Egg White Ovomucin Shows Anti-Adhesive and Anti-fouling Properties

by

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A thesis submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in

Food Science and Technology

Department of Agricultural, Food and Nutritional Science

University of Alberta

ABSTRACT

Ovomucin, a member of mucin family, is a glycoprotein accounting for 2-4% of egg white protein. Ovomucin is rich in sialic acid (2.6-7.4%, w/w), which has been suggested as an essential nutrient for infants. Ovomucin is insoluble at neutral pH or in the absence of denaturing agents. The overall objective of this thesis was to explore the potential of egg white ovomucin as value-added anti-adhesive and anti-fouling ingredients.

To improve ovomucin's water solubility, ovomucin was hydrolyzed by various proteases with yields and degree of hydrolysis (DHs) ranged from 42.6% (flavourzyme) to 97.4% (protease N), and 2.4% (flavourzyme) to 46.3% (pronase), respectively. Ovomucin hydrolyzed by pronase and protex 26L showed molecular weight (Mw) distribution less than 40 kDa while the others with Mw larger than 200 kDa. Allergenicity of all ovomucin hydrolysates was significantly reduced ($P<0.05$) in comparison to ovomucin extracts. The content of sialic acid in hydrolysates ranged from 0.1% (protex 26L) to 3.7% (pronase).

The anti-adhesive potential of ovomucin/ovomucin hydrolysates was firstly determined by hemagglutination assay (HA). HA results demonstrated that ovomucin hydrolysates, but not intact ovomucin, prevented two K88 enterotoxigenic *Escherichia coli* (ETEC) strains adhesion to porcine erythrocytes. Ovomucin hydrolysate prepared by acid protease II exhibited the best anti-agglutinating activity against both ETEC strains; this hydrolysate was fractionated by cation exchange chromatography and reverse-phase HPLC. The most active fractions, F3(9) and F7(1), with minimal inhibitory concentration of 0.03 g/L and 0.25 g/L, against strains ECL13795 and ECL13998, respectively, were subjected to structure characterization. Six glycopeptides identified were all derived from α-ovomucin, composed of a pentasaccharide core of two N-acetylglucosamine and three

mannose residues ($GlcNAc_2Man_3$) and a bisecting N-acetylglucosamine (GlcNAc). The anti-adhesive activity of ovomucin hydrolysates was further validated in porcine small intestinal epithelial cells (IPEC-J2) using both plate counting and Syto 9 staining methods. Interaction study suggested that the anti-adhesive activity of ovomucin hydrolysates was due to competitive binding to ETEC through $K88_{ac}$ fimbriae as decoy receptors. The peptide sequences of ovomucin glycopeptides play a role in binding to $K88_{ac}$ fimbriae while the glycan moieties are indispensable for this binding. The terminal β-linked galactose from ovomucin glycopeptides could be one of the binding sites for $K88_{ac}$ fimbriae.

The anti-fouling property of ovomucin was studied by determining the adsorption of bovine serum albumin (BSA) on ovomucin-coated polystyrene surface. Ovomucin significantly inhibited BSA adsorption. To further confirm the anti-fouling property, directly surface force measurements between ovomucin and model proteins (BSA and ovomucin) were carried out using surface force apparatus (SFA). The SFA results suggested that pure repulsive forces were measured under both symmetric (ovomucin vs. ovomucin) and asymmetric configurations (BSA vs. ovomucin) at all tested pHs (2.0, 6.0 and 7.2) and ionic strengths (0.1, 10, and 150 mM NaCl) during approaching and separation. Atomic force microscope imaging, zeta potential and dynamic light scattering results suggested that the electrostatic and steric repulsions could be the main forces responsible for the anti-fouling property of ovomucin.

This thesis demonstrated the potential applications of ovomucin as an ingredient for follow-up formula due to its high sialic acid content, an anti-adhesive agent against infection, and an anti-fouling surface.

PREFACE

This thesis is an original work by Xiaohong Sun and has been written according to the guidelines provided by the Faculty of Graduate Studies and Research at the University of Alberta. The concept of the thesis originated from my supervisor Dr. Jianping Wu and the research was funded by the grants from Alberta Livestock Meat Agency Inc. (ALMA) and Natural Sciences and Engineering Research Council (NSERC) of Canada to Dr. Wu.

This thesis is consisted of seven chapters: Chapter 1 provides a general introduction and thesis objectives; Chapter 2 is literature review regarding egg white proteins, bacterial adhesion to host cells, anti-adhesive therapy of infectious diseases, and anti-fouling surfaces; Chapter 3 has been published as Xiaohong Sun, Michael Gänzle, Catherine J. Field and Jianping Wu, "Effect of proteolysis on the sialic acid content and bifidogenic activity of ovomucin hydrolysates" in *Food Chemistry*; Chapter 4 has been published as Xiaohong Sun, Michael Gänzle and Jianping Wu, "Identification and Characterization of Glycopeptides from Egg Protein Ovomucin with Anti-Agglutinating Activity against Porcine K88 Enterotoxigenic *Escherichia coli* Strains" in *Journal of Agricultural and Food Chemistry*; Chapter 5 is in preparation for submission as Xiaohong Sun, Michael Gänzle and Jianping Wu, "Glycopeptides from Egg White Ovomucin Inhibit $K88_{ac}$ Enterotoxigenic *Escherichia coli* Adhesion to Porcine Small Intestinal Epithelial Cell-line" to *Molecular Nutrition and Food Research*. Chapter 6 entitled as "Effect of pHs and Ionic Strengths on the Anti-fouling Property of Egg White Protein Ovomucin" is in preparation for submission; Chapter 7 gave some concluding remarks and future research directions.

For chapters 3-5, Drs Jianping Wu and Michael Gänzle contributed to experimental design, data interpretation, manuscripts preparation and edits. I was responsible for

literature search required for the study, experimental designs, performing experiments, data analysis, and drafting the manuscripts. Dr. Catherine J. Field was the co-applicants for the application and helped with manuscript edits (Chapter 3). In Chapter 6, Drs Jianping Wu and Hongbo Zeng both supervised this work and were involved in experimental design, data interpretation, manuscripts preparation and edits. Mr. Jun Huang contributed to performing experiments, analyzing data, and manuscript edits of surface force measurements and atomic force microscope imaging parts. I was responsible for literature search required for the study, experimental designs, performing experiments, data analysis, and drafting the manuscript.

DEDICATION

This thesis is dedicated to the loving memory of my farther Mr. Libin Sun.

You will always be the brightest star in my sky, lighting up my way.

ACKNOWLDEGMENTS

Time flies, I have spent more than five years in my Ph.D. study. This long journey is fully filled with success, failure, happiness, frustration, and encouragement, which make it become a memorable and extraordinary experience for me. I am lucky to have great supports and help from many people during the entire doctorate program, whom I am forever grateful to.

First and foremost, I would like to express my sincere gratitude to my supervisor Dr. Jianping Wu for providing me an opportunity to work with him at the beginning, and for his enormous support, encouragement, guidance, and patience throughout these years. I will never forget his tremendous efforts on my PhD study: countless of discussions, meetings, emails, and manuscript edits. I am very much thankful to my supervisory committee members Drs Michael Gänzle and Hongbo Zeng, for providing me the opportunity to use their laboratory facilities, being always willing to help, and giving constructive advice and guidance. Special thanks go to Dr. Hoon Sunwoo for nicely being both of my candidacy examiner and defense examiner. My deepest appreciation goes to the external examiner Dr. Andreas Hensel, Institute of Pharmaceutical Biology and Phytochemistry, University of Münster, for devoting his time to read and evaluate this thesis.

I would like to specially thank Maria Offengenden for mentoring me in the lab when I just started my project and for being always helpful even after she left from lab. I am also thankful to Dr. Subhadeep Chakrabarti for teaching me cell culture techniques and it was a pleasant experience to work with him. I am grateful to Dr. Sandra Galle for guidance on carbohydrate metabolism of bifidobacteria. I would also like to extend my sincere thanks

to Drs. Wenlin Yu and Jianhui Li for helping me settle down when I firstly arrived in Canada.

I am very grateful for the support from Mrs. Jody Forslund, the Graduate Program Administrator of the Department of Agricultural, Food and Nutritional Sciences (AFNS), the department technical support from Mr. Gary Sedgewick. My special thanks go to Robin Miles, AFNS Stores Manager and Purchasing Technologist, who is always efficient and helpful.

My sincere thanks go to past and present members of Dr. Wu' research group, for their support and company, for the joyful memories we shared, and for all the hard working days we were together. They are all great colleagues in the laboratory and great friends in daily life. Many thanks go to my numerous friends in Department of AFNS specially: Yuan Fang, Yuanyao Chen, Fuyong Li, Yang Xu, Hui Li, Hua Chen, Xiaoyan Chen, Tingting Ju, Qing Li, Chen Yang, and many more, who helped me make this journey more eventful.

I would also like to thank Drs. Xiqun Zheng, Xiaolan Liu and Jian Ren from Qiqihar University, for always being supportive and caring about me. They are not only my mentors, but also life-long friends. I am so blessed to have the opportunity to spend a wonderful year together with Dr. Xiaolan Liu in Edmonton, and I will always remember her encouraging words, endless support, useful advice and we are family.

Finally, I would not be able to come so far without the support of my family. Words cannot express my gratitude towards my loving parents Mr. Libin Sun and Mrs. Qiuzhi Tian for their unconditional love, support, trust and understanding. Especially thanks my mother for being so strong to go through the tough situation with me, and for taking all the

responsibilities for taking care of my father when he was badly sick. I am also grateful to my relatives and many friends in China who helped our family during the hard days.

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LIST OF ABBREVIATIONS

3’SL: 3’-sialyllactose sodium salt

A. actinomycetemcomitans: *Actinobacilllus actinomycetemcomitans*

AAs: Amino acids

ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)

ACN: Acetonitrile

AFM: Atomic force microscopy

AFSSA: French Agency for Food Safety

APTES: 3-aminopropyltriethoxysilane

BabA: Blood group Ag-binding adhesin

BCIP/NBT: Bromochloroindolyl phosphate/nitro blue tetrazolium

BSA: Bovine serum albumin

BSM: Bovine submaxillary mucin

C. albicans: *Candida albicans*

C. jejuni: *Campylobacter jejuni*

CBB: Coomassie brilliant blue

cGMP: Casein glycomacropeptide

CVs: Column volumes

DH: Degree of hydrolysis

DHB: 2,5-dihydroxybenzoic acid

DLS: Dynamic light scattering

DMEM: Dulbecco’s modified eagle medium

DMSO: Dimethyl sulfoxide

DPPH: 2,2-di(4-tert-octylphenyl)-1-picrylhydrazyl

DTT: Dithiothreitol

E. coli: *Escherichia coli*

ECM: Extracellular matrix

EDTA: Ethylenediaminetetraacetic acid

EGF: Epidermal growth factor

ELISA: Enzyme-linked immunosorbent assay

EPEC: Enteropathogenic *E. coli*

EPS: Exopolysaccharides

ESI: Electrospray ionization

ETEC: Enterotoxigenic *Escherichia coli*

FBS: Fetal bovine serum

FECO: Fringes of equal chromatic order

FITC: Fluorescein isothiocyanate

FnBPs: Fibronectin-binding proteins

FPLC: Fast performance liquid chromatography

FTF: Fructosyltransferase

Gal: Galactose

GalNAc: N-acetylgalactosamine

GI: Gastrointestinal

GlcNAc: N-acetylglucosamine

GTF: Glucosyltransferase

H. influenza: *Haemophilus influenza*

H. pylori: *Helicobacter pylori*

HA: Hemagglutination assay

HDL: High-density lipoproteins

HEY: Hyperimmunized chicken egg yolk

HIV: Human immunodeficiency virus

HMOs: Human milk oligosaccharides

HPAEC/PAD: High-performance anion-exchange chromatography with pulsed amperometric detection

HPLC: High-performance liquid chromatography

HSA: Human serum albumin

IAA: Iodoacetamide

IPEC-J2: Porcine small intestinal epithelial cells

ITS: insulin-transferrin-selenium

LB: Luria-Bertani

LC: Liquid chromatography

LNF-II: Lacto-*N*-fucopentaose II

LPS: Lipopolysaccharides

LT: Heat-labile enterotoxins

MAC: Minimum anti-agglutinating concentration

MALDI: Matrix-assisted laser desorption/ionization

Man: Mannose

MS: Mass spectrometry

MSCRAMMs: Microbial surface components recognizing adhesive matrix molecules

Mw: Molecular weight

NDM: Non-dialyzable material

NDV: Newcastle disease virus

Neu5Ac: N-acetylneuraminic acid

Neu5Gc: N-glycolylneuraminic acid

nLc4Cer: Neolactotetraosylceramide

NR: Nitrogen recovery

OD: Optical density

P. intermedia: *Prevotella intermedia*

P. nigrescens: *Prevotella nigrescens*

Paa: Porcine attaching and effacing-associated factor

PAA-*b*-PMMA: Poly(acrylic acid-b-methyl methacrylate)

PAGE: Polyacrylamide gel electrophoresis

PAS: Periodic acid-Schiff

PB: Phosphate buffer

PBS: Phosphate-buffered saline

PBST: Phosphate buffered saline containing Tween 20

PEG: Poly(ethylene glycol)

PGM: Pig gastric mucin

pI: Isoelectric point

PLL-g-PEG: Poly(L-lysine)-graft-PEG

pNPP: p-nitrophenyl phosphate

RP-HPLC: Reverse-phase high performance liquid chromatography

S. enteritidis: *Salmonella enteritidis*

S. epidermidis: *Staphylococcus epidermidis*

S. fyris: *Salmonella fyris*

S. mutans: *Streptococcus mutans*

S. pneumoniae: *Streptococcus pneumoniae*

SD: Standard deviation

SDS: Sodium dodecyl sulfate

SEM: Standard error of the mean

SFA: Surface force apparatus

sIgA: Secretory IgA

SR-180: Sarcoma-180 cells

ST: Heat-stable enterotoxin

TFA: Trifluoroacetic acid

TNBS: 2,4,6-Trinitrobenzenesulfonic acid solution

TOF: Time of flight

UPEC: Uropathogenic *E. coli*

UPLC: Ultra-performance liquid chromatograph

UTI: Urinary traction infection

V. cholera: *Vibrio cholera*

αMM: Methyl α-D-mannopyranoside

CHAPTER 1 – General Introduction and Thesis Objectives

1.1 General introduction

Ovomuicn, a glycoprotein accounting approximately for 2-4% of egg albumen protein, is a member of mucin family and the primary contributor to the viscous nature of fresh egg white (Omana, Wang, & Wu, 2010). Ovomucin is composed of a carbohydrate-poor and a carbohydrate-rich subunit containing 11-15% and 50-57% (w/w) carbohydrate, respectively (Robinson & Monsey, 1971; Wang & Wu, 2012). On average, ovomucin consists of 33% of carbohydrate content (Mine, 1995a). Based on its solubility, ovomucin can be classified into an insoluble form and a soluble form with molecular weights of 23,000 kDa and 8,300 kDa, respectively (Tominatsu & Donovan, 1972). Generally, ovomucin is insoluble at neutral pH or in the absence of denaturing agents, which may limit its future application. Protein hydrolysis is widely applied to improve protein functionalities including solubility and bioactivities (Hammershøj, Nebel, & Carstens, 2008; Hiidenhovi, Hietanen, Mäkinen, Huopalahti, & Ryhänen, 2005). Therefore, preparation of ovomucin hydrolysates is a promising strategy to broaden the applications of ovomucin.

Ovomucin is rich in sialic acids (2.6-7.4%, w/w), which are a family of acylated derivatives of a nine-carbon carboxylated monosaccharide (Robinson & Monsey, 1971; Tang, Liang, Cai, & Mou, 2008). Sialic acid in ovomucin is N-acetylneuraminic acid (Neu5Ac), which is identical to the sialic acid found in human glycans but different from the N-glycolylneuraminic acid (Neu5Gc) present in glycans of other mammals (Schauer, Srinivasan, Coddeville, Zanetta, & Guérardel, 2009). Neu5Ac is thought to play crucial

roles in cognition and memory development in infants and has been suggested as an essential nutrient for infants (Tang, Liang, Cai, & Mou, 2008; Wang & Brand-Miller, 2003). Herein, ovomucin hydrolysates have potential to serve as a value-added ingredient to increase sialic acid content in follow-up formula.

Prebiotic was defined as "*a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon*" (Glenn & Roberfroid, 1995). Prebiotic can be regarded as one of the most promising nutritional supplements and functional foods (Wichienchot, Thammarutwasik, Jongjareonrak, Chansuwan, Hmadhlu, Hongpattarakere, et al., 2011). Some reports already demonstrated that mucin can serve as an endogenous prebiotic to stimulate bifidobacteria growth, since mucin is rich in carbohydrate (Hoskins, Agustines, McKee, Boulding, Krians, & Niedermeyer, 1985; Killer & Marounek, 2011; Ruas-Madiedo, Gueimonde, Fernández-García, de los Reyes-Gavilán, & Margolles, 2008). Ovomucin is a mucin-type glycoprotein and contains ~33% of carbohydrates. Thus, ovomucin may possess bifidogenic activity.

Adhesion of pathogens to the epithelial cells or to mucosal surfaces is the initial step in the infection process and glycoconjugates on host cell surfaces usually function as receptors for pathogens (Ofek, Bayer, & Abraham, 2013). Antibiotic regimens are commonly used to prevent or treat bacterial infection, especially in farm animals. However, the use of antibiotics is less favored due to the fear of developing bacterial antibiotic resistance (Docic & Bilkei, 2003; Nagy & Fekete, 1999). A promising alternative strategy to prevent infectious diseases is to use anti-adhesive agents to interfere the adhesion and colonization of bacteria to host tissues. Because ovomucin has similar structures to

mammalian mucin and is rich in carbohydrates, ovomucin may have the potential to act as receptor analogs and prevent pathogens adhesion to host tissues.

Undesired accumulation of proteins on surfaces is a serious issue affecting numerous applications, such as biosensors, biomedical implants, and food processing industry (Banerjee, Pangule, & Kane, 2011; Wong, Han, Timachova, Veselinovic, Hyder, Ortiz, et al., 2012). The typical approach to prevent protein adsorption is to coat the surface with an anti-fouling material that resists the non-specific interactions (Benhabbour, Liu, Sheardown, & Adronov, 2008). The main function of the mucous layer, covering epithelial cells, is to function as a natural anti-fouling surface to prevent undesirable adhesion to host tissues (Cone, 2009). This layer is mainly composed of water and mucins (Bansil & Turner, 2006). Due to the similar structures to mammalian mucins, ovomucin may also exhibit anti-fouling property.

1.2 Hypotheses and objectives

In the light of the above background information, we hypothesized that 1) Ovomucin hydrolysates could be a value-added ingredient in follow-up formula, functional foods and nutraceuticals, and 2) Ovomucin may function as an anti-fouling surface. In order to investigate the above hypotheses, the following objectives were addressed:

1) To prepare and characterize ovomucin hydrolysates (Chapter 3);
2) To test the effect of proteolysis of ovomucin on the sialic acid content and bifidogenic activity of ovomucin hydrolysates (Chapter 3);
3) To evaluate the anti-adhesive potential of ovomucin/ovomucin hydrolysates (Chapters 4 and 5);

4) To determine the anti-fouling activity of ovomucin and explore the underlying mechanism mainly by surface force apparatus (SFA) (Chapter 6).

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CHAPTER 2 – Literature Review

2.1 Egg white proteins

Hen egg has a long history of use as a human food (Vadehra, Nath, & Forsythe, 1973). Egg consists of three main components: eggshell (9.5%), egg white (63.0%), and yolk (27.5%) (Corrweill & Geiger, 1977). Egg white is composed of 88% water, 11% protein, which represents approximately 50% of the total egg protein, and 1% minor components including carbohydrates, ash, as well as trace amount of lipids (Abeyrathne, Lee, & Ahn, 2013; Stevens, 1991). During the development of hen embryo, egg white provides necessary nutrients to embryo and also protects embryo against bacterial contamination (Stevens, 1991). In the food industry, egg white protein is well recognized for its exceptional functional properties, such as foaming and emulsifying properties (Abeyrathne, Lee, & Ahn, 2013). The major proteins of egg white include ovalbumin (54%), ovotransferrin (12%), ovomucoid (11%), lysozyme (3.5%), and ovomucin (3.5%). Ovoinhibitor (1.5%), ovoglycoprotein (1.0%), ovoflavoprotein (0.8%), ovomacroglobulin (0.5%), avidin (0.05%), and cystatin (0.05%) are the minor proteins in egg albumen (Kovacs-Nolan, Phillips, & Mine, 2005).

In this review, the chemical compositions and representative characteristics of major egg white proteins are discussed.

2.1.1 Ovalbumin

Ovalbumin, a member of the serpin family although it exhibits no protease inhibitory activity, is the predominant protein in egg white with an isoelectric point (pI) of 4.5, accounting for 54% of total egg albumen proteins (Huntington & Stein, 2001; Stevens,

1991). Ovalbumin is a glycoprotein with a molecular weight (Mw) of 45 kDa, comprising 385 of amino acid residues (Nisbet, Saundry, Moir, Fothergill, & Fothergill, 1981). The N-terminal amino acid is acetylated glycine and the C-terminal residue is proline (Narita & Ishii, 1962; Nisbet, Saundry, Moir, Fothergill, & Fothergill, 1981). The amino acid sequence of ovalbumin has six cysteines with a single disulfide bond between Cys74 and Cys121, making it the only egg white protein with free SH groups (Thompson & Fisher, 1978). Ovalbumin has been classified into 3 isomers, A_1, A_2 and A_3, containing two, one, and no phosphate group, respectively, in a ratio of 85:12:3 in egg white (Abeyrathne, Lee, & Ahn, 2013). Ovalbumin contains 3.5% of carbohydrate (w/w), which forms a single carbohydrate chain covalently linked to Asn293 (Li-Chan & Nakai, 1989). Its glycans are found to be mainly high-mannose (Man) and hybrid compositions, predominantly $Man_5GlcNAc_2$ (N-acetylglucosamine) and $Man_6GlcNAc_2$, which adopt bi- to penta-antennary structures and largely lack the terminal galactose (Gal) (Harvey, Wing, Küster, & Wilson, 2000; Vadehra, Nath, & Forsythe, 1973).

During storage, native ovalbumin easily changes to S-ovalbumin (Smith, 1964; Smith & Back, 1965); although there were no changes in their chemical compositions (Castellano, Barteri, Bianconi, & Bruni, 1996; Smith & Back, 1968). S-ovalbumin shows higher thermal stability, more compact configuration, and larger surface hydrophobicity (Castellano, Barteri, Bianconi, & Bruni, 1996; Nakamura & Ishimaru, 1981). Circular dichroism and Fourier transform infrared spectroscopies results suggested that S-ovalbumin decreased 2-5% of α-helix while concomitantly increased anti-parallel β-sheet (Huntington, Gettins, & Patston, 1995; Kint & Tomimatsu, 1979).

2.1.2 Ovotransferrin

Ovotransferrin, accounting for about 12-13% of total egg albumin proteins, is made of monomeric glycoprotein consisting of 686 amino acid residues with a Mw of 78 kDa (Guha Thakurta, Choudhury, Dasgupta, & Dattagupta, 2003; Wu & Acero-Lopez, 2012). Ovotransferrin has only one carbohydrate chain attached to Asn473 composed of 4 residues of Man and 8 residues of GlcNAc, making up 2.6% of ovotransferrin mass (Williams, 1968; Williams, Elleman, Kingston, Wilkins, & Kuhn, 1982; Wu & Acero-Lopez, 2012). The glycan composition of ovotransferrin lacks Gal and sialic acid which is different from serum transferrin (Williams, 1968).

As a member of the transferrin family, ovotransferrin consists of two similarly sized and homologous lobes (N- and C-lobes) (Oe, Doi, & Hirose, 1988). Each lobe contains one single iron-binding site located within the inter-subdomain cleft (Kurokawa, Mikami, & Hirose, 1995). It was thought that the antimicrobial activity of ovotransferrin was due to its iron-binding property since ovotransferrin could sequester and deprive iron necessary for the growth of microorganisms (Bullen, Rogers, & Griffiths, 1978; Valenti, Antonini, Von Hunolstein, Visca, Orsi, & Antonini, 1983). However, metal ion saturated ovotransferrin also showed the antimicrobial activity, which suggested its antimicrobial activity is very likely iron independent (Tranter & Board, 1982). Ovotransferrin showed potential to be used as an infant formula ingredient for prevention or treatment of acute diarrheas due to its antimicrobial activity (Del Giacco, Leone, & Ferlazzo, 1985).

2.1.3 Ovomucoid

Ovomucoid constitutes 11% of egg white proteins with a Mw of 28 kDa and an pI of 4.1 (Gujral, 2015). The molecule consists of 186 amino acids representing three

structurally independent tandem domains with amino acid sequences of 1-68, 69-130, and 131-186, respectively, cross-linked by three disulfide bridges (Kato, Schrode, Kohr, & Laskowski, 1987). The secondary structure of ovomucoid includes 26% of α-helix, 46% of β-structure, 10% of β-turns, and 18% of random coil (Watanabe, Matsuda, & Sato, 1981). Ovomucoid is a highly glycosylated protein containing 20-25% of carbohydrate (w/w) which is attached to the polypeptide chain through asparagine residues (Yamashita, Tachibana, Hitoi, & Kobata, 1984).

The trypsin inhibitory activity of ovomucoid has been well studied. The results suggested that each ovomucoid molecule binds one molecule of trypsin. The trypsin inhibitory activity of ovomucoid is heat stable (Abeyrathne, Lee, & Ahn, 2013). More than 90% of the activity still remained after heating ovomucoid at 80 °C for 30 min in an acidic or neutral pH (Lineweaver & Murray, 1947). When the heating at 100 °C for 15 min at pH 6.0, 30% of the activity retained (Stevens & Feeney, 1963). In addition, the trypsin inhibitory activity of ovomucoid was not affected after pepsin hydrolysis (Kovacs-Nolan, Zhang, Hayakawa, & Mine, 2000).

Ovomucoid is considered as the dominant food allergen in egg white, which has multiple IgE and IgG epitopes throughout its three-domain structure (Bernhisel-Broadbent, Dintzis, Dintzis, & Sampson, 1994). It has been demonstrated that more IgG and IgE in sera derived from egg allergic patients could bind to the third domain of ovomucoid than to the first and second domains (Zhang & Mine, 1998). Only epitopes on the ovomucoid polypeptide backbone are responsible for the IgE binding while carbohydrate residues have a minor effect on allergenicity (Besler, Steinhart, & Paschke, 1997). Specifically, charged amino acids (aspartic acid, glutamic acid, and lysine) and some hydrophobic (leucine,

phenylalanine, and glycine) and polar (serine, threonine, tyrosine, and cystein) amino acids of ovomucoid are important for antibody (IgG and IgE) binding (Mine & Zhang, 2002).

2.1.4 Lysozyme

Lysozyme, also known as muramidase or N-acetylmuramic hydrolase, is a 14.4 kDa protein with 129 amino acids and an pI of 10.7 (Stevens, 1991). It represents 3.4 % of the total egg white proteins (Gujral, 2015). Lysozyme consists of a single polypeptide chain that is cross-linked by four disulfide bridges (Mine, 1995a). The peptide sequence of lysozyme was determined by Canfield in 1963 (Canfield, 1963). The conformation and structure of hen egg lysozyme were also well studied by X-ray analysis in 1960s (Blake, Koenig, Mair, North, Phillips, & Sarma, 1965; Blake, Mair, North, Phillips, & Sarma, 1967).

Lysozyme is considered as a natural defense factor against bacterial infection due to its antimicrobial activity, especially Gram-positive bacteria, such as *Staphylococcus aureus*, *Micrococcus lysodeikkticus*, *Bacillus cereus*, *Bacillus stearothermophilus*, *Clostridium thermosaccarolyticum*, and *Clostridium tyrobutyricum* (Cunningham, Proctor, & Goetsch, 1991). However, lysozyme is relatively ineffective against Gram-negative bacteria (Mine, Ma, & Lauriau, 2004), since lysozyme can lyse the cell wall of Gram-positive bacteria by hydrolyze the β-linkage between N-acetylneuraminic acid (Neu5Ac) and GlcNAc of the peptidoglycan (Mine, Ma, & Lauriau, 2004). Some studies have been conducted on broadening the antimicrobial spectrum of lysozyme, including conjugating lysozyme with EDTA (ethylenediaminetetraacetic acid) or dextran (Nakamura, Kato, & Kobayashi, 1990; Samuelson, Rupnow, & Froning, 1985), partially denatured or denatured lysozyme (Ibrahim, 1998; Ibrahim, Higashiguchi, Koketsu, Juneja, Kim, Yamamoto, et al., 1996),

combining with other natural preservatives, such as nisin, lactoferrin, and glycine (Hauben, Wuytack, Soontjens, & Michiels, 1996; Mine, Ma, & Lauriau, 2004).

2.2 Egg white ovomucin

Ovomuicn, a glycoprotein accounting approximately for 2-4% of the total egg albumen proteins, is a member of mucin family and the primary contributor to the gel-like property of fresh egg white (Brooks & Hale, 1959; Omana, Wang, & Wu, 2010). Based on its solubility, ovomucin can be classified into an insoluble form and a soluble form with Mws of 23,000 kDa and 8,300 kDa, respectively (Tominatsu & Donovan, 1972). Soluble ovomucin has been found in both thick and thin egg white, while insoluble ovomucin is only present in thick egg white (Hiidenhovi, 2007; Sato & Hayakawa, 1977). Generally, ovomucin is highly insoluble at neutral pH or in the absence of denaturing agents.

2.2.1 Structure and composition of ovomucin

Light scattering study suggested ovomucin adopted a random coiled structure (Tominatsu & Donovan, 1972), which provided evidence that ovomucin may have a highly polymerized macromolecular structure via disulfide bond formation as that of mammalian mucins (Bansil & Turner, 2006). Two subunits (α- and β-ovomucin) were separated from ovomucin by gel filtration chromatography and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), with Mws of 220 kDa and 400~700 kDa, respectively (Akio, Takayuki, Kunihiko, Katsuya, & Masao, 1991; Itoh, Miyazaki, Sugawara, & Adachi, 1987).

α-ovomucin is considered as a carbohydrate poor subunit since it contains 11-15% (w/w) of carbohydrate (Robinson & Monsey, 1971). The primary amino acid sequence of α-ovomucin was determined from cloned cDNA, which consists of 2087 amino acids with

a relative Mw of 230 kDa (Watanabe, Shimoyamada, Onizuka, Akiyama, Niwa, Ido, et al., 2004). β-ovomucin is a carbohydrate-rich subunit containing 50-57% (w/w) of carbohydrate (Robinson & Monsey, 1971). The complete amino acid sequence of β-ovomucin has not been deduced so far. Only part of the amino acid sequence of β-ovomucin (827 amino acids) has been revealed with a Mw of 91,836 Da (Hiidenhovi, 2007). The amino acid analysis demonstrated that β-ovomucin is predominant of hydroxyl amino acids, such as threonine and serine (Itoh, Miyazaki, Sugawara, & Adachi, 1987; Robinson & Monsey, 1971).

On average, ovomucin consists of 33% of carbohydrate content, including Man, Gal, N-acetylgalactosamine (GalNAc), GlcNAc, Neu5Ac and sulfated saccharides (Donovan, Davis, & White, 1970; Mine, 1995a). It is worthwhile to mention that ovomucin is rich in sialic acid (Neu5Ac, 2.6-7.4%, w/w), which are a family of acylated derivatives of a nine-carbon carboxylated monosaccharide and play important roles in biological processes and in infant nutrition (Robinson & Monsey, 1971; Tang, Liang, Cai, & Mou, 2008). There are at least three types of glycan structures, including Gal, GalNAc, Neu5Ac and sulfate (molar ratio 1:1:1:1); Gal and GlcNAc (molar ratio 1:1); Man and GlcNAc (molar ratio 1:1) that are attached to the protein backbone of ovomucin (Bansil & Turner, 2006; Kato, Fujinaga, & Yagishita, 1973). The N-glycosylation of α- and β-ovomucin was characterized by nano LC (liquid chromatography) ESI (electrospray ionization)-MS (mass spectrometry), MS/MS and MALDI (matrix-assisted laser desorption/ionization) MS (Offengenden, Fentabil, & Wu, 2011). The results suggested that N-linked glycans were present in both ovomucin subunits. Eighteen potential N-glycosylated sites were found in α-ovomucin and two were detected in β-ovomucin. Moreover, MALDI MS results

indicated that the most abundant N-glycan structure in α-ovomucin was composed of $GlcNAc_2Man_3$ core, a bisecting GlcNAc and another three GlcNAc antennae located on the mannoses of the core.

2.2.2 Biological properties of ovomucin and its derived components

In addition to providing necessary energy and essential nutrients, food is thought to play an important role in human health. Therefore, there has been a growing interest to explore the biological activities of foods and to develop health-promoting functional foods. Research in the field of bioactive food proteins and food-derived peptides has intensified during the past three decades (Hartmann & Meisel, 2007). The biological activities of ovomucin and its derived glycopeptides have also been explored as summarized in Table 2.1.

2.2.2.1 Antioxidant activity

The antioxidant activity of ovomucin hydrolysates has been investigated using ABTS (2,2'-azino-bis[3-ethylbenzothiazoline-6-sulphonic acid]) and DPPH (2,2-di[4-tert-octylphenyl]-1-picrylhydrazyl) radical scavenging assays (Chang, Ha, Han, Seol, Kim, Jeong, et al., 2013). The results suggested that the antioxidant activity was affected by the incubation time but not by different proteases used. After partial purification of the ovomucin hydrolysate by ultrafiltration and reverse-phase high-performance liquid chromatography (HPLC), two antioxidant peptides, LDEPDPL and NIQTDDFRT, were identified by MS.

2.2.2.2 Anti-tumor effect

The anti-tumor effect of β-ovomucin on Sarcoma-180 cells (SR-180) was reported both *in vitro* and *in vivo* studies. When SR-180 cells were incubated with β-ovomucin *in*

vitro, the cell proliferation rate was apparently reduced. In addition, SR-180 cells treated with β-ovomucin showed morphological changes associated with cell necrotic degeneration. Furthermore β-ovomucin only showed cytotoxic effect on SR-180 cells, not on normal cells (Yokota, Ohishi, & Watanabe, 1999b). Later, β-ovomucin was found to suppress the growth of subcutaneously xenografted SR-180 cells in mice. Since massive accumulations of neutrophils, macrophages and lymphocytes were found at the margin of the β-ovomucin-treated tumor cells, the anti-tumor activity of β-ovomucin was suggested due to activating the immune system (Yokota, Ohishi, & Watanabe, 1999a).

Anti-tumor activities were also identified from α-ovomucin using a double grafted tumor system. Meth-A fibrosarcoma tumor cells were simultaneously inoculated to the right and left flanks of BALB/c mice. Two highly glycosylated fragments from pronase-treated ovomucin with Mws of 220 and 120 kDa were injected into the right tumor separately. Both fragments completely cured the right (treated) tumor and slightly inhibited the growth of the left (untreated) tumor. This study postulated the same mechanism as above: the immune system was possibly activated by ovomucin fragments (Watanabe, Tsuge, Shimoyamada, Ogama, & Ebina, 1998). Similarly, another α-ovomucin derived glycosylated fragment with a Mw of 70 kDa could also completely inhibit the growth of Meth-A fibrosarcoma tumor cells (Oguro, Watanabe, Tani, Ohishi, & Ebina, 2000).

2.2.2.3 Immunomodulating property

The immunomodulating property of ovomucin was studied in 1990s. Firstly, it was reported that ovomucin enhanced the proliferation of mouse spleen lymphocytes, stimulated by lipopolysaccharides (Otani & Maenishi, 1994). Later, Tanizaki et al found that the sulphated glycopeptides of ovomucin exhibited good macrophage-stimulating

activity; the O-linked glycans of the sulphated glycopeptides was identified as the responsible components for this macrophage-stimulating activity (Tanizaki, Tanaka, Iwata, & Kato, 1997).

2.2.2.4 Cholesterol-lowering effect

The cholesterol-lowing effect of ovomucin was firstly suggested *in vitro*; using casein as a control, ovomucin elevated bile acid-binding capacity, lowered micellar cholesterol solubility, and suppressed cholesterol uptake in Caco-2 cells. Feeding ovomucin to rats (Male rats of the Wistar strain) increased fecal excretion of bile acids, decreased serum total cholesterol in rats and reduced the concentration of total lipids in liver. Direct interaction between cholesterol mixed micelles and ovomucin in the jejunal epithelia was proposed as the mechanism underlying the cholesterol-lowering effect of ovomucin (Nagaoka, Masaoka, Zhang, Hasegawa, & Watanabe, 2002).

2.2.2.5 Anti-agglutinating/anti-adhesive activity

Hemagglutination assay (HA) is a widely used method to test bacterial adhesion; erythrocytes mimic the gut wall, thus anti-agglutinating activity could indicate the anti-adhesive potential. The anti-agglutinating activity of ovomucin against influenza virus was reported in late 1940s, and its possible mechanism was proposed as the interaction between influenza virus enzyme and ovomucin (Gottschalk & Lind, 1949b; Lind, 1949). Further research showed that ovomucin or ovomucin derived components exhibited anti-agglutinating activity against bovine rotavirus, hen newcastle disease virus (NDV), and human influenza virus, due probably to the high affinity of ovomucin with the viruses (Tsuge, Shimoyamada, & Watanabe, 1996a; 1996b; 1997b). The authors also found that

the Neu5Ac residue in the β-ovomucin greatly contributed to the binding of ovomucin to NDV (Tsuge, Shimoyamada, & Watanabe, 1997a).

Since the binding of ovomucin to viruses was important for its anti-agglutinating activity, the binding activity of ovomucin/ovomucin glycopeptides to some bacteria was tested (Kobayashi, Hattori, Hara-Kudo, Okubo, Yamamoto, Takita, et al., 2004). Glycopeptides from pronase-treated ovomucin showed binding activity to *Escherichia coli* (*E. coli*) O157:H7. After treating ovomucin glycopeptides with sialidase, the desialylated glycopeptides lost the binding activity, suggesting that sialic acid played a significant role in its binding to *E. coli* O157:H7 (Kobayashi, et al., 2004). In addition, ovomucin inhibited the adhesion and colonization of *Helicobacter pylori* (*H. pylori*) to the mouse stomach *in vivo*, which had potential to prevent or treat diseases caused by infection of *H. pylori*, such as peptic ulcers (Kodama, Gifu, Nobutake, Kimura, & Saitama, 2001).

