University of Alberta

N-glycosylation and gelling properties of ovomucin from egg white

by

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A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Master of Science

in

Food Science and Technology

Department of Agricultural, Food and Nutritional Science

Fall 2011

Edmonton, Alberta

Dedication

To Ron, my shining sun on cloudy days

Abstract

Ovomucin is a bioactive egg white glycoprotein responsible for its gel-like properties and is believed to be involved in egg white thinning, a natural process that occurs during storage. Ovomucin is composed of two subunits: a carbohydrate-rich β-ovomucin and a carbohydrate-poor α-ovomucin. N-glycosylation of ovomucin was studied by nano LC ESI-MS, MS/MS and MALDI MS. Both proteins were N-glycosylated and site-occupancy of 18 potential N-glycosylation sites in α-ovomucin and two sites in β-ovomucin was determined. N-glycans of α-ovomucin were bisected complex-type glycans, while $GlcNAc_6Man_3$ was the most abundant glycan.

In the second part, rheology and microstructure of aqueous ovomucin dispersions were studied. Ovomucin formed a fibrous network with weak gel properties in distilled water at room temperature and shear thinning behaviour, suggesting that ovomucin can be used as a thickener and stabilizer in various applications. In addition, influence of salt on ovomucin gel and its isoelectric point was determined.

Acknowledgements

I would like to thank my supervisor Dr. Jianping Wu for his advice, encouragement and guidance throughout my program that made the completion of this work possible.

I highly appreciate the advice and suggestions I received from my supervisory committee members Dr. Mirko Betti and Dr. Michael Gaenzle. I would also like to thank Dr. Betti for allowing my access to his rheometer and Dr. Yang Liu for being my external examinator.

Special thanks to Messele Fentabil for contributing his experience and knowledge in the area of mass spectrometry, MS results analysis and writing; to Dr. Jiapei Wang and Dr. Morshedur Rahman from whom I learned a lot.

I am very grateful to all the wonderful people in the lab for their tremendous help and support during this time: Sahar Navidghasemizad, Kaustav Majumder, Jiandong Ren, Alexandra Acero-Lopez, April Milne, Dr. Shengwen Shen, Chamila Nimalaratne, Nandika Bandara, Dr. Aman Ullah, Dr. Chanchan Wang, Dr. Satyanarayana Bejjani, Dr. Sunjong You, Sen Li, Wenlin Yu, Yuchen Gu, Mejo Remanan, Ali Akbari, Justina Zhang, Xiuran Wang, Bushra Malik and Qi Yi Li.

I would also like to thank Dr. Urmila Basu, Dr. Spencer Proctor and Kristina MacNaughton for access and help with Zetasizer, Dr. Jocelyn Ozga and Alena Jin, Dr. Randy Whittal and Jing Zheng from Mass Spectrometry Laboratory, Department of Chemistry; and Dr. Ming Chen for his help with electron microscopy.

I would like to express my appreciation to the Poultry Research Centre (PRC), National Science and Engineering Research Council of Canada (NSERC) and Alberta Livestock Meat Agency Inc. (ALMA) for providing financial support for the research and the Graduate Student Association for the very helpful childcare subsidies.

I am thankful to my parents Anatoly and Inna Tolchinsky, my grandmother Irina, brother Igor and my in-laws Regina and Dr. Yevsey Offengenden for their encouragement. Above all I would like to thank my husband Bernard Offengenden for his encouragement, support and understanding and my son Ron for always making me smile and lifting my spirits.

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Abbreviations

Asn: asparagine

Asp: aspartic acid

CK: cystine knot

CS domain: cysteine rich domain

DLS: dynamic light scattering

DTT: dithiothreitol

EGF: epidermal growth factor

ER: endoplasmic reticulum

ESI: electro-spray ionization

Fuc: fucose

Gal: galactose

GalNAc: N-acetylgalactosamine

Glc: glucose

GlcNAc: N-acetylglucosamine

Glu: glutamic acid

Hex: hexose

HexNAc: N-acetylhexosamine.

LC: liquid chromatography

MALDI: matrix-assisted laser desorption/ionization

Man: mannose

MS: mass spectrometry

OSM: ovine submaxillary mucin

PGM: pig gastric mucin

PNGase F: peptide N-glycosidase

PTS domain/region: mucin domain

RER: rough endoplasmic reticulum

SDS: sodium dodecyl sulfate

SEA domain: sperm protein, enterokinase, and agrin domain

SEM: scanning electron microscopy

Ser: serine

TEM: transmission electron microscopy

Thr: threonine

TOF: time of flight

Trp: tryptophan

VWF: von-Willebrand factor

Chapter 1. Literature review

1.1 The mucins: Genes and structure

1.1.1 The mucin family

Mucosal cells in the digestive, respiratory, reproductive, urinary tracts and the eye are protected by a glycocalyx of glycolipids and glycoproteins and a layer of viscous mucus gel – a physical barrier that provides lubrication and protection against microorganisms, chemical and mechanical damage. Mucins are a family of glycoproteins characterized by a presence of a large amount of O-linked glycans on a special domain rich in serine and threonine [1-3]. Currently there are over 20 genes in the mucin family according to Human Genome Organization Gene Nomenclature Committee (HGNC) (http://www.genenames.org/genefamilies/MUC). Human mucin genes are designated as MUC, while those from other species as Muc [4]. The mucins are classified into secreted gel-forming, secreted non-gel forming and membrane bound mucins. Gel-forming mucins are the major components in the mucus that are responsible for its ability to form protective gels. The mucin composition in the mucus and their glycosylation can change according to physical location and the physiological state of the organism and therefore respond to various conditions such as bacterial colonization [3]. The membrane-bound mucins are constituents in the glycocalyx, where they limit access to the cell surface, serve as receptors for bacterial adhesion and involved in signal transduction [1-3]. The

following sections will deal with the structure of the gel-forming secreted and the membrane bound mucins.

1.1.2 Secreted gel-forming mucins

There are five secreted gel-forming mucins: MUC2, MUC5AC, MUC5B, MUC6 that are located on 11p15 [5] and a relatively recently discovered MUC19 that is located on 12q12 [6]. MUC2 is a main component of the intestinal and colonic mucus, secreted from goblet cells. Its transcription is influenced by various factors such as microorganisms and their by-products, hormones, toxins and cytokines [2]. MUC5AC and MUC6 are main constituent in the gastric mucus [2], although MUC5AC is a major component in ocular mucins [7], respiratory tract and cervical secretions as well [8]. MUC19 is expressed in salivary glands and is the largest gel-forming mucin with over 7000 amino acids [6]. Gel-forming secreted mucins share a common domain structure of the polypeptide. A schematic representation of the domain organization of secreted gel-forming mucins is presented in Fig. 1-1.

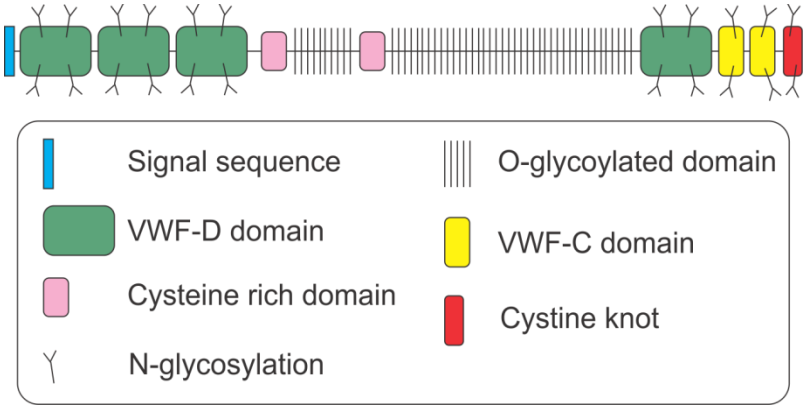

Figure 1-1: Schematic domain representation of MUC2, as an example of domains present in gel-forming secreted mucins. Adapted from Dekker *et al.*[5].

The mucin domains, also termed PTS regions due to high abundance of proline, threonine and serine, are composed of repeated tandem peptides where the length, number and amino acid sequence differ among mucins. These domains provide attachment sites for O-glycans that confer these domains with semi-rigid extended conformation devoid of secondary structure [5, 9]. The PTS domain shows little conservation; therefore evolutionary studies of mucins are carried out by studying the cysteine rich domains located on both sides of the PTS domain [10]. The amino terminal and the carboxy-terminal domains contains VWF D, C and CK domains, rich in cysteines that are involved in formation of intra- and inter- chain disulfide bonds. Therefore, these domains are believed to be involved in dimerization and further polymerization of the mucin to form long polymers, essential for gel formation [6, 9, 11, 12]. The first step in mucin polymerization is the formation of disulfide – linked dimers of two mucin monomers through their cysteine knots that are located in their carboxy-terminal region. The dimers undergo further polymerization by forming interchain disulfide bonds with their VWF-D domains [9]. This oligomerization leads to formation of a linear polymer, although the formation of branched oligomers through D domains was suggested as well [13].

1.1.3 Membrane-bound mucins

Membrane-bound mucins constitute the glycocalyx where they present beyond other glycocalyx components due to their linear structure. This configuration prevents access to these components and enhances the mucosal barrier [3]. Like the gel-forming secreted mucins, membrane-bound mucins possess a heavily O-

glycosylated extracellular mucin domain rich in serine and threonine. However, they contain other domains that do not appear in gel-forming mucins such as SEA (sperm protein, enterokinase, and agrin) modules, EGF (Epidermal Growth Factor) modules, a membrane-spanning domain and a cytoplasmic tail [14]. During biosynthesis the membrane-bound mucins undergo proteolysis in the SEA domain, possibly by an autocatalytic process [15]. As a result the extracellular domain is noncovalently linked to an extracellular stem that is linked to the transmembrane domain [14] and can be released into the mucus by additional proteolysis or shearing forces in the SEA domain [1].

Membrane-bound mucins vary in size from smallest mucin - MUC1 to large mucins such as MUC3A, MUC3B, MUC4, MUC11, MUC12, MUC16 and MUC17. MUC4 has a NIDO, AMOP and VWF-D domains which do not appear in other membrane-bound mucins [16]. Many membrane-bound mucins are associated with cancer, such as MUC1. It is expressed in most epithelial cells but over expressed in significant number of breast, ovarian and other carcinomas. MUC16, the largest membrane bound mucin, is used as a marker in ovarian cancer, progression of which is associated with increased levels of MUC16 in the serum [4].

1.2 Mucin glycosylation

1.2.1 Glycosylation types in mucins

Protein glycosylation is a complex co- and post translational modification. It is an enzymatic process influenced by various factors that depend on the species, cell type and its physiological state [17, 18]. Glycan types are defined based on the

linkage type of the glycans to the protein. The most common are N-linked glycans attached to the peptide through a nitrogen of an asparagine and O-linked glycans that are connected through an oxygen of serine or threonine [19]. Both N- and O-glycans are present on mucins, the former on the WVF D, C and CK domains, while the later on the mucin domains. In addition, C-mannosylation of the cysteine rich (CS) domain was suggested and involves covalent attachment of mannose to a carbon of the first tryptophan in a sequence of Trp-X-X-Trp. However, C-mannosylation was not observed on a mucin in vivo [13].

1.2.2 N-glycosylation

N-glycosylation is a co-translational modification of proteins that takes place in the endoplasmic reticulum (ER). The biosynthetic pathway of N-glycosylation in mammalian cells initiates with a transfer of a fourteen monosaccharides core - $Glc_3Man_9GlcNAc_2$ to an asparagine on the nascent polypeptide through N-glycosidic bond. The asparagine has to be a part of a consensus sequence of Asn-X-Ser/Thr (where X is any amino acid except proline) in order to be recognized by the oligosaccharyltranferase enzyme. However, presence of a consensus sequence does not guarantee its glycosylation as 10-30% of the potential glycosylation sites are not occupied. Site occupancy is influenced by various factors such as the identity of X (proline prevents glycosylation, while the presence of Trp, Asp and Glu reduce its efficiency), enzymes levels, primary and secondary structure of the protein and the presence of disulfide bonds [20, 21]. The core $Glc_3Man_9GlcNAc_2$ oligosaccharide is subjected to further processing after its attachment to the peptide: three glucosyl residues are removed by α-

glucosidases in the ER and further removal of sugars by exoglycosidases and addition of sugars by glycosyltransferases takes place in the ER and Golgi. Sugars added in the Golgi include N-acetylglucosamine, galactose, sialic acid and fucose [18, 21]. As a result, although N-glycans have a common core structure of $Man_3GlcNAc_2$ they are classified into high-mannose, complex and hybrid types. The $Man_3GlcNAc_2$ core of high-mannose type glycans are substituted by only mannose residues, while no mannose is attached to the core of complex-type glycans. Instead, the trimannosyl on the core is substituted by N-acetylglucosamines forming two to five antennae that can be substituted by other monosaccharides such as galactose, sialic acid and fucose. Hybrid-type glycans have mannose residues attached to one of the core mannoses, while the other is substituted by GlcNAc antennae [22].

N-glycans are important for inducing correct folding of proteins during synthesis and contribute to stability of folded proteins, however are not essential for maintaining a folded structure of already folded proteins [21].

In gel-forming mucins N-linked glycosylation occurs at the cysteine-rich non-mucin domains: D-, C- and CK domains [13]. N-glycans are involved in efficient mucin oligomerization in the rough endoplasmic reticulum (**RER**) by inducing proper protein folding that facilitates formation of disulfide bonds by disulphide isomerase, as shown in the rat gastric mucin [23]. In addition to the presence of glycans, mucin oligomerization also depends on the presence of glycans on specific sites, especially near areas that are important for folding. This was demonstrated for rat intestinal mucin – MUC2, where specific N-glycosylation

site near the cystine knot (CK) affected the rate of dimerization. Dimer formation was accelerated when that glycan was missing, however the products were unstable and dissociated after secretion [12].

1.2.3 O-glycosylation

Mucin type O-glycans are attached via α-GalNAc to a hydroxyl group of serine and threonine. O-glycosylation occurs in the Golgi and is initiated by a transfer of GalNac to the protein by N-acetyl-α-D-galactosaminyltransferases, up to 20 isoforms of which exist. The expression of these enzymes is tissue specific and their activity is specific to the attachment site on the protein; therefore they influence O-glycosylation site occupancy and density [24]. Unlike N-glycosylation, no consensus sequence is required for O-glycosylation, although presence of proline near the site of glycosylation is favourable [25]. Further substitution of GalNAc with Gal, GlcNAc and GalNAc leads to formation of at least eight core structures which can undergo elongation and termination by Fuc, Gal, GlcNAc, GalNAc, sialic acid and sulphate, leading to a wide variety of O-glycans, some of which are blood group and tissue antigens [26].

O-glycosylation of mucins (maturation) occurs in the Golgi after N-glycosylation and oligomerization in the RER [23]. The high degree of O-glycosylation of a domain rich in serine and threonine is the main characteristic of the mucins, where the glycans can comprise over 50% of their molecular weight. [27].

O-glycans in mucins play many important roles and highly influence their biological and physical properties. O-glycosylation of mucins can change as a result of disease. Cancer-associated O-glycans show increased sialylation,

decreased sulfation, truncated structures such as GalNAc and Galβ1-3GalNAc (T and Tn antigens, respectively) also in sialylated form [28]. In addition, O-glycans may serve as receptors for bacterial adhesins and therefore involved in bacterial interactions with mucins present in mucus and on cell surfaces [3].

