in an ordinary lab setup as evidence for easy scale up.

1.3. Chapter Format

There are five chapters in the thesis and a summary of each chapter is given as follows:

Chapter 1 Gives a brief introduction to the egg white proteins and their current extraction methods. The drawbacks of those methods have been highlighted and the plausible solution explained. It also summarizes the working of siphon filter for separation of protein precipitates which has been explained in detail in chapter 4.

Chapter 2 Provides a background study based on the literature review of the previous works on egg white protein extraction.

Chapter 3 Extraction of Egg white proteins- Optimization

This chapter will cover the previous methods of extraction in detail and how those were modified/improved by optimizing the conditions of the process. This will mainly involve the optimization trials of lysozyme and ovomucin along with minor modifications for ovotransferrin and ovalbumin.

Chapter 4 Extraction of egg white proteins- Scale up

After the extraction of egg white proteins on a small scale, the scale up of the operation will be discussed. In this chapter, siphon filtration as a method of separating resin particles, OVM, and OVT aggregates from the supernatants in their respective steps will be uncovered

Chapter 5 Thesis Conclusion

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2. LITERATURE REVIEW

2.1. Eggs

Eggs are a perfect food, containing almost every nutrient essential to sustaining life. They contain everything that a bird needs to initiate and complete all the stages of embryonic development. Chicken eggs are a very common food commodity that we see in kitchens and can be used in various ways. Eggs are one of the few foods that are consumed throughout the world because their proteins have a complete amino acid profile. They may also be used as a thickener or emulsifier in baking (Wu 2014).

Eggs consist of 3 main components: eggshell, egg white and yolk **(Figure 2.1)**. Egg's shell is built of 8000-10,000 pores which ensure that oxygen can penetrate inside, and carbon dioxide and other gases can escape out. The shell represents about 10% of weight of egg and consists of calcium carbonate and calcium phosphate mainly. When the eggshell is cracked open, we see 2 portions – the yellow one is called the yolk whereas the white/transparent portion is egg white (Wu 2014). Yolk represents about 28% of weight of the egg. The fat content of yolk consists primarily of triglycerides, cholesterol, and phospholipid lecithin. The amount of fat and cholesterol as well as the composition of fat is influenced by the diet of the hen. The spermatogenic membrane (VM) is a multi-layered structure that protects and shapes the egg yolk and separates it from the egg white (Mann 2008). Along with chalazae, the VM keeps the egg yolk in the central part of the egg, thus preventing its fusion with the shell membrane (Damaziak et al. 2020). In addition, it acts as a diffusion barrier by transporting water and nutrients between the egg yolk and the egg white. It protects the embryo during the first 96 hours of incubation against the strongly alkaline nature of the egg white (Bellairs et al. 1963; Mann 2008). Whole egg is composed of water (75%), proteins (12%), lipids (12%), and carbohydrates and minerals (Kovacs-Nolan et al. 2005)

2.2. Egg white and its proteins

Egg white predominantly comprises of water (89%) and protein (10%), with the rest consisting of carbohydrates (0.4%), ash (0.5%), and trace amounts of lipids (0.03%) (Kovacs-Nolan et al. 2005). The protein composition of egg white by weight is ovalbumin (OVA, 54%), ovotransferrin (OVT, 11%), ovomucin (OVM, 3.5%), and lysozyme (LSZ, 3.4%) which are the major proteins and therefore, the most studied. The rest of the fractions are minor proteins, ovomucoid (11%), G2 globulin (4.0%), G3 globulin (4.0%), ovoinhibitor (1.5%), ovoflavoprotein (0.8%), ovoglycoprotein (1.0%), ovomacroglobulin (ovostatin) (0.5%), cystatin (0.05%), and avidin (0.05%) (Hacohen et al. 2018). The four major proteins of egg white have numerous applications in the fields of food, nutraceuticals, vaccines, pharmaceuticals, and antimicrobials (Wu 2019). Some of these egg white proteins in their isolated forms also have the potential to prevent the spread of COVID-19 (Mann and Ndung'u 2020).

2.3. Properties and applications of the major egg white proteins

2.3.1. Ovalbumin (OVA)

OVA, one of the first proteins extracted from egg white, is a phosphoglycoprotein with a molecular weight of 45 kDa and comprising 386 amino acid residues (Nisbet et al. 1981). It has four cysteine residues and a single cystine disulphide bridge. When egg white proteins are separated by electrophoresis, three ovalbumin bands appear (Lush 1961); these correspond to the dephosphorylated, mono- and di-phosphorylated forms, and the sites of phosphorylation have been identified as serine residues 68 and 344. In addition, ovalbumin has two further sites of modification: the N-terminus is acetylated, and the carbohydrate moiety is linked through asparagine 292 (Nisbet et al. 1981). An interesting feature of the structure of ovalbumin that

became evident from its sequence is its homology with a group of proteinase inhibitors known as serpins. It was found to have 30% sequence homology with the archetype member of the family α_1-antitrypsin (Hunt and Dayhoff 1980). Most members of the serpin family have what is described as a stressed (S) and a relaxed (R) conformation. Proteolytic cleavage converts them from the S to the R conformation. It is also found that the S and R forms exhibit different heat stabilities. Ovalbumin is also susceptible to proteolysis; when treated with subtilisin it is cleaved at residues 346 and 352, releasing a hexapeptide and the major fragment which comprises 346 amino acid residues (Stevens 1991).

OVA has been widely used for its emulsification as well as for its nutritional properties (Mine et al. 1991). It can be used as a drug carrier, substituting for human blood albumin (Abeyrathne et al. 2013a). OVA was also reported to have tumor necrosis releasing factors, which can apply in tumor suppression (Kovacs-Nolan et al. 2000). Some of the peptides derived from ovalbumin not only showed strong ACE-inhibitory effects but also lowered blood lipid content (Manso et al. 2008). Vasodilation effect (Miguel et al. 2007) was observed from the peptides derived from egg white as well as from ovalbumin (Abeyrathne et al. 2013a). The peptides ovokinin and ovokinin 2 to 7 showed the most prominent ACE- inhibitory activities (Yu et al. 2012; Abeyrathne et al. 2013a).

2.3.2. Ovotransferrin (OVT)

Ovotransferrin, as the name suggests, is a protein that belongs to the transferrin family (Superti et al. 2007). OVT, initially known as conalbumin, comprises of 686 amino acid residues and has a molecular weight of 76 kDa (Williams 1968). It is a single glycopeptide chain that is folded into two globular lobes linked by an alpha helix structure (Lee et al. 1980; Mazurier et al. 1983). This protein is 40-70% homologous to mammalian (serum) transferrin and lactoferrin when comparing

their domains (lobes) (Mazurier et al. 1983). Each lobe has the capability to reversibly bind one Fe^{3+} ion concomitantly with one bicarbonate anion (Dorland et al. 1979). The order of iron binding is pH dependent; at pH 6.0 it binds first to the C-domain, but at pH 8.5 it first binds to the N-domain (Stevens 1991). OVT exists in 2 forms namely, apo-ovotransferrin (iron free) and holo-ovotransferrin (iron bound) where the Apo form is less stable and is easily destroyed by chemical and physical treatments as compared to Holo form (Azari and Feeney 1958)

Due to its iron binding property, OVT deprives the iron necessary for the growth of microorganisms and hence has been used in many food items as an antimicrobial ingredient (particularly, against gram negative bacteria) (Alderton et al. 1946; Bullen et al. 1978; Del Giacco et al. 1985). In the presence of a carbonate ion or by pH increase, this antimicrobial activity of OVT further elevates (Valenti et al. 1981; MacGillivray et al. 1998; Baron et al. 2014). Both the forms of OVT (apo as well as holo) exhibit antimicrobial/antibacterial effect. OVT is also a O_2^- scavenging molecule as it demonstrates superoxide dismutase (SOD), like superoxide anion scavenging activity, which is promoted by copper/manganese binding as well as with catechin(Ibrahim et al. 2007; You et al. 2014). Reduction-induced auto-cleaved OVT has anticancer properties which is evident from its treatment of human cancers and health benefits in nutraceuticals (Ibrahim and Kiyono 2009). Recently, it was found that OVT can also be used as a drug carrier to specifically target active compounds directly to pathogens, intracellularly infected cells, and even to cancer cells (Ibrahim et al. 2015, 2020).

2.3.3. Ovomucin (OVM)

OVM is a high molecular weight sulfated glycoprotein that confers the gel-like properties to egg white, thus maintaining the viscosity and structural integrity of egg albumen (Omana et al. 2010a).

OVM has a varying molecular weight that depends on its form (Hiidenhovi 2007a). A single unit (monomer) of OVM has a molecular weight of 163 kDa (Donovan et al. 1970) whereas native, soluble, and insoluble OVM have the molecular weights 8000 kDa, 8300 kDa, and 23000 kDa, respectively (Lanni et al. 1949; Tominatsu and Donovan 1972; Hayakawa and Sato 1976). Ultracentrifugation can separate these fractions into precipitate (consisting of the insoluble fraction majorly) and supernatant (consisting of the soluble fraction majorly) (Kato et al. 1970a). Furthermore, OVM has two subunits: α, a mixture of α1 and α2, and β. The α subunit is carbohydrate poor and has around 15% dry matter whereas the β subunit is carbohydrate rich and has around 50% dry matter. The molecular weight of α subunit is around 200 kDa (α1=150kDa, α2=220kDa) whereas the molecular weight of β subunit varies between 500 and 700 kDa (Kato and Sato 1971; Itoh et al. 1987). Due to this, OVM's electrophoretic profile is unique as it shows 3 distinct bands in an SDS-PAGE gel for the fractions α1, α2, and β subunits (Itoh et al. 1987). The carbohydrate content in ovomucin is around 33% and comprises of 10-12% hexosamine, 15% hexose and 2.6-8% sialic acid (Mine 1995). OVM is hardly soluble at neutral pH but can be solubilized in alkaline water by homogenization (blending), sonication or in the presence of dissociating/reducing agents (Robinson and Monsey 1971; Itoh et al. 1987). Sonication however reduces the molecular weight of OVM from 8300 kDa to 230 kDa and the intrinsic viscosity from 365 ml/g to 15 mL/g which could be due to soluble ovomucin dissociating into smaller molecules through disruption of interaction between carbohydrate rich and poor components (Hayakawa and Sato 1976).

As mentioned earlier, OVM is a gelling agent of egg white and is responsible for egg white thinning, the most significant transformation in egg white during storage (Hiidenhovi 2007a). Egg white thinning is mostly due to the degradation of OVM complex (Kato et al. 1971; Robinson and

Monsey 1972). The β subunit as well as certain fragments like 220kDa, 120kDa and highly glycosylated 70kDa of OVM exhibits cytotoxic effect on tumor cells and shows growth inhibiting and cell damaging effects on sarcoma cells; hence, these can be used for their anticancer properties (Yokota et al. 1999; Oguro et al. 2000; Lee and Paik 2019). Due to the presence of sialic acid, OVM and its peptides are anti-inflammatory and have shown to reduce inflammation of skin tissues (Spence et al. 2013; Sun et al. 2016). Ovomucin also showed a strong binding for bovine rotavirus (RV) and influenza virus (IV) through α and β subunits and newcastle disease virus (NDV) through β subunit only, thereby showing the anti-hemagglutination property (Tsuge et al. 1996b, a, 1997). Furthermore, OVM acts as an antibacterial agent as it was reported that ovomucin glycopeptide, due to sialic acid, binds to *Escherichia coli* O157:H7 and could prevent food borne illness. It can also be used as a probe to detect the presence of this pathogen in food (Kobayashi et al. 2004). Lastly, OVM can also help control hypercholesterolemia as it can directly interact with cholesterol mixed micelles in the jejunal epithelia (Nagaoka et al. 2002). Hence, OVM and its various peptides are used for their biological and nutraceutical properties (Omana et al. 2010a).

2.3.4. Lysozyme (LSZ)

Lysozymes are a group of enzymes extensively spread around in various aspects of life like organs, tissues, secretions of vertebrates as well as bacteria, phages, and plants (Van Herreweghe and Michiels 2012). Their molecular weights may differ depending on the source, but they all exhibit the same property of lysing the bacterial cell walls instantly through the action as muramidase, hence the name ‘lyso’ (lysis) and ‘zyme’ (enzyme) (Hartsell 1947). Egg white has LSZ with a molecular weight of 14.2 kDa, one of the lowest molecular weight egg white proteins, and an isoelectric point ranging 10.5-11 (Alderton et al. 1945). Hen lysozyme is a monomeric, secretory protein containing four disulfide bonds: Cys 6-127, 30-115,64-80, and 76-94, which cause high

thermal stability of the enzyme together with six helix regions; its tertiary structure is quite stable in aqueous solutions (Cegielska-Radziejewska et al. 2008; Ibrahim 2018). The lysozyme molecule is ellipsoid with dimensions of 4.5 x 3.0 x 3.0 nm and consists of two domains or lobes, linked by a long α-helix between which lies the active site of the enzyme (Cegielska-Radziejewska et al. 2008; Ibrahim 2018).

LSZ has been used as a food preservative for a while now as well as an active ingredient in antimicrobial food package film more recently, as it facilitates a prolonged storage for plenty of foods including fresh vegetables, meat, fruit, dairy and seafood (Mine et al. 2004; Bayarri et al. 2014; Abdul Manab et al. 2016). LSZ extracted from chicken egg white impedes the growth of *Clostridium tyrobutyricum* spores in cheese (Scott et al. 1987). LSZ has been reported to delay the microbial growth in alcoholic beverages like beer thus enhancing the shelf life and acting as a natural preservative (Makki and Durance 1996). LSZ also has a lot of significance in pharmaceutics as it is extensive used in wound healing, eye drops, anticancer medications, and so on (Sava et al. 1989; Shi et al. 2021; Xiao et al. 2021). The FAO, WHO, and many countries such as Japan, Austria, Australia, Belgium, Denmark, Finland, France, Germany, Italy, Spain, the United Kingdom (Japan being the largest consumer) have recognized the nontoxicity of lysozyme and have permitted its use in some foods as well as in pharmacological and therapeutic applications (Cunningham et al. 1991). It was estimated that more than 100 tonnes of lysozyme per annum is extensively used as a food preservative and also for pharmacological and therapeutic applications (Scott et al. 1987). Clearly, LSZ has various applications in the food industry and hence, efficient extraction schemes of this protein are of importance.

In order to exploit the aforesaid advantages of all the major egg white proteins namely OVA, OVT, OVM, and LSZ as described above, a cost-effective, efficient, and scalable extraction of all these proteins is essential.

2.4. Extraction methods of the major egg white proteins

2.4.1. Lysozyme

There are various sources from which lysozyme can be extracted like saliva, tears, mucus, human milk, and chicken egg white. However, chicken egg white is the most suitable source for the extraction due to its widespread availability. The extraction of lysozyme from this source is difficult, as only about 3.5% of egg white is lysozyme (Shahmohammadi 2018). Various techniques such as precipitation, thermal and reductants treatment (Chang et al., 2000), chromatography, ultrafiltration, magnetic chitin adsorption (Šafařík & Šafarařík ová, 1993), are used in lysozyme purification processes (Shahmohammadi, 2018) **(table 2.1)**.

2.4.1.1. Precipitation

This is one of the oldest, easiest, fastest, and the most economical approach of obtaining lysozyme (Shahmohammadi 2018). The earliest reported method of lysozyme extraction from chicken egg white was by precipitation using acetone and was carried out by Wolff (Meyer et al. 1936). The method involved precipitation of egg white by using colloidal iron, evaporating the supernatant to a lesser volume and precipitating lysozyme with acetone, giving a lysozyme with no nitrogen/sulfur (Meyer et al. 1936; Abraham 1939). The aqueous solution of this precipitate was dialyzed several times to finally obtain a lyophilized white powder, soluble in water, but insoluble

in the common organic solvents (Meyer et al. 1936). This method was further modified by Meyer et al., 1936 who used acetone to precipitate all the egg white proteins except LSZ, and then used flavianic acid to precipitate the LSZ from the supernatant **(Figure 2.2)**. Further, a solvent-free method was designed where the lysozyme could be crystallized directly from the egg white by using a 5% NaCl solution and yields of 60-80% be obtained (Alderton et al. 1945). But this method required a very long crystallization time (72 hours) and a large quantity of salt remained in the egg white along with other proteins. However, crystallization of lysozyme with 5% NaCl at pH 9.5 is still the most classical method of lysozyme preparation with the only drawback of long crystallization time (1 week).

2.4.1.1.1. Selective precipitation using reverse micellar surfactants

LSZ has been selectively precipitated from chicken egg white using reverse micellar anionic surfactant like di-(2-ethylhexyl) sodium sulfosuccinate (Aerosol-OT or AOT). AOT forms an insoluble reverse micellar complex with lysozyme in the core (aqueous phase) and precipitates it from the egg white solution (Shin et al. 2003). The maximum yield of lysozyme from egg white achieved by this method was reported to be around 50% at a pH of around 6 (Shin et al. 2003). This method however required a 50-fold dilution of egg white using a phosphate buffer which makes it expensive and less feasible. A similar method of extraction was demonstrated by Noh & Imm, 2005 but with a cationic surfactant –cetyldimethylammonium bromide (CDAB). A 96% yield of lysozyme from reconstituted freeze-dried egg white was reported by this method at pH 9 and 50 mM CDAB as the optimum conditions. The advantages of extraction of LSZ by this method include high yield, lower time consumption, and the economy of the process (Noh and Imm 2005). However, both methods involve the use of organic chemicals/solvents like isooctane/hexanol/acetone (Shin et al. 2003; Noh and Imm 2005); the effect of these chemicals on

the remaining egg white proteins like OVA, OVM, and OVT that are excluded from the aqueous phase and reside in the organic phase, remain unknown.

2.4.1.1.2. Use of reductants and heat treatment

In a normal scenario, heating egg white above 70°C would induce disulfide-exchange reactions and interactions between sulfhydryl or disulfide and disulfide-containing proteins like lysozyme, ovomucin, and ovomucoid; this would trigger polymerization, denaturation, and gelation of these proteins, resulting in precipitation of lysozyme hence showing its thermal instability (Kato et al. 1975; King 1977; Matsuda et al. 1982; Peng et al. 1982). However, the addition of reductants such as ascorbic acid, cystine, and cysteine in the egg white prevents those reactions to various extents, protects lysozyme from interacting with other proteins, and prevents it from being denatured when heat treated (Chang et al. 2000). In such a case, the other proteins precipitate leaving lysozyme mostly unaffected and in the supernatant phase that can be separated. This was especially seen with the addition of 1% ascorbic acid or 1% cystine where the recoveries were reported to be 78% and 75% respectively (Chang et al. 2000). Using ascorbic acid, the pH of the solution was reported to be around 4.5 at which lysozyme has the maximum thermal stability; hence, the yield was slightly higher in this case due to the acidity it provides to the egg white solution (Cunningham and Lineweaver 1967; Branchu et al. 1999). This method of extracting lysozyme is quick, efficient, and economical. However, it denatures all the other proteins in egg white due to the heat, thereby rendering them unusable.

2.4.1.1.3. Aqueous two-phase system

The aqueous two-phase method is not precipitation per se but a selective separation of lysozyme from other egg white proteins. This method involved the use of 16.1% (w/w) PEG (M.W 6000), 12% (w/w) sodium sulfate, and 0.5M sodium perchlorate to form the aqueous biphasic system which was then used for the partitioning and separation of lysozyme from the mixture of egg white proteins. Egg white was diluted 7.6 times and the entire process was carried out 25°C, pH 10 to extract 70% of the lysozyme from egg white (Su and Chiang 2006). The advantages of using this procedure are that it is environmentally biocompatible and has a low processing time. On the flip side, its drawbacks include difficulties in lysozyme recovery from the upper phase and in scaling up the process due to the use of the PEG system (Shahmohammadi 2018).

2.4.1.1.4. Precipitation of non-lysozyme proteins by ethanol

LSZ can also be obtained from chicken egg white through the precipitation of non-lysozyme protein impurities by incubation in the presence of ethanol (Gemili et al. 2007). Accordingly, it was reported that a partial purification of lysozyme could be achieved using 4–6-h or 6–8-h incubation of diluted egg white in the presence of 30 and 40% ethanol (Gemili et al. 2007). A 6-hour incubation in the presence of 30% ethanol was more suitable for batch lysozyme isolation, whereas that in the presence of 40% ethanol was suitable for continuous process; the respective yields obtained were 88% and 83% (Gemili et al. 2007). The LSZ yields obtained by ethanol precipitation of egg white proteins were higher than those obtained by (a) selective precipitation using AOT surfactant (Shin et al. 2003), (b) thermal precipitation of egg white protein impurities at 70°C in the presence of ascorbic acid used as reductant(Chang et al., 2000), and (c) aqueous 2 phase system (Su and Chiang 2006).

Nevertheless, the use of organic solvents/chemicals and heat treatment are the major drawbacks of these methods. Hence, we need to focus on some other physical/chemical property of egg white lysozyme that would be distinct from other egg white proteins. Two such properties are its high isoelectric point (pI) and low molecular weight; the pI of lysozyme is 10.5-11 which is higher than any other egg white protein and its molecular weight is 14.6 kDa (Strang 1984). The first allows a straightforward approach for the isolation of lysozyme by ion-exchange chromatography in a single step, while the second enables an unambiguous qualitative analysis by SDS-polyacrylamide electrophoresis (Strang 1984).

2.4.1.2. Ion Exchange Chromatography

The earliest available literature on the extraction of lysozyme from egg white using ion exchange chromatography dates back to 1984 where the students were asked to conduct the extraction in the most economical way (Strang 1984). They could either use an anion exchanger (diethylaminoethyl cellulose) to remove all the other proteins or a cation exchanger (Carboxymethyl cellulose) to selectively extract lysozyme. The most economical approach was using carboxymethyl cellulose cation exchanger. This is because it would require lesser resin dosage since lysozyme is only 3.5% of the egg white protein as compared to using a lot of anion exchange resins to remove all the other proteins. A wide range of lysozyme yield was reported (84-100%) and this extraction had 4 purification steps using 0.1M glycine/NaOH buffer, with the last step being the main elution with 0.1M glycine/NaOH buffer and 0.5M NaCl, all at pH 10 (Strang 1984). Considering the economics of the process for extracting lysozyme from 1 egg white, it was reported to cost £7.50 (that would make it £26.25 for 100 g egg white) back in 1983-84. However, over the years, these methods have been modified to further reduce the cost, make the handling of resin easier, and obtain a more

efficient process overall by emphasizing the extraction of other proteins which will be discussed further.

In order to explore the compatibility of the ion exchange for lysozyme extraction, it was attempted with another cation exchanger resin, Duolite C-464, a macroporous weak acid type resin (Li-Chan et al. 1986). A high lysozyme yield in the range of 90-95% was obtained; retention of the whipping and gelling properties in the lysozyme-separated egg white, ease of column operation, and capacity of column utilization were reported (Li-Chan et al. 1986). SDS-PAGE results showed the high purity of the recovered lysozyme and simultaneous recovery of avidin under the eluting conditions. This was the first ever reported automated and continuous process for lysozyme extraction from egg white using Duolite 464 as a cation exchange resin ever reported (Li-Chan et al. 1986).

