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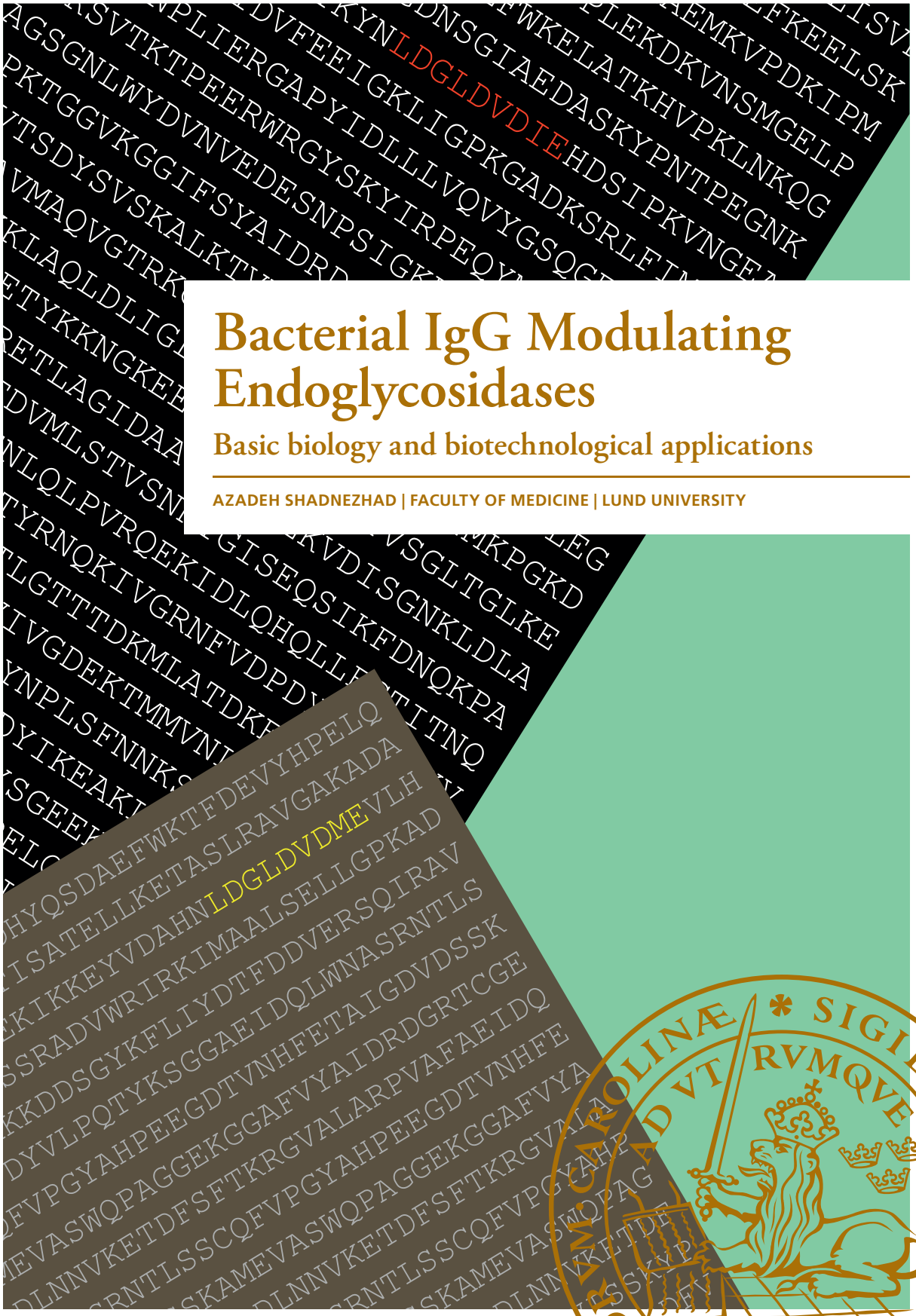
Shadnezhad, Azadeh

2017

Document Version:
Publisher's PDF, also known as Version of record



Bacterial IgG Modulating Endoglycosidases

Basic biology and biotechnological applications

AZADEH SHADNEZHAD | FACULTY OF MEDICINE | LUND UNIVERSITY

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Basic biology and biotechnological applications

Azadeh Shadnezhad

LUND
UNIVERSITY

DOCTORAL DISSERTATION
By due permission of the Faculty of Medicine, Lund University, Sweden.
To be defended at Segerfalkssalen (BMC A10), on February 3rd 2017 at 13:15.

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Organization LUND UNIVERSITY	Document name Doctoral dissertation
Faculty of Medicine Department of Clinical Sciences Division of Infection Medicine	Date of issue: 20170203
Author(s): Azadeh Shadnezhad	Sponsoring organization

Title and subtitle: Bacterial IgG Modulating Endoglycosidases- Basic biology and biotechnological applications

Abstract

Immunoglobulin G (IgG) is the most abundant antibody in human blood and plays a fundamental role in the adaptive immune system. Asparagine linked oligosaccharides (*N*-glycans) are essential for structure and biological function of IgG. Bacteria produce glycosidases that can hydrolyze carbohydrates on glycoproteins such as IgG and thereby impede stability and effector functions. One such endoglycosidase secreted by *Streptococcus pyogenes* is EndoS. EndoS hydrolyzes glycans on the Fc region of IgG leading to a significant alteration in IgG binding to its receptors and conseqeuntly the effector functions of IgG. In this thesis I show that enzymatically inactive EndoS is a useful tool to purify structurally intact and glycosylated IgG, in contrast to the golden standard protein G that binds IgG irrespective of structural integrity and glycosylation status. Furthermore, I have identified and characterized the EndoS-like enzymes EndoSd in the animal pathogen *Streptococcus dysgalactiae*, CP40 in the animal and zoonotic pathogen *Corynebacterium pseudotuberculosis*, and EndoCd in the human pathogen *Corynebacterium diphtheriae*. EndoSd hydrolyzes the β1-4 linkage between the two *N*-acetylglucosamines (GlcNAcs) in the chitobiose core of the *N*-linked glycan of IgG. Furthermore, CP40 from *C. pseudotuberculosis* that was previously suggested to be a protease, is an endo-β-*N*-acetylglucosaminidase that cleaves *N*-linked glycans on human, horse and sheep IgGs. In addition, I found that most *C. diphtheriae* strains express CP40 enzymes that were denoted EndoCd. Most EndoCd allleles were predicted to be enzymatically active, while a few might be inactive. Endoglycosidases are produced by many pathogenic bacteria so identification and characteriazation of them may help to extend our knowledge about pathogenicity and provide novel ideas on how to combat bacterial infection. Furthermore, wild type or engineered enzymes may also be evaluated as biotechnological tools and potential biopharmaceuticals against autoimmunity and other immunogical disorders.

Key words: *Streptococcus dysgalactiae*, *Corynebacterium*, endoglycosidases, EndoS, EndoSd, CP40, EndoCd, IgG

Classification system and/or index terms (if any)

Supplementary bibliographical information	Language English
ISSN and key title 1652-8220 Lund University, Faculty of Medicine Doctoral Dissertation Series 2017:20	ISBN 978-91-7619-401-0
Recipient's notes	Number of pages 155 / Price
	Security classification

Signature  Date 2016.12.12

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Cover photo: The sequence of EndoSd and CP40 proteins by Aidin Shadnezhad

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ISBN **978-91-7619-401-0**
ISSN **1652-8220**

Printed in Sweden by Media-Tryck, Lund University
Lund 2016

توانا بود هر که دانا بود

ز دانش دل پیر برنا بود*

* *Capable is who is wise*

Happiness from wisdom will arise

Ferdowsi

Contents

Persian summary

ایمونوگلوبولین‌ها یا آنتی‌بادی‌ها گروهی از گلیکوپروتئین‌ها (کربوهیدرات + پروتئین) در سیستم ایمنی بدن هستند که قادر به تشخیص عوامل خارجی (آنتی‌ژنها) بوده و بطور اختصاصی با آنها واکنش می‌دهند و نقش بسزایی در دفاع بدن در مقابل باکتری‌ها ایفا می‌کنند. آنتی‌بادی‌ها در سرم و مایع بافتی همه پستانداران یافت می‌شوند. زنجیره گلایکان یا کربوهیدرات در ساختار و عمل آنتی‌بادیها دخالت دارد، به صورتی که نبود آن باعث عدم توانایی آنتی‌بادی در واکنش با آنتی‌ژن می شود.