Besides their biological roles, O-glycans are important in protecting the mucin from proteolysis by reducing accessibility of proteases. In addition, they increase the solubility and hydration of mucin peptide and induce a specific conformation to the molecule. Native mucin exists as a relatively stiff, highly expanded random coil configuration that derives from steric interactions between the O-linked GalNAc and the peptide. In contrast, fully deglycosylated mucin was more flexible with a conformation typical to denatured globular proteins. This expanded random coil structure is believed to be very important for the viscoelastic properties of the mucins due to formation of entanglement networks [29, 30].

1.3 The biophysical properties of gel-forming secreted mucins

1.3.1 The structure and conformation of mucins in solution

Mucins from different species and organs exhibit similar structure of long extended threads [31]. Long linear threads polydisperse in size were obtained from pig gastric mucin, human cervical and bronchial mucins [32], while in vitro study of MUC5AC from a cell line showed similar structures with high degree of oligomerization and extended conformation. These 10 μm-long threads became approximately 570 nm long upon reduction, exhibiting stiff coil conformation [8]. As other mucins, polymer length of ocular mucins showed heterogeneity and

decreased upon reduction with dithiothreitol (DTT) [7]. As mentioned above, this conformation is highly influenced by the presence of O-linked carbohydrates [29, 30]. However, not only the carbohydrates are responsible for the conformation of mucins: the cysteine-rich non-mucin domains are believed to be involved in conformational changes that occur in mucin as a result of changes in pH and ionic strength. In dynamic light scattering (DLS) studies conducted on pig gastric mucin (PGM) in dilute solutions (≤5 mg/mL), a conformation change at pH 4 was observed. While at pH 7 the mucin existed in random-coil conformation with persistence length of 8 nm, a transition towards an anisotropic extended conformation occurred as the pH decreased below 4 and reached a persistence length of 43 nm at pH 2 [33]. In addition, acidification induced a conformation change in the structure of the unglycosylated regions of PGM in dilute solution (1 mg/mL) resulting in a loss of the tertiary structure in this region and exposure of hydrophobic residues. These changes were suggested to be important for the gelling ability of PGM at higher concentrations [34].

1.3.2 The gelling properties of secreted gel-forming mucins

The most important property of the mucins is their ability to form viscoelastic gels [9], which is influenced by various internal factors such as molecular weight, the carbohydrate side chains, degree of polymerization and concentration as well as environmental conditions such as pH and ionic strength.

1.3.2.1 Influence of mucin concentration on gel formation

A transition from sol to gel with an increase in mucin concentration was observed for human tracheobronchial mucin at concentration of approximately 14 mg/mL. Below this concentration the mucin exhibited behaviour of a viscoelastic fluid, while at high concentration of above 20 mg/mL tracheobronchial mucin formed a typical weak gel [35].

1.3.2.2 Influence of polymeric structure and carbohydrate chains on gellation

Mucin gels are highly influenced by the polymeric structure of the mucin. Disruption of the polymeric structure by enzymatic digestion and reduction induces mucin solubilisation and transition from a weak gel towards a viscous liquid occurs.

In addition to covalent bonds, gelling properties are influenced by the presence of noncovalent interactions suggested to originate from the carbohydrate side chains of the mucin [36]. Different mucins differ in lengths of the oligosaccharide side chains that can be as short as two monosaccharides in ovine submaxillary mucin (OSM) [29] or as long as 20 sugars in human tracheobronchial mucins [35]. This variation in length has little influence on the size of mucin in solution [37], however has great impact on the viscoelastic properties of the mucin gel [30]: submaxillary mucin with only two sugar residues showed a mechanical spectrum characteristic of an entanglement network, in contrast to a weak gel of a mucin with five-sugar residues [38]. In addition, the elasticity of tracheobronchial mucins increased with increasing length and branching of the glycan side chains at comparable concentration and molecular weight [35].

1.3.2.3 pH-dependent gellation of porcine gastric mucin

Although the carbohydrate side chains play an important role in the gelling properties of mucin, hydrophobic interaction between the carbohydrate-poor regions of the mucins seem to be the major factor that induces pH-dependent gellation of pig gastric mucin.

This ability of gastric mucins to form a gel at low pH is important in protecting the epithelium from the acidic environment of the stomach. Pig gastric mucin undergoes an increase in viscosity and aggregation at low pH [39]. DLS [33] and bulk rheology [40] studies determined that a pH dependent sol-gel transition of concentrated solutions of porcine gastric mucin took place at pH 4 and involved an increase in surface hydrophobicity and a change towards a more extended conformation of the mucin. According to proposed model, at neutral pH, hydrophobic regions in the non-glycosylated mucin parts are hidden in folds that are stabilized by electrostatic interactions. These folds are stabilized by salt bridges between acidic amino acids (Asp, Glu) with pKa≈4 and positively charged amino acids on the protein. Upon acidification, disruption of these bridges leads to exposure of the hydrophobic regions which in turn associate to form a gel [11, 33].

1.3.2.4 Influence of salts on mucin gels

Salts have negative influence on mucin gels, leading to a decrease in their rheological properties [35, 39, 40]. This was explained by screening of electrostatic interactions as a result of salt addition. As a result, a decrease in repulsion between the carbohydrate side-chains occurred, leading to a less

extended conformation of the protein. In addition, stability of salt bridges is affected and influences protein folding and its ability to form intermolecular interactions. Overall, addition of salt decreases cross-linking and entanglement on the mucin chains [40].

1.4 Biological roles and applications

1.4.1 Mucins in interaction with bacteria

The mucus covering epithelial cells is the first defence line against pathogens, physical and chemical damage [2]. The viscous properties of the mucus gel are conferred by the gel-forming mucins and constitute a physical barrier that can trap microorganisms and incorporate antibacterial substances. The microorganisms can be further eliminated by movement of the outer mucus layer [1]. In the stomach and the colon the mucus is organized in two layers: the first layer closer to the lumen is loose and subjected to degradation by the intestinal microflora and to removal with the intestinal content, while the second is strongly attached to epithelial cells [41]. In the colon, this inner layer is dense and free of bacteria that might present in the outer, less dense layer. The major component in both layers is MUC2 mucin and it was suggested that the outer layer is formed from the inner layer by proteolysis [42]. Lower density of the outer layer enables its colonization by commensal bacteria that was suggested to be selected based on the glycan profile of the mucin [41]. O-glycans of human intestinal tract mucins exhibited core 3 as the most abundant structure. However glycan length and terminal epitope varied in different regions: glycans from the ileum were fucosylated, while those from distal colonic mucins were sialylated and sulphated. This

diversity was suggested to be important in region-specific bacterial colonization in the intestine [43]. In addition, O-glycosylation of MUC2 from sigmoid colon showed little variability between individuals, while high variability was observed in glycans from mucins in other locations. Uniformity of MUC2 O-glycosylation in the distal colon might be involved in determination of the commensal bacteria which is similar between individuals. This stands in contrast with other locations where the glycans of the mucins are involved in trapping of pathogens and require a broad spectrum of glycans [44].

In addition to determining commensal microbiota, the glycans of secreted mucins provide with 'decoy ligands' for binding of pathogens and prevent their adhesion and invasion into epithelial cells [3]. These properties of the mucus and the mucins can find a potential application as a way to prevent invasion of various pathogens, such as *Campylobacter jejuni* - an enteric pathogen in humans but a commensal in chicken. It was demonstrated that chicken intestinal mucus can inhibit *C. jejuni* penetration into human epithelial cells, possibly due to the 'trapping' ability of chicken mucus, which is different from human mucus [45].

1.4.2 Biomedical applications of mucins

1.4.2.1 Mucoadhesion and drug delivery

Mucins play an important role in drug delivery, as diffusion through mucus and interactions with it highly influence drug absorption [11]. The attractive interaction at the interface between a pharmaceutical and a mucosal membrane is defined as mucoadhesion. Mucoadhesion was suggested to occur in several steps.

First step involves wetting and swelling of the dosage, followed by establishing non-covalent bonds within the mucus. Afterwards, interpenetration into the mucus and entanglement that is followed by formation of covalent and non-covalent bonds occurs. Mucoadhesive polymers should be able to form hydrogen bonds and possess strong anionic or cationic charges, high molecular weight, chain flexibility and appropriate surface energy [46]. One such polymer is chitosan – a cationic polysaccharide widely used in biomedical and drug delivery applications. The mucoadhesive properties of chitosan were attributed to electrostatic attractive forces, hydrogen bonds and hydrophobic interaction [47]. In addition, gelatin-mucin water-swelling microspheres for controlled release of a drug by rectal drug delivery were described. Inclusion of mucin into the delivery system improved drug entrapment and release in comparison to gelatine alone [48].

1.4.2.2 Mucin coatings on polymeric materials

Mucins are able to form a coating on hydrophobic surfaces through the hydrophobic non-glycosylated domains that adsorb to the surface, while the hydrophilic glycosylated domains are faced towards the aqueous medium. This can be utilized for changing the properties of surfaces and prevent adhesion and colonization by pathogens. Coating of bovine submaxillary gland mucin decreased the hydrophobicity of the surface and suppressed adhesion of *Staphylococcus aureus* and *S. epidermis*, colonization by which imposes a risk in surgical procedures and implants [49]. In another study, bovine salivary gland mucin, porcine gastric mucin and human salivary mucins coated on polymeric biomaterial suppressed adhesion of neutrophils to the surface [50].

1.4.2.3 Mucins as biomarkers for cancer

Both secreted and surface-bound mucins carry modified glycan structure as a result of cancer. The major changes in glycosylation involve the formation of truncated and modified sialylated glycans. Both mucins such as MUC1 and MUC16 and carbohydrate structures such as SLe^a and STn antigens can be utilized as cancer biomarkers [51].

1.5 Ovomucin – the egg mucin

1.5.1 Ovomucin – the gelling agent of the egg white

The avian egg is a reproductive cell that contains all the nutrients needed for embryo growth and development. The unfertilized egg is composed of three main components: the egg shell and its membranes that provide the egg with its shape and prevent penetration of microorganisms, the egg albumen (egg white) and the yolk, a major source of nutrients with a blastodisc on its top [52]. The egg white is a viscous liquid that surrounds the yolk and protects it from mechanical shock and penetration of pathogens [53]. It is composed of two liquid fractions (thin whites) and a gel fraction (thick white) between. The viscosity of the thick white gives the egg white its viscous character and is conferred by a glycoprotein ovomucin [54]. In addition to the thick white, ovomucin is also present in thin white and chalaza [55]. However, ovomucin is unevenly distributed between the thick and the thin white. While most ovomucin is contained in the thick white, some ovomucin is present in the thin white in a soluble form [52]. Ovomucin is composed of two glycoproteins: a protein rich α-ovomucin with 15% carbohydrates and a carbohydrate rich β-ovomucin with approximately 57% carbohydrates [56].

Genetically, ovomucin can be regarded as a mucin-type glycoprotein, as it is a product of two genes located on a locus on chromosome 5 in the chicken. This locus is similar to the human 11p15 that contains MUC6, MUC2, MUC5B and MUC5AC, however in addition to the Muc6, Muc2, Muc5ac and Muc5b genes it contains an additional gene termed ovomucin that corresponds to α-ovomucin. α-ovomucin has a domain structure similar to Muc2, but lacks the PTS domain, while β-ovomucin is an orthologue of MUC6 [57].

1.5.2 The protein-rich subunit: α-ovomucin

Molecular mass of α-ovomucin is 254 kDa, as estimated from its amino acid sequence and carbohydrate composition of 9% [58, 59]. Other α-ovomucin components with molecular weights of 150 and 220 kDa denoted as α1 and α2-ovomucins, respectively [55] and α-ovomucin component with molecular weight of 350 kDa [60] were reported as well. In addition, α-ovomucin was identified in areas corresponding to molecular weights of 250-100 kDa in a mass spectrometric analysis of egg white [61]. The differences between these α-ovomucins remain unclear although it is possible that these might result from glycan heterogeneity and proteolysis.

The peptide of α-ovomucin is composed of 2087 amino acids with relative molecular mass of 230.9 kDa and possesses a domain structure. These are cysteine rich domains that are similar to D, C and cystine knot domains of human pre-pro-von Willebrand factor and human mucins such as MUC2. These domains may be involved in dimerization and polymerization of ovomucin through disulfide bonds [9, 58].

α-ovomucin contains 9.2-14.7% carbohydrates, predominantly N-acetylglucosamine and mannose, with small amounts of galactose and sialic acid. Due to its relatively high contents of mannose and N-acetylglucosamine, α-ovomucin is believed to contain N-linked glycans [55] that could be attached to the protein through 21 potential N-glycosylation sites [58], however their structures and glycosylation sites are unknown.

1.5.3 The carbohydrate-rich subunit: β-ovomucin

Up to date only a partial 827 amino acid sequence of β-ovomucin was submitted by Watanabe et al. (http://www.uniprot.org/uniprot/Q6L608). The molecular mass of β-ovomucin was reported to be 400-610 kDa, with high amount of threonine, serine and proline [55, 60, 62]. It is unknown whether β-ovomucin contains a mucin-like PTS domain, although O-glycosylated fragments rich is serine and threonine with resistance to proteolysis support its existence [59].

The carbohydrates of β-ovomucin are mainly composed of galactose, glucosamine, galactosamine, sialic acid and sulphate suggesting their O-glycosidic linkage to serine and threonine on the protein [19, 59]. Several O-glycans released by β-elimination were characterized as appear in Fig. 1-2 [63-65]. It is most likely that these O-glycans originated from β-ovomucin – the main source of N-acetylgalactosamine in ovomucin.

O-glycans play important roles in ovomucin. First, they protect ovomucin from proteolysis, that usually takes place in the carbohydrate poor regions [59, 66]. Second, as in other mucins, the glycans may confer ovomucin with its linear, random coil character [67] and contribute to its hydration [29]. Third, terminal

sialic acid contributes to the negative charge of ovomucin and its ability to interact with other proteins such as lysozyme. The interaction between sialic acid and positively charged lysyl amino groups in lysozyme were suggested to be involved in the formation of the ovomucin-lysozyme complex. In addition, sialic acid contributes to the viscosity of ovomucin [68, 69].

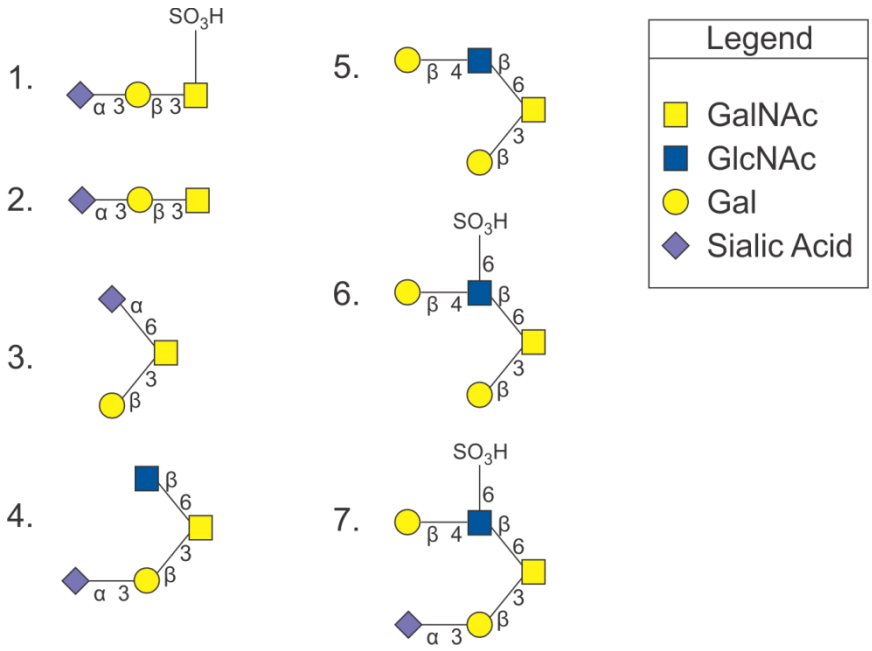

Figure 1-2: Structures of O-glycans of ovomucin. (1) Sialylated and sulfated core 1 structure [65]. (2, 3) Sialylated core 1 structures; (4 - 7) Core 2 structures [63, 64].