2.3 Bacterial adhesion to host cells

Adhesion of pathogens to host cells or tissue surfaces is considered as an essential first step in the infection process (Savage, 1977). Meanwhile, bacterial adhesion to host cell surfaces gains a number of advantages. Firstly, adherent bacteria have the ability to withstand cleansing mechanisms operating on mucosal surfaces, such as secretions, salivation, excretion, coughing and sneezing, to eliminate the unattached bacteria (Ofek & Doyle, 1994b). Secondly, adherent bacteria have better access to nutrients released by host cells (Zafriri, Oron, Eisenstein, & Ofek, 1987). Thirdly, adhesion to host cells protects bacteria from deleterious substances in the surrounding milieu, such as anti-microbial agents and antibodies (Ofek & Doyle, 1994b).

The barrier provided by epithelial surfaces of the integument and the gastrointestinal (GI), respiratory, and urinary tracts is considered as the first front line of host defense, which forms a critical interface between the internal and external environments (McCormick, 2006). One of the primary functions of epithelial cells is to protect the underneath tissue from invasion or infection by pathogens and toxins (Van Lommel, 2003). Many epithelial cells are covered by a gel-like mucus layer, especially in the GI and respiratory tracts (Neutra, 1987). Mucus is mainly composed of water and mucins that are large filamentous glycoproteins (averagely 2×10^6 Da) secreted by goblet cells (Forstner, Taichman, Kalnins, & Forstner, 1973). The mucus layer protects epithelium from dehydration, mechanical damage and invading of infectious agents, including pathogenic bacteria, viruses and parasites (Kim & Khan, 2013). This review focuses on discussion of bacterial adhesion to mucus layer and epithelial cells.

2.3.1 Brief introduction of bacterial adhesion

Bacterium, epithelium and mucus layer are all negatively charged in the physiological environment. When a bacterium adheres to a host cell, the repulsive forces must be overcome by various interactions which are varied depending on the distances between the bacterium and cell surface (Ofek & Doyle, 1994b). Specifically, only van der Waals interaction is present when the distance is more than 50 nm (first region); both van der Waals and Coulombic interactions are significant at a distance ranging from 10 to 20 nm (second region); complementary interactions (specific adhesin-host receptor interaction) are required at a distance of 1-5 nm (third region). In addition, hydrophobin-hydrophobin (hydrophobic interaction) and charge-charge (electrostatic) interactions may also

contribute to form the eventually irreversible adhesion (Busscher & Weerkamp, 1987; Ofek & Doyle, 1994b; Shoaf-Sweeney & Hutkins, 2008).

Once a bacterium moves to within 5 nm of the host cell surface, bacterial adhesion occurs following a two-step kinetic model (Hasty, Courtney, Sokurenko, & Ofek, 1994). In the first step, bacteria have to overcome repulsive forces and reversibly adhere to the host cell surface through weak hydrophobic interactions generated by hydrophobins on the bacterial surface and hydrophobic groups on the host cell surface (Rosenberg, Greenstein, Barki, & Goldberg, 1996). In the second kinetic step, a strong irreversible adhesion between a specific bacterial surface molecule (bacterial adhesin) and a complementary host receptor is developed (Ofek & Doyle, 1994b; Shoaf-Sweeney & Hutkins, 2008).

2.3.2 Bacterial adhesins

Fimbriae (pili) are hair-like appendages, consisting of protein subunits that are commonly anchored in the outer membrane of Gram-negative bacteria. Fimbriae carry adhesins at their tips, along their sides or entire length (Ofek & Doyle, 1994b). The binding of pathogenic bacteria to host receptors is usually mediated by adhesins (Bouguénec, 2005). Adhesins are responsible for recognizing and binding to specific receptors on host cell surfaces (Soto & Hultgren, 1999). In a broad sense, adhesins can be classified into fimbrial and afimbrial adhesins.

2.3.2.1 Fimbrial adhesins

As the name implies, fimbrial adhesins are carried by fimbriae. Typical examples of fimbrial adhesins are observed in the pyelonephritis-associated pili, type 1 fimbriae of uropathogenic *E. coli* (UPEC), and type IV pili of *Pseudomonas aeruginosa*, enteropathogenic *E. coli* (EPEC), and pathogenic *Neisseriae* (Hauck, 2006;

Shoaf-Sweeney & Hutkins, 2008). Few fimbrial adhesins have been noticed in Gram-positive bacteria so far (Ton-That & Schneewind, 2004). Fimbrial adhesins mostly function as lectins and recognize the carbohydrate moieties of glycoproteins and glycolipids on the surface membrane of host cell (Hauck, 2006).

2.3.2.2 Afimbrial adhesins

Afimbrial adhesins are either embedded into or attached to the outer surface of bacteria, which are not commonly localized on surface protrusions (Hauck, 2006). Non-fimbrial adhesins have been found in both Gram-negative and Gram-positive bacteria. Afimbrial adhesins mediate the adhesion of bacteria to host receptors through direct protein-protein interactions (Hauck, 2006). Some of the most well-studied afimbrial adhesins are fibronectin-binding proteins (FnBPs) expressed by *Streptococcus pyogenes* and *Staphylococcus aureus* which are surface proteins anchored in the bacterial cell wall (Patti, Allen, McGavin, & Hook, 1994).

2.3.3 Specific bacterial adhesin-host receptor interactions

Since bacterial adhesins binding to host receptors is a critical step in bacterial adhesion, identification and characterization of direct molecular interactions between adhesins and receptors are significant in developing novel strategies to interfere bacterial adhesion and to further prevent infection (Shoaf-Sweeney & Hutkins, 2008). Two main types of specific adhesin-receptor interactions have been well studied, which will be discussed below in detail.

2.3.3.1 Lectin–carbohydrate interactions

Lectins are carbohydrate binding proteins that many bacteria employ as adhesins (Ofek & Doyle, 1994b; Shoaf-Sweeney & Hutkins, 2008). The most well-documented

bacterial adhesin-host receptor interactions are those involving bacterial lectins and complementary oligosaccharide ligands on the host cell surface (Shoaf-Sweeney & Hutkins, 2008). Lectins are usually carried by fimbriae in Gram-negative bacteria and embedded into the peptidoglycan layer or anchored in the cytoplasmic membrane in Gram-positive bacteria (Ofek & Doyle, 1994a; Shoaf-Sweeney & Hutkins, 2008). Glycoprotein and glycolipids generally serve as the receptor molecules of bacterial lectins (Rauvala & Finne, 1979). Several approaches have been applied to determine the carbohydrate specificity of bacterial lectins; for example, to test the effects of removing certain carbohydrates (monosaccharides, simple glycosides and oligosaccharides) of receptors on bacterial adhesion (Yollken, Peterson, Vonderfecht, Fouts, Midthun, & Newburg, 1992; Hirmo, Kelm, Schauer, Nilsson, & Wadström, 1996; Kobayashi, et al., 2004; Ofek, Mirelman, & Sharon, 1977). To further understand the binding specificity, the glycol-conjugated receptors have to be isolated to characterize their primary and fine structures (Fang, Gan, & Marquardt, 2000; Teneberg, Ångström, & Ljungh, 2004).

In some cases, carbohydrates on bacteria surface can function as adhesins. For example, the Gram-positive pathogen *Streptococcus* employs hyaluronic acid capsular polysaccharide as the adhesin to bind to the host cell lectin CD 44 (Cywes, Stamenkovic, & Wessels). Lipopolysaccharides (LPS) anchored to the outer membranes of Gram-negative pathogens have been demonstrated to act as adhesins of various bacterial species, such as *Campylobacter jejuni* (*C. jejuni*), *E. coli* and *H. pylori* (Jacques, 1996). LPS can bind to host cells, mucus and the extracellular matrix (ECM), laminin (Cohen, Arruda, Williams, & Laux, 1985; Morrison, Lei, Kirikae, & Chen, 1993; Valkonen, Wadström, & Moran, 1994).

2.3.3.2 Protein–protein interactions

Pathogenic bacteria frequently express surface proteins specifically interacting with the ECM of host cells to form protein-protein interactions that contribute significantly to bacterial adhesion (Patti, Allen, McGavin, & Hook, 1994; Westerlund & Korhonen, 1993). ECM is a stable structure that underlies epithelial and endothelial cells, composed of collagen, laminin, fibronectin, and proteoglycan (Westerlund & Korhonen, 1993). ECM become available for bacterial binding when the host cell surface is compromised (Shoaf-Sweeney & Hutkins, 2008). The surface proteins (bacterial adhesins) that bind to ECM are defined as microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) (Shoaf-Sweeney & Hutkins, 2008). The best-characterized protein–protein interactions are those involving FnBPs (members of MSCRAMMs) from staphylococci and streptococci and fibronectin (Shoaf-Sweeney & Hutkins, 2008). Fibronectin is a dimeric glycoprotein with a Mw of 440 kDa, which is composed of two variant protein chains linked by C-terminal disulfide bonds. Each monomer includes three distinct types of modules (Henderson, Nair, Pallas, & Williams, 2011; Joh, Wann, Kreikemeyer, Speziale, & Höök, 1999). Amino acid sequences of the fibronectin-binding domains from staphylococci and streptococci FnBPs have been determined as FnbpA, FnbA, FnbB, and Sfb (Jönsson, Signäs, Müller, & Lindberg, 1991; Lindgren, McGavin, Signäs, Guss, Gurusiddappa, Höök, et al., 1993; Signäs, Raucci, Jönsson, Lindgren, Anantharamaiah, Höök, et al., 1989; Talay, Valentin-Weigand, Jerlström, Timmis, & Chhatwal, 1992). Although the amino acid sequences of binding domains may vary considerably, the overall structures of these FnBPs are similar (Joh, Wann, Kreikemeyer, Speziale, & Höök, 1999). Namely, the primary binding domain is localized to a region containing 3 to 4 repeats

(35~40 amino acids) and positioned outside the cell wall-spanning domains (Jönsson, Signäs, Müller, & Lindberg, 1991; Patti, Allen, McGavin, & Hook, 1994). Immunological studies have been conducted to suggest how the FnBPs interact with fibronectin. It appears that binding domains of the FnBPs have a largely unorganized structure and an ordered structure occurs only upon binding to fibronectin (House-Pompeo, Xu, Joh, Speziale, & Höök, 1996; Joh, Wann, Kreikemeyer, Speziale, & Höök, 1999).

2.3.4 An example: adhesion of porcine enterotoxigenic *E.coli* (ETEC) K88 strains to mucus or small intestine of piglets

2.3.4.1 K88 fimbriae

ETEC strains expressing K88 fimbriae specifically adhere to the small intestine of neonatal and post-weaning piglets causing diarrhea (Moonens, Van den Broeck, De Kerpel, Deboeck, Raymaekers, Remaut, et al., 2015). The K88 fimbriae include three antigenic variants, designated as $K88_{ab}$, $K88_{ac}$ and $K88_{ad}$, mainly mediating ETEC adhesion to host receptors (Ofek & Doyle, 1994c). These three variants show a related yet distinct receptor binding specificity since they have the same major structure component, FaeG, while the primary sequences and conformation of their FaeG subunits are different (Moonens, et al., 2015). It has been demonstrated that the amino acid residues from 125 to 163 of the FaeG are significant for K88 variant-specific binding (Zhang, Fang, & Francis, 2009). The K88 fimbriae are composed of the multiple repeated major subunit (FaeG) and some minor subunits, while only FaeG is responsible for the fimbrial binding specificity (Hoschützky, Bühler, Ahrens, & Jann, 1991; Zhang, Fang, & Francis, 2009). FaeG serves as the adhesin of the K88 fimbriae, which is not only located at the tip, but all along the pilus structure (Bakker, Willemsen, Simons, van Zijderveld, & de Graaf, 1992).

To better understand the binding activity of K88 fimbriae, attention has been directed towards the structure and carbohydrate binding sites of FaeG subunit. The crystal structure of the chloroplast-targeted FaeG has been determined, suggesting that FaeG contains an immunoglobulin-like core including strands A_1, A_2, B_1, B_2, C, D, E_1, E_2, F and G, and an extra domain introduced between strands D and E_1 (D'-D''-α_1-α_2), which is believed to host the carbohydrate binding site of FaeG (Van Molle, Joensuu, Buts, Panjikar, Kotiaho, Bouckaert, et al., 2007). Lactose was employed as a model chemical to test the carbohydrate binding sites of $FaeG_{ad}$. The crystal structure of $FaeG_{ad}$ bound to lactose presented that two small peptides Phe^{150}-Glu^{152} and Val^{166}-Glu^{170} from $FaeG_{ad}$ specifically interact with the terminal galactose in lactose (Moonens, et al., 2015).

2.3.4.2 K88 fimbrial adhesin receptors

Receptors of K88 fimbrial adhesin have been identified from the brush borders of epithelial cells, intestinal membranes, and mucus, which appear to be either glycoproteins or glycolipids (Blomberg, Krivan, Cohen, & Conway, 1993; Jin & Zhao, 2000b). It was suggested that $K88_{ad}$ adhesin preferentially binds to glycolipids, while $K88_{ab}$ and $K88_{ac}$ adhesins preferentially bind to glycoproteins (Grange, Erickson, Levery, & Francis, 1999).

The first two specific receptors for $K88_{ac}$ adhesin have been identified from the brush borders of porcine intestinal epithelial cells in 1990s, which are glycoproteins with Mws of 210 and 240 kDa (Erickson, Willgohs, McFarland, Benfield, & Francis, 1992). These two receptors were characterized as O-linked mucin-type sialoglycoconjugates, showing similar amino acid compositions that were rich in threonine (49%) and proline (25%) (Erickson, Baker, Bosworth, Casey, Benfield, & Francis, 1994). Three other receptors specific for $K88_{ac}$ adhesin have been recognized from the porcine small intestinal mucus

by affinity chromatography, with sizes of 26, 41, and 80 kDa (Fang, Gan, & Marquardt, 2000; Jin, Marquardt, Baidoo, & Frohlich, 2000).

Glycoproteins with Mws of 25, 35, 40-42, and 60 kDa in the small intestinal mucus, and 16, 23, 35, 40-70, 74, and 91 kDa in the brush border membranes have been identified as the receptors for $K88_{ab}$ adhesin (Grange & Mouricout, 1996; Laux, McSweegan, Williams, Wadolkowski, & Cohen, 1986; Metcalfe, Krogfelt, Krivan, Cohen, & Laux, 1991; Staley & Wilson, 1983). Among these receptors, the 74 kDa glycoprotein specifically bound to $K88_{ab}$, which was characterized as a mucosal transferrin (Grange & Mouricout, 1996). An intestinal neutral glycosphingolipid has been recognized as a phenotype-specific receptor for the $K88_{ad}$ adhesin, which appears to be neolactotetraosylceramide (nLc4Cer, Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer) (Grange, Erickson, Levery, & Francis, 1999).

It has been clearly demonstrated that all three K88 adhesin variants recognize the carbohydrate moiety of the glycoconjugated receptors. However, the fine carbohydrate structures of the K88 adhesin receptors have not been characterized (Jin & Zhao, 2000b). Several studies demonstrated that terminal β-linked Gal of both glycoprotein and glycolipid is the minimum determinant required for binding to K88 fimbriae (Blomberg, Krivan, Cohen, & Conway, 1993; Grange, Erickson, Anderson, & Francis, 1998; Payne, O'Reilly, & Williamson, 1993). In addition, the sequence of Galβ1-3GalNAc and Galβ1-3/4GlcNAc was found to be the essential binding sites for $K88_{ac}$ adhesin, and that of Galα1-3Gal for $K88_{ab}$ adhesin (Willemsen & de Graaf, 1992). The neuraminidase digestion assay demonstrated that terminal sialic acid of host receptors was not crucial for the binding of $K88_{ac}$ adhesin (Grange, Erickson, Anderson, & Francis, 1998).

2.4 Anti-adhesive therapy of infectious diseases

Since adhesion of pathogens to host cells is an important early event for infection, one of the ultimate goals for studying the bacterial adhesion is to develop effective anti-adhesive therapy to interfere or inhibit pathogen adhesion to host cells, accordingly, prevent and/or treat infectious diseases (Ofek, Bayer, & Abraham, 2013). Much research effort has been devoted to developing anti-adhesive agents, such as receptor and adhesin analogs, adhesin-based vaccines, and probiotics (Bernet, Brassart, Neeser, & Servin, 1994; Kelly, Younson, Hikmat, Todryk, Czisch, Haris, et al., 1999; Lane, Mariño, Naughton, Kavanaugh, Clyne, Carrington, et al., 2012; Moon & Bunn, 1993). Generally, the major function of anti-adhesive agents is to block the interactions between bacterial adhesins and host receptors (Ofek, Hasty, & Sharon, 2003). Compared to chemotherapy or antibiotic treatments, anti-adhesive therapy is mild and safe since it does not kill or inhibit the growth of pathogens (Karlsson, 1998). Although resistant strains may also develop in the anti-adhesive therapy, they will be diluted by the sensitive strains which result in a significantly lower rate compared to antibiotic-resistant strains (Ofek, Hasty, & Sharon, 2003). Since the early 1980s, there has been an increasing interest in identification of anti-adhesive agents that naturally exist in dietary sources because they are relatively safe. Furthermore, some of the most effective anti-adhesive components identified so far are present in foods (Ofek, Bayer, & Abraham, 2013). We only focus on the anti-adhesive activity of dietary constituents in this review, which will be discussed based on the phases of clinical research.

2.4.1 *In vitro* study

2.4.1.1 Anti-agglutinating activity to indicate anti-adhesive potential

The anti-agglutinating activity of egg white ovomucin against influenza virus was recognized by HA in late 1940s, probably through interaction of ovomucin with an enzyme at the surface of influenza virus, therefore preventing the virus agglutinating erythrocytes (Gottschalk & Lind, 1949a, 1949b). In the last decade, the polysaccharide fractions from roots of *Panax ginseng* were found to inhibit the activity of *H. pylori* and *Porphyromonas gingivalis* to agglutinate erythrocytes (Belogortseva, Yoon, & Kim, 2000; Lee, Lee, Chung, & Kim, 2004). Additionally, bovine milk oligosaccharides indicated the anti-adhesive potential against seven ETEC strains isolated from diarrheic calves (Martín, Martín-Sosa, & Hueso, 2002). The potential anti-adhesive activity of exopolysaccharides produced by strains of *Lactobacillus reuteri* against K88 ETEC strains was also reported (Wang, Gänzle, & Schwab, 2010).

In general, the obvious advantages of HA are simple and efficient, which can provide results within a few hours. However there are limitations. Firstly, analysis of HA relies on manual interpretation which can be subjective, leading to discrepancies among different researchers (Wood, Laurie, & Engelhardt, 2013). Secondly, HA cannot provide quantitative values for the extent of bacterial adhesion and therefore is suitable only for preliminary experiment.

2.4.1.2 Cell culture

Cell culture assay is a common method to test the anti-adhesive activity of compounds *in vitro*, which is more biological relevant compared to HA method. In this section, the

anti-adhesive activity determined by cell culture assay was discussed based on individual foodstuff and summarized in Table 2.2.

A. Human milk

Human milk is well known to be beneficial in inhibiting adherence of certain pathogens to host cells and subsequently preventing bacterial infections (Ashkenazi, 1994; Kunz & Rudloff, 1993). The composition of human milk is complex and variable, and the anti-adhesive activity has been attributed mainly to oligosaccharides and to less extend to mucin, κ-casein, and lactoferrin (Table 2.2).

Besides lactose and lipids, oligosaccharides are the third largest component of human milk (Coppa, Zampini, Galeazzi, Facinelli, Ferrante, Capretti, et al., 2006). It has been demonstrated that human milk oligosaccharides (HMOs) can act as receptor analogs to block bacteria adherence to host cells and subsequently prevent infectious diseases; many species such as *E. coli*, *Vibrio cholera* (*V. cholera*), *Salmonella fyris* (*S. fyris*), *Candida albicans* (*C. albicans*), *Streptococcus pneumoniae* (*S. pneumoniae*), *Haemophilus influenza* (*H. influenza*) have been studied (Andersson, Porras, Hanson, Lagergård, & Svanborg-Edén, 1986; Brassart, Woltz, Golliard, & Neeser, 1991; Coppa, et al., 2006; Cravioto, Tello, Villafan, Ruiz, del Vedovo, & Neeser, 1991). It was suggested that the effective anti-adhesive activity of HMOs is associated with their complex compositions because oligosaccharides mixture has more chance to bind to bacterial multi-adhesins and has stronger interactions (high affinity) with bacteria than single glycan (Coppa, et al., 2006).

Mucins (MUC 1 and MUC 4) are important anti-adhesive agents in human milk, which are glycoproteins with large Mws (>200 kDa) (Liu, Yu, Chen, Kling, & Newburg,

2012; Schroten, Plogmann, Hanisch, Hacker, Nobi-Bosch, & Wahn, 1993). Most studies indicated that the carbohydrate moieties of mucins, which resemble the host cell surface receptors for pathogens, are responsible for the anti-adhesive activity (Ruvoën-Clouet, Mas, Marionneau, Guillon, Lombardo, & Le Pendu, 2006; Schroten, Plogmann, Hanisch, Hacker, Nobis-Bosch, & Wahn, 1993). Nevertheless, recent study found that the protein backbones of mucins may function as receptor analogs as well to bind to *Salmonella enterica* serovar Typhimurium (Liu, Yu, Chen, Kling, & Newburg, 2012). Human milk MUC1 showed anti-adhesive activity against poxviruses as MUC1 possibly aggregate poxviruses prior to their entry into host cells, which indicated the potential to inhibit other enveloped viruses such as human immunodeficiency virus (HIV) (Habte, Kotwal, Lotz, Tyler, Abrahams, Rodriques, et al., 2007).

Lactoferrin, a glycoprotein, is known to inhibit adhesion of *Actinobacilllus actinomycetemcomitans* (*A. actinomycetemcomitans*), *Prevotella intermedia* (*P. intermedia*), and *Prevotella nigrescens* (*P. nigrescens*) to fibroblasts and epithelial cells (Alugupalli & Kalfas, 1995). The possible mechanisms underlying this action are that lactoferrin binds to both bacteria and host cells and subsequently blocks specific adhesin-ligand and non-specific charge-dependent interactions (Alugupalli & Kalfas, 1997). Caseins, which represent for almost half of the protein contents in human milk, are crucial as nutritional and bioactive glycoproteins for breast-fed infants. Fucose containing moieties of κ-casein are responsible for inhibition of *H. pylori* to human gastric mucosa (Strömqvist, Falk, Bergström, Hansson, Lönnerdal, Normark, et al., 1995). Secretory IgA (sIgA) induces a 94 kDa plasmid-encoded outer membrane protein that mediates EPEC adhesion

to HEp-2 cells and thus prevents the localized adherence of EPEC to HEp-2 cells (Cravioto, Tello, Villafan, Ruiz, del Vedovo, & Neeser, 1991).

B. Cranberry/cranberry juice

Cranberry (*Vaccinium macrocarpon*) has long been recognized for its use against urinary tract infection (Camesano, Liu, & Pinzon-Arango, 2007), probably through the inhibition of adherence of urinary tract *E. coli* to human uroepithelial cells (Schmidt & Sobota, 1987; Sobota, 1984). It was postulated that the anti-adhesive activity of cranberry juice was due to its ability to acidify urine and thus to inhibit bacterial growth and adhesion (Kunin, 1987; Ofek, Goldhar, & Sharon, 1996). However, there was no significant pH decrease in urine after consumption of cranberry juice (Di Martino, Agniel, David, Templer, Gaillard, Denys, et al., 2006).

Both type 1 fimbriae and P fimbriae identified from UPEC strains are associated with bacterial adhesion and infection (Arthur, Johnson, Rubin, Arbeit, Campanelli, Kim, et al., 1989). Zafriri et al. (Dina Zafriri, Ofek, Adar, Pocino, & Sharon, 1989) and Howell et al. (Howell, Vorsa, Marderosian, & Foo, 1998) later implicated that fructose and proanthocyanidins in cranberry could inhibit the adherence of *E. coli* with type 1 fimbriae and P fimbriae to urinary tract, respectively. The authors speculated that the fimbriae are the target of inhibitory action, although the anti-adhesive mechanism has not been elucidated.

Further study showed that proanthocyanidins could even inhibit the adherence of multi-drug resistant P fimbriated *E. coli* strains to uroepithelial cells (Gupta, Dwivedi, Mahdi, Nagana Gowda, Khetrapal, & Bhandari, 2012). Atomic forces microscopy (AFM), a nanotechnology-based tool, has been employed to understand the possible molecular

mechanisms of anti-adhesive property of cranberry juice. The AFM results suggested that cranberry juice components modified the protein conformation of P fimbriae and made it compressed, thus being unable to attach to uroepithelial cells (Liu, Black, Caron, & Camesano, 2006). In addition, cranberry juice cocktail significantly decreased nanoscale adhesion forces between P fimbriated *E.coli* and human uroepithelial cells (Liu, Pinzón-Arango, Gallardo-Moreno, & Camesano, 2010). Besides proanthocyanidins, recent study also proved that cranberry phenolic compounds and their microbial-derived metabolites, such as simple phenols, benzoic, phenylacetic and phenylpropionic acids, had anti-adhesive property against UPEC (Llano, Esteban-Fernández, Sánchez-Patán, Martín-Álvarez, Moreno-Arribas, & Bartolomé, 2015).

Approximately 50% of the world's population are infected with *H. pylori,* which can cause diverse GI diseases including duodenal ulcer and gastric cancer (Zafra-Stone, Yasmin, Bagchi, Chatterjee, Vinson, & Bagchi, 2007). Some *in vitro* studies discovered that cranberry could decrease *H. pylori* adhesion to gastric mucosa and cultured gastric epithelial cells, and at the same time inhibited the growth of *H. pylori* (Burger, Weiss, Sharon, Tabak, Neeman, & Ofek, 2002; Chatterjee, Yasmin, Bagchi, & Stohs, 2004; Shmuely, Domniz, & Yahav, 2016). A high Mw, non-dialyzable material (NDM), isolated from cranberry was believed to be responsible for the above-mentioned properties (Shmuely, Burger, Neeman, Yahav, Samra, Niv, et al., 2004; Shmuely, Ofek, Weiss, Rones, & Houri-Haddad, 2012). Further studies hypothesized that NDM from cranberry blocked the sialyllactose-specific adhesin of *H. pylori* thereby preventing *H. pylori* adhesion to host tissues (Burger, Weiss, Sharon, Tabak, Neeman, & Ofek, 2002; Camesano, Liu, & Pinzon-Arango, 2007). Another study suggested that polyphenols from cranberry played

key roles in inhibiting *H. pylori* growth since they induced *H. pylori* to change into a coccoid form (Matsushima, Suzuki, Masui, Kasai, Kouchi, Takagi, et al., 2008). Cranberry also showed potential to increase susceptibility of *H. pylori* to clarithromycin, a proven antibiotic against *H. pylori* infection, which means a combination of clarithromycin and cranberry showed synergistic effect against *H. pylori* infection (Chatterjee, Yasmin, Bagchi, & Stohs, 2004; Shmuely, et al., 2004).

Cranberry components, such as proanthocyanidins or NDM, exert benefit for oral health by reducing oral diseases including dental caries and periodontitis (Gardner, 2014). The mechanisms of reducing oral diseases are complicated but were thought to be due to anti-adhesive activity of cranberry components that could inhibit oral pathogens (especially mutans streptococci) adhesion and biofilm formation on teeth surfaces (Bodet, Grenier, Chandad, Ofek, Steinberg, & Weiss, 2008; Koo, Nino de Guzman, Schobel, Vacca Smith, & Bowen, 2005; Weiss, Kozlovsky, Steinberg, Lev-Dor, Greenstein, Feldman, et al., 2004). For example, Yamanaka et al. evaluated the effect of cranberry juice on the adhesion and biofilm formation of oral streptococci strains to saliva-coated hydroxyapatite beads (mimic the teeth surfaces). The results showed that when the bacteria were momentarily exposed to cranberry juice, both their adherence and biofilm formation were significantly decreased; this activity was related to the reduction of the bacterial surface hydrophobicity but not due to an antibacterial activity. The high molecular mass components of cranberry juice were believed to be responsible for inhibiting the biofilm formation. Specifically, proanthocyanidins and flavonols from cranberry may be the active constituents against *Streptococcus mutans* (*S. mutans*) (Duarte, Gregoire, Singh, Vorsa, Schaich, Bowen, et al., 2006; Yamanaka, Kimizuka, Kato, & Okuda, 2004). Other studies proposed different

mechanism; cranberry could inactivate the enzymes of glucosyltransferase (GTF) and fructosyltransferase (FTF) which are indispensable for adhesion and biofilm formation of mutans streptococci (Steinberg, Feldman, Ofek, & Weiss, 2004).

C. Chicken egg yolk

Sialic acids are a family of acylated derivatives of a nine-carbon carboxylated monosaccharide, playing an important role in many biological processes, such as acting as a recognition site for microorganisms, toxins and hormones and involving in intermolecular and intercellular interactions (Spichtig, Michaud, & Austin, 2010; Spyridaki & Siskos, 1999). The major distributions of sialic acid in hen egg are egg white ovomucin, chalaza and egg yolk membrane (Juneja, Koketsu, Nishimoto, Kim, Yamamoto, & Itoh, 1991). Ovomucin has showed anti-adhesive potential due to the presence of sialic acid (Kobayashi, et al., 2004), and it was hypothesized that sialylated fractions of egg yolk may have anti-adhesive activity. Several studies proved this hypothesis, for example, sialyloligosaccharides and sialylglycopeptides (conjugated with carboxymethyl cellulose) from egg yolk could inhibit rotavirus adhesion to MA-104 cells (an established cell line derived from rhesus monkey kidney) and prevent the binding of *Salmonella enteritidis* (*S. enteritidis)* and *E. coli* to Caco-2 cell line, respectively (Koketsu, Nitoda, Juneja, Kim, Kashimura, & Yamamoto, 1995; Sugita-Konishi, Kobayashi, Sakanaka, Juneja, & Amano, 2004).

Antibodies are another compounds from chicken egg yolk with anti-adhesive activity. Especially, egg-yolk antibodies (anti-adhesin antibodies) exhibited good protective effect against the adhesion of ETEC K88 strains to piglet intestinal mucus *in vitro* and showed the prophylactic potential for infectious intestinal diseases in piglets (Jin, Baidoo,

Marquardt, & Frohlich, 1998; Wiedemann, Linckh, Kühlmann, Schmidt, & Lösch, 1991; Yokoyama, Peralta, Diaz, Sendo, Ikemori, & Kodama, 1992). In addition, nonimmunized egg yolk powder without specific antibodies was able to prevent foodborne pathogens adhesion as well, such as *S. enteritidis*, *Salmonella typhimurium*, and *E. coli* O157:H7; high-density lipoproteins (HDL) was identified as another active anti-adhesive fraction from egg yolk (Kassaify, Li, & Mine, 2005).

D. Plant polysaccharides

Oligosaccharides, especially HMOs, have attracted intensive attention for their anti-adhesive activity against different pathogens (Coppa, et al., 2006; Newburg, 2000), which led to a hypothesis that polysaccharides may have the similar anti-adhesive property. From the last decade, researchers started to explore the anti-adhesive polysaccharides from different plant origins, such as green tea and *Panax ginseng* (Ji-Hye Lee, 2004; Lee, Shim, Chung, Lim, & Kim, 2009a; Lee, Shim, Lee, Kim, Chung, & Kim, 2006). Most identified polysaccharides could prevent *H. pylori* adhesion to human stomach tissue and were characterized as acidic polysaccharides containing uronic acids (Lee, Shim, Lee, Kim, Yang, Chung, et al., 2006; Wittschier, Faller, & Hensel, 2009; Xu, Ruan, Li, Guo, Ren, Shuang, et al., 2010). Nevertheless, one recent study proposed that the neutral side chains (arabinogalactans and/or arabinans) might be critical for inhibiting adhesion of *H. pylori* to human gastric adenocarcinoma epithelial cells (Inngjerdingen, Thöle, Diallo, Paulsen, & Hensel, 2014). However, there is very limited structure-activity relation study. Furthermore, polysaccharides may lose their anti-adhesive activities after subjecting to enzymatic hydrolysis, so the anti-adhesive activity of polysaccharides needs to be further confirmed

by *in vivo* studies (Lee, Shim, Chung, Lim, & Kim, 2009b). However, no *in vivo* studies have been conducted yet to demonstrate the clinical relevance of *in vitro* findings.

E. Plant proteins or peptides

ECM (mainly proteins) of host cells, such as collagen, laminin, and fibronectin, could function as receptors for bacterial adhesin to form protein-protein interactions and contribute significantly to bacterial adhesion (Patti, Allen, McGavin, & Hook, 1994; Westerlund & Korhonen, 1993). To test if peptides derived from the plant protein have anti-adhesive activity against *H. pylori* or not, pea protein isolate was subjected to pancreatic trypsin digestion. After fractionation and purification, two anti-adhesive peptides (S3 and S5) were identified by MALDI-TOF (time of flight)-MS. The peptides act as receptor analogs and specifically interact with *H. pylori* outer membrane protein adhesin BabA (blood group Ag-binding adhesin). The peptide sequence (11 amino acids) and possible tertiary structure are indispensable for anti-adhesive activity (Niehues, Euler, Georgi, Mank, Stahl, & Hensel, 2010). Another study demonstrated that a proteinaceous fraction (Globulin 3, 66 kDa) of wheat bran may prevent the adhesion of ETEC K88 strains to porcine epithelial cells (González-Ortiz, Bronsoms, Quarles Van Ufford, Halkes, Virkola, Liskamp, et al., 2014). However, it was not known whether the presence of other components such as carbohydrate in the wheat bran extract might be attributable to the anti-adhesive activity against K88 ETEC.

2.4.2 Animal study

Although numerous compounds from dietary sources showed anti-adhesive activity against pathogens *in vitro* studies as reviewed above, it is imperative to conduct *in vivo* study to test the efficacy and safety of this anti-adhesive therapy (Tables 2.3). The efficacy

of anti-adhesive agents to protect animals against bacterial infection has been firstly reported in late 1970s (Aronson, Medalia, Schori, Mirelman, Sharon, & Ofek, 1979; Fader & Davis, 1980). In these two studies, methyl α-D-mannopyranoside (αMM), an anti-adhesive carbohydrate, was found to significantly reduce the rate of urinary tract infection in mice and rats caused by type 1 fimbriaed *E. coli* and *Klebsiella pneumoniae*, respectively. Subsequently, more potential anti-adhesive compounds were tested with different pathogenic bacteria and animals, especially HMOs or bovine milk glycoconjugates.

In milk, approximately 70% of oligosaccharides are fucosylated and 30% are sialylated, both showed anti-adhesive activity *in vitro* and were further tested by animal studies. Firstly, fucosylated oligosaccharides of human milk protected sucking mice from diarrhea caused by heat-stable enterotoxin (ST) of *E. coli*. It was postulated that the minor fraction of fucosylated oligosaccharides, but not the major commercially available fucosylated oligosaccharides, act as analogs to the ST receptor in the host tissues (Newburg, Pickering, McCluer, & Cleary, 1990). In another study, fucosylated oligosaccharides of human milk inhibited the colonization of *C. jejuni* to mice because specific milk fucosyl α1,2-linked oligosaccharides interfered the interactions between *C. jejuni* and intestinal H(O) antigen (Ruiz-Palacios, Cervantes, Ramos, Chavez-Munguia, & Newburg, 2003). Furthermore, the anti-adhesive activity of 3'-sialyllactose sodium salt (3'SL), a sialylated oligosaccharide that occurs naturally in human and bovine milk, was tested in primates (rhesus monkeys), which aroused particular interest (Mysore, Wigginton, Simon, Zopf, Heman-Ackah, & Dubois, 1999). One group of six rhesus monkeys naturally infected by *H. pylori* were treated with 3'SL. Two of them were cured; one cleared the

infection transiently; other three remained infected. Another group of six animals treated by the combination of 3'SL and conventional anti-ulcer drugs, while this combination did not increase cure rate. These results suggested that anti-adhesive therapy is safe and partially confirmed the anti-adhesive activity of 3'SL *in vivo*.

Similarly, sialylated glycoconjugates from bovine milk inhibited the *H. pylori* infection in BALB/cA mouse model. The results demonstrated that both the gastric colonization by *H. pylori* and inflammation score were remarkably decreased in all mice treated with bovine milk glycoconjugates compared to the control animals (Wang, Hirmo, Willén, & Wadström, 2001). Another sialylated compound from bovine milk is casein glycomacropeptide (cGMP), which is a glycoprotein originating during cheese making. It showed promising potential to improve gut health of piglets by preventing the adhesion of ETEC K88 to the intestinal mucosa and by stimulating the growth of lactobacilli in the intestine (Hermes, Molist, Pérez, Ywazaki, Davin, Nofrarias, et al., 2013).

The protective effect of chicken egg yolk compounds were examined in animals as well. For example, oral administration of sialyloligosaccharides and sialylglycopeptides to *Salmonella* infected BALB/c mice effectively prevented bacteria proliferating in spleen and inhibited *Salmonella*-induced infection, which was due to preventing the entry of bacteria into the gut but not related to activating macrophages (Sugita-Konishi, Sakanaka, Sasaki, Juneja, Noda, & Amano, 2002). Egg-yolk antibodies obtained from immunized hens with fimbrial antigens of K88 ETEC strain (adhesin-based vaccines) were found to protect neonatal and early-weaned piglets from ETEC infection. The K88 ETEC induced diarrhea in 3-day-old piglets was cured by feeding with egg-yolk antibodies (anti-adhesin antibodies) after 24 h. The 21-day-old weaned piglets had transient diarrhea, positive body

weight gains and 100% survival after treating with egg-yolk antibodies, which gave a remarkable improvement compared to the control animals (severe diarrhea, dehydration and death) (Marquardt, Jin, Kim, Fang, Frohlich, & Baidoo, 1999).

2.4.3 Clinical trial

Typical dietary constituents that prevent bacterial/virus adhesion and/or infection in clinical trial were listed in Table 2.4. Recently, the preventive effect of D-mannose powder on recurrent urinary traction infection (UTI) in women was tested by a randomized clinical trial for 6 months (Kranjčec, Papeš, & Altarac, 2014). 308 women with a history of recurrent UTI while no other significant comorbidities were randomly assigned to three groups: 2 g of D-mannose powder in 200 mL of water daily, 50 mg Nitrofurantoin (standard prophylaxis) daily, and the control (no intervention group). The results revealed that the recurrent rates of D-mannose group, Nitrofurantoin group and no prophylaxis group were 14.6%, 20.4% and 60.8%, respectively.