Lately, there have been attempts to extract all the major egg white proteins with high purity and in least number of steps possible (Guérin-Dubiard et al. 2005; Omana et al. 2010b). Omana et al., 2010a processed the egg white to remove ovomucin first using the 2 step method (Omana and Wu 2009), and then performed anion exchange chromatography of the 100 mM NaCl supernatant fraction from 1st step of ovomucin isolation; they produced a flow-through fraction and three other fractions indicating ovotransferrin, ovalbumin, and flavoproteins. The flow-through fraction was further separated into lysozyme, ovoinhibitor, ovotransferrin, and an unidentified fraction. Chromatographic separation of 500 mM supernatant from 2nd step of ovomucin isolation led to the fractions including lysozyme, ovotransferrin, and ovalbumin. This co-extraction procedure provides a total yield of 71.0% proteins (Omana et al. 2010b).

In the most recent study, lysozyme was separated from 2 × diluted egg white using Amberlite FPC 3500 ion-exchange resin (1 g/10 mL of egg white) (Abeyrathne et al. 2013b, 2014). It was reported that the purity of lysozyme and ovalbumin was > 97% and 87%, respectively, and the yield of

lysozyme and ovalbumin was >88.9% and >97.7%, respectively (Abeyrathne et al. 2013b). This protocol was found to be simple and effective in separating lysozyme and ovalbumin, and the resin proved to be more convenient for handling on an industrial scale in comparison to the previous CMC resin (Abeyrathne et al. 2013b). Finally, a combined scalable extraction was designed for sequential separation method of all the major egg white proteins (LSZ, OVM, OVT, OVA) keeping the extraction protocol for lysozyme the same as described above (Abeyrathne et al. 2014). For both laboratory and scale-up preparations, the yield of lysozyme was >82%. The purity of separated proteins except for ovomucin was >90%. However, this method required a long contact time (24hrs i.e., overnight) for the resin with egg white solution, a lower mixing temperature (4°C), and a higher quantity of resin, all of which increase the cost of operation.

2.4.1.3. Ultrafiltration

The previously mentioned methods have some limitations of scale up either due to the raw materials/chemicals required or expensive setup. Hence, they are more suitable for lab scale or pilot scale operation. Ultrafiltration (UF) process, being a physical separation method, has various advantages including ease of operation, maintainable high efficiency and purity, affordability, and minimum use of chemicals that alter the nature of proteins (Balakrishnan and Agarwal 1996); it is also more easily scalable as compared to chromatography and electrophoresis.

In 1997, in one experiment the fractionation of single, ternary, and natural egg-white solutions was compared. Unmodified and UV modified polysulfone ultrafiltration membranes were used to fractionate ternary mixtures of lysozyme, ovalbumin and conalbumin and natural egg white protein solutions. Salts (NaCl and KCl) were used in the experiment to alter the ionic strength and cause repulsion between proteins and to alter hydrodynamic diameter of proteins. This influenced the retention and permeation of proteins and thus affected the UF based fractionation of proteins

especially from ternary and egg white solutions as observed in their study (lower retention and flux reduction). In comparison with unmodified membrane, the modified membranes had intensified initial water current and more negative zeta potentials. Because of the formation of carboxylate and sulphonate groups, the UV modified membranes were more hydrophilic. It was demonstrated that the behavior of ovalbumin which has the highest amount among other proteins, chiefly specifies the separation characteristics of the mixtures. The results obtained from the fractionation of solutions of ternary model proteins and natural egg whites revealed that in ultrafiltration of the natural egg-white solutions, retention was lower and flux reduction was smaller. Maybe this occurred due to the interactions of proteins or/and salts exists in the natural egg white solutions. Overall, it was concluded that the behavior of proteins in the mixtures was very similar to that of the single protein (Ehsani et al. 1997).

Most of the reported articles have mentioned the separation of lysozyme from single or binary model protein solutions (Balakrishnan and Agarwal 1996; Ehsani et al. 1997). However, some of the recent articles have mentioned the separation of lysozyme from chicken egg white. One of the articles compares the separation of LSZ from chicken egg white using two different membranes- Biomax 30 kDa polyethersulfone and Ultracel Amicon YM using parameter scanning ultrafltration with carrier phase ultrafltration (CPUF). The purity of LSZ after separation from other egg white proteins was found to be higher (>94%) in case of Biomax 30kDa membrane as compared to YM membrane (~85%).

Nevertheless, a latest study on protein fractionation by ultrafiltration has highlighted the clogging issue of the membrane pores (Batrinescu et al. 2020). It was reported that the permeate flow decreased gradually from one cycle to another due to the internal clogging of the membrane when treating aqueous protein solutions like wastewater from fermentation industries. Further, a

decrease in the permeate flux by over 50% was stated for the ultrafiltration of protein solutions with a suspended yeast content and was majorly due to the clogging on the membrane's surface (Batrinescu et al. 2020).

2.4.2. Ovomucin

Ovomucin can be extracted by only a few methods which can be broadly classified into 3 categories: isoelectric precipitation, chromatography, and ultracentrifugation (Hiidenhovi 2007b). Over the years, these methods have been modified or combined to achieve desired goals of scale up and high yield or purity (Omana et al. 2010a) **(table 2.2)**. The most common and widely used method of preparing OVM is by isoelectric precipitation (Hiidenhovi 2007b). In fact, the first method of extracting ovomucin ever reported was by precipitation (Eichholz 1898). Eichholz diluted egg white with distilled water in the ratio 1:4 and separated the mucous layer by centrifugation. This mucous layer thought of containing ovomucin along with other protein impurities was washed several times with distilled water, boiled in rectified spirit, washed in ether, collected and dried, and finally purified ovomucin powder was obtained (Eichholz 1898).

2.4.2.1. Isoelectric precipitation

Various researchers over the years have explored this method with some alterations. In one of the studies, after the removal of lysozyme by crystallization by the procedure of Alderton & Fevold , crude OVM was extracted from lysozyme devoid egg white (MacDonnell et al. 1951). The lysozyme-free supernatant was altered to pH 4.2-4.5 and diluted with 10-15 volumes of water to precipitate the ovomucin, which flocculated and quickly settled (MacDonnell et al. 1951).

The previous method however did not mention the yield/purity of the extraction; so, there was another method reported which was a modification of the method by MacDonnell et al. In this

method by Donovan et al., LSZ was first crystallized and separated, and the supernatant was used for OVM extraction **(Figure 2.3)**.White ovomucin powder with a yield of 425 mg/100 mL egg white was obtained (Donovan et al. 1970).

In another study, precipitation of ovomucin was achieved at a pH other than its isoelectric point of 4.5-4.8. Kato et al. started the process by ultracentrifugation of the thick egg white for 60 min at 59000 x g to obtain a precipitate and a supernatant. The precipitate was washed several times with water, whereas the supernatant was further processed by changing the pH to 6 and then obtaining another precipitate by centrifugation at 1000 x g for 15 min (Kato et al. 1970a).

The extraction of high purity OVM by the previously mentioned protocols had always been a challenge due to the interactions of OVM with LSZ and OVA which are coprecipitated along with it as the major impurities (Donovan et al. 1970; Kato et al. 1970a; Robinson and Monsey 1971). The viscous OVM precipitate obtained by the previously mentioned protocols, however, has other egg white proteins as impurities which cannot be removed by repeated water and 2% KCl washing procedures (Kato et al. 1970b). It was found that with KCl washing led to a loss of OVM resulting in a lower yield (Robinson and Monsey 1969; Lyndrup 1973) whereas without KCl washing the purity was low (64%) (Hiidenhovi et al. 2002). Over the years, these processes have been modified and recently, a new method of obtaining high purity (>90%) OVM from egg white was reported (Omana and Wu 2009). In this method, egg white was first diluted with 3x volume of 100 mM NaCl solution to weaken the interactions between OVM and the other proteins and then the pH was adjusted to 6; the solution was held overnight at 4°C. The solution was then centrifuged at 15,300 x g for 10 min at 4°C to obtain a precipitate and a supernatant. The precipitate was then re-

suspended in a 500 mM NaCl solution whilst mixing for 4 h followed by overnight sedimentation at 4°C. This solution was then centrifuged at 15,300 x g for 10 min at 4°C and the precipitate thus obtained was lyophilized to obtain OVM powder. This was the only method to have reported a purity >90% along with a yield of 400.02 mg/100 g egg white (Omana and Wu 2009). This method is commonly known as the 2-step method for OVM extraction.

Any isoelectric precipitation method for OVM has a few common steps as mentioned in Figure 2.4 (Hiidenhovi 2007b).

2.4.2.2. Extraction by chromatography

Most of the isoelectric precipitation methods produce OVM with other proteins as impurities which are difficult to remove as discussed earlier; hence, chromatographic methods were explored (Young and Gardner 1972). The first ever extraction of OVM reported by chromatography was by gel filtration chromatography (GFC)(Young and Gardner 1972). Egg white was blended and diluted in 0.85% saline and 10 mL of this solution was applied to 2.5 x 25 cm Sepharose 4B column which had been previously equilibrated with 0.85% saline buffer. Elution was performed using the 0.85% saline buffer at a flow rate of about 50 mL per hour and all the protein in the void volume was collected and stored below 2°C. OVM was eluted out in the first fraction due to its high molecular weight. When sufficient volume of protein was collected, the OVM solution was concentrated by ultrafiltration (Young and Gardner 1972). They reported a sialic acid content of 10.7 mg per 100 mg protein in the OVM sample fraction.

This method was further explored but in most of the studies (Awadé et al. 1994; Hiidenhovi et al. 1999), it involved the use of the organic chemical (potent anionic detergent) sodium dodecyl

sulfate (SDS) (Pincus 2012) along with β-Mercaptoethanol which is a denaturing agent and cleaves the disulfide bonds (Gavini and Parameshwaran 2020). However, one of the studies prepared OVM for further applications where the obtained crude OVM after isoelectric precipitation was dissolved in phosphate buffered saline, which was further passed on to preparative gel filtration chromatography to obtain 97% pure ovomucin (Hiidenhovi et al. 1999)

Nevertheless, gel filtration chromatography was only reported to be used for preparing small quantities of OVM (Young and Gardner 1972) and there exist no evidence for its large-scale production.

2.4.2.3. Ultracentrifugation

Ovomucin has also been extracted by ultracentrifugation of egg white in a dissociating solvent (Sleigh et al. 1973) **(Figure 2.5)**. Although this method is simple and quick, a very low yield was reported. Also, the scale up of such a method would be cumbersome due to the need for a very high centrifugal speed.

The biggest challenge with OVM is obtaining large quantities of it with high purity as it tends to interact with LSZ, OVA, and OVT (Kato et al. 1976). Some methods like the 2 step method by Omana & Wu, as explained previously, have proven to solve this issue by enabling the preparation of OVM with high purity (more than 90%). This method however requires centrifugation at 15,300 x g (10000 RPM) which is not feasible with very large-scale extractions unless done in multiple batches (using high RPM, low fluid capacity centrifuges) or by compromising over RPM which could result in an incomplete separation.

2.4.3. Ovotransferrin

The first ever isolation of OVT from egg white was reported to be done by precipitation (Fraenkel-Conrat and Feeney 1950). OVA was crystallized by forming a saturated ammonium sulfate

solution and separated, and this OVA free supernatant was adjusted to pH 3 using acetic acid to precipitate crude OVT. The crude OVT was then reprecipitated to further purify it (Fraenkel-Conrat and Feeney 1950). However, the product had low purity and its electrophoretic behavior differed from that of the native protein.

Another precipitation method to obtain OVT was by using 50% ethanol in 0.02 M NaCl (Warner and Weber 1951). In this method, OVA was first crystallized and separated out according to the protocol by Sorensen et al., 1915. The OVA free supernatant that had a pH of 4.7 and was slightly less than half saturated with ammonium sulfate was used for OVT extraction. Then, 8 g of ammonium sulfate was added to this solution for every 100 mL of filtrate. The OVT precipitate thus obtained was dialyzed with distilled water. The precipitate was separated out and the supernatant was adjusted to pH 6 and made 0.02 M in NaCl and then 50% ethanol was added slowly to obtain the precipitate. To this mixture was added excess iron in the form of ferric nitrate in 0.1 M sodium citrate, along with an excess of sodium bicarbonate. The mixture containing the small precipitate which developed when the iron was added, was adjusted to pH 9.2 with ammonia. It was exhaustively dialyzed against 0.002 M ammonia at cold temperature and a small yellow precipitate was separated. The supernatant was adjusted to pH 6, made 0.02 M in NaCl, and the ethanol concentration increased till very slight turbidity. The solution was then seeded with crystals and allowed to stand overnight. The crystalline precipitate was stirred with the solution and the ethanol concentration was gradually raised. The pink or red crystals that settled left a slightly colored solution and were dissolved in water and recrystallized twice by the same method (Warner and Weber 1951). A yield of around 44% was reported after three repetitive crystallization steps. This method required large quantities of ethanol and other chemicals and the overall process itself was tedious due to repetitive crystallizations and yet the yield was quite low.

Ion exchange chromatography is the most researched and commonly used method to obtain OVT from egg white and is backed by multiple studies (Rhodes et al. 1958; Azari and Baugh 1967; Al-Mashikiii and Nakai 1987; Chung et al. 1991; Awadé et al. 1994; Vachier et al. 1995; Croguennec et al. 2001; Ko and Ahn 2008; Tankrathok et al. 2009; Omana et al. 2010b) **(table 2.3)**. For instance, one of the studies highlights the extraction of OVT with a high yield (about 80%) using carboxymethyl cellulose (CMC) as a cation exchanger (Azari and Baugh 1967) at pH 4.7 for binding OVT. The advantages of this procedure involve the simple and rapid extraction of OVT from egg-white without involving the preliminary crystallization of other proteins, dialysis, and unnecessary ammonium sulfate fractionation steps for the removal of other proteins as is with the method by Warner and Weber 1951, explained earlier. However, this method uses large quantities of CMC (500 g CMC for 1 L egg white) for the direct isolation of ovotransferrin from egg white (Azari and Baugh 1967).

Over the years, different chromatographic procedures have been developed to separate OVT from other egg white proteins. But most of these methods developed are feasible at a laboratory scale only. For example, a purity of 80% determined by HPLC was obtained using anion exchange chromatography on a Q Sepharose Fast Flow column followed by a second step of CM-Toyopearl 650 M cation exchange chromatography (Tankrathok et al. 2009); however, the yield of OVT by this method was low (21%). In another recent study, an 80% pure OVT was isolated from OVM-free egg white prepared by 100 mM NaCl; the OVM-free sample was passed through an anion-exchange column (Q Sepharose FF column) followed by cation-exchange chromatography (SP Sepharose FF column) (Omana et al. 2010b). However, the yield of OVT by these chromatographic methods is very low and these methods have difficulties in handling and the resins are not appropriately designed for large-scale extraction of OVT.

Ammonium sulfate and citric acid precipitation is a modification of the earlier method and is commonly used for the extraction of large quantities of OVT (Abeyrathne et al. 2014). The process involves a 1:1 dilution of egg white for separation of LSZ by cation exchange chromatography using Amberlite FPC 3500 resin (Abeyrathne et al. 2013b) and then the removal of OVM by isoelectric precipitation (Abeyrathne et al. 2014). This LSZ and OVM free egg white is then used for OVT extraction. 5.0% (wt./vol) ammonium sulfate and 2.5% (wt./vol) citric acid are added to this solution giving a pH 2.90 after addition; the solution is then held at 4°C for 12 h. Crude OVT is obtained as the precipitate and OVA as the supernatant. This crude OVT is further purified by resuspending in distilled water and then reprecipitating by the addition of 2.0% (wt./vol) ammonium sulfate and 1.5% citric acid (wt./vol) combination (final pH 3.35). OVT with high purity (~95%) is achieved with yield ~83% in both lab scale (280 g egg white) and large-scale extractions (3 kg egg white).

2.4.4. Ovalbumin (OVA)

OVA is one of the first proteins to be extracted from egg white in its pure form by salting out using ammonium sulfate and acetic acid (Hopkins 1900; Chick and Martin 1913; Sorensen et al., 1915) or sodium sulfate (Kekwick and Cannan 1936). According to the literature, egg-white was beaten to a froth with an equal amount of saturated ammonium sulphate solution and the mixture was allowed to stand overnight (Hopkins 1900). The precipitated protein was separated from the supernatant and 10% acetic acid (glacial acetic acid diluted to ten times its bulk) was added to the supernatant till a precipitate was obtained. This precipitate (OVA) was separated and held for a few hours for crystallization to occur. Although the reported yield was around 90% (50 g OVA from 1 kg egg white), no purity data is available. Also, these methods use large quantities of ammonium sulfate that leads to a byproduct with a high salt concentration. Moreover, a high purity

is reached by multiplication of the precipitation/solubilization cycles which is usually four to five times for OVA, according to these methods.

Due to the shortcomings of the crystallization/precipitation methods, ion exchange chromatography was explored for the purification and characterization of OVA (Rhodes et al. 1958; Mandeles 1960; Levison et al. 1992; Jacobs et al. 1993; Awade et al. 1994; Vachier et al. 1995). These chromatographic methods had high purities (90-100%). However, these studies had reported very low yields even after OVA being the major egg white protein. For instance, one of the studies describes the extraction of large quantities of OVA using an anion exchanger and that the purity of the extract was around 83%, but the yield was reported to be as low as 41% (Croguennec et al. 2000).

These methods have been optimized in the past and the most common method utilized is the salting out process (Abeyrathne et al. 2014) **(table 2.4)**. As described earlier for OVT extraction using ammonium sulfate and citric acid by the method of Abeyrathne et al., (2014) OVA is obtained from the LSZ, OVM and OVT devoid supernatant. This supernatant is heated at 70°C for 15 minutes to precipitate the impurities and thereby increase the purity of OVA in the supernatant. In this method, the yields for small and large-scale OVA extraction were found to be 98% and 99% respectively. However, the purity reduced from 94% to 88% while scale up, even though a special purification heat treatment was provided (Abeyrathne et al. 2014).

2.5. Conclusion

The aforementioned methods of extraction of the major egg white proteins that are currently used have several drawbacks as explained above. Organic solvents like ethanol used for OVT extraction

can denature some egg white proteins making them unfit for use (Ko and Ahn 2008). Also, organic solvents are flammable and need special care while handling. The current extraction methods require centrifugal separation in multiple steps and sometimes centrifugal speeds like 10000 RPM or even higher, especially for the extraction of OVM (Sleigh et al. 1973; Kato et al. 1975; Omana and Wu 2009). This limits the extraction batch size and increases the equipment cost, operating cost, and ultimately the cost of the finished product. This demands for a novel, economical, and robust idea of separation of the protein precipitates from the supernatant. Vacuum filtration can be one of the plausible solutions but has high operating cost due to pump and maintenance of the filter membranes. Another possible solution which looks promising but needs to be explored further is siphon filtration as it helps overcome most of the shortcomings of the earlier methods discussed above.

In many African villages where there is no power supply, a tool called 'ceramic siphon filter' is used to remove dirt, bacteria, protozoa and viruses from the water (Barnes et al. 2009). A siphon is any tube (generally 'U' shaped) which causes a liquid to flow upward, above the surface of a reservoir, with no pump, but powered by the fall of the liquid as it flows down the tube under the pull of gravity, then discharging at a level lower than the surface of the reservoir from which it came. Adding a filter of desired porosity at the inlet of the tube makes it a siphon filter. This filter can be modified according to the particles that we want to separate. This method of separation has never been attempted before with protein precipitates.

2.6. References

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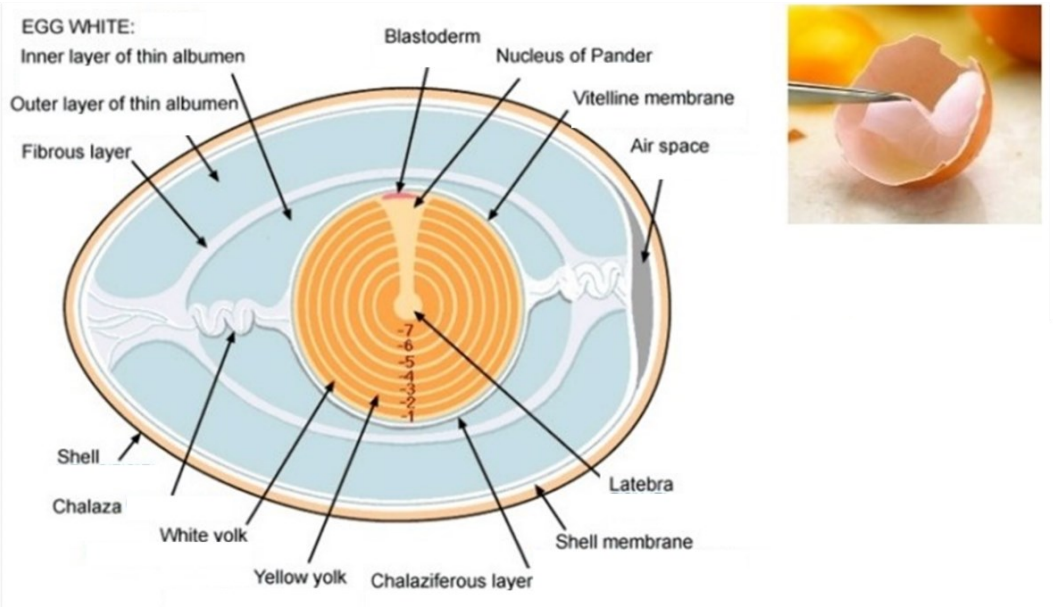

Figure 2.1: Cross Section of Egg (Adegbenjo et al. 2020)

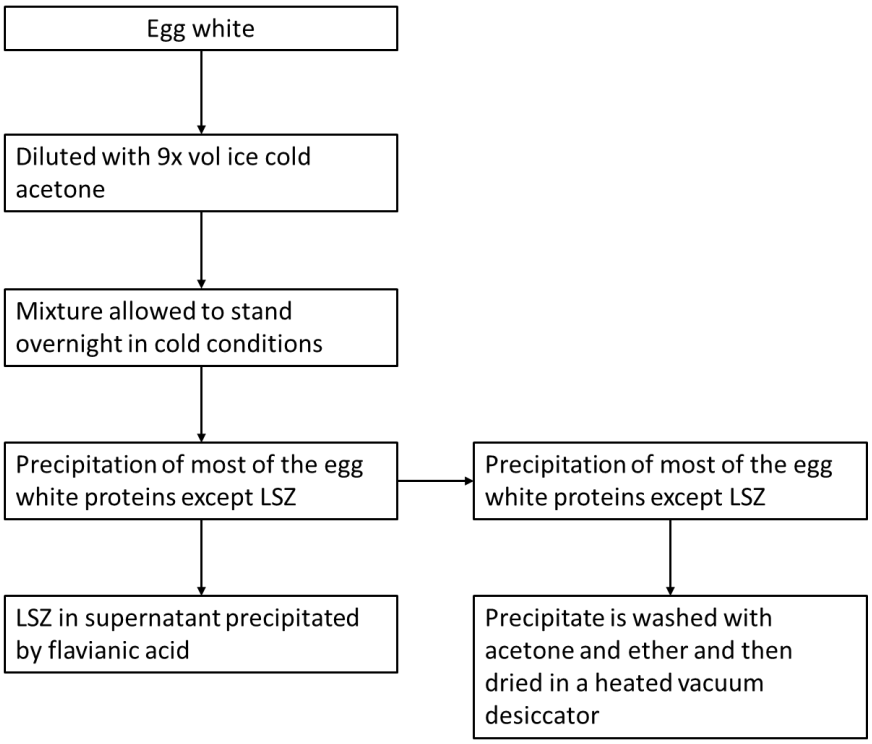

Figure 2.2: Meyer's process for LSZ extraction (Meyer et al. 1936)