1.5.4 Ovomucin polymerization and rheology

Ovomucin is a linear molecule with random coil character and high degree of aggregation and polymerization [67], resulting in very high molecular weight of 240,000 kDa at pH 6.2 [70]. Treatment with reducing agents causes to

depolymerisation of ovomucin into subunits [67] and reduction in its viscosity [69, 71]. The presence of mucin-like non-glycosylated domains responsible for mucin polymerization [27] in α-ovomucin suggests their involvement in polymerization of ovomucin [58], although the existence of such domains in β-ovomucin is not known.

Ovomucin gel has a non-Newtonian character. A transition to Newtonian behaviour was obtained upon reduction, sonication, proteolysis and treatment with chaotropic agents and SDS, suggesting involvement of covalent and non-covalent interactions in gel formation. In addition, the polyanionic character of ovomucin conferred by sialic acid was suggested to be responsible for a viscosity decrease upon increasing ionic strength and removal of sialic acid [68, 69, 71]. Further studies on ovomucin gel rheology were not performed.

1.5.5 Egg white thinning – comparable process to the formation of 2 mucus layers?

The gel-like structure of albumen in fresh eggs becomes more liquid upon storage, a process accompanied by a rise in pH from 7.6 to 9.5 and termed "egg white thinning". As this process also occurs during incubation, it was suggested that it is facilitates transport of proteins [52] and water movement from the albumen into the embryo [53]. Several explanations to the process were suggested such as enzymatic proteolysis, interactions with lysozyme, and reduction of disulfide bonds, resulting in depolymerization [52, 72].

The organization of the egg white into thick and thin fractions reminds of the organization of colonic mucus into a dense layer that is attached to the epithelium

and a loose, more diluted layer. The loose layer was suggested to form from the thick layer by the action of proteolytic enzymes, possibly of internal origin [42]. It is interesting to note that the main mucin in the colon is MUC2 [42], similar to α-ovomucin – the main component of the thin egg white [58]. It is possible that egg white thins by a similar mechanism that is involved in the formation of the outer layer of colonic mucin, suggesting that degradation of α-ovomucin by internal proteolytic enzymes might be responsible for egg white thinning, although this enzyme was not found.

1.5.6 Biological properties

One of the main roles of the egg white is to protect the developing embryo against microorganisms. It is not surprising, therefore, that in addition to its gelling properties ovomucin has many anti-bacterial activities such as inhibition of stomach colonization by *Helicobacter pylori* [73] and the ability to bind *E. coli* O157:H7 through sialylated glycopeptides [74]. Anti-viral activities included hemagglutination inhibition activity against bovine rotavirus that required presence of a macromolecule containing both α- and β-ovomucins and against hen Newcastle disease virus by β-ovomucin only, while sialic acid contributed to the binding [75, 76]. In addition, anti-tumor activities of β-ovomucin were reported [77, 78].

1.5.7 Applications

Eggs are a rich source of nutrients such as proteins, lipids, minerals and vitamins contained in both the yolk and the albumen. In addition, eggs contain many bioactive components with nutritional, health and biotechnological benefits [79].

As outlined in COST action 923 “Multidisciplinary Hen Egg Research” of the Council of the European Union (http://register.consilium.europa.eu/pdf/en/02/st00/st00231.en02.pdf), extending the use of eggs beyond traditional food uses has economical benefits such as increasing viability of egg production and creating new employment opportunities, in addition to public health benefits. Along with other bioactive egg white proteins [80], the vast array of bioactivities of ovomucin suggests its potential for applications as a bioactive component in foods and nutraceuticals [72]. However, in addition to previously reported bioactivities, ovomucin has many other potential applications – an outcome of its unique structural properties. The structural similarity of ovomucin to mucin-type glycoproteins, combined with the fact that it can easily be extracted from eggs, suggests that there is a great potential in utilizing it as a mucin in application mentioned in part 1.4, such as an anti-adhesive coating for biomaterials and in drug delivery applications. However, utilization of ovomucin as a mucin-type glycoprotein requires further characterization of its structural and functional properties.

1.5.8 Objectives

The major functional property of mucins is their ability to form viscoelastic gels, which is influenced by factors such as O-glycosylation of the mucin domains [29, 30], pH [39] and their polymeric structure [36]. Mucin polymerization is influenced by N-glycosylation of the non-mucin domains [12, 23], as described in 1.2.2. It is known that α-ovomucin is similar to the non-mucin domains [58] and that β-ovomucin is an orthologue of human MUC2 [57]; however N-glycosylation

of these two glycoproteins in unknown. Therefore, the first objective of this study is to characterize N-glycosylation of ovomucin. Specific objectives are:

1. Determination of site-specific glycosylation and glycosylation site occupancy;
2. To assign characterized glycosylation sites into domain and to determine conserved glycosylation sites. This will allow better understanding of possible roles of N-glycans especially in polymerization of ovomucin - a main prerequisite for its gel-forming ability [67, 69];
3. Determination of the N-glycan profile and glycan structures.

Ovomucin is the gelling agent of egg white [54]; therefore gelation is its most important functional property. However, its ability to form gel and its rheological properties are not well studied. Therefore, the second objective of this study is to investigate the gelling properties of ovomucin. Specific objectives are:

1. To determine the rheological properties of ovomucin gel under different environmental factors such as salt concentrations and heating;
2. To understand ovomucin's gel forming ability;
3. To determine influence of pH and NaCl on ovomucin dispersions.

1.6 Literature Cited

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Chapter 2. N-glycosylation of ovomucin from hen egg white[1]

2.1 Introduction

Egg white is a gelatinous fluid surrounding the yolk in avian eggs. The hen egg white proteins, constituting approximately 11% of albumen's weight, provide the developing embryo with nutrients and protect it from physical damage and bacterial infection [1, 2]. Ovomucin, a glycoprotein that is responsible for the gel properties of the egg white [3], comprises approximately 3.5% of the egg white proteins [4]. Its high viscosity provides mechanical protection from penetration of pathogens into egg yolk [1]. Ovomucin also possesses anti-bacterial, anti-viral and anti-tumor activities [4-6]. It is composed of two components: a carbohydrate-rich β-ovomucin with approximately 60% carbohydrates and a carbohydrate-poor α-ovomucin with approximately 15% carbohydrates that form a linear high molecular weight polymeric structure via disulfide bonds [7, 8]. From rheological [8], structural [9] and genetic [10] aspects, ovomucin is similar to other gel forming mucins [11]. Mucins are high molecular weight glycoproteins responsible for the viscoelastic and gel-like properties of the mucus that covers epithelial cells thus protecting them from dehydration and microbial infection [12]. Characterized by a high amount of O-linked glycans linked via GalNAc to serine or threonine residues, mucin genes belong to the MUC gene family [13]. In addition to a central O-glycosylated domain (referred also as PTS domain), which contributes to the ability of mucins to interact with various microorganisms and

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their properties, non-PTS domains such as von-Willebrand-factor (VWF) domains, C-terminal domain and cysteine rich domains are present. These domains are conserved in mucins, contain N-linked carbohydrates and are important for their dimerization and polymerization [11, 14, 15].

Gel forming mucins in the chicken are coded from a locus that is similar to a corresponding locus of human mucins of MUC2, MUC5AC, MUC5AB and MUC6 located on chromosome 11 [10, 13]. It was determined that β-ovomucin is an orthologue of human MUC6; while α-ovomucin, a protein similar to MUC2 with a missing PTS domain, is coded by an additional gene that is inserted between Muc2 and Muc5ac [10]. Similar to non-PTS domains in other mucins, α-ovomucin contains von-Willerbrand factors D, C and cystine knot domains [9]. The reason for an additional mucin-type protein with only the non-PTS N-glycosylated domains in the egg white is unknown; however, it is possible that these domains along with the N-linked carbohydrates have a particular role in the egg white, such as hatching [9].

Several O-linked carbohydrates released from ovomucin by reductive β-elimination were characterized previously. These partially sulfated short oligosaccharides composed of 3-5 units of N-acetylgalactosamine, galactose, N-acetylglucosamine, and sialic acid [16-19], might contribute to the gelling properties of ovomucin in a similar manner to other mucins [8, 20], and to its ability to interact with various microorganisms. In contrast, information about N-linked glycans of ovomucin is not available. N-glycans are covalently linked to asparagines on the protein that are located within a consensus sequence of Asn-

Xaa-Ser/Thr, where Xaa can be any amino acid with the exception of proline. They share a common pentasaccharide core and can be classified as high-mannose-type, complex-type, and hybrid-type [21]. Many egg white proteins are N-glycosylated. Ovalbumin, the major egg white protein [2] has high mannose and hybrid type N-glycans [22-24]. Another major egg white protein is ovomucoid, an inhibitor of proteolytic enzymes [2]. It possesses complex type N-glycans composed of the pentasaccharide core and a bisecting GlcNAc, some of which have pentaantennary structure and galactose in a terminal position [25, 26]. α-ovomucin contains 24 potential N-glycosylation sites in its amino acid sequence [9]. The 827 amino acid fragment of β-ovomucin submitted to EMBL/GenBank/DDBJ databases by Watanabe et al. (http://www.uniprot.org/uniprot/Q6L608) contains 9 potential N-glycosylation sites; however, neither the N-glycan structures nor the N-glycosylation sites of ovomucin were investigated. In the present study, mass spectrometric techniques (LC-ESI MS, MS/MS and MALDI-TOF MS) were utilized to determine the major N-glycan structures, site occupancy and site-specific N-glycosylation of ovomucin.

2.2 Materials and Methods

2.2.1 Materials

Fresh eggs from White Leghorn laid within 24 h were obtained from the Poultry Research Centre of the University of Alberta (Edmonton, Canada). Sodium chloride was purchased from Acros Organics (Morris Plains, NJ, USA). Laemmli sample buffer, 20% SDS solution, molecular weight protein marker for SDS-

PAGE and iodoacetamide were purchased from BIO-RAD (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Hydrochloric acid and sodium hydroxide were bought from Fisher Scientific Inc. (Fisher Scientific, Ottawa, ON, Canada). Proteomics grade PNGase F from *Elizabethkingia meningosepticum*, ammonium bicarbonate, 2-mercaptoethanol, dithiothreitol and dimethyl sulfoxide were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sequencing grade modified trypsin was purchased from Promega (Madison, WI, USA). Acetonitrile (HPLC grade) was from ACROS (Fair Lawn, NJ, USA). Water used for the experiments was obtained from the Milli-Q water supply system (Millipore Corporation, Billerica, MA, USA).

2.2.2 SDS-PAGE

Egg white from fresh egg was manually separated from the yolk at the day of collection (within 24 hours of laying), vortexed and diluted to give an aqueous solution of 20% v/v egg white with 5% SDS and 1% β-mercaptoethanol. This solution was incubated overnight at 25 $^{\circ}$C, diluted in a ratio of 1:1 with Laemmli sample buffer containing 5% β-mercaptoethanol and loaded on 4-20% Mini-PROTEAN TGX precast gel (Bio-Rad Laboratories, Inc.). Loading volume was 20 µL and a molecular weight standard (Bio-Rad) was loaded to a separate well. SDS-PAGE was performed [27] in a Mini-PROTEAN tetra cell with a PowerPac Basic electrophoresis apparatus (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at constant voltage of 200 V. The gel was stained with Commassie Brilliant Blue and scanned in Alpha Innotech gel scanner (Alpha Innotech Corp., San Leandro, CA, USA) with FluorChem SP software.

2.2.3 In-gel PNGase F digestion

PNGase F digestion was performed according to Sigma-Aldrich technical bulletin for PNGase F with slight modifications. To briefly describe, excised gel bands of ovomucin subunits were transferred into low-retention microcentrifuge tubes (Fisher Scientific, Ottawa, ON, Canada) and destained by double incubation with 200 µL of 200 mM ammonium bicarbonate with 40% acetonitrile for 30 min. After drying in a Speed Vac (Savant Automatic Environmental SpeedVac, AES2000, Savant Instruments, Inc., Farmingdale, NY), 15 µL of prepared PNGase F solution (prepared by adding 100 µL of water to a vial containing 50 units of enzyme) was added, centrifuged briefly and incubated overnight at 37 °C. After overnight incubation, released carbohydrates were extracted with 200 µL of water four times, collected and lyophilized. Deglycosylated gel pieces were dried in a SpeedVac and subjected to digestion with trypsin.

2.2.4 In-gel trypsin digestion

Excised gel pieces containing the protein of interest with and without PNGase F treatments (destaining was carried out as for the PNGase F treated sample), were digested following a modified protocol [28] based on the method of Shevchenko et al. [29]. Briefly, the proteins in the gel pieces were reduced with 10 mM DTT, alkylated with 50 mM iodoacetamide, washed with 100 mM ammonium bicarbonate and acetonitrile, and dried in a SpeedVac. Dried gel bands were subjected to overnight proteolytic digestion at 37 °C with trypsin. Digestion was performed with 0.8 µg enzyme (solution of 20 ng/µL in 50 mM ammonium bicarbonate). After incubation, peptides were extracted into new microcentrifuge

tubes with 30 µL of 100 mM ammonium bicarbonate followed by extraction with 30 µL solution containing 5% formic acid and 50% acetonitrile in water twice and dried to ~15 µL in a SpeedVac.

2.2.5 Nano LC-ESI MS and MS/MS Analysis

The tryptic digest was analyzed by a hybrid quadrupole orthogonal acceleration time-of-flight mass spectrometer, QToF Premier (Waters, Milford, MA), online connected to Waters nanoACQUITY ultra high performance liquid chromatography (UPLC) system. The tryptic digest containing (glyco-) peptides (5 µL) was loaded onto a nanoAcquity UPLC system with peptide trap (180 µm x 20 mm, Symmetry® C18 nanoAcquity™ column, Waters, Milford, MA) and a nano analytical column (75 µm × 100 mm, Atlantis™ dC_{18} nanoAcquity™ column, Waters, Milford, MA). Desalting of the trapped sample was achieved by flushing the trap column with a solution of 1% acetonitrile and 0.1% formic acid in water (Solvent A) at a flow rate of 10 µL/min for 3 min. Peptides were separated with a gradient of 1- 65% solvent B (acetonitrile, 0.1% formic acid) over 35 min at a flow rate of 300 nL/min. The column was connected to a QToF premier (Waters Corporation) for ESI-MS and MS/MS analysis of the effluent.

2.2.6 Permethylation of N-glycans and MALDI-TOF MS and MS/MS

Permethylation of released N-glycans was performed according to Dell et al. [30] using the sodium hydroxide/dimethyl sulfoxide slurry method. Lyophilized permethylated glycans were dissolved in 20 µL of methanol and 0.8 µL of sample solution was spotted to an 800 µm Bruker's anchorChip™ MALDI target (Bruker Daltonics, Billerica, MA, USA). Then, 0.8 µL of matrix solution was then spotted

on top of the sample and left to dry. 2, 5-Dihydroxybenzoic acid (DHB) was used as the matrix compound. Matrix solution was prepared in 50% acetonitrile at a final concentration of 3.5 mg/mL. MALDI MS and MS/MS were performed on an ultraflexXtreme^{TM} MALDI-TOF/TOF (Bruker Daltonics, Billerica, MA, USA) mass spectrometer in positive MS or lift mode.