The association between consumption of HMOs and prevention of infectious diseases in breast-fed infants were determined by analyzing the data and banked samples from breast-feeding mother-infant pairs. In a pilot study, 49 mother-infant pairs provided breast milk sample 2 weeks postpartum and infant health was assessed at the age of 2, 6, 12, and 24 weeks by maternal report. LNF-II (lacto-*N*-fucopentaose II), a major HMO, was selected to represent the level of total oligosaccharides consumed in human milk. The authors concluded that consumption of HMOs through breastfeeding was associated with lower reported respiratory and GI diseases in infants (Stepans, Wilhelm, Hertzog, Rodehorst, Blaney, Clemens, et al., 2006); however, small sample size (49) and short experimental time (24 weeks) made the results inconclusive. Another similar study was

conducted on 93 mother-infant pairs with extended length from birth to 2 years. Milk samples collected 1 to 5 weeks postpartum were measured for oligosaccharide content and diarrhea data obtained weekly. The results suggested that HMOs are clinically relevant to protect infants against diarrhea caused by *Campylobacter* and caliciviruses (Morrow, Ruiz-Palacios, Altaye, Jiang, Guerrero, Meinzen-Derr, et al., 2004).

3'SL was tested in rhesus monkeys to show its safety and partially confirmed the anti-adhesive activity against *H. pylori* (Mysore, Wigginton, Simon, Zopf, Heman-Ackah, & Dubois, 1999). However, 3'SL did not suppress or eradicate *H. pylori* colonization in humans although it is safe and well tolerated to patients with *H. pylori* infection (Parente, Cucino, Anderloni, Grandinetti, & Porro, 2003). This randomized, double-blind study included three groups and lasted for 4 weeks: two treatment groups were given 3'SL at doses of 10 g/day and 20 g/day, while the third group (21) was given placebo. During the treatment, no serious adverse events were observed in any of the three groups; the three groups did not significantly differ in demographic or clinical patient characteristics.

The efficacy of hyperimmunized chicken egg yolk (HEY) immunoglobulin in children with rotavirus diarrhea was assessed in a randomized, double-blind study. 79 children with proven rotavirus diarrhea were allocated to two groups: one group treated with HEY and another received placebo for 4 days. Treatment with HEY against rotavirus-induced diarrhea in children had a positive impact on clearance of rotavirus from stools, but the diarrheal duration was not reduced (Sarker, Casswall, Juneja, Hoq, Hossain, Fuchs, et al., 2001).

Most of the current clinical trials involve in evaluating the efficacy of cranberry product in the treatment of UTI in different subpopulations, such as sexually active adult

women, elderly patients, children, and patients suffering from different medical conditions. Cranberry components are the only anti-adhesive agents from dietary source, which have been approved to be marketed as urinary health promoters by French Agency for Food Safety (AFSSA) (Shmuely, Ofek, Weiss, Rones, & Houri-Haddad, 2012). Several excellent reviews on the current clinical status of the preventive effects of cranberry components against UTI have been published before 2013 (Guay, 2009; Jepson, Craig, & Williams, 2013; Shmuely, Ofek, Weiss, Rones, & Houri-Haddad, 2012; Vasileiou, Katsargyris, Theocharis, & Giaginis, 2013; Wang, Fang, Chen, Liu, Yu, Wu, et al., 2012). Therefore, only representative clinical trials reported in the last three years were selected in this review.

In general, the existing clinical studies suggested that the preventive efficacy of cranberry against UTI is moderate at best and variable among subpopulations. In 2014 and 2015, two studies testing the effectiveness of cranberry capsules to treat/prevent UTI in the specific subpopulation were conducted. In the first study, the targeted subpopulation were women undergoing elective benign gynecological surgery. This randomized, double-blind, placebo-controlled trial included 160 patients from the same hospital which were randomized into cranberry treated group (received 2 cranberry juice capsules 2 times a day, equivalent to 28 ounce of cranberry juice) and placebo group. The treatment lasted for 6 weeks after surgery and results showed that the use of cranberry extract capsules during the postoperative period significantly reduced the occurrence of UTI from 38% to 19% (Foxman, Cronenwett, Spino, Berger, & Morgan, 2015). The quantity of active ingredient (proanthocyanidins) in the capsules was not reported in this study, which is important for evaluating the efficacy of cranberry product. The second randomized, double-blind,

placebo-controlled trial was performed on the vulnerable older populations who were divided into a group that took twice daily either cranberry capsules (9 mg proanthocyanidins/capsule) or placebo for 12 months. Cranberry capsules treatment helped people with high UTI risk at baseline reduce 26% of the incidence of clinically defined UTI, but not strictly defined UTI (Caljouw, Hout, Putter, Achterberg, Cools, & Gussekloo, 2014). Additionally, more recent study suggested that the consumption of cranberry juice (240 mL daily for 24 weeks) lowed the incidence of clinical UTI in women with recent history of UTI (Maki, Kaspar, Khoo, Derrig, Schild, & Gupta, 2016). Although these studies favored the cranberry use, but the optimum dosage of cranberry components has not been established. A well-designed dose-efficacy research is needed in future. However, not all clinical trials gave positive results for the therapeutic/prophylactic efficacy of cranberry components on the UTI. For example, a multicenter, randomized, placebo-controlled, double-blind trial was conducted on 171 multiple sclerosis patients with urinary disorders in 2014. After 1 year, the daily administration of cranberry extract containing 36 mg of proanthocyanidins did not prevent UTI occurrence compared to placebo group (Gallien, Amarenco, Benoit, Bonniaud, Donzé, Kerdraon, et al., 2014).

In addition to test the efficacy of cranberry to prevent/treat infectious diseases, the cost-effectiveness of cranberry prophylaxis was evaluated compared to antibiotics (trimethoprim-sulfamethoxazole, TMP-SMX) in premenopausal women with recurrent UTI (110 women followed up the entire study). This randomized clinical trial lasted for 12 months and indicated that taking cranberry to prevent UTI is not only less effective but also more expensive than using antibiotics (Bosmans, Beerepoot, Prins, ter Riet, & Geerlings, 2014). The cost of cranberry prophylaxis may be not accurate since the optimal

dosage of cranberry is not known yet. However, this work at least provided some clues to the future development of anti-adhesive agents with regard to cost and efficacy.

2.4.4 Major challenges and future directions

Although there is a great potential to develop anti-adhesive based therapy against infectious diseases, there are many challenges. Most anti-adhesive studies were performed using *in vitro* model, therefore the safety and efficacy of these compounds have not been established *in vivo*. Only a few compounds, such as cranberry product, have been tested by *in vivo* study and clinical trials. Furthermore, the anti-adhesive components from dietary sources have not been identified and the molecular mechanism underlying the action is not known. Some pathogens carry multiple adhesins with different receptor binding specificities, making it impossible to block all bacterial adhesins by single anti-adhesive agent and causing low affinity of anti-adhesive agents. Often a high concentration of anti-adhesive agents is required to exert the function, which may be impractical for widespread applications. There is also a need to reduce the cost of production of anti-adhesive agents; for example, biotechnology is more cost-effective than isolation or synthesis of HMOs for commercial uses.

Undoubtedly, anti-adhesive compounds from dietary source hold great promise for anti-adhesive therapy. Future research is needed to discover new anti-adhesive compounds from foodstuffs with commercial potential that are safe, cost-effective, and scalable. Purification of the anti-adhesive compounds for practical application is not always encouraged as a complex nature may provide greater benefit for binding to bacteria with multi-adhesins, in addition to its increased cost of production; while characterization of the main components responsible for the activity is inevitable to unravel the anti-adhesive

mechanism, to study the structure-activity relation, the binding sites and major driving forces between inhibitors and bacterial adhesins.

2.5 Biofouling and anti-fouling surfaces

2.5.1 The adverse effects of biofouling on different applications

Biofouling, an undesired accumulation of biomacromolecules (e.g. proteins) or organisms (e.g. bacteria) on wetted surfaces, is a serious issue affecting numerous applications, such as biosensors, biomedical implants, and food industry (Banerjee, Pangule, & Kane, 2011). Biofouling is considered to be one of the major causes for the failure of *in vivo* biosensors (Wisniewski & Reichert, 2000). As soon as a device is implanted into the body, body will have some immediate responses to heal the injury. For example, blood will flow the injured area, resulting in adsorption of blood proteins on the device surface. Protein adsorption on surface could reduce the sensitivity of biosensor or even make it malfunction because the mass transfer between biosensor and the analyte of interest is significantly decreased (Yeh, Kizhakkedathu, Madden, & Chiao, 2007).

Protein adsorption plays a vital role in the bacterial colonization and subsequent biofilm formation since a layer of protein could serve as a conditioning film (Banerjee, Pangule, & Kane, 2011). Colonization and formation of biofilm on medical implants (such as catheters or prosthetic joints) cause device-related infections, which has long been a significant problem in the biomedical field, both during implant surgery and after the implantation (Hetrick & Schoenfisch, 2006; Pham, Bhadra, Truong, Crawford, & Ivanova, 2015). Implant-related infections may result in a high incidence of revision surgeries, and sometimes fatality, placing a greater financial burden on the healthcare system of many countries (Beloin, Renard, Ghigo, & Lebeaux, 2014; Pham, Bhadra, Truong, Crawford, &

Ivanova, 2015; Veerachamy, Yarlagadda, Manivasagam, & Yarlagadda, 2014). Taking US as an example, the direct medical costs associated with such infections was estimated more than $3 billion annually about one decade ago, which is expected to increase nowadays (Darouiche, 2004).

In the food industry, biofouling formation, especially biofilm, in pipes, equipment and cooling systems causes enormous economic losses through increasing maintenance costs and reducing equipment operational efficiencies (Brooks & Flint, 2008; Moreira, Fulgêncio, Alves, Machado, Bialuch, Melo, et al., 2016). Biofilm can also be a source of microbial contamination, causing food-borne infectious diseases and posing a threat to human health. Food-borne diseases are still a global public health concern nowadays, and new ones continue to emerge (Newell, Koopmans, Verhoef, Duizer, Aidara-Kane, Sprong, et al., 2010; Srey, Jahid, & Ha, 2013). In addition, microbial contamination negatively impacts on food storage, which results in undesirable changes to the sensory and quality of food (Endersen, O'Mahony, Hill, Ross, McAuliffe, & Coffey, 2014). Currently, it is estimated that 25% of the total food produced annually wasted due to microbial contamination (Endersen, O'Mahony, Hill, Ross, McAuliffe, & Coffey, 2014).

2.5.2 Anti-fouling surfaces

To address the biofouling problem, much attention has been directed towards the development of anti-fouling strategies which are mainly based on either preventing biocontaminations from attaching or degrading/killing them (Banerjee, Pangule, & Kane, 2011). One promising approach is to prepare anti-fouling surfaces based on different polymers such as poly(ethylene glycol) (PEG), amphiphilic polymer, zwitterionic polymer, and antimicrobial polymer. Here we will briefly discuss the PEG-based anti-fouling

surfaces (most commonly used) and mucins as anti-fouling surfaces (related to my research).

2.5.2.1 PEG-based anti-fouling surfaces

PEG is one of the most well-documented anti-fouling polymers (Lowe, O'Brien-Simpson, & Connal, 2015). Surfaces based on PEG have been used in a variety of applications, such as biosensors and tissue engineering (Liu, Chen, Tsai, Lin, & Hsiue, 2007; Weimer, Walsh, & Wang, 2000). The unique physical and biochemical properties of PEG are critical for its anti-fouling applications, like non-toxicity, non-immunogenesis, non-antigenicity and good biocompatibility (Krishnan, Weinman, & Ober, 2008; Ye & Zhou, 2015; Yu, Zhang, Wang, Brash, & Chen, 2011). PEG and its derivatives showed good anti-fouling activity against platelet adhesion and protein adsorption including bovine serum albumin and fibrinogen (Murthy, Shell, & Grunlan, 2009; Sharma, Popat, & Desai, 2002; Tan, McClung, & Brash, 2013; Unsworth, Sheardown, & Brash, 2005; Zhang, Kang, Neoh, & Huang, 2001). Although the mechanisms of anti-fouling property have not been completely understood, it was postulated that steric barrier, chain length, surface density, refractive index, and hydrophilic nature are responsible for protein resistance (Jeon, Lee, Andrade, & De Gennes, 1991; Ostuni, Chapman, Holmlin, Takayama, & Whitesides, 2001; Unsworth, Sheardown, & Brash, 2005).

It was hypothesized that, since a layer of adsorbed protein can act as a conditioning film for bacterial adhesion, surfaces with anti-fouling property should prevent bacterial adhesion (Ostuni, Chapman, Liang, Meluleni, Pier, Ingber, et al., 2001). Therefore, much research efforts have been devoted to examine this hypothesis and demonstrated that surfaces based PEG could resist bacterial adhesion (Fernández, van der Mei, Lochhead,

Grainger, & Busscher, 2007; Kingshott, Wei, Bagge-Ravn, Gadegaard, & Gram, 2003; Park, Kim, Han, Kim, Lee, Suh, et al., 1998). The forces of interaction between bacteria and PEG-coated surfaces were measured by AFM in order to indicate the possible anti-fouling mechanism. The data suggested that PEG chains not only interfered the long-range attractive forces between bacteria and substrates, but also exhibited large steric repulsion forces (Razatos, Ong, Boulay, Elbert, Hubbell, Sharma, et al., 2000). In addition, the polymer chain length is an important factor in preventing bacterial adhesion (Roosjen, van der Mei, Busscher, & Norde, 2004).

Many researchers have attempted to improve the anti-fouling activity of PEG polymer. A polymer composition (PEG and cationic polycarbonate) revealed significantly stronger anti-fouling activity than individual PEG coating (Ding, Yang, Lim, Hsu, Engler, Hedrick, et al., 2012). Combination of chemical modification and surface texturing has been reported recently as an efficient way to improve anti-fouling activity of biomaterials (Xu & Siedlecki, 2015). PEG-grafted submicron textured biomaterial surfaces greatly increased the activity to reduce both platelet adhesion and bacterial adhesion/biofilm formation.

However, surfaces based on PEG cannot entirely prevent bacterial adhesion/colonization. Recent study demonstrated that the polymer brush coating of poly(L-lysine)-graft-PEG (PLL-g-PEG) on titanium did not resist *Staphylococcus epidermidis* (*S. epidermidis*) colonization and biofilm formation. Because the biofilm formed by *S. epidermidis* was rich in polysaccharides and extracellular DNA, which could interact with PEG brushes and completely remove the polymer from titanium surface (Xu & Siedlecki, 2015). Furthermore, PEG has several obvious limitations: susceptible to oxidation in complex media, not suitable for long-term applications, not biodegradable,

bioaccumulation in the lysosomes of healthy cells, provoking an immune response in some circumstances (Duncan & Gaspar, 2011; Herold, Keil, & Bruns, 1989; Lowe, O'Brien-Simpson, & Connal, 2015; Schellekens, Hennink, & Brinks, 2013).

2.5.2.2 Mucins as anti-fouling surfaces

The main function of the mucous layer, covering epithelial cells, is to serve as a natural anti-fouling surface to prevent undesirable adhesion to host tissues (Cone, 2009). This layer is mainly composed of water and mucins, a member of heavily glycosylated and gel-forming high molecular-weight glycoproteins (Bansil & Turner, 2006). Therefore, much attention has been directed towards exploring the potential of mucin as anti-fouling surface to suppress cell and bacterial adhesion, including bovine submaxillary mucin (BSM) and pig gastric mucin (PGM).

Mucins (BSM and PGM) were reported to adsorb on hydrophobic substrates and form a mucin layer that prevents the surface adhesion to mammalian epithelial cells, fibroblasts, and myoblasts. This cell-repellent effect of mucin coatings could help improve the biocompatibility of biomedical implants. The authors suggested that the glycan moieties and critical surface density of mucins are indispensable to achieve the cell-repulsion (Crouzier, Jang, Ahn, Stocker, & Ribbeck, 2013).

Mucin coatings have exhibited the activity to prevent bacteria adhesion to polymeric material surfaces. In one study, BSM was coated on a surface consisting of a poly(acrylic acid-b-methyl methacrylate) (PAA-*b*-PMMA) diblock copolymer. BSM coating reduced the adhesion of *S. epidermidis* and *E. coli*; increasing the mucin film thickness resulted in further decrease of the density of adhering *S. epidermidis* but not *E. coli*. BSM coating had affinity to *C. albicans* but did not resist *C. albicans* adhesion (Bushnak, Labeed, Sear, &

Keddie, 2010). In another study, BSM was coated to four polymeric materials, PMMA, silicone, polyurethane and polystyrene. *S. aureus* and *S. epidermidis* were applied to contaminate the BSM coated surfaces and corresponding bare surfaces. A correlation was established in all BSM-coated polymers: the more BSM adsorption, the less bacteria adhesion. The inhibitory percentages of bacterial adherence on BSM-coated PMMA, polystyrene, polyurethane, and silicone were about 97, 90, 92 and 70%, respectively (Shi, Ardehali, Caldwell, & Valint, 2000). Coatings of PGM and BSM on polystyrene surfaces suppressed the adhesion of *Streptococcus. pneumoniae* and *S. aureus*. The authors further suggested that glycan moieties of mucins are indispensable for bacterial repulsion possibly because glycans are key contributors in the critical thickness and softness of the mucin coatings which are associated with increasing bacterial repulsion (Co, Crouzier, & Ribbeck, 2015).

2.5.3 General conclusion and perspectives

Fabrication and identification of anti-fouling surfaces are promising approach to address the biofouling problem in numerous applications. Although PEG is one of the most well-documented anti-fouling polymers, it is susceptible to oxidation, not suitable for long-term applications, and not biodegradable, which would limit its applications. Research on anti-fouling property of many components is still at infancy. Novel anti-fouling surfaces is expected to improve its stability and biodegradability; development of promising natural anti-fouling surfaces with scale-up potential and cost-effective is highly expected.

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Table 2.1 Reported bioactivities of ovomucin and its derived components

Active components	Bioactivity	Experimental assay	Published year
Two peptides: LDEPDPL and NIQTDDFRT	Antioxidant activity	ABTS and DPPH	2013
β-ovomucin	Cytotoxic effect on tumor cells and inhibit tumor cell proliferation	*In vitro*	1999
β-ovomucin	Suppress the growth of subcutaneously xenografted SR-180 cells and cure the tumor	*In vivo*	1999
Glycopeptides with Mw 220 and 120 kDa,	Cure Meth-A fibrosarcoma tumor cells and inhibit tumor growth	*In vivo*	1998
Glycopeptides with Mw 70 kDa	Complete inhibit the growth of Meth-A fibrosarcoma tumor cells	*In vivo*	2000
Ovomucin	Enhance the proliferation of mouse spleen lymphocytes	*In vitro*	1994
O-linked glycans of the sulphated glycopeptides	Macrophage-stimulating activity	*In vitro*	1997
Ovomucin	Hypocholesterolemic action	*In vitro* and *In vivo*	2002
Ovomucin	Anti-agglutinating activity against influenza virus	*In vitro* (Hemagglutination)	1949
Ovomucin or ovomucin glycopeptides	Anti-agglutinating activity against bovine rotavirus, hen newcastle disease virus (NDV), and human influenza virus	*In vitro* (Hemagglutination)	1996, 1997
Ovomucin glycopeptides	Binding activity to *Escherichia coli* O157:H7	*In vitro*	2004
Ovomucin	Inhibit the adhesion and colonization of *Helicobacter pylori* to the mouse stomach	*In vivo*	2001

Table 2.2 Anti-adhesive activity of dietary constituents tested *in vitro*

Anti-adhesive compound	Bacterium or virus affected	Mechanism underlying the effect	Published year
	Human milk		
Acidic oligosaccharides Neutral oligosaccharides	*E. coli*, *V. cholerae*, *S. fyris*	Oligosaccharides as receptor analogs, and the complexity of oligosaccharides plays important roles in this protecting effect	2006
Fucoα(l-2)Galβ-bearing complex carbohydrates	*C. albicans*	Carbohydrates as receptor analogs	1991
Carbohydrates moieties of the neolactoseries of glycolipids	*S. pneumoniae, H. influenzae*	Oligosaccharides as receptor to block bacterial adhesion, which is not affected by immunoadsortption	1986
MUC 1 and MUC 4	*Salmonella enterica* serovar Typhimurium	Mucin as receptor analogs, *S. enterica* may bind to sialylated glycans or the protein backbone of mucin	2012
MUC 1	poxvirus	Mucin aggregates the poxvirus and therefore inhibits virus entering into host cells.	2007
MUC 1 and MUC 4	Norwalk virus capsids	2-linked fucosylated residues act as receptor analogs and block the binding of virus to host cells	2006
Mucins (>200 kDa)	*S-fimbriated E. coli*	Carbohydrate residues of mucin are responsible for this anti-infective mechanism, which depends on the period of lactation	1993
Lactoferrin	*A. actinomycetemcomitans*, *P. intermedia*, *P. nigrescens*	Lactoferrin binds to both bacteria and host cells, and subsequently blocks specific adhesin-ligand and non-specific charge-dependent interactions	1997
κ-casein (glycoprotein)	*H. pylori*	Fucose containing glycan moieties as receptor decoys	1995
sIgA, oligosaccharides	EPEC	sIgA induces a response to EPEC adherence factor; fucosylated tetra-and pentasaccharides as receptor analogs	1991

Continued Table 2.2

Anti-adhesive compounds	Bacterium or virus affected	Mechanism underlying the effect	Published year
Cranberry/cranberry juice			
Fructose	Type 1 fimbriated *E.coli*	Fructose interferes Type 1 fimbriated *E.coli* binding to urinary tract	1989
Proanthocyanidins	P fimbriated *E.coli*	Prevent P fimbriated *E.coli* from adhering to the urinary tract	1998
Cranberry juice components	P fimbriated *E.coli*	Modify the protein conformation of P fimbriae and made it compressive	2006
Cranberry juice cocktail	P fimbriated *E.coli*	Decrease nanoscale adhesion forces between P fimbriated *E.coli* and human uroepithelial cells	2010
Cranberry phenolic compounds and microbial-derived metabolites	UPEC ATCC®53503	Not mentioned	2015
Non-dialyzable material (NDM) from cranberry	*H.pylori*	NDM blocked the sialyllactose-specific adhesin	2007 2002
polyphenols	*H.pylori*	Inhibit *H.pylori* growth by changing *H.pylori* into a coccoid form	2008
High molecular mass components, proanthocyanidins, flavonols	Oral streptococci strains	Decrease bacterial adherence and biofilm formation due to reducing the surface hydrophobicity, not antibacterial effect	2004 2006
NDM	Mutans streptococci	Inactivate the enzymes of glucosyltransferase (GTF) and fructosyltransferase (FIF)	2004

Continued Table 2.2

Anti-adhesive compounds	Bacterium or virus affected	Mechanism underlying the effect	Published year
Chicken egg yolk			
Sialyloligosaccharides and sialylglycopeptides	Rotavirus, *S. enteritidis*, *E. coli*	Prevent the bindings of Rotavirus or pathogens to cell lines	2004 1995
Egg-yolk antibodies	Enterotoxigenic *E. coli* (ETEC) K88	Inhibit the adhesion of ETEC K88 to piglet intestinal mucus	1998
High-density lipoproteins (HDL)	*S. enteritidis*, *S. typhimurium*, *E. coli* O157:H7	Not mentioned	2005
Plant polysaccharides			
Acidic polysaccharides containing uronic acids	*H. pylori*	Prevent *H.pylori* adhesion to human stomach tissue	2010 2009 2006
Neutral side chains of arabinogalactans/arabinans	*H. pylori*	Prevent *H.pylori* adhesion	2014
Plant proteins or peptides			
Peptides from pea protein	*H. pylori*	Peptides act as receptor analogs	2010
Globulin from wheat bran	ETEC K88	Not mentioned	2014

Table 2.3 Dietary constituents that prevent bacterial adhesion and/or infection in animal study

Anti-adhesive compound	Bacterium affected	Animal, site of infection	Published year
Methyl α-D-mannopyranoside (αMM)	Type 1 fimbriaed *E. coli*	Mice, urinary tract	1979
	Klebsiella pneumoniae	Rate, urinary tract	1980
Fucosylated oligosaccharides of human milk	Heat-stable enterotoxin of *E. coli*	Sucking mice, gastrointestinal tract	1990
Fucosyl 1,2-linked oligosaccharides of human milk	*C. jejuni*	Mice, gastrointestinal tract	2003
3'-sialyllactose sodium salt (3'SL)	*H. pylori*	Rhesus monkeys, gastrointestinal tract	1999
Sialylated glycoconjugates from bovine milk	*H. pylori*	Mice, gastrointestinal tract	2001
Casein glycomacropeptide (cGMP)	ETEC K88	Piglet, gastrointestinal tract	2013
Sialyloligosaccharides and sialylglycopeptides from hen egg yolk	*Salmonella enteritidis*	Mice, gastrointestinal tract	2002
Egg-yolk antibodies	ETEC K88	Piglet, gastrointestinal tract	1999

Table 2.4 Dietary constituents that prevent bacterial/virus adhesion and/or infection in clinical trial

Anti-adhesive compound	Experimental design, sample size	Subpopulation	Result	Published year
D-mannose powder	Randomized, 308	Women with recurrent urinary tract infection (UTI)	Lower the risk of recurrent UTI	2014
Human milk oligosaccharides	Analyzing data from collecting samples, 49	Infant (0 to 24 weeks old)	Less reported respiratory and gastrointestinal infection	2006
2-linked fucosylated oligosaccharide of human milk	Analyzing data from collecting samples, 93	Infant (0 to 2 years old),	Prevent diarrhea from breast-fed infants	2004
3'-sialyllactose sodium salt (3'SL)	Randomized, double-blind, 60	Patients with *H. pylori* infection	Safe but not suppress or eradicate *H. pylori* colonization	2003
Egg-yolk antibodies	Randomized, double-blind, 79	Children with rotavirus diarrhea	Clearance of rotavirus from stools, but not reducing the diarrheal duration	2001
Cranberry capsules	Randomized, double-blind, placebo-controlled, 160	Women undergoing elective benign gynecological surgery	Reduce the occurrence of UTI from 38% to 19%	2015
Cranberry capsules	Randomized, double-blind, placebo-controlled, 928	Vulnerable older populations	Reduce the incidence of clinically defined UTI, but not strictly defined UTI	2014
Cranberry extract containing proanthocyanidins	Multicenter, randomized, placebo-controlled, double-blind, 171	Multiple sclerosis patients with urinary disorders	Not prevent UTI occurrence	2014

CHAPTER 3 – Effect of Proteolysis on the Sialic Acid Content and Bifidogenic Activity of Ovomucin Hydrolysates

3.1 Introduction

Egg white is widely used in the food industry due to its excellent gelling, foaming and emulsifying properties (Wang & Wang, 2009). Ovomucin, a glycoprotein accounting for 2-4% of the total egg white protein, is a major contributor to egg white functionality (Kato, Oda, Yamanaka, Matsudomi, & Kobayashi, 1985). It is composed of a carbohydrate-poor and a carbohydrate-rich subunit, containing 11-15% and 50-57% (w/w) carbohydrate, respectively (Robinson & Monsey, 1971; Wang & Wu, 2012). Based on its solubility, ovomucin can be classified into an insoluble form and a soluble form with molecular weights of 23,000 kDa and 8,300 kDa, respectively (Tominatsu & Donovan, 1972). Generally, ovomucin is highly insoluble at neutral pH or in the absence of denaturing agents.

Protein hydrolysis is widely applied to improve protein functionalities including solubility and bioactivities. The solubility of ovomucin was increased at increasing degree of hydrolysis (DH); ovomucin hydrolysates showed relatively high foaming ability at DH of 15%-40% (Hammershøj, Nebel, & Carstens, 2008; Hiidenhovi, Hietanen, Mäkinen, Huopalahti, & Ryhänen, 2005). Glycopeptides prepared from pronase digestion of ovomucin showed anti-adhesive activity against *E.coli* O157:H7 (Kobayashi et al., 2004), and anti-tumor activity in a double grafted tumor system (Oguro, Watanabe, Tani, Ohishi, & Ebina, 2000). In addition, two peptides (LDEPDPL and NIQTDDFRT) with radical scavenging activity were identified from ovomucin hydrolysate (Chang et al., 2013).

Ovomucin is rich in sialic acid (2.6-7.4%, w/w), which plays important roles in various biological processes and in infant nutrition (Robinson et al., 1971; Tang, Liang, Cai, & Mou, 2008). Sialic acid acts as recognition sites for microorganisms, toxins and hormones, protects cells from enzymatic hydrolysis and immunological attacks, and is involved in intermolecular and intercellular interactions (Spichtig, Michaud, & Austin, 2010). As an important component of brain gangliosides and polysialylated neural cell adhesion molecules, sialic acid is thought to play crucial roles in cognition and memory development in infants. Sialic acid has been suggested as an essential nutrient for infants (Wang & Brand-Miller, 2003). The sialic acid in ovomucin is N-acetylneuraminic acid, which is identical to the sialic acid found in human glycans but different from the N-glycolylmuramidic acid present in glycans of other mammals. Ovomucin may thus serve as source of sialic acid in human nutrition (Schauer, Srinivasan, Coddeville, Zanetta, & Guérardel, 2009). However, the effect of hydrolysis on the sialic acid content in the hydrolysates has not been studied. Furthermore, sialic acid-containing substances were suggested to promote the growth of bifidobacteria (Idota, Kawakami, & Nakajima, 1994), indicating the bifidogenic potential of ovomucin hydrolysate. Interestingly, porcine gastric mucin, a member of mucin family, was reported to have bifidogenic activity (Killer & Marounek, 2011). Thus, it is interesting to explore whether ovomucin hydrolysates support the growth of bifidobacteria.

The objectives of the study were to determine the effect of proteolysis of ovomucin on the sialic acid content and bifidogenic activity of ovomucin hydrolysates. Ovomucin hydrolysates were prepared by proteolysis and the hydrolysis yield, nitrogen recovery, amino acid composition, peptide profile, Mw distribution, degree of hydrolysis, sialic acid

contents, and the growth of bifidobacteria with ovomucin as sole carbohydrate source were studied. Since egg is a major allergen, effect of hydrolysis on allergenicity of ovomucin extracts was also determined.

3.2 Materials and methods

3.2.1 Materials and chemicals

Fresh eggs from White Leghorn were obtained within 24h from the Poultry Research Centre of the University of Alberta (Edmonton, Canada) and used in the same day for extraction of ovomucin. 2,4,6-Trinitrobenzenesulfonic acid solution (TNBS), Sodium Dodecyl Sulfate (SDS), trifluoroacetic acid (TFA) were all obtained from Sigma-Aldrich (St. Louis, MO, USA). Tween 20 and Coomassie Brilliant Blue (CBB) R-250 were obtained from Bio-Rad (Bio-Rad Laboratories, Inc., Hercules, CA). Hydrochloric acid and sodium hydroxide were bought from Fisher Scientific Inc. (Fisher Scientific, Ottawa, ON, Canada). Standard proteins (ovalbumin, ovotransferrin, ovomucoid, and lysozyme) were provided by Neova Technologies Inc. (Abbotsford, BC, Canada). Milli-Q water was prepared by the Milli-Q water supply system (Millipore Corporation, Billerica, MA, USA).

3.2.2 Extraction of ovomucin from egg white

Ovomucin was extracted as we previously reported (Wang et al., 2012). In brief, fresh egg white was diluted with 3 times of Milli-Q water, and stirred for 120 min. After adjusting the pH to 5.0 by 2N HCl, the slurry was placed in cold room (4 °C) for 24 hours and centrifuged at 15,344 g for 10 min at 4 °C (Beckman Coulter, Rotor JA10, USA). The precipitate was collected, lyophilized and stored at -20 °C until further analysis.

3.2.3 Hydrolysis of ovomucin by different enzymes

To prepare hydrolysate, ovomucin extract was dispersed into Milli-Q water to make 1% (w/v, ovomucin/water) slurry. The pH and temperature of the slurry was adjusted to appropriate conditions of individual enzymes (Table 3.1) and then enzyme was added at a level of 2% (w/w, enzyme/substrate). Hydrolysis was performed on a Titrando (842, Metrohm, Herisan, Switzerland) equipped with a circulating water bath during hydrolysis to maintain constant pH and temperature during hydrolysis for 3 h for pepsin and pancreatin or 4 h for the other 12 enzymes. After incubation, the suspension was heated at 95 °C for 15 min in water bath, cooled to ambient temperature on ice and then centrifuged at 15,344×g for 20 min at 4 °C to remove the precipitate (Beckman Coulter, Rotor JA14, USA). The supernatant was collected and lyophilized. All hydrolysis were conducted in duplicate. The hydrolysis yield and the nitrogen recovery (NR) were calculated as follows:

$$\text{Hydrolysis yield (\%)} = \frac{\text{Sample weight in supernatant}}{\text{Sample weight before hydrolysis}} \times 100$$

$$\text{Nitrogen recovery (\%)} = \frac{\text{Nitrogen content in supernatant}}{\text{Nitrogen content in original substrate}} \times 100$$

3.2.4 Characterization of ovomucin and ovomucin hydrolysates

3.2.4.1 The purity of the prepared ovomucin

The purity of the ovomucin extract was determined by a High-load 16/60 column (Superdex 200 preparatory grade) coupled with Fast Performance Liquid Chromatography (FPLC) (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) as previously reported (Omana & Wu, 2009). The prepared ovomucins were dissolved in 100 mM sodium phosphate buffer (pH 7.0) containing SDS (50 g/L) and β-mercaptoethanol (10 mL/L) at a concentration of 5 g/L. Samples were filtered through a 0.45 μm filter (PVDF, Mandel

Scientific Company Inc.) prior to FPLC analysis. The injection volume was 3 mL, and the column was eluted with 100 mM phosphate buffer (pH 7.0) containing 5 g/L of SDS and 1 mL/L of β-mercaptoethanol at a flow rate of 1 mL/min. The eluate was monitored by a UV detector at 280 nm. The percentages of other proteins (ovalbumin, ovomucoid, and lysozyme) in ovomucin extracts were calculated from their respective standard curves, and the purity of ovomucin was calculated by subtracting the amount of other proteins (Hiidenhovi, Mäkinen, Huopalahti, & Ryhänen, 2002; Omana et al., 2009).

3.2.4.2 Degree of hydrolysis (DH) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

DH was measured according to the TNBS method (Adler-Nissen, 1979). SDS-PAGE was carried out by 7.5% ready-to-use gels (Bio-Rad Laboratories, Inc., Hercules, CA). Protein and carbohydrate bands in the gel were stained with CBB R-250 and periodic acid-Schiff (PAS) reagents (Glycoprotein Detection Kit, Sigma Chemical Co.), respectively (Kobayashi et al., 2004).

3.2.4.3 Quantification of sialic acid in ovomucin hydrolysate

Samples were firstly acid hydrolyzed by 0.1 M HCl at a concentration of 2 g/L, at 80 °C for 1 h (Rohrer, Thayer, Weitzhandler, & Avdalovic, 1998). The amount of sialic acid in ovomucin hydrolysates was determined by the high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC/PAD) using Dionex ICS-3000 Ion Chromatography system on a Carbopac PA20 column (3 × 150 mm, Dionex, Oakville, Canada). N-acetylneuraminic acid was used as standard (Rohrer et al., 1998). The injection volume was 10 uL and the column temperature was kept at 30 °C. The column was eluted using a gradient, composed of 100 mM NaOH (A) and 1 M sodium

acetate in 100 mM NaOH (B), 7-30% B from 0 to 7.5 min, 30% B from 7.5 to 9 min, 30%-7% B from 9.0 to 9.5 min, and 7% B from 9.5 to 16.5 min, at a flow rate of 0.35 mL/min.

3.2.4.4 Amino acid composition

Amino acid composition was analyzed by a HPLC method on a Supelcosil 3 micron LC-18 reverse phase column (4.6 x 150 mm; Sigma-Aldrich, St. Louis, MO, USA) equipped with a guard column (4.6 x 50 mm; Sigma-Aldrich, St. Louis, MO, USA) packed with Supelco LC-18 reverse phase packing (20-40 μm) using a Fluorichrom detector (excitation 340 nm emission 450 nm). Samples were hydrolyzed under nitrogen atmosphere in 6 N HCl at 110 °C for 24 h to determine all the amino acids except cysteine, methionine, proline, and tryptophan (Jarrett, Cooksy, Ellis, & Anderson, 1986). Methionine and cycteine were determined by oxidation using performic acid hydrolysis (Moore, 1963) and tryptophan was released by alkaline hydrolysis before qualification (Ravindran & Bryden, 2005). Amino acid composition was expressed as g of amino acid/100 g of hydrolysate.

3.2.4.5 Size exclusion chromatography

Molecular weight (Mw) distribution of ovomucin hydrolysate was analyzed by a TSK-Gel G3000SWxl gel filtration column (7.8 mm×30 cm, 7 μm, Tosoh Bioscience LLC, Montgomeryville, PA, USA) coupled with a guard column (6.0 mm ×4.0 cm, 7 μm, Tosoh Bioscience LLC, Montgomeryville, PA, USA) attached to a Waters 600 HPLC system (Waters Corporation, Milford, MA, USA). Samples, at a concentration of 10 g/L, were prepared by dissolving hydrolysates in 100 mM sodium phosphate buffer (pH 6.7) containing 0.05% (w/v) of sodium azide and 100 mM of sodium sulfate. The samples were

automatically injected by a Waters 2707 autosampler at a volume of 25 µL, and the column was eluted isocratically with 100 mM sodium phosphate buffer (pH 6.7) containing 0.05% (w/v) of sodium azide and 100 mM of sodium sulfate at a flow rate of 0.5 mL/min for 40 min, and monitored by a Waters 2998 photodiode array at 280 nm (Wang, Omana, & Wu, 2012).

3.2.4.6 Peptide profiles of ovomucin hydrolysates

Hydrolysates were dissolved in water (HPLC grade) containing 0.1% trifluoroacetic acid (TFA), and separated on an Acquity Ultra-Performance Liquid Chromatograph (UPLC) system with an Acquity UPLC BEH C_{18} column (100 mm × 2.1 mm i.d., 1.7 µm, Waters, Milford, MA, USA) using a linear gradient of 100%-60% of solvent A (water in 0.1% TFA) within 30 min; while solvent B was acetonitrile in 0.1% TFA. The sample concentration was 10 mg/mL and injection volume was 10 µL. Separation was performed at 40 °C at a flow rate of 0.2 ml/min. The elution was detected at 220 nm.