بسیاری از باکتری‌ها قادر به تولید آنزیم‌هایی به نام گلیکوزیداز (glycosidase) هستند که زنجیره کربوهیدراتی را از آنتی‌بادیها جدا نموده و آنها را غیرفعال می‌سازند. EndoS یکی از این گلیکوزیدازها است که توسط باکتری استرپتوکوکوس پایوژنز (*Streptococcus pyogenes*) تولید می‌شود.

استرپتوکوک‌ها باکتری‌های کروی‌شکلی هستند که برخی از آنها به طور طبیعی در بدن انسان بوده و گروهی از آنها موجب بیماری عمده‌ای در انسان می‌شوند. استرپتوکوک‌ها بر اساس کربوهیدرات موجود در دیواره سلولی به گروه‌های مختلفی طبقه‌بندی می‌شوند که با حروف انگلیسی نامگذاری شده‌اند. استرپتوکوکوس پایوژنز در رده‌بندی، گروه A به حساب می‌آید که باعث سندرم شوک سمی و تب مخملک در انسان می‌شوند.

هدف این تحقیق یافتن آنزیم‌هایی مشابه با EndoS در گروه های دیگر استرپتوکوک می باشد. ما موفق به یافتن آنزیم مشابهی دراسترپتوکوک‌های گروه C و G شده‌ایم و آن را EndoSd نام نهاده‌ایم. این آنزیم فعالیتی مشابه با EndoS دارد در حالی‌که درانتهای زنجیره پروتئینی خود ۷۸ اسید آمینه بیشتر دارد.

در تحقیق دیگری ما آنزیم‌های CP40 و EndoCd مشابهی در دو سویه کورینه باکتریوم (*Corynebacterium*) یافته‌ایم که عمل مشابهی را بر روی آنتی‌بادیها انجام می‌دهند.

CP40 آنزیمی مترشحه از باکتری *Corynebacterium pseudotuberculosis* است که میزبان آن حیوانات اهلی هستند. این باکتری در گوسفند و بز باعث عفونت لنفی واگیردار می‌شود و به سرعت به حیوانات سالم منتقل می گردد و باعث مرگ آنها می‌شود. از این رو از لحاظ اقتصادی باعث لطمه بزرگی به دامداران می‌گردد. این باکتری به‌ندرت در انسان ایجاد بیماری می‌کند. EndoCd آنزیمی است که باکتری خطرناک *Corynebacterium diphtheriae* که عامل عفونت دیفتری در انسان است آن را تولید می‌کند.

از آن‌رو که امکان جداسازی گلیکوزیدازها از محیط کشت باکتریایی در آزمایشگاه وجود دارد ما متمایل به استفاده از این آنزیمها درعلم پزشکی هستیم. همچنین گاهی در بیماری‌های خاص مثل بیماری‌های اتوایمن (که سیستم دفاعی بدن علیه سلول‌های بدن انسان آنتی بادی می سازد و آنها را از بین می برد) و یا برای جلوگیری از رد پیوندها ناچار به سرکوب سیستم ایمنی بدن انسان هستیم، استفاده از گلیکوزیدازها گزینه مناسبی برای توقف عملکرد آنتی بادی‌ها به نظر می‌رسد. ما در پی ساختن داروهایی هستیم که به کمک آنها بتوانیم در صورت نیاز، سیستم ایمنی بدن را سرکوب کنیم تا از آسیب جدی به بدن جلوگیری به عمل بیاوریم. علاوه بر این تهیه واکسن مناسب برای بسیاری از بیماریها تا کنون میسر نشده است. تحقیقات نشان می دهند که استفاده از آنزیم CP40 باعث تحریک سیستم ایمنی در حیوانات اهلی شده و باعث عدم گسترش بیماری می‌شود. در ضمن از آنجایی که این آنزیم‌ها قادر به اتصال به آنتی‌بادی‌ها هستند می توان از آنها برای خالص سازی آنتی‌بادیها استفاده کرد به صورتی که با ایجاد جهش ژنی باعث عدم فعالیت آنزیم بر روی آنتی‌بادی شویم در حالیکه آنزیم هنوز قادر به اتصال به آنتی‌بادی است.

شناخت و یافتن گلیکوزیدازها علاوه بر یافتن مکانیسم‌هایی که باکتری برای ایجاد بیماری از آنها استفاده می‌کند دریچه ای را برای یافتن داروهای اختصاصی و جلوگیری از پیشرفت بیماری و مرگ و میر در اثر عفونت در مقابل دانشمندان باز می‌کند.

Acknowledgements

To my supervisor
Mattias

Thank you, for allowing me the space and freedom I needed to work and for continued support and guidance from day one. Your extraordinary human quality was a real privilege for me. I have been extremely lucky to have a supervisor like you! I could not have imagined having a better supervisor! Although I am not as patient as you, if I would be a supervisor one day, I will try to behave like you. ☺

To my co-supervisor
Andreas

Thank you for always having time for me and the scientific discussions, also for teaching me how to work with HPLC. I have learned a lot about everything from you.

To my past and present colleagues
Eleni, Maria, Jonathan, Julia, Sara, Markus, Raja

Thank you for interesting discussions, your suggestions, act of kindness and mostly being good friends. I have really enjoyed working with you.

To Rolf

Thank you for taking care of my samples during weekends and your valuable suggestions, kind advice and your friendship. I was trying to be at work before 8AM like you, but I never could! ☺

To my co-authors
Barbara and Niclas

Thank you for welcoming to me to your lab in Gothenburg and kind assistance for UHPLC performance.

To my students
Simon, Fredrik, Jonathan A, Malin

Thank you for helping me in the lab, and making the lab work easier. I have learned that it is not easy to be a good supervisor! ☺

To B14 leaders
Lars, Heiko, Anita

Thank you for creating wonderful environment.

To my Iranian colleagues
Mina, Mojgan, Zahra, Syrina

Thank you! I never forget your kindness. Thank you Mina for keeping us happy during stressful time in the lab. Thank you for the great breakfast, birthday cakes, flowers, laughs and ....

To my fika colleagues
Elisa, Swati, Clement, Malin, Anas, Anele, Sandra(s), Suado, Sinead, Mohammad, Suvi, Tirthankar, Finja and Laura

Thank you for the great time and non-specific discussions, laughs, movie nights, karaoke and party time in and out of the lab.

To friends outside the lab
Banafsheh, Shahram, Sheida, Sepideh, Shwan, Vahid and Leila

You are all awesome! Thank you for your help and support whenever I needed! You always make me laugh and joyful. ☺

To my Swedish family
Yousef, Maria, Arvin, Nicky

Thank you for your warm welcome to your place in Sweden! Thank you Yousef! I am happy to have such an uncle! Thank you Maria! You were always like a sister toward me! Thank you to my lovely cousins Arvin and Nicky! You are Great! I never forget your kindness and the first day in Copenhagen when you came to pick me up!

To my mom and dad

Success is in my stride, because I have parents like you by my side. Thank you for everything!

To my brother and his wife
Farhad and Aida

Thank you for all the fun we are having together!

To Aidin

I could never have come this far without you. Thank you very much for your endless support. I never forget how you were trying to fix my computer problems when we were so far away! ☺

List of papers

Paper I

EndoSd: an IgG glycan hydrolyzing enzyme in *Streptococcus dysgalactiae* subspecies *dysgalactiae*.