2.2.7 Bioinformatic methods

Database search was performed by Mascot search engine (Matrix Science). The mass spectra were submitted to Mascot search engine as a pkl file. The parameters used for database search were as follows: type of search defined was MS/MS Ion Search, Carbamidomethylation (C) was defined as a fixed modification, while Deamidation (NQ) (for PNGase F treated samples) and Oxidation (M) were defined as variable modifications. Sequence alignment was performed by ClustalW in Uniprot database accessible from http://www.uniprot.org, motifs search was performed by ScanProsite [31, 32], accessed from http://ca.expasy.org/prosite.

2.3 Results

2.3.1 Ovomucin subunits separation and identification by SDS-PAGE

Reduced fresh egg white was subjected to SDS-PAGE analysis. Three bands potentially containing ovomucin at approximately 350-400, 250 and 150 kDa were labeled as 1, 2 and 3 respectively, as shown in Fig. 2-1. These bands were excised and treated consecutively with PNGase F and trypsin. The peptides obtained were analyzed by nano LC-ESI MS and MS/MS. The mass spectra were submitted for database search using Mascot search engine, as explained in

Materials and Methods section. Mascot search engine detected α-ovomucin in bands 1 and 2, while β-ovomucin was detected in band 1 only. No ovomucin was detected in band 3, where the primary protein was characterized as ovostatin.

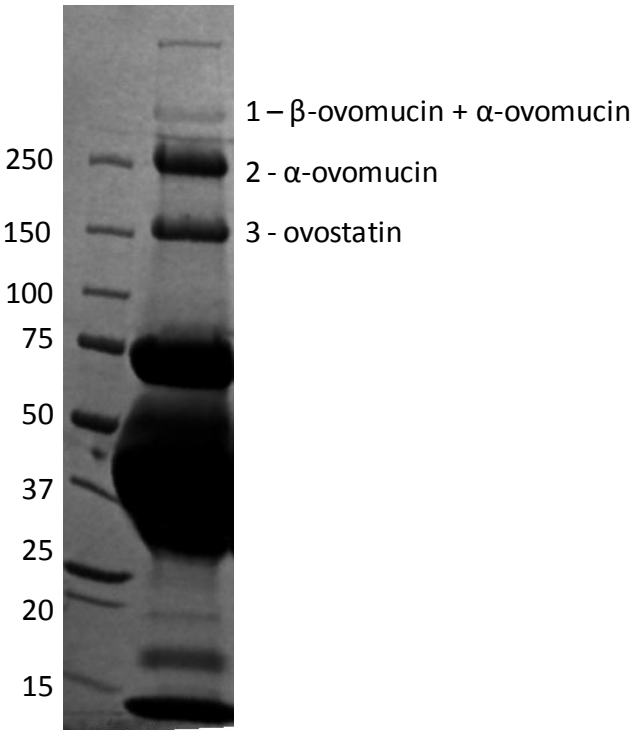

Figure 2-1: SDS-PAGE separation of ovomucin subunits. Molecular weight markers (in kDa) appear on the left, analyzed bands containing ovomucin, labeled 1, 2 and 3, on the right.

2.3.2 Identification of N-glycosylation sites of α-ovomucin

In order to characterize the potential N-glycosylation sites, PNGase F treated and trypsinized samples were analyzed. The enzyme PNGase F removes N-linked carbohydrates from proteins while deamidating the originally glycosylated asparagine into aspartic acid that results in an increase in molecular mass by

0.9840 Da [33]. This increase in mass was exploited to identify glycosylation sites of α and β-ovomucin.

Tryptic peptides obtained from digestion of deglycosylated ovomucins were subjected to nano LC-ESI MS and MS/MS. The data was analyzed by Mascot database search engine to identify tryptic peptides containing deglycosylated asparagines, supplemented by manual analysis of the data. By using this approach, 18 of the potential glycosylation sites of α-ovomucin (see Table 2-1 for details) and two sites on β-ovomucin (Table 2-1) were characterized. Two sites on α-ovomucin were not glycosylated (N^{69}, N^{673}), one site – N^{1639} existed in both glycosylated and not glycosylated forms, while all other identified sites on α-ovomucin and β-ovomucin were glycosylated. A representative MS and MS/MS data of one of the glycosylation sites containing a deamidated asparagine, Asn^{772}, is presented in Fig. 2-2; data of a not-glycosylated site, Asn^{637}, is represented in Fig. 2-3.

2.3.3 Determination of site specific N-glycan structures by LC-MS/MS

Glycosylation site occupancy and glycan heterogeneity on a particular site were determined from glycopeptide analysis. Tryptic glycopeptides obtained from in-gel digested α-ovomucin without PNGase F treatment were subjected to nano LC-ESI MS and MS/MS analysis. Glycopeptides were detected by searching for diagnostic glycan ions such as HexNAc (m/z 204) and Hex-HexNAc (m/z 366) resulting from fragmentation [33]. Representative MS/MS data of glycopeptides is presented in Fig. 2-4.

Table 2-1: Glycosylation sites in α-ovomucin, obtained from LC-MS/MS data of tryptic (glyco)peptides by MASCOT database and manual spectrum analysis.

Site	Peptide Position	Mass[(a)]	*m/z*Obs	Sequence	Glyco-sylation
N^{69}	54-78	3019.3[c]	1007.1^{3+}	FTFPGTC*TYVFASH-C*NDSYQDFNIK	-
N^{381}	368-385	2069.8	1035.9^{2+}	VYSSGGTYSTPC*QNC*TC*K	+
N^{528}	510-529	2374.2	594.5^{4+} 792.4^{3+}	VQMKPVMQLSITVDHSYQNR	+
N^{599}	589-618	3396.6	850.2^{4+}	FAQHWC*ALLSNTSSTF-AAC*HSVVDPSVYIK	+
N^{673}	657-677	2506.1[c]	1253.5^{2+}	QGIC*DPSEEC*PETM-VYNYSVK	-
N^{680}	678-684	987.4	494.7^{2+}	YC*NQSC*R	+
N^{772}	763-787	2767.1	1384.6^{2+} 923.4^{3+}	DC*PAPMYYFNC*SSA-GPGAIGSEC*QK	+
N^{855}	853-862	1322.5	662.3^{2+}	QWNC*TDNPC*K	+
N^{1036}	1028-1047	2316.0	1159.0^{2+}	ITSTC*SNINMTDLC*ADQPFK	+
N^{1219}	1215-1230	1844.9	923.4^{2+}	TYPLNETIYSQTEGTK	+
N^{1371}	1370-1392	2746.2	1374.1^{2+}	FNESWDFGNC*QIATCL-GEENNIK	+[(b)]
N^{1452}	1451-1467	2009.0	1005.4^{2+}	ENC*TYVLVELIQPSSEK	+
N^{1567}	1565-1580	1924.8	963.4^{2+}	YYNNTMGLC*GTC*TNQK	+
N^{1639}	1636-1645	1340.7[c] 1341.6	671.4^{2+} 671.8^{2+}	IIWNLTEC*HR	-/+
N^{1792}	1775-1802	3576.5	895.1^{4+}	EAWEHDC*QYC*TC*DEETL NISC*FPRPC*AK	+
N^{1807}	1803-1810	917.5	$916.5^{1+(b)}$	SPPINC*TK	+ [(b)]
N^{1841}	1837-1842	749.4	749.4^{1+}	TC*IINK	+
N^{1964}	1960-1984	2815.2	$1408.1^{2+(b)}$	APYDNC*TQYTC*TES-GGQFSLTSTVK	+ [(b)]

[a] – Calculated mass (deamidated Asn) with modified Cys.

[b] – Peptides detected manually (not detected by Mascot database search) in PNGase F untreated sample

[c]– Calculated mass (Asn not deamidated) with modified Cys.

* - Carbamidomethylated cysteines

Table 2-2: Glycosylation sites from β-ovomucin, obtained from LC-MS/MS data of tryptic peptides by MASCOT database.

Site	Peptide Position	Mass[(a)]	*m/z*Obs	Sequence	Glycosylation
N^{238}	238-249	1477.6	739.8^{2+}	NC*TC*STLSEYSR	+
N^{945}	944-952	1182.6	592.3^{3+}	YNMTLIWNK	+

[a] – Calculated mass (deamidated Asn) with modified Cys.

* - Carbamidomethylated cysteines

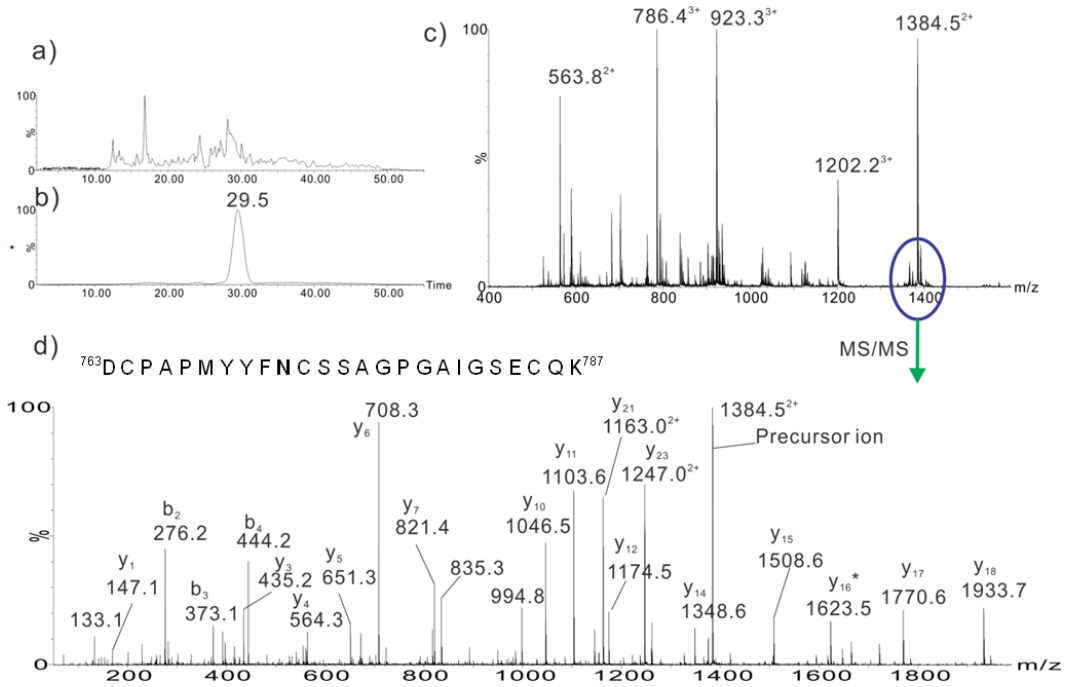

Figure 2-2: LC-MS and MS/MS ion chromatogram of a peptide ^{763}DC*PAPMYYFNC*SSAGPGAIGSEC*QK^{787} with deglycosylated asparagine - N^{772}. (a) Ion chromatogram of a sample untreated with PNGase F. The peptide was not detected in this sample, due to glycosylation. (b) Sample treated with PNGase F. The peptide eluted at 29.5 min. (c) MS extracted from PNGase F treated sample. The doubly (m/z 1384.5^{2+}) and the triply (m/z 923.4^{3+}) charged forms of the peptide were detected. (d) MS/MS of the doubly charged ion (m/z 1384.5^{2+}) shows b and y ions as labeled. The mass difference between y16 and y15, which corresponds to Asn, is 115 Da instead of 114 Da as a result of deglycosylation with PNGase F. Cysteines marked with an asterisk are carbamidomethylated.

(m/z 1253.5^{2+}) shows b and y ions as labeled. The mass difference between y4 and y5, which corresponds to Asn, is 114 Da which is not deamidated by PNGase F. Cysteines marked with an asterisk are carbamidomethylated.

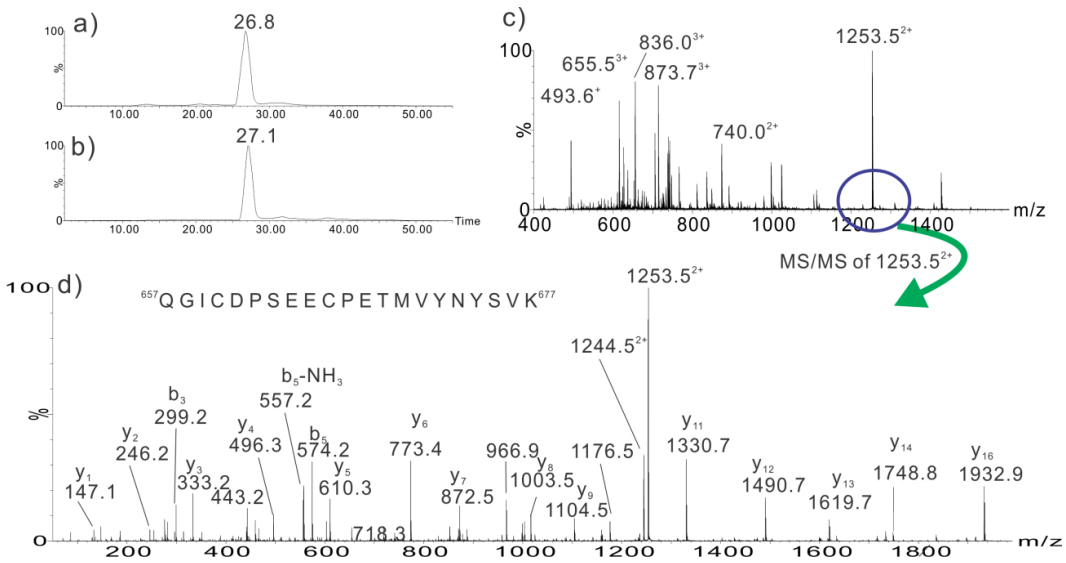

Figure 2-3: LC-MS and MS/MS ion chromatogram of a peptide ^{657}QGIC*DPSEEC*PETMVYNYSVK677 which does not contain a glycan on its potential glycosylation site – N^{673}. Ion chromatogram of (a) untreated and (b) treated samples with PNGase F. The peptide eluted around 27 min in both samples, which implies that the peptide is not glycosyalted. (c) MS extracted from PNGase F treated sample: The doubly (m/z 1253.5^{2+}) and the triply (m/z 836.0^{3+}) charged forms of the peptide were detected. (d) MS/MS of the doubly charged ion.

MS/MS data of a doubly charged glycopeptide ion at m/z 1227.5 is presented in Fig. 2-4a. In the MS/MS data, the peak at m/z 748.4 corresponds to a peptide predicted by ExPAsy proteomics server (www.expasy.org), TC*IINK, which contains the potential N-glycosylation site, Asn^{1841}. The peaks at m/z 951.5 and 1154.6 correspond to the peptide with one and two N-acetylglucosamines, respectively. The peak at m/z 1316.6 corresponds to $GlcNAc_2Man$ attached to the peptide. The peak at m/z 1519.7 has the same structrue as the previous peak with additional HexNAc that indicates the presence of a bisecting GlcNAc. The peak at 1843.8 corresponds to the pentasaccharide core of $GlcNAc_2Man_3$ with a bisecting

GlcNAc. From the mass of the glycopeptide (2453.0 Da) calculated based on the detected doubly charged ion at m/z 1227.5 and the peptide mass (747.4 Da), it was determined that the glycan part has a mass of 1705.5 Da and is composed of $GlcNAc_2Man_3$ + $HexNAc_4$. Fig. 2-4b and c show MS/MS of two triply charged glycopeptides of m/z 1016.4 and 1084.5 with similar MS/MS spectra. The glycopeptides have a common peptide at m/z 671.4 (doubly charged ion of IIWNLTEC*HR). MS/MS of the 1016.4 ion shows that the glycopetide has the same glycan structure ($GlcNAc_2Man_3$ + $HexNAc_4$) as the glycopeptides in Fig. 2-4a, while the ion at 1084.5 has one more HexNAc. Following the same strategy, five different glycosylation sites carrying different glycans were identified (Table 2-3). Although sulphated glycans were reported previously for O-glycans of ovomucin [16, 17, 19], there was no evidence of sulfation in N-glycans of α-ovomucin.