3.2.5 Enzyme-Linked Immunosorbent Assay (ELISA)

Allergenicity of ovomucin hydrolysate was evaluated using a half-sandwich ELISA format (Li, Offengenden, Fentabil, Gänzle, & Wu, 2013), to determine the IgE binding activity. Freeze dried ovomucin hydrolysates were dissolved in 50 mM sodium carbonate buffer (pH 9.6) at a concentration of 5 g/L, then 100 µL of the sample was applied onto each well of the 96-well flat bottomed microtiter plates (Costar, Corning, Inc, NY) and incubated at 4 °C overnight. The plate was washed 4 times with phosphate buffered saline containing 0.05% Tween 20 (PBST, pH 7.2, 200 µL/well), then blocked with 150 µL/well of 2% bovine albumin serum (BSA, Sigma Chemical Co.) in 50 mM sodium carbonate buffer at pH 9.6 for 2 hours at 37 °C with gentle shaking. The plate was washed again with

PBST for three times. Human plasma from patients with egg-white allergy (PlasmaLab International, Everett, WA, USA) were diluted 1:25 in PBST containing 1% BSA, then 100 μL/well was applied to the plate and incubated overnight at room temperature with gentle shaking. After washing, the plate was incubated overnight with 100 μL/well of goat anti-human IgE secondary antibody (Sigma Chemical Co.) at ambient temperature with gentle shaking, which was diluted 1:2000 in PBST containing 1% BSA. Finally, the plate was washed again with PBST before the addition of 100 μL/well of p-nitrophenyl phosphate (pNPP, Sigma Chemical Co.) for developing color at room temperature with shaking. After 45 min, the reaction was stopped by adding 25 μL/well of 3 M sodium hydroxide and absorbance was read at 405 nm. The acidified egg white was used as positive control (Kovacs-Nolan, Zhang, Hayakawa, & Mine, 2000) and all the final results were expressed after subtraction of blank value (<0.10).

3.2.6 Metabolism of ovomucin hydrolysates by bifidobacteria

3.2.6.1 Bacterial strains and growth conditions

The reference bacterial strains used in the study were *Bifidobacterium adolescentis* (American Type Culture Collection, ATCC 15703), *Bifidobacterium longum* (ATCC 15707), *Bifidobacterium bifidum* (ATCC 15696, ATCC 29521 and ATCC 35914), and *Bifidobacterium infantis* (ATCC 15697). The bacteria were fermented in modified MRS medium supplied with selected ovomucin hydrolysates as sole carbohydrate source at 37 °C for 48 h under anaerobic condition (10% of CO_2, 5% of H_2, and 85% N_2) (Schwab & Gänzle, 2011). The growth was measured as turbidity at 600 nm at interval of 0, 8, 24, and 48 h.

3.2.6.2 Analysis of metabolite and degradation of carbohydrates in ovomucin hydrolysates

The concentration of lactate was analyzed at fermentation intervals of 0, 8, 24, and 48 h by HPLC with a refractive index detector and an Aminex HPX-87 column (300 mm×7.8 mm, Bio-Rad) at 70 °C. 5 mM of H_2SO_4 was used as the solvent and the flow rate was 0.4 ml/min. For sample preparation, equal volumes of 7.5% perchloric acid were added to the supernatants of fermentation liquor to precipitate protein after overnight incubation at 4 °C. The precipitates were removed by centrifugation at 8,603×g for 5 min at room temperature (Galle, Schwab, Arendt, & Gänzle, 2010).

After fermentation, carbohydrates in ovomucin medium were hydrolyzed by incubating for 2 h at 95 °C in 2 M HCl. Resulting monosaccharides were determined on a Dionex ICS-300 system equipped with a CarbopacPA20 column (Dionex, Oakville, Canada) using water (A) and 200 mM NaOH (B) as eluents at a flow rate of 0.25 mL/min with the following gradient: 0 min 6% B and 20 min 100% B. Mannose, galactose, galactosamine hydrochloride, and glucosamine hydrochloride (all purchased from Sigma Chemical Co.), which are present in ovomucin, were applied as external standards (Galle et al., 2010; Schwab et al., 2011).

3.2.7 Statistical analysis

All experiments were performed at least in triplicate and the results were expressed as mean ± standard deviation (SD). All statistical analysis was carried out using IBM SPSS statistics 19 (SPSS Inc, USA). Significant differences were defined at a 5% level ($P<0.05$).

3.3 Results and discussion

3.3.1 Preparation of ovomucin hydrolysates

This study employed the one-step method for preparation of ovomucin (Wang et al., 2012). The purity of ovomucin extract was 55.7%, which was lower than the sample prepared by a 2-step method (Omana et al., 2009) but comparable to the purity of ovomucin preparations reported elsewhere (Hiidenhovi et al., 2002). The contents of ovalbumin, ovomucoid, and lysozyme were 20.0±1.5%, 3.3±0.1%, and 20.9±4.4%, respectively.

Previous studies on hydrolysis of ovomucin were usually performed over 24 h period with yields ranging from 67% to 98% (Hammershøj et al., 2008**;** Kobayashi et al., 2004; Oguro et al., 2000). Our preliminary study showed 4 hours of digestion reach the plateau of ovomucin hydrolysis. The peptide yield prepared using our method ranged from 42.6% (flavourzyme) to 97.4% (protease N) and the nitrogen recovery (NR) ranged from 41.6% (flavourzyme) to 93.8% (protease N) (Table 3.1); these results compare favorably to previous results. Most ovomucin hydrolysates exhibited similar NR and yield values except for pepsin-pancreatin and pronase hydrolysates. The NRs of the pepsin-pancreatin hydrolysate and pronase hydrolysate (67.8% and 85.8%, respectively) was significantly lower (P<0.05) than their yields (83.4% and 91.1%, respectively), indicating the presence of a higher proportion of carbohydrate moieties in these hydrolysates.

Degree of hydrolysis (DH) ranged from 2.4 to 46.3% (Table 3.1). DH showed a positive correlation with the yield of hydrolysates. However, Protease N showed the highest yield (97.4%) but with a relatively low DH (8.5%). A high yield associated with a low DH was probably due to a lack of cleavage sites of the substrate for Protease N

although it could partially hydrolyze ovomucin and improve its solubility (Hiidenhovi et al., 2005).

3.3.2 Molecular weight distribution of ovomucin hydrolysates

Different enzymes displayed different protease activity against ovomucin. As shown in Figure 3.1A, major bands over 150 kDa in ovomucin extracts were noticeably degraded by protex 26L, protease M, pepsin/pancreatin, acid protease II, pepsin, protease P, and pronase. Protease 26L and pronase were the two most efficient enzymes as the dominant polypeptides released by these two enzymes were all below 40 kDa (Figure 3.1A). PAS staining showed the presence of glycopeptides bands larger than 100 kDa in most hydrolysates with the exception of protease 26L and protease M hydrolysates although acid protease II and pepsin hydrolysates gave relatively light bands (Figure 3.1B).

The Mw distribution of ovomucin hydrolysates were further analyzed by size exclusion chromatography (Figure 3.2A). Hydrolysates prepared by protease 6L, flavourzyme, trypsin VI, alcalase 2.4L, protease A, protex 51FP were composed mainly of protein fragments larger than 200 kDa, indicating a low degree of degradation of ovomucin. On the other hand, hydrolysates prepared by pepsin followed by pancreatin, protease P, protease M, protex 26L and pronase are composed of protein fragments with Mw less than 12.4 kDa. In general, a higher DH resulted in the hydrolysates with smaller Mw.

3.3.3 Sialic acid content of ovomucin hydrolysates

The ovomucin extract contains 3.2% of sialic acid, which was in the range of the reported data (2.6% to 7.4%, w/w) (Feeney, Rhodes, & Andersos, 1980; Sleigh, Melrose, & Smith, 1973). Sialic acid contents in ovomucin hydrolysates ranged from 0.1% to 3.7% (Table 3.1). Hydrolysates prepared by pronase, protease N, protease P, trypsin VI, and

alcalase 2.4L showed relatively high content of sialic acid, whereas those of protex 26 L and acid protease II were extremely low (0.1% and 0.3%, respectively). The extreme low level of sialic acid in these two hydrolysates might be due to the applied acid pH (pH 3.0) during digestion and high temperature (95 °C) during deactivating enzyme, which were known to release or degrade sialic acid (Spichtig et al., 2010).

3.3.4 Amino acid composition

Amino acid composition of ovomucin and ovomucin hydrolysates are listed in Table 3.2. All ovomucin hydrolysates contained similar amounts (g/100g of ovomucin) of essential amino acids except the hydrolysate prepared by pepsin and pancreatin. Pepsin-pancreatin hydrolysate had relatively lower amounts of essential amino acids probably due to its lower content of protein (Table 3.1). Ovomucin extract in the present study contained more essential amino acids except His compared to the published data as shown in Table 3.2 (Robinson et al., 1971). After hydrolysis, the amino acid composition of ovomucin hydrolysates did not change except for Phe and Cys. The amount of essential amino acids in all the hydrolysates meets the FAO/WHO/UNU reference pattern for adult, and some of them were also higher than the model value for infant, which suggested the potential of ovomucin hydrolysates as ingredients in nutritional formulations.

3.3.5 Peptide profiles of ovomucin hydrolysates

The peptide profiles were analyzed by reverse phase HPLC. Ovomucin hydrolysates displayed very different profiles, suggesting different cleavage sites of enzymes (Figure 3.2B). Elution from reverse phase column can be used as an index of the hydrophobicity; peptides prepared with pepsin-pancreatin, protease M, pronase, protease A and protease P eluted early while hydrolysates prepared by alcalase 2.4L, pepsin, protex 6L, and trypsin

VI were comparatively more hydrophobic (Figure 3.2B). Protex 26L and protease N hydrolysates were evenly distributed over the elution time. Hydrolysates prepared by pepsin, protex 26L and acid protease II showed low Mw (Figure 3.2A) but eluted later in chromatography, suggesting high hydrophobicity, possibly related to low glycosylation.

3.3.6 IgE binding ability of ovomucin hydrolysates

Egg proteins are potential food allergens (Mine & Zhang, 2002). Enzymatic hydrolysis is an efficient method to reduce potential protein allergenicity (Ena, Van Beresteijn, Robben, & Schmidt, 1995), therefore the effect of hydrolysis on the allergenicity was determined. The IgE-binding abilities of ovomucin hydrolysates were determined using four sera from patients with confirmed egg allergies. All the patient sera bound the acidified egg white (positive control), serum #14474 showed the highest binding activity (Figure 3.3D). After protease digestion, IgE binding ability of ovomucin hydrolysates were all reduced ($P<0.05$) or even below the detection limit. Hydrolysates from flavourzyme, protex 51FP, protex 6L, and protease A were determined to have lower DH but retained IgE-binding activity; especially, the hydrolysate prepared by protex 6L revealed high binding activity with both sera 17912 (Figure 3.3A) and 14474 (Figure 3.3D). The decreased IgE binding of ovomucin hydrolysates possibly results from the hydrolysis of epitopes by proteases (Mills, Madsen, Shewry, & Wichers, 2003). Generally, a low degree of hydrolysis corresponded to a relatively high IgE binding ability of hydrolysates.

3.3.7 Metabolism of ovomucin hydrolysates by bifidobacteria

Six bifidobacteria strains were fermented in media with ovomucin hydrolysate as the sole carbon source. Compared to the growth of bifidobacteria in negative control (MRS without added sugars), only *B. infantis* displayed growth in the ovomucin medium. Among

the tested ovomucin hydrolysates, only pepsin-pancreatin hydrolysate significantly promoted the growth of *B. infantis* after 24 h of incubation and increased the production of lactate shown in Figure 3.4(A-B) ($P<0.05$).

The degradation of carbohydrates in media by bifidobacteria was also evaluated. Only galactose and mannose in pepsin-pancreatin hydrolysate were decreased by 9.5% and 14.9%, respectively, after fermentation by *B. infantis*. Glycopeptides present in the ovomucin hydrolysate were thus only partially used as carbon source by *B. infantis*. Firstly, ovomucin is rich in sialic acid and sialic acid occupies the terminal position of the glycans within ovomucin, which could resist proteolytic effect and inhibit the action of some endoglycosidases (Schauer, 2004). Among the six tested strains, only *B. infantis* was reported to have gene clusters encoding sialidases to release of the sialic acid (LoCascio et al., 2007), and could secrete fucosidase, β-galactosidase and β-N-acetylhexosaminidase as well (Kitaoka, 2012). Some species of *Bifidobacterium* have been demonstrated to metabolize human milk oligosaccharides (HMO) as sole carbon source, such as *B. bifidum* and *B. infantis* (Asakuma et al., 2011), and *B. bifidum* was clarified to degrade HMO by α-fucosidases (Ashida et al., 2009). The structures of ovomucin glycans differ substantially from HMO; in particular, ovomucin contains no fucose. Enzymes produced by bifidobacteria for degradation of HMO (Sela & Mills, 2010) may thus not hydrolyze glycans from ovomucin. It is likely, however, that ovomucin is fermented in the large intestine by other members of the intestinal microbiota (Sela et al., 2010; Klaassens et al., 2009).

3.4 Conclusions

Ovomucin extract was hydrolyzed by 14 proteases within 4 h, in comparison to 24 h reported in literature. Yields and DHs ranged from 42.6% (flavourzyme) to 97.4% (protease N), and 2.4% (flavourzyme) to 46.3% (pronase), respectively. These hydrolysates showed a wide range of Mw distribution while only two hydrolysates prepared by pronase and protex 26L showing Mw less than 40 kDa. The content of sialic acid in these hydrolysates ranged from 0.1% (protex 26L) to 3.7% (pronase). IgE binding of ovomucin hydrolysates was significantly reduced ($P<0.05$) in comparison to ovomucin extracts. Ovomucin hydrolysates did not generally support growth of *Bifidobacterium* spp *in vitro*, but may be degraded within the infant gastrointestinal tract by potential protocooperation among different bifidobacteria strains or other members of gut microbiota and form short chain fatty acid.

3.5 References

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Table 3.1 Enzymatic conditions and characterization of ovomucin hydrolysates

Enzymes	pH	Temp (°C)	Origin	Supplier	Protein content (w/w, %)	Yield (%)	Nitrogen recovery (%)	Sialic acid content (w/w, %)	Degree of hydrolysis (%)
Protease N	7.5	55	*Bacillus subtilis*	Amano Pharmaceutical Co.	81.8±0.4	97.4±0.6	93.8±0.4	2.1	8.5±1.5
Protex 51FP	7.5	50	*Aspergillus oryzae*	Genencor Division of Danisco	82.9±1.5	66.3±0.3	64.7±1.2	1.7±0.1	3.6±1.8
Protex 6L	9.5	60	*Bacillus licheniformis*	Genencor Division of Danisco	83.1±0.1	54.6±3.4	53.5±0.1	0.8±0.2	3.1±0.8
Trypsin VI	8.0	37	Porcine pancreas glands	Neova Technologies Inc.	85.6±0.4	84.4±1.7	85.1±0.4	2.0±0.3	3.8±0.7
Protex 26L	3.0	50	*Aspergillus niger*	Genencor Division of Danisco	83.4±0.2	70.8±2.1	69.5±0.2	0.1	11.8±3.5
Protease P	7.0	45	*Aspergillus melleus*	Amano Pharmaceutical Co.	80.1±0.2	88.5±1.3	83.5±0.2	2.4±0.1	24.1±1.3
Alcalase 2.4L	8.0	50	*Bacillus licheniformis*	Sigma Chemical Co.	81.8±0.3	75.8±0.9	73.1±0.3	2.1	4.0±0.3
Protease M	5.0	45	*Aspergillus oryzae*	Amano Pharmaceutical Co.	80.8±0.4	49.0±1.0	46.6±0.2	1.7±0.1	17.4±0.5
Pepsin+ Pancreatin	2.0/6.5	37	Porcine pancreas and gastric	Sigma Chemical Co.	69.0±0.7	83.4±1.7	67.8±0.7*	1.7±0.2	17.6±3.7
Acid protease II	3.5	45	*Rhizopus niveus*	Amano Pharmaceutical Co.	84.6±1.0	58.1±1.2	57.9±0.7	0.3	7.4±1.1
Pepsin	2.0	37	Porcine gastric	Sigma Chemical Co.	85.9±1.7	81.0±1.5	81.9±1.6	0.8±0.1	6.1±3.4
Flavourzyme	7.0	50	*Aspergillus oryzae*	Sigma Chemical Co.	82.9±2.0	42.6±0.6	41.6±1.0	0.5±0.2	2.4±1.0
Protease A	7.5	50	*Aspergillus oryzae*	Amano Pharmaceutical Co.	84.1±0.1	59.1±0.7	58.5±0.1	1.3±0.1	3.8±0.6
Pronase	7.5	50	*Streptomyces griseus*	Roche Diagnostics GMBH	80.0	91.1±0.3	85.8±0.1*	3.7	46.3±2.2

‡ Data were expressed as Mean ± SD; *Nitrogen recovery was significantly different from the corresponding yield (P<0.05)

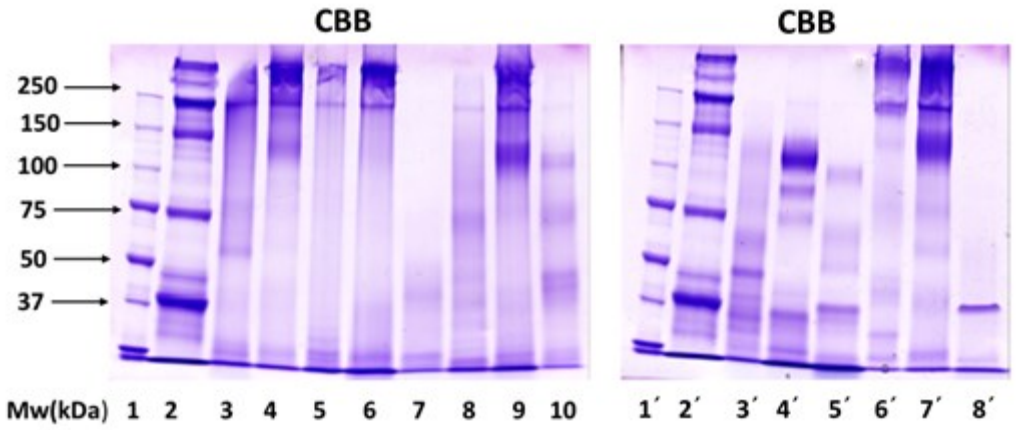
Table 3.2 Amino acid profiles of ovomucin (OvoM) and ovomucin hydrolysates (g/100g of sample)

	OvoM-Protease N	OvoM-Protex 51FP	OvoM-Protex 6L	OvoM-Trypsin VI	OvoM-Protex 26L	OvoM-Protease P	OvoM-Alcalase 2.4L	OvoM-Protease M	OvoM-Pepsin & Pancreatin
Essential amino acids									
Leu	5.0	5.0±0.3	5.5±0.7	6.0	5.0	5.0	5.5±0.7	5.0	4.0
Ile	4.0	4.0±0.3	4.0	4.0	4.0	4.0	4.0	4.0	3.0
Lys	5.0	5.1±0.4	5.0	5.5±0.7	5.0	5.0	5.0	5.0±1.4	4.0
Met	2.5±0.1	2.6±0.3	2.0±0.8	2.7±0.1	1.3±0.8	2.0±0.8	2.1±0.8	1.8±0.1	1.7±1.0
(Met + Cys)	18.0±0.9	19.4±1.6	16.3±5.0	16.5±0.1	15.4±0.3	17.0±0.9	15.8±0.4	14.7±0.3	16.7±0.3
Phe	4.0	4.2±0.3	4.0	4.0	4.0	4.0	4.0	4.0	3.0
(Phe + Tyr)	6.5±0.7	7.1±0.6	7.0	6.0	6.5±0.7	6.5±0.7	6.5±0.7	6.0	5.5±0.7
Thr	4.5±0.7	4.6±0.4	4.5±0.7	5.0	4.0	5.0	5.5±0.7	5.0	4.0
Val	5.0	4.9±0.3	5.0	5.0	4.0	4.5±0.7	5.0	5.0	4.0
Trp	2.5±0.8	2.1±0.4	1.9±0.1	1.7±0.6	2.0±0.1	1.7	1.6±0.1	1.6±0.4	1.7±0.1
His	1.5±0.7	1.4±0.1	2.0	1.5±0.7	1.0	1.0	2.0	2.0	1.0
Non-essential amino acids									
Ala	3.0	3.0±0.1	3.0	4.0	3.0	3.0	3.5±0.7	3.0	3.0
Arg	4.5±0.7	3.7	4.0	5.0	4.5±0.7	4.0	3.5±0.7	3.0	3.0
Asp[e]	8.5±0.7	10.2±1.3	9.0	10.0	11.5±0.7	10.5±0.7	10.0±1.4	9.0	8.0±2.8
Cys-s[f]	15.5±0.8	16.8±1.3	14.3±4.2	13.9±0.2	14.1±0.6	15.0±1.7	13.7±1.1	12.9±0.4	15.0±0.7
Glu[g]	7.0±1.4	9.8±1.5	8.5±0.7	9.0	9.0±1.4	9.0	10.0±1.4	9.0	7.5±2.1
Gly	3.0	2.8±0.5	3.0	4.0	3.0	3.0	3.0	3.0	2.5±0.7
Ser	4.0	4.1±0.1	4.0	4.5±0.7	4.5±0.7	4.0	4.5±0.7	4.0	3.5±0.7
Tyr	2.5±0.7	2.9±0.8	3.0	2.0	2.5±0.7	2.5±0.7	2.5±0.7	2.0	2.5±0.7
Pro	ND[a]	ND[a]	ND[a]	ND[a]	ND[a]	ND[a]	ND[a]	ND[a]	ND[a]

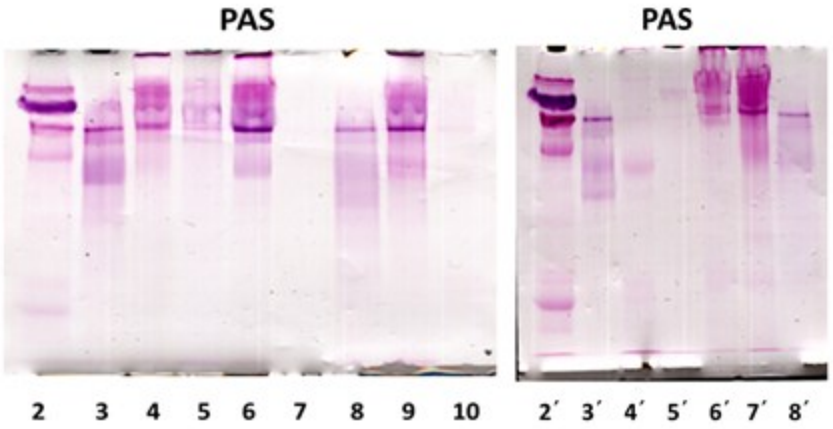
Continued Table 3.2

	OvoM-Acid Protrease II	OvoM-Pepsin	OvoM-Flavourzyme	OvoM-Protease A	OvoM-Pronase	OvoM	OvoM (3)	FAO/WHO/UNO[b] (g/100g of protein)	
								Infant	Adult
Essential amino acids									
Leu	6.0	5.6±0.2	6.0	5.5±0.2	5.6±0.8	5.3±0.6	4.0	6.6	1.9
Ile	4.5±0.7	4.3±0.1	5.0	4.1±0.3	4.5±0.9	4.1±0.3	2.6	2.8	1.3
Lys	5.5±0.7	4.9±0.3	6.0	5.4±0.1	5.2±0.4	5.3±0.8	4.3	5.8	1.6
Met	1.9±0.4	1.6±0.5	1.9±0.6	1.3±0.1	1.0±0.4	2.4±0.8	1.4		
(Met + Cys)	17.0±0.1	15.9±4.0	15.6±1.5	14.6±0.1	11.3±2.1	20.3±3.5	5.5	2.5[c]	1.7[c]
Phe	4.0	4.2±0.2	5.0	4.5±0.1	4.2±0.5	3.9±0.3	3.1		
(Phe + Tyr)	7.5±0.7	6.8±0.3	8.0	6.9±0.1	6.4±0.7	6.3±0.4	5.6	6.3[d]	1.9[d]
Thr	5.0	4.4±0.1	5.5±0.7	4.9±0.3	5.2±0.6	4.7±0.1	4.6	3.4	0.9
Val	5.0	4.8	5.5±0.7	5.1±0.5	5.1±0.8	4.5±0.3	3.3	3.5	1.3
Trp	2.0±0.4	1.9±0.4	1.7±0.1	1.7	2.7±0.9	1.9±0.1	0.9	1.1	0.5
His	2.0	1.3±0.1	2.0	2.1±0.2	1.7±0.3	1.3±0.2	1.7	1.9	1.6
Non-essential amino acids									
Ala	3.5±0.7	3.6±0.1	3.0	3.4±0.4	3.2±0.2	3.5±0.3	1.8		
Arg	5.0	5.0±0.3	3.5±0.7	4.0±0.4	4.6±0.2	4.9±0.8	2.6		
Asp[e]	11.0	10.1±1.9	10.0	9.6±0.1	10.2±1.9	10.4±0.6	5.6		
Cys-s[f]	15.1±0.3	14.4±3.5	13.7±0.9	13.3±0.1	10.3±1.6	17.9±4.2	4.1		
Glu[g]	10.0	8.0±1.7	11.0	10.0±1.1	9.0±1.2	9.2±1.1	7.0		
Gly	4.0	3.5±0.1	3.5±0.7	3.8±0.7	3.3±0.2	3.3±0.5	1.8		
Ser	5.0	4.3±0.4	4.5±0.7	4.2±0.5	4.4±0.4	4.5±0.3	4.3		
Tyr	3.5±0.7	2.7±0.5	3.0	2.5±0.2	2.3±0.2	2.4±0.1	2.5		
Pro	ND[a]	ND[a]	ND[a]	ND[a]	ND[a]	ND[a]	3.3		

[a] Not determined
[b] FAO/WHO/UNU energy and protein requirements (1985).
[c] Requirements for Methionine + Cysteine.
[d] Requirements for Phenylalanine + Tyrosine.
[e] Aspartic acid + Asparagine.
[f] Cysteine + Cystine.
[g] Glutamic acid + Glutamine.

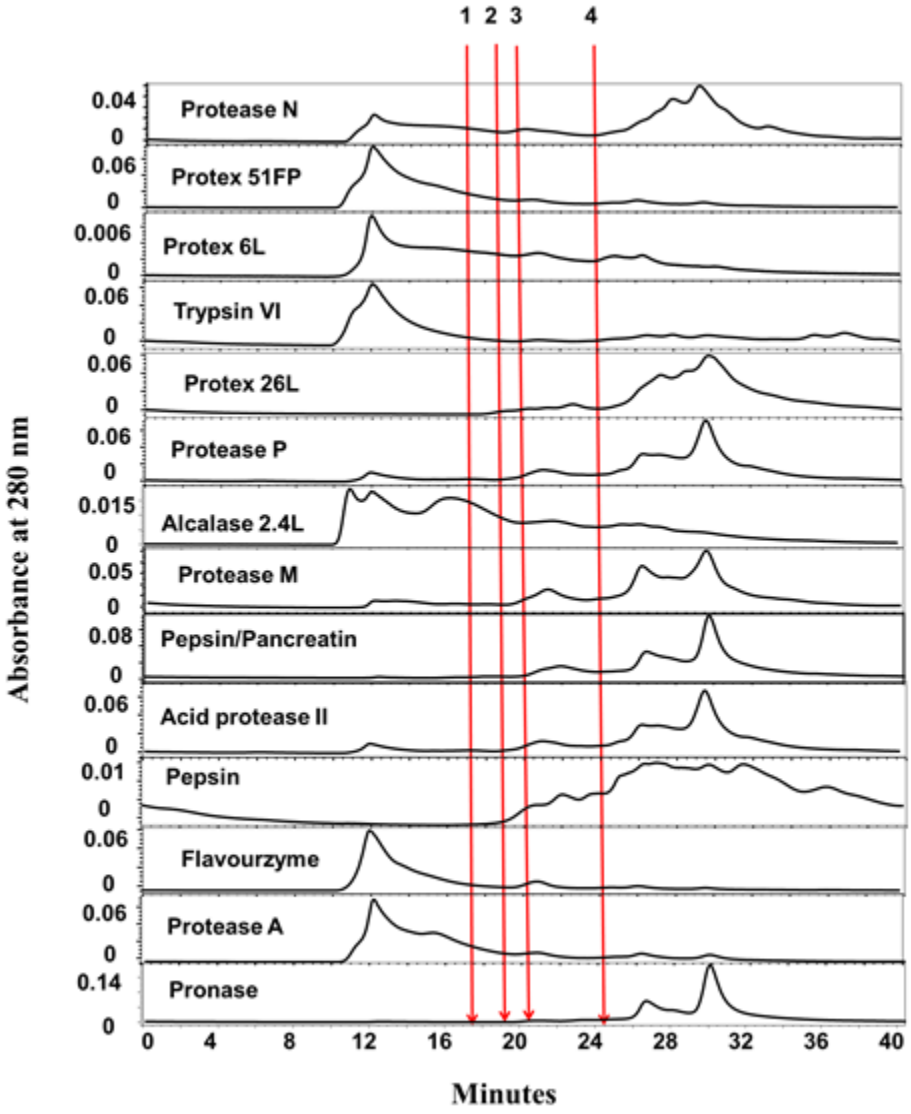


A



B

Figure 3.1: SDS-PAGE analysis of ovomucin hydrolysates. The gels were stained with (A) CBB and (B) Schiff reagents. Lanes 1 to 10 were molecular weight (Mw) marker (1), ovomucin reduced by β-mercaptoethanol (2), ovomucin digested by protease N (Ovomucin-Protease N) (3), Ovomucin-Protex 51FP (4), Ovomucin-Protex 6L (5), Ovomucin-Trypsin VI (6), Ovomucin-Protex 26L (7), Ovomucin-Protease P (8), Ovomucin-Alcalase 2.4L (9), and Ovomucin-Protease M (10). Lanes 1′ to 8′ were Mw marker (1), Ovomucin reduced by β-mercaptoethanol (2), Ovomucin-pepsin and pancreatin (3), Ovomucin-Acid protease II (4), Ovomucin-Pepsin (5), Ovomucin-Flavourzyme (6), Ovomucin-Protease A (7), and Ovomucin-Pronase (8).


1 2 3 4
Protease N
Protex 51FP
Protex 6L
Trypsin VI
Protex 26L
Protease P
Alcalase 2.4L
Protease M
Pepsin/Pancreatin
Acid protease II
Pepsin
Flavourzyme
Protease A
Pronase
0.04
0
0.06
0
0.006
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0.06
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0.06
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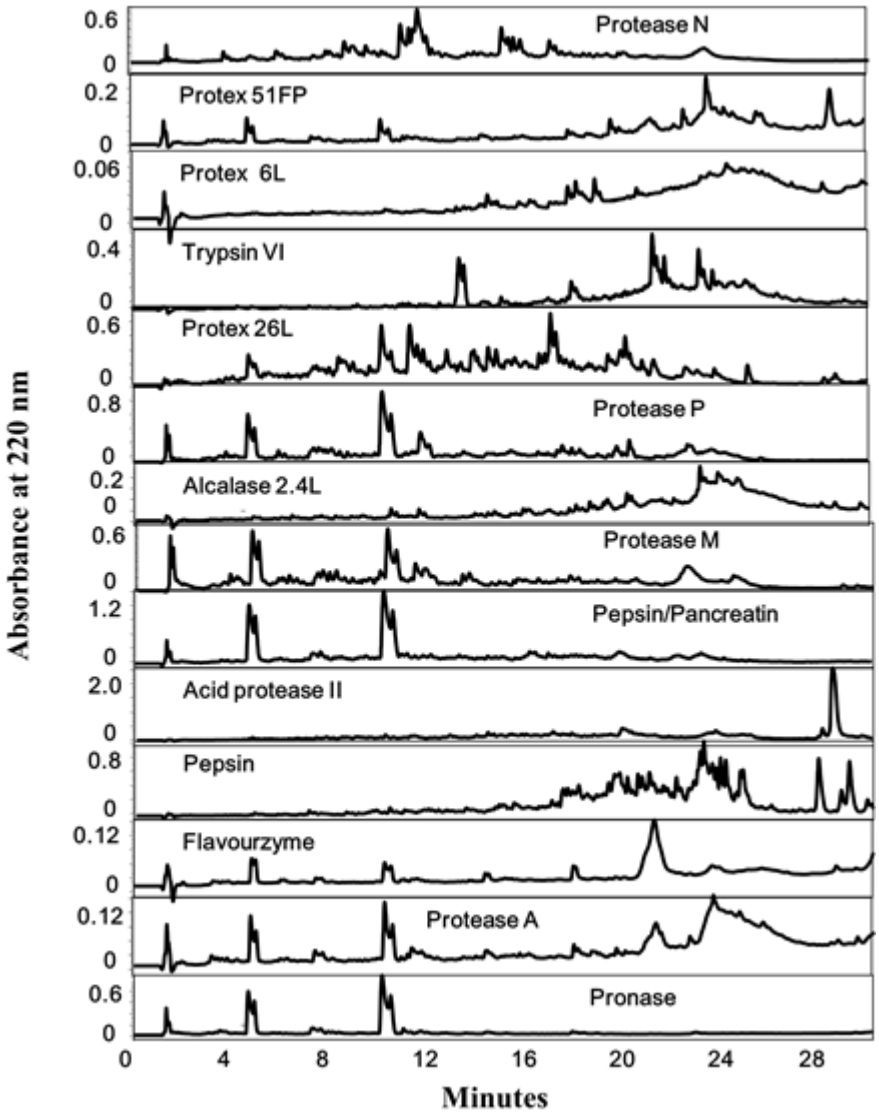
Absorbance at 280 nm
Minutes

A



B

Figure 3.2: (A) Gel filtration chromatograms of ovomucin hydrolysates and (B) HPLC elution profiles of ovomucin hydrolysates on an Acquity UPLC BEH C18 column. (A) The sample was eluted isocratically with 100 mM sodium phosphate buffer (pH 6.7) containing 0.05% (w/v) of sodium azide and 100 mM of sodium sulfate at a flow rate of 0.5 mL/min for 40 min. The cut-off value 1 to 4 were protein standards with molecular weights of 200, 150, 66, and 12.4 kDa, respectively. The proteases in the picture represented the corresponding ovomucin hydrolysates, **(B)** HPLC was carried out with a linear gradient of 100%-60% of solvent A (water in 0.1% TFA) within 30 min at 0.2 ml/min at 220 nm; solvent B was acetonitrile in 0.1% TFA. The proteases in the picture represented the corresponding ovomucin hydrolysates.

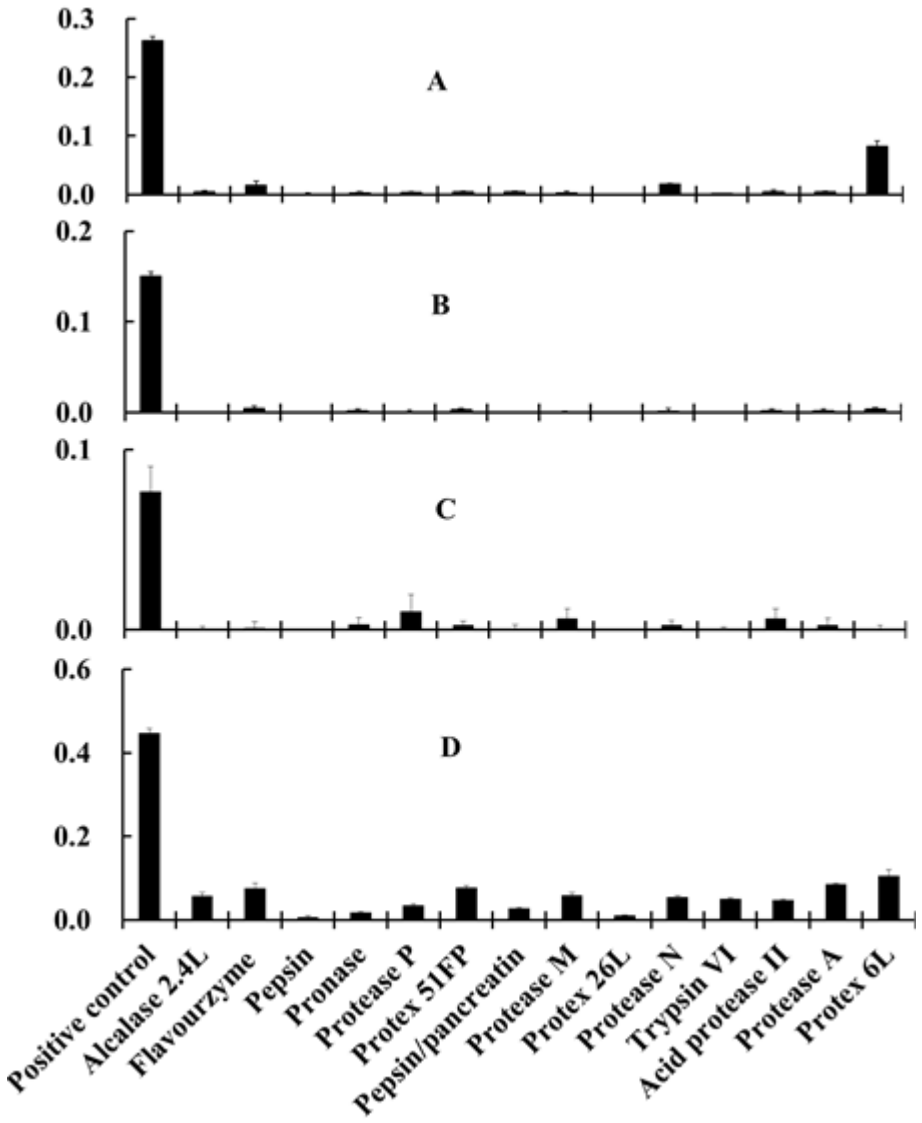


Figure 3.3: IgE binding activity of ovomucin hydrolysates (A) serum 17912, (B) serum 12388, (C) serum 14982, and (D) serum 14474. The positive control was acidified egg white. The proteases in the picture represented the corresponding ovomucin hydrolysates.