Azadeh Shadnezhad, Andreas Naegeli, Jonathan Sjögren, Barbara Adamczyk, Fredrik Leo, Maria Allhorn, Niclas G Karlsson, Anders Jensen & Mattias Collin

Future Microbiology 2016 vol 11, 721-736

Paper II

A new approach for IgG purification using enzymatically inactive EndoS from *Streptococcus pyogenes*

Maria Allhorn, **Azadeh Shadnezhad**, Helena Björklund, Victoria Ptasinski, Katarzyna Tarnawska, Anders I Olin, Mattias Collin

Manuscript

Paper III

CP40 from *Corynebacterium pseudotuberculosis* is an endo-β-*N*-acetylglucosaminidase

Azadeh Shadnezhad, Andreas Naegeli and Mattias Collin

BMC Microbiology 2016 vol 16(1): 261

Paper IV

Identification of CP40-like endoglycosidases in *Corynebacterium diphtheriae*

Azadeh Shadnezhad, Mattias Collin

Manuscript

Introduction

For all multicellular organisms including plants, animals and humans it is essential to have an efficient immune system that can defend against microbial infections. Higher eukaryotic organism including humans have an immune system that has two major branches to eliminate microbes, innate immunity (also called natural or native) and the adaptive immunity (also called specific or acquired). Individuals with defects in the functions of the immune system are generally more susceptible to serious bacterial and viral infections, even though there is substantial redundancy. Through co-evolution bacterial pathogens have acquired many different systems to evade the effects of the immune system on different levels. For instance, pathogenic bacteria secrete extracellular enzymes such as proteases and glycosidases that degrade or modify proteins of the immune system and thereby avoid recognition and/or elimination.

Streptococcus and *Corynebacterium* are two genera of Gram-positive bacteria. While many streptococcal and corynebacterial species are part of the normal flora in animals and humans, some are important pathogens or opportunists. These are for example *Streptococcus pyogenes* and *Corynebacterium diphtheriae*, that are human pathogens, and *Corynebacterium pseudotuberculosis* and *Streptococcus dysgalactiae* subspecies *dysgalactiae* (SDSD) that are animal pathogens.

The main subject of this study was to identify new endoglycosidases in SDSD and corynebacteria and investigate if they are active against glycoproteins of the human immune system. The aim of this study was to increase the understanding of how such glycosidases might be involved in bacterial immune evasion, but also evaluate if some of theme could be developed into biotechnological tool for purifying and/or modification of glycoproteins of the immune system.

This thesis summary gives an overview about protein, and especially antibody, glycosylation, different antibody glycoforms, and exemplifies analytical workflows for glycan analysis. Furthermore, I will discuss the relationship

between bacterial glycosidases and immune evasion, and introduce the concept of enzymatic antibody glycan hydrolyzing enzymes as a biotechnological tools and potential biopharmaceuticals against immunological disorders.

Glycosylation

Glycosylation is the most common post-translational protein modification, and most of the key molecules in the innate and adaptive immune system are glycoproteins. The carbohydrate moieties (glycans) of glycoproteins are found in different structures and compositions and effect the chemical, physical and biological properties of glycoproteins (Rudd *et al.*, 2001).

Glycans are important for protein folding and assembly. Glycans located on the surface of proteins can protect proteins from proteolysis in the serum (Rudd *et al.*, 2001). Glycans can also determine the orientation and location of the binding faces of the proteins to which they are attached (Kuttner-Kondo *et al.*, 1996).

Glycoprotein diversity

Protein glycosylation is a diverse modification and natural glycoproteins consist of mixtures of glycoforms. Consequently, the same peptide backbone with different glycosylation pattern makes the structure of the glycoproteins more heterogenous than non-glycosylated proteins (Goodfellow *et al.*, 2012). In human, glycans are classified into two groups based on the nature of their linkage: *N*-linked glycans start with an *N*-acetylglucosamine residue attached to the amide nitrogen of asparagine residue by the recognition of a glycosylation sequence (Asn-X-Ser/Thr), and *O*-linked glycans that begin with an *N*-acetylgalactosamine attached to a hydroxyl group of serine or a threonine. *O*-linked glycans are short while *N*-linked glycans usually contain several branches (Varki *et al.*, 2009).

Glycosidases and glycoside hydrolases (GHs)

Enzymes that hydrolyze carbohydrates on *N*- and *O*-linked glycans are either exo- or endoglycosidases. Exoglycosidases hydrolyze the terminal residue on the

glycan chain and hydrolyze one specific residue, whereas endoglycosidases act within the glycan structure and can release several residues in one reaction (Sjögren & Collin, 2013). Glycosyltransferases are enzymes that catalyze glycosidic linkage to form a glycoside. These enzymes use activated sugar phosphates as glycosyl donors, and catalyze glycosyl group transfer to a nucleophilic group, usually an alcohol (Bojarová & Kren, 2009).

Enzymes that hydrolyze the glycosidic bonds are called glycoside hydrolases (GHs) and are subdivided into families based on amino acid sequence similarities. Today, there are more than 130 families described in the Carbohydrate-Active Enzymes (CAZy) database (CAZy, 2016; http://www.cazy.org).

Biosynthesis of *N*-glycosylation

Glycans attached to the amino acid sequences are ubiquitous in eukaryotic organisms. Glycosylation takes place in the endoplasmic reticulum (ER) and Golgi apparatus of the cells as a post-translational modification (Varki *et al.*, 2009). Biosynthesis of all *N*-linked glycans occurs in rough ER with the addition of a large preformed oligosaccharide precursor. This structure is linked by a pyrophosphoryl residue to a dolichol lipid that is embedded in the ER membrane. Dolichol acts as a carrier for the oligosaccharide precursor. The precursor contains three glucose (Glc), nine mannose (Man), and two *N*-acetylglucosamine (GlcNAc) molecules ($Glc_3Man_9(GlcNAc)_2$) (Figure 1). This structure is conserved in all eukaryotes. The glycan precursor is transferred *en bloc* from the dolichol carrier by oligosaccharide-protein transferase to an asparagine residue on a newly synthesized polypeptide. The substrates for transferases are Asn-X-Ser and Asn-X-Thr. Before entering to the Golgi, glycan residues are trimmed down to the core mannose and three glucoses. At least one mannose sugar will be removed from the *N*-glycan in the precursor and $Man_8(GlcNAc)_2$ that enters the Golgi apparatus. The removal of glucose prepares the glycoprotein structure for correct folding before transition to Golgi (Hossler *et al.*, 2007). In the Golgi, first mannose residues are removed and then different transferases add glycan residues one by one to the different positions of the glycan precursors (Figure 1) (Varki *et al.*, 2009).

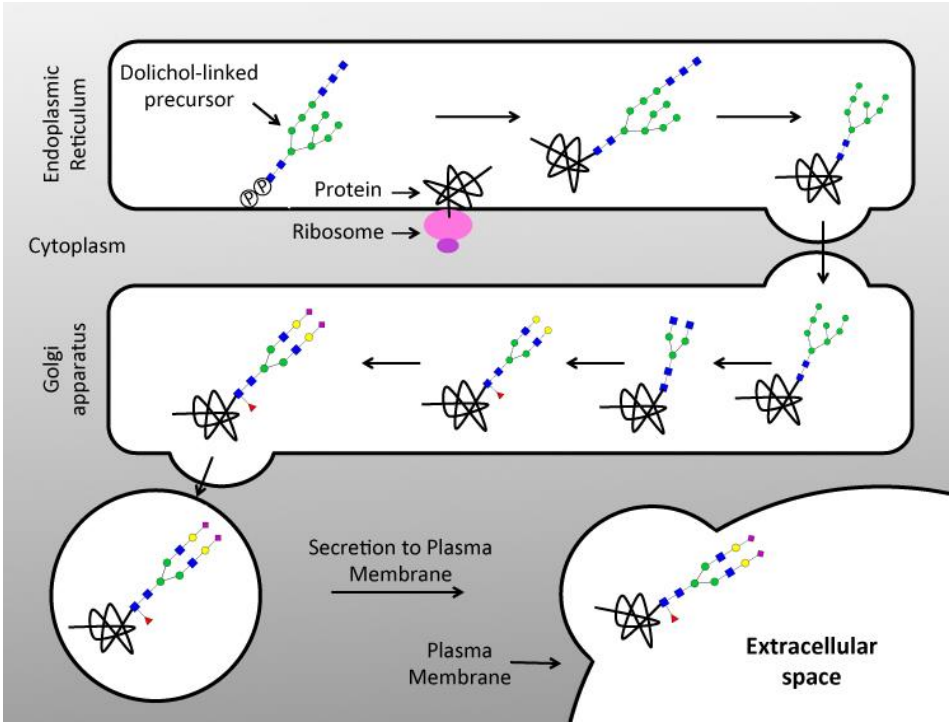

Figure 1. Biosynthesis pathway of *N*-glycosylating a protein
The biosynthesis of *N*-glycan starts in the endoplasmic reticulum and Dol-P-P glycan is transferred to the newly built protein and the precursor is transferred to the Golgi apparatus. Then the mannose residues are trimmed and after galactosylation and sialylation the glycan is integrated into the cytoplasmic membrane or secreted to the extracellular space.