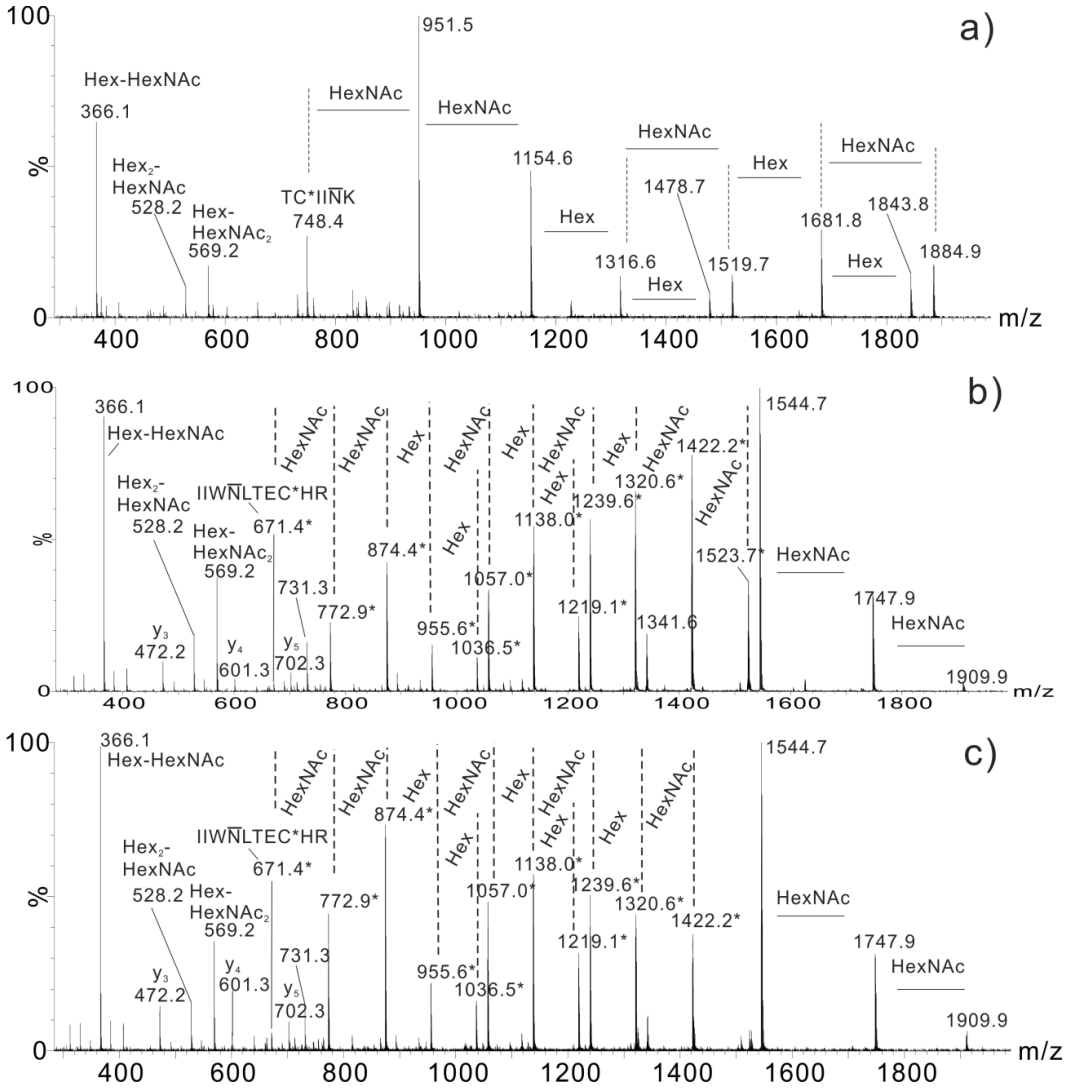

Figure 2-4: LC-MS/MS ion chromatogram of representative glycopeptides.(a) LC-MS/MS of a doubly charged ion at m/z 1227.5 corresponding to a peptide TC*IINK and a glycan $GlcNAc_2Man_3$+$HexNAc_4$. (b) LC-MS/MS of a triply charged glycopeptides ion at m/z 1016.4 corresponding to a peptide IIWNLTEC*HR carrying a glycan $GlcNAc_2Man_3$+$HexNAc_4$. (c) MS/MS of a triply charged glycopeptides ion at m/z 1084.5 corresponding to a peptide IIWNLTEC*HR carrying a glycan $GlcNAc_2Man_3$+$HexNAc_5$. Cysteines marked with an asterisk are carbamidomethylated.

Table 2-3: Tryptic glycopeptides from α-ovomucin and site specific N-glycosylation

Site	Peptide	m/z Obs	Peptide mass	Glycan mass	Perm. mass [(a)]	Glycan Structure
N^{1639}	IIWNLTEC*HR	1016.4^{3+}	1341.7	1705.5	2151.6	Core + $HexNAc_4$
		1084.5^{3+}	1341.7	1908.6	2396.7	Core + $HexNAc_5$
N^{1219}	TYPLNETIYS-QTEGTK	1183.8^{3+}	1844.9	1705.5	2151.6	Core + $HexNAc_4$
N^{1841}	TC*IINK	1227.5^{2+}	748.4	1705.5	2151.6	Core + $HexNAc_4$
		1328.5^{2+}	748.4	1908.6	2396.7	Core + $HexNAc_5$
		1430.6^{2+}	748.4	2111.8	2641.8	Core + $HexNAc_6$
		1409.6^{2+}	748.4	2070.7	2600.7	Core + Hex $HexNAc_5$
		1511.6^{2+}	748.4	2273.8	-	Core + Hex $HexNAc_6$
N^{1807}	SPPINC*TK	1210.0^{2+}	916.5	1502.4	1906.5	Core + $HexNAc_3$
		1311.0^{2+}	916.5	1705.5	2151.6	Core + $HexNAc_4$
		1412.6^{2+}	916.5	1908.6	2396.7	Core + $HexNAc_5$
		1010.0^{3+}	916.5	2111.8	2641.8	Core + $HexNAc_6$
N^{599}	FAQHWC*AL-LSNTSSTFAA-C*HSVVDPS-VYIK	1276.3^{4+}	3396.6	1705.5	2151.6	Core + $HexNAc_4$
		1225.5^{4+}	3396.6	1502.4	1906.5	Core + $HexNAc_3$

* - Carbamidomethylated cysteines

[a] – Perm. Mass refers to mass of permethylated glycans as $[M+Na]^+$ molecular ions detected by MALDI-TOF MS (see Fig. 2-5).

2.3.4 Determination of N-Glycan profile of α-ovomucin by MALDI-TOF MS

Glycans were released from α-ovomucin (band 2 in Fig. 2-1) by treatment with PNGase F, permethylated and analyzed by MALDI-TOF MS and MS/MS. MALDI-TOF MS spectrum of permethylated glycans is presented in Fig. 2-5. The peaks at m/z 1906.5, 2151.6, 2396.7, 2600.7 and 2641.8 correspond to the complex type N-glycans obtained from LC-ESI MS and MS/MS analysis of glycopeptides presented in Table 2-3. MALDI-TOF MS results revealed two major glycan structures at m/z of 1906.5 and 2151.6 that are composed of a pentasaccharide core+$HexNAc_3$ and the core+$HexNAc_4$, respectively. The peaks at m/z 2109.6 and 2355.7 had the same composition as the peaks at m/z of 1906.5

and 2151.6, respectively with an additional hexose on each. The glycan structures (see Fig. 2-5) for these and other peaks are based on the findings that ovomucin had a bisecting GlcNAc from LC-ESI MS/MS results, known N-glycan structures of other egg white proteins [23-26, 34] and carbohydrate composition of α-ovomucin that contained mainly N-acetylglucosamine (3.1-8.5%), mannose (2.9-4.6%), small amount of galactose (1.1-4.3%) and sialic acid (0.3-1.3%) [7, 35-37]. A glycan with composition of Core+ Hex $HexNAc_6$ with m/z of 1511.6 on site N^{1841} (Table 2-3) was not detected by MALDI-TOF/MS, possibly due to its low abundance which resulted in a weak signal.

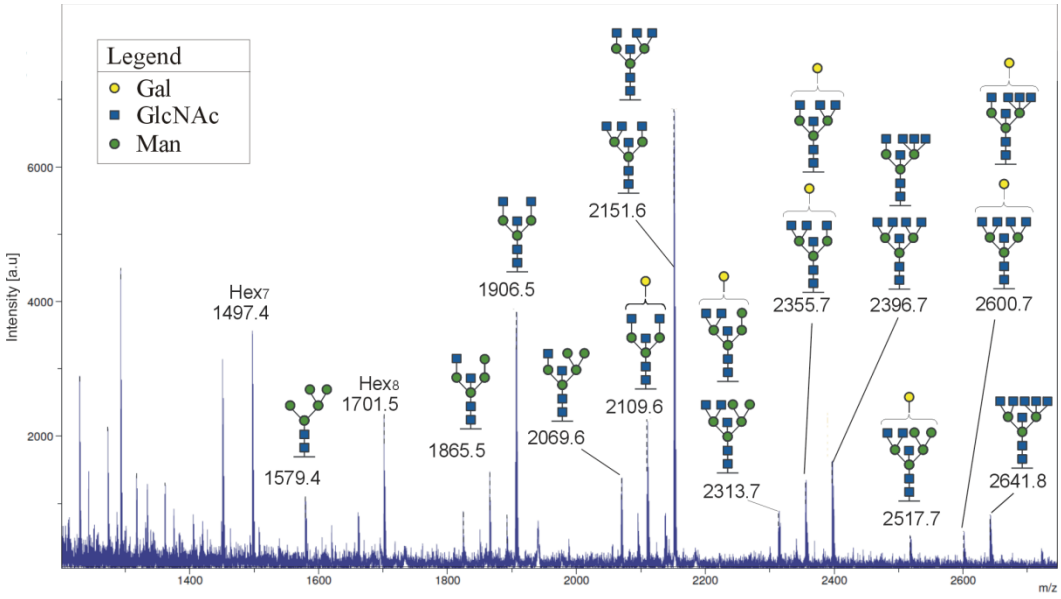

Figure 2-5: MALDI-TOF ion chromatogram of permethylated glycans released by in-gel digestion with PNGase F from SDS-PAGE band 2 that contained α-ovomucin. Possible glycan structures are presented.

2.3.5 Motifs search in α-ovomucin sequence and alignment of its N-glycosylation sites with human MUC2

The sequence of α-ovomucin was subjected to a search by ScanProsite against its database to identify motifs. ScanProsite identified four VWF D domains, two VWF C domains and a cystine knot. VWF D domains contained 6 glycosylation sites, one glycosylation site was found in VWF C2 domain and no glycosylation sites were found in the cystine knot, as illustrated in Fig. 2-6. To determine N-glycosylation sites conservation, alignment of α-ovomucin sequence with the sequence of human MUC2 was performed by ClustalW. Four glycosylation sites were conserved among the two proteins: N^{1371}, N^{1452}, N^{1567} and N^{1964} as presented in Fig. 2-6.

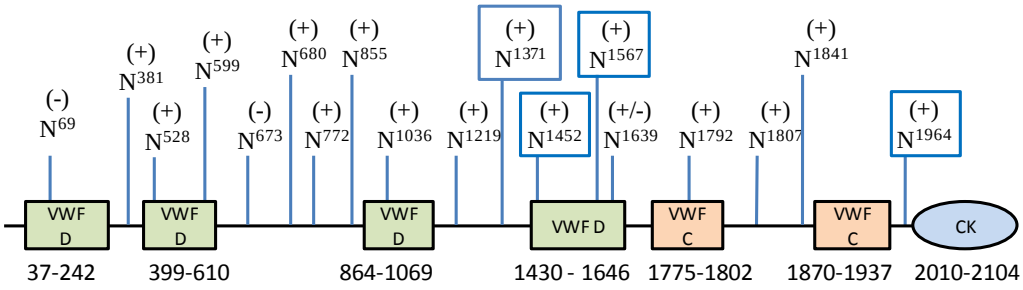

Figure 2-6: Location of N-glycosylation sites on α-ovomucin. Domains were assigned by ScanProsite. Glycosylation status as was determined in results section (Table 2-1) is presented as well as conserved glycosylation sites are surrounded by a square (aligned with human MUC2 by ClustalW).

2.4 Discussion

Ovomucin, a glycoprotein responsible for the gel properties of fresh egg white, is composed of two components: β-ovomucin, with molecular mass of 400-610 kDa

[35, 38, 39] and α-ovomucin - a polypeptide of 2087 amino acids with an estimated molecular mass of 254 kDa [9, 37]. Two components of α-ovomucin - α1 and α2-ovomucins with estimated molecular weights of 150 and 220 kDa respectively were reported [35] and another α-ovomucin component with molecular weight of 350 kDa was reported as well [39]. These results confirmed the presence of β-ovomucin in the 350-400 kDa gel region, in agreement with previous reports [35, 38]. α-ovomucin was present in gel bands that corresponded to molecular weights of ~350 kDa and 250 kDa; however, it was not detected in 150 kDa region where the major protein was ovostatin. The absence of α1-ovomucin in the 160 kDa fraction obtained by gel filtration was reported previously [39]. However, LC-MS/MS analysis of egg white revealed α-ovomucin on SDS-PAGE gel in the area of 100-250 kDa [40]. In the present experiment, fresh eggs were analyzed and no further ovomucin purification was performed to minimize the impact of sample preparation on the structure of ovomucin. Depolymerization of ovomucin complex through reduction of disulfide bonds was suggested as a cause for egg white thinning, occurring during storage [4]. Degradation of α-ovomucin could possibly occur during egg storage or ovomucin preparation, leading to α-ovomucin migration to lower molecular weight regions on SDS-PAGE. Therefore, previous observations of a smaller α-ovomucin in ovomucin extracts might be due to degradation of α-ovomucin occurred during egg sample storage or ovomucin preparation.

N-glycans play important roles in folding, oligomerization, sorting and transport of proteins [41]. In order to understand how glycosylation influences the

biological function of a protein, it is indispensable not only to elucidate the composition and structures of the glycans but also to identify the sites on the protein that are glycosylated. Protein glycosylation depends on various factors such as its 3D structure, location of the potential site, its accessibility by glycosylating enzymes and glycosylation of other sites on the protein, leading to 10-30% of the potential sites to be not occupied [42]. Glycosylation status of 18 glycosylation sites, out of the potential 24 [9] were determined for the first time. 15 sites were occupied by glycans, two sites (N^{69} and N^{673}) were not glycosylated and one site (N^{1639}) existed in both glycosylated and non-glycosylated forms. Only 2 of the potential N-glycosylation sites in β-ovomucin were identified, possibly due to a high amount of densely O-glycosylated regions as characteristic of mucus glycoproteins [10, 43] that prevented cleavage. Therefore other approaches should be applied for further investigation of β-ovomucin.