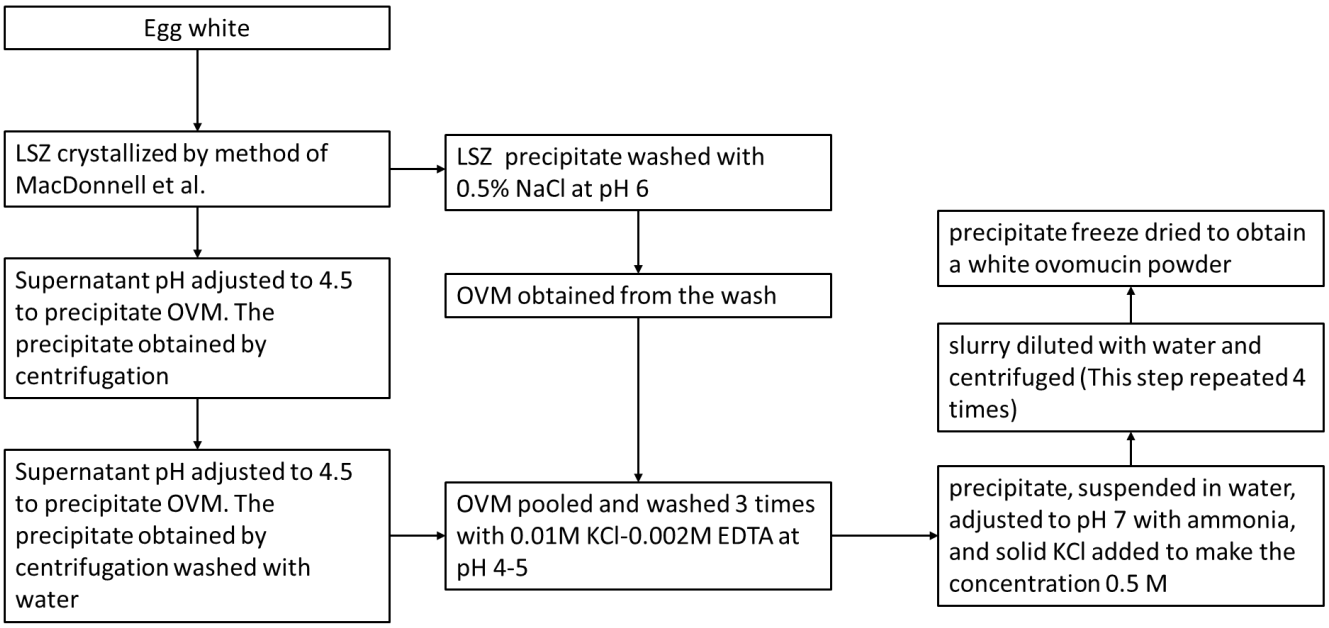

Figure 2.3: Donovan's method for OVM extraction (Donovan et al. 1970)

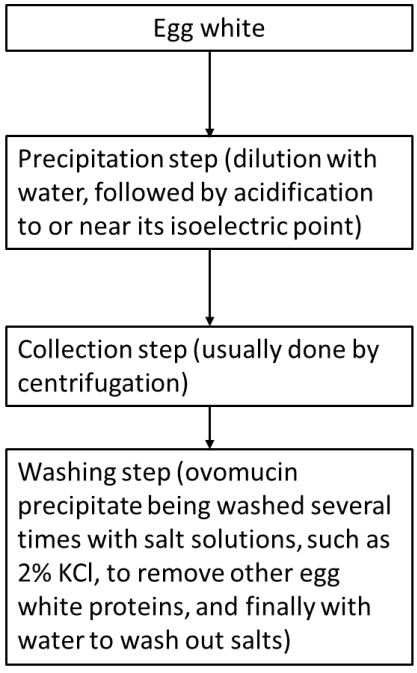

Figure 2.4: General steps for isoelectric precipitation of OVM (Hiidenhovi 2007)

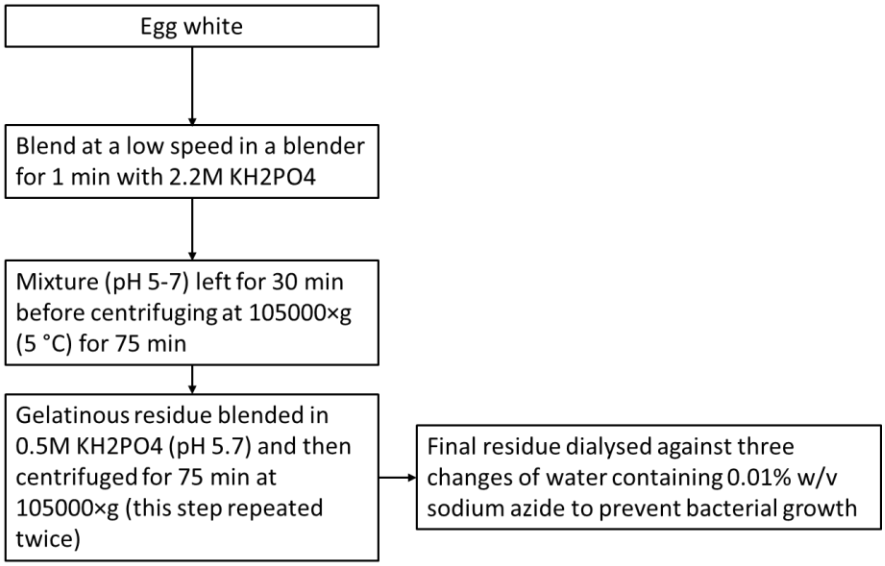

Figure 2.5: OVM separation from egg white using ultra-centrifugation (Sleigh et al. 1973)

Table 2.1: LSZ extraction methods with yields and purity- Summary

Process description	Conditions	Yield	Purity	Costing	References
Reverse micellar surfactant-Cetyldimethylammonium bromide (CDAB)	Sodium borate buffer (50 mM, pH 9, no added KCl) and organic phase containing 50 mM CDAB.	96%	N/A	Low- Simple experimental setup, less time consuming, However, requires organic solvents	(Noh and Imm 2005)
Extraction from egg white using reductants and Thermal Treatment	Reduction conditions- 1.0% ascorbic acid at 70 °C for 5 min.	78%	N/A	Low- Simple experimental setup and raw material requirement	(Chang et al. 2000)
Aqueous two phase separation	16.1% (w/w) PEG (M.W 6000), 12% (w/w) sodium sulfate, and 0.5M sodium perchlorate at 25°C, pH 10	70%	N/A	Low- Simple experimental setup and raw material requirement. However, requires organic solvents	(Su and Chiang 2006)
Precipitation of non-lysozyme proteins by ethanol incubation	6h incubation with 30% ethanol (for batch extraction), 40% ethanol (for continuous extraction)	83% (for batch), 88% (for continuous)	N/A	Low- Simple experimental setup and raw material requirement. However, requires organic solvents	(Gemili et al. 2007)
Cation exchange chromatography using CMC resin	Washing buffer- 0.1M glycine/NaOH buffer (pH 10), Elution buffer- 0.1M glycine/NaOH buffer with 0.5M NaCl (pH 10)	84-100%	N/A	Low- Simple experimental setup and raw material requirement. Also, does not involve any organic solvents and the resin can be regenerated	(Strang 1984)

Cation exchange chromatography using CMC resin- Duolite C-464	Washing buffer- phosphate buffer Elution buffer- phosphate buffer with NaCl or pH based elution	90-95%	N/A	Low- Simple experimental setup and raw material requirement. Also, does not involve any organic solvents and the resin can be regenerated. However, the resin has been discontinued	(Li-Chan et al. 1986)
Cation exchange chromatography using CMC resin- Amberlite FPC 3500	Washing buffer- Distilled water, 0.1 M glycine-NaOH buffer, pH 9.3 Elution buffer- 0.1 M glycine-NaOH buffer, pH 9.3, containing 0.5 M NaCl	88-89%	96%	Low- Simple experimental setup and raw material requirement. Also, does not involve any organic solvents and the resin can be regenerated	(Abeyrathne et al. 2014)

Table 2.2: OVM extraction methods with yields and purity- Summary

Process description	Conditions	Yield	Purity	Costing	References
Earliest reported method (precipitation)	Mucous layer separated by centrifugation, followed by washing with distilled water, boiled in rectified spirit, washed in ether to remove impurities	N/A	N/A	Moderate- Requires centrifuge. However, requires organic solvents	(Eichholz 1898)
Extraction at pH 4.5 by precipitation	After LSZ crystallization, pH is adjusted to 4.5	425mg/100g EW	N/A	Moderate- Requires centrifuge	(Donovan et al. 1970)
Gel permeation chromatography- Superose 6 HR column	Elution buffer- 0.05 M Tris HCl buffer (pH 9) containing 0.2 M NaCl, room temperature	80%	80%	Expensive due to use of chromatography columns	(Awade et al. 1994)
2 step method	Precipitation at pH 6 in the presence of 0.1 M NaCl. Reprecipitation of the 1st precipitate in ddH2O, pH 6 and 0.5M NaCl	400.2mg/100g EW	>90%	Moderate- Requires centrifuge	(Omana and Wu 2009)

Table 2.3: OVT extraction methods with yields and purity- Summary

Process description	Conditions	Yield	Purity	Costing	References
CM-Cellulose column	Step wise elution by pH and ionic strength variation at room temperature	87%	N/A	Expensive due to use of chromatography columns	(Rhodes et al. 1958)
CMC resin packed bed vacuum filtration with adjusted flow rate (vacuum) followed by crystallization	All proteins eluted by citrate buffer. OVT eluted by ammonium sulfate buffer	~95%	N/A	Moderate- Due to the usage of vacuum filtration setup instead of chromatography column	(Rhodes et al. 1958)
Immobilized metal affinity chromatography (IMAC)-Sepharose 6B activated and crosslinked using 1,4-butanediol diglycidyl ether (BGE) and coupled with iminodiacetic acid (IDA)	Half of the column saturated with CuCl2 for binding OVT. Starting buffer and washing buffer for removing unbound proteins- (0.05 M Tris-acetate/0.5 M NaCI, pH 8.2), Elution buffer- 0.05 M acetate-Tris/0.5 M NaCl, pH 4.0. All at room temperature	N/A	94-98%	Expensive due to use of chromatography columns	(Al-Mashikiii and Nakai 1987)
Anion exchange chromatography (Q-Sepharose Fast Flow)	Elution by 0.05 M Tris-HCl 0.02 M NaCl buffer, pH 9.0, room temperature	90%	80%	Expensive due to use of chromatography columns	(Awadé et al. 1994)
Anion exchange chromatography by High-Prep 16/10 column (Q Sepharose FF)	Binding at pH 8 and elution using a gradient 01.4-0.5M NaCl. All at room temperature	80%	100%	Expensive due to use of chromatography columns	(Omana et al. 2010b)
Ammonium sulfate and citric acid based precipitation	OVT is precipitated by salting out and centrifuged at 3400g to separate the precipitate. Performed at 4° C	82-83%	~95%	Low- This was one of the few methods that were less equipment and setup demanding and was scalable.	(Abeyrathne et al. 2014)

Table 2.4: OVA extraction methods with yields and purity- Summary

Process description	Conditions	Yield	Purity	Costing	References
Precipitation and crystallization	Saturated ammonium sulfate and 10% acetic acid followed by crystallization	90%	N/A	Moderate- Requires centrifuge. However, requires organic solvents	(Hopkins 1900)
Anion exchange chromatography- Q Sepharose fast flow	Wash buffer- Tris-HCl 20 mM, pH 8.2 Elution buffer- Tris-HCl 20 mM, pH 8.2 with 0.5 or 0.14 M NaCl	41%	94%	Expensive due to use of chromatography columns	(Croguennec et al. 2001)
Ammonium sulfate and citric acid based precipitation	OVT is precipitated by salting out and centrifuged at 3400g to separate the precipitate. Performed at 4° C. The supernatant that remains after OVT separation is OVA	98-99%	88-94%	Moderate- Requires centrifuge. No organic solvents required	(Abeyrathne et al. 2014)

3. EXTRACTION OF THE MAJOR EGG WHITE PROTEINS- A STUDY ON THE OPTIMIZATION OF LYSOZYME AND OVOMUCIN EXTRACTION

3.1. Introduction

The major proteins of egg white are ovalbumin (OVA, 54%), ovotransferrin (OVT, 11%), ovomucin (OVM, 3.5%), and lysozyme (LSZ, 3.4%), by weight. These proteins have many uses in foods, dietary supplements, vaccines (delivery system), pharmaceuticals, antibacterial agents, preventing the spread of COVID 19 and so on, after they are isolated from egg white (Gómez et al. 2006; Chang et al. 2017; Wu 2019; Mann and Ndung'u 2020).

Egg white lysozyme (LSZ), with a molecular weight of 14.2 kDa, is one of the lowest molecular weight egg white proteins with an isoelectric point in the range of 10.5-11 (Alderton et al. 1945). Due to these properties LSZ is easier to extract as compared to other egg white protein and can be scaled up easily with high purity via ion exchange chromatography using a suitable resin (Abeyrathne et al. 2013a). Abeyrathne et al. (2014) demonstrated the extraction of LSZ with the resin Amberlite™ FPC3500. The yield of the isolated LSZ was more than 82% and its purity was more than 90%. This resin proved to be more convenient for handling on an industrial scale than the previously used CMC resin (Abeyrathne et al. 2013b). However, this method involved a larger quantity of resin (0.5 g/10mL diluted egg white solution), a very long contact time (24hrs or overnight) of resin with the egg white solution, and a low temperature condition, all of which need further optimization. Also, the process involved the use of step wise washing/elution using a buffer which was needed to be investigated further by attempting the same without a buffer (or by using ddH_2O alone in order to avoid the addition of more chemicals to the process).

OVM, with a pI of 4.5-5, has a varying molecular weight that ranges from 163 kDa (single monomer) to 23000 kDa (insoluble OVM) (Lanni et al. 1949; Donovan et al. 1970; Tominatsu and Donovan 1972; Hayakawa and Sato 1976). OVM extraction methods are broadly classified into 3 categories: chromatography, centrifugation, and precipitation. These methods have been modified

or combined to achieve the desired purity and yields. The biggest challenge with OVM is to obtain large quantities with high purity as it tends to interact with LSZ, OVA as well as with OVT (Kato et al. 1976). Nevertheless, methods like the 2 step method by Omana & Wu, 2009 have proven to solve this issue to some extent as OVM with high purity (>90%) can be prepared. This method was further optimized for different conditions like pH, salt concentrations at the second extraction, extraction volume, settling time, centrifugation force and operation temperatures (Wang and Wu 2012). However, this method of extraction was never attempted on lysozyme devoid egg white, and no optimization study is available for the same. Hence, our objective was to investigate if using lysozyme devoid egg white would help improve the purity of the OVM as all the previously reported studies have LSZ as an impurity in the extracted OVM. Also, in this study, the pH conditions, single vs double precipitation step (without salt) and the need for salt (NaCl) for OVM precipitation were examined and compared with the 2 step method by Omana and Wu 2009.

Ovalbumin, (OVA) was one of the first proteins to be extracted in its pure form from egg white by salting out, using large quantities of ammonium sulfate and acetic acid (Chick and Martin 1913). Over the years, many different methods have been developed for the extraction of OVA like precipitation by salting out or by using a solvent, at isoelectric pH, and liquid chromatography (Sorensen and Hoyrup 1915; Croguennec et al. 2000). The most common method is salting out using ammonium sulfate and citric acid (Abeyrathne et al. 2014) where OVT is obtained as a precipitate and OVA as supernatant. This method however requires heating of the supernatant containing OVA in order to precipitate some impurities (Abeyrathne et al. 2014). The yields for small-scale (280g EW) and large-scale (3625g EW) OVA extractions were found to be 98% and 99% respectively, however, the purity reduced from 94% to 88% with scale up. Our study's

objective was to explore this process without the application of any heat. The overall objectives of this study were:

a. To optimize the resin dosage and contact time for LSZ extraction at room temperature
b. To use the LSZ devoid egg white obtained from step a to further optimize the extraction of OVM
c. To use LSZ and OVM devoid egg white for OVT and OVA extraction

3.2. Materials and methods

3.2.1. Materials

Large sized chicken eggs were purchased from a local supermarket and used the very same day. Amberlite FPC 3500 (styrene-divinylbenzene, total exchange capacity >2.6 mEq/g, H^+-form), PYREX™ vacuum filtration flask, and hydrochloric acid were purchased from Fisher Scientific, (Ottawa, Ontario, Canada). Sodium Chloride (ACS reagent, >99.0%) and citric acid (anhydrous) were purchased from Sigma – Aldrich (Oakville, Ontario, Canada). Sodium dodecyl sulfate-polyacrylamide was purchased from Bio-Rad Laboratories, Inc. (Hercules, California, USA). Ammonium sulfate was purchased from Acros Organics (Geel, Belgium). 2-Mercaptoethanol was obtained from MP Biomedicals, LLC (Solon, OH, USA). BCA (Pierce™ BCA Protein Assay Kit) for protein analysis was from Thermofisher (Waltham, Massachusetts, United States). The purity of protein was calculated by converting the density of protein bands in the scanned gel using the ImageJ software (NIH, Bethesda, MD, USA) as the percent of the total gel density. The actual protein content of egg white from the literature (10%) was used to calculate the yields (Stevens 1991). Flash 2000 Organic Elemental analyzer (for nitrogen content), Yamato DKN 810

mechanical convection oven (for dry matter), and Barnstead Thermolyne furnace (for ash content) were used for overall proximate analysis. For desalting/concentrating the samples, Vivaspin 6 (desalting centrifuge tubes-5 and 10kDa), Vivaspin 20 (3kDa, 30kDa) from Sartorius and the Tangential Flow Filtration (TFF) unit from Millipore (Burlington, Massachusetts, USA) were used.

3.2.2. Methods

3.2.2.1. LSZ extraction optimization

100 g egg white was diluted with ddH_2O in the ratio 1:3 and mixed well till the pH stabilized. This solution was used as the starting material for all LSZ extraction optimizations (resin dosage, contact time and washings)

For LSZ resin dosage optimization, Amberlite FPC 3500 weak acid cation exchange resin was added (2, 4, 6, 8, 10, 12g) **(Figure 3.1)** to this solution. pH vs time curves were plotted for each resin dosage over a time of 6 hours to monitor the time course required for the pH to stabilize as this is an indicator of the ion exchange reaction nearing completion (exchange of ions influence pH drop) **(Figure 3.2)**. This time was found to be 180 minutes for all the resin dosages. This was a preliminary analysis for determining the contact time.

Then the experiments were conducted again with different resin dosages (2, 4, 6, 8, 10, 12g) but this time keeping contact time as 180 minutes. After 180 minutes the resin was separated from the remaining solution using vacuum filtration and the resin was washed thrice with 500 mLddH_2O by stirring in a beaker and monitoring the pH of the wash (washing was stopped when pH of

wash=pH of ddH_2O= around pH 5-6). The resin was then mixed with 40 mL 0.8M NaCl elution buffer (volume and molarity based on the number of binding sites on resin), a pH drop was observed, and the mixing was continued till the pH stabilized (approximately 20 minutes for elution and pH stabilization) **(Figure 3.3)**. Equal volumes (0.5 mL) of each of these solutions of 40mL were drawn for BCA and SDS-PAGE analysis. SDS-PAGE was performed using 12% separating gel. For SDS-PAGE analysis, these samples were diluted 1:1 with 2x laemmli buffer and equal 'volumes' of these buffer samples were loaded in the wells to observe band intensity differences (a stronger band intensity would indicate that the 40mL sample had higher protein concentration and vice versa) and hence the trend would be observed. Further proximate analysis was conducted from the desalted (performed using Vivaspin 3kDa MWCO as mentioned in the materials) freeze-dried (40mL) samples. The same starting material (diluted egg white) was used for resin contact time optimization. For contact time optimization, Amberlite FPC 3500 weak acid cation exchange resin was added as per the optimized dosage quantity and allowed to mix for 30, 60, 180 minutes as well as for 24 hours to compare the results with previously reported studies. After the required contact times, the resin was separated from the remaining solution using vacuum filtration and the resin was repeatedly washed with excess ddH_2O (pH 5-6). The resin was then mixed with 40mL 0.8M NaCl elution buffer (volume and molarity based on the number of binding sites on resin), a pH drop was observed, and the mixing was continued till the pH stabilized (**Figure 3.6**). From here, the sampling and analysis were carried out in the same way as described earlier for LSZ resin dosage optimization.

Finally, for determining the number of washes for the resin, the same starting material was used (diluted egg white). Amberlite FPC 3500 weak acid cation exchange resin was added as per the optimized dosage quantity and allowed to mix for the optimized contact time. After the required

contact times, the resin was separated from the remaining solution using vacuum filtration and the resin was washed thrice with ddH_2O and these three washes were analyzed by SDS-PAGE **(Figure 3.9)**. This was done to optimize the number of washes by checking for the presence of impurities (proteins other than LSZ).

3.2.2.2. OVM extraction optimization (using LSZ devoid egg white)

After the extraction of LSZ at the optimized resin dosage and contact time conditions, the LSZ-devoid egg white solutions were used for OVM extraction optimization. Precipitation at different pH (4.75, 6, 7) was carried out, and accordingly, two treatment groups were performed. In the first group, the precipitation was performed only once at this pH, whereas in the second group the precipitation was performed twice (reprecipitation of the 1st precipitate). This reprecipitation was simply done by taking the first precipitate and resuspending it in 10x volume of ddH_2O. The pH was adjusted accordingly, and the solution was allowed to stand overnight for precipitation to occur and the precipitate was separated by centrifugation. Furthermore, the usage of NaCl in 1x and 2x precipitation at the optimized pH was studied **(Figure 3.12 and Figure 3.13)**.

SDS-PAGE and proximate analysis (crude protein/nitrogen content) were conducted to check for the best conditions for extraction of OVM from LSZ devoid egg white.

3.2.2.3. Overall extraction scheme

(See **Figure 3.16** for the overall extraction process flowchart)

Process description- 100g egg white was diluted with ddH_2O in the ratio (1:3) and the initial pH was noted. 2g Amberlite FPC 3500 resin was added and allowed to mix with the diluted egg white solution for 3 hours. After the required contact time, the resin particles were separated by vacuum filtration. The resin was washed thrice with ddH_2O, and the elution was performed by mixing it

with 0.8M NaCl solution till pH stabilization. This solution was then desalted (using Vivaspin 3kDa as explained earlier) and freeze dried to obtain LSZ powder.