N-glycan types

N-linked glycans are classified in three different categories: high-mannose, hybrid or complex-type structures, all carrying the core $Man_3GlcNAc_2$ structure. High mannose structures consist of 5 to 9 mannose. In complex-type structures, 2 GlcNAc are attached to the tri-mannosyl core and a combination of high mannose structure and complex-type build hybrid type structure (Figure 2)(Hossler *et al.*, 2007).

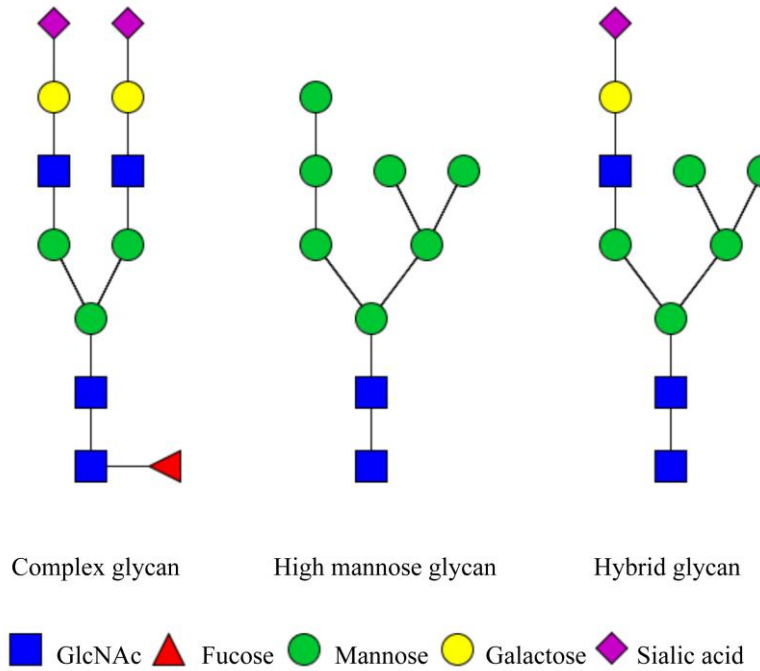

Figure 2. Types of glycans attached to asparagine in *N*-linked glycans
The structure of different *N*-glycan chains attached to the protein backbone is shown schematically. Monosaccharide structures conform to the standard colors and shapes as devised by the Consortium for Functional Glycomics. GlcNAc: *N*-acetylglucosamine

Glycan analysis

Many different methods have been developed to analyze glycan structures such as high/ultra performance liquid chromatography (HPLC/UPLC), nuclear magnetic resonance (NMR), mass spectrometry (MS), capillary electrophoresis (CE) and lectin arrays. Samples should be prepared using different chemicals, equipment and techniques depending on the analytical method. For most of the techniques glycans must be released and separated from the proteins, and glycans may require labeling. For example for analysis of glycans using MALDI-TOF-MS samples do not need to be labeled, while for HPLC and UPLC they must be labeled (Figure 3).

The first essential step for preparing the sample is releasing glycan from the protein backbone of the glycoprotein. The most common way to release *N*-glycan is hydrolysis of the glycoprotein with peptide-*N*-glycosidase F (PNGaseF), an amidase that hydrolyzes the bonds between *N*-acetylglucosamines of *N*-linked glycans and the asparagines they are attached to. The enzymatic reaction is

performed for at least 10h for complete or nearly complete release of glycans. After digestion, molecular weight cut-off filters can be used to separate proteins and the released glycan glycans that subsequently are subjected to labeling. To detect glycans conjugation to fluorescent tags are used. The most common fluorescent label is 2-aminobenzamide (2-AB). 2-AB is an ideal label since it is stable, non selective, allows for quantitative measurement of the relative amounts of the individual glycans, and is compatible with a variety of glycoanalytical methods (Royle *et al.*, 2008). Separation and labeling are important to prepare a high quality glycan for analysis. Therefore, it is necessary to remove contaminants such as salts and also excess of label from samples before chromatography. There are different methods for sample cleanup such as gel filtration, liquid-liquid extraction, solid phase extraction (SPE) (Nakano *et al.*, 2003; Royle *et al.*, 2008). Among the cleanup methods, SPE method is usually used since there is a great selection of stationary phases. SPE methods are also well suited for high-throughput set ups.

Since the nature of glycans is hydrophilic, hydrophilic interaction chromatography (HILIC) is a liquid based method that can be used for *N*-glycan separation and differentiation. In glycan separation with HILIC methods, the glycan structure, size, linkage, branches and charge can influence the separation (Huang *et al.*, 2016). To calibrate HILIC columns, dextran ladder can be used and every glycan profile is assigned with glucose units values (Campbell *et al.*, 2008).

A combination of liquid chromatography and mass spectrometry (LC/MS) is a developed approach to characterize glycans (Huang *et al.*, 2016). In this thesis released IgG glycans treated with exoglycosidases were labeled with 2-AB and after HILIC separation analyzed with HPLC or UHPLC. The chromatograms of released glycans from each reaction were obtained from HPLC according to their retention time and compared with the published IgG chromatograms that were detected by UHPLC coupled by MS. The glycan structures are presented according to the Consortium for Functional Glycomics (Figure 2) (Harvey *et al.*, 2009).

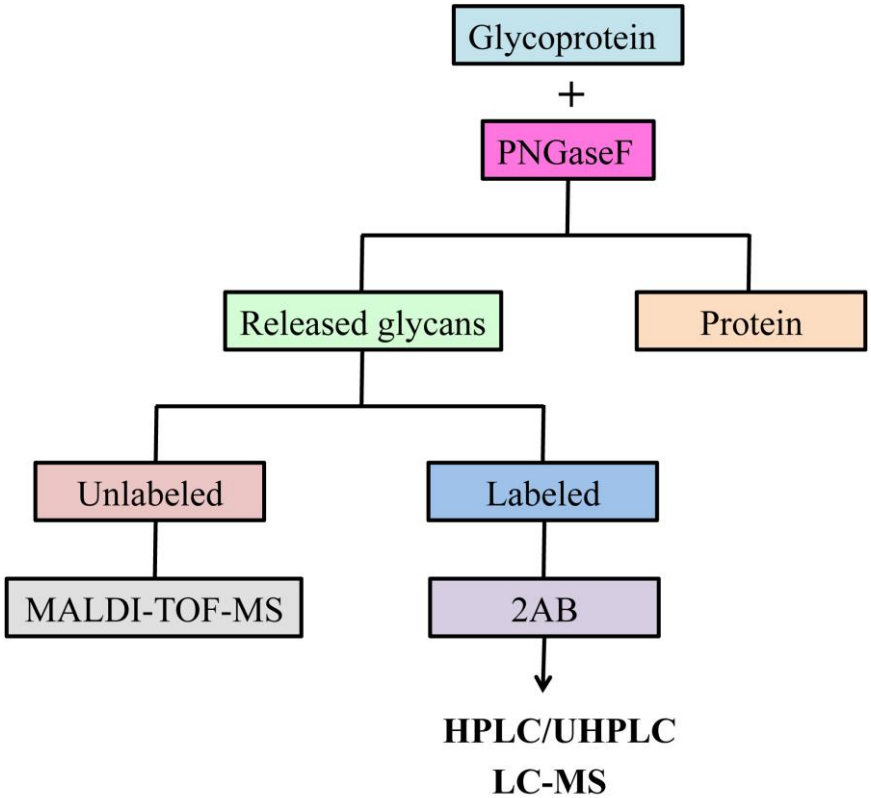

Figure 3. Schematic flowchart of the most common experimental methods for analyzing *N*-glycans
The chart shows the overview of glycan preparation for analyzing. The glycoproteins are incubated with PNGaseF and released glycans are subjected for labeling or MALDI-TOF-MS. The glycans labeled with 2AB are subjected to HPLC/HPLC or LC-MS. (2AB: 2-aminobenzamide)