Glycopeptide analysis of 5 glycosylation sites revealed glycan heterogeneity with up to five glycans on each site (see Table 2-3), except for N^{1219} on which only one glycan type was determined, possibly due to the large mass of the glycopeptides that prevented it from being detected. This is not surprising, as glycoforms are very common due to the fact that glycosylation results from a series of not always complete enzymatic reactions [44].

Identified glycans from α-ovomucin glycopeptides had a pentasaccaride core of $GlcNAc_2Man_3$, which is characteristic to N-linked glycans, and a bisecting GlcNAc. Bisected type N-glycans are common in other egg white proteins such as ovotransferrin, ovomucoid and chicken riboflavin binding protein [24-26, 34]. As

glycosylation is species and tissue specific [42], it was assumed that N-glycans from α-ovomucin may have similar structures to the above egg white proteins. Taking that into account, possible glycans structures for the peaks obtained by MALDI-TOF MS (Fig. 2-5) were proposed and it was determined that the most abundant glycan is composed of $GlcNAc_2Man_3$ core, a bisecting GlcNAc and another 3 GlcNAc antennae located on the mannoses of the core. The fact that this glycan was present on all glycopeptides identified by LC-MS/MS supports this observation.

N-glycans from α-ovomucin were released by PNGase F from SDS-PAGE band corresponding to a molecular weight of 250 kDa. However, this band contained ovalbumin as a contaminant, although with a much lower score than that of α-ovomucin, as can be seen in Table 2-4. Therefore, it is likely that the N-glycan profile contained small amounts of glycans from ovalbumin. Several relatively weak peaks at m/z 1579.4, 2069.6, 2313.7 and 2517.7 that can possibly correspond to abundant glycans from ovalbumin [24] were identified. In addition to the glycan structures that were detected by both LC-MS/MS and MALDI-TOF MS (m/z 1906.5, 2151.6, 2396.7, 2600.7 and 2641.7), three additional peaks at m/z 1865.5, 2109.6 and 2355.7 were detected. While the later two could result from galactose addition to the abundant glycans (m/z 1906.5 and 2151.6, respectively) it is unknown whether the peak at m/z of 1865.5 is a glycan from ovomucin or other source.

Table 2-4: Proteins identified in band 2 by Mascot[(a)].

Protein	NCBI accession number	Mascot score
Ovomucin α-subunit	gi 12583679	4972
PNGase F	gi 157833480	1122
Ovalbumin	gi 129293	693
Ovotransferrin	gi 1351295	238
Ovotransferrin - Chain A, N-terminal lobe, iron loaded open form	gi 6729806	238
Trypsin	gi 136429	137

[a] - Only proteins with scores higher than 80 were included.

It was found that α-ovomucin possesses sequence similarity of 33% with the N-terminal D-domain, 27% with the C-terminal D4 domain, 34% with C1 domain and 29% with the cystine knot of human pre-pro-VWF domains [9]. It was shown that specific N-glycosylation sites on VWF in D1, D', B1 and CK domains influence its synthesis and secretion [45]. ScanProsite was used in order to assign specific N-glycosylation sites into domains. Seven sites were found within four VWF D domains and one site within VWF C2 domain as shown in Fig. 2-6. When aligned with human MUC2 (*results not shown*), four conserved glycosylation sites (N^{1371}, N^{1452}, N^{1567}, N^{1964}) were found, one site more than in previously reported alignment [9]. This conservation might suggest that these specific sites play an important role in the properties of α-ovomucin, possibly in the same way as specific N-linked glycans are involved in dimerization of rat MUC2 [15]. However, studies of tyrosinase related protein family show that N-glycosylation site conservation does not guarantee conserved roles in glycoprotein family among different species [46]. Therefore, the roles of these conserved

glycosylation sites in α-ovomucin, the exact structures of its N-glycans along with the role of α-ovomucin in the egg white and the factors contributing to its degradation should be further investigated.

2.5 Literature Cited

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Chapter 3. Weak gel properties of ovomucin from hen egg white

3.1 Introduction

Proteins and polysaccharides serve various roles in foods, cosmetics and pharmaceuticals, such as thickeners, gelling, coating and emulsifying agents. Gelation of proteins can be achieved through their denaturation and destabilization in various ways, such as heating, acidification and enzymatic treatments [1]. The forthcoming association and the balance between attractive and repulsive interactions determine the gel character [2]. Gel forming ability is also characteristic to some polysaccharides such as carrageenan, agarose, gellan gum and others. They produce thermo-reversible cold setting gels through transition from a disordered to ordered conformation and formation of helices that further aggregate to form a gel [3]. Besides acting as gelling agents, polysaccharides are frequently used as thickeners by creating entanglement networks above the critical overlap concentration (C*) [4].

Eggs are widely used in food systems for their nutritional values and functional properties such as foaming, emulsifying and gelling [5]. The gelling properties of the egg white are exploited in foods during cooking. Egg white gels are usually formed by heating that leads to denaturation of the globular proteins: ovotransferrin, lysozyme and ovalbumin. Gel quality is influenced by pH and ionic strength: best gels are obtained at a high pH and low ionic strength [6]. Microstructure of a heat induced transparent gel with excellent functional properties obtained at pH 11 revealed a fine homogeneous filamentous network [7]. Gelation of egg white proteins by acidification was reported as well [8].

However, even in the native state at room temperature egg white has a gel-like nature. The viscous fraction (thick white) has a weak gel character that is conferred by the glycoprotein ovomucin [9] which comprises approximately 3.5% of the egg white proteins [10]. Ovomucin is composed of two subunits - a carbohydrate poor α-ovomucin and a carbohydrate rich β-ovomucin with approximately 15% and 60% carbohydrates that can be separated after treatment with reducing agents [11, 12]. Ovomucin has linear and flexible filamentous structure [13, 14] formed by a high degree of polymerization through disulfide bonds, in a similar manner to mammalian mucins [10].

Mucins are high molecular weight glycoproteins responsible for the viscoelastic nature of mucus secretions. They are characterized by a high amount of O-linked carbohydrates that are attached to serine and/or threonine on the mucin domain (a domain rich in serine, threonine and proline). O-linked carbohydrates induce an extended random-coil configuration to the mucin molecules [15] that increases their solution size and therefore influences their viscoelastic properties [16]. The mucin polypeptide contains also non-mucin domains at the N- and C- termini. These domains are rich in acidic amino acids, contain large amount of cysteins, small amount of N-glycans and are believed to be involved in mucin polymerization [17, 18]. The polymeric structure of the mucin, the extended conformation and the non-covalent interactions in its both carbohydrate and protein parts are important for the functionality of mucins - their ability to form viscoelastic gels [16, 19-21].

Ovomucin shares genetic and functional similarity to the gel-forming mucins: α-ovomucin resembles the non-mucin cysteine rich domains of the mucins [22], while β-ovomucin is an orthologue of human MUC6 [23] with high amount of serine and threonine that provide docking points for O-linked carbohydrates [24] which are sialylated and sulphated [25-27]. Ovomucin has a linear and flexible random-coil structure [14] while ovomucin gel has non-Newtonian properties which are highly influenced by its polymeric structure and non-covalent interactions [28]. Additional rheological data on ovomucin gelation is not available.

Rheological characterization of ovomucin is important for determining its possible uses and applications. The purpose of this study was to investigate the rheological and gel-forming properties of ovomucin gels and dispersions and determine the influence of factors such as ionic strength, pH and temperature on its rheology.

3.2 Materials and Methods

3.2.1 Materials

Fresh eggs from White Leghorn laid within 24 h were obtained from the Poultry Research Centre of the University of Alberta (Edmonton, AB, Canada). Sodium chloride was purchased from Acros Organics (Morris Plains, NJ, USA). Hydrochloric acid and sodium hydroxide were bought from Fisher Scientific Inc. (Fisher Scientific, Ottawa, ON, Canada). Water was obtained from the milliQ water supply system (Millipore Corporation, Billerica, MA, USA)

3.2.2 Ovomucin preparation

Ovomucin was prepared by a 2-step isoelectric precipitation method [29]. Egg white was separated manually from fresh eggs and homogenized by a magnetic stirrer. The solution was diluted with 3 x volume of 100 mM NaCl, stirred for 30 min and the pH was adjusted to 6.0. After keeping the solution overnight at 4 ºC, it was centrifuged at 10,000 rpm for 10 min at 4ºC in Avanti J-E centrifuge (Beckman Coulter, Inc., Fullerton, CA, USA). The precipitate was re-suspended in 500 mM NaCl solution and the pH of the dispersion was adjusted to 6.0. The dispersion was stirred and placed at 4 ºC overnight. After overnight settling, the precipitate obtained by centrifuging at 10,000 rpm for 10 min at 4 ºC was dialyzed against milliQ water and lyophilized.

3.2.3 Gel filtration chromatography

Ovomucin purity was determined by gel filtration chromatography according to Hiidenhovi *et al*. [30, 31] with some modifications. Ovomucin sample of 2 mg/mL in 100 mM sodium phosphate buffer pH 7.0 containing 50 mg/mL SDS and 10 μL/mL β-mercaptoethanol was solubilized overnight. The sample was filtered through a 0.22 μm mixed esters of a cellulose syringe filter (Fisher Scientific, Ottawa, ON, Canada) and 3 mL were loaded to two High-load 16/60 columns connected in series (Superdex 200 and 75 preparatory grade, GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The columns were connected to AKTAexplorer Fast Performance Liquid Chromatography system (GE Healthcare Bio-Sciences AB). The columns were eluted with 100 mM phosphate buffer pH 7.0 containing 5 mg/mL SDS and 1 μL/mL β-mercaptoethanol at flow rate of 0.4

mL/min; absorbance of the eluate was monitored at 280 nm. The purity was calculated by subtracting the amount of contaminating proteins, determined according to standard protein curves [30].

3.2.4 Gel preparation

Gels of 10 mg/mL were prepared in distilled water or appropriate NaCl solution (1, 10 and 100 mM) by a T25 digital ULTRA-TURRAX homogenizer (IKA® Works Inc., Wilmington, NC, USA) at 15,000 rpm for 1 min at room temperature. The gels were centrifuged at 2200g for 30 sec in a microcentrifuge (Fisher Scientific, Ottawa, ON, Canada) to eliminate air bubbles. Heat treated gel was prepared in distilled water as described above and heated at 95 ºC in a water bath for 10 minutes. All the gels were placed at 4 ºC for 24 hr after preparation.

3.2.5 Rheological measurements

Rheological measurements were performed in a Physica MCR rheometer (Anton Paar GmbH, Va., USA) equipped with a parallel plates geometry (diameter of 2.5 cm). The gap between the plates was set to 0.5 mm and the temperature was set to 25 ºC using a Peltier plate of the rheometer. The sample was covered with low viscosity silicone oil and equilibrated between the plates for 20 min before measurements. Amplitude sweeps were performed at constant angular frequency of ω=1 rad/sec. Frequency sweeps were performed within the linear viscoelastic region.

3.2.6 Zeta (ζ) potential measurements

Ovomucin dispersions of 0.1% in distilled water and in 10 mM NaCl were prepared by T25 digital ULTRA-TURRAX homogenizer (IKA® Works Inc., Wilmington, NC, USA) at 15,000 rpm for 1 min at room temperature. After pH adjustments with 0.1 mM NaOH and 0.1 mM HCl, the dispersions were sonicated in an ultrasonic bath for 15 minutes and centrifuged at 2200 g for 30 sec in a microcentrifuge (Fisher Scientific, Ottawa, ON, Canada). ζ potential was measured in folded capillary cells in a Malvern Zetasizer Nano – ZS (Malvern, Worcestershire, UK).

3.2.7 TEM and SEM

Ovomucin gel was prefixed in 2.5% glutaraldehyde in Millonig's phosphate buffer [32] at room temperature for 1 hr. The sample was washed in the same buffer three times and postfixed in 1% OsO_4 at room temperature for 1 hr, washed in distilled water and dehydrated in a series of 50-100% ethanol. For SEM the sample was critical point dried (SeeVac Inc., Florida, USA), sputter-coated with gold (Edwards, Model 5150B sputter coater) and observed in HITACHI S-2500 (Hitachi, Tokyo, Japan) electron microscope. For TEM, the sample was embedded in Spurr's medium, stained with 2% uranyl acetate and 2% lead acetate after ultra thin section and observed at 80 kV in FEI Morgagni 268 Transmission Electron Microscope (FEI, Eindhoven, The Netherlands) equipped with Gatan digital camera.

3.3 Results and Discussion

3.3.1 Purity of prepared ovomucin

Purity of ovomucin prepared by a 2-step isoelectric precipitation method was determined by gel filtration chromatography presented in Fig. 3-1. Ovomucin was 92.6 % pure and contaminated by ovalbumin, lysozyme and ovomucoid.

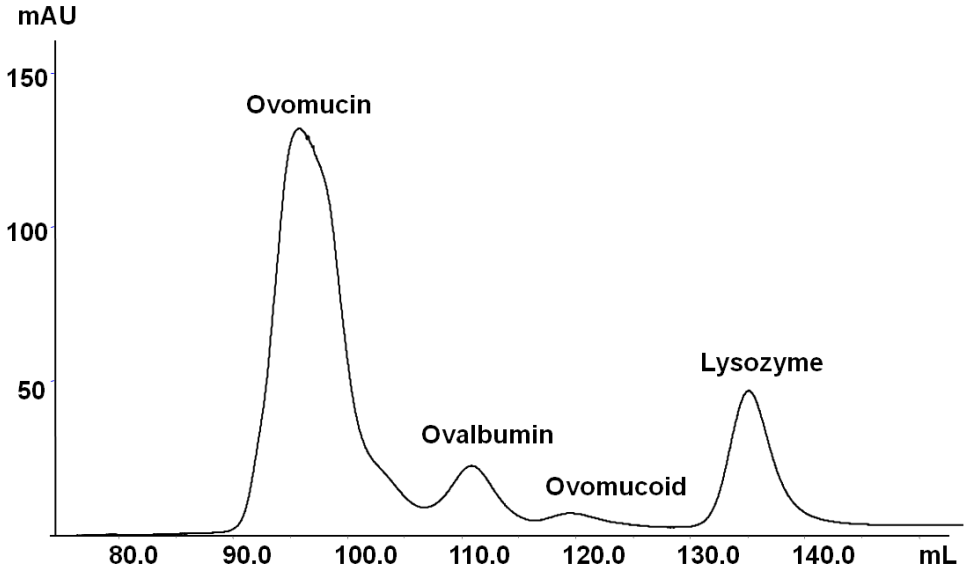

Figure 3-1: Gel filtration chromatography of ovomucin prepared by a 2-step isoelectric precipitation method.

3.3.2 Rheological properties of ovomucin gel

Frequency sweep of 1% ovomucin dispersion prepared in distilled water at room temperature is presented in Fig. 3-2. The mechanical spectrum of ovomucin dispersion reveals gel-like properties as the storage modulus G' is greater than the loss modulus G'' over the whole examined range. Similar rheological behavior was obtained for PGM in its gel state at pH 2 [33]; however unlike PGM, ovomucin formed gel at neutral pH.