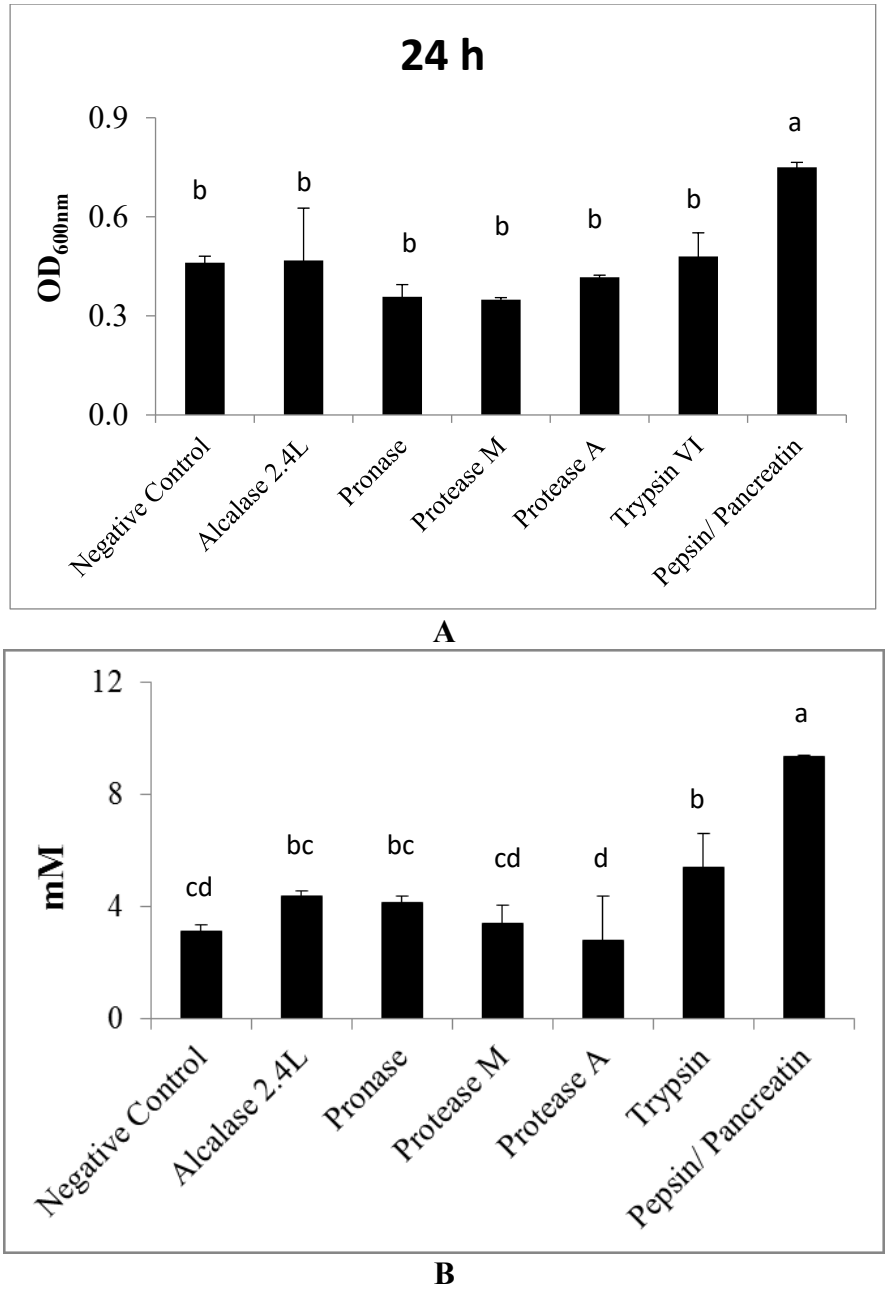


Figure 3.4: (A) The OD values (600 nm) of *B. infantis* and (B) Production of lactate by *B. infantis.* (A) The OD values (600 nm) at 24 h when *B. infantis* fermented in modified MRS with ovomucin hydrolysates as sole carbohydrate source at 37 °C for 48 h under anaerobic condition (10% of CO_2, 5% of H_2, and 85% N_2). The proteases in the picture represented the corresponding ovomucin hydrolysates. The bars with different letters above were significantly different (P<0.05), **(B)** The production of lactate (48 h minus 0 h) by the strain of *B. infantis*. The other information was same to that in Figure 3.4A.

CHAPTER 4 – Identification and Characterization of Glycopeptides from Egg Protein Ovomucin with Anti-Agglutinating Activity against Porcine K88 Enterotoxigenic *Escherichia coli* Strains

4.1 Introduction

Diarrhea in newborn and post-weaning piglets increases the mortality and morbidity rates, causing substantial economic losses to the swine industry. Enterotoxigenic *Escherichia coli* (ETEC) strains are the primary cause of piglet diarrhea (Nagy & Fekete, 1999). ETEC strains express fimbrial adhesins (mainly K88 or F18 fimbrial adhesin) that specifically recognize the intestinal epithelial glycoprotein receptors, thereby colonize the small intestine microvilli (Kulkarni, Weiss, & Iyer, 2010), subsequently secrete and transfer heat-stable and/or heat-labile enterotoxins to the target cells, leading to diarrhea (Nagy & Fekete, 2005). Adhesion to mucosal tissue or epithelial cells of the host is the initial and prerequisite step in ETEC pathogenesis (Moonens, et al., 2015). Antibiotic regimens are commonly used to prevent or treat bacterial diarrhea of farm animals. However, the use of antibiotics in farm animals is less favored and even banned for use in Europe for the fear of developing bacterial antibiotic resistance and transferring antibiotic resistance to human pathogens (Chen, Woodward, Zijlstra, & Gänzle, 2014; Docic & Bilkei, 2003; Nagy & Fekete, 1999). Therefore, it is imperative to find alternative approaches to control bacterial infection in farm animals.

A promising strategy to prevent infectious diseases is to use anti-adhesive agents to interfere the initial stage of adhesion and colonization of bacteria to host tissues or to detach bacteria from the tissues at the early stages of infection (Ofek & Sharon, 2002;

Sharon & Ofek, 2000). Since anti-adhesive agents are not bactericidal, it is less likely to develop resistant strains (Sharon, 2006; Ofek & Sharon, 2002). The anti-adhesive effect of human milk oligosaccharides on adherence of *Campylobacter jejuni*, *E. coli*, *Vibrio cholera*, and *Salmonella fyris*, have been demonstrated (Coppa, et al., 2006; Hickey, 2012; Ruiz-Palacios, Cervantes, Ramos, Chavez-Munguia, & Newburg, 2003). Proanthocyanidin from cranberry was also reported to interfere with adherence of *E. coli* to uroepithelial cells, thus preventing recurrent urinary tract infections in women (Sengupta, Alluri, Golakoti, Gottumukkala, Raavi, Kotchrlakota, et al., 2011; Camesano, Liu, & Pinzon-Arango, 2007). Although anti-adhesive agents are not commercially available against ETEC infection, the potential of exopolysaccharides (EPS) produced by *Lactobacillus spp*., proteins in porcine milk fat globule membrane and some natural feed ingredients has been demonstrated (González-Ortiz, Hermes, Jiménez-Díaz, Pérez, & Martín-Orúe, 2013; Novakovic, Huang, Lockerbie, Shahriar, Kelly, Gordon, et al., 2015; Wang, Gänzle, & Schwab, 2010).

Ovomucin is a mucin-type glycoprotein from egg white, accounting for 2-4% of total egg albumen protein (Kato, Oda, Yamanaka, Matsudomi, & Kobayashi, 1985). Ovomucin is composed of a carbohydrate-poor and a carbohydrate-rich subunits containing 11-15% and 50-57% (w/w) of carbohydrates, respectively (Robinson & Monsey, 1971). Carbohydrates in ovomucin include mannose (Man), galactose (Gal), *N*-acetylgalactosamine (GalNAc), N-acetylglucosamine (GlcNAc), sialic acid (N-acetylneuraminic acid) and sulfated saccharides (Donovan, Davis, & White, 1970). Sialic acid was thought to be responsible for the binding activity of ovomucin or ovomucin glycopeptides to enterohemorrhagic *Escherichia coli* O157:H7, new castle disease virus and influenza virus (Kobayashi, et al., 2004; Lind, 1949; Tsuge, Shimoyamada, &

Watanabe, 1997a). Due to its structural similarity to mucin, ovomucin may have the potential to act as receptor analogs and compete for bacterial adhesion. Therefore, the objectives of this study were to determine the anti-adhesive potential of ovomucin or ovomucin derived hydrolysates against porcine K88 ETEC using hemagglutination assay, a widely used model system to test the activity of bacterial adhesion to identify peptide sequences (Martín, Martín-Sosa, & Hueso, 2002; Wang, Gänzle, & Schwab, 2010) and possible glycan structures from the most potent fractions of ovomucin hydrolysate, and to elucidated the possible binding sites of ovomucin hydrolysate to K88 ETEC.

4.2 Materials and methods

4.2.1 Materials and chemicals

Fresh eggs from White Leghorn laid within 24 h were obtained from the Poultry Research Centre of the University of Alberta (Edmonton, Alberta, Canada). Hydrochloric acid, sodium hydroxide, HPLC-grade water, methanol, acetonitrile (ACN) were bought from Fisher Scientific Inc. (Fisher Scientific, Ottawa, ON, Canada). Dithiothreitol (DTT), iodoacetamide (IAA), proteomics grade PNGase F from *Elizabethkingia meningosepticum*, ammonium acetate, ammonium carbonate, HPLC grade dimethyl sulfoxide (DMSO), formic acid, and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Mass spectrometry-grade trypsin gold was obtained from Promega (Madison, WI, USA). Porcine whole blood was bought from Innovative Research Inc. (Novi, MI, USA). Milli-Q water was prepared by the Milli-Q water supply system (Millipore Corporation, Billerica, MA, USA).

4.2.2 Extraction of ovomucin from egg white

Ovomucin was extracted according to the method of Wang and Wu with modifications (Wang & Wu, 2012). In brief, fresh egg white was homogenized by stirring for 45 min before mixing with 3 times of Milli-Q water, followed by further stirring 60 min. After adjusting pH to 5.0 by 2N HCl, the above slurry was placed in cold room (4 °C) for 24 h. Ovomucin was recovered by centrifugation at 15,344 g for 10 min at 4 °C (Beckman Coulter, Rotor JA10, USA), lyophilized and stored at -20 °C until further analysis.

4.2.3 Hydrolysis of ovomucin by different enzymes

Ovomucin was hydrolyzed by 14 enzymes as listed in Table 4.1. Enzymatic hydrolysis was performed at recommended pHs and temperatures, at the ratios of ovomucin/water (w/v) and enzyme/substrate (w/w) were 1% and 2%, respectively, as reported in our previous study (Sun, Gänzle, Field, & Wu, 2016). The hydrolysis time was 4 h except pepsin (3 h) and pancreatin (3 h). All hydrolysis were conducted in duplicate.

4.2.4 Bacterial strains and culture conditions

Two porcine ETEC strains with K88 antigen, ECL13795 (O149; virotype STb:LT:EAST1:F4) and ECL13998 (O149; virotype STa:STb:LT:EAST1:F4:Paa), were kindly provided by the *Escherichia coli* Laboratory at the University of Montreal (Montreal, Quebec, Canada). Strains were cultured at 37 °C aerobically overnight on Minca agar (Guinée, Veldkamp, & Jansen, 1977). Cultures were recovered with 1 mL of phosphate buffer (PB, 150 mM, pH 7.2). The concentration of cell suspension was determined by optical density at 600 nm (OD_{600}). An OD_{600} of 1.2 corresponds to 10^9 cfu mL^{-1}. The final bacteria concentrations were between 10^{10} cfu mL^{-1} and 10^{11} cfu mL^{-1} for both strains (Chen, Woodward, Zijlstra, & Gänzle, 2014).

4.2.5 Hemagglutination assay

Porcine whole blood was washed three times by PB (150 mM, pH 7.2) to obtain erythrocytes, which were then resuspended in PB at 5% (v/v). Hemagglutination assay was conducted in V-bottomed 96-well polystyrene microtiter plates (Corning, Corning, NY, USA). After adding 25 μL of bacteria suspension (10^{10} cfu mL^{-1} to 10^{11} cfu mL^{-1}) or PB (negative control) or serially diluted PB solution containing ovomucin hydrolysates or reuteran (as the positive control) at concentrations ranging from 20 to 0.16 g/L to each well, the 96-well plate was incubated for 5 min at room temperature. And then 25 μL of 5% erythrocytes suspension in PB was added to the wells and mixed gently. Microtiter plates were inspected visually after 2 h of incubation at 4 °C. The applied two porcine ETEC strains agglutinated porcine erythrocytes but were resistant to mannose at 10 g/L. The minimum anti-agglutinating concentration (MAC) of the tested sample was defined as the lowest concentration achieving complete inhibition of porcine erythrocytes agglutination by ETEC strains (Wang, Gänzle, & Schwab, 2010). Hemagglutination assay was performed at least three independent assays in triplicate and the MACs could be repeated at least three times.

4.2.6 Ion exchange chromatography

Ion exchange chromatography was performed on a cation exchange column (16×100 mm, Hiptrp 16/10 SP FF, GE Healthcare, Sweden) coupled with an ÄKTA explorer 10XT system (GE Healthcare, Uppsala, Sweden). Freeze-dried ovomucin hydrolysate prepared by acid protease II was dissolved in 0.01 M ammonium acetate (pH 4.0) at a concentration of 10 g/L. After loading 10 mL sample, the column was firstly washed with 2 column volumes (CV) of 0.01 M ammonium acetate (pH 4.0), increased to 12% 0.5 M ammonium

carbonate over 3 CV and then to 100% 0.5 M ammonium carbonate over 7 CV at a flow rate of 4 mL/min. The elution was monitored at 280 nm, and fractions were collected based on peaks and pooled, freeze dried, and analyzed for anti-agglutinating assay as above (Majumder & Wu, 2011).

4.2.7 Reverse-phase high performance liquid chromatography (RP-HPLC)

Two active fractions 3 and 7 (Figure 4.1) from ion exchange chromatography were further purified by reverse-phase high performance liquid chromatography on a Xbridge C_{18} column (10×150 mm, 0.5 μm, Waters Inc., Milford, MA, USA) coupled with a guard column (40×10 mm, Waters Inc., Milford, MA, USA). Samples were automatically injected by Waters 2707 autosampler at a volumn of 900 μL. Fractions 3 and 7 were eluted with a linear gradient from 99% solvent A (0.1% TFA in HPLC-grade water) and 1% solvent B (0.1% TFA in ACN) to 99% solvent B over 55 min and 20 min, respectively, at a flow rate of 5 mL/min. Elution of fraction 3 was collected in 2 min-interval from 5 to 55 min (25 fractions), while that of fraction 7 was collected two subfractions based on peaks (Majumder & Wu, 2011).

4.2.8 Preparation of samples for mass spectrometry

Two most active sub-fractions, 3(9) and 7(1) from HPLC fractionation were further subjected to mass spectrometry (MS) analysis to identify peptide sequences and possible glycan structures. Samples were firstly reduced with 50 mM DTT, alkylated with 65 mM IAA, and dried in a SpeedVac (Savant Automatic Environmental SpeedVac, AES2000, Savant Instruments, Inc., Farmingdale, NY, USA). Then 15 μL of PNGase F solution (500 units/mL) was added and incubated at 37 °C overnight. The released N-glycans and de-N-glycosylated peptides were separated by passing through a 3 mL Sep-Pak C18

cartridge (Waters, Milford, MA, USA). N-glycans was permethylated by the sodium hydroxide/DMSO slurry method (Dell, Khoo, Panico, McDowell, Etienne, Reason, et al., 1993). Based on the preliminary SDS-PAGE results (*data not shown*), fraction 7(1) contained two protein bands and their molecular weights were about 25 kDa and 15 kDa, respectively; while the protein bands of fraction 3(9) were not shown, due probably to their small molecular weights. Thus, only de-N-glycosylated peptides obtained from fraction 7(1) were subjected to trypsin digestion at 37 °C prior to MS analysis.

4.2.9 Nano LC-ESI-MS/MS analysis

De-N-glycosylated peptides were subjected to liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MSMS) analysis using a Waters QToF Premier equipped with a nanoAcquity Ultra High Performance Liquid Chromatography (UPLC) system (Waters, Milford, MA, USA). Samples were loaded onto a trap column (75 µm x 20 mm, Acclaim PepMap® 100 column, Thermo/Dionex, Bannockburn, IL, UK) and desalted by flushing trap with 2% ACN in water with 0.1% formic acid (Solvent A) and 100% ACN with 0.1% formic acid (Solvent B) at a flow rate of 3 µL/min for 6 min, and then eluted on a nano analytical column (75 µm × 150 mm, Acclaim PepMap® 100 column, Thermo/Dionex, Bannockburn, IL, UK) at a flow rate of 0.35 µL/min using a linear gradient of solvent B from 2-8% over 5 min, 8-55% over 35 min, 55-95% over 8 min, kept at 95% of solvent B for 5 min and then decreased to 2% over 2 min.

4.2.10 MALDI-TOF MS analysis of permethylated N-glycans

Permethylated N-glycans were dissolved in methanol. An aliquot of 2 µL of sample was mixed with a matrix solution and 1 µL was spotted onto a Bruker Daltonics MTP Ground steel target and air dried (Bremen, Germany). The matrix solution consisted of a

20 g/L 2,5-dihydroxybenzoic acid (DHB) in methanol. Mass spectra were obtained in the positive reflectron mode of ionization using a Bruker Daltonics ultrafleXtreme MALDI TOF/TOF mass spectrometer (Bremen, Germany).

4.2.11 Database search

Database search was performed by ProteinScape Server (Bruker Daltonik, Bremen, Germany) using an in-house Mascot server (Version 2.2, Matrix Science, London, UK). Settings for database search were as follows: parent ion and MS/MS tolerance were set to 0.1 Da and 0.2 Da, respectively; no enzyme was specified for enzymatic cleavage; carbamidomethylation on cysteine was selected as fixed modifications and oxidation on methionine and deamidation on glutamine and asparagine were defined as variable modification. Confidence of positive protein identification was judged by high protein and peptide scores in the search results.

The possible glycan compositions and structures were searched within database GlycomeDB by GlycoQuest using Glycan QTOF CID searching method (Version 1.1) which was integrated in ProteinScape Sever (Bruker Daltonik, Bremen, Germany). The major parameters used for database search were as follows: derivatization was selected as permethylation (PerMe); ions was defined as up to 1 Na^+; negative and positive charges were both defined as 1; reducing end was defined as methylation (Me); MS tolerance was set to 0.8 Da.

4.3 Results

4.3.1 Anti-agglutinating activity of ovomucin and ovomucin hydrolysates

K88 fimbriae specifically recognize carbohydrate moieties of glycoconjugated receptors expressed on host cells (Jin & Zhao, 2000a). Protein components of receptors

also appear to play an important role in bacterial adhesion because pretreatment of the receptor with pronase or trypsin lost binding activity to K88 fimbriae (Chandler, Mynott, Luke, & Craven, 1994; Laux, McSweegan, Williams, Wadolkowski, & Cohen, 1986). As a glycoprotein, ovomucin extract did not exhibit anti-agglutinating activity against both ETEC strains (ECL13795 and ECL13998) at concentrations ranging from 1.25 to 20 g/L.

Ovomucin was further enzymatically hydrolyzed to release various glycopeptides using 14 enzymes (You & Wu, 2011). Our preliminary screening showed that ovomucin hydrolysates, at a concentration of 20 g/L, prepared by trypsin VI, protex 6L, protex 51FP, flavourzyme and pepsin did not inhibit erythrocyte agglutination with ETEC strains. As shown in Table 4.1, ovomucin hydrolyzed by protease P, acid protease II, protex 26L and pancreatin+pepsin showed strong anti-agglutinating activity against both ETEC strains at MAC of 0.31 or 0.63 g/L; all ovomucin hydrolysates did not show bactericidal activity against both ETEC strains.

4.3.2 Purification of anti-agglutinating fractions from ovomucin-acid protease II hydrolysate

Acid protease II hydrolysate, showed the best anti-agglutinating activity against both ETEC strains, was chosen to identify the responsible peptide sequences and glycans for binding to ETEC. Purification of the acid protease II hydrolysate by cation-exchange chromatography resulted in nine fractions (Figure 4.1A). Among these nine fractions, fractions 3 and 4 showed anti-agglutinating activity against strain ECL13795, fractions 3 and 5 showed weak anti-agglutinating activity against strain ECL13998, while fraction 7 exhibited the highest anti-agglutinating activity against strain ECL13998 (Figure 4.1B). Interestingly, we found that fraction 6 only showed anti-agglutinating activity against

strain ECL13998 at the concentrations of 0.5 and 0.25 g/L, but a dose response curve could not be established, similar to results of other studies (González-Ortiz, Hermes, Jiménez-Díaz, Pérez, & Martín-Orúe, 2013; Llano, Esteban-Fernández, Sánchez-Patán, Martín-Álvarez, Moreno-Arribas, & Bartolomé, 2015). Fractions 3 and 7, the most active fractions, were further subjected to RP-HPLC fractionation as shown in Figure 4.2 (A and C), where 25 subfractions and 2 subfractions were collected, respectively. The MACs of fractions 7(1) and 7(2) against strain ECL13998 were 0.25 and 0.50 g/L, respectively (Figure 4.2D). Among the subfractions from fraction 3, subfraction 3(9) gave the strongest activity at the MAC of 0.03 g/L (Figure 4.2B). Thus, fraction 7(1) (MAC 0.25 g/L) and fraction 3(9) (MAC 0.03 g/L) were chosen as the most potent fractions against strains ECL13998 and ECL13795, respectively, to characterize their peptide sequences and possible glycan structures. However, neither of these two fractions showed good anti-agglutinating activity against both ETEC strains.

4.3.3 Identification of peptides and possible glycan structures from ovomucin

Peptides from fractions 7(1) and 3(9) were subjected to nano LC-ESI MS/MS analysis. Fraction 7(1) consisted of egg white lysozyme and α-ovomucin, and six peptides were identified from α-ovomucin as shown in Table 4.2. These peptides were in three motifs of α-ovomucin peptide sequence: 177 to 240 (64 amino acids, 7.52 kDa), 993 to 1003 (11 amino acids, 1.14 kDa), and 1400 to 1491 (92 amino acids, 10.57 kDa). Fraction 3(9) included both α-ovomucin and egg white ovalbumin; six peptides were identified from α-ovomucin (Table 4.2). The motifs of identified peptide sequences in α-ovomucin were 138 to 201 (64 amino acids, 7.46 kDa), 1223 to 1291 (69 amino acids, 7.32 kDa), and 1514 to 1519 (6 amino acids, 0.76 kDa).

MALDI-TOF MS spectra of permethylated glycans of fractions 7(1) and 3(9) were presented in Figure 4.3. In Figure 4.3A, six glycans were determined at m/z of 1865.76, 1906.78, 2069.87, 2110.90, 2151.91 and 2356.03, of two major glycans at m/z of 2151.91 and 1906.78 were previously reported (Offengenden, Fentabil, & Wu, 2011). The possible glycan structures were proposed according to GlycoQuest; since the glycan peptides are derived from α-ovomucin containing a pentasaccharide core of $GlcNAc_2Man_3$ and a bisecting N-acetylglucosamine (GlcNAc) (Itoh, Miyazaki, Sugawara, & Adachi, 1987; Offengenden, Fentabil, & Wu, 2011; Robinson & Monsey, 1971), the proposed glycan structures were all composed of $GlcNAc_2Man_3$ core and a bisecting GlcNAc, and peaks of m/z 1865.76, 2110.90 and 2356.03 exhibited terminal β-linked galactose (Figure 4.3A). The possible glycan structures in Figure 4.3B for the thirteen peaks obtained from fraction 3(9) were presented. Similarly, all the glycan structures were composed of $GlcNAc_2Man_3$ core and a bisecting GlcNAc except peak of m/z 1580.09 which did not have a bisecting GlcNAc; of six proposed glycan structures contained terminal β-linked galactose. The peaks of m/z 1662.15, 2846.89 and 3050.97 in this study were not identified from ovomucin before (Offengenden, Fentabil, & Wu, 2011).

4.4 Discussion

There are three variants of fimbriae K88 ($K88_{ab}$, $K88_{ac}$, and $K88_{ad}$), which show different, yet related, carbohydrate-binding specificities (Grange, Mouricout, Levery, Francis, & Erickson, 2002). Both ETEC strains used in this study expressed $K88_{ac}$ fimbriae, the most prevalent type. Interestingly, they showed different binding activities to ovomucin hydrolysates. For example, alcalase hydrolysate had a MAC value of 5 g/L against strain ECL13795 but did not show inhibitory effect on strain ECL13998; ovomucin hydrolyzed

by protease A, protease M or protease N had good binding activity to strain ECL13998, but not to strain ECL13795. K88 fimbriae were believed to mediate the binding of these two ETEC strains to targeted receptors of erythrocytes or ovomucin hydrolysates (Jin & Zhao, 2000a). Strain ECL13998, but not ECL13795, contains porcine attaching and effacing-associated factor (Paa), a non-fimbrial adhesin, and the distinct binding activities may be related to the different binding specificities of these two strains due to the presence of Paa. Some results suggested that Paa contributes to the early stages of A/E lesions (Bardiau, Szalo, & Mainil, 2010; Batisson, Guimond, Girard, An, Zhu, Oswald, et al., 2003). However, ETEC do not exhibit A/E lesions, so the precise adhesive mechanism of Paa in ETEC strains remains unclear (Leclerc, Boerlin, Gyles, Dubreuil, Mourez, Fairbrother, et al., 2007).

Previously reported anti-adhesive agents often have low affinity to bacterial binding sites (Sharon & Ofek, 2000), therefore many anti-adhesive compounds such as EPS synthesized by *Lactobacillus reuteri*, wheat bran, casein glycomacropeptide, and locust bean, showed their MACs as high as of ~10 g/L (González-Ortiz, Hermes, Jiménez-Díaz, Pérez, & Martín-Orúe, 2013; Wang, Gänzle, & Schwab, 2010). In this work, 4 out of 14 ovomucin hydrolysates, including protease P, acid protease II, protex 26L and pancreatin+pepsin hydrolysates, showed potent anti-agglutinating activity against both ETEC strains at MACs of 0.31 or 0.63 g/L. The most active subfractions from ovomucin-acid protease II hydrolysate exhibited even lower MACs; namely, fractions 3(9) and 7(1) showed MACs of 0.03 g/L and 0.25 g/L, respectively, against strains ECL13998 and ECL13795. These data indicated the promising potential of ovomucin hydrolysates as anti-adhesive agents against infectious diseases.

Fraction 7(1) contains both hen egg white lysozyme and α-ovomucin; since lysozyme does not contain any carbohydrates, while K88 fimbriae could only recognize specific carbohydrate moieties, therefore it is very likely glycopeptides derived from α-ovomucin were responsible for this anti-agglutinating activity. Fraction 3(9) was contaminated with ovalbumin according to nano LC-ESI MS/MS analysis, so it is likely that the N-glycan profile in Figure 4.3B contained certain amounts of glycans from ovalbumin. Ovalbumin contains 3.5% of carbohydrates by weight, which mainly consists of mannose and GlcNAc but largely lack the terminal galactose (Harvey, Wing, Küster, & Wilson, 2000; Tomiya, Awaya, Kurono, Endo, Arata, & Takahashi, 1988). Therefore, the proposed glycan structures with terminal β-linked galactose in Figure 4.3(A-B) were most likely derived from α-ovomucin. Furthermore, pure hen egg white lysozyme and ovalbumin did not show any anti-adhesive activity against K88 ETEC strains (*data not shown*); therefore, α-ovomucin derived glycopeptides are indispensable for preventing ETEC strains adhesion.

Many studies suggested that β-galactose is essential for the attachment of K88 fimbriae (Grange, Erickson, Anderson, & Francis, 1998; Jin & Zhao, 2000a; Payne, O'Reilly, & Williamson, 1993), hence, terminal β-linked galactose from α-ovomucin could be one of the binding sites for $K88_{ac}$ fimbriae. The very low MAC value of fraction 3(9) against strain ECL13795 was due probably to its complex peptide profile (Figure 4.2A) that might have synergistic effects and block more than one bacterial binding ligand. Interestingly, crude acid protease II-ovomucin hydrolysate had better anti-agglutinating activity (MAC 0.31 g/L) than that of FPLC fraction 7 (MAC 1 g/L) for strain ECL13998, further suggesting the presence of synergistic effects of various glycopeptides. Similarly, a

mixture of human milk oligosaccharides showed stronger anti-adhesive activity than that of an individual oligosaccharide (Coppa, et al., 2006).

In conclusion, our study showed ovomucin hydrolysates, but not intact ovomucin, could interfere K88 ETEC adhesion to porcine erythrocytes, indicating the potential of ovomucin derived glycopeptides, in particular prepared by acid protease II, as anti-adhesive agents against infectious diseases. Glycopeptides derived from α-ovomucin, composed of a pentasaccharide core of $GlcNAc_2Man_3$ and a bisecting GlcNAc, possibly containing terminal β-linked galactose, are responsible for this anti-agglutinating activity.

4.5 References

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Table 4.1 Minimum anti-agglutinating concentration (MAC) of ovomucin hydrolysates against ETEC strains (n=9)

Number	Hydrolysates	Enzyme origin	MAC against ETEC strain ECL13795 (g/L)	MAC against ETEC strain ECL13998 (g/L)
1	Protease A	*Aspergillus oryzae*	>20*	2.5
2	Protease P	*Aspergillus melleus*	0.31±0.27	0.63±0.29
3	Protease M	*Aspergillus oryzae*	>20*	5±2.5
4	Protease N	*Bacillus subtilis*	5	0.31±0.30
5	Acid protease II	*Rhizopus niveus*	0.31±0.20	0.31±0.25
6	Protex 6L**	*Bacillus licheniformis*	>20	>20
7	Protex 26L	*Aspergillus niger*	0.31±0.31	0.63±0.22
8	Protex 51FP**	*Aspergillus oryzae var*	>20	>20
9	Pronase	*Streptomyces griseus*	0.94±0.18	2.5
10	Trypsin VI**	*pork pancreas glands*	>20	>20
11	Pancreatin+pepsin	*Porcine pancreas/ Porcine gastric*	0.63±0.22	0.63±0.20
12	Flavourzyme**	*Aspergillus oryzae*	>20	>20
13	Alcalase 2.4L	*Bacillus licheniformis*	5	>20*
14	Pepsin**	*Porcine gastric*	>20	>20

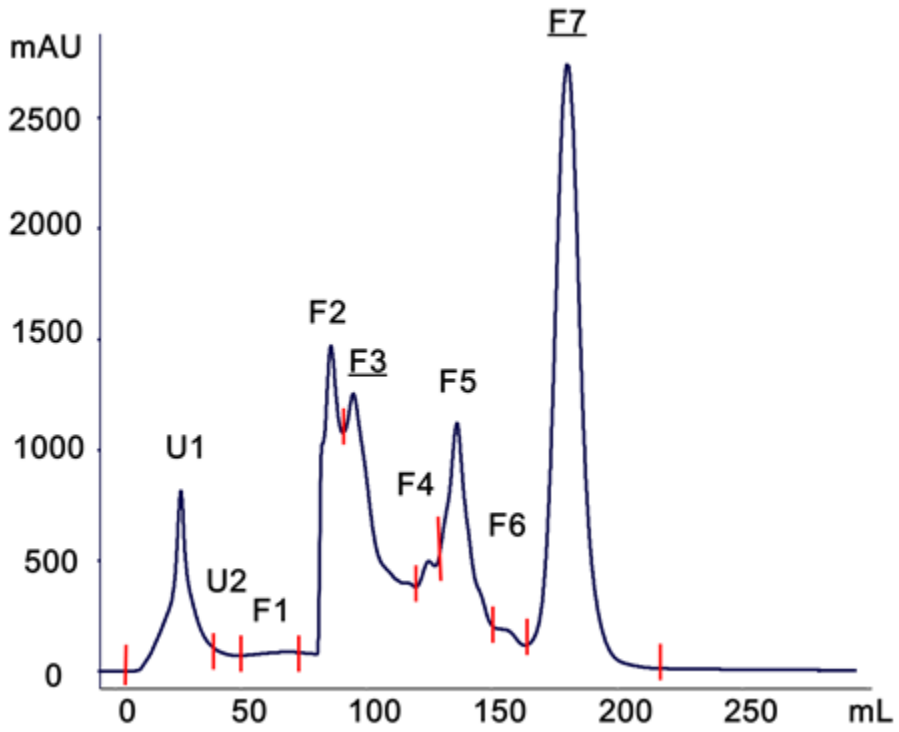
* No anti-agglutinating activity at concentrations ranging from 1.25 to 20 g/L

** No anti-agglutinating activity at concentration of 20 g/L in the preliminary experiment

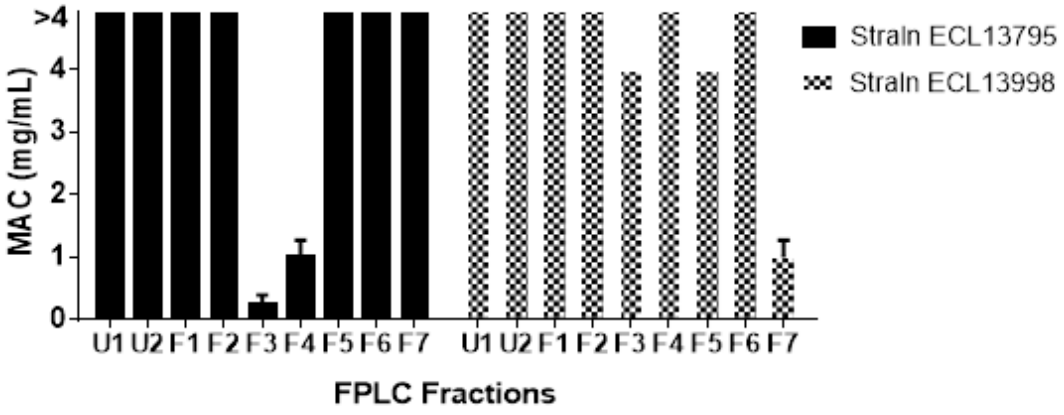
Table 4.2 Identified α-ovomucin peptides from fractions 7(1) and 3(9) by performing nano LC-ESI MS/MS analysis

Peptide location in α-ovomucin	Calculated mass	m/z measured	Peptide sequence*
	Fraction 7(1)		
177-182	692.4	693.5	LILDGY
228-233	791.4	792.6	MCKKLL
228-240	1569.8	785.8	MCKKLLSRFGNCP
993-1003	1254.5	628.3	VCGLCGDFDGR
1400-1404	612.4	613.4	PQQLK
1486-1491	678.4	679.5	SMSLLI
	Fraction 3(9)		
138-143	643.4	644.4	LGLTLK
196-201	703.3	704.4	EDPSEK
1223-1227	627.2	628.3	YSQTE
1,249-1,262	1534.7	768.4	CSTLSVPAQEQLMQ
1280-1291	1321.5	661.7	TDNGQLIQMGEN
1,514 - 1,519	764.4	765.4	LFNDKK

* The possible N-glycosylation site (N) and O-glycosylation sites (S and T) are underline

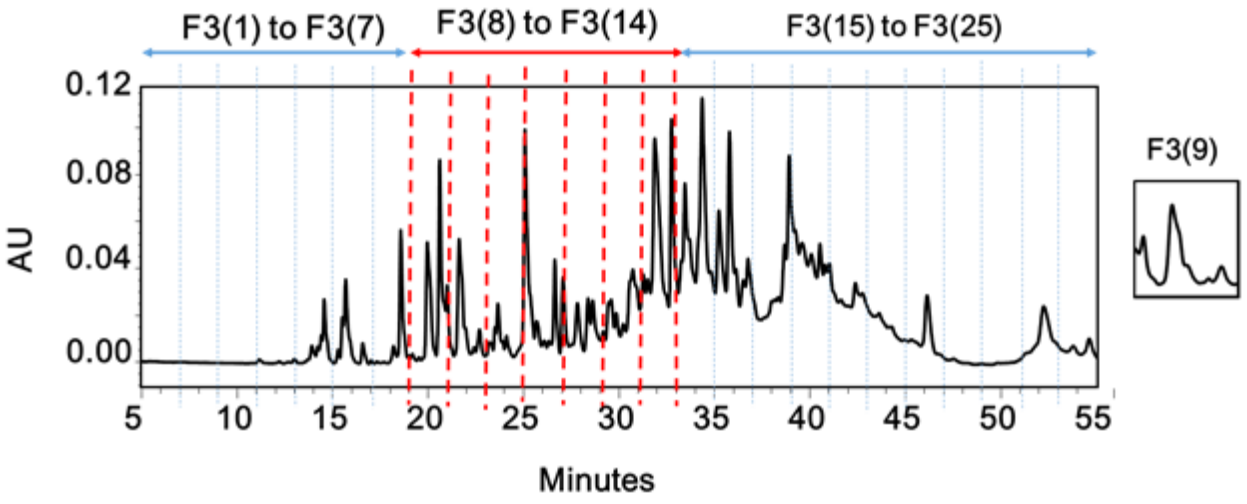


A

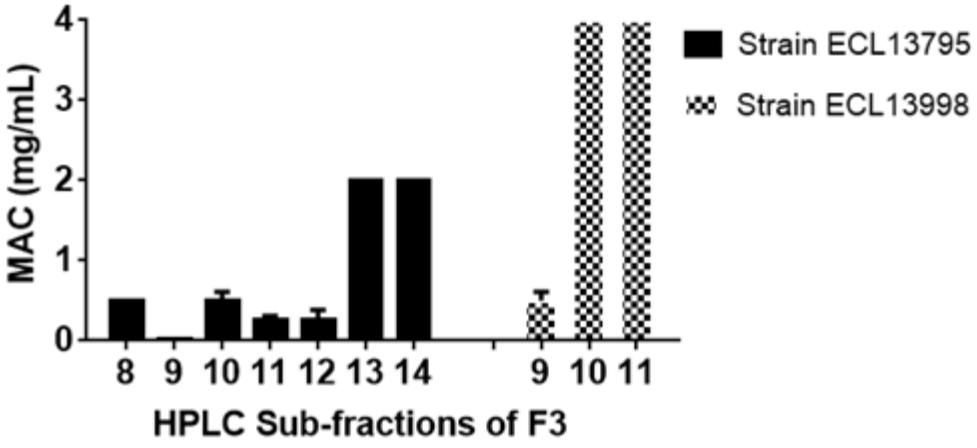


B

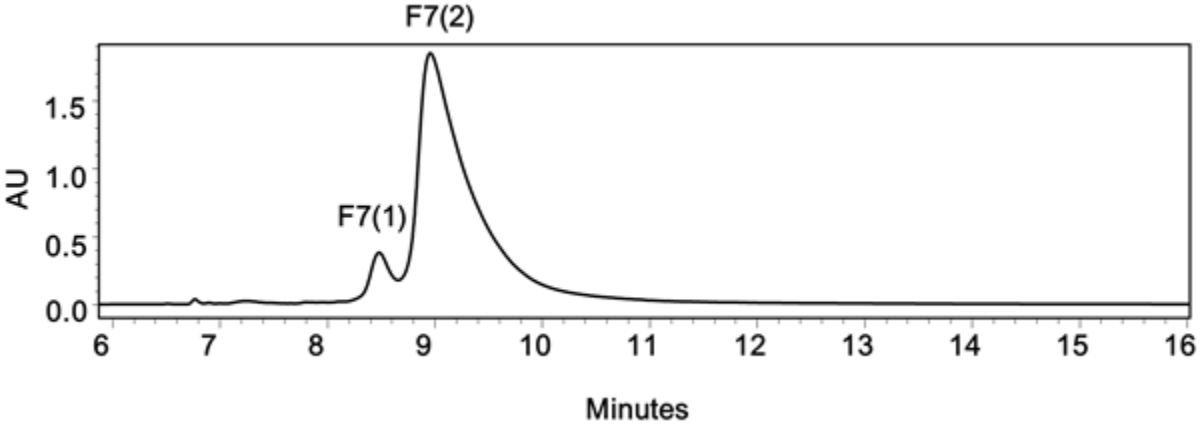
Figure 4.1: (A) Cation exchange chromatogram of acid protease II hydrolysate and (B) Minimum anti-agglutinating concentration (MAC) against ETEC strains of the FPLC fractions from acid protease II hydrolysate (n=9). (A) Sample was dissolved in 0.01 M ammonium acetate (pH 4.0) at 10 g/L, and was eluted with 2 column volume (CV) of 0.01 M ammonium acetate (pH 4.0), 3CV of a gradient to 12% 0.5 M ammonium carbonate, 7CV of 12% to 100% of 0.5 M ammonium carbonate, at a flow rate of 4 mL/min. The elution was monitored at 280 nm, **(B)** MAC>4 means there was no anti-agglutinating activity within the concentrations from 0.02 g/L to 4 g/L (2 time serial dilution).