Immunoglobulins and glycosylation

Immunity is the ability of the host to resist a particular infection for instance by producing antibodies and specific cells against the pathogens and attack them. The immune system prevents infection by coordinating innate and adaptive immunity. The first response of the immune system to infectious foreign substances is provided by innate immunity and this response enhances adaptive immune response (Hoebe *et al.*, 2004). Physical, chemical, and microbiological barriers in the host are involved in the innate immune system. The elements, in this immunity are neutrophils, monocytes, macrophages, complement, cytokines, and acute phase proteins, which provide immediate host defense. However, the antigen specific response is generated by the adaptive immune system through T lymphocytes and B lymphocytes (Parkin & Cohen, 2001). In the early stages of infection activated macrophages release cytokine that activates neutrophils that are migrating to the site of infection and remove organisms by phagocytosis. Complement system factors are activated in a cascade sequence and via three pathways: classical, alternative and lectin pathways. It has an important role to detect and kill invading pathogens and also in clearance of apoptotic and necrotic cells (Schatz-Jakobsen *et al.*, 2016). The antigen-antibody complex activates the classical pathway of complement. This activation leads to the formation of C3 convertase via the recruitment of other proteins. Hydrolysis of C3 by C3 convertase results in signal amplification, further complement activation and cleavage of C5. The cleavage of C5 further activates a cascade leading to the assembly of the complement membrane attack complex and cell lysis. The lectin pathway is very similar to classical pathway, however it initiates with binding of mannose binding lectin (MBL) to the carbohydrates on the glycoproteins of microorganisms.

In addition there is a third, alternative pathway, leading to complement activation. Similar to the classical pathway, it is initiated by hydrolysis of C3, however in this pathway factor D cleaves factor B into Ba and Bb. Bb binds to C3 and forms

C3Bb that is C3 convertase. The C3 convertase subsequently binds to properdin and forms C3bBbP. This compound can form the C5 convertase by binding to an additional C3b. The rest of the alternative complement cascade is similar to classical pathway (Schatz-Jakobsen *et al.*, 2016).

Lymphocytes are the most important members of the adaptive immune system. Both B cells and T cell recognize antigens by their receptors. These lymphocytes are produced in bone marrow. At the early development stage T cells migrate to the thymus to be developed while B cell will be developed in Bone marrow. When these cells encounter an antigen, it leads to activation and differentiation that occurs in the lymphoid tissues. After differentiation T cells are migrating to the site of infection and B cells produce antibodies. Antigen is presented to the T cells in the MHC molecules (Major histocompatibility complex) by antigen presenting cell such as dendritic cells. T cells will be activated and move to the site of infection and initiate cytotoxic attack, or stimulate an inflammatory response (Parkin & Cohen, 2001).

Recognition of antigen by an Ig in the B cell receptor induces B cell to proliferate. B cells may differentiate to plasma cells that produce antibodies or into memory B cells which responses quickly when it subsequently encounters the same antigen. On the other hand antigen-presenting cells such as dendritic cells activate T cells. Activated T cells lead to activate B cells and it leads the production of Igs (Clark & Ledbetter, 1994).

Opsonization and phagocytosis are two important processes that the immune system uses to eliminate antigens. Opsonization involves the binding of antibody to the antigen. After phagocytes (such as macrophages) are attracted to the antigen-antibody complex and bind to the Fc part of IgG phagocytosis occurs. Phagocytosis is a process where the phagocyte engulf a particle and digest it (Parkin & Cohen, 2001). Macrophages use this mechanism to eliminate microbial agents.

Immunoglobulins (Ig) are the most abundant proteins of the adaptive immune system. There are five distinct classes of Igs in humans: IgG, IgM, IgA, IgE, and IgD (Arnold *et al.*, 2007).

Igs consist of two identical light chains (LC) and two identical heavy chains (HC). Igs are characterized by a distinctive set of glycoforms that vary in different classes of Igs. There is also diversity in *N*-linked glycosylation part in Igs both in Fab (antigen-binding fragment) and Fc (crystallizable fragment) (Arnold *et al.*, 2007). The hinge region of Ig can be *O*- or *N*-linked glycosylated. There are no conserved glycosylation sites on LCs. The glycans are multifunctional, playing crucial structural roles such as maintaining solubility and conformation and effector functions by providing optimal binding of the Fc to Fc receptors (FcR) (Mimura *et al.*, 2000; 2001).

IgG is the major immunoglobulin in human serum. This glycoproteins composed of 82–96% protein and 4–18% carbohydrate (Vidarsson *et al.*, 2013)

IgG is divided into four subclasses: IgG1, IgG2, IgG3, and IgG4 differ in their γ-chain sequences and disulfide bridging patterns. 2-3% of IgG mass constitute carbohydrates (Jefferis, 2009). Among all subclasses IgG1 is the most abundant serum IgG (Arnold *et al.*, 2007). Different subclasses of IgG have different effector functions and responses to different types of antigens, for example IgG2 mostly respond to bacterial capsular polysaccharide antigens and IgG3 is involved in response to viral infections. IgG4 can be induced by allergens in addition to IgE. IgG1 can be induced by soluble protein antigens and membrane proteins (Vidarsson *et al.*, 2013). The flexibility and length of hinge regions differs among different subclasses of IgG. IgG3 has longer hinge region provide more flexibility than the other IgG subclasses. The flexibility of the IgGs affects antigen-binding capacity and immune complex structure. It also can affect the orientation and movement of Fab to cover the binding site of C1q and FcγR (Roux *et al.*, 1997).

The molecular weight of IgG is about 150 kDa and it structurally composed of two 50 kDa γ heavy (H) chains and two 25 kDa κ or λ light (L) chains, linked together by inter-chain disulfide bonds. The heavy chain of IgG consists of three constant domains: CH1, CH2, CH3 (Vidarsson *et al.*, 2013).

Only 10–20% of the Fabs have *N*-glycosylation sites in the binding region but there is a single conserved *N*-linked glycosylation site (Asn-297) in each of the Cγ2 (CH2) domains (Vidarsson *et al.*, 2013). This glycan is involved in the quaternary structure and the stability of the Fc (Mimura *et al.*, 2000).

The Asn-297 site on each heavy chain, contains one of a family of 32 glycans with several naturally occurring variants of this glycan that can be specified to the three subsets: IgG-G0, -G1, and -G2 that demonstrate the number of terminal galactose residue on each arm. The degree of sialylation on Asn-297 varies as well as absence of bisecting GlcNAc. Glycans containing the galactose residues without terminal sialic acid are termed G0. Glycans lacking one galactose and fully galactosylated are termed G1 and G2 respectively (Figure 4) (Butler *et al.*, 2003). The galactosylation of the α1,6 arm is predominantly preferred, in compare with the α1,3 arm (Jefferis *et al.*, 1990).

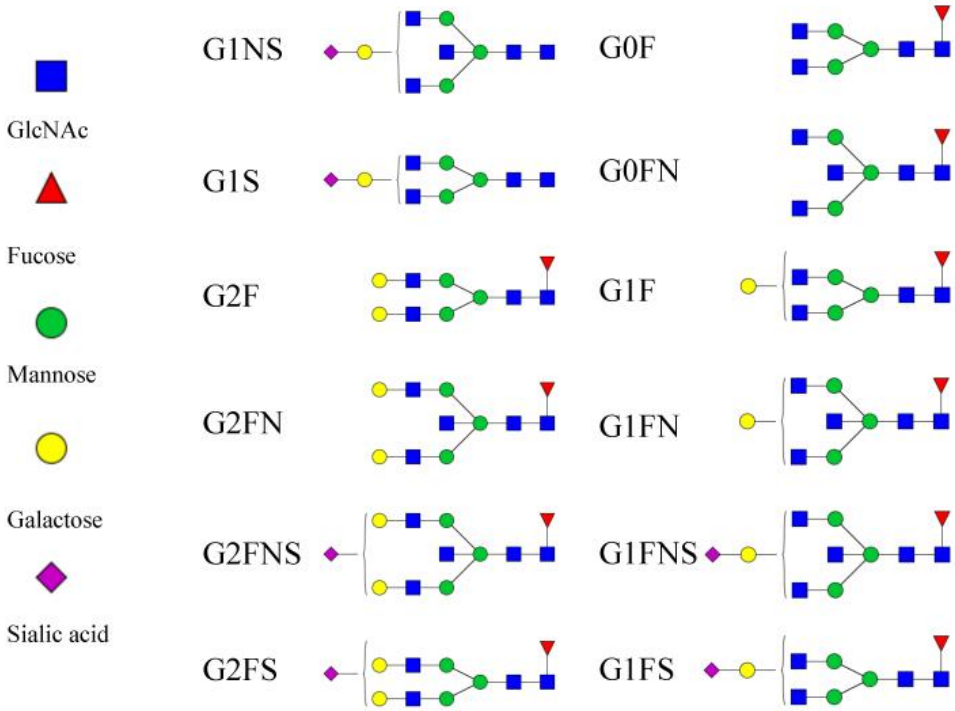

Figure 4. The most prominent *N*-glycan structures attached to the Asn-297 on IgG.
The schematic structure of G0 that ends with two GlcNAc, G1 with one galactosylation site and G2 with two galactosylation sites attached to the core mannose is presented. F and S are showing the proportion of fucosylated, sialylated, structures respectively and N corresponds to proportin of bisecting *N*-acetylglucosamine structure. GlcNAc: *N*-acetylglucosamine.