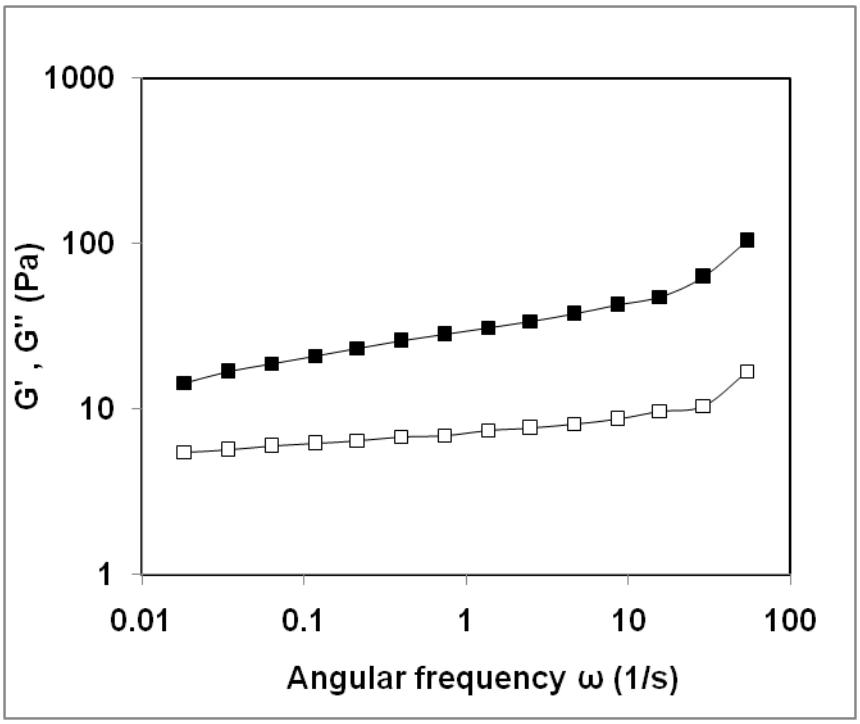

Figure 3-2: Frequency sweep of 1% ovomucin dispersion in distilled water showing weak gel properties with the storage modulus G' (■) higher than the loss modulus G'' (□). Both moduli are slightly frequency dependent.

The gelation mechanism of PGM involves a pH dependent sol-gel transition at pH 4. According to the proposed model, protonation of Asp and Glu at pH 4 triggers a conformation change in the non-glycosylated regions of the protein exposing hydrophobic regions. As a result, intermolecular domains are formed between the non-glycosylated regions of the protein through hydrophobic interactions [20]. The importance of hydrophobic interactions in contributing to ovomucin's non-Newtonian character was demonstrated previously, as ovomucin gel formed in the presence of chaotropic agents showed a decrease in viscosity and loss of the non-Newtonian properties [28]. The ability of ovomucin to form gel at neutral pH

might derive from the fact that ovomucin contains a protein-rich subunit (α-ovomucin) in addition to the glycan-rich subunit (β-ovomucin). The presence of α-ovomucin that possesses structural similarity to the non-glycosylated regions of mucins [22] in the ovomucin complex may provide sufficient amount of hydrophobic domains to form a gel at neutral pH.

The storage and the loss moduli are slightly frequency-dependent and G' is less than 10 times G'' (Fig. 3-2); therefore it was concluded that ovomucin possesses weak gel character under these conditions [34]. Weak gel behavior is characteristic to polysaccharides such as xanthan gum – a typical weak gel [35, 36]. Xanthan gum is a bacterial polysaccharide composed of 1,4-linked β-D glucose residues with trisaccharide side chains of mannose and glucuronic acid, that is widely used as thickener and stabilizer in various applications [37]. Xanthan gum showed a power-law behavior of the dynamic viscosity with an exponent of -0.9 and no zero shear rate plateau [35]. In addition, no Cox-Merz superposition was observed with the complex viscosity higher than the steady shear viscosity [35]. As xanthan gum, there was no Cox-Merz superposition for ovomucin weak gel, as can be seen from the Cox-Merz plot in Fig. 3-3, and the complex viscosity showed shear thinning behavior, as it decreased with increasing frequency. The complex viscosity exhibited power law behavior with an exponent of -0.80. This value is comparable to the value of -0.81 obtained for psyllium weak gel – a polysaccharide with a xylan backbone heavily substituted by short arabinose branches [38]. Weak gel rheological behavior was also observed in pig colonic and submaxillary mucins where the carbohydrate side chains were

suggested to be partially responsible for the noncovalent interactions [19, 39]. As in these mucins, the complex viscosity (η*) of ovomucin gel showed a shear thinning behavior (Fig. 3-3) and an absence of a Newtonian plateau, indicating that ovomucin gel involves intermolecular interaction in addition to the entanglements among ovomucin chains. The absence of the zero shear rate plateau indicates a solid-like behavior at rest and an ability to flow when shear is applied, suggesting that ovomucin can function as a stabilizing agent in suspensions and in emulsions [6].

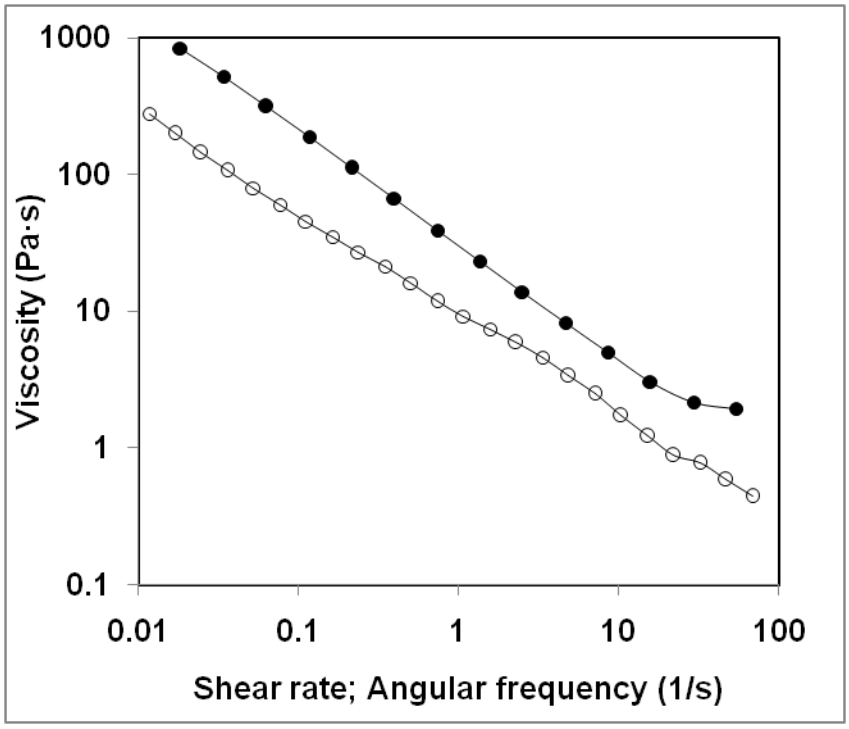

Figure 3-3: Cox-Merz plot of 1% ovomucin dispersion in water showing the lack of Cox-Merz superposition. (●) Complex viscosity - η*. (○) Steady shear viscosity - η.

3.3.3 Effect of heating on ovomucin rheology

The mechanical spectrum of heat-treated ovomucin gel is presented in Fig. 3-4.

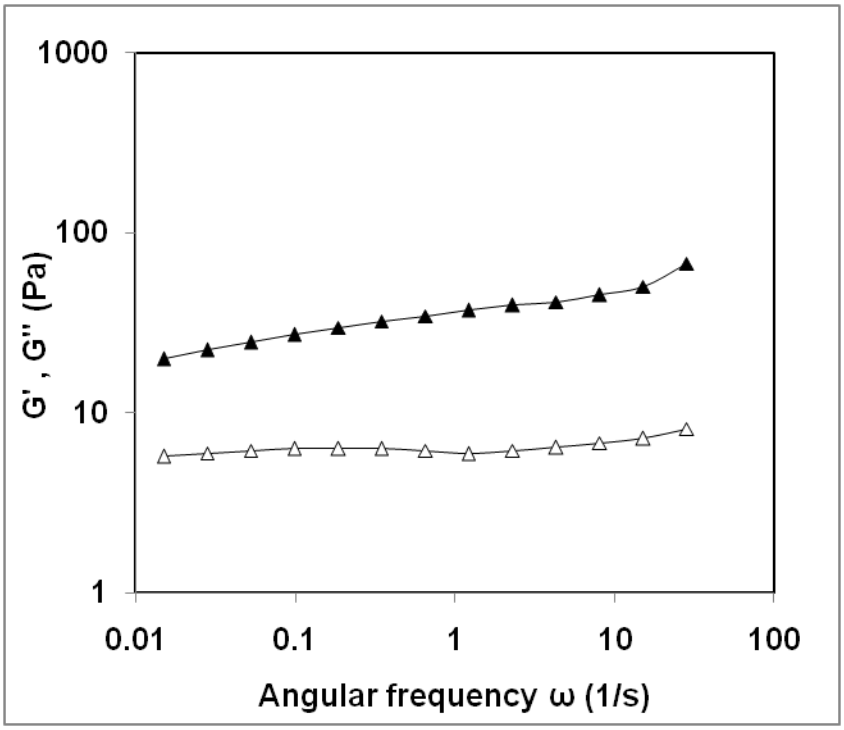

Figure 3-4: Frequency sweep of 1% ovomucin dispersion heated at 95 ºC for 10 min. The storage (▲) and loss (△) moduli are almost identical to those obtained without heating (Fig. 3-2).

The storage and loss moduli of the heated gel did not change significantly in comparison to the unheated one (Fig. 3-2), although the moduli were slightly less frequency dependent, suggesting the formation of a stronger gel. It was previously reported that ovomucin did not undergo thermal denaturation as no endotherm was observed in a DSC study, possibly due to its random coil conformation [40]. In addition, glycosylation confers thermal stability to proteins [41] and it is known that ovomucin is heavily glycosylated [42]. Tsuge et al. [43] studied the effect of

heating for 10 min at various pH and temperatures on the conformation and biological activity of ovomucin solutions. They determined that no change in the secondary structure of ovomucin at pH 6 and 8 occurred as a result of heating at 100 ºC. Although the ability of ovomucin to bind to Newcastle disease virus decreased significantly due to release of sialic acid on heating, binding of anti-ovomucin antibodies (influenced by ovomucin's tertiary structure) at pH 6 decreased only slightly [43]. Partial release of sialic acid could occur in this sample as well; however it did not influence ovomucin's conformation and its gel rheology. Therefore, it was concluded that heating for 10 min at 95 ºC did not cause damage to ovomucin's gel.

3.3.4 Effect of salts on ovomucin gel rheology

The influence of NaCl on ovomucin dispersions was determined by rheological measurements. As can be seen in Fig. 3-5, addition of NaCl negatively influences ovomucin gel. Adding NaCl even at 1 mM significantly decreased G' and weakened gel; ovomucin gel was close to the sol-gel transition at 10 mM NaCl and there was no gel formation at 100 mM NaCl. Instead, an unstable dispersion of ovomucin that precipitated within a short time was formed (*data not shown*). The results are in good agreement with previous data showing negative influence of NaCl on viscosity of ovomucin preparations [44], as a characteristic to a flexible and linear polyelectrolyte of high molecular weight [13]. Other mucins also exhibited similar properties in the presence of salts. Pig gastric mucin underwent a decrease in viscosity at increased ionic strength [45] and a transition from gel to solution [33]; this was due to the screening of electrostatic interactions

that caused to a decrease in cross-linking and entanglement of the gel as well as a less extended conformation of the sugar side chains [33]. From these results it is evident that ovomucin can form a gel at 10 mM NaCl, and that higher salt concentrations cause to aggregation of ovomucin.

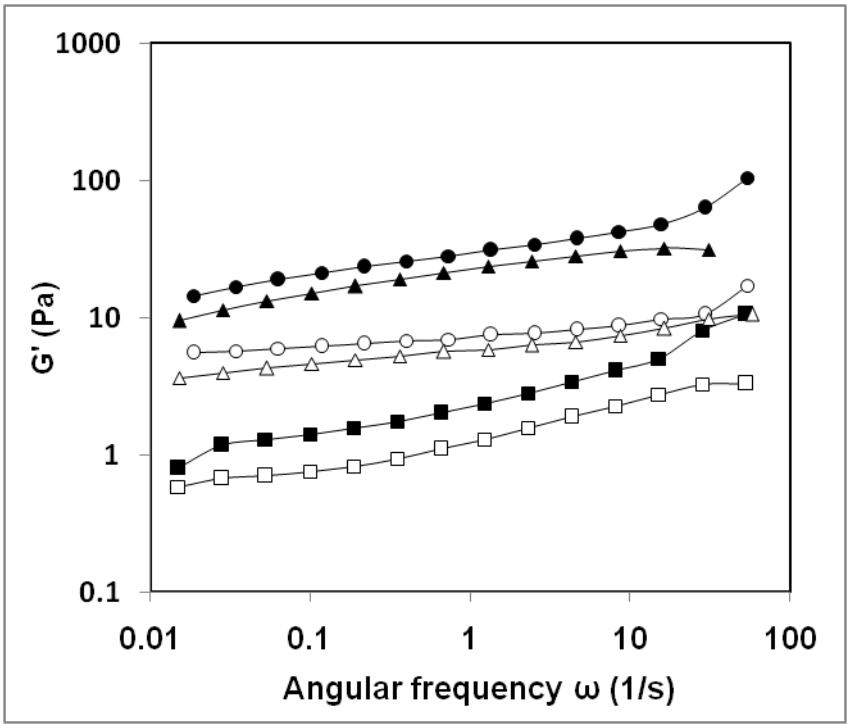

Figure 3-5: Frequency sweep of 1% ovomucin dispersion in various NaCl concentrations demonstrating the negative effect of salt on ovomucin gel rheology. G' (●) and G'' (○) of 1% ovomucin in water; G' (▲) and G'' (△) of 1% ovomucin in 1 mM NaCl; G' (■) and G'' (□) of 1% ovomucin in 10 mM NaCl.

Although ovomucin aggregation was suggested to be important for its gel formation [14], it seems that excessive aggregation (as can be seen from the SEM image in Fig. 3-5c) leads to a disruption of the fine ovomucin network resulting in coarser network and a decrease in gel quality until precipitation occurs. Therefore

it seems that a delicate balance between attractive and repulsive forces is essential to form ovomucin gel, disruption of which as a result of addition of salts causes to destabilization of the network and aggregation.

3.3.5 Microstructure of ovomucin gel

SEM and TEM micrographs of 1% ovomucin gel in distilled water and 10 mM NaCl are presented in Fig. 3-5. As can be seen in Fig. 3-5a, ovomucin dispersion of 1% w/v in distilled water exhibits a network of fibrous nature. This is the first report on ovomucin gel microstructure. TEM images of several ovomucin strands that were obtained previously showed fibrous structure of the molecule with possible longitudinal association of at least two strands [14]. This type of association will stiffen the molecule and enable formation of the network as seen in Fig. 3-5a. The TEM image (Fig. 3-5b) shows a network of ovomucin chains, similar to those obtained previously [14] with varying degree of aggregation that is also observable on the SEM images (Fig. 3-5c). It is unknown whether these dense aggregates are present in the native gel or result from the sample preparation that involved placement in buffer and dehydration with ethanol. Drastic aggregation occurred during dehydration step of ovomucin gel in 10 mM NaCl, a result of the destabilizing effect of salts. SEM and TEM of ovomucin aggregates formed as a result are presented in Fig. 3-5d and 3-5e respectively and provide an insight on the aggregated state of ovomucin. As can be seen in the SEM image in Fig. 3-5d, globular aggregates are interconnected by ovomucin fibers as they appear in the non-aggregated state in Fig. 3-5a. I believe that the non-aggregated regions seen in Fig. 3-5a and 3-5c closely represent the native

state of ovomucin in the gel, as these regions of ovomucin network show similarity to a network of 2% porcine gastric mucin solution obtained by SEM [46], although partial aggregation could have occurred as appear in Fig. 3-5b during sample preparation.