The LSZ devoid egg white was then used for OVM extraction. 1.75g NaCl was added to this diluted egg white solution and allowed to mix for 30 min, followed by pH adjustment to 6 (modified 2 step method) (Omana and Wu 2009). This solution was then kept overnight at 4° C and centrifuged at 15,300 x g (10000 RPM) for 10 minutes at 4°C. The OVM pellets thus obtained were resuspended in 10x volume of 0.5M NaCl solution, and the pH was adjusted to 6. This solution was then allowed to stand overnight at 4°C and then centrifuged at 15,300g (10000RPM) for 10 min at 4°C. The purified OVM pellets thus obtained after 2nd precipitation were desalted (Vivaspin 20, 30kDa) and freeze dried to obtain OVM powder.

The LSZ and OVM devoid egg white solution (pH 6) was finally used for the extraction of OVT. Ammonium sulfate and citric acid were added to this solution at 5% wt/vol and 2.5% wt/vol respectively. The solution was stirred continuously, till the pH stabilized at 2.90. The solution was then held overnight at 4°C and then centrifuged at 3400xg, 30 minutes at 4°C. The precipitate (OVT pellets) was separated and resuspended in ddH_2O with 2% wt./vol ammonium sulfate and 1.5% wt/vol citric acid. This solution was held for 24 hours at 4°C and then centrifuged at 3400xg, 30 minutes at 4°C to separate the purified OVT pellets from the supernatant. The OVT pellet solution and OVA solution were desalted (tangential flow filtration unit as mentioned in the materials) and freeze dried to obtain the respective protein samples.

All the samples were analysed by BCA, SDS-PAGE and proximate (crude protein content, dry matter and ash)

3.2.2.4. Purity and yield calculation

The freeze-dried samples were analyzed for crude protein content by BCA analysis as well as proximate analysis using Flash analyzer. The samples were then run for SDS-PAGE to determine the purity of the protein of interest by scanning the band intensity using a gel scanner and the ImageJ software (NIH, Bethesda, MD) as percentage of the total gel density. This purity percentage represented the quantity of the target protein amongst other egg white proteins (impurities) present but did not account for the moisture and ash content. The moisture and ash content in the freeze-dried powder/samples were further measured using the proximate analysis method. The crude protein weight and the purity of the protein were then used to determine the quantity of pure protein extracted from a particular amount of egg white. This quantity was then represented as the yield in two ways – 1. mg or g of pure protein per 100 g of egg white, 2. quantity of pure protein divided by the theoretical quantity of that protein present in egg white to calculate the percentage yield.

3.2.2.5. Statistical Analysis

All the experiments were conducted in triplicates, and the results were expressed as mean value ± standard deviation and analyzed by one-way analysis of variance (ANOVA) with Tukey's Test to determine the significant differences among the means ($p < 0.05$), by Prism and Minitab software.

3.3. Results

3.3.1. LSZ resin dosage optimization

Since pH is used as an indicator of charge stabilization in an ion exchange reaction (Kunin 1958; Cisilotto et al. 2020), the pH for different resin dosages was monitored for an extended contact period of 6 hours. The time required for the pH to stabilize was found to be 180 minutes **(Figure 3.1)**. There was a drastic drop in pH observed for the first 30 minutes after which it began to stabilize. However, minor fluctuations were observed until 120 minutes (data not shown), which stabilized completely after 180 minutes and remained stable for the next 3 hours. This trend was observed for all resin dosages **(Figure 3.2)**. Thus, the resin dosage optimization experiments were performed keeping a constant contact time of 180 minutes. A detailed time optimization experiment was performed later in the study.

The resin dosage experiment was then performed with a constant contact time of 180 minutes and the analyses were performed as described in the methods section above. It was found that as the resin dosage increased, the band intensity decreased significantly ($p<0.05$) **(Figure 3.4 and Table 3.1)** indicating a lesser binding of lysozyme with the resin. This could be due to the competition between the proteins for binding to the resin. The resin dosage was found to influence the pH – as the resin dosage increased, the pH dropped more. The initial pH of egg white was 8.80 and on addition of 2, 4, 6, 8, 10, and 12g resin, the pH dropped to 6.44, 5.70, 5.34, 5.16, 4.87, and 4.80, respectively. For cation exchange resin reaction, the pH of the solution should be lower than the isoelectric point (pI) of the target protein. LSZ has a pI of 10.5 whereas OVT, OVM, and OVA have a pI of 6, 4.7, and 4.5, respectively. This could mean that at higher resin dosages (>2g), as the solution becomes more acidic, OVT becomes positively charged and competes with LSZ to bind to the available resin sites. Hence, a resin dosage of 2g seems suitable as the pH usually drops to around 6.44 keeping only LSZ positively charged for cation exchange while OVT is still

negatively charged and so are other proteins. Furthermore, as the resin dosage increases, the LSZ yield was found to decrease significantly ($p<0.05$) **(Table 3.1 and Figure 3.5)**. The yield from the optimized resin dosage of 2g was 0.203 ± 0.020g per 100g of EW. The band intensities and yields for 10 and 12g resin dosage were found to be extremely low, indicating a poor LSZ binding and hence, these results have been excluded from the table and chart. The optimized resin dosage was found to be lower than that suggested by Abeyrathne et al. (2013b, 2014) which could help in a cost-effective scale-up.

3.3.2. LSZ contact time optimization

This study was conducted to further check the optimum contact time with 2g optimized resin dosage and confirm if our estimated contact time of 180 minutes from the pH-time graph was correct. From the SDS-PAGE gel band intensities **(Figure 3.7 and Table 3.2**), we can see that as the contact time increased from 30 minutes to 180 minutes, the band intensity increased significantly ($p<0.05$). Further, a long contact time of 24 hours was also checked in order to confirm the trend and compare to previously reported results. A lower band intensity and yield indicated that a prolonged contact time of 24 hours does not have a significant effect ($p>0.05$) on the extraction of LSZ as compared to the 30 minutes contact time **(Table 3.2 and Figure 3.8)**. Also, contact times less than 180 minutes have significantly lower yield ($p<0.05$) as compared to that at 180 minutes. Hence, the combination 2g resin dosage and 180 minutes contact time per 100g egg white or 400 mL diluted egg white solution (1 part egg white and 3 parts ddH_2O) was used for further experiments. The yield from the optimized reaction conditions of 2g resin dosage and 180 minutes contact time was 0.203 ± 0.020 g per 100g of EW.

3.3.3. LSZ (resin) wash optimization

Out of the 4 washes performed **(Figure 3.10)**, wash 1 had the most impurities consisting mainly OVA, OVT as well as OVM. Wash 2 and 3 consisted only OVA. There was a significant reduction ($p<0.05$) in the protein (impurities) concentration after each wash **(Table 3.3 and Figure 3.11)**. Wash 4 did not show any detectable protein content either with BCA or by SDS-PAGE and hence has been excluded from the table and chart.

3.3.4. OVM extraction optimization (using LSZ devoid egg white)

The optimization of OVM extraction in this study was done using LSZ-devoid egg white which has never been attempted before. There was no significant difference ($p>0.05$) in the purity of OVM at pH 4.75, 1 step as compared to pH 6, 2 step, hence, the latter was chosen for OVM extraction **(Table 3.4)**. This is because of 2 reasons - 1. The yield of OVM at pH 6, 2-step extraction was significantly higher ($p<0.05$) than the yield at pH 4.75, 1-step extraction; 2. The quantity of HCl required to adjust the pH after LSZ extraction is lesser to adjust it to pH 6 rather than to pH 4.75 since the solution is already at around pH 6.45 after LSZ extraction. Also, the purity of OVM in 2-step extraction increased significantly ($p<0.05$) as compared to 1-step extraction at pH 6 and pH 7 **(Table 3.4)**.

Double precipitation at pH 6 was thereby selected as the preferred condition for extraction as explained above. The requirement for the addition of salt (increasing the ionic concentration of the solution) to reduce the interactions between OVM and other proteins and prevent coprecipitation of other proteins (Donovan et al. 1970; Kato et al. 1970a; Omana and Wu 2009) was tested after this. There was a significant reduction in the LSZ impurity after the addition of NaCl in case of 1 step precipitation at pH 6 ($p<0.05$) **(Figure 3.14 and Table 3.4)**. There was only 1.22 ± 0.17% LSZ in 1 step salt precipitation calculated using the SDS-PAGE gel band intensity method. The

use of salt was also tested by adding it in the 2nd precipitation step keeping the pH 6. This further significantly reduced the impurities due to OVT and LSZ ($p<0.05$), whereas there were still 10.18 ± 0.60% OVA as impurity **(Figure 3.14 and Table 3.4)**. Thus, doing a 2-step salt precipitation at pH 6 using LSZ-devoid egg white enabled us to obtain OVM with high purity (~89%), no LSZ and OVT impurities but only some OVA impurity **(Figure 3.14)**. The yield of OVM was found to be the highest at pH 6, 2 step salt extraction conditions as compared to other extraction conditions **(Table 3.4 and Figure 3.15)**. This yield (305mg/100g EW) was however lesser than that reported by Omana and Wu 2009 which was 400.02mg/100g EW but the purity was comparable and LSZ was not present in our OVM sample by 2 step salt method, as determined by SDS-PAGE scanned gel.

3.3.5. Overall extraction yield and purity

In this study we modified the process established by Abeyrathne et al. 2014, by optimizing various parameters like the cation exchanger resin dosage, contact time of the resin, OVM extraction conditions from LSZ-devoid egg white, and finally, eliminating the heating step for the purification of OVA and obtaining OVT as a precipitate **(Figure 3.16)**. By optimizing the overall extraction scheme, we could achieve a yield of 203mg/100g EW (59% yield) for LSZ, 305mg/100g EW (89% yield) for OVM, 900mg/100g EW (76% yield) for OVT, and 5.35g/100g EW (99% yield) for OVA **(Table 3.5)**. The purity of LSZ, OVM, OVT and OVA were found to be 95%, 89%, 73%, and 93%, respectively, as can be seen from the scanned SDS-PAGE gel **(Figure 3.17)**. Our yields and purity for LSZ were found to be comparable to that by Tankrathok et al. 2009, whereas for OVM the purity (but not yield) was very similar to that by Omana and Wu 2009. The yields for OVA and OVT were very similar to that obtained by Abeyrathne et al. 2013a, 2014. The moisture content of the extracted proteins LSZ, OVM, OVT, and OVA was found to be 10.29%, 2.63%,

4.27%, and 4.54%, respectively whereas the ash content was 9.80%, 7.14%, 6.60%, and 9.46% respectively **(Table 3.5)**.

3.4. Discussion

3.4.1. LSZ extraction

LSZ has an isoelectric point of 10.5 (greater than all the other major egg white proteins), making ion exchange chromatography a very widely used method of extraction (Strang 1984; Li-Chan et al. 1986; Jiang et al. 2001). Many resins like Duolite C-464, phospho cellulose (PC), carboxymethyl cellulose (CMC) and carboxymethyl Sepharose (CMS) have been used in the past for studying the LSZ binding; these resins had the highest capacities for adsorption of LSZ from an aqueous buffer (Li-Chan et al. 1986). Out of these resins, Duolite C-464 was further explored for LSZ extraction from diluted egg white (Li-Chan et al. 1986). The yield was reported to be around 90-95%. However, Li-Chan et al. (1986) reported LSZ contained OVM as an impurity which was not observed in our LSZ samples. This OVM impurity could be due to the LSZ-OVM interaction in the pH range of 7.2-10.4, as a result of the use of alkaline pH buffers in the reported extraction protocol (Powire 1985; Li-Chan et al. 1986). They used alkaline buffer (sodium phosphate buffer) to keep the pH of the resin wash and elution above 7 whereas our method did not involve the use of any alkaline buffers, which could be a possible reason for not having any OVM impurity in our case. We optimized the ddH_2O washing steps in order to increase the purity of LSZ by clearing out the proteins that were weakly binding to the resin. However, we did have OVT as an impurity which could not be eliminated by ddH_2O washing alone. This could mean that some amount of OVT must have bound to the resin after 3 hours contact time due to the final pH

of the solution (pH around 6.40) being close to the isoelectric point of OVT (6.00). The percentage of OVT as an impurity in our LSZ samples was around 5-8%, calculated based on band intensity of the SDS-PAGE scanned gel **(Figure 3.17)**. In certain cases this OVT impurity was not seen (as it was with our resin contact time experiments, **Figure 3.7**) which could be due to the variation in the isoelectric points of OVT which varies between 5.8-6.8 (Warner and Weber 1951). The process by Li-Chan et al. (1986) required a very long extraction time (approx. 10 hours) whereas our process could be accomplished in 5 hours (total time including washing and elution, not the contact time alone), further highlighting the efficiency of the process. This extraction time can be compared to many other studies involving the use of bead column chromatography for protein extractions, and in most scenarios, the bead columns required more time, were high pressure driven and laborious which causes the process to be less efficient (Klein 2000; Zou et al. 2001; Chiu et al. 2007). An alternative to the bead columns could be membrane based chromatography as was investigated by Chiu et al. (2007), however the purity of LSZ extracted from egg white by this method was reported to be very low (around 78%). Also, increasing the flow rate from 1 mL/min to 10 mL/min to speed up the process, it was found that the yield reduced from 81% to 54% (Chiu et al. 2007). Although membranes can be reused many times, similar to the resins in our method that can be regenerated and reused, the membranes however need to be constructed prior to using them and it involves multiple steps further complicating the process design and material requirements. Also, this method involved the use of organic solvent (ethanol) for removing other proteins from egg white except LSZ and OVA, unlike our method. Another study achieved a purity and yield of 87% and 55% respectively by anion exchange chromatography (Tankrathok et al. 2009). This method involved the use of Tris-HCl buffer, pH 9.0, containing 10 mM β-mercaptoethanol (organic compound) for diluting the egg white. Tankrathok et al. (2009) removed

OVM first by centrifugation alone (12000 g, 20 minutes) and then used OVM devoid egg white for LSZ extraction. Since the objective of their study didn't involve the extraction of high purity OVM, it was eliminated in the first step without any optimization, and no data is available on OVM's purity or yield. In one study, high purity and yield OVM was extracted by 2-step salt method and the supernatant was further used for the extraction of LSZ and other proteins (Omana et al. 2010b). This was one of the studies where high purity proteins were extracted, with LSZ purity and yield being 100% and around 70% respectively; however, it involved the use of chromatography column and expensive resins. In our process, since we used salt (NaCl) for high purity OVM extraction, just like the 2 step method by Omana and Wu (2009), this salt would hinder in LSZ – resin binding (which was observed in initial trials with our resin, data not shown here). Hence, the first protein we extracted in our process was LSZ using a relatively inexpensive resin and without packing it in a column. Furthermore, the egg white was diluted with ddH_2O alone. We did not use any buffer solutions or ionic salts in this dilution and hence no altering of pH or ionic strength of the original egg white solution as compared to other methods (Li-Chan et al. 1986; Guérin-Dubiard et al. 2005; Tankrathok et al. 2009; Omana et al. 2010a). Moreover, our process is a more optimized and improved version of the method designed by Abeyrathne et al. (2013b). This is because we are using lower resin quantity per 100g egg white, lesser contact time and have optimized the washing steps in order to demonstrate and ensure the removal of impurities in a step wise manner. Although our yield of LSZ (~60%) was not as high as their study (Abeyrathne et al. 2014), but we could achieve a purity (~95%) comparable to this study as well as some others (Guérin-Dubiard et al. 2005; Tankrathok et al. 2009; Omana et al. 2010b; Luding et al. 2011; Abeyrathne et al. 2014). Our yield of LSZ was comparable to other studies (Tankrathok et al. 2009; Omana et al. 2010b). This lower yield did not increase either by increasing the resin

dosage or contact time as it has been reported in our study **(Figure 3.4, Table 3.1 and Figure 3.7, Table 3.2).**

3.4.2. OVM extraction

There have been a very few studies conducted on OVM extraction and the yield and purity data are limited too. After the removal of LSZ from the egg white, the LSZ-devoid egg white was used for OVM extraction optimization study. This has never been investigated before as most of the articles discussed above had separated OVM first and then other egg white proteins (Guérin-Dubiard et al. 2005; Tankrathok et al. 2009; Omana et al. 2010b; Luding et al. 2011). Since OVM tends to precipitate and clog in the column during chromatography, these studies focused on separating it prior to chromatographic separation of other proteins (Guérin-Dubiard et al. 2005). However, our process does not involve any chromatography column-based separation; hence we had the option to extract OVM after LSZ extraction. Also, since we have first extracted most of the LSZ, it does not appear as a major impurity in our OVM sample, compared to OVM prepared by 2 step salt precipitation (Omana and Wu 2009) **(Figure 3.14)**. Hence we do not require any further purifications like successive water or KCl washings and gel permeation chromatography (Brooks and Hale 1959; Kato et al. 1970a; Hiidenhovi et al. 1999, 2002). However, OVM prepared by adjusting pH and precipitation conditions (lanes 3-9) other than the 2-step salt method (lane10) **(Figure 3.14)** still shows LSZ as an impurity, which could be due to the residual LSZ left in the LYZ-devoid sample; addition of NaCl was reported to reduce the interaction and to improve LSZ extraction from the OVM, thus giving us a high purity OVM (Donovan et al. 1970; Kato et al. 1970a, 1976, 1985; Omana and Wu 2009; Omana et al. 2010b). A wide range of purities have been reported in literature: 65% (Hiidenhovi et al. 2002), 80% (Awade et al. 1994), 82.2% (Abeyrathne et al. 2014), and 97-99% when a combination of isoelectric precipitation and gel filtration

chromatography was applied (Hiidenhovi et al. 2003). However, the latter method involved the use of chromatography column which would limit the scale up potential. Another study also reported s high purity OVM extraction by 2 step precipitation method with the use of salt (100mM NaCl in first step, 500mM NaCl in second step) (Omana and Wu 2009). This was one of the few studies to have such a high purity (94.6%) and yield (400mg/100g egg white) whilst showing a promising potential for scale up. Our study was based on a value of 3.4% OVM in egg white protein which is closer to the value published in recent findings (Stadelman 1999; Rao et al. 2016; Stadelman et al. 2017). Our study, upon using the 2-step method, could achieve a yield of about 86% (326 mg crude protein from 100g egg white) at a purity of 89.77% **(table 3.5)**. Also, in this study we were able to clearly see 3 distinct OVM bands at around 135, 180 and above 245 kDa **(Figure 3.17)**. The molecular weight of α-ovomucin varies from 150 to 220 kDa, whereas β-ovomucin varies from 400 to 523 kDa (Omana et al. 2010a). The numerous bands visible in SDS-PAGE gel could be the products of the OVM subunits due to the reducing action of β-mercaptoethanol on the disulfide bonds during the sample preparation for the SDS-PAGE.

3.4.3. Ovotransferrin and Ovalbumin

After the removal of LSZ and OVM, the major proteins remaining in the supernatant are OVT and OVA. Now, either OVA or OVT can be precipitated and the other one would remain in the supernatant. There have been attempts to precipitate OVA by using large quantities of ammonium sulfate and acetic acid, or sodium sulfate as the salting out agent (Hopkins 1900; Chick and Martin 1913; Sorensen and L. 1915; Kekwick and Cannan 1936). However, these methods required a lot of salt which remains in the supernatant at the end of the process as excess chemical which then needs to be treated. Hence, instead of precipitating OVA, Abeyrathne et al. (2013b), devised a process for salting out OVT using lesser quantity (5% wt/vol) of ammonium sulfate, and adding

(2.5% wt/vol) citric acid for bringing the pH to 2.90 (acidic precipitation) (Abeyrathne et al. 2013b, 2014). This precipitate contained some coprecipitated OVA that needed to be separated. Hence the precipitate was resuspended and reprecipitated using 2% wt/vol ammonium sulfate and 1.5% citric acid combination (final pH 3.35). By this method they were able to achieve a purity and yield of 96.2% and 83.39% for OVT and 94.2% and 98.54% for OVA respectively. However, their method involved the use of heat treatment after the first OVT precipitation step. In their study, they compared in one of the SDS-PAGE gels, the difference in untreated supernatant and heat treated supernatant (Abeyrathne et al. 2014)). Upon evaluating the scanned SDS-PAGE gel results published in their study, it was seen that this heat treatment could be avoided, and the process needed to be attempted and analyzed without heat treatment which we attempted in our study. We found that we could achieve OVA purity of 93.34% as shown in **Figure 3.17**, and yield of 99.61%, which were very comparable to the previously reported studies (Abeyrathne et al. 2013b, 2014). However, for OVT we achieved a purity and yield of 73.63% and 78.27% respectively, lower than that reported in the literature, which could be due to co-precipitation of some of the OVA along with OVT (Abeyrathne et al. 2013b, 2014).

3.5. Conclusions

Various strategies have been used to optimize the extraction protocols and to improve the existing methods. In the present study we focused mainly on optimizing and improving the methods of extraction of LSZ and OVM along with modifying the already existing procedure for OVT and OVA extraction. We could successfully separate these four major egg white proteins and were able to achieve the desired/ hypothesized objectives of our study. However, further insight is needed especially with the extraction of OVT as the purity was found to be lower than that reported

in previous literatures, meanwhile keeping the process design simple and scalable as the goal of optimizing these protocols is to ease the scale up and make the overall process economic and sustainable.