The functional role of IgG glycans

Human serum contains a variety of complex-type glycoforms attached to the Fc on IgG. It has been shown that although removal of the core fucose residue from the IgG Fc glycan does not have any effect on IgG binding to C1q, it enhances antibody-dependent cellular cytotoxicity (ADCC) (Shields *et al.*, 2002). Afucosylated IgG has higher affinity to FcγRIIIa on natural killer cells (Ferrara *et al.*, 2011; Mizushima *et al.*, 2011). Crystallographic data shows that carbohydrate–carbohydrate interactions generate complexes between FcγRIIIa and nonfucosylated Fc. One of the GlcNAc in FcγRIIIa is interacting with nonfucosylated Fc and stabilizes the complex between glycoproteins (Mizushima *et al.*, 2011). There are some ways to get afucosylated recombinant mAbs that is favorable for threating cancers. For example, knocking out the genes such as *FUT8* that is involved in transferring fucose to the nascent glycan precursor.

Another way could be overexpression of the enzymes that generates antibodies with bisecting GlcNAc that subsequently inhibits fucosylated structures (Becker & Lowe, 2003). Although fucosylation status of IgG affects binding of IgG to FcγR, bisecting GlcNAc is also important in interaction of IgG to FcγR. Fc glycoforms carrying a bisecting GlcNAc significantly increases the binding affinity of Fc to FcγRIIIa and enhances ADCC consequently independent of core fucosylation (Zou *et al.*, 2011).

Agalactosylation on IgG in human serum was found in patients with inflammatory diseases such as rheumatoid arthritis (RA), tuberculosis and Crohn disease (Collin & Ehlers, 2013; Parekh *et al.*, 1989). It was already shown that increasing G0 serum IgG correlates to RA (Parekh *et al.*, 1989; Scherer *et al.*, 2010). These G0 antibodies develop pro-inflammatory response via activating the complement pathway via mannose-binding lectin (MBL) and FcγR (Nimmerjahn *et al.*, 2007). Improvement of pristane-induced arthritis during pregnancy is related to galactosylation status of IgG. The level of galactosylated IgG ncreases during pregnancy and improves RA. Not only agalactosylated IgG, but also lack of terminal sialic acid increases the inflammatory activity of G0 antibody (Scherer *et al.*, 2010; Thompson *et al.*, 1992).

Anti-inflammatory activity of IgG is dependent on terminal sialic acid on CH2 domain and IgGs in patients suffering from autoimmune diseases such as RA show decreased level of sialylation (Quast *et al.*, 2015). High frequency of IgG lacking galactose and sialic acid is a sign of a pro-inflammatory state (Fokkink *et al.*, 2014).

Intravenous immunoglobulin (IVIG) is a purified IgG from serum of thousands of donors and is widely used as a therapeutic agent for inflammatory diseases. Galactosylated and sialylated IVIG has anti-inflammatory effect on RA (Kaneko *et al.*, 2006). Agalactosylated IgG can be used as a biomarker to distinguish prostate cancer (Kazuno *et al.*, 2016). The *N*-linked glycans on the Fc portion of each heavy chain can be symmetric or asymmetric. It has been suggested that high mannose structures on the heavy chain of IgG cause faster clearance mAb in human (Goetze *et al.*, 2011). However Liu *et al.* have recently shown that the effect on clearance on therapeutic antibodies was not proportional to the degree of modification and both symmetric or asymmetric glycan structures cleared at the same rate (Liu & Flynn, 2016).

Principles for therapeutic antibodies

Therapeutic antibodies are monoclonal antibodies all belonging to the IgG class that are used in immunotherapy (Jacobs *et al.*, 2016). They stimulate immune

system against specific antigen. The first therapeutic antibody was the CD3-specific monoclonal antibody muromonab. This antibody was approved by US Food and Drug Administrator to be used in human, for treatment of acute transplant rejection (Leavy, 2010). These days researchers are applying antibody engineering method to get recombinant version of mAbs. In this method the genes encoding mAbs are cloned into eukaryotic expression vectors and any mAb can be obtained using diverse cell lines in a reproducible form (Chames *et al.*, 2009). IgG is the important antibody used in recombinant monoclonal antibody (mAb) therapeutics that has developed to treat severe diseases such as cancer and autoimmunity (Le *et al.*, 2016).

For expression of IgG recombinantly, mammalian cells cultures such as Chinese hamster ovary (CHO), mouse NSO or SP2/0 plasma cell line are mostly used, but utilizing yeast and plant expression systems has also been investigated (Jefferis, 2005; Uçaktürk, 2012). Production of the mAb in a unicellular ciliate has recently been performed. The produced mAb showed enhanced ADCC that is the result of unusual *N*-linked glycosylation including afucosylated, high mannose glycans (Calow *et al.*, 2016).

The IgG will be matured by posttranslational modifications that influence the structure and function of IgG. The *N*-linked glycan on the Fc region of IgG is important for clinical activity of therapeutic antibodies. For optimization and improvement of the efficiency of therapeutic antibodies, changing the glycosylation pattern of IgG would be helpful. Fc glycoforms can influence pharmacokinetic behavior of mAb (Chiu & Gilliland, 2016). Some parameters play critical role on glycosylation pattern of mAb biopharmaceuticals such as culture conditions, type of host cell and clone selection (Loos & Steinkellner, 2012).

Over the past 10 years several kinds of therapeutic antibodies have been developed and the market for monoclonal antibodies has grown exponentially. Nowadays these antibodies are used to treat severe diseases such as cancers and autoimmune diseases. Researchers are modifying current therapeutic antibodies to enhance their specificity, immune effector functions, increase their half-life and optimize their binding capacity interact with selected Fcγ receptors (Sondermann *et al.*, 2013). The most abundant antibody in the blood is IgG, and IgG1 is the predominating subclass. Therefore, it is not surprising that most of currently licensed therapeutic antibodies are of the IgG1 subclass (Jefferis, 2009).

The most common and known therapeutic antibodies are: trastuzumab, bevacizumab, infiliximab, adalimumab, and rituximab. Trastuzumab is a human epidermal growth factor receptor 2 (HER2)-specific antibody and bevacizumab is a vascular endothelial growth factor A (VEGFA)-specific antibody. Both trastuzumab and bevacizumab are used in treatment of numerous cancers.

Infiliximab, the tumor necrosis factor (TNF)-specific antibody, and adalimumab are nowadays used to treat Crohn's disease, as well as rheumatoid arthritis and plaque psoriasis. Rituximab, a CD20-specific antibody also is used to treat rheumatoid arthritis and non-Hodgkin's lymphoma (Leavy, 2010).

Variation in glycosylation of mammalian IgGs

IgGs are present in the serum of all mammalians, but the *N*-glycosylation patterns on the CH2 domain of IgGs vary between species (Raju *et al.*, 2000) and it effects the biological and physiochemical functions of IgG (Garbe & Collin, 2012). The glycosylation pattern on IgG is species-specific in different mammalian IgGs (Raju *et al.*, 2000). Human IgG contains oligosaccharides with terminal *N*-acetyl-neuraminic acid (NANA), while sheep, goat, cow, horse, rhesus and mouse IgGs contain *N*-glycolyl-neuraminic acid (NGNA) instead. The IgG of dog, rat, rabbit and guinea pig contain both NANA and NGNA (Raju *et al.*, 2000).