F3(1) to F3(7)
F3(8) to F3(14)
F3(15) to F3(25)
F3(9)
AU
0.12
0.08
0.04
0.00
5
10
15
20
25
30
35
40
45
50
55
Minutes

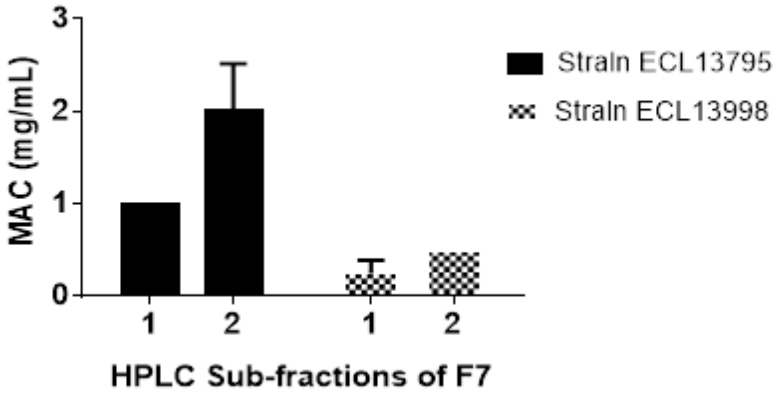
A


MAC (mg/mL)
4
3
2
1
0
Strain ECL13795
Strain ECL13998
8
9
10
11
12
13
14
9
10
11
HPLC Sub-fractions of F3

B


F7(2)
F7(1)
AU
1.5
1.0
0.5
0.0
6
7
8
9
10
11
12
13
14
15
16
Minutes

C



D

Figure 4.2: (A) RP-HPLC chromatogram of fraction 3 (F3) from cation exchange chromatography of acid protease II hydrolysate, (B) Minimum anti-agglutinating concentration (MAC) against ETEC strains of the HPLC subfractions from F3 (n=9), (C) RP-HPLC chromatogram of fraction 7 (F7) from cation exchange chromatography, and (D) MAC against ETEC strains of the HPLC subfractions from F7 (n=9). (A) The sample was eluted with a linear gradient from 99% solvent A (0.1% TFA in HPLC-grade water) and 1% solvent B (0.1% TFA in acetonitrile) to 99% solvent B over 55 min at a flow rate of 5 mL/min. The elution was monitored at 280 nm. Fractions were collected in 2 min-interval from 5 to 55 min (25 fractions). Fractions 8 to 14 showed anti-agglutinating activity, and fraction 3(9) was the most potent fraction against strain ECL13795, **(B)** The tested concentrations were from 0.02 g/L to 4 g/L (2 time serial dilution), and the anti-agglutinating activity of F3(8), F3(12), F3(13) and F3 (14) against strain ECL 13998 was not determined, **(C)** All the chromatographic conditions were the same as that of fraction 3 except the running time was 20 min. Two fractions were collected, and fraction 7(1) gave the best anti-agglutinating activity against strain ECL 13998, **(D)** The tested concentrations were from 0.02 g/L to 4 g/L (2 time serial dilution).

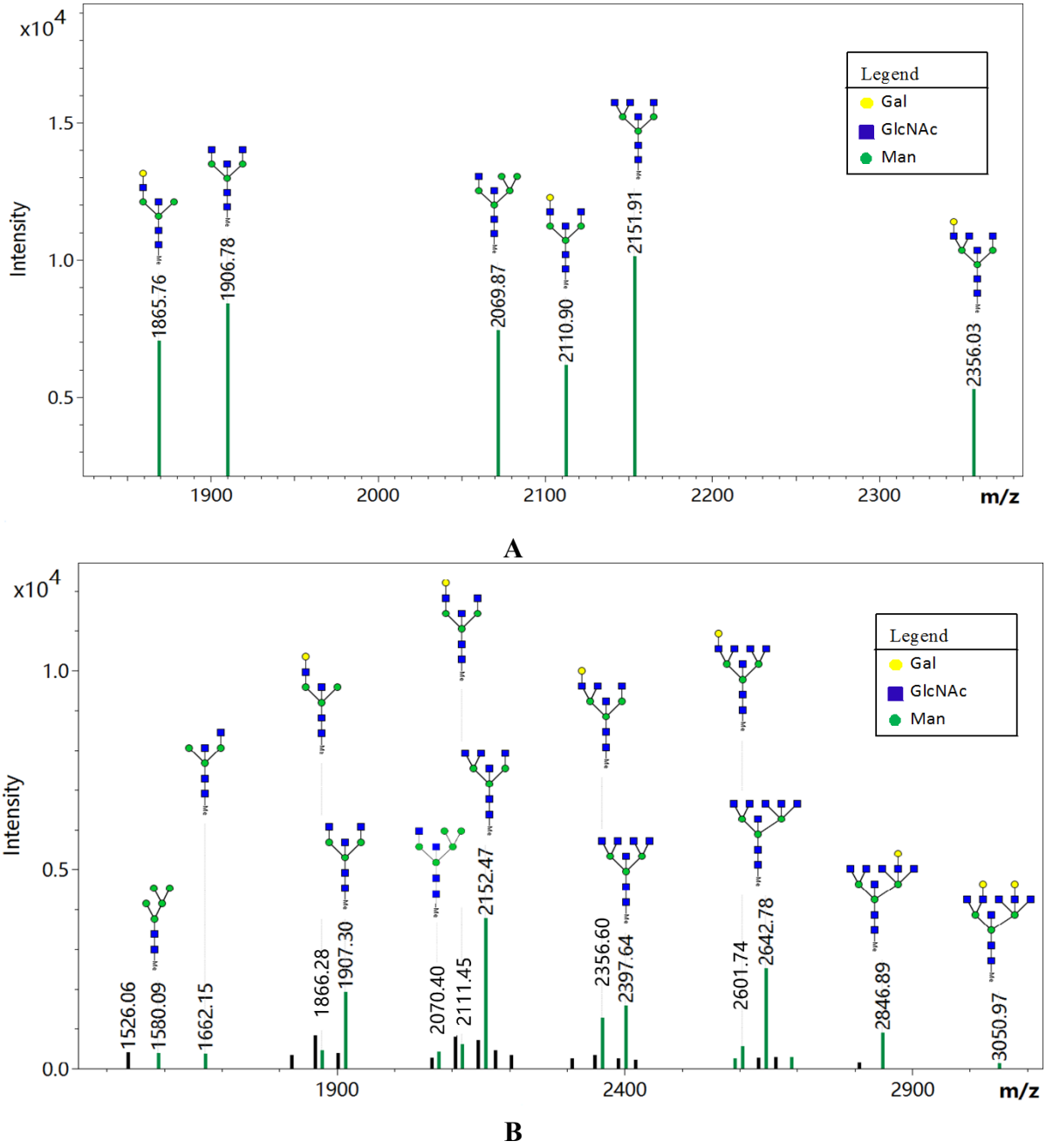


Figure 4.3: MALDI-TOF ion chromatogram of permethylated glycans released from (A) fraction 7(1) and (B) fraction 3(9) by PNGase F. The possible glycan structures were based on database GlycomeDB by GlycoQuest in ProteinScape Sever.

CHAPTER 5 – Glycopeptides from Egg White Ovomucin Inhibit $K88_{ac}$ Enterotoxigenic *Escherichia coli* Adhesion to Porcine Small Intestinal Epithelial Cell-line

5.1 Introduction

Enterotoxigenic *Escherichia coli* (ETEC) is a primary contributor to bacterial diarrhea in man and farm animals, causing substantial mortality and morbidity (Nagy & Fekete, 1999; Black, Merson, Rahman, Yunus, Alim, Huq, et al., 1980). ETEC adherence to the microvilli of small intestinal epithelial cells is the initial and prerequisite step for infection (Nagy & Fekete, 1999; Moonens, et al., 2015). Taking the pathogenesis of piglet diarrhea as an example, porcine ETEC firstly specifically recognizes the glycoconjugate receptors located on the intestinal epithelium (mainly by K88 fimbriae) and adheres to the microvilli of small intestine, subsequently produces heat-stable (ST) and/or heat-labile enterotoxins (LT) to disrupt intestinal fluid homeostasis and to cause hypersecretion of fluid and electrolytes through activation of adenylate cyclase (by LT) or guanylate cyclase (by ST) in small intestinal mucosal cells, and cause diarrhea (Croxen & Finlay, 2010; Kulkarni, Weiss, & Iyer, 2010; Nagy & Fekete, 2005). Therefore, inhibition or prevention of the initial adhesion of ETEC to host epithelial cells, often termed as anti-adhesive therapy, is emerging as an alternative approach to antibiotic regimens against bacterail infection (González-Ortiz, Bronsoms, Quarles Van Ufford, Halkes, Virkola, Liskamp, et al., 2014; González-Ortiz, Pérez, Hermes, Molist, Jiménez-Díaz, & Martín-Orúe, 2014; González-Ortiz, Hermes, Jiménez-Díaz, Pérez, & Martín-Orúe, 2013). Compared to

antibiotic regimens, anti-adhesive therapy is mild, gentle and less likely to develop resistant strains (Ofek & Sharon, 2002; Sharon & Ofek, 2000).

Since the early 1980s, there has been an increasing interest in identification of anti-adhesive agents that naturally exist in dietary sources, such as human milk, chicken egg yolk and plant polysaccharides (Inngjerdingen, Thöle, Diallo, Paulsen, & Hensel, 2014; Kassaify, Li, & Mine, 2005; Liu, Yu, Chen, Kling, & Newburg, 2012). Only a few compounds, such as human milk oligosaccharides (HMOs) and cranberry product, have been tested for their *in vivo* efficacies. HMOs have been demonstrated to exert anti-adhesive effects on *Campylobacter jejuni*, *E. coli*, *Vibrio cholera*, and *Salmonella fyris* (Coppa, et al., 2006; Hickey, 2012; Ruiz-Palacios, Cervantes, Ramos, Chavez-Munguia, & Newburg, 2003). However, the synthesis of HMOs as anti-adhesive agents is costly and impractical to be used widely. Proanthocyanidin from cranberry was identified as the major component to interfere with adherence of uropathogenic *E. coli* (UPEC) strains to epithelial cells, responsible for its prevention of recurrent urinary tract infections in women (Maki, Kaspar, Khoo, Derrig, Schild, & Gupta, 2016; Shmuely, Ofek, Weiss, Rones, & Houri-Haddad, 2012). Cranberry extract is the only anti-adhesive agent from dietary source, which has been approved to be marketed as urinary health promoters by French Agency for Food Safety (AFSSA) (Shmuely, Ofek, Weiss, Rones, & Houri-Haddad, 2012).

Ovomucin, a glycoprotein from egg white accounting for 2-4% of total egg albumen protein, shows structural similarity to mammalian mucin (Kato, Oda, Yamanaka, Matsudomi, & Kobayashi, 1985). On average, ovomucin contains 33% (w/w) of carbohydrate including mannose (Man), galactose (Gal), N-acetyl-D-galactosamine (GalNAc), N-acetyl-D-glucosamine (GlcNAc), sialic acid (N-acetylneuraminic acid) and

sulfated saccharides (Mine, 1995a; Robinson & Monsey, 1971). Ovomucin and its derived glycopeptides were reported to have antioxidant, anti-tumor and immunomodulating activities (Chang, Ha, Han, Seol, Kim, Jeong, et al., 2013; Tanizaki, Tanaka, Iwata, & Kato, 1997; Watanabe, Tsuge, Shimoyamada, Ogama, & Ebina, 1998). In particular, ovomucin or ovomucin glycopeptides could bind to enterohemorrhagic *E. coli* O157:H7, new castle disease virus and influenza virus (Kobayashi, et al., 2004; Gottschalk, & Lind, 1949; Tsuge, Shimoyamada, & Watanabe, 1997a), which indicated their potential to act as decoy receptors and subsequently prevent adhesion. Recently, we have determined the anti-adhesive potential of ovomucin hydrolysates against porcine $K88_{ac}$ ETEC by hemagglutination assay (Sun, Gänzle, & Wu, 2017). As a simple method, hemagglutination assay is suitable for preliminary test. However, hemagglutination assay cannot provide quantitative values for the extent of bacterial adhesion (Wang, Gänzle, & Schwab, 2010) and erythrocytes have distinct features and morphology from epithelial cells. For example, erythrocytes lack of mucus layer and microvilli, which are believed to be the targeted sites for bacterial adhesion (Nagy & Fekete, 2005; Vergauwen, 2015).

Therefore, there is a need to study the anti-adhesive activity of ovomucin hydrolysates against $K88_{ac}$ ETEC using porcine small intestinal epithelial cell line (IPEC-J2) (Koh, George, Brözel, Moxley, Francis, & Kaushik, 2007). IPEC-J2 cells were isolated from the jejunum of a neonatal unsuckled piglet and were used extensively as an *in vitro* model for investigating adhesion and pathogenesis of ETEC in last decade due to their similar morphology and functionality to the intestinal epithelial cells *in vivo* (Schierack, Nordhoff, Pollmann, Weyrauch, Amasheh, Lodemann, et al., 2006; Koh, George, Brözel, Moxley, Francis, & Kaushik, 2007; Vergauwen, 2015). The objectives of this study were to further

determine the anti-adhesive potential of ovomucin hydrolysates, to discuss the possible anti-adhesive mechanism by testing the effect of ovomucin hydrolysate on the $K88_{ac}$ fimbriae binding to IPEC-J2 cells, and to characterize the responsible anti-adhesive glycopeptides from ovomucin hydrolysate.

5.2 Materials and methods

5.2.1 Materials and chemicals

Fresh eggs from White Leghorn laid within 24 h were obtained from the Poultry Research Centre of the University of Alberta (Edmonton, Alberta, Canada). Sodium hydroxide, HPLC-grade water, acetonitrile, Dulbecco's modified eagle medium (DMEM)/Ham's F-12 (1:1), fetal bovine serum (FBS), penicillin-streptomycin, insulin-transferrin-selenium (ITS), epidermal growth factor (EGF), CO_2-independent medium, Luria-Bertani (LB) medium, Syto 9 green fluorescent nucleic acid stain, and tryptic soy broth were bought from Thermo Fisher Scientific Inc. (Ottawa, ON, Canada). Dithiothreitol (DTT), iodoacetamide (IAA), proteomics grade PNGase F from *Elizabethkingia meningosepticum*, citric acid, Triton X 100, HPLC grade dimethyl sulfoxide (DMSO), glycine, sodium periodate, sodium dodecyl sulfate (SDS), and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Milli-Q water was prepared by the Milli-Q water supply system (Millipore Corporation, Billerica, MA, USA).

5.2.2 Preparation of ovomucin hydrolysates

Ovomucin was extracted as we previously reported (Sun, Gänzle, Field, & Wu, 2016; Wang & Wu, 2012). Briefly, fresh egg white was mixed with 3 times of Milli-Q water and stirred for 120 min. Then ovomucin was precipitated at 4 °C by adjusting pH to 5.0,

centrifuged and lyophilized. Subsequently, the ovomucin extract was hydrolyzed by six kinds of enzymes; the enzyme names and origin were listed in Table 5.1. Enzymatic hydrolysis was performed according to the previous study (Sun, Gänzle, Field, & Wu, 2016). All enzymes were applied at their recommended pHs and temperatures, and the ratios of ovomucin/water (w/v) and enzyme/substrate (w/w) were 1% and 2%, respectively. The hydrolysis times of pepsin and pancreatin were 3 h, and those of other enzymes were all 4 h.

5.2.3 Bacterial strains and culture conditions

Two porcine $K88_{ac}$ ETEC strains, ECL13795 (O149; virotype STb:LT:EAST1:F4) and ECL13998 (O149; virotype STa:STb:LT:EAST1:F4:Paa), were kindly provided by the *Escherichia coli* Laboratory at the University of Montreal (Montreal, Quebec, Canada). Strains were cultured at 37 °C aerobically overnight (16 h) on Minca agar (Guinée, Veldkamp, & Jansen, 1977). Cultures were recovered with phosphate buffer (PB, 150 mM, pH 7.2). The bacteria concentration was about 10^9 cfu mL^{-1} for both strains and used in the inhibition assay.

5.2.4 Cell-culture growth

Porcine small intestinal epithelial cell line (IPEC-J2) was kindly donated by Professor Anthony Blikslager from the North Carolina State University (Raleigh, North Carolina, USA). Cells were grown in complete medium consisting of DMEM/Ham's F-12 (1:1), supplemented with 5% of FBS, 1% of penicillin-streptomycin, 1% of ITS and 5 ng/mL of EGF, which were maintained in an atmosphere of 5% CO_2 at 37 °C until confluence.

After cell confluence, the IPEC-J2 cells were seeded into 96-well cell culture plate (Greiner Cellstar, Sigma-Aldrich). Two days prior to the inhibition assay, standard culture

media containing antibiotics was replaced by an antibiotic-free and CO_2-independent medium (Hermes, Manzanilla, Martín-Orúe, Pérez, & Klasing, 2011; Paszti-Gere, Szeker, Csibrik-Nemeth, Csizinszky, Marosi, Palocz, et al., 2012).

5.2.5 Inhibition assay

The inhibition assay was modified from the method described by Hermes et al. (Hermes, Manzanilla, Martín-Orúe, Pérez, & Klasing, 2011). After IPEC-J2 cells getting confluence in CO_2-independent medium within 96-well cell culture plate, cells were washed with 200 μL of sterile 150 mM PB at pH 7.2 once, then 200 μL of the pre-incubated (at room temperature for 30 min) bacteria-ovomucin sample mixture (1:1, v/v) was applied to IPEC-J2 cells. After incubating at 37 °C for 30 min, cells were washed by 200 μL of sterile 150 mM PB at pH 7.2 for three times to exclude the non-adherent bacteria. The adherent bacteria were released from the cells by incubating in 200 μL of 0.1% Triton X100 for 30 min at room temperature. Finally, the released bacteria were diluted by LB broth and counted on LB agar. 10 g/L of casein glycomacropeptides (cGMP, Arla Foods Ingredients, Inc., Aarhus, Denmark) was used as the positive control (Hermes, Molist, Pérez, Ywazaki, Davin, Nofrarias, et al., 2013). Inhibition assay was performed at least two independent assays in triplicate, and the results were expressed as mean ± standard error of the mean (SEM). The inhibitory percentage of the bacteria adherence to cells was used to indicate the anti-adhesive activity:

$$\text{Inhibitory percentage} = \frac{\text{negative control} - \text{treated sample}}{\text{negative control}} \times 100$$

Another method to confirm the anti-adhesive activity was using Syto9 staining (130 nM, 5 min) adherent bacteria on the cells. Five random fields for each sample were examined with an Axio Imager M1m microscope (Carl Zeiss Inc., Oberkochen, German) at

a ×40 magnification and the corresponding images were captured by an AxioCam M1m camera (Carl Zeiss Inc., Oberkochen, German). The number of bacteria adherent to the cells was assessed by a randomized, blind evaluation from 5 persons. The scores ranged from 0 to 5: the more bacteria, the higher score.

5.2.6 Purification of $K88_{ac}$ fimbriae from ETEC strains

Porcine $K88_{ac}$ ETEC strains were cultured in tryptic soy broth for 48 h at 37 °C with 150 rpm shaking. The cultures were centrifuged at 5,311 g for 15 min at 4 °C, and the resulting pellets were washed with 150 mM PB (pH 7.2). The washed pellets were resuspended in the same PB (10 times the pellet volume), subjected to heating at 65 °C for 30 min to release the fimbriae from bacteria, and immediately homogenized by an Ultra Turrax (IKA, Staufen, Germany) at 24, 000 rpm for 30 min. The bacteria were removed by centrifugation (20,000 g, 4 °C, 30 min), and the supernatant containing $K88_{ac}$ fimbriae was firstly filtered through a 0.45 μm filter (PVDF, Mandel Scientific Company Inc.), then concentrated twofold using a SpeedVac (Savant Instruments, Inc., Farmingdale, NY, USA). Subsequently, the fimbriae in the supernatant were precipitated by adjusting to pH 4.0 by 2.5% (w/v) citric acid and incubated at 4 °C for 2 h, followed by centrifugation (20,000 g, 4 °C, 30 min) to collect fimbriae. The precipitated fimbriae were redissolved in 150 mM PB (pH 7.2), and this pH 4.0 precipitation was conducted two times. Finally, $K88_{ac}$ fimbriae were dissolved in 150 mM PB at pH 7.4 to keep a net negative charge (Erickson, Willgohs, McFarland, Benfield, & Francis, 1992; Fang, Gan, & Marquardt, 2000).

The $K88_{ac}$ fimbriae were firstly purified by ion exchange chromatography using a HiPrep 16/10 QFF anion exchange column (GE Healthcare, Uppsala, Sweden). The column was equilibrated with 3 column volumes (CVs) of buffer A (150 mM PB at pH

7.4). Then, 5 mL of $K88_{ac}$ fimbriae (5 g/L in buffer A) were loaded onto the column. The unbound fractions were eluted with 2 CVs buffer A and $K88_{ac}$ fimbriae were eluted with a 10 CVs linear gradient from 0 to 100% buffer B (150 mM PB, pH 7.4, containing 1 M NaCl) at 5 mL/min. The elution was monitored at 280 nm (Broeck, Cox, & Goddeeris, 1999). Subsequently, $K88_{ac}$ fimbriae fraction from IEC was further purified by gel filtration chromatography using a Hiload 16/600 Superdex 75 preparatory grade column (GE Healthcare, Uppsala, Sweden). $K88_{ac}$ fimbriae were eluted isocratically with 10 mM PB containing 150 mM NaCl (pH 7.4) at 1 mL/min. The elution was detected at 215 nm.

5.2.7 SDS-polyacrylamide gel electrophoresis (PAGE) and western blotting

SDS-PAGE was carried out by using 4-20% ready-to-use gels (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Protein bands in the gel were stained with CBB R-250 and the precision plus protein unstained standards (Bio-Rad Laboratories, Inc., Hercules, CA, USA) were used.

To further confirm the protein band of the major subunit (FaeG) of $K88_{ac}$ fimbriae, western blotting was performed to recognize FaeG using primary antibody against *E. coli* O149:K91, $K88_{ac}$ (Abbotstown) (APHA Scientific, Surrey, UK). Goat anti-rabbit IRDye 680RD from Licor Biosciences (Lincoln, NE, USA) was used as the secondary antibody. Protein bands were detected by Licor Odyssey BioImager (Licor Biosciences).

5.2.8 Dot-blot assay

Specific binding of purified $K88_{ac}$ fimbriae to ovomucin hydrolysate was tested by dot-blot assay at room temperature using Zoom Blot^{TM} strip plate with white wells (Vitrozm, San Francisco, CA, USA) based on the manufacturer's protocol and reported methods (González-Ortiz, et al., 2014). Ovomucin-protex 26L hydrolysate or BSA

(negative control) was immobilized on mixed cellulose esters membranes (30 ug/dot) for 30 min. After blocking with 50 μL of 10% (w/v) BSA in phosphate-buffered saline (PBS) with 0.05% Tween (BSA/PBST) for 30 min, purified $K88_{ac}$ fimbriae in 1% BSA/PBST (2 ug/dot) were applied to the wells and incubated for 1 h. Then the wells were rinsed with 100 μL of 1% BSA/PBST, and incubated with 4 μL of antibody against *E. coli* O149:K91, $K88_{ac}$ (Abbotstown) (dilute 1:400) for 5 min. After washing, the wells were incubated with 2 μL of alkaline phosphatase-conjugated anti-rabbit IgG (dilute 1:400) (Promega, Madison, WI, USA) for 5 min, completely rinsed, and the bound proteins were visualized within 10 min by Bromochloroindolyl phosphate/nitro blue tetrazolium (BCIP/NBT) substrate (Sigma, St. Louis, MO, USA).

To investigate the effects of protein and glycan moieties of the ovomucin hydrolysate on the specific binding to $K88_{ac}$ fimbriae, ovomucin-protex 26L hydrolysate was hydrolyzed by pronase (Roche Diagnostics GMBH, Mannheim, Germany) which could randomly cleave the protein backbone of ovomucin-protex 26L hydrolysate, for 4 h (pH 7.4, 50 °C, 5% (w/w) of enzyme/substrate) or treated by 25 mM of sodium periodate in 50 mM sodium acetate (pH 4.7) at room temperature for 16 h in darkness (Paulp, Thangam, Gunasekaran, & Kannan, 2011). These two treated samples were tested in dot-blot assay.

5.2.9 Affinity chromatography

Approximately 10 mg of purified $K88_{ac}$ fimbriae were coupled to 1 g of cyanogen bromide-activated Sepharose 4B (Sigma, St. Louis, MO, USA) according to Sigma's manual and the unreacted binding sites were blocked by 0.2 M glycine at pH 8.0. This column was equilibrated with 5 CVs of buffer A (5 mM PB containing 150 mM NaCl, pH 7.2). Then about 30 mg of ovomucin-protex 26L hydrolysate (10 g/L) were loaded onto the

column. After incubation at 37 °C for 1 h, the unbound samples were washed out by 10 CVs of buffer A. The affinity fractions were firstly eluted by 5 CVs of 5 mM PB containing 1 M NaCl, pH 7.2 (low affinity fraction), followed by 5 CVs of 0.1 M glycine-HCl, pH 2.5 (high affinity fraction). These two affinity fractions were collected, dialyzed (molecular weight cut-off of 3000 Da) against water and lyophilized (Jin, Marquardt, Baidoo, & Frohlich, 2000; Novakovic, et al., 2015).

5.2.10 Identification of peptide sequences and possible glycan structures by mass spectrometry (MS)

The high affinity fractionation was further subjected to MS analysis to identify peptide sequences and possible glycan structures. The procedures were similar to our previous study (Sun, Gänzle, & Wu, 2017). Briefly, after reducing with 50 mM of DTT and alkylating with 65 mM of IAA, the N-glycans of high affinity fraction were released by PNGase F solution (500 units/mL, 37 °C, overnight). The released N-glycans and de-N-glycosylated peptides were separated by passing through a 3 mL Sep-Pak C18 cartridge (Waters, Milford, MA, USA). N-glycans were permethylated by the sodium hydroxide/DMSO slurry method (Dell, et al., 1993). De-N-glycosylated peptides were analyzed by liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MSMS) using a Waters QToF Premier equipped with a nanoAcquity Ultra High Performance Liquid Chromatography system (Waters, Milford, MA, USA). Permethylated N-glycans were tested using a Bruker Daltonics ultrafleXtreme matrix-assisted laser desorption/ionization (MALDI) TOF/TOF (time of flight) MS (Bremen, Germany). Database search was performed by ProteinScape Server (Bruker Daltonik, Bremen, Germany) using an in-house Mascot server (Version 2.2, Matrix Science, London, UK).

The possible glycan compositions and structures were searched within database GlycomeDB by GlycoQuest using Glycan QTOF CID searching method (Version 1.1).

5.3 Results

5.3.1 Determination of the anti-adhesive activity of ovomucin hydrolysates against $K88_{ac}$ ETEC strains

Six ovomucin hydrolysates, determined by hemagglutination assay, were subjected to anti-adhesive assay using IPEC-J2 cells at a concentration of 10 g/L. As shown in Table 5.1, ovomucin-protex 26L and pepsin/pancreatin hydrolysates showed anti-adhesive activity against both ETEC strains, pronase and protease P hydrolysates exerted activity only against strain ECL13998, while acid protease II and protease N hydrolysates did not inhibit either ETEC strains.

We next determined the effects of different concentrations of ovomucin-protex 26L and pepsin/pancreatin hydrolysates on the anti-adhesive activity. As shown in Table 5.2, the minimum anti-adhesive concentrations of cGMP (positive control), ovomucin-pepsin/pancreatin hydrolysate and ovomucin-protex 26L hydrolysate were 10 g/L, 5 g/L and 2.5 g/L, respectively. Therefore, ovomucin-protex 26L and ovomucin-pepsin/pancreatin hydrolysates exerted better anti-adhesive activity against $K88_{ac}$ ETEC strains than cGMP. Ovomucin-protex 26L hydrolysate was applied in the following experiments due to its best anti-adhesive activity.

We further used Syto9 staining to image ETEC adherence to IPEC-J2 cells to confirm the anti-adhesive activity of ovomucin hydrolysate. Syto9 stains the nucleic acids of both ETEC and IPEC-J2 cells, whereas ETEC and IPEC-J2 cells could be differentiated according to their distinct morphologies (ETEC are small dots). Representative

fluorescence images were shown in Figure 5.1; adding ovomucin hydrolysate reduced adherence of ETEC to IPEC-J2 cells. The average scores of negative control, ovomucin-protex 26L hydrolysate (10 g/L) and cGMP (10 g/L) were 4, 1, and 2 points, respectively. The higher score, the more bacteria adherence to cells. Therefore, Syto9 staining method also suggested that ovomucin-protex 26L hydrolysate showed better anti-adhesive activity than that of cGMP.

5.3.2 Identification of major subunit FaeG of $K88_{ac}$ fimbriae

It is well accepted that K88 ETEC strains adherence to IPEC-J2 cells are mainly mediate by K88 fimbriae (Jin & Zhao, 2000), while FaeG, with a Mw of 25-26 kDa, is the major subunit of $K88_{ac}$ fimbriae responsible for the receptor-binding specificity (Broeck, Cox, & Goddeeris, 1999; Jacobs, Roosendaal, van Breemen, & De Graaf, 1987). To make sure FaeG subunit is the major component of the purified $K88_{ac}$ fimbriae from ETEC, purified $K88_{ac}$ fimbriae from ETEC were further characterized by SDS-PAGE and western blotting. The Mw of major protein band was in the range of 25-26 kDa (Figure 5.2A) and the protein band with the Mw of 25-26 kDa was further confirmed against FaeG antibody by western blotting (Figure 5.2B).

5.3.3 Ovomucin-protex 26L glycopeptides as decoy receptors of $K88_{ac}$ fimbriae

In dot-blot assay, the final blue dots represent that the tested compounds could be recognized by $K88_{ac}$ fimbriae and interact with $K88_{ac}$ fimbriae. In Figure 5.3, the dots of negative control were colorless, which suggested that BSA could not bind to $K88_{ac}$ fimbriae; accordingly, blue color was observed in the dots of ovomucin-protex 26L hydrolysate, confirmed an interaction between the ovomucin hydrolysate and $K88_{ac}$ fimbriae. Treating ovomucin-protex 26L hydrolysate with pronase to further breakdown

the protein backbones (Temporini, Perani, Calleri, Dolcini, Lubda, Caccialanza, et al., 2007), reduced the blue color intensity suggesting that peptide sequences of ovomucin hydrolysate play roles in the binding to $K88_{ac}$ fimbriae. This interaction suggested that ovomucin hydrolysate could bind to ETEC through $K88_{ac}$ fimbriae and function as a decoy receptor, therefore preventing ETEC adhesion to cells.

Treating the ovomucin-protex 26L hydrolysate with sodium periodate, to completely destroy its glycan moieties by oxidation of the vicinal hydroxyl groups into dialdehydes without altering peptide backbones (Petersen, Vieths, Aulepp, Schlaak, & Becker, 1996), led to complete loss of the blue color indicating that the carbohydrate moieties are indispensable for $K88_{ac}$ fimbriae interacting with ovomucin-protex 26L hydrolysate.

5.3.4 Characterization of the responsible $K88_{ac}$ fimbriae-binding glycopeptides from ovomucin-protex 26L hydrolysate

Ovomucin-protex 26L hydrolysate was subjected to purification on an affinity column containing $K88_{ac}$ fimbriae-coupled cyanogen bromide-activated Sepharose 4B. Two $K88_{ac}$ fimbriae-binding fractions isolated from ovomucin-protex 26L hydrolysate were analyzed by SDS-PAGE (Figure 5.4A). The high affinity fraction showed one protein band with an approximate Mw of 15 kDa and the low affinity fraction contained a ~250 kDa band and a minor 15 kDa band, which is the carry-over from the high affinity fraction. The high affinity fraction was subjected to further structural characterization.

The high affinity fraction was deglycosylated and then subjected to nano LC-ESI MS/MS analysis. As shown in Table 5.3, this fraction consisted of egg white lysozyme and α-ovomucin. Nine peptides were identified from α-ovomucin and four peptides were from egg white lysozyme. These identified peptides locate in a wide range of α-ovomucin (from

883 to 1716 AAs) and egg white lysozyme (from 5 to 102 AAs). Considering the Mw range of deglycosylated high affinity fraction (2.0 kDa to 4.5 kDa) as measured by MALTI-TOF (*chromatogram not shown*), four out of nine identified peptides from α-ovomucin were in this range, including VTPSFQGKVCGLCGDFDGRSRND (2.6 kDa), SQTEGTKCGNAFCGPNGMIIE (2.3 kDa), DETGCCEVFECQCICSGWGNEH (2.7 kDa), and ALDWKAPVSTNRYCNPGISEPVK (2.6 kDa). Only two identified peptides from lysozyme were in above Mw range: GLDNYRGYSLGNWVCAAKFE (2.3 kDa) and FESNFNTQATNRNTDGSTDYGILQINSR (3.2 kDa). In our previous study, VCGLCGDFDGR and SQTE sequences have been identified from α-ovomucin (Sun, Gänzle, & Wu, 2017), which belong to the present peptide sequences of VTPSFQGKVCGLCGDFDGRSRND and SQTEGTKCGNAFCGPNGMIIE), respectively.

The glycans prepared from the high affinity fraction were permethylated and then analyzed by MALDI-TOF MS as presented in Figure 5.4B. Twelve permethylated glycans were determined at m/z of 1579.67, 1661.73, 1865.82, 1906.85, 2069.93, 2110.96, 2151.98, 2356.10, 2397.12, 2601.23, 2642.26, and 3050.52. All of the above identified glycans were in agreement with the reported N-glycans in α-ovomucin (Offengenden, Fentabil, & Wu, 2011; Sun, Gänzle, & Wu, 2017). The possible glycan structures were searched within database GlycomeDB by GlycoQuest using Glycan QTOF CID searching method (Offengenden, Fentabil, & Wu, 2011).

5.3.5 Validation of the anti-adhesive activity of the high affinity fraction

The anti-adhesive activity of the high affinity fraction against both ETEC strains was tested. The results showed that the inhibitory percentages (mean ± SEM) against strain

ECL 13795 of cGMP (10 g/L) and high affinity fraction (about 1 g/L) were 50±11 and 22±6, respectively. However, the high affinity fraction did not show anti-adhesive activity against strain ECL 13998 at the concentration of 1 g/L.

5.4 Discussion

Cell culture method is thought to be more biological relevant for testing anti-adhesive activity of compounds *in vitro* compared to the hemagglutination assay. The anti-adhesive activities against $K88_{ac}$ ETEC strains of six ovomucin hydrolysates tested in this study using IPEC-J2 cells were not completely in alignment with their anti-agglutinating results (Sun, Gänzle, & Wu, 2017). Particularly, ovomucin-acid protease II hydrolysate exerted the most potent anti-agglutinating activity, while it did not show anti-adhesive activity at the concentration of 10 g/L in the cell study. This discrepancy is due to different characteristics between erythrocytes and epithelial cells as mentioned in the introduction. Additionally, it was reported that K88 ETEC can only bind to the erythrocyte receptors at 4 °C and it was observed that lower number of bacteria adhere to erythrocytes than to epithelial cells (Gaastra & de Graaf, 1982; Gibbons, Jones, & Sellwood, 1975).

The anti-adhesive activity of ovomucin hydrolysates compared favorably to previously reported anti-adhesive agents, which indicated their potential to act as anti-adhesive agents. The minimum anti-adhesive concentration of ovomucin-protex 26L hydrolysate was 2.5 g/L, in comparison, natural extracts from locust bean, exopolysaccharide and wheat bran ranged from 2 to 10 g/L (González-Ortiz, Pérez, Hermes, Molist, Jiménez-Díaz, & Martín-Orúe, 2014; González-Ortiz, Hermes, Jiménez-Díaz, Pérez, & Martín-Orúe, 2013), human milk

oligosaccharides ranged from 3 to 10 g/L (Coppa, et al., 2006; Ruiz-Palacios, Cervantes, Ramos, Chavez-Munguia, & Newburg, 2003).

K88 fimbriae are believed to mainly mediate the adhesion of K88 ETEC strains to IPEC-J2 cells (Jin & Zhao, 2000). In an attempt to understand the possible anti-adhesive mechanism of ovomucin hydrolysates, $K88_{ac}$ fimbriae were purified, then dot-blot assay was conducted. Dot-blot assay suggested that ovomucin hydrolysates function as decoy receptors for $K88_{ac}$ fimbriae, competitively binds to $K88_{ac}$ ETEC with receptors on IPEC-J2 cells and subsequently prevent bacterial adhesion. The glycan moiety of ovomucin hydrolysate is indispensable for its binding to $K88_{ac}$ fimbriae, which is consistent with the recognized carbohydrate-binding specificity of $K88_{ac}$ fimbriae (Grange, Erickson, Anderson, & Francis, 1998; Grange, Mouricout, Levery, Francis, & Erickson, 2002). Moreover, the protein backbone of ovomucin-protex 26 L hydrolysate contributes to the binding as well because further cleaving the peptide into smaller fragments reduced $K88_{ac}$ fimbriae binding to ovomucin hydrolysate. This finding could be supported by the identified $K88_{ac}$ fimbriae-binding proteins (Novakovic, et al., 2015).