3.6. References

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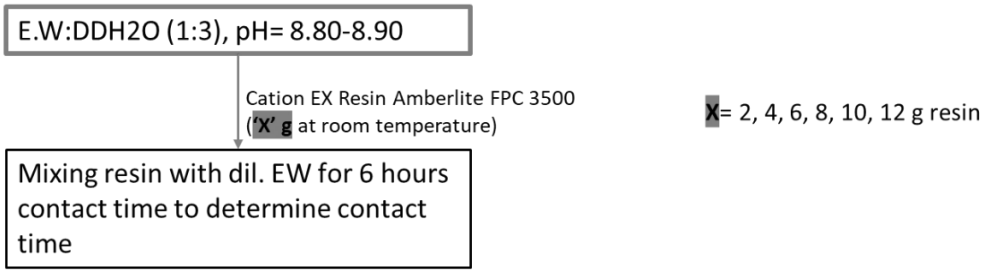

Figure 3.1: Influence of resin dosage on pH

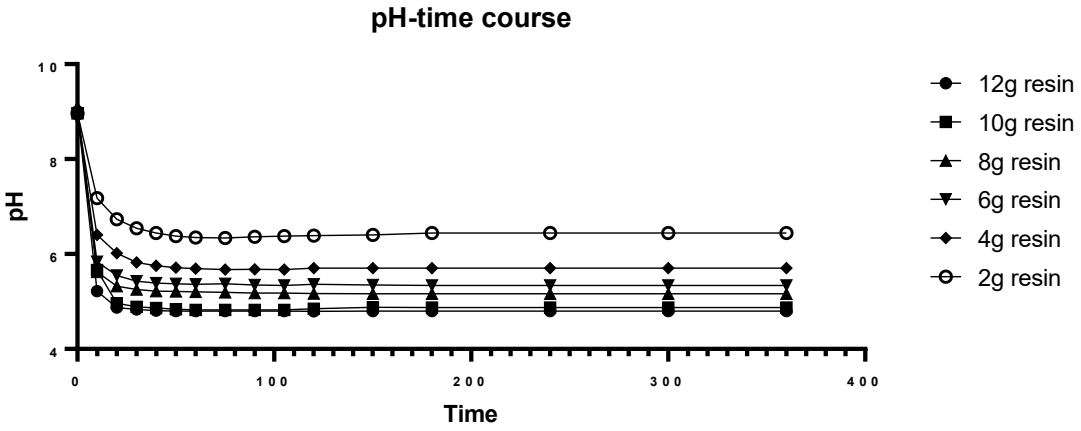

Figure 3.2: pH-time course for different resin dosage

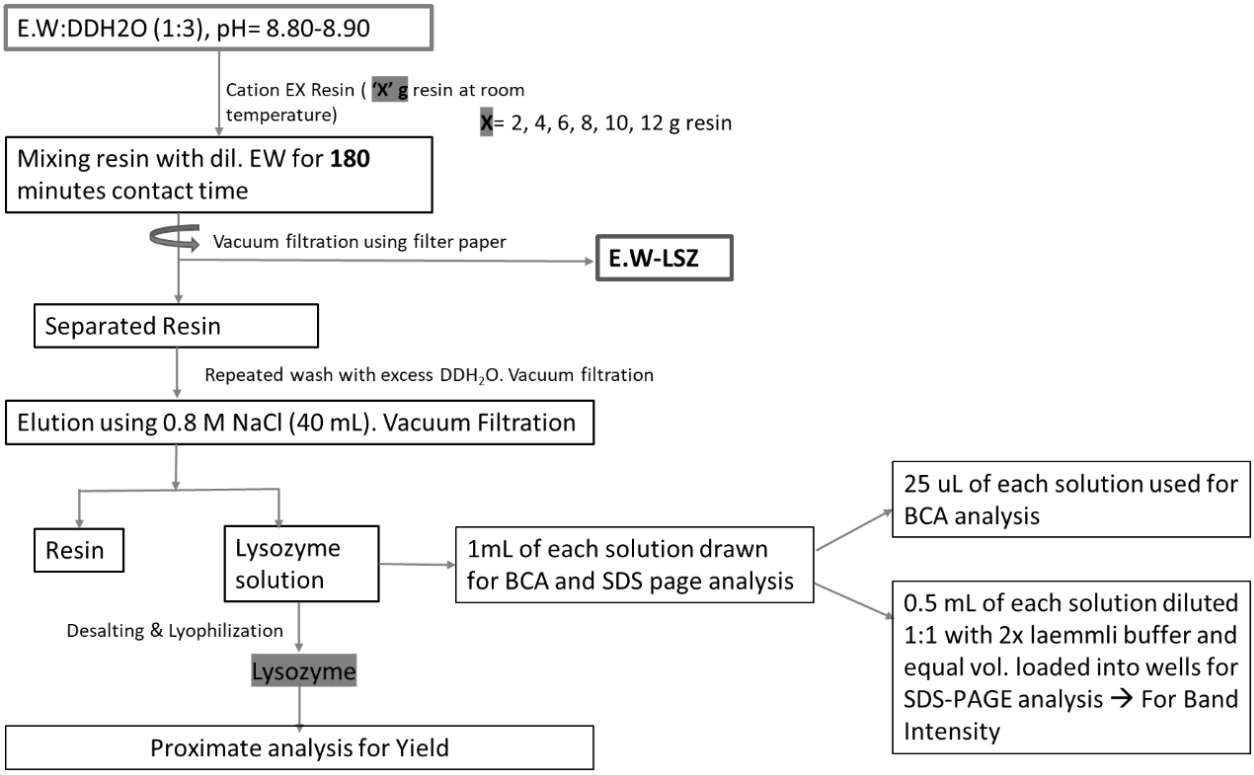

Figure 3.3: LSZ resin dosage optimization process

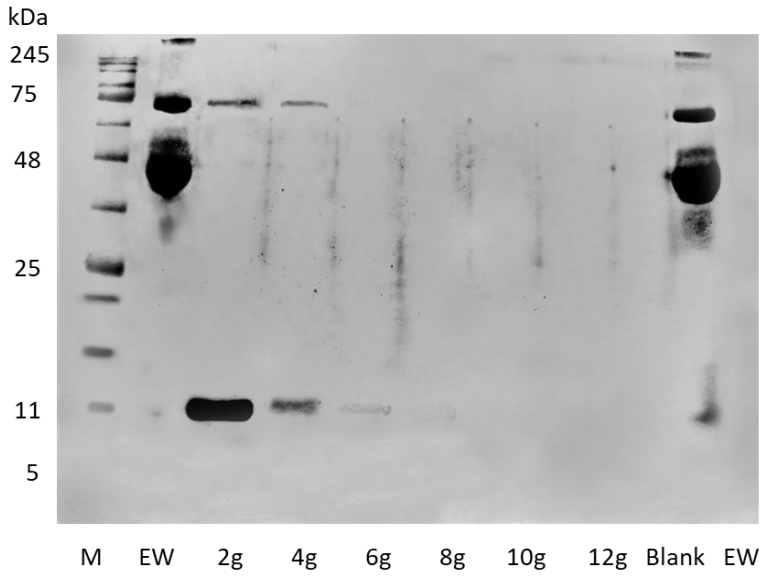

Figure 3.4: SDS-PAGE analysis for resin dosage optimization (lane 1 – marker, lane 2 – egg white, lane 3 – 2g resin dosage, lane 4 – 4g resin dosage, lane 5 – 6g resin dosage, lane 6 – 8g resin dosage, lane 7 – 10g resin dosage, lane 8 – 12g resin dosage, lane 9 – blank, lane 10 – egg white

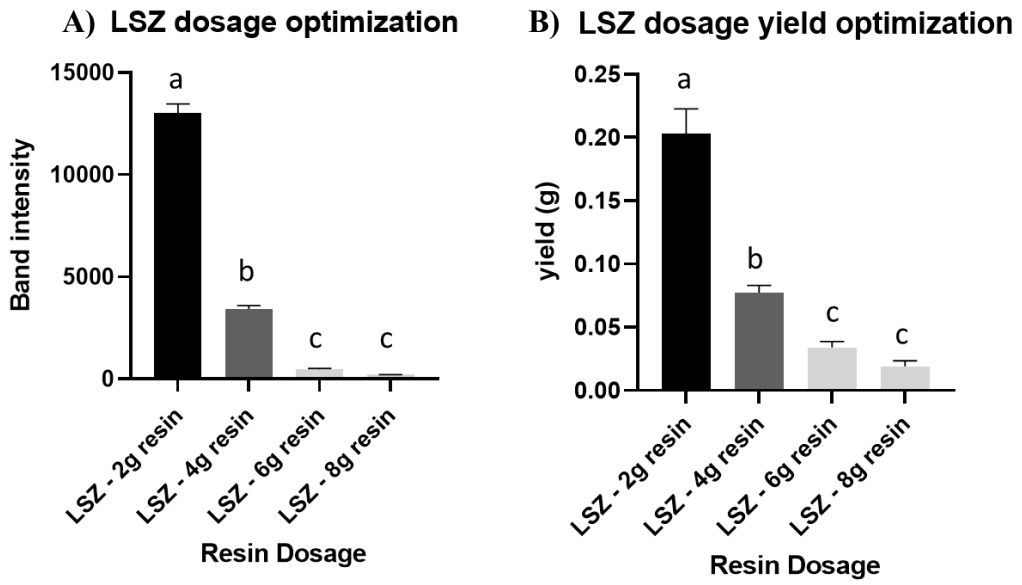

Figure 3.5: LSZ resin dosage optimization based on – A) Band intensity B) Yield (g)

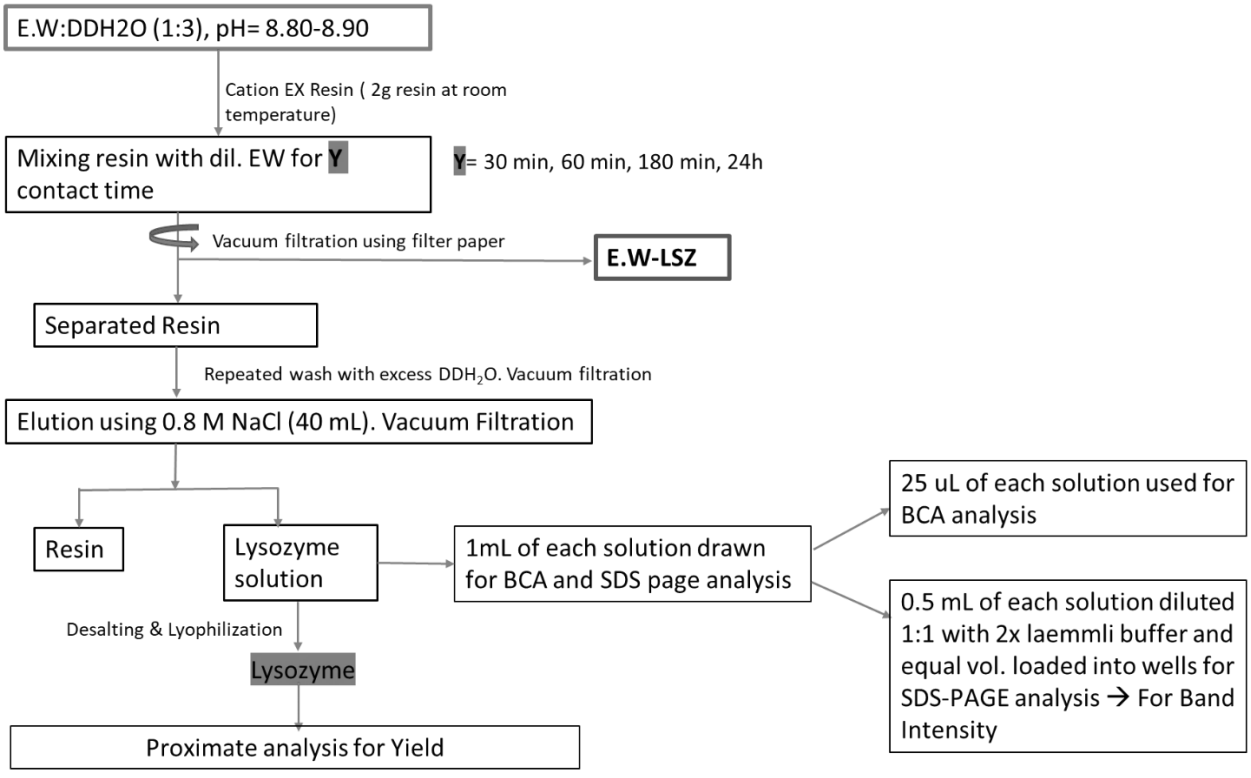

Figure 3.6: LSZ resin contact time optimization process

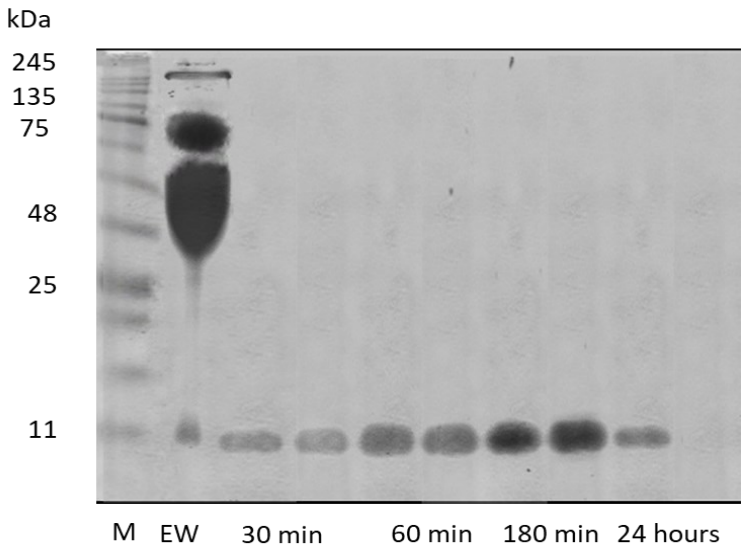

Figure 3.7: SDS-PAGE analysis for resin contact time optimization- (lane 1 – marker, lane 2 – egg white, lane 3 & 4 – 30 minutes, lane 5 & 6– 60 minutes, lane 7 & 8 – 180 minutes^, lane 9– 24 hours)

^The sharpest band intensity was observed at 180 min after which it was found to be reducing (data not shown). However, the band after a prolonged contact time of 24 hours has been included.

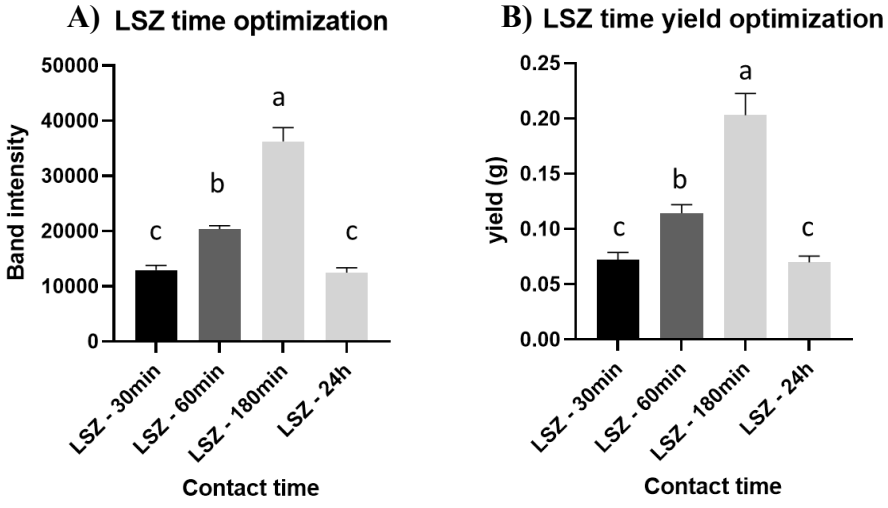

Figure 3.8: LSZ resin contact time optimization based on – A) Band intensity B) Yield (g)

^The sharpest band intensity & highest yield were observed at 180 min after which these were found to be reducing (data not shown). However, the data after a prolonged contact time of 24 hours has been included.

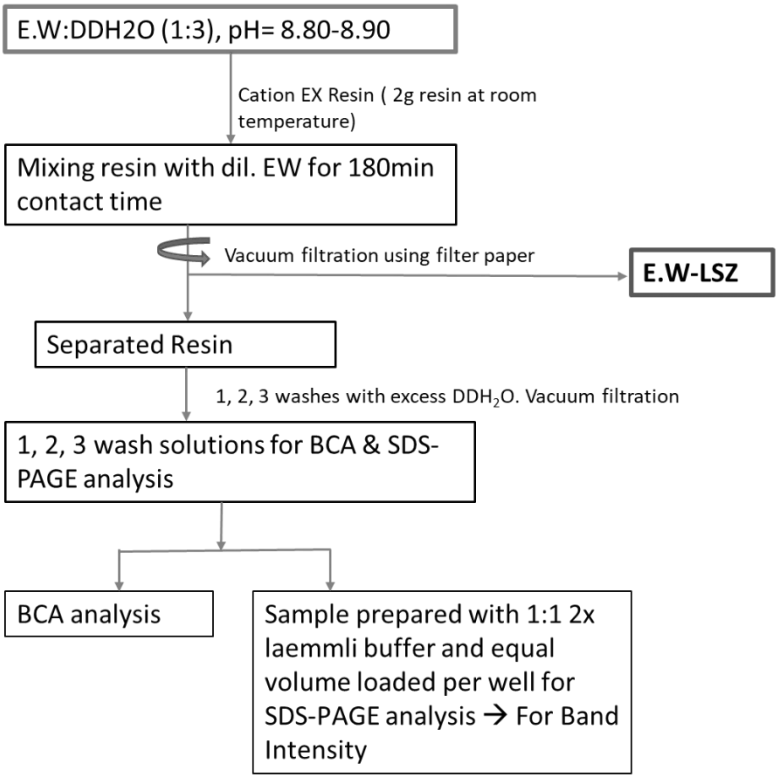

Figure 3.9: LSZ resin wash optimization process

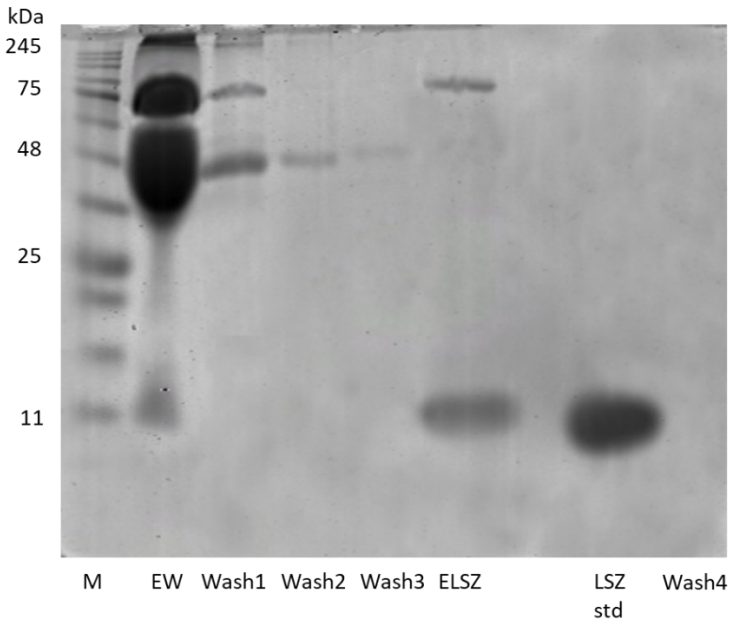

Figure 3.10: SDS-PAGE analysis for resin ddH_2O wash optimization- (lane 1 – marker, lane 2 – egg white, lane 3 – 1st wash, lane 4– 2nd wash, lane 5 – 3rd wash, lane 6– Eluted LSZ, lane 7– blank, lane 8– LSZ standard, lane 9– 4th wash)

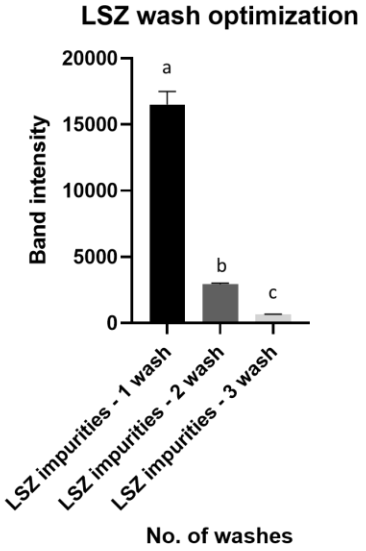

Figure 3.11: LSZ resin wash optimization

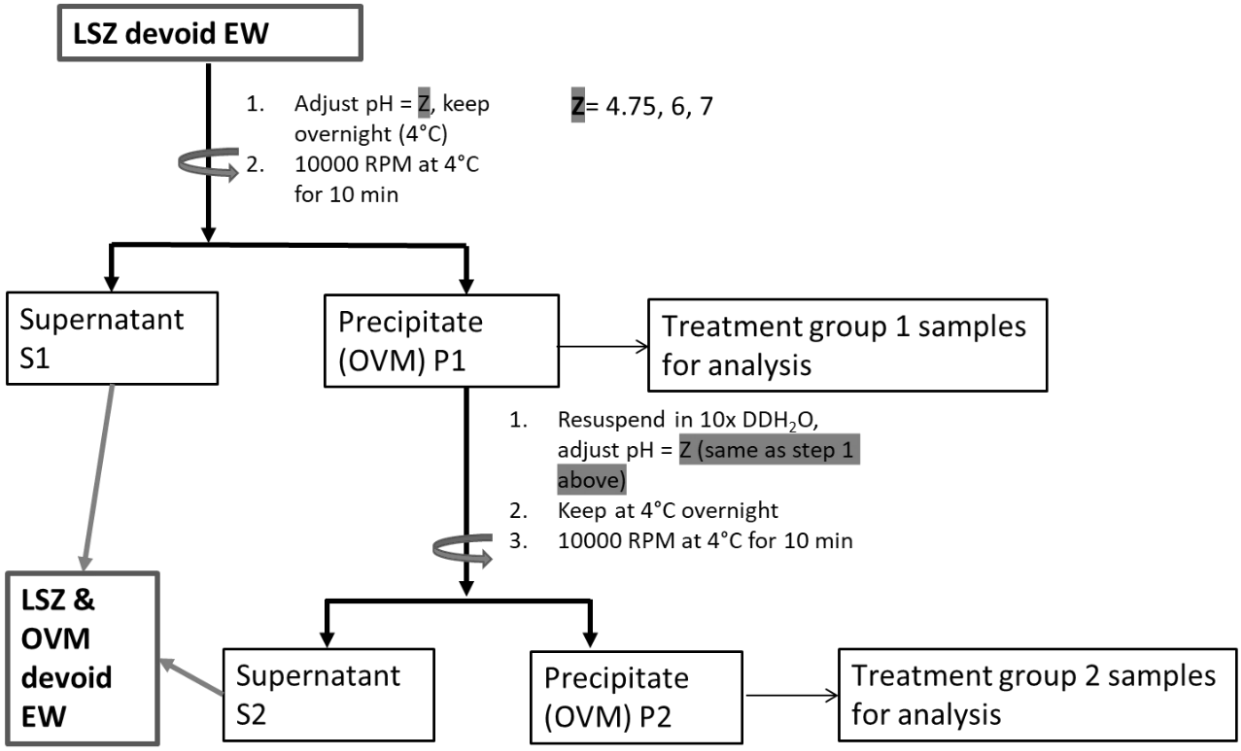

Figure 3.12: OVM extraction conditions optimization (without salt) process

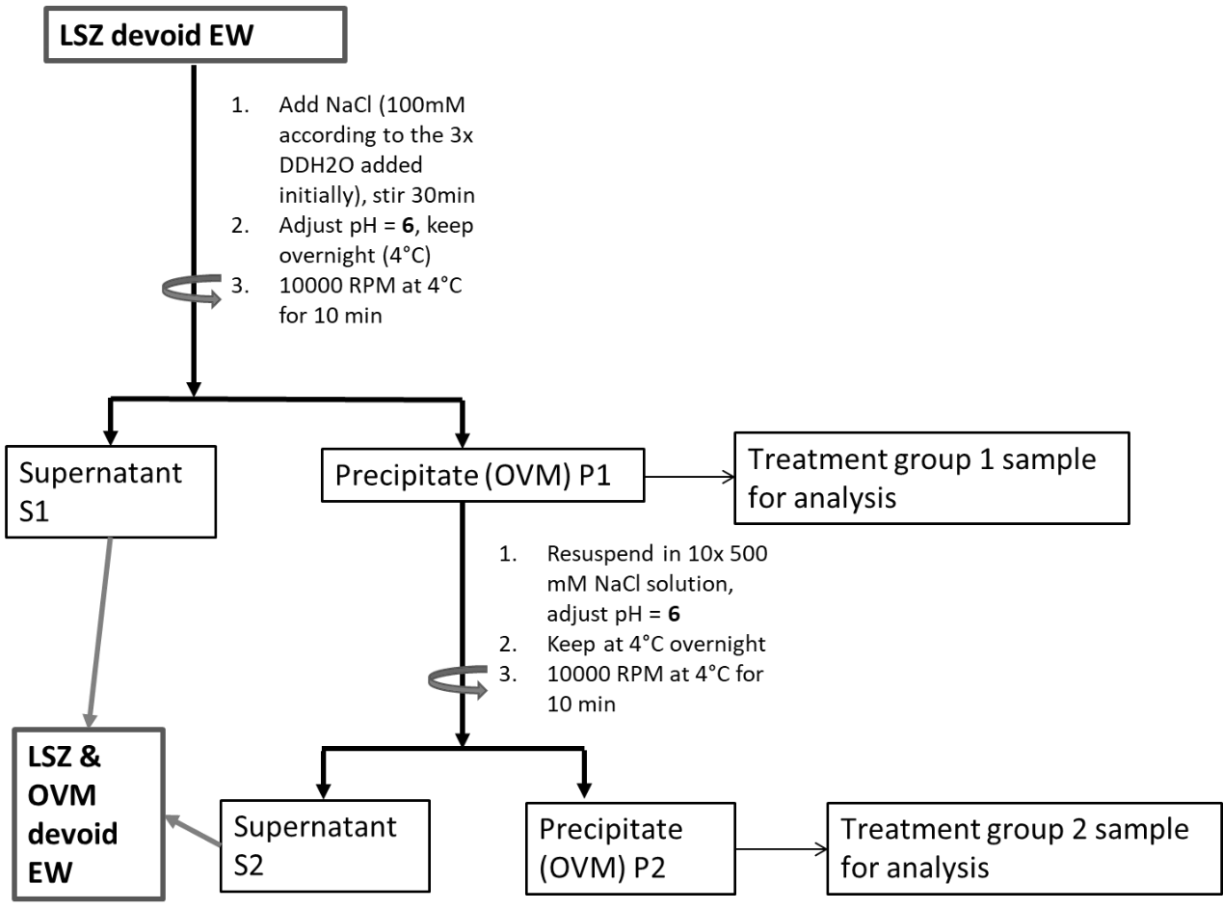

Figure 3.13: OVM extraction conditions optimization (with salt) process

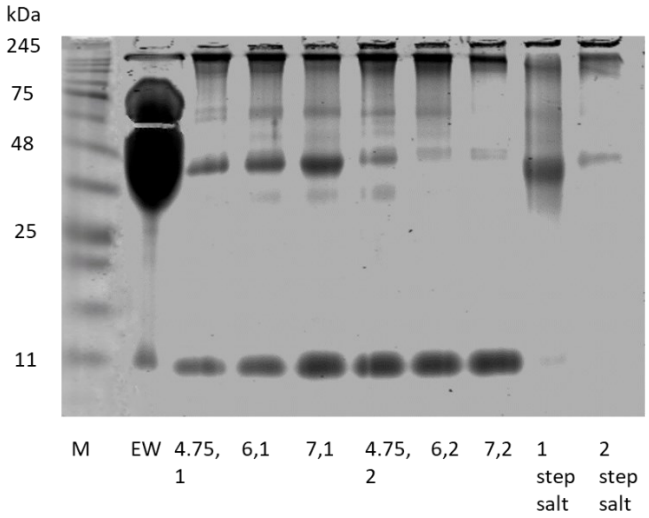

Figure 3.14: SDS-PAGE analysis for OVM extraction conditions optimization (with and without salt)- (lane 1 – marker, lane 2 – egg white, lane 3 – pH 4.75, 1 step ppt, lane 4– pH 6, 1 step ppt, lane 5 – pH 7, 1 step ppt, lane 6– pH 4.75, 2 step ppt, lane 7– pH 6, 2 step ppt, lane 8– pH 7, 2 step ppt, lane 9– pH 6, 1 step ppt with salt, lane 10– pH 6, 2 step with salt)