In mammalian IgGs triantennary structure of oligosaccharides consisting of three GlcNAc are found, but in human those structures are not present, and there are just biantennary structures with bisecting GlcNAc. *N*-linked glycans on dog, cat, mouse and horse IgG does not contain bisecting GlcNAc. There is also a very low amount of galactosylated glycans on dog, horse and rat IgGs (Lewis *et al.*, 2008; Raju *et al.*, 2000). Mammalian IgG have different subclasses and for example horse IgG has seven subclasses. Among different subclasses of IgG in various animals some of them contain *O*-linked glycans like for example horse IgG3 that contains *O*-linked glycans attached to the heavy chain, and rabbit IgG and mouse IgG2b that contain *O*-linked glycans attached to the hinge region (Lewis *et al.*, 2008). *O*-linked glycosylation of IgG at hinge region protects IgG from proteases such as pepsin and papain (Parham, 1983).

The importance of Fc glycosylation

Antibody-receptor interaction

The Fc glycans are important for optimal Fcγ receptor (Fcγ R) binding (Arnold *et al.*, 2007). The FcγRs are located on the surface of most of leukocytes including macrophages, neutrophils, and lymphocytes. These receptors are divided into three distinct classes: FcγRI (CD64), FcγRII (CD32), and FcγRIII (CD16). All of these FcγR classes belong to the immunoglobulin superfamily and have an IgG-binding α-chain with an extracellular portion composed of Ig-like domains. The stability of Fc is dependent on the glycans at Asn-297 (Shields *et al.*, 2000). It has been shown that non-covalent interactions of multiple amino acid residues with the Fc

hexasaccharide that contains primary and secondary GlcNAc are necessary for optimal recognition of the protein-binding site by FcγRI (Lund *et al.*, 1996). Fc glycan confers an open conformation to the antibody structure allowing binding to the FcγR (Sondermann *et al.*, 2000). Both human FcγRIIIa and FcγRIIIb express a conserved glycan at position 162 that cause glycan-glycan interaction and stabilize the conformation of immune complex (Mizushima *et al.*, 2011). Ferrara *et al*. have suggested that core fucosylation, affects binding of IgG to receptor. Nonfucosylated antibodies have increased affinity to FcγRIII. The reason for this is that the interactions formed between the receptor carbohydrate at Asn-162 and regions of the Fc that are only accessible when the fucose is absent. Consequently, de-fucosylation of the *N*297-linked glycan in the Fc part of the IgG increases ADCC activity. This demonstrates the importance of glyco-engineering of antibody for improved clinical efficacy (Ferrara *et al.*, 2006).

Complement activation

IgG can activate complement. Complement activation is initiated through binding of IgG to non-self antigens and then binding of C1q to the Fc portion of IgG in the immune complex. This binding is followed by deposition of C3b to opsonize the target and form the membrane attack complex and finally disruption of the targeted lipid bilayer. IgG activation complement constitute the classical pathway (Sarma & Ward, 2010). The composition of the CH2 domain of IgG is important for binding of C1q and activation of complement, so different subclasses of IgG behave differently in activation of complement where IgG1 and IgG3 act more efficiently in this regard (Morgan *et al.*, 1995).

Alteration of opsonizing antibodies

Opsonization and phagocytosis are two significant host defense mechanisms involved in the battle against bacterial infections. After entering an antigen to the human body the adaptive immune system of the host, produces opsonizing antibodies directed towards surface-exposed antigens on the microorganism. By modification of the glycan structure on IgG, IgG would not be able to bind on FcγR and thereby prevent opsonization and phagocytosis. So bacteria could through such a mechanism evade adaptive immune system by alteration of opsonizing antibodies and it increases bacterial survival in human blood (Collin *et al.*, 2002).

Correlation with disease and contribution to pathogenicity

IgG plays an important role in chronic inflammatory processes leading to the destruction of healthy tissues during autoimmune diseases. Removing the sugar moiety leads to a dramatic reduction of the affinity of IgGs for cellular FcγRs and a loss in proinflammatory activity. Modifying antibody glycosylation *in vivo* might

be a promising strategy to reduce autoantibody-induced tissue destruction and chronic inflammation (Albert *et al.*, 2008).

Lack or altering the *N*-glycan on IgG can be correlated to specific disease. The galactosylation level is changing in patients with rheumatoid arthritis. Lack of galactose in oligosaccharide chain is associated with this disease. It has been shown that increased expression of agalactosylated IgG correlates directly to pathogenicity in murine collagen-induced arthritis. In this model most of the oligosaccharide chains on IgG are terminated in *N*-acetylglucosamine (G0) rather than galactose/sialic acid. These results indicate that agalactosylated IgG autoantibodies are pathogenic (Rademacher *et al.*, 1994).

Cellular and complement-mediated autoimmune hemolysis is highly dependent of the binding of anti-RBC IgG to FcRs. Monocytes bind and phagocytize anti-RBC antibodies via FcγR and also mediate immune RBC destruction to shorten RBC survival. This ability of monocytes is highly related to the glycosylation status of the Fc domain on the anti-RBC antibodies (Allhorn *et al.*, 2010).

Since deglycosylation of IgG reduces effector function such as complement activation and FcR binding, IgG mediated autoimmune diseases could potentially alleviated through IgG modification (Collin *et al.*, 2001b).

The benefits of recombinantly synthesized glycoforms

The knowledge of the molecular and structural features of glycoforms that are involved in pathological processes helps to develop specific glycoforms (Dalziel *et al.*, 2014). Alteration of cellular glycosylation pathways can decrease the symptoms of congenital metabolic disorders. For example disruption of viral replicative cycle dependent on glycosylation develops anti-influenza and potential new classes of anti-inflammatory drugs. Recombinant cellular biosynthetic technologies aids to produce defined glycoforms for developing therapeutic glycoproteins. A very important approach in usage of glycoforms in drug discovery is the development of monoclonal antibodies with engineered glycosylation (Dalziel *et al.*, 2014). Modulating glycans on glycoprotein by using bacterial glycosidases was shown to be promising method in preclinical studies for treating autoimmunity and cancer. Advanced technologies in glycobiology elucidates unusual features of antibodies and may offer new therapeutic opportunities for curing antibody-dependent diseases (Dalziel *et al.*, 2014).

Bacterial virulence and glycosidases

Streptococcus pyogenes

Streptococcus pyogenes (group A *Streptococcus*, GAS) is the causative agent of a broad spectrum of diseases. It is a Gram-positive bacterium that develops skin infections such as impetigo, infection in respiratory tract such as pharyngitis and also systemic disease such as toxic shock syndrome (Collin & Olsén, 2003). This pathogen is an important cause of morbidity and mortality and causes more than 500,000 death on a global scale annually (Carapetis *et al.*, 2005). The classification of *Streptococcus* in groups was based on specific and unique carbohydrate antigens on the bacterial cell wall (Lancefield, 1933). The group carbohydrate for GAS contains a polyrhamnose backbone with an immunodominant GlcNAc side chain, which helps for rapid diagnosis in streptococcal infections. Without the GlcNAc side chain, antibodies can kill the bacteria by opsonophagocytosis and protect the host from systemic infection (van Sorge *et al.*, 2014). These bacteria produce numerous virulence factors to defend themselves against the immune system and survive.

Streptococcal capsule

In GAS, a hyaluronic acid capsule encloses the bacterium. This high molecular weight polymer capsule is made of hyaluronan, alternating residues of *N*-acetylglucosamine and glucuronic acid, that helps bacteria to resist phagocytosis (Dale *et al.*, 1996). The human virulent GAS strains contain both M protein and capsule and cause invasive infections. There is hyaluronan in human connective tissue, which is chemically identical to the one in encapsulated GAS. So this component does not stimulate the immune system and help bacteria to develop the infection.