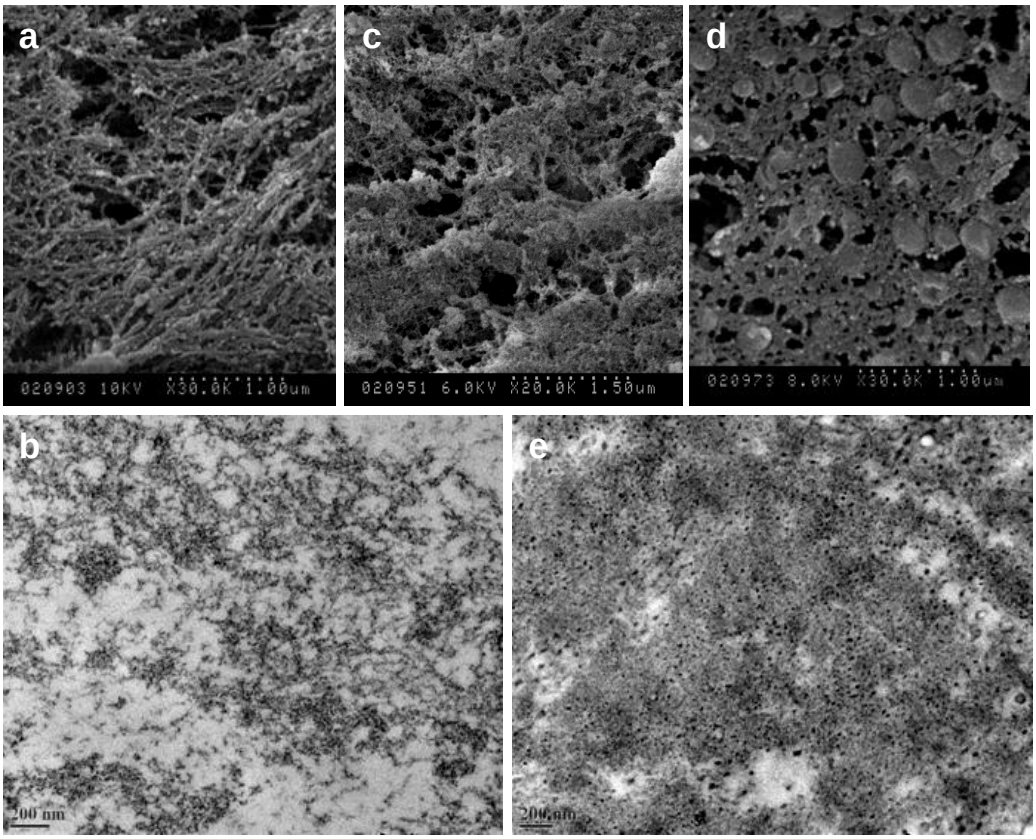

Figure 3-6: SEM and TEM micrographs of 1% ovomucin gels. (a) SEM image of ovomucin gel in water showing fine network of fibres. (b) TEM image of ovomucin gel in water, showing areas with fine network and more aggregated regions. (c) SEM image showing aggregates connected by fine network of strands, showing an intermediate state between expanded and more aggregated network. (d) SEM image of aggregated state of ovomucin gel in 10 mM NaCl (aggregated during sample preparation). Large spherical aggregated are connected by thin fibers, corresponding to the fibers seen in (c). (e) TEM image of fully aggregated ovomucin gel in 10 mM NaCl. Densely packed ovomucin correspond to the globules that appear in (d).

3.3.6 ζ potential of ovomucin dispersions: effect of NaCl and pH.

Ovomucin charges in 0.1% dispersions in water and 10 mM NaCl at various pH were assessed by measuring ζ potential as presented in Fig. 3-7.

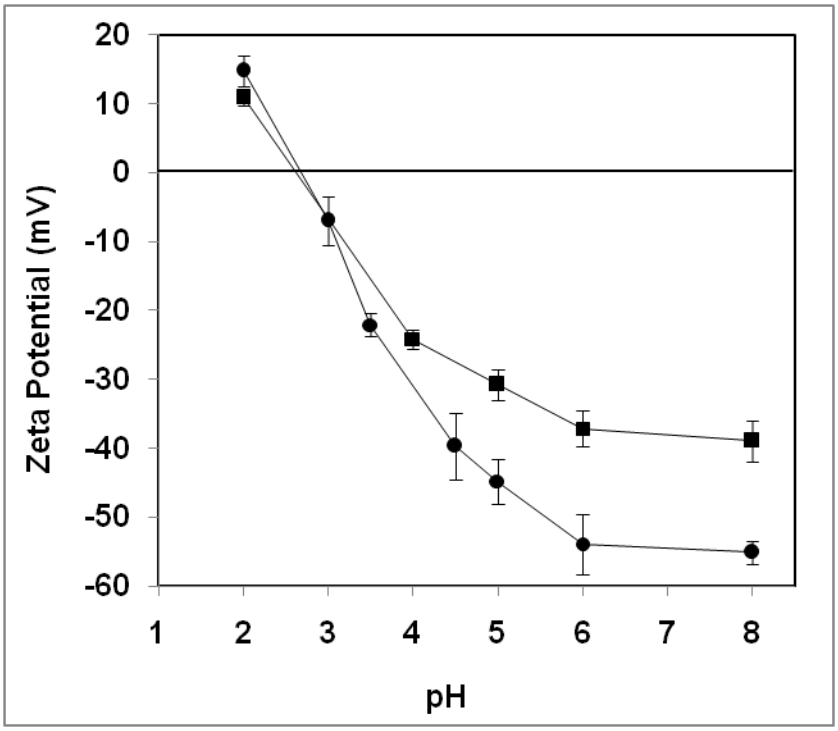

Figure 3-7: ζ-potential of 0.1% ovomucin dispersions in water (●) and 10 mM NaCl (■). The absolute values of ζ-potential of ovomucin in salt are significantly lower than ovomucin in water at pH above 4 corresponding to precipitation in that sample. Both samples reach isoelectric point at approximately the same pH.

Ovomucin is a negatively charged molecule at pH above its pI of 4.5-5 [13]. However, these results suggest that the isoelectric point of ovomucin is 2.6, where the ζ potential is 0 (calculated from a curve fitted to the ζ potential at pH 6 to 2). This can be explained by a high content of aspartic acid and glutamic acid [29]

with pK_a of approximately 4 [20, 47, 48] and the presence of sialic acid and sulphate [12] with pK_a of approximately 2 and 1 respectively [20, 47, 48].

During acidification of dilute ovomucin solution in water no aggregation was observed until approximately pH 4-4.5, where the zeta potential was -40 mV, as can be seen in Fig. 3-8.

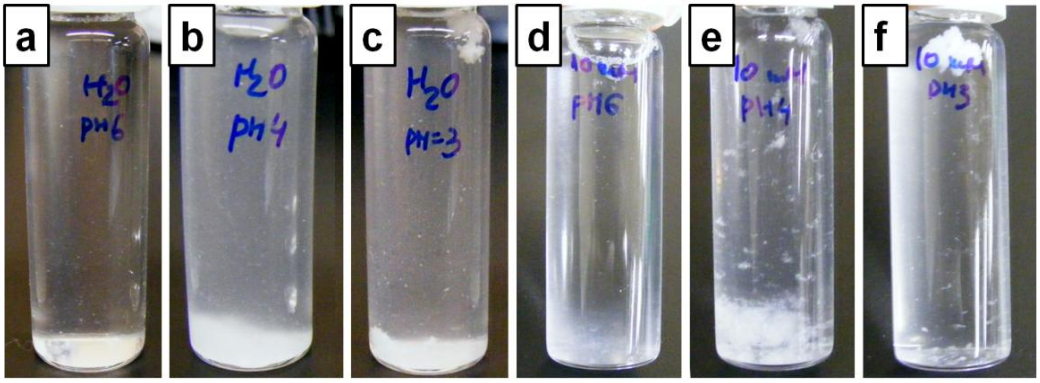

Figure 3-8: Appearance of 0.1% ovomucin dispersions in water and 10 mM NaCl at various pH. (a) Ovomucin solution in water at pH 6. Clear solution, no precipitation was observed. (b) Ovomucin solution in water at pH 4: turbid solution, precipitate started to form (c) Ovomucin solution in water at pH 3: aggregates were visible at the top and bottom. (d) Ovomucin solution in 10 mM NaCl at pH 6: fine precipitate was formed at the bottom. (e) Ovomucin solution in 10 mM NaCl at pH 4: ovomucin precipitated. (f) Ovomucin solution in 10 mM NaCl at pH 3: large aggregate was formed.

This could be a result of protonation of the Asp and Glu residues and increase in hydrophobic interactions as a result of conformation change, as was suggested for PGM [20]. As was mentioned before in 3.3.2, it seems that the relatively high

amount of the non-glycosylated protein regions in ovomucin is responsible for this.

Ovomucin solution in 10 mM NaCl was not stable and precipitation occurred throughout the whole measured pH range. Therefore it was concluded that at ζ potential of approximately -40, ovomucin reaches its instability region. This instability and precipitation at high ζ potential values can be an indication to the strong attractive forces present in ovomucin, possibly derived from its hydrophobic regions. The nature of the precipitate observed in the 10 mM NaCl sample at neutral pH was different from the one obtained at acidification, the former composed of finer aggregates, in comparison to the large aggregates formed at pH 4.5. Therefore, it seems that aggregation induced by salt resulted from a decrease in repulsion forces as a result of charge shielding, rather than by a change in conformation that occurred at pH 4-4.5.

In both samples, further acidification induced positive charge of ovomucin. This stands in contrast with results obtained for pig gastric mucins where the molecules remained negative at pH below 2 [47, 49].

3.4 Conclusions

The results from this study demonstrate that ovomucin can form a weak gel at room temperature in distilled water with similar rheological properties to those of PGM at low pH where it existed as a gel. Gel formation at room temperature at pH close to neutrality is important for the function of ovomucin as the gelling agent of egg white [9]. By forming a gel, ovomucin contributes to the protective properties of the egg white against pathogen penetration to the embryo [50], in the

same way the gel forming mucins protect epithelial cells [21]. This ability to form a gel at neutral pH might be attributed to the presence of α-ovomucin – a protein rich subunit that might be responsible for an increase in amount of hydrophobic domains available for intermolecular interaction and gel formation. The rheological profile of ovomucin and its shear thinning behavior was also similar to that of xanthan gum. This suggests that ovomucin can be used as a thickener and stabilizer in various applications, especially when heat stability is needed, as ovomucin exhibited little change in its rheological properties due to heating. Ovomucin gel has a structure of a fibrous network prone to aggregation as seen by electron microscopy. The results suggest that electrostatic interactions are extremely important for the stability of ovomucin in both dilute and concentrated solution due to strong hydrophobic interactions in its protein backbone. In addition the isoelectric point of ovomucin was determined to be 2.6 and that it was found that ovomucin in dilute solution undergoes aggregation at pH 4-4.5.

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Chapter 4. Final remarks

4.1 Ovomucin – a mucus glycoprotein from egg

Ovomucin, the gelling agent of the egg white shares many structural and functional characteristics with secreted mucins from human and other species. Genetically, ovomucin genes are located on a cluster encoding secreted mucins that is similar to the human 11p15 locus with additional gene for α-ovomucin [1]. Functionally, ovomucin confers the thick egg white with viscous properties that provide protection for the embryo against pathogens and physical damage [2] in the same manner that the mucins provide with a viscous layer that protects epithelial cells [3]. During incubation the thick egg white liquefies, allowing transfer of water and proteins from the albumen into the yolk. A similar liquefaction of the thick egg white occurs during storage of the egg and termed as the egg white thinning [2, 4]. Being a mucus-type glycoprotein that can be easily extracted, ovomucin has a great potential to be applied as a bioactive component and in other applications. However, this requires better understanding of the structure of its subunits and its gel-forming abilities.

4.2 Summary of the present research

Aqueous dispersion at room temperature of freeze dried ovomucin prepared by a 2-step isoelectric precipitation method had a gel-like appearance. Rheological measurements showed that it had ‘weak gel’ properties, similar to those of pig gastric mucin at low pH where it presented as a gel [5]. Ovomucin gel has a fibrous character prone to aggregation in the presence of salts. Therefore, it is

suggested that electrostatic interactions that are shielded by salt addition are important for stabilizing ovomucin and preventing its aggregation and further precipitation. It was suggested that the ability of ovomucin to form a gel at mild conditions (room temperature in distilled water) derived from the increased amount of hydrophobic regions that resulted from the presence of α-ovomucin that formed a network in a similar manner to that suggested for pig gastric mucin [6]. The role of α-ovomucin in the egg white is unknown, as it contains only the non-mucin domains with the O-glycosylated mucin domain missing [7]. However, it is possible that α-ovomucin provides additional non-mucin domains that contribute to the gel-like structure of the egg white. As was determined in Chapter 2, α-ovomucin contains at least 15 N-glycans that might provide it with a suitable conformation that enables network formation. The importance of the N-glycans of ovomucin for its gel-forming ability is further strengthened by the presence of four conserved N-glycosylation sites with MUC2 that were found glycosylated. This suggests that these glycans serve an important role in the gelling ability of ovomucin, such as influencing dimerization and polymerization between α- and β-ovomucin subunits. It is likely that like other mucins α-ovomucin forms dimers via its cystine knots in its carboxy terminus and polymers via its amino terminal region, however not this nor the way it polymerizes with β-ovomucin are known. In addition it was determined that the N-glycans of α-ovomucin are of bisected complex type, similar to the glycans of other egg white proteins.

Another possible role of α-ovomucin is its involvement in the process of the egg white thinning. SDS-PAGE of fresh egg white was performed, while α-ovomucin

was not identified in a 150 kDa SDS-PAGE band, where α-ovomucin was previously reported [8]. It is possible that the presence of this protein in these regions resulted from its degradation during egg storage or purification processes. Cleavage of α-ovomucin in a specific site, similar to the cleavage of the membrane-bound mucins in the SEA module [9], may be responsible for the egg white thinning, although further studies are required to confirm that.

4.3 Applications and further research

The ability of ovomucin to form a heat-stable weak gel at room temperature suggests that ovomucin has a potential to be applied as a thickener and stabilizer in systems where heating is undesirable, or heat stability is required that can react to various conditions such as changes in pH and ionic strength. More specifically, ovomucin has a potential to act as a mucoadhesive biopolymer for drug delivery due to its capability to form hydrogen bonds, its high molecular weight, chain flexibility and negative charge that are required for mucoadhesion [10]. In this case the drugs can be incorporated in the spaces between ovomucin chains that were seen on TEM and SEM images. In addition, as ovomucin is structurally similar to other mucins, it has a potential to act as a coating of polymeric materials for biomedical applications to suppress bacterial adhesion, as was suggested for mucins [11]. For these and other applications the interactions of ovomucin with other polymers and proteins should be studied and further understanding of the structure and functions of its subunits is required. While the amino acid sequence and the glycosylation state of 18 out of 24 potential N-glycosylation sites of α-ovomucin with its N-glycans profiles are now known, the

structure of β-ovomucin remains vague. First, besides a fragment of 872 amino acids its sequence is not known, so is the presence of a PTS and other domains and N- and O-glycosylation sites. Second, although some glycan structures of β-ovomucin were reported, full profile of its glycans should be obtained. In addition, the way α- and β-ovomucin polymerize, the reason for the presence of α-ovomucin in the area of 150 kDa on SDS-PAGE and whether its suggested degradation plays a role in egg-white thinning should be determined.

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