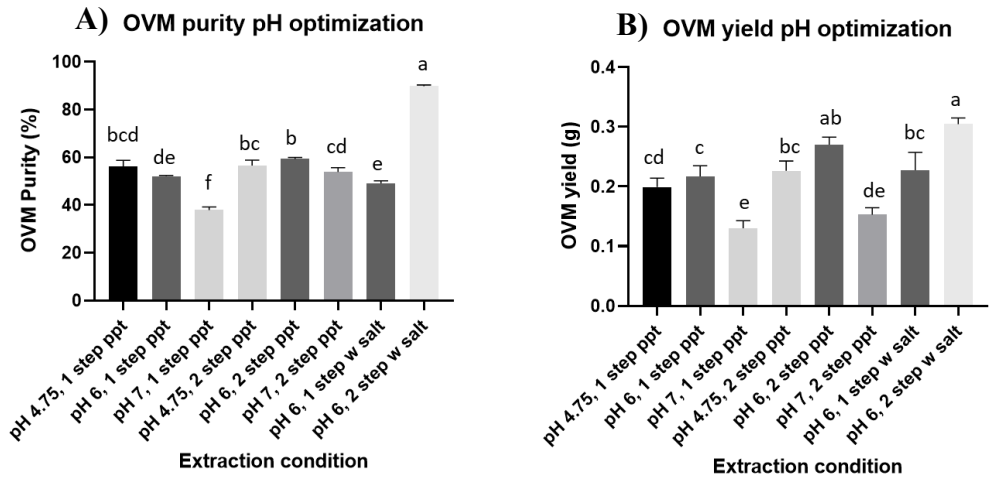

Figure 3.15: OVM extraction conditions optimization (with and without salt) – A) Purity (%) B) Yield (g)

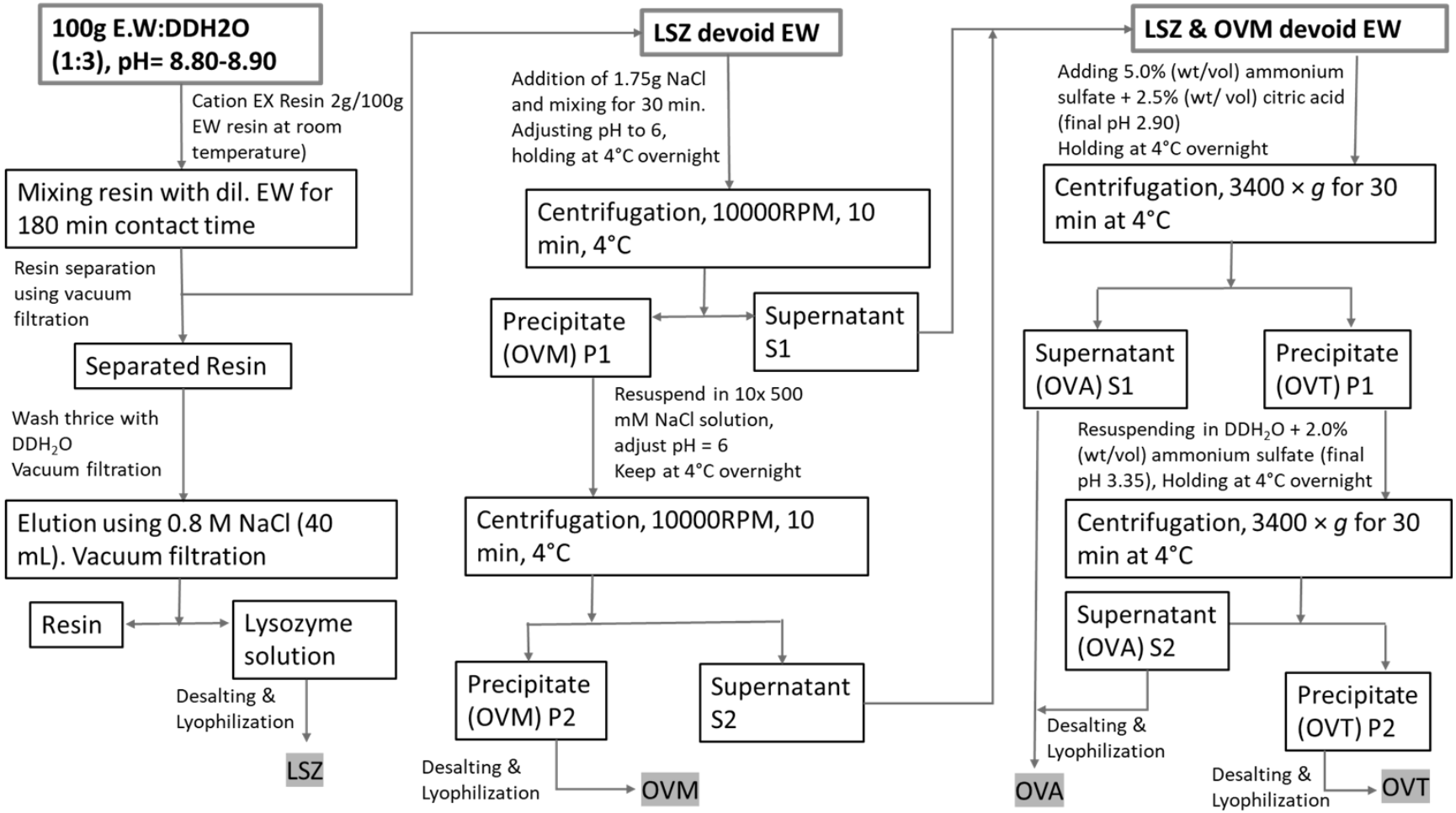

Figure 3.16: Overall extraction process

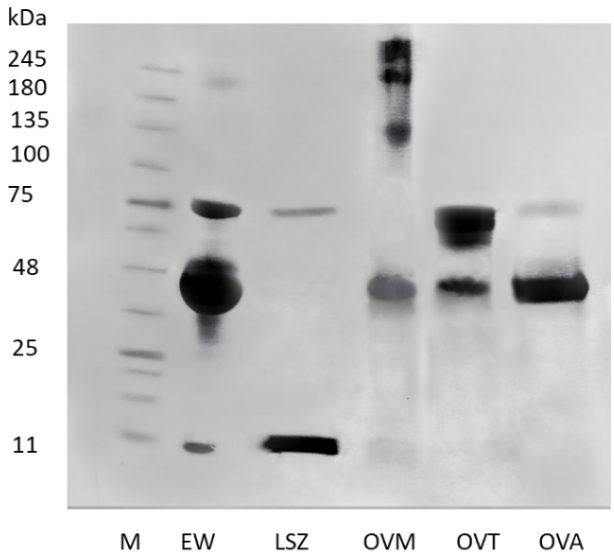

Figure 3.17: SDS-PAGE analysis for overall extraction- (lane 1 – marker, lane 2 – egg white, lane 3 – LSZ, lane 4– OVM, lane 5 – OVT, lane 6– OVA)

Table 3.1: LSZ resin dosage optimization

Sample	Band Intensity	Yield (g) per 100 g EW
LSZ - 2g resin	13018 ± 455^{a}	0.203 ± 0.020^{a}
LSZ - 4g resin	3432.8 ± 161.8^{b}	0.077 ± 0.0057^{b}
LSZ - 6g resin	473.7 ± 50.9^{c}	0.034 ± 0.0046^{c}
LSZ - 8g resin	205.49 ± 7.65^{c}	0.019 ± 0.0044^{c}

Given values are mean values and standard deviation of triplicate trials. Values in the same column with different letters were significantly different at $p < 0.05$

Table 3.2: LSZ resin contact time optimization

Sample	Band Intensity	Yield (g) per 100 g EW
LSZ - 30min	12952 ± 837^{c}	0.072 ± 0.0065^{c}
LSZ - 60min	20364 ± 628^{b}	0.114 ± 0.0076^{b}
LSZ - 180min	36247 ± 2489^{a}	0.203 ± 0.020^{a}
LSZ - 24h	12481 ± 870^{c}	0.070 ± 0.0056^{c}

Given values are mean values and standard deviation of triplicate trials. Values in the same column with different letters were significantly different at $p < 0.05$

Table 3.3: LSZ resin wash optimization

Sample	Band Intensity
LSZ impurities - 1 wash	16506 ± 986^{a}
LSZ impurities - 2 wash	2941.6 ± 85.6^{b}
LSZ impurities - 3 wash	642.9 ± 27^{c}

Given values are mean values and standard deviation of triplicate trials. Values in the same column with different letters were significantly different at $p < 0.05$

Table 3.4: OVM extraction conditions optimization (with and without salt)

Sample	^OVM Purity %	OVT Impurity %	OVA Impurity %	LSZ Impurity %	^Yield (g)
pH 4.75, 1 step ppt	56.21 ± 2.46^{bcd}	2.84 ± 0.27^{b}	19.36 ± 0.81^{d}	19.33 ± 1.12^{e}	0.199 ± 0.015^{cd}
pH 6, 1 step ppt	52.03 ± 0.39^{de}	4.72 ± 0.34^{a}	22.23 ± 0.78^{c}	22.63 ± 1.09^{d}	0.217 ± 0.017^{c}
pH 7, 1 step ppt	37.85 ± 1.37^{f}	4.47 ± 0.30^{a}	25.10 ± 0.76^{b}	35.27 ± 1.51^{bc}	0.130 ± 0.013^{e}
pH 4.75, 2 step ppt	56.50 ± 2.28^{bc}	1.95 ± 0.10^{c}	9.38 ± 1.08^{e}	33.82 ± 1.11^{c}	0.226 ± 0.017^{bc}
pH 6, 2 step ppt	59.31 ± 0.65^{b}	1.83 ± 0.08^{c}	2.25 ± 0.14^{f}	36.68 ± 0.78^{b}	0.270 ± 0.013^{ab}
pH 7, 2 step ppt	53.90 ± 1.69^{cd}	-	2.34 ± 0.23^{f}	46.14 ± 0.93^{a}	0.153 ± 0.012^{de}
pH 6, 1 step w salt	49.03 ± 1.07^{e}	3.11 ± 0.14^{b}	46.34 ± 2.08^{a}	1.22 ± 0.17^{f}	0.227 ± 0.030^{bc}
pH 6, 2 step w salt	89.77 ± 0.54^{a}	-	10.18 ± 0.60^{e}	-	0.305 ± 0.010^{a}

Given values are mean values and standard deviation of triplicate trials. Values in the same column with different letters were significantly different at $p < 0.05$

^The purity percentage represents the quantity of the target protein amongst other egg white proteins (impurities) present but does not account for the moisture and ash content. The crude protein weight and the purity of the protein are used to determine the quantity of pure protein extracted from a particular amount of egg white. This quantity is then represented as the yield in "g of pure protein per 100 g of egg white"

Table 3.5: Overall extraction results

EW Protein	^Purity %	^Yield %	^Moisture %	^Ash %	Crude Protein Weight (g)
LSZ	95.23 ± 2.78	62.83 ± 4.22	10.29 ± 0.31	9.80 ± 0.61	0.224 ± 0.024
OVM	89.77 ± 3.65	86.11 ± 3.84	2.63 ± 0.46	7.14 ± 0.35	0.326 ± 0.019
OVT	73.63 ± 1.24	78.27 ± 2.91	4.27 ± 0.27	6.60 ± 0.41	1.275 ± 0.037
OVA	93.34 ± 4.11	99.61 ± 3.05	4.54 ± 0.33	9.46 ± 0.43	5.76 ± 0.188

Given values are mean values and standard deviation of triplicate trials

^The purity percentage represents the quantity of the target protein amongst other egg white proteins (impurities) present but does not account for the moisture and ash content. The moisture and ash content in the freeze-dried powder/samples were measured using the proximate analysis method. The crude protein weight and the purity of the protein were then used to determine the quantity of pure protein extracted from a particular amount of egg white. This quantity was then represented as “quantity of pure protein divided by the theoretical quantity of that protein present in egg white” to calculate the percentage yield.

4. PILOT SCALE EXTRACTION OF MAJOR EGG WHITE PROTEINS - APPLICATION OF SIPHON FILTRATION AS A SEPARATION TECHNIQUE FOR SCALE-UP

4.1 Introduction

The major egg white proteins are ovalbumin (OVA, 54%), ovotransferrin (OVT, 11%), ovomucin (OVM, 3.5%), and lysozyme (LSZ, 3.4%), by weight. Owing to the very high isoelectric point of LSZ as compared to other egg white proteins, ion exchange chromatography seems to be the best method for the extraction of LSZ (Abeyrathne et al. 2013b, 2014). Abeyrathne et al. 2014 demonstrated the extraction of LSZ with the resin Amberlite™ FPC3500. The yield of the isolated LSZ was more than 82% and its purity was more than 90%. OVM extraction methods are broadly classified into 3 categories: chromatography, centrifugation, and precipitation (Hester 2016). The biggest challenge with OVM is to obtain large quantities with high purity as it tends to interact with LSZ, OVA as well as with OVT (Kato et al. 1976). The methods like the 2 step method by Omana & Wu, 2009 have proven to solve this issue to some extent as OVM with high purity (>90%) can be prepared. For OVA and OVT, the most common method is salting out using ammonium sulfate and citric acid (Abeyrathne et al. 2014) where OVT is obtained as a precipitate and OVA as supernatant. Due to the shortcomings of the currently used methods of extracting these proteins, their scalability and ease of operation are very limited. This is mainly due to the factors like the need for expensive and bulky equipment like chromatography columns, ultracentrifuge units and so on. The use of centrifuge in case of OVM extraction limits the scalability of the process as it requires a high centrifugal speed of 10000 RPM which is difficult to be achieved on an industrial scale (Sleigh et al. 1973; Kato et al. 1975; Omana and Wu 2009). Our study aimed to explore the use of siphon filtration for separating the precipitates of OVM and OVT as well as for the separation of resin particles from egg white after LSZ extraction. Although this alone cannot be a replacement for centrifugation but combining it with a longer sedimentation

time to allow the protein particles to nucleate and precipitate in larger quantities could help ease the separation (Glatz et al. 1986). In many African villages where the power supply is unavailable, a tool called 'ceramic siphon filter' is used to remove dirt, bacteria, protozoa and viruses from the water (Barnes et al. 2009). A siphon is "any tube (generally 'U' shaped) which causes a liquid to flow upward, above the surface of a reservoir, with no pump, but powered by the fall of the liquid as it flows down the tube under the pull of gravity, then discharging at a level lower than the surface of the reservoir from which it came" (Ramette and Ramette 2011; Rizzuan et al. 2021). Adding a filter of desired porosity at the inlet of the tube makes it a siphon filter (siphon+filter). This filter can be modified according to the particles that need to be separated. This method of separation has never been attempted before with protein precipitates. Also, doing so could help with the scale up and handling of large quantities of egg white in a very efficient way as there would be no special requirements for excessive centrifugal capacity or speed; moreover, a siphon filter operates without electricity or any moving parts, under the influence of gravity and atmospheric pressure (lower operational, maintenance and setup costs) (Ramette and Ramette 2011; Rizzuan et al. 2021). This chapter will compare the scale up batches (1 Kg and 20 Kg egg white) to lab scale batch (100 g) for siphon filtration as the separation technique. This would be the first ever study about the scalability of the process to a pilot scale (extracting proteins from10 kg or more of egg white is considered as pilot scale) (Metz and Data 2015).

4.2 Materials and Methods

4.2.1 Materials

Liquid egg white was purchased from EggSolutions EPIC Inc (Lethbridge, AB, Canada). Amberlite FPC 3500 (styrene-divinylbenzene, total exchange capacity >2.6 mEq/g, H^+-form) and hydrochloric acid were purchased from Fisher Scientific. Sodium chloride (ACS reagent, >99.0%)

and citric acid (anhydrous) were purchased from Sigma–Aldrich and Aquabond (Toronto, Ontario, Canada) respectively. Sodium dodecyl sulfate-polyacrylamide was purchased from Bio-Rad Laboratories, Inc., Hercules, CA. Ammonium sulfate was purchased from Acros Organics. 2-Mercaptoethanol was obtained from MP Biomedicals, LLC (Solon, OH, USA). KA Werke Eurostar Power-B Overhead Stirrer was used for blending/mixing the solutions. Standard proteins (ovalbumin, ovotransferrin, ovomucoid, and lysozyme) were obtained from Neova Technologies Inc. (Abbotsford, BC, Canada). BCA (Pierce™ BCA Protein Assay Kit) for protein analysis was from Thermofisher (Waltham, Massachusetts, United States). The purity of protein was calculated by converting the density of protein bands in the gel picture using the ImageJ software (NIH, Bethesda, MD) as the percent of the total gel density. The actual protein content of egg white from literature (10%) was used to calculate the yields (Stevens 1991). Pall Corporation Ultrafiltration (New York, United States) unit was used for concentrating/desalting. Flash 2000 Organic Elemental analyzer (for nitrogen content), Yamato DKN 810 mechanical convection oven (for dry matter), and Barnstead Thermolyne furnace (for ash content) were used for overall proximate analysis. Materials used for the siphon filter were: 3/16" ID 7/16" OD Clear Vinyl Tubing Food Grade Multipurpose Tube, Stainless steel Mesh (100 and 400 mesh sizes), and Waterproof Silicone Sealing Tape from Amazon.ca.

4.2.2 Methods

4.2.2.1 Checking OVM presence in the supernatant after prolonged holding/sedimentation

100g egg white was diluted with ddH_2O in the ratio 1:3 and mixed well till pH stabilized (~8.90). Amberlite FPC 3500 weak acid cation exchange resin was added as per the optimized dosage quantity (2g) and allowed to mix for the optimized contact time (180 min). After the required

contact time, the resin was separated from the diluted egg white solution using a 100 mesh (0.149 mm) siphon filter (resin particle size is 0.3–1.18 mm). This solution was LSZ devoid egg white solution.

1.75g NaCl was added to this solution and allowed to mix for 30 minutes, then the pH was adjusted to 6 (modified 2 step method). The day of NaCl addition and pH adjustment was regarded as Day 0. From this point onwards, aliquots of the supernatant were collected each day for 7 days (Day 0 to Day 6) **(Figure 4.1)**. BCA analysis and SDS-PAGE were performed to check for the band intensity of OVM (to check if the band intensity reduces overtime, indicating the sedimentation/further precipitation of OVM).

4.2.2.2 Checking OVT presence in the supernatant after prolonged holding/sedimentation

The LSZ and OVM devoid egg white solution was used for the extraction (precipitation) of OVT. 5% (wt/vol) ammonium sulfate and 2.5% (wt/vol) citric acid (final pH 2.90) were added to the LSZ and OVM devoid egg white solution. The day of this addition was regarded as Day 0. From this point onwards, aliquots of the supernatant were collected each day for 3 days (Day 0 to Day 2) **(Figure 4.4)**. BCA analysis and SDS-PAGE were performed to check for the band intensity of OVT (to check if the band intensity reduces overtime, indicating the disappearance/further precipitation of OVT).

4.2.2.3 Selection of filter for siphon filtration (supernatant analysis)

This study was carried out to find the best possible filter for separation of the precipitates/pellets from the supernatant after the optimized sedimentation time. These trials were performed for the separation of OVM (1st step) precipitate/pellets from the supernatant and the aim was to have as few OVM precipitate/pellets as possible in the supernatant that is siphoned out. Siphon filtration was performed using 4 different types of filters whatman filter, teabag filter, nitrocellulose

membrane filter and 400 mesh stainless steel filter **(Figure 4.7)**. These filters were attached to the tip of the tube/pipe in the same manner- they were folded in the form of a hemisphere (to allow maximum surface area or orifices for diffusion of supernatant across them) and tied tightly on the tip with waterproof tape (to avoid any gaps or pores that would interfere with the functioning of the siphon filter). The supernatant was siphoned out and collected in a separate container kept about 1.5 m below the first container. The tip of the siphon filter was moved along with the level of the solution. These supernatants were then analyzed using SDS-PAGE in order to check for the band intensity of OVM in each case.