The responsible ovomucin glycopeptides binding to $K88_{ac}$ fimbriae were purified by affinity chromatography. The MS results presented that the fraction with high affinity to $K88_{ac}$ fimbriae is comprised of both hen egg white lysozyme and α-ovomucin. However, hen egg white lysozyme did not bind to $K88_{ac}$ fimbriae in the dot-blot assay and did not inhibit the adhesion of both ETEC strains to IPEC-J2 cells either (*data not shown*), hence, α-ovomucin derived glycopeptides are believed to be responsible for binding to $K88_{ac}$ fimbriae. Based on the reported galactosyl-binding specificity of $K88_{ac}$ fimbriae, terminal β-linked galactose of the identified glycans from α-ovomucin could be one of the binding sites

for $K88_{ac}$ fimbriae (Grange, Erickson, Anderson, & Francis, 1998; Payne, O'Reilly, & Williamson, 1993; Sellwood, 1980).

Our dot-blot assay suggested that ovomucin-protex 26L hydrolysate (3 g/L) act as decoy receptors for competitive binding to ETEC through $K88_{ac}$ fimbriae, therefore preventing ETEC adhesion to IPEC-J2 cells. However, the high affinity at a concentration of 1 g/L exerted anti-adhesive activity against strain ECL 13795 (O149; virotype STb:LT:EAST1:F4) but not strain ECL 13998 (O149; virotype STa:STb:LT:EAST1:F4:Paa). Another explanation is that $K88_{ac}$ fimbria is not the only factor for mediating strain ECL 13998 adhesion to cells; for example, porcine attaching and effacing-associated factor (Paa), a non-fimbrial adhesin, may contribute to K88 ETEC adherence to cells (Batisson, et al., 2003; Leclerc, et al., 2007). Paa has been implicated in porcine ETEC-associated diarrhea, however, the specific role of paa in ETEC pathogenesis is unknown (Leclerc, et al., 2007), and our results provided a clue for future study on this area.

In conclusion, the present study confirmed that ovomucin hydrolysates, in particular prepared by protex 26L, could effectively inhibit porcine $K88_{ac}$ ETEC adhesion to IPEC-J2 cells, indicating their potential as anti-adhesive agents against infectious diseases. Glycopeptides in ovomucin hydrolysate could competitively bind to ETEC through $K88_{ac}$ fimbriae and subsequently interfere bacterial adhesion to cells. The peptide sequences of α-ovomucin glycopeptides play a role in the binding to $K88_{ac}$ fimbriae while the glycan moieties are indispensable for this binding. Terminal β-linked galactose of the identified glycans from α-ovomucin could be one of the binding sites for $K88_{ac}$ fimbriae. However, ovomucin hydrolysate binding to $K88_{ac}$ fimbriae did not play a crucial role in the

anti-adhesive activity against strain ECL 13998 probably because both $K88_{ac}$ fimbriae and paa are responsible for bacterial adhesion to cells. The anti-adhesive efficacy of ovomucin hydrolysates needs to be determined in animal models in future.

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Table 5.1 Anti-adhesive activity of ovomucin hydrolysates (10 g/L) against ETEC strains

Ovomucin hydrolysates	Enzyme origin	Inhibitory percentage (%) against strain ECL13795[a)]	Inhibitory percentage (%) against strain ECL13998
Acid protease II	*Rhizopus niveus*	No activity	No activity
Protease P	*Aspergillus melleus*	No activity	50±7
Protex 26L	*Aspergillus niger*	67±8	67±4
Pepsin/pancreatin	Porcine gastric/Porcine pancreas	57±4	77±4
Pronase	*Streptomyces griseus*	No activity	61±4
Protease N	*Bacillus subtilis*	No activity	No activity

[a)] Data were expressed as Mean ± SEM

Table 5.2 Effect of different concentrations on anti-adhesive activity

	Inhibitory percentage (%) against strain ECL13795[a)]	Inhibitory percentage (%) against strain ECL13998
Ovomucin-pepsin/pancreatin hydrolysate		
10 g/L	64±11	76±12
5 g/L	59±12	67±7
2.5 g/L	No activity	No activity
1.25 g/L	No activity	No activity
Ovomucin-protex 26L hydrolysate		
10 g/L	68±5	68±6
5 g/L	38±6	54±9
2.5 g/L	33±9	47±6
1.25 g/L	No activity	No activity
Casein glycomacropeptides (cGMP, as positive control)		
10 g/L	56±7	60±6
5 g/L	No activity	No activity

[a)] Data were expressed as mean ± SEM

Table 5.3 Identified peptides from deglycosylated high affinity fraction by performing nano LC-ESI MS/MS analysis [a)]

Peptide location in protein	Calculated mass	m/z measured	Peptide sequence [b)]
α-ovomucin			
883-891	1114.5	1115.5	FLGDCDYIL
985-1007	2571.2	643.8	VTPSFQGKVCGLCGDFDGRSRND*
1017-1029	1557.7	779.8	MSIQEFGNSWKIT
1224-1244	2270.0	1136.0	SQTEGTKCGNAFCGPNGMIIE*
1345-1351	700.4	701.5	NATSLVP
1376-1392	1966.9	984.4	FGNCQIATCLGEENNIK
1417-1438	2733.0	912.0	DETGCCEVFECQCICSGWGNEH*
1599-1621	2601.3	651.3	ALDWKAPVSTNRYCNPGISEPVK*
1706-1716	1264.6	633.4	VYKPCGEAKRN
Hen egg white lysozyme			
5-16	1398.7	467.3	GRCELAAAMKRH
17-36	2319.1	774.0	GLDNYRGYSLGNWVCAAKFE*
35-62	3162.5	1055.1	FESNFNTQATNRNTDGSTDYGILQINSR*
85-102	1907.0	636.7	LSSDITASVNCAKKIVSD

[a)] Only peptides with scores higher than 10 were included.
[b)] The possible N-glycosylation site (N) and O-glycosylation sites (S and T) are underlined.
* In the molecular weight range (2.0 to 4.5 kDa) of deglycosylated high affinity fraction.

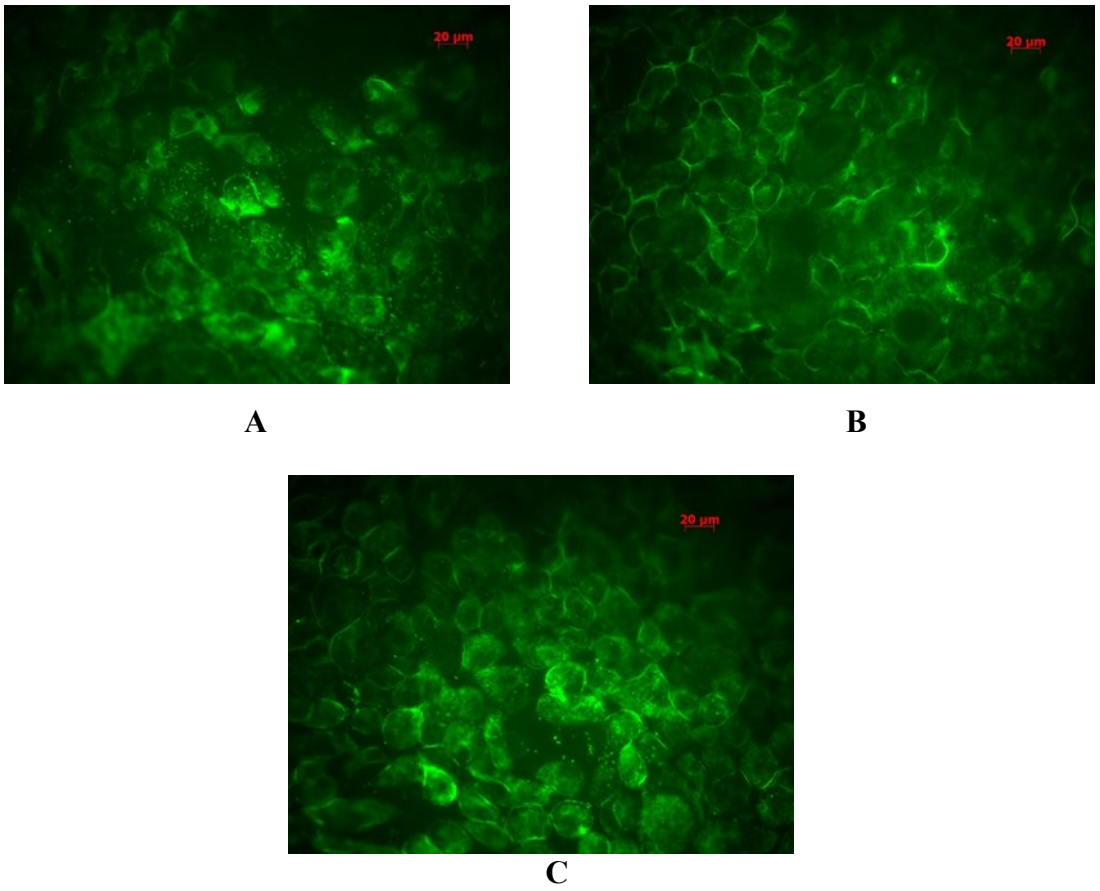

Figure 5.1: Representative fluorescence microscopy images (×40 magnification) of Syto9 staining adherent bacteria on IPEC-J2 cells. **(A)** untreated negative control, (**B)** treated by 10 g/L of ovomucin-protex 26L hydrolysate, (**C)** treated by 10 g/L of cGMP (positive control). All the images were captured under the same photographic conditions.

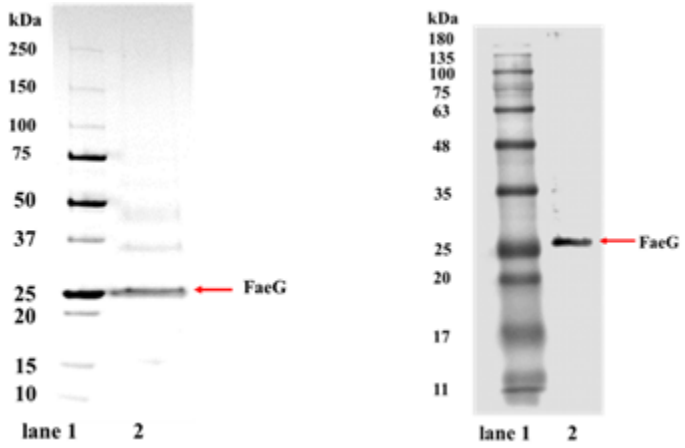

Figure 5.2: (A) SDS-PAGE analysis of purified K88$_{ac}$ fimbriae and (B) identification of FaeG by western blotting. (A) 4-20% ready-to-use gel were used and proteins were stained with CBB R-250, **(B)** Identification of FaeG by western blotting. Primary antibody against *E. coli* O149:K91, K88$_{ac}$ (Abbotstown) and second antibody goat anti-rabbit IRDye 680RD were used. Protein bands were detected by Licor Odyssey BioImager. Lane 1 was molecular weight marker and lane 2 was purified K88$_{ac}$ fimbriae in both Figure 5.2 A-B.

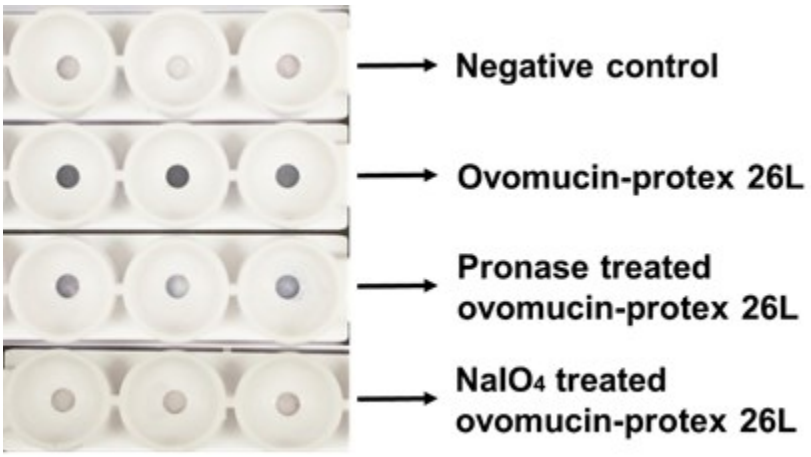

Figure 5.3: Dot-blot assay to determine the bindings between the tested compounds and FaeG. Dot-blot assay was conducted to test: (1) The interactions between purified $K88_{ac}$ fimbriae and ovomucin-protex 26L hydrolysate; (2) The effect of peptide sequences and glycan moieties of ovomucin-protex 26L hydrolysate on these interactions. Briefly, the tested samples were firstly immobilized on membranes. After blocking, purified $K88_{ac}$ fimbriae, primary antibody against *E. coli* O149:K91, $K88_{ac}$ (Abbotstown), and secondary antibody alkaline phosphatase-conjugated anti-rabbit IgG were applied to the wells in sequence. Finally, if tested samples can bind to $K88_{ac}$ fimbriae, the dot would form blue color by BCIP/NBT substrate.

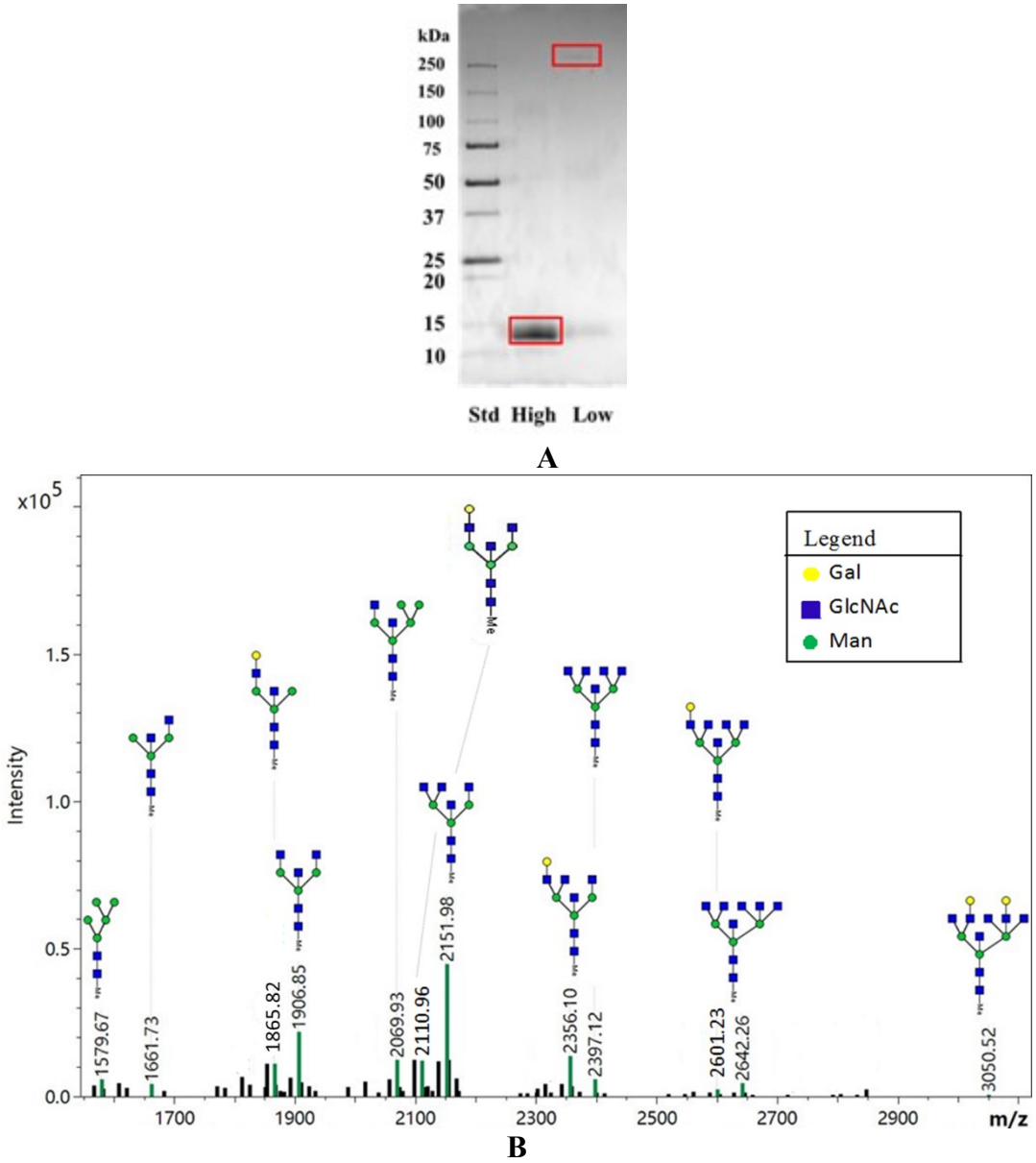

Figure 5.4: (A) SDS-PAGE analysis of high and low affinity fractions from ovomucin-protex 26L hydrolysate and (B) MALDI-TOF ion chromatogram of permethylated glycans released from the high affinity fraction by PNGase F. (A) These two fractions were purified by affinity chromarography using $K88_{ac}$ fimbriae. Abbrieviation used: Std (molecular weight marker), High (high affinity fraction), Low (low affinity fraction), **(B)** Proposed glycan structures based on literatures (Offengenden, Fentabil, & Wu, 2011).

CHAPTER 6 – Effect of pHs and Ionic Strengths on the Anti-fouling Property of Egg White Protein Ovomucin

6.1 Introduction

Undesired accumulation of proteins on surfaces is a serious issue affecting numerous applications, such as biosensors, biomedical implants, and food processing industry (Banerjee, Pangule, & Kane, 2011; Wong, et al., 2012). Specifically, protein adsorption on surface reduces the sensitivity of biosensor, decreases the efficacy of biomedical implants and may also result in undesirable host responses, such as blood coagulation, thrombus formation, platelet activation (Amiji, 1993; Banerjee, Pangule, & Kane, 2011; Wong, et al., 2012). Protein adsorption also plays a vital role in the bacterial colonization and subsequent biofilm formation since the layer of protein could serve as a conditioning film (Banerjee, Pangule, & Kane, 2011). The device-related infections due to pathogenic bacteria adherence and biofilm formation on medical implants (such as catheters or prosthetic joints) has resulted in a high incidence of revision surgeries, and sometimes fatality (Beloin, Renard, Ghigo, & Lebeaux, 2014; Veerachamy, Yarlagadda, Manivasagam, & Yarlagadda, 2014). Therefore, from both medical and economic point of view, development of anti-fouling surface to inhibit protein adsorption and subsequently prevent bacterial colonization is imperative. The typical approach to prevent protein adsorption is to coat the surface with an anti-fouling material that resists the non-specific interactions (Benhabbour, Liu, Sheardown, & Adronov, 2008). Many anti-fouling surfaces have been explored, such as amphiphilic polymer-based surfaces, zwitterionic polymer-based surfaces, and bactericidal polymer-based surfaces (Ye & Zhou, 2015).

Poly(ethylene glycol) (PEG) based materials are the most widely used for inhibition of protein adsorption due to their non-toxicity, non-immunogenesis, non-antigenicity and good biocompatibility (Krishnan, Weinman, & Ober, 2008; Ye & Zhou, 2015; Yu, Zhang, Wang, Brash, & Chen, 2011). However, PEG is susceptible to autoxidation in the presence of oxygen and forms aldehyde groups that may react with proteins, which makes PEG lose anti-fouling property (Banerjee, Pangule, & Kane, 2011; Herold, Keil, & Bruns, 1989). Thus, it is necessary to identify alternative substances with anti-fouling property.

The mucous layer covering epithelial cells functions as a natural anti-fouling surface to prevent undesirable adhesion to host tissues (Cone, 2009). This layer is mainly composed of water and mucins, a member of heavily glycosylated and gel-forming high molecular-weight glycoproteins (Bansil & Turner, 2006). Therefore, much attention has been directed towards exploring the potential of mucin as anti-fouling coatings, especially bovine submaxillary mucin (BSM). BSM coatings could suppress cells and bacteria adhesion to biomaterials, which indicated its potential to serve as an anti-fouling surface in biomedical applications (Bushnak, Labeed, Sear, & Keddie, 2010; Crouzier, Jang, Ahn, Stocker, & Ribbeck, 2013; Shi, Ardehali, Caldwell, & Valint, 2000).

Egg is an easily available and relatively cheap commodity; egg white contains approximately 3.5% of ovomucin, a mucin-type glycoprotein in egg white (Nakamura & Sato, 1964; Omana, Wang, & Wu, 2010). Ovomucin has similar structures as mammalian mucins: it has a long linear chain of polymerized macromolecules with a randomly coiled structure and subunits are linked by disulfide bonds, with a molecular weights of 23,000 kDa (Gallagher & Corfield, 1978; Tominatsu & Donovan, 1972). Ovomucin consists of a carbohydrate-poor (α-ovomucin) and a carbohydrate-rich (β-ovomucin) subunit, containing

11-15% and 50-57% (w/w) of carbohydrate, respectively (Itoh, Miyazaki, Sugawara, & Adachi, 1987; Robinson & Monsey, 1971). On an average, ovomucin contains 33% (w/w) of carbohydrate, including mannose (Man), galactose (Gal), N-acetyl-D-galactosamine (GalNAc), N-acetyl-D-glucosamine (GlcNAc), N-acetylneuraminic acid (Neu5Ac) and sulfated saccharides (Mine, 1995a; Robinson & Monsey, 1971). Its structural similarity to mammalian mucins led us to hypothesize that ovomucin may have anti-fouling property.

The objectives of this study were to test the adsorption of bovine serum albumin (BSA) on the ovomucin-coated polystyrene surface *in vitro*, to directly measure the surface force between ovomucin and model proteins (BSA and ovomucin) at different pHs and ionic strengths using a surface force apparatus (SFA) (Zeng, Tian, Anderson, Tirrell, & Israelachvili, 2007), and to further reveal the anti-fouling mechanism behind by characterizing surface morphology using atomic force microscopy (AFM), and determining the zeta potential and average hydrodynamic size of proteins.

6.2 Materials and Methods

6.2.1 Materials and chemicals

Fresh eggs from White Leghorn laid within 24h from the Poultry Research Centre of the University of Alberta (Edmonton, AB, CA) were used for extraction of ovomucin. Coomassie Brilliant Blue (CBB) R-250 was purchased from Bio-Rad (Bio-Rad Laboratories, Inc., Hercules, CA). 3-aminopropyltriethoxysilane (APTES) (98%) was obtained from Alfa Aesar (Ward Hill, MA, USA). Sodium chloride, bovine serum albumin (BSA), sodium chloride, sodium phosphate monobasic, sodium phosphate dibasic, sodium dodecyl sulfate (SDS), albumin-fluorescein isothiocyanate (FITC) conjugate and β-mercaptoethanol were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Hydrochloric acid was bought from Fisher Scientific Inc. (Fisher Scientific, Ottawa, ON, CA). Standard proteins (ovalbumin, ovomucoid, and lysozyme) were provided by Neova Technologies Inc. (Abbotsford, BC, CA). Mica sheets (ruby mica blocks, grade 1) were purchased from S&J Trading Inc. (Floral park, NY, USA). Milli-Q water was prepared by the Milli-Q water supply system (Millipore Corporation, Billerica, MA, USA), which was filtered through 0.2 μm PTFE filters (Mandel Scientific Company Inc., Guelph, ON, CA) prior to use.

6.2.2 Extraction of ovomucin from egg white

Ovomucin was extracted by the method established by our lab (Omana & Wu, 2009). Briefly, crude ovomucin was firstly precipitated by 100 mM of NaCl solution at pH 6.0. This slurry was kept in a cold room (4 °C) overnight and then centrifuged at 15,300 g for 10 min at 4 °C (Beckman Coulter, Inc., Fullerton, CA, USA). The precipitate was collected and re-suspended in 500 mM of NaCl solution (pH 6.0). After stirring for 4 h, the suspension was settled at 4 °C overnight. Finally, the precipitate was separated by centrifugation at 15,300 g for 10 min at 4 °C (Beckman Coulter, Inc., Fullerton, CA, USA), dialyzed for 24 h, freeze-dried and stored at -20 °C until use.

6.2.3 Determination of the purity of ovomucin by gel filtration chromatography

The purity of the extracted ovomucin was measured by a High-load 16/60 gel filtration column (Superdex 200 preparatory grade) coupled with Fast Performance Liquid Chromatography (FPLC) (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) as reported (Omana & Wu, 2009). Ovomucins was dissolved at a concentration of 5 g/L in 100 mM of sodium phosphate buffer at pH 7.0 containing 50 g/L of SDS and 10 mL/L of β-mercaptoethanol. The injection volume was 3 mL, and ovomucin was eluted with 100

mM phosphate buffer (pH 7.0) containing 5 g/L of SDS and 1 mL/L of β-mercaptoethanol at a flow rate of 1 mL/min, which was monitored by a UV detector at 280 nm. Since there is no commercial ovomucin standard, the purity of ovomucin was calculated by subtracting the amount of other proteins (ovalbumin, ovomucoid, and lysozyme).

6.2.4 Determination of the anti-fouling property of ovomucin *in vitro*

The adsorption of BSA on an ovomucin-coated surface of the 96-well polystyrene microplate was tested to validate the anti-fouling property of ovomucin. Firstly, 200 μL of ovomucin (about 100 μg/mL) in 5 mM phosphate buffer with 150 mM NaCl at pH 7.2 was coated on the polystyrene surface for 24 h at room temperature. After washing with phosphate buffer, 200 μL of BSA-FITC (fluorescein isothiocyanate) conjugate at the concentration of 200 μg/mL was applied and incubated for 24 h at 37 °C. The polystyrene surface was rinsed lightly with 200 μL of phosphate buffer twice, then the fluorescence intensity was applied to indicate the amounts of BSA adsorbed on the surface, which was determined by SpectraMax M3 (Molecular devices, Sunnyvale, CA, USA) with an excitation at 495 nm and an emission at 520 nm. The adsorption of BSA on the non-coated polystyrene surface was measured as control (Haraguchi, Kubota, Takada, & Mahara, 2014).

6.2.5 Surface force measurement

6.2.5.1 Ovomucin solution preparation

Ovomucin was firstly suspended at the concentration of 5 mg/mL in 5 mM phosphate buffer with 150 mM NaCl at pH 7.2 and magnetically stirred overnight at 1,200 rpm. Then the suspension was centrifuged at 5,331 g for 15 min twice and the final ovomucin concentration in supernatant was diluted to 88 μg/mL, which was determined at 280 nm by

a Nanodrop 1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA).

6.2.5.2 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed by using 4-20% ready-to-use gels (Bio-Rad Laboratories, Inc., Hercules, CA) to test the compostions of the above ovomucion solution, and the loaded amount of sample was 10 μg per well. Protein bands in the gel were stained with CBB R-250 for visualization.

6.2.5.3 Surface preparation for surface force measurement

Surface force measurements were mainly conducted based on three kind of surfaces: 3-aminopropyltriethoxysilane (APTES)-coated mica, ovomucin-coated APTES-mica, and BSA-coated APTES-mica. Mica surface was freshly cleaved in the laminar flow hood (Thermo Electron Corporation, Waltham, MA, USA). APTES is an amino silane that is routinely employed for charge reversal or to create coupling layers on oxide surfaces (Pick, Argento, Drazer, & Frechette, 2015), in our case, the APTES coating was applied to enhance the adsorption amount of ovomucin and BSA proteins on mica surface. The APTES modified mica surface was prepared based on vapour deposition method which has been reported previously (Liu, Li, Zhou, Wei, Song, & Wang, 2005; Lu, Wang, Faghihnejad, Zeng, & Liu, 2011). Briefly, mica surface was treated by water plasma (Harrick Plasma, 40W) and 50 μL of APTES droplets was placed together in a desiccator. Then the desiccator was kept under vacuum (100 mTorr) for 12 hours to allow surface deposition and APTES-mica reaction. Later, those APTES treated mica surfaces were kept under vacuum for another 24 h without APTES source for stabilization. To prepare

ovomucin-coated APTES-mica and BSA-coated APTES-mica substrates, 500 μL of 88 μg/mL of ovomucin or 200 μg/mL of BSA solution was dipped onto the APTES-mica surface, incubated for 20 min at 23 °C, washed with Milli-Q water.

6.2.5.4 Surface force apparatus (SFA) technique

SFA (Surforce LLC, Santa Barbara, VA, USA) was used to measure the intermolecular interaction between ovomucin coated APTES-mica surface with different substrates (APTES coated-mica, BSA-coated APTES-mica and ovomucin-coated APTES-mica). The SFA technique allows accurate determination of forces between two surfaces as a function of separation distance with force sensitivity down to 1 nN and the distance resolution down to 0.1nm. Two back silvered mica surface with the thickness around 5 μm were first glued onto cylindrical silica disks with the curvature radius around 2 cm. After coating with desired materials, the two disks were further mounted in the SFA chamber in a crossed-cylinder configuration. Then buffer solutions with different pHs (2.0, 6.0, and 7.2) and ionic strengths (0.1 mM, 10 mM, and 150 mM) were injected between the two surfaces, respectively. The pHs and ionic strengths were selected based on the physiological environment. The interaction force F between the curved surfaces at different pHs and ionic strengths was measured as a function of absolute surface separation distance D, which was tested in real-time by multiple beam interferometry (fringes of equal chromatic order (FECO)). All the force measurements were conducted at 23 °C (Lu, Wang, Faghihnejad, Zeng, & Liu, 2011).

6.2.6 Atomic force microscopy (AFM) imaging

AFM (MFP-3D Asylum Research, Santa Barbara, CA, USA) was employed to image the surface morphology of three different substrates, including ovomucin-coated

APTES-mica, BSA-coated APTES-mica, and BSA-ovomucin-coated APTES-mica substrates. Preparation of ovomucin-coated APTES-mica and BSA-coated APTES-mica substrates was the same as that introduced in section 6.2.5.3. BSA-ovomucin-coated APTES-mica substrate was prepared by coating BSA on ovomucin-coated APTES-mica surface. The substrates were imaged in tapping mode in air with an AFM tip (Bruker Nano, N-type silicon, tip radius < 20 nm, with a nominal resonance frequency of 300−400 kHz and spring constant of ~40 N/m) (Lu, Danner, Waite, Israelachvili, Zeng, & Hwang, 2013).

6.2.7 Particle size and zeta potential measurements

The zeta potential of ovomucin and BSA at different pHs and ionic strengths was determined at 23 °C by a Zetasizer NanoZS (model ZEN1600, Malvern Instruments Ltd., Malvern, UK). The average hydrodynamic size was measured by dynamic light scattering (DLS) using the same Zetasizer Nano ZS. The concentration of BSA was 200 μg/mL, which was same as that in SFA measurements. To prepare ovomucin samples, the method was introduced in section 6.2.5.1, and the only difference in this assay was using buffers with different pHs and ionic strengths to solubilize ovomucin.

6.2.8 Statistical analysis

All experiments were conducted in triplicate and the results of particle size, zeta potential and fluorescence intensity of adsorbed BSA were expressed as mean ± standard deviation (SD). All statistical analysis was carried out using IBM SPSS statistics 19 (SPSS Inc, USA), and significant differences were defined at a 5% level ($P<0.05$).

6.3 Results

6.3.1 Ovomucin purity

The gel filtration chromatogram of extracted ovomucin was shown in Figure 6.1A, where proteins were separated based on the size of molecules: larger proteins eluted sooner from the column. Since molecular weights of ovomucin subunits were large (150, 220, and 400 kDa corresponding to α_1, α_2, and β ovomucin, respectively) (Itoh, Miyazaki, Sugawara, & Adachi, 1987), they eluted earlier (53 mL) than ovalbumin (45 kDa, at 64 mL), ovomucoid (28 kDa, at 70 mL) and lysozyme (14.4 kDa, at 80 mL), respectively (Omana & Wu, 2009). The purity of ovomucin was 89% with 9% of ovalbumin and 2% of lysozyme as the contaminants, which was comparable to the published data (Omana & Wu, 2009).

6.3.2 Determination of the compositions of ovomucin solution by SDS-PAGE

Since only small amount of ovomucin can be solubilized in 5 mM phosphate buffer with 150 mM NaCl at pH 7.2, and as mentioned above, ovalbumin and lysozyme were contaminated proteins. In order to confirm the presence of ovomucin in the prepared solution in section 6.2.5.1, SDS-PAGE was employed to determine the compositions of the prepared ovomucin solution. In Figure 6.1B, we can clearly see the bands of α-ovomucin (250 kDa) and β-ovomucin (400-720 kDa), which demonstrated that ovomucin is the major component in the solution used through this study (Hiidenhovi, Ovomucin, Lopez-Fandino, Anton, & Schade, 2007). There was a faint lysozyme band at 14.4 kDa suggesting lysozyme was presence in the ovomucin solution as a minor component.

6.3.3 Validation of the anti-fouling property of ovomucin *in vitro*

The anti-fouling activity of ovomucin was validated *in vitro* by using BSA as a model protein. The concentrations of ovomucin and BSA in this assay were both the same as those in the following SFA measurements and the used phosphate buffer (5 mM phosphate buffer with 150 mM NaCl at pH 7.2) was applied to mimic the physiological environment. The fluorescence intensities of the adsorbed BSA on ovomucin-coated and non-coated polystyrene surface were 0.67±0.75 and 10.90±2.18, respectively. These results showed that ovomucin-coated polystyrene surface could significantly decrease BSA adsorption and even completely resisted BSA adsorption in several replications.

6.3.4 Surface forces measurements

In order to better understand the interaction mechanism for the protein-inhibiting behavior of ovomucin-coated surfaces, the surface forces measurements were performed between ovomucin-coated APTES-mica surface and opposing protein-coated APTES-mica surfaces with different pHs and ionic strengths, including BSA-coated APTES-mica and ovomucin-coated APTES-mica. The concentration of ovomucin used in present study was 88 μg/mL, which was in the range with published studies on BSM (Perez, & Proust 1986; Zhu, DeGraaf, Winnik, & Leckband, 2004). Generally, during a typical approach-separation force run, the two surfaces were first moved toward each other until a "hardwall" distance, i.e., the thickness of confined protein film between two opposing mica surfaces that does not change with an increasing compressive load, is achieved. Then the two surfaces were kept in contact for 1 min and then separated (Lu, Hwang, Liu, & Zeng, 2012).

The surface forces between ovomucin and positively charged APTES coated-mica at pH 7.2 with 150 mM NaCl were measured as a control and shown in Figure 6.2. The approaching force curve shows long range repulsion with a repulsion force range of about 100 nm. The film thickness was measured to be around ~24 nm for ovomucin coated APTES-mica surface, which was close to the thickness of BSM coated on mica (Zhu, DeGraaf, Winnik, & Leckband, 2004). However, slight adhesion force with the magnitude less than 0.5 mN/m with large adhesion hysteresis was measured during separation. And very weak adhesive force was observed possibly due to electrostatic interaction between the two surfaces since ovomucin carried net negative charge and APTES had positive charge at pH 7.2 (Omana, Wang, & Wu, 2010).

Figure 6.3(A-E) showed the force-distance profiles of an ovomucin film coated on APTES-mica interacting with BSA coated on APTES-mica surface at varied ionic strengths (0.1, 10 or 150 mM) and pHs (2.0, 6.0 or 7.2). It was observed that when the ionic strengths increased from 0.1 mM to 10 mM and 150 mM at pH 7.2 (Figure 6.3(A-C)), the ranges of the repulsion decreased from 140 nm to 60 nm. The change of repulsive force range suggested the electrostatic repulsion plays an important role in the surface forces between ovomucin and BSA surfaces. In Figure 6.3(C-E), the hard wall thickness measured by SFA technique remained at 32 nm upon changing pHs whereas the adhesion hysteresis showed difference. Specifically, there was no noticeable hysteresis at pH 6.0 (Figure 6.3D) and hysteresis increased at pH 2.0 (Figure 6.3E) as compared to that at pH 7.2 (Figure 6.3C). Overall, only repulsion forces were measured between ovomucin and BSA surfaces at all the tested ionic strengths and pHs, which further demonstrated the anti-fouling property of ovomucin.

In order to better understand the protein inhibiting behavior of ovomucin, a symmetric configuration experiment employing the SFA technique has been also designed. The normal force-distance profiles of two ovomucin films coated on APTES-mica surfaces in a symmetric geometry with different ionic strengths and pHs were presented in figure 6.4(A-E). We found that when the pH was fixed at 7.2 and ionic strengths decreased from 150 mM to 10 or 0.1 mM, repulsive force ranges were almost no change and remained at 120 nm. On the other hand, when solution pH varied from 7.2 to 6.0, the repulsive force ranges clearly decreased from 120 nm (Figure 6.4C) to 80 nm (Figure 6.4D). Further decreasing buffer pH to 2.0 resulted in an increase of the measured repulsive force range to 120 nm again, which was shown in Figure 6.4E. More importantly, the approaching and separation force curves were almost overlapped at pHs 7.2 and 6.0 and showed only pure repulsion (Figure 6.4(C-D)). The repulsion measured between two ovomucin surfaces with very small hysteresis is resemble to that between two polymer brush surfaces. When the buffer pH decreased from 6.0 to 2.0, large hysteresis was observed (Figure 6.4(D-E)). However, the measured hardwall thickness stayed at 40 nm, suggesting the conformation could have changed during constantly loading and unloading process. In general, forces between two ovomucin films coated on APTES-mica surfaces were repulsive at all measurements, during both approaching and separations, which were not influenced by changing ionic strengths and pHs.

6.3.5 AFM imaging

The topographical features of protein films (BSA, ovomucin and ovomucin plus BSA) coated APTES-mica surface were imaged in air by an AFM operating in tapping mode. As shown in figure 6.5A, the AFM image of BSA film immobilized on APTES-mica surface

showed a smooth surface with barely particles. However, after coating with ovomucin film (Figure 6.5B), the AFM image revealed large particles, and surface roughness increased as compared to Figure 6.5A. The BSA coated on ovomucin-APTES-mica surface was also imaged by AFM and shown in Figure 6.5C. The sizes and amounts of particles did not increase, probably due to both the protein resistant behavior of ovomucin and smaller size of BSA. The AFM results also indicated that the aggregated particles could contribute to the repulsive force measured during approaching.

6.3.6 Hydrodynamic size and zeta potential measurements

The hydrodynamic sizes and zeta potential of ovomucin and BSA at different pHs and ionic strengths were listed in Table 6.1. When the pH decreased from 7.2 to 2.0 at a fixed ionic strength (150 mM), the zeta potential of ovomucin particles changed from negative (-13.9±1.3 mV) to neutrality (-0.8±0.9 mV) probably because the isoelectric point of ovomucin is around pH 5.0 (Omana & Wu, 2009). In addition, the lower ionic strengths (10 mM and 0.1 mM) at fixed pH 7.2, and the higher negative zeta potential values of ovomucin were observed (-34.2±1.9 and -36.4±1.1 mV). Positive charge of the BSA surface (13.8±1.5 mV) was only observed at pH 7.2 with 150 mM of ionic strength, and at other pH and ionic strength conditions, BSA surface all presented negative charge, and especially, at pH 7.2 and 0.1 mM NaCl condition, BSA had the highest negative value (-17.1±2.6 mV).