4.2.2.4 Overall extraction scheme

Overall extraction process using siphon filtration (see **Figure 4.10** for the overall extraction process flowchart) –

100 g egg white was diluted with ddH_2O in the ratio (1:3) and the initial pH was noted. Amberlite FPC 3500 resin was added at 2 g/100g egg white and allowed to mix with the diluted egg white solution for 3 hours. After the required contact time, the resin particles were separated by siphoning out the solution using a 100-mesh siphon filter. The resin was washed thrice with ddH_2O, and the elution was performed using 0.8M NaCl solution. The elute was then desalted and freeze dried to obtain LSZ powder.

The LSZ devoid egg white was used for OVM extraction. 1.75g NaCl per 400 mL diluted egg white solution was added and allowed to mix for 30 min, followed by pH adjustment to 6. This solution was then held for 3 days and on day 4 siphon filtration was performed to separate the aggregates/precipitates from the supernatant. The OVM pellet solution thus obtained was resuspended in 10x volume of 0.5M NaCl solution (account the volume of solution present along with the pellet while resuspending). This solution was then allowed to stand for another 2 days (a

clear precipitation was seen) and then the supernatant was siphoned out. The purified OVM pellets thus obtained after 2nd precipitation were desalted and freeze dried to obtain OVM powder.

The LSZ and OVM devoid egg white solution (pH 6) was finally used for the extraction of OVT. Ammonium sulfate and citric acid were added to this solution at 5% wt/vol and 2.5% wt/vol respectively. The stirring was continued, and the pH was monitored till it stabilized to 2.90. The solution was then held for 1 day and on day 2 siphon filtration was performed to separate the supernatant (high purity OVA solution) from the precipitate (OVT with some impurities). This impure OVT was then resuspended in ddH_2O and 2% wt./vol ammonium sulfate and 1.5% wt./vol citric acid were added for reprecipitation. This solution was held for 24 hours and then siphon filtration was performed to separate the purified OVT pellets from the supernatant. The OVT pellet solution and OVA solution were desalted and freeze dried to obtain the respective protein samples.

4.2.2.5 Yield and purity calculation

The freeze-dried samples were analyzed for crude protein content by BCA analysis as well as proximate analysis using Flash analyzer. The samples were then run for SDS-PAGE to determine the purity of the protein of interest by scanning the band intensity using a gel scanner and the ImageJ software (NIH, Bethesda, MD) as percentage of the total gel density. This purity percentage represented the quantity of the target protein amongst other egg white proteins (impurities) present but did not account for the moisture and ash content. The moisture and ash content in the freeze-dried powder/samples were further measured using the proximate analysis method. The crude protein weight and the purity of the protein were then used to determine the quantity of pure protein extracted from a particular amount of egg white. This quantity was then represented as the yield in two ways – 1. mg or g of pure protein per 100 g of egg white, 2. quantity

of pure protein divided by the theoretical quantity of that protein present in egg white to calculate the percentage yield.

4.2.2.6 Statistical analysis

All the experiments were conducted in triplicates, and the results were expressed as mean value ± standard deviation and analyzed by one-way analysis of variance (ANOVA) with Tukey's Test to determine the significant differences among the means ($p < 0.05$), by Prism and Minitab software.

4.3 Results

4.3.1 Checking OVM presence in the supernatant after prolonged holding/sedimentation

The purpose of prolonged sedimentation time was to check for sufficient time required for the collision of protein aggregates to form larger aggregates, and hence cause an increase in the cluster sizes that would help in precipitation and separation from the supernatant (Bell et al. 1983; Petenate and Glatz 1983; Glatz et al. 1986; Fisher and Glatz 1988; Kirk et al. 2021). By keeping equal protein loading per well in SDS-PAGE analysis, the change in protein composition of the supernatant was observed over time – a lower band intensity of OVM would indicate a lesser presence of it in the supernatant which is desirable for our study. At day 0 the OVM band intensity was 43233 ± 1982; the band intensity showed a significantly reducing trend ($p<0.05$) over a period of 7 days **(Figure 4.2 and Table 4.1)**. On day 4 and day 6 the band intensities were 27910 ± 1677 and 25433 ± 2959, respectively, whereas, for the control (centrifuge) it was 22042 ± 1705 **(Figure 4.3)**. There was no significant difference ($p>0.05$) between the band intensities from day 4 to day 6 and, when compared to the control **(Table 4.1)**. Hence, day 4 was selected as the optimum holding time for the separation of OVM precipitate.

4.3.2 Checking OVT presence in the supernatant after prolonged holding/sedimentation

As explained earlier for OVM presence in the supernatant, the presence of OVT was checked for in the supernatant after prolonged holding time. The OVT band intensity reduced significantly ($p<0.05$) over a period of 3 days indicating its disappearance from the supernatant over time **(Figure 4.5, Figure 4.6, and Table 4.2)**. The OVT precipitation (holding) time was lesser than that for OVM as mentioned earlier. This could be due to the higher salt content and/or salt type (due to Hofmeister series for salting out, as ammonium sulfate has stronger salting out effect) in case of OVT precipitation. Also, upon the analysis of this supernatant, we found that it consists of high purity OVA (>90%) further giving strong evidence of the absence of OVT from the supernatant after a holding time of 3 days.

4.3.3 Selection of filter for siphon filtration (supernatant analysis)

This study was conducted to check for the best filter for siphon filtration. OVM aggregate separation was tested with different filters. The separation would be considered efficient if the target protein is not abundantly found in the supernatant after separation i.e., lower intensity bands of OVM in the supernatant. Upon SDS-PAGE analysis of the supernatant, it was found that the band intensity of OVM was low and not significantly different ($p>0.05$) in case of nitrocellulose membrane filter (0.45 μm) and 400 stainless steel mesh (37 μm) indicating a very efficient separation as compared to the control (centrifuge) **(Figure 4.8, Figure 4.9, and Table 4.3)**. However, there was a technical limitation in case of the nitrocellulose membrane due to its low pore size which resulted in a very long separation time. The band intensity was lower for nitrocellulose membrane than for stainless steel mesh (although no significant difference – $p>0.05$); however, the latter was chosen as the preferred filter since the separation could be achieved in 40 seconds for 400mL solution while it took around 80 minutes with the nitrocellulose

membrane. In addition to having higher intensity bands as compared to the nitrocellulose membrane filter and stainless-steel mesh filter, the whatman filter and teabag filter had other drawbacks. The whatman filter had the problem of swelling and tearing due to excessive water absorption and hence was not suitable for scale-up which involves long separation time. The teabag filter was unable to maintain a hemispherical shape in the solution as its structure collapsed due to suction during siphoning. Hence, these filters were concluded to be not feasible for scale-up.

4.4 Discussion

Over the years, there have been many studies on the lab scale extraction of egg white proteins (Eichholz 1898; Hopkins 1900; Meyer et al. 1936; Alderton et al. 1945; Croguennec et al. 2000; Guérin-Dubiard et al. 2005; Omana and Wu 2009; Omana et al. 2010b). Many of these studies have claimed their process/protocol as ‘scalable’ or ‘having a potential for industrial scale up’. However, very limited studies have demonstrated a systematic and step wise scale up to support these claims. One of these studies has proven the feasibility of scale up to 3kg which can be regarded as a pilot scale and not an industrial scale (Abeyrathne et al. 2014; Metz and Data 2015). In order to prove the scalability of the process to an industrial level, without affecting the purity and yield of proteins, whilst ensuring the simplicity of the process, there was a need to improvise certain process parameters as we have demonstrated in this study to show the ease of scale up. Some major changes in this study were to increase the sedimentation time during precipitation, to allow the particles/aggregates of proteins to nucleate and grow, and to use a customized siphon filter to accomplish the separation of resin particles as well as aggregated/nucleated precipitates (Bell et al. 1983; Petenate and Glatz 1983; Glatz et al. 1986; Fisher and Glatz 1988; Barnes et al. 2009; Kirk et al. 2021). This can be compared to the traditional approach similar for the separation of cheese and whey in cheese processing using cheesecloth, but it has never been attempted so far

with protein or specifically egg protein separation (Ong et al. 2018). Also, some proteins like OVT, upon precipitation appear like curd 'cheese' and form a thick aggregate layer which can be separated easily by siphon filtration without the use of centrifuges and thereby no electricity consumption. Our study was thus performed at 3 scales – 100g, 1kg, 20kg. This was the first ever study to be conducted to prove the industrial level scale up (>10kg) of egg white extraction (Metz and Data 2015). The setup of the whole experiment was such that the scale up became easy and cost-effective. It was possible to conduct all the scale up batches, including the ones with 20kg egg white, in a normal lab setting, owing to the minimal experimental setup and requirements **(Figure 4.12)**.

The purity of LSZ was >90% for all 3 batch sizes (100 g, 1 kg, 20 kg) and the yields were 58%, 60% and 60%, respectively **(Figure 4.11 and Table 4.4)**, which were very similar to those obtained by Tankrathok et al. 2009 . However, their study used chromatography column which would increase the cost of extraction on scale-up, whereas our study was based on the batch ion exchange reaction, similar to the extraction designed by Abeyrathne et al. 2013, 2014. Our yields were lower than those reported by Abeyrathne et al. 2013, 2014, however, the purity of scale up batches were similar; also the lab scale purity was comparable to many of the previous studies (Guérin-Dubiard et al. 2005; Tankrathok et al. 2009; Omana et al. 2010b; Luding et al. 2011). As explained in the previous chapter, we have further optimized the extraction conditions for an efficient scale up as part of this study. This was achieved, by using a lower resin dosage, avoiding the use of pH gradient buffers for washing and elution steps, and by using siphon filter (100mesh) for separating resin particles from egg white, instead of using a centrifuge or vacuum filtration unlike the study conducted by Abeyrathne et al. 2013, 2014

In case of OVM, the purities for 100 g, 1 kg and 20 kg were 85%, 81% and 76%, and the yields were 83%, 81% and 76% respectively **(Figure 4.11 and Table 4.4)**. Although the purities and yields reduced with scale-up, this decrease was not significant ($p>0.05$) **(Table 4.4)**. Our yield of OVM (333mg/100g EW) from the modified 2 step method was lesser as compared to that claimed by 2 step method by Omana and Wu (2009) (400.2mg/100g EW). The scale-up study for the process by Omana and Wu (2009) is not available and hence, a comparison of our scale-up cannot be made. A very high yield of >100% was reported in the scale up study conducted by Abeyrathne et al. 2014. This was clearly an overestimate and was further justified in the study. However, we could easily scale up the extraction of OVM by only pH adjustment, and centrifugation was not required to be used as we could undertake the separation of the aggregates from the supernatant by a combination of prolonged holding time and the use of siphon filtration (400 mesh).

In case of OVT extraction, for 100 g, 1 kg and 20 kg, the purities were 69%, 72%, and 67%, respectively, where there were no significant differences in the purity ($p>0.05$) upon scale-up **(Figure 4.11 and Table 4.4)**. However, there was a significant difference in the yields ($p<0.05$) **(Table 4.4)**. Our extraction protocol does not require any heat treatment, unlike the earlier reported methods (Abeyrathne et al. 2013b, 2014), since the supernatant obtained in the end is high purity OVA (>90%), although the yield of OVA reduced significantly ($p<0.05$) on scaling up from 1 kg to 20kg **(Table 4.4)**. Nevertheless, we could achieve yields for OVT that were comparable to or even higher than the earlier reported studies which were conducted on small scale extraction volumes (Awadé et al. 1994; Vachier et al. 1995; Guérin-Dubiard et al. 2005; Ko and Ahn 2008; Tankrathok et al. 2009; Omana et al. 2010b). The major limitations for these earlier methods were difficulties in scale-up process because of high resin costs, slow separation speed, and low yields (Hester 2016). This was also seen with OVA column chromatography-based separation where the

yields were reported to be lower than 90% , in some cases as low as 41% (Rhodes et al. 1958; Croguennec et al. 2000)

The yields of LSZ and OVM reported in our study were lower than that reported by (Abeyrathne et al. 2014). However, the yields for OVA and OVT were found to be quite similar. Due to the use of siphon filter for separation, our process does not involve equipment, maintenance, and operational costs associated with other comparable separation methods like centrifugation. Moreover, previous studies that have used chromatographic separation techniques claimed that the process is easily scalable but have not proven the scalability to compare the results. Although siphon filtration may not be able to completely replace the conventional methods, it has proven to be scalable in a much easier and cost-effective manner due to the aforementioned reasons.

4.5 Conclusion

In this study we modified the procedure we described in chapter 3, in order to make it suitable and easy for scale up. We introduced new separation technique, namely siphon filtration, which could be attempted with other protein separation and tested for scale up. This siphon filtration is like the traditional approach towards protein aggregate/precipitate separation, like using cheesecloth. However, we designed it appropriately, in this study to suit our experimental and scale up requirements. We were able to successfully scale up the process without significantly affecting the purity and yield of the extraction. However, further insight is needed to speed up the rate of sedimentation, separation speed and to further make the process more efficient.

4.6 References

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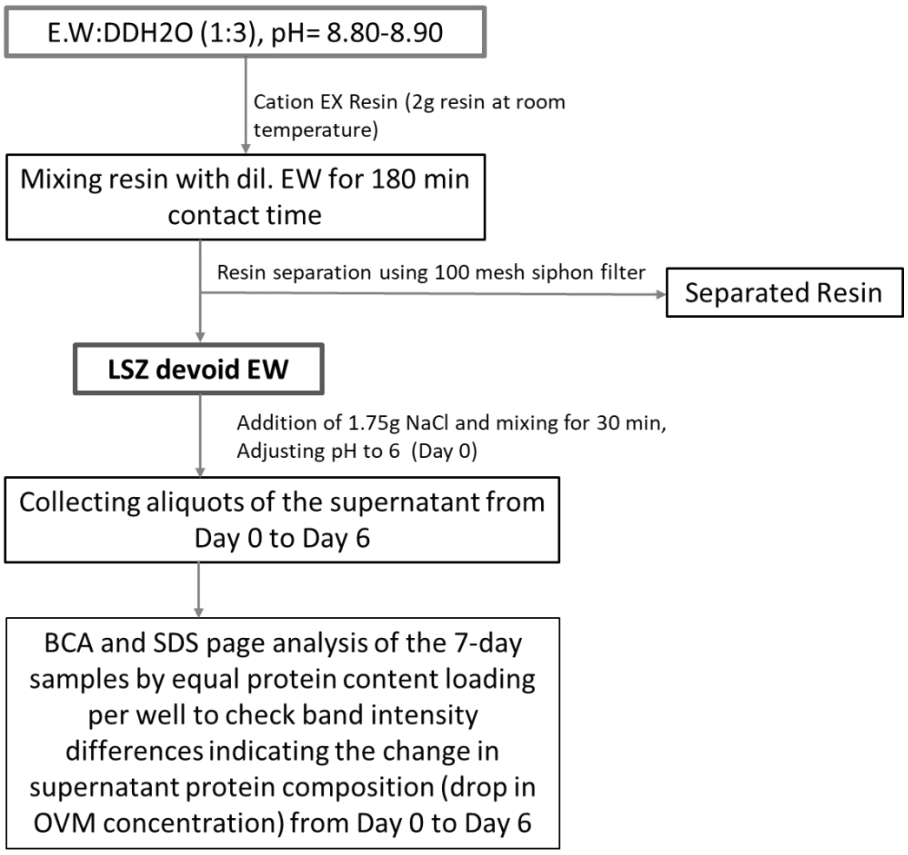

Figure 4.1: Protocol for checking the presence of OVM in the supernatant

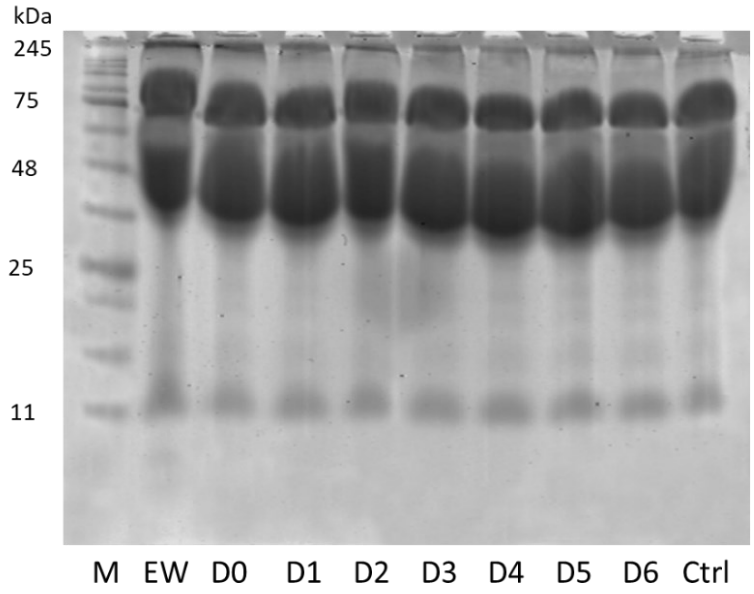

Figure 4.2: SDS-PAGE analysis for OVM presence (lane 1 – marker, lane 2 – egg white, lane 3 – Day 0, lane 4 – Day 1, lane 5 – Day 2, lane 6 – Day 3, lane 7 – Day 4, lane 8 – Day 5, lane 9 – Day 6, lane 10 – Control (centrifuge). SDS-PAGE was performed using 12% separating gel. The samples were analysed for protein content by BCA protein analysis and loaded accordingly into the wells as described in protocol (Figure 4.1).

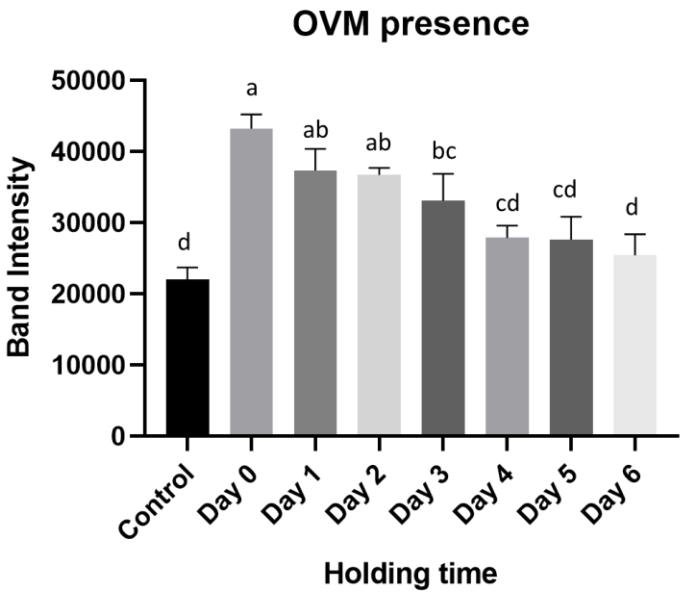

Figure 4.3: Bar graph for comparing the supernatants after specific holding time. The band intensity is of OVM that remains in the supernatant after a particular holding time

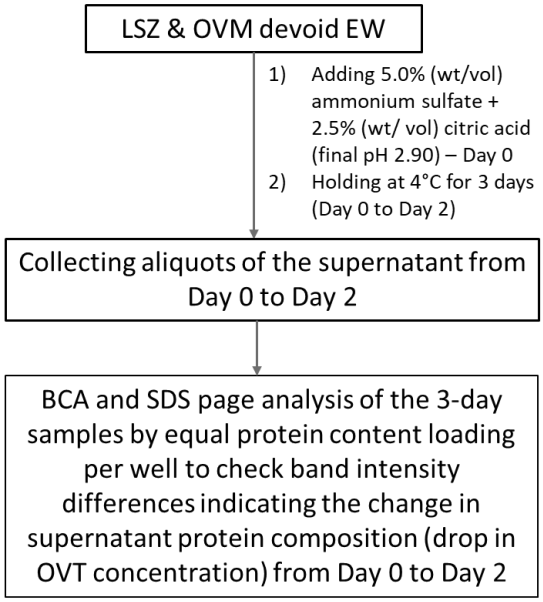

Figure 4.4: Protocol for checking the presence of OVM in the supernatant

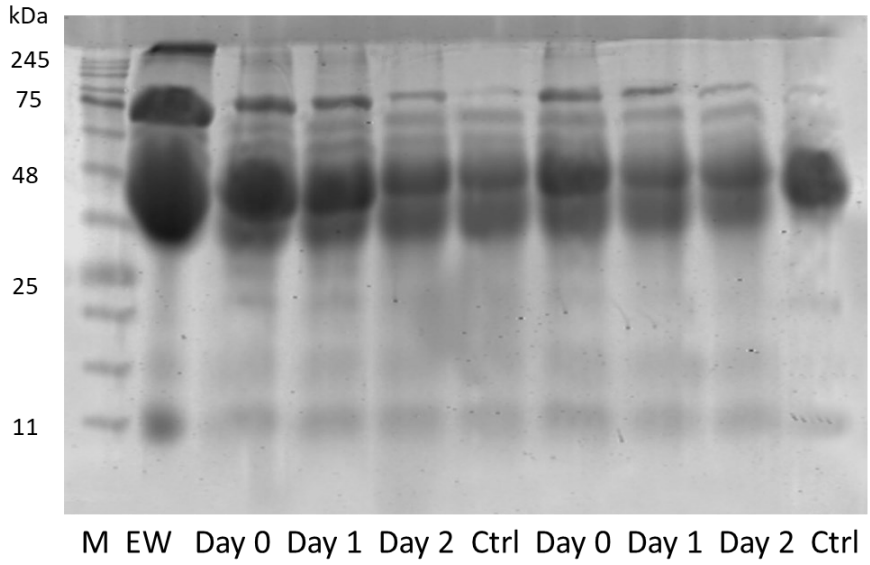

Figure 4.5: SDS-PAGE analysis for OVT presence (lane 1 – marker, lane 2 – egg white, lane 3 – Day 0, lane 4 – Day 1, lane 5 – Day 2, lane 6 – Control (centrifuge), lane 7 – Day 0, lane 8 – Day 1, lane 9 – Day 2, lane 10 – Control (centrifuge). SDS-PAGE was performed using 12% separating gel. The samples were analysed for protein content by BCA protein analysis and loaded accordingly into the wells as described in protocol (Figure 4.4).