The hydrodynamic size of ovomucin at pH 7.2 and 150 mM NaCl was 19.4±4.5 nm, while two distributions of hydrodynamic size were observed: one is 25.5±8.8 nm (80%) and another is 441.1±78.0 nm (20%) at decreased pH (6.0), suggesting aggregation of ovomucin occurred. As the pH further reduced to 2.0, the hydrodynamic size of ovomucin

was reduced to 3.3±0.5 nm, which was in contradiction with that mucin aggregates and forms gel at low pH as reported (Cao, Bansil, Bhaskar, Turner, LaMont, Niu, et al., 1999; Offengenden & Wu, 2013). This could be a result of a low ovomucin concentration used in the present study (46 μg/mL). To test the effect of ionic strengths on the hydrodynamic size of ovomucin at a fixed pH of 7.2, the ionic strengths were chosen as 0.1 mM, 10 mM and 150 mM. The DSL results suggested that a higher ionic strength reduced the aggregation behavior of ovomucin, which was consistent with SFA results that at higher salt concentration the repulsive force range clearly decreased. While changing pHs and ionic strengths did not significantly influence on the hydrodynamic sizes of BSA particles, which were ranging from 5.9±0.5 nm to 8.7±0.0 nm, and BSA was considered to be present as monomer (Li, Yang, & Mei, 2012).

6.4 Discussion

Human serum albumin (HSA), the most abundant blood plasma protein, is known to cause biofouling since it may adsorb on hydrophobic, negatively charged coating surface of biomedical applications (Junter, Thébault, & Lebrun, 2016; McArthur, McLean, Kingshott, St John, Chatelier, & Griesser, 2000). BSA shares 76% of sequence homology with that of HSA (Rezwan, Meier, Rezwan, Vörös, Textor, & Gauckler, 2004); thus, BSA was selected as the model protein to test the anti-fouling activity of ovomucin.

The effects of ionic strengths on the hydrodynamic size of ovomucin could be explained by the polyampholytes nature of ovomucin since ovomucin carries both acidic and basic groups (Offengenden & Wu, 2013). At pH 7.2, ovomucin was under the condition of high charge asymmetry and with net negative charge since ovomucin is rich in aspartic and glutamic acids in protein moiety, as well as sialic acid and sulphate in the

glycan part (Smith, Reynolds, Buckingham, & Back, 1974; Robinson & Monsey, 1971). These negative charges cause repulsion that could stretch the ovomucin chains. Therefore, the addition of salt screened the repulsion, resulting in a decrease in the ovomucin particle size (Dobrynin, Colby, & Rubinstein, 2004; Offengenden & Wu, 2013).

Only repulsive forces were detected by SFA between ovomucin and BSA surfaces during approaching and separations at all the applied pHs and ionic strengths, suggesting that ovomucin coated APTES-mica surface has good anti-fouling behavior. And the repulsive force range decreasing at high ionic strength was mainly due to the changes of surface charge, which was shown in Table 6.1. When fixed salt concentration and varied pHs, the hysteresis in the force curves at pH 2.0 (Figure 6.3E) increased as compared to that at pH 7.2 (Figure 6.3C). This could be mainly due to several reasons. Firstly, the ovomucin conformation changed when pH decreased, which was also demonstrate by DLS size measurement. Secondly, ovomucin became neutral (-0.8±0.9 mV) at pH 2.0, the decrease of surface charge of ovomucin-coated APTES-mica surface would lead to the decreasing of electrostatic repulsion even when BSA was positively charged (13.8±1.5 mV at pH 2.0). Moreover, ovomucin chains were less extended and less compressible at pH 2.0, which could also decrease the steric repulsion (Efremova, Huang, Peppas, & Leckband, 2002). Thus, electrostatic and steric repulsions were proposed as the main driving mechanism of anti-fouling behavior of ovomucin against BSA.

Similarly, the pure repulsion was measured between ovomucin and ovomucin surfaces at different ionic strengths and pHs, suggesting the ovomucin layer possesses polymer brush like property. Ovomucin contains hydrophobic, hydrophilic, charged and uncharged groups along its protein backbone and glycan side-chains (Omana, Wang, & Wu, 2010).

The aspartic and glutamic acid in the protein part as well as sialic acid and sulphate in the glycan moiety all carry negative charge at pH 7.2, which could be responsible for the electrostatic repulsions of ovomucin (Offengenden & Wu, 2013). In addition, the increased hysteresis between ovomucin and ovomucin at pH 2.0 was probably due to the protonation of the aspartic and glutamic acids leading to a decrease in electrostatic repulsions (Offengenden & Wu, 2013). Moreover, ovomucin contained protein rich subunit (α-ovomucin) with high amount of hydrophobic regions, which is different from other mammalian mucins (Watanabe, et al., 2004). Protonation of aspartic and glutamic acids at pH 2.0 induced the ovomucin conformation changing to expose hydrophobic protein domains (cysteine-rich domains) exteriorly (Cao, et al., 1999). Thus, hydrophobic interactions may contribute to the hysteresis between ovomucin and ovomucin surfaces. Overall, the electrostatic and steric repulsions should play important roles in the surface forces between ovomucin and ovomucin as well.

A protein adsorption assay using BSA as a model molecule was conducted which was designed to simulate the physiological environment. The results demonstrated that ovomucin-coated polystyrene surface significantly reduced or even completely inhibit BSA adsorption, which validate the protein resistant property of ovomucin in this work.

Moreover, the pure repulsive forces between ovomucin film and protein surfaces further demonstrated the potential of ovomucin as an anti-fouling surface. Measurement of the anti-fouling property of ovomucin *in vivo* and determination of the anti-adhesive activity of ovomucin-coated surfaces against bacteria and cells needs to be studied in future.

6.5 Conclusions

Overall, the present work demonstrated that ovomucin exhibited anti-fouling property at different pHs and ironic strengths due to electrostatic and steric repulsions, suggesting its potential as an anti-fouling surface for the first time. This study would open new windows for developing cheap, easy available and effective anti-fouling surfaces and expand the ovomucin application to a wider range of areas, such as biosensors, biomedical implants and food processing industry.

6.6 References

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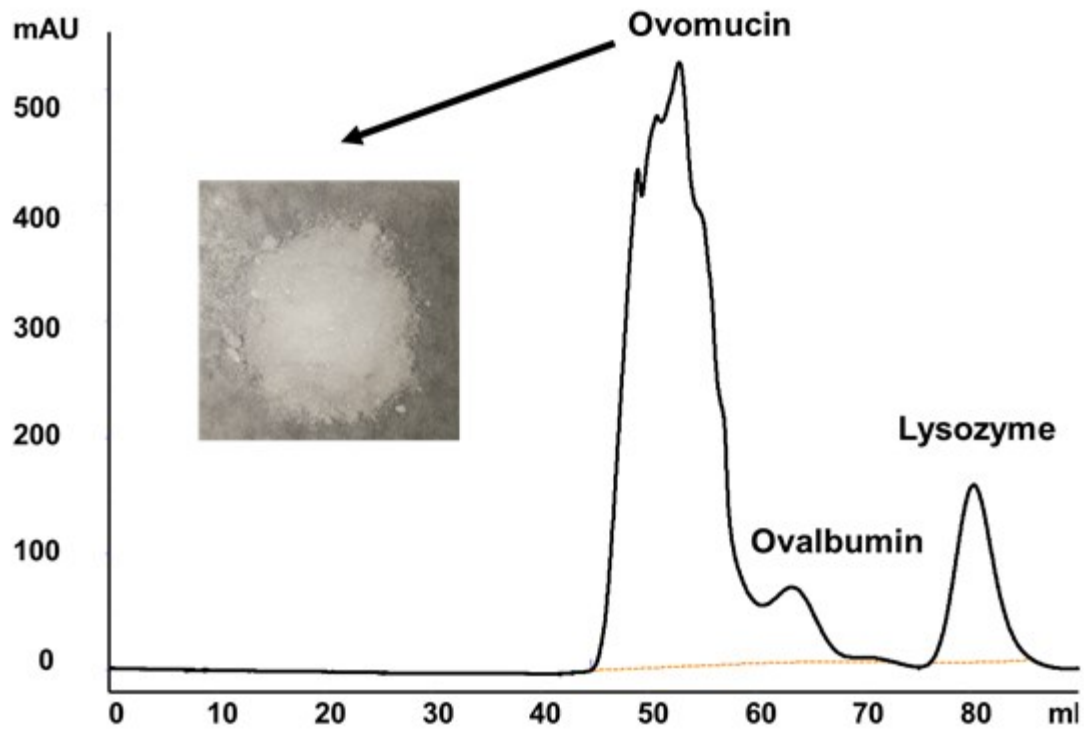
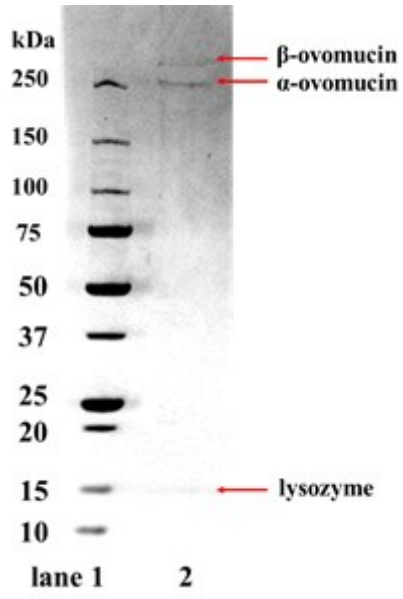
Table 6.1 Hydrodynamic sizes and zeta potential of ovomucin and BSA at different pHs and ionic strengths

Sample	Concentration (μg/mL)	Zeta potential (mV)	Size (nm)/Percentage	
			Peak 1	Peak 2
Ovomucin 150 mM pH 7.2	326	-13.9±1.3	19.4±4.5 (100%)	N.D.*
Ovomucin 150 mM pH 6.0	201	-14.7±0.7	25.5 ± 8.8 (80%)	441.1 ± 78.0 (20%)
Ovomucin 150 mM pH 2.0	46	-0.8±0.9	3.3 ± 0.5 (100%)	N.D.
Ovomucin 10 mM pH 7.2	300	-34.2±1.9	68.8 ± 14.2 (30%)	717.5 ± 105.0 (70%)
Ovomucin 0.1 mM pH 7.2	222	-36.4±1.1	79.7 ± 16.4 (20%)	924.7 ± 198.8 (80%)
BSA 150 mM pH 7.2	200	-10.5±0.4	8.7 ± 0.0 (100%)	N.D.
BSA 150 mM pH 6.0	200	-9.3±1.4	8.7 ± 0.0 (100%)	N.D.
BSA 150 mM pH 2.0	200	13.8±1.5	7.6 ± 1.1 (100%)	N.D.
BSA 10 mM pH 7.2	200	-9.1±0.7	5.9 ± 0.5 (100%)	N.D.
BSA 0.1 mM pH 7.2	200	-17.1±2.6	5.9 ± 0.5 (100%)	N.D.

*Not detected



A



B

Figure 6.1: (A) Gel filtration chromatogram of ovomucin sample and (B) SDS-PAGE pattern of the ovomucin solution prepared in section 6.2.5.1. (A) The sample was eluted isocratically with 100 mM phosphate buffer (pH 7.0) containing 5 g/L of SDS and 1 mL/L of β-mercaptoethanol at a flow rate of 1 mL/min, which was monitored by a UV detector at 280 nm, **(B)** The protein samples (10 µg/well) were run in SDS-PAGE and the gel (4-20%) was stained with CBB R250. Lanes 1 was molecular weight marker and lane 2 was ovomucin solution.

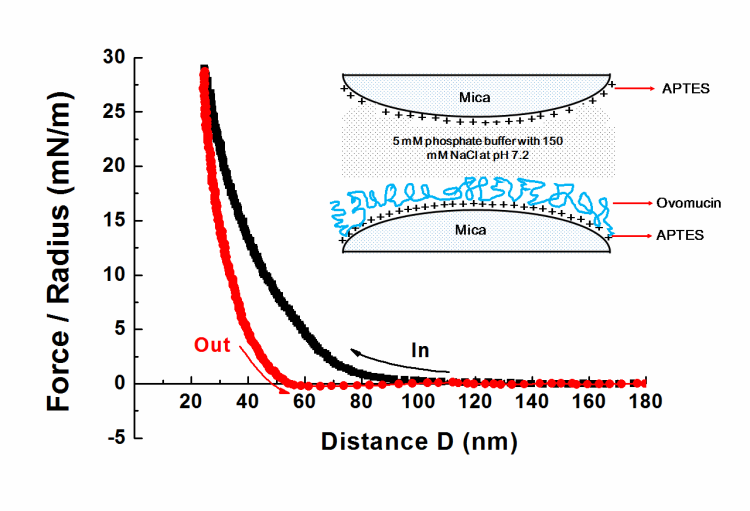
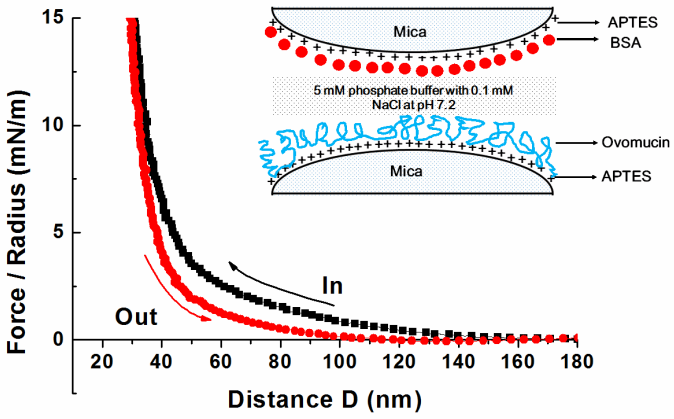
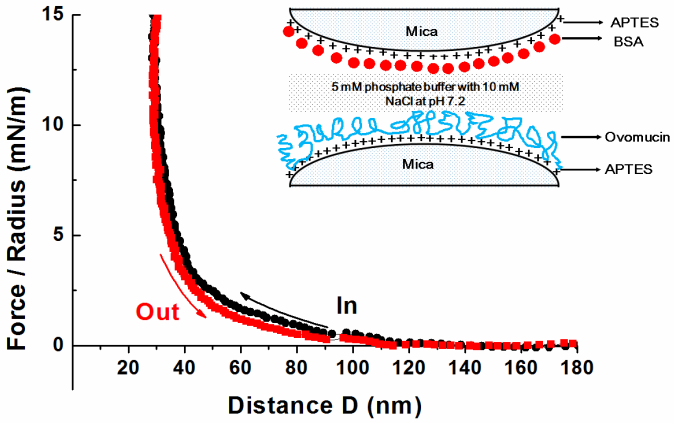


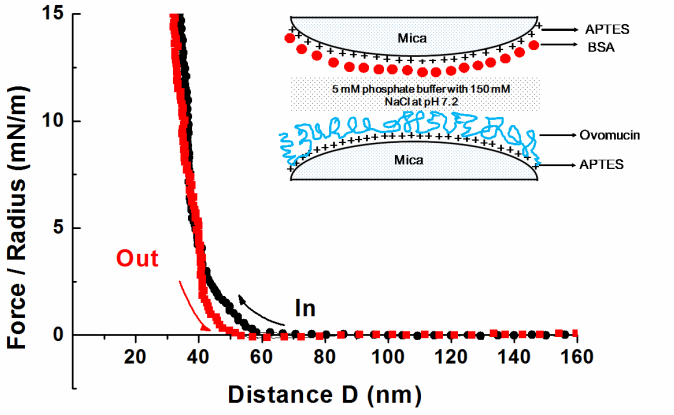
Figure 6.2: Surface force curve between ovomucin coated APTES-mica and APTES-mica surfaces at pH 7.2 with 150 mM NaCl.


Force / Radius (mN/m)
Distance D (nm)
Out
In
Mica
APTES
BSA
5 mM phosphate buffer with 0.1 mM NaCl at pH 7.2
Ovomucin
Mica
APTES

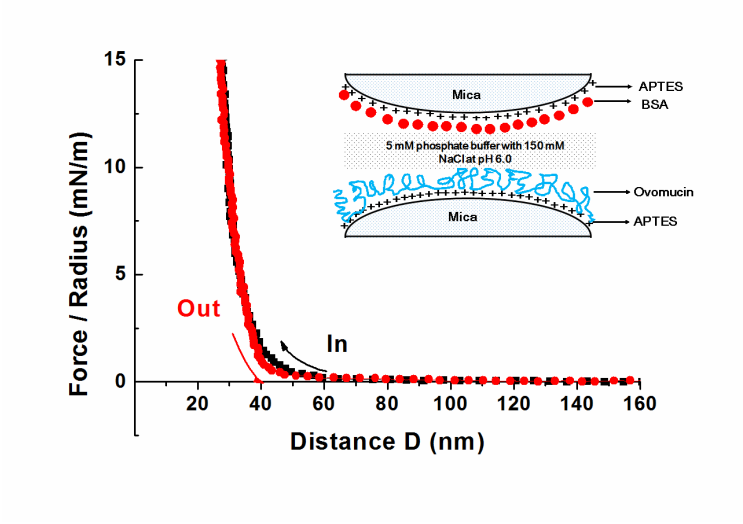
A


Force / Radius (mN/m)
Distance D (nm)
Out
In
Mica
APTES
BSA
5 mM phosphate buffer with 10 mM NaCl at pH 7.2
Ovomucin
Mica
APTES

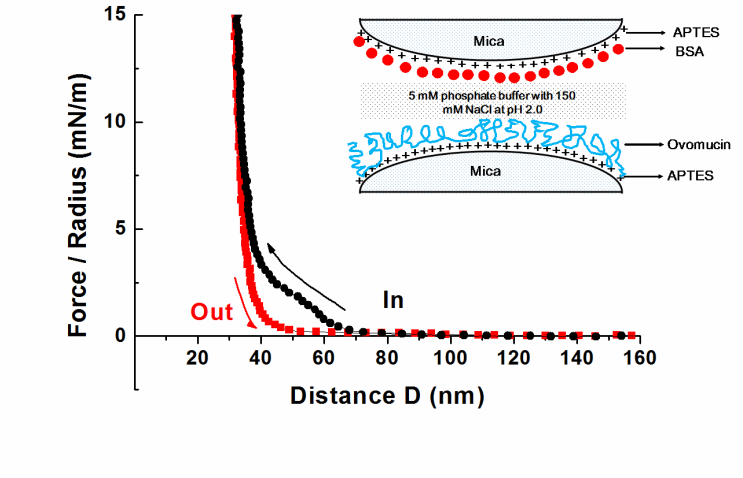
B


Force / Radius (mN/m)
Distance D (nm)
Out
In
Mica
APTES
BSA
5 mM phosphate buffer with 150 mM NaCl at pH 7.2
Ovomucin
Mica
APTES

C

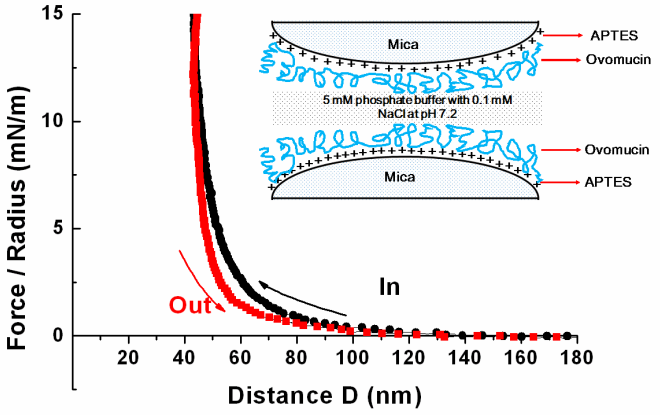


D

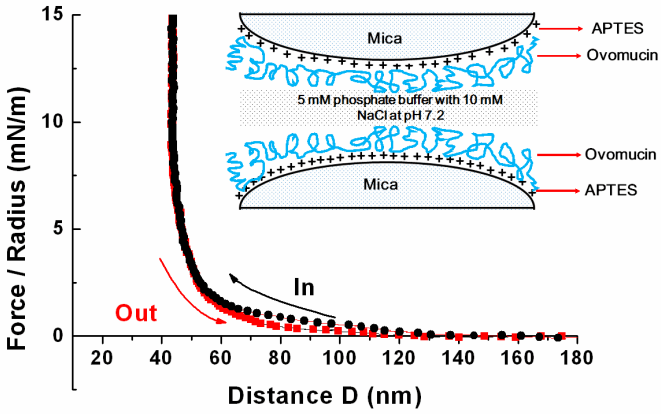


E

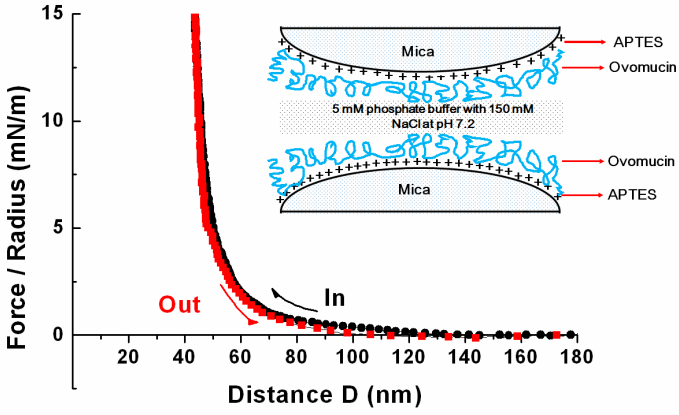
Figure 6.3: Surface force curves between ovomucin coated APTES-mica and BSA coated APTES-mica surfaces: (A) pH 7.2, 0.1 mM NaCl, (B) pH 7.2, 10 mM NaCl, (C) pH 7.2, 150 mM NaCl, (D) pH 6.0, 150 mM NaCl, (E) pH 2.0, 150 mM NaCl.


Force / Radius (mN/m)
Distance D (nm)
Mica
APTES
Ovomucin
5 mM phosphate buffer with 0.1 mM NaCl at pH 7.2
Ovomucin
APTES
Mica
In
Out

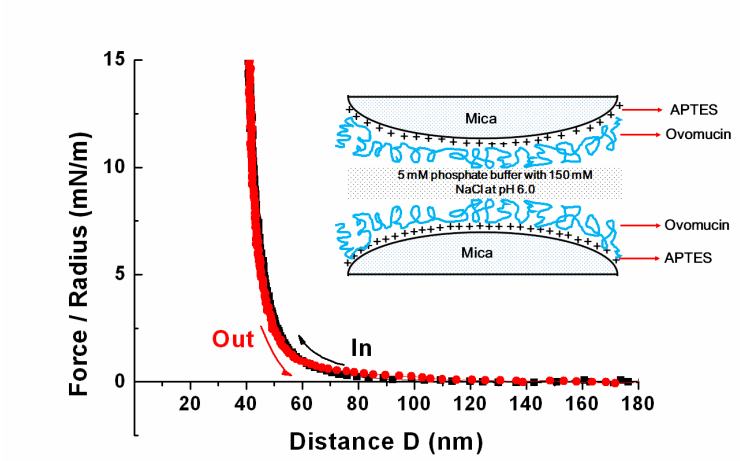
A


Force / Radius (mN/m)
Distance D (nm)
Mica
APTES
Ovomucin
5 mM phosphate buffer with 10 mM NaCl at pH 7.2
Ovomucin
APTES
Mica
In
Out

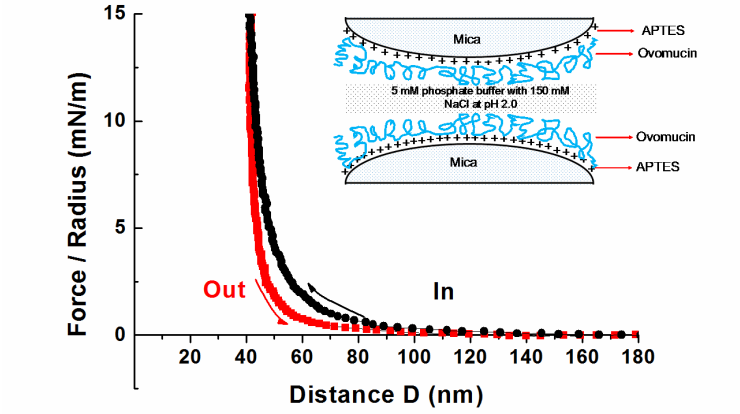
B


Force / Radius (mN/m)
Distance D (nm)
Mica
APTES
Ovomucin
5 mM phosphate buffer with 150 mM NaCl at pH 7.2
Ovomucin
APTES
Mica
In
Out

C



D



E

Figure 6.4: Surface force curves between two ovomucin coated APTES-mica surfaces in a symmetric geometry: (A) pH 7.2, 0.1 mM NaCl, (B) pH 7.2, 10 mM NaCl, (C) pH 7.2, 150 mM NaCl, (D) pH 6.0, 150 mM NaCl, (E) pH 2.0, 150 mM NaCl.

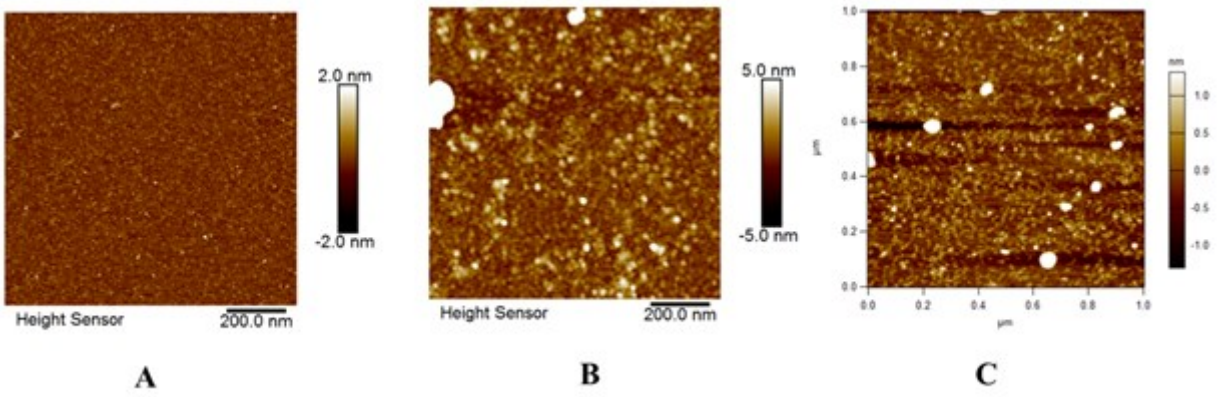


Figure 6.5: AFM images of protein-coated on APTES-mica surface in air: (A) 200 µg/mL of BSA, (B) 88 µg/mL of ovomucin, (C) BSA coated on ovomucin-APTES-mica surface.

CHAPTER 7 –Concluding Remarks and Future Directions

7.1 Key findings of the present research

There are four research chapters in the thesis, including effect of proteolysis on the sialic acid content and bifidogenic activity of ovomucin hydrolysates (chapter 3), the anti-adhesive potential of ovomucin/ovomucin hydrolysates and the possible anti-adhesive mechanism (chapters 4 and 5), and the anti-fouling property of ovomucin (chapter 6). The key findings of each study are listed below:

1. **Chapter 3**: Ovomucin, a glycoprotein accounting for 2-4% of the total egg white protein, is a major contributor to egg white functionality (Kato, Oda, Yamanaka, Matsudomi, & Kobayashi, 1985). On average, ovomucin contains ~33% of carbohydrates (Mine, 1995b). Ovomucin is rich in sialic acid (2.6-7.4%, w/w), which is a family member of acylated derivatives of a nine-carbon carboxylated monosaccharide (Robinson & Monsey, 1971). Sialic acid (N-acetylneuraminic acid, Neu5Ac) is thought to play crucial roles in cognition and memory development in infants and has been suggested as an essential nutrient for infants (Tang, Liang, Cai, & Mou, 2008; Wang & Brand-Miller, 2003). Furthermore, sialic acid-containing substances were suggested to promote the growth of bifidobacteria (Idota, Kawakami, & Nakajima, 1994), indicating the bifidogenic potential of ovomucin. However, ovomucin's limited water solubility might limit its future applications. Therefore, the objectives of the study were to prepare and characterize ovomucin hydrolysates, to determine the effect of proteolysis of ovomucin on the sialic acid content, and to test the bifidogenic activity of ovomucin hydrolysates. The results showed that hydrolysis

yields and DHs ranged from 42.6% (flavourzyme) to 97.4% (protease N), and 2.4% (flavourzyme) to 46.3% (pronase), respectively. These hydrolysates showed a wide range of molecular weight (Mw) distribution while only two hydrolysates prepared by pronase and protex 26L showing Mw less than 40 kDa. The content of sialic acid in these hydrolysates ranged from 0.1% (protex 26L) to 3.7% (pronase). IgE binding of ovomucin hydrolysates was significantly reduced ($P<0.05$) in comparison to ovomucin extracts. Ovomucin hydrolysates did not generally support growth of *Bifidobacterium* spp *in vitro*, but may be degraded within the infant gastrointestinal tract by potential protocooperation among different bifidobacteria strains or other members of gut microbiota and form short chain fatty acid. Overall, in chapter 3, fourteen ovomucin hydrolysates were prepared and characterized. This study demonstrated that ovomucin hydrolysates had potential to use as a value-added ingredient to increase sialic acid content in follow-up formula.

2. **Chapter 4**: Diarrhea in newborn and post-weaning piglets increases the mortality and morbidity rates, causing substantial economic losses to the swine industry. Enterotoxigenic *Escherichia coli* (ETEC) strains are the primary cause of piglet diarrhea (Nagy & Fekete, 1999). Adhesion to mucosal tissue or epithelial cells of the host is the initial and prerequisite step in ETEC pathogenesis (Moonens, et al., 2015). A promising strategy to prevent infectious diseases is to use anti-adhesive agents to interfere the initial stage of adhesion and colonization of bacteria to host tissues (Ofek & Sharon, 2002; Sharon & Ofek, 2000). Due to its structural similarity to mucin, ovomucin may have the potential to act as receptor analogs and compete for bacterial adhesion (anti-adhesion). This study aimed to determine whether ovomucin or

ovomucin hydrolysates could prevent adhesion to two porcine K88 ETEC strains. Adhesion was determined *in vitro* using hemagglutination assay. The results demonstrated that ovomucin hydrolysates, but not intact ovomucin, prevented ETEC adhesion to porcine erythrocytes. Ovomucin hydrolysate prepared by acid protease II exhibited the best anti-agglutinating activity against both strains; this hydrolysate was fractionated by cation exchange chromatography and reverse-phase HPLC. The most active fractions, F3(9) and F7(1), with minimal inhibitory concentration of 0.03 g/L and 0.25 g/L, against strains ECL13795 and ECL13998, respectively, were subjected to structure characterization. Six glycopeptides identified were all derived from α-ovomucin, composed of a pentasaccharide core of two N-acetylglucosamine and three mannose residues ($GlcNAc_2Man_3$) and a bisecting N-acetylglucosamine (GlcNAc). The terminal β-linked galactose from these glycopeptides could be one of the binding sites for $K88_{ac}$ fimbriae. In conclusion, chapter 4 suggested that ovomucin hydrolysates had anti-adhesive potential against ETEC strains.

3. **Chapter 5**: As a simple method, hemagglutination assay is suitable for preliminary test. However, hemagglutination assay cannot provide quantitative values for the extent of bacterial adhesion (Wang, Gänzle, & Schwab, 2010) and erythrocytes have distinct features and morphology from epithelial cells (Nagy & Fekete, 2005; Vergauwen, 2015). Therefore, we determined the anti-adhesive activity of ovomucin hydrolysates against $K88_{ac}$ ETEC using porcine small intestinal epithelial cell line (IPEC-J2) in this chapter. The objectives of this study were to further investigate the anti-adhesive potential of ovomucin hydrolysates, to discuss the effect of ovomucin hydrolysate on the $K88_{ac}$ fimbriae binding to IPEC-J2 cells, and to characterize the

responsible anti-adhesive glyocpeptides. The anti-adhesive activity of ovomucin hydrolysates against ETEC adhesion to IPEC-J2 cells was determined by plate counting and Syto 9 staining methods. Ovomucin-protex 26L hydrolysate showed the best anti-adhesive activity at a minimum effective concentration of 2.5 g/L. Dot-blot assay suggested that the anti-adhesive activity of ovomucin hydrolysates was due to competitive binding to ETEC through $K88_{ac}$ fimbriae as decoy receptors. The responsible glycopeptides in ovomucin-protex 26 L hydrolysate was purified by affinity chromatography and four peptide sequences and twelve possible glycans were identified. The peptide sequences play a role in competitive binding to $K88_{ac}$ fimbriae while the glycan moieties are indispensable for this binding. Chapter 5 further indicated that ovomucin glycopeptides showed potent anti-adhesive activity, which had potential to be used as anti-adhesive agents against infectious diseases. The anti-adhesive efficacy of ovomucin hydrolysates may associate with the presence of distinct adhesins on the surfaces of ETEC, such as K88 fimbriae and porcine attaching and effacing-associated factor (paa).

4. **Chapter 6**: Undesired accumulation of proteins on surfaces is a serious issue affecting numerous applications, such as biosensors, biomedical implants, and food processing industry (Banerjee, Pangule, & Kane, 2011; Wong, et al., 2012). Therefore, development of anti-fouling surface to inhibit protein adsorption is imperative. The main function of the mucous layer, covering epithelial cells, is to function as a natural anti-fouling surface to prevent undesirable adhesion to host tissues (Cone, 2009). This layer is mainly composed of water and mucin, a member of heavily glycosylated and gel-forming high molecular-weight glycoproteins (Bansil & Turner, 2006). Structural

similarity between mammalian mucins and ovomucin led us to hypothesize that ovomucin may have anti-fouling property. In this study, the anti-fouling property of ovomucin was studied by determining the adsorption of bovine serum albumin (BSA) on ovomucin-coated polystyrene surface. Ovomucin significantly inhibited BSA adsorption to polystyrene surface. Pure repulsive forces were measured by the surface force apparatus (SFA) under both symmetric (ovomucin vs. ovomucin) and asymmetric configurations (BSA vs. ovomucin) at all tested pHs (2.0, 6.0 and 7.2) and ionic strength (0.1, 10, and 150 mM NaCl) during approaching and separation, which further proved the potential application of ovomucin as an anti-fouling surface. Atomic force microscope imaging, zeta potential measurement and dynamic light scattering indicated that the electrostatic and steric repulsions could be the main underlying mechanism of the anti-fouling property of ovomucin. Overall, chapter 6 demonstrated that ovomucin exhibited anti-fouling property at different pHs and ironic strengths, suggesting its potential as anti-fouling surface for the first time.

Although the ovomucin hydrolysate exhibited the best anti-agglutinating activity (ovomucin-acid protease II) is different from that of the anti-adhesive activity (ovomucin-protex 26L hydrolysates) as reported in chapters 4 and 5, respectively, interestingly, the responsible glycopeptides from these hydrolysates showed similarity. Two identical peptides (VCGLCGDFDGR and SQTE) from α-ovomucin, and twelve identical permethylated glycans were determined.

In conclusion, this thesis demonstrated that ovomucin hydrolysates are rich in sialic acids, have a good water solubility, and show potent anti-adhesive activity against ETEC strains; therefore ovomucin hydrolysates have the potential to be developed as a

value-added ingredient in follow-up formula, functional foods and nutraceuticals. In addition, our research suggested that ovomucin could be a promising candidate for anti-fouling surfaces.

7.2 Significance of this research

The potential of egg white ovomucin as a value-added ingredient and as an anti-fouling surface hold promises to grow the egg industry beyond the food uses. As a rich source of sialic acid, this thesis demonstrated that some ovomucin hydrolysates are also rich in sialic acid. Sialic acid has been suggested as an essential nutrient for infants (Wang, & Brand-Miller, 2003). Additionally, the sialic acid in ovomucin is Neu5Ac, the identical one found in human glycans but different from the Neu5Gc present in glycans of other mammals (Schauer, Srinivasan, Coddeville, Zanetta, & Guérardel, 2009).

Antibiotics are commonly used to prevent or treat bacterial diseases; study the potential of ovomucin hydrolysates as anti-adhesive agents could provide a promising alternative approach to control bacterial infection. Anti-adhesive agents are milder and safer than antibiotics since anti-adhesive agents are not bactericidal and less likely to develop resistant strains (Ofek & Sharon, 2002; Sharon, 2006). Development of ovomucin hydrolysates as anti-adhesive agents hold great promise for anti-adhesive therapy because ovomucin hydrolysates have commercial potential that are safe, affordable, and scalable. Hen egg is abundant and has a long history of use as a human food.

Understanding the anti-adhesive mechanism of ovomucin hydrolysates and characterizing the responsible anti-adhesive glycopeptides are crucial to build up the fundamental knowledge on the structure-activity relationship and to develop effective therapy in the future. Moreover, the present study suggested porcine attaching and

effacing-associated factor (Paa), a non-fimbrial adhesin, may contribute to K88 ETEC adherence to IPEC-J2 cells for the first time. Due to the specific role of paa in ETEC pathogenesis is unknown (Leclerc, et al., 2007), future research is needed.

This thesis also suggested the potential of ovomucin as an anti-fouling surface for the first time. This work would open new windows for developing cheap, abundant and effective anti-fouling surfaces, which could be used in biosensors, biomedical implants and food processing industry.

7.3 Recommendations for future research

Based on the key findings of the thesis, the recommended further studies are outlined below:

1. Ovomucin hydrolysates did not generally support the growth of *Bifidobacterium* spp *in vitro*, *in vivo* study should be conducted to further investigate the bifidogenic activity of ovomucin hydrolysates to test if ovomucin hydrolysates could be degraded within the gastrointestinal tract by potential protocooperation among different bifidobacteria strains or other members of gut microbiota.
2. Ovomucin hydrolysates exerted potent anti-adhesive activity against porcine ETEC strains in this work, and further research is required to test the anti-adhesive efficacy of ovomucin hydrolysates against human pathogens.
3. The efficacy and safety of ovomucin hydrolysates as anti-adhesive agents need to be determined in animal models in the future.
4. The current research identified several possible glycan structures from ovomucin glycopeptides which bind to $K88_{ac}$ fimbriae, and it is interesting to further clarify the required glycan structures of ovomucin glycopeptides interacting with $K88_{ac}$ fimbriae.

In addition, the major driving forces between ovomucin glycopeptides and $K88_{ac}$ fimbriae are worthy to be explored in future, which can help us better understand the anti-adhesive mechanism.

5. The present work indicated ovomucin's potential as an anti-fouling surface using BSA as model protein, while it is essential to evaluate the anti-fouling activity of ovomucin against complex biological media *in vivo*. Furthermore, it is necessary to determine the anti-fouling activity of ovomucin, i.e., evaluating the activity of inhibiting cells and bacteria adsorption on ovomucin-coated surfaces.

7.4 References

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