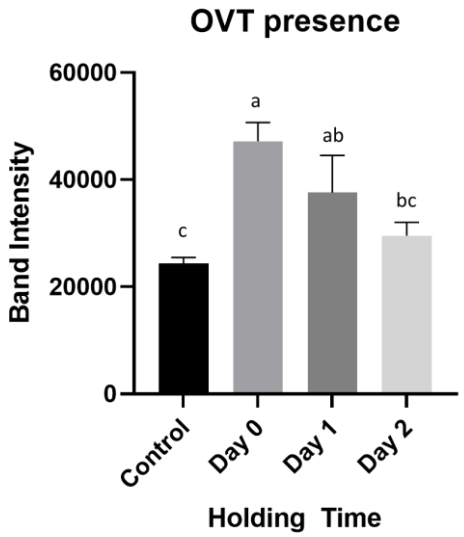

Figure 4.6: Bar graph for comparing the supernatants after specific holding time. The band intensity is of OVT that remains in the supernatant after a particular holding time

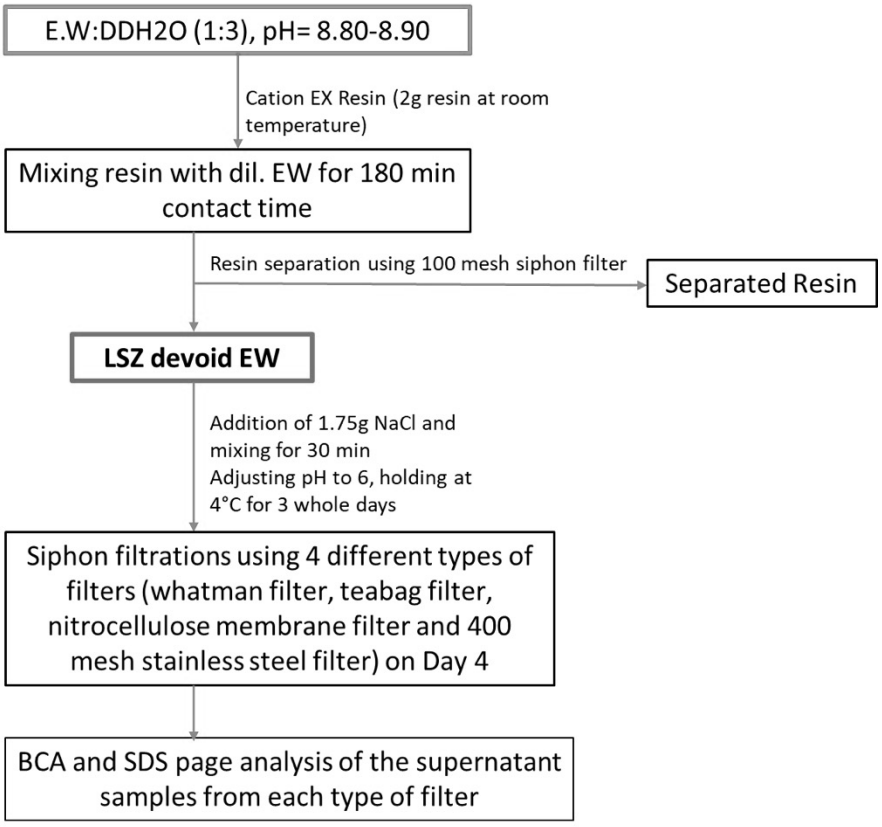

Figure 4.7: Protocol for selecting the filter for siphon filtration. The supernatant after optimized holding time and after the application of siphon filtration (using different filters) was analysed for the presence of OVM

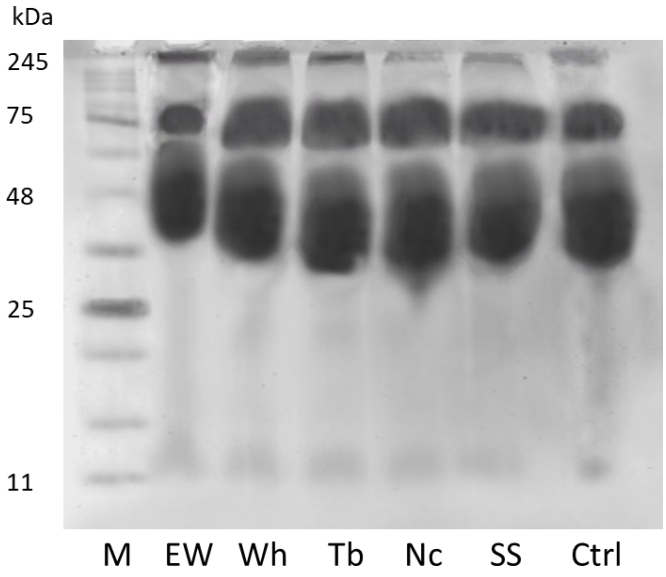

Figure 4.8: SDS-PAGE Gel for types of filters for siphon filtration (lane 1 – marker, lane 2 – egg white, lane 3 – Whatman filter paper, lane 4 – Teabag filter, lane 5 – Nitrocellulose membrane filter, lane 6 –Stainless steel filter, lane 7 – Control (centrifuge). SDS-PAGE was performed using 12% separating gel. The samples were analysed for protein content by BCA protein analysis and loaded accordingly into the wells as described in protocol (Figure 4.7).

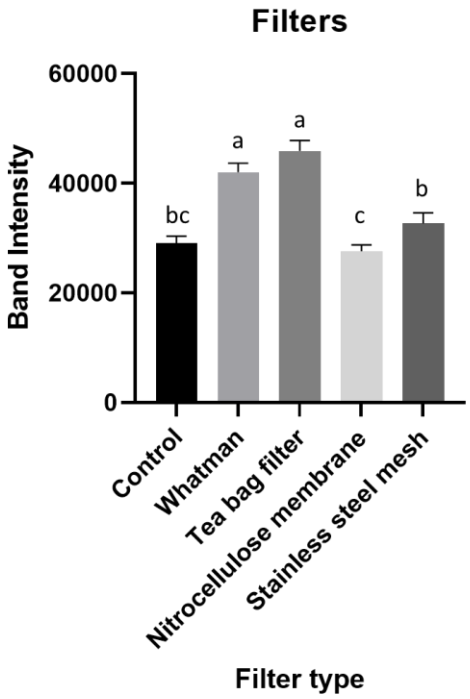

Figure 4.9: Bar graph for comparing the supernatants after the optimized holding time and the application of siphon filtration (using different filters). The band intensity is of OVM that remains in the supernatant after performing siphon filtration

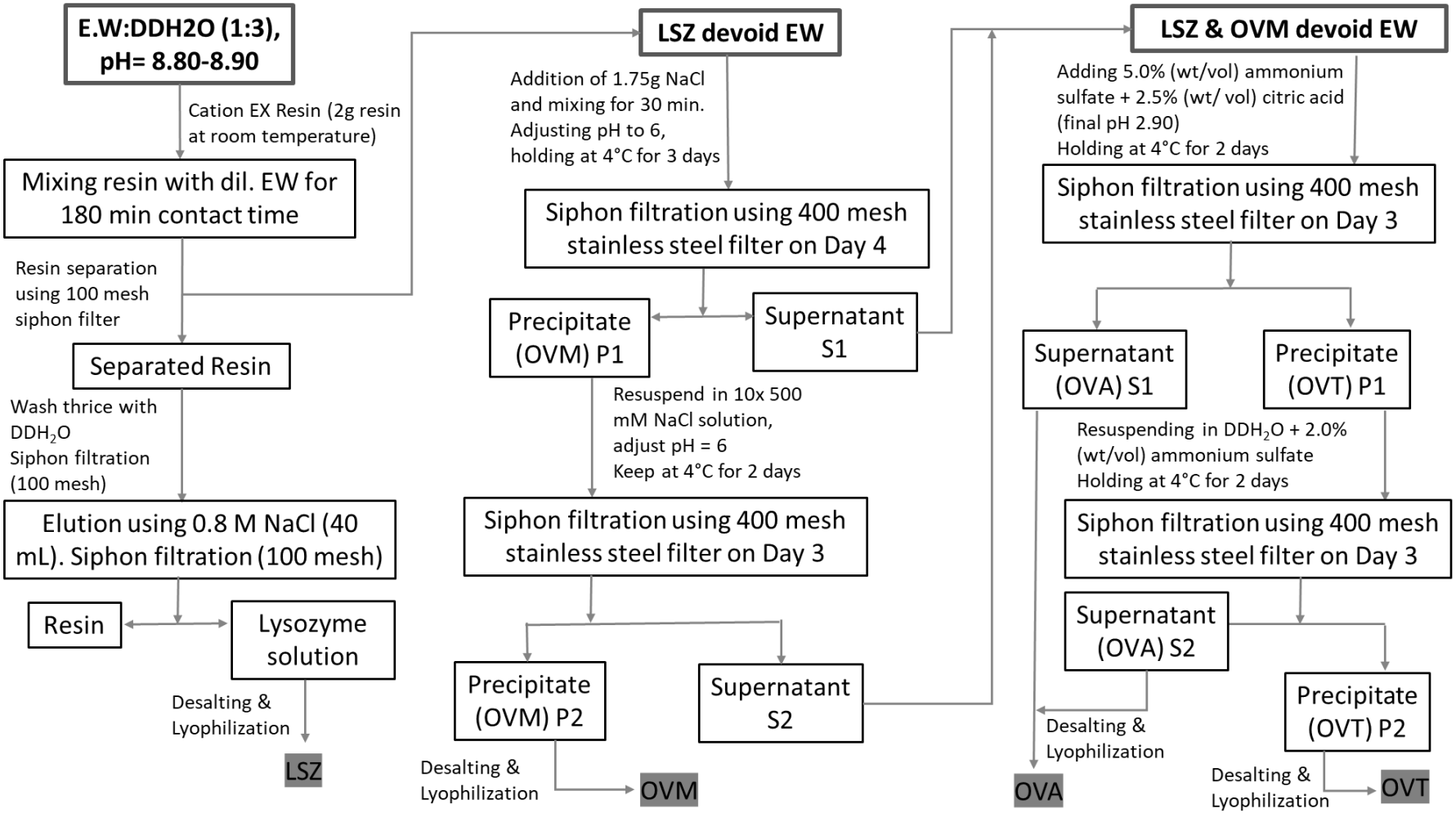

Figure 4.10: Overall extraction process using siphon filtration

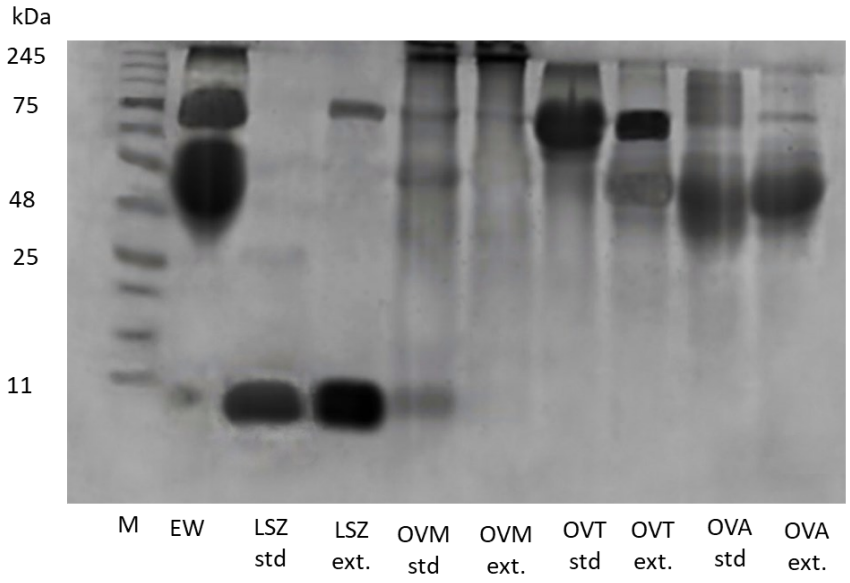

Figure 4.11: SDS-PAGE analysis for overall extraction using siphon filtration (lane 1 – marker, lane 2 – egg white, lane 3 – LSZ standard, lane 4 – LSZ extracted, lane 5 – OVM standard, lane 6 – OVM extracted, lane 7 – OVT standard, lane 8 – OVT extracted, lane 9 – OVA standard, lane 10 – OVA extracted. SDS-PAGE was performed using 12% separating gel. The samples were analysed for protein content by BCA protein analysis and loaded with equal protein content into the wells.

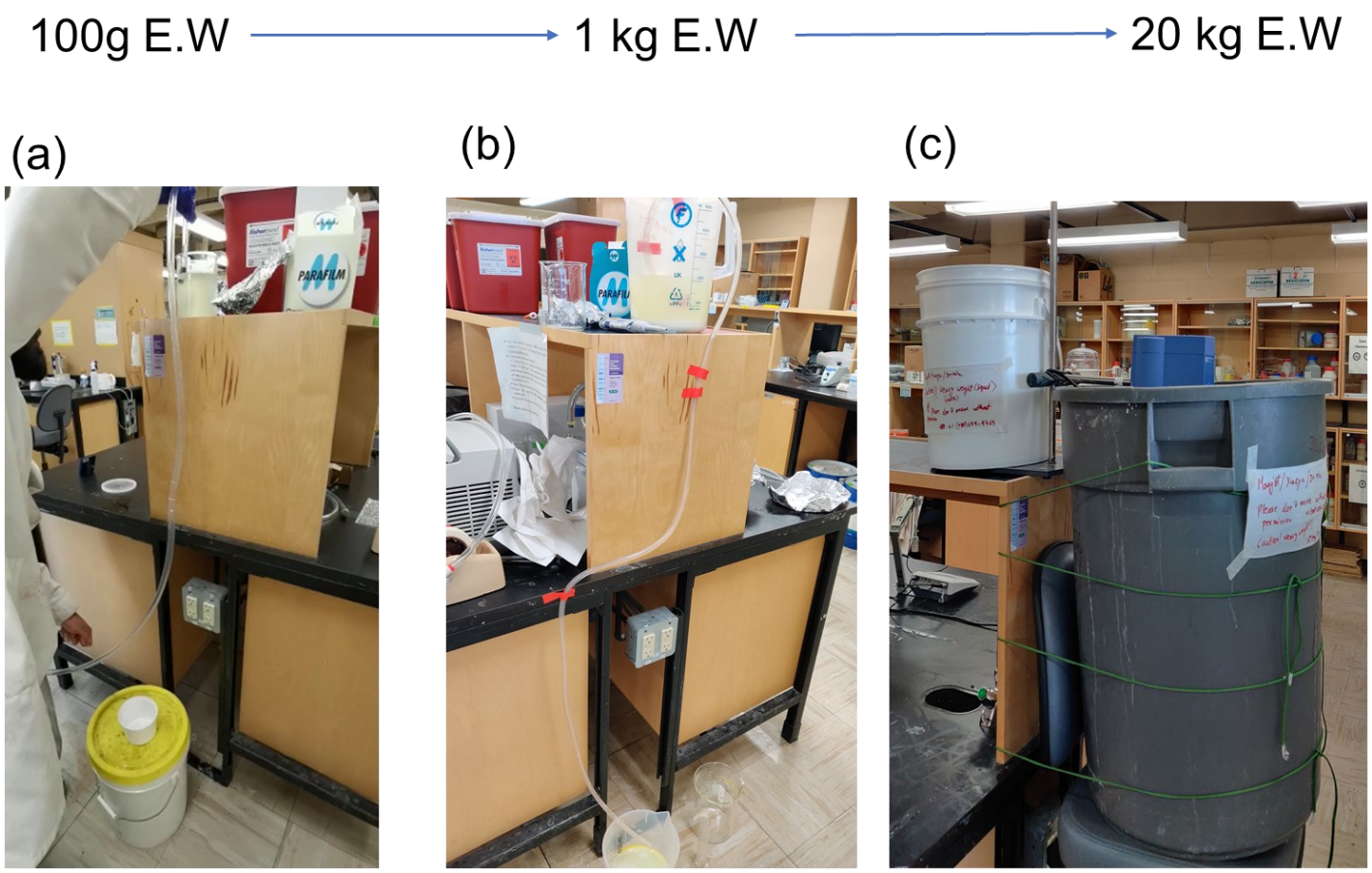

Figure 4.12: Step wise scale up in a normal lab setting, showcasing the minimal experimental needs. The tubes in (a) and (b) are siphon filter pipes. In (a) and (b), the supernatants are being separated from the precipitates without the using of centrifuge or electricity, by the force of gravity due to siphoning. In (c), the resin is being mixed with the egg white solution for LSZ extraction

Table 4.1: Band intensities of OVM in the supernatant after specific holding times

Sample	Band Intensity
Control (centrifuge)	22042 ± 1705^{d}
Day 0	43233 ± 1982^{a}
Day 1	37345 ± 3054^{ab}
Day 2	36749 ± 988^{ab}
Day 3	33116 ± 3791^{bc}
Day 4	27910 ± 1677^{cd}
Day 5	27632 ± 3234^{cd}
Day 6	25433 ± 2959^{d}

Given values are mean values and standard deviation of triplicate trials. Values in the same column with different letters were significantly different at $p < 0.05$. A lower OVM band intensity would indicate optimum holding time

Table 4.2: Band intensities of OVT in the supernatant after specific holding times

Sample	Band Intensity
Control (centrifuge)	24396 ± 1062^{c}
Day 0	47187 ± 3464^{a}
Day 1	37554 ± 6980^{ab}
Day 2	29548 ± 2488^{bc}

Given values are mean values and standard deviation of triplicate trials. Values in the same column with different letters were significantly different at $p < 0.05$. A lower OVT band intensity would indicate optimum holding time

Table 4.3: Band intensities of OVM that remains in the supernatant after siphon filtration using different types of filters

Sample	Band Intensity
Control (centrifuge)	29140 ± 1234^{bc}
Whatman	42034 ± 1652^{a}
Tea bag filter	45904 ± 1903^{a}
Nitrocellulose membrane	27646 ± 1145^{c}
Stainless steel mesh	32665 ± 1966^{b}

Given values are mean values and standard deviation of triplicate trials. Values in the same column with different letters were significantly different at $p < 0.05$. A lower OVM band intensity would indicate a better separation of precipitated protein from supernatant.

Table 4.4: Overall extraction using siphon filtration

Sample	100 g			1 kg			20 kg		
	Weight (g)	**^Purity (%)**	**^Yield (%)**	**Weight (g)**	**^Purity (%)**	**^Yield (%)**	**Weight (g)**	**^Purity (%)**	**^Yield (%)**
LSZ	0.220 ± 0.022	92.50 ± 1.69^{a}	58.41 ± 3.19^{a}	2.250 ± 0.101	90.50 ± 0.72^{a}	60.05 ± 2.01^{a}	45.27 ± 2.29	91.16 ± 1.33^{a}	60.66 ± 2.21^{a}
OVM	0.333 ± 0.009	84.97 ± 3.15^{a}	83.22 ± 3.13^{a}	3.500 ± 0.121	81.23 ± 6.93^{a}	81.23 ± 3.61^{a}	69.51 ± 8.62	76.75 ± 9.33^{a}	77.69 ± 2.95^{a}
OVT	1.303 ± 0.042	69.42 ± 4.49^{a}	75.83 ± 4.28^{ab}	14.03 ± 0.426	72.23 ± 4.06^{a}	84.53 ± 4.37^{a}	263.29 ± 20.0	66.98 ± 2.55^{a}	73.35 ± 2.89^{b}
OVA	5.650 ± 0.211	94.65 ± 3.78^{a}	99.1 ± 0.67^{a}	58.67 ± 4.26	91.02 ± 2.42^{a}	98.9 ± 0.86^{a}	51.35* ± 0.60	95.88 ± 1.36^{a}	91.16 ± 0.46^{b}

Given values are mean values and standard deviation of triplicate trials. Values in the same row with different letters were significantly different at $p < 0.05$

^The purity percentage represents the quantity of the target protein amongst other egg white proteins (impurities) present but does not account for the moisture and ash content. The crude protein weight and the purity of the protein were then used to determine the quantity of pure protein extracted from a particular amount of egg white. This quantity was then represented as "quantity of pure protein divided by the theoretical quantity of that protein present in egg white" to calculate the percentage yield.

5. THESIS CONCLUSION

The overall objectives of this study were to extract the four major proteins (lysozyme, ovomucin, ovotransferrin and ovalbumin) from egg white by optimizing/modifying the currently used methods in such a way that the scale up of the process becomes economical and sustainable without affecting the yield and purity while scaling up. The key findings of each study are listed below:

5.1 Extraction of egg white proteins- Optimization

The four major proteins of egg white have numerous applications in the fields of food, nutraceuticals, vaccines, pharmaceuticals, antimicrobials, and so on once they are extracted from egg white (Wu 2019). In order to minimize the complexity and cost of operations, the currently employed methods of extraction needed optimization/modifications. For the extraction of LSZ, the resin dosage, contact time and washing conditions were optimized, whereas for OVM, LSZ devoid egg white was used for finding the best extraction conditions by varying pH and precipitation steps (1x and 2x). Also, in case of OVT and OVA extraction, the heating step was eliminated from the method by Abeyrathne et al. 2013, 2014 which further helps reduce the number of processing steps and overall cost of operation/setup. Also, there were minor modifications like conducting extraction of LSZ at room temperature and without the use of any pH gradient washing solvents. By designing the process in such a way, we could achieve a solvent free and heat free extraction, and the yields and purity were comparable to the earlier methods.

5.2 Pilot scale extraction of the major egg white proteins- Application of siphon filtration as a separation technique for scale-up

Based on the previous studies, the extraction of high purity OVM from egg white requires high speed centrifuge (at least 15,300g or 10000 RPM) which limits the scalability and ease of operation (Sleigh et al. 1973; Kato et al. 1975; Omana and Wu 2009). This is also due to the factors like the

need for expensive and bulky equipment like chromatography columns, ultracentrifuge units and so on. Our study aimed to explore the use of siphon filtration for separating the precipitates of OVM and OVT as well as the separation of resin particles from the egg white after LSZ extraction. Although this alone cannot be a replacement for centrifugation but combining it with a longer sedimentation time to allow the protein particles to nucleate and precipitate in larger quantities could help ease the separation (Glatz et al. 1986). Doing so helped with the scale up and handling of large quantities of egg white in a very efficient way as there were no special requirements for centrifugal capacity or speed and the siphon filter operates without electricity or any moving parts (lower operational, maintenance and setup costs).

5.3 Significance for food industry and future research

The application of siphon filtration for separation of protein aggregates has not been explored extensively. This method of separation although traditional, could help in separation of aggregates when combined either with prolonged sedimentation time or by heat denaturation as was done in a study on whey separation for cheese making using cheese cloth bag (Ong et al. 2018). Cheese making involves curding of milk by addition of lactic acid bacteria that induces coagulation and 'curd' like protein network formation. This is then separated by using cheese cloth bag. This was also seen with OVT precipitation in case of egg white, as the OVT aggregates appear to have 'curd' like structure. However, we avoided the use of cheesecloth as the aggregates would get entrapped in the cloth layers and then recovering the aggregates would be troublesome.

5.4 Limitations and approaches to overcome them

The theoretical content of the target egg white proteins was used in all calculations based on the data available from the literature. However, actual content of these proteins in the egg white was not measured which could be done in the future to have more accurate yield calculations. Siphon filtration has not yet been explored thoroughly for different proteins separations; hence limited literature is available. Siphon filtration as a separation technique could result in a lower yield in certain cases but would help reduce the equipment and processing cost and ease the scale up. Also, by further redesigning of the filter (filter tips) and by increasing separation pressure by using suction pumps a better separation may be achieved. The scale-up was performed from 100 g to 20 kg which can be considered pilot scale-up (200x), which was unique for this study as no literature is available for a stepwise scale up to 20kg egg white. However, further work is required to check for the scalability to industrial scale like 100 kg or 200 kg egg white so that siphon filtration can be evaluated for its industrial design, cleaning, maintenance, and automated operation. Since this study covered a wide range of protein extractions and their respective scale-ups, only SDS-PAGE was performed to reduce the complexity of sample analysis. However, analytical techniques like FPLC or HPLC can further be used to understand the presence of other minor proteins as well as the composition of trace elements present in the extracted protein samples which could result in more accurate purity and yield calculations.

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