Streptococcal exotoxins

GAS often produce bacteriophage encoded pyrogenic exotoxins known as superantigen that are associated with streptococcal toxic shock syndrome (STSS) (Bisno *et al.*, 2003). It belongs to streptococcal pyrogenic exotoxins (Spe) family and are involved in severe invasive infections (Ferretti *et al.*, 2016a). Superantigens are highly potent immunostimulators able to stimulate T lymphocytes and activate them to release T cell mediators and pro-inflammatory cytokines. They are able to bind to the major histocompatibility (MHC) class II molecules that leads to activation of a large number of T cells and secretion of specific cytokines, such as TNFα, interleukin 1β, and T cell mediators such as interleukin 2 and interferon γ (Commons *et al.*, 2014; Ferretti *et al.*, 2016b).

Streptococcal proteases

SpeB

SpeB is a secreted streptococcal cysteine protease and best-characterized streptococcal virulence factor (Nelson *et al.*, 2011). Virulence factors are molecules produced by pathogens that enhance colonization, immunoevasion, immunosuppression, and contribute to pathogenicity. These molecules also help bacteria to enter and exit out of the cell and receive nutrition from the host. It is secreted in an inactive 40 kDa zymogen form. It will be cleaved autocatalytically into a 28 kDa mature form and becomes enzymatically active after reduction (Elliott, 1945). Active SpeB degrades several host proteins such as fibronectin (Elliott, 1945). This protein is be able to cleave IgG from the hinge region in a papain like manner (Collin & Olsén, 2001a). It was also shown that SpeB *in vitro* cleaves the heavy chains of IgA, IgM, and IgD into fragments and degrades the heavy chain of IgE (Collin & Olsén, 2001b) but the physiological relevance of this has been questioned (Persson *et al.*, 2013). It was shown that SpeB cleaves and degrades IgGs only in a reduced state, i.e., semimonomeric molecules and not under physiological conditions (Persson *et al*., 2013).

IdeS

IdeS is a secreted cysteine proteinase and a virulence factor from GAS with unique specificity for immunoglobulin G. This protease is able to cleave IgGs bound to *Streptococcus* cell wall and protect bacteria against phagocytosis. This enzyme cleaves IgG below the hinge region (Pawel-Rammingen *et al.*, 2002). In many applications this enzyme is used as a tool for analyzing monoclonal antibody structure and post-translational modifications. It have been used for structural characterization of monoclonal antibodies to facilitating the optimization of therapeutic mAb production (Kirley *et al.*, 2016). IdeS cleaves not only the soluble IgG but also the IgG molecule when it is present in the B cell receptors (BCR) attached to the B cell membrane (Järnum *et al.*, 2015). Because of the unique degree of specificity of IdeS, it can be used for complete, rapid but temporary removal of IgG from the entire plasma *in vivo* to inactivate Fc-mediated effector function in transplantation and autoimmune diseases (Winstedt *et al.*, 2014).

Group C and G streptococci

According to the Lancefield classification, different *Streptococcus* are clustered in specific groups based on their carbohydrate group antigen that is an integrated part of the cell wall. *Streptococcus dysgalactiae* and *Streptococcus equi* are mainly clustered in group C and G streptococci (GCS, GGS) and similar to group A are

belonging to the pyrogenic group of streptococci. GCS, GGS frequently have been isolated from humans in the clinical microbiology laboratory (Trell *et al.*, 2016) These groups are evolutionary related to GAS and share many virulence-associated genes and cause a similar spectrum of diseases (Bramhachari *et al.*, 2010). Targeted microarray containing 216 GAS virulence genes has suggested that half of the GAS virulence genes used in the experiments were identified in GCS, GGS, however there was not any statistical differences between GCS samples versus GGS (Davies *et al.*, 2007). *S. equi* subsp. *equi* is an animal pathogen, a member of GCS, and is the causative agent of strangles in horses (Harrington *et al.*, 2002). Strangles is the most contagious illness among horses and spreads rapidly to the lymph nodes and cause lymphadenitis and infiltration in polymorphonuclear leukocytes. The disease is associated with the *S. equi* M-like protein (Galan & Timoney, 1985). The bacteria express a homolog of IdeS named IdeE, which is very similar to IdeZ an IdeS-like protein in *S. equi* subsp. *zooepidemicus* (Lannergård & Guss, 2006).

Streptococcus dysgalactiae is divided into two subspecies: *Streptococcus dysgalactiae* subsp. *equisimilis* (SDSE) and *Streptococcus dysgalactiae* subsp. *dysgalactiae* (SDSD). SDSE are human isolates and β-hemolytic while SDSD are α- hemolytic and animal isolates (Facklam, 2002; Jensen & Kilian, 2012). SDSD and SDSE are able to produce M protein and superantigens similarly to GAS (Bisno *et al.*, 1996; Commons *et al.*, 2014). SDSE cause similarly to GAS localized infection such as respiratory tract, skin infections and severe infections such as arthritis, and necrotizing fasciitis (Bramhachari *et al.*, 2010). There is a significant overlap in the virulence factors and infection spectrum between GAS and SDSE. This could in part be explained by horizontal gene transfer from GAS to SDSE (Davies *et al.*, 2007). SDSD is one of the most important animal pathogens and is associated with bovine mastitis that results in substantial economical loss in dairy industries. So prevention of the diseases causes by SDSD is of commercial interests. Although SDSD is not known as human pathogen but caries a lot of important virulence genes responsible for GAS pathogenesis in humans (Rato *et al.*, 2011). Many of GAS virulence genes are distributed in SDSD by mobile genetic elements (MGEs) such as M1 and M3, which are carried by bacteriophages. SDSD has therefore been suggested to be an emerging zoonotic pathogen (Rato *et al.*, 2011).

Streptococcal IgG binding proteins

M protein

One of the most important virulence factors in GAS is the cell wall-anchored M protein (Bisno *et al.*, 2003). The M protein appears as fibrils on the cell surface because of its α-helical coiled-coil configuration. This structure contains a complex of two polypeptide chains that comprise four repeat blocks of amino acids. The carboxy (C) terminal of the M proteins is highly conserved and located in the cell wall of the bacteria, however the amino (N) terminal of the proteins shows diversity among different clinical isolates and is known as a hypervariable region in this protein. There are many serotypes of GAS that identified based on their M proteins. M1 producing GAS are the most frequently isolated bacteria from patients (Bisno *et al.*, 2003). The ability of M protein to bind fibrinogen protects bacteria against phagocytosis (Wang *et al.*, 1995). M protein has ability to bind to IgG Fc and contribute to resistance to phagocytosis by human granulocytes. Some M proteins can bind IgA as well (Podbielski *et al.*, 1996). The M protein also interferes with the alternative pathway of the complement and impedes opsonization (Fischetti, 1989).

Protein G

Protein G is a cell wall-anchored protein from group C and G streptococci. It is about 65 kDa in group G and about 58 kDa in group C streptococci. Protein G has affinity to both IgG and human serum albumin, however in some strains of group G it only binds to IgG. This protein is binding to the CH2-CH3 interface of the Fc region of IgG. The albumin binding site is located in the N-terminal of the protein and IgG binding site in C-terminal (Sjöbring *et al.*, 1991). This protein is also able to bind Fab of IgG (Derrick & Wigley, 1992).

The C-terminal of protein G has three domains binding to IgG (Olsson *et al.*, 1987). This protein has become a standard tool for purification of IgG, however to decrease the albumin contamination in the sample albumin-binding site on protein G has been removed. The cell wall-anchored region of protein G has also been removed from the protein to decrease the unspecific binding of proteins (Zhang *et al.*, 2015). Protein G is able to bind to all 4 subclasses of human IgG (Aybay, 2003).

Protein H

Protein H is another cell wall-anchored in *S. pyogenes*. This 40 kDa protein only binds Fc the region of IgG and not Fab region. It cannot bind other immunoglobulins. Protein H can bind weakly to the protease inhibitor α-macroglobulin (Åkesson *et al.*, 1990). This protein can similarly to protein G bind to serum albumin. It binds to albumin with higher affinity compare with protein H

to IgG. The binding site of protein H on IgG is overlapping with the binding site for protein G. (Frick *et al.*, 1994). Similar to streptococcal M protein that contains C repeats, protein G has C repeats responsible for binding to serum albumin. The IgG binding site of protein H is located in N-terminal and albumin binding site is close to the cell wall.

Protein H produced by GAS can bind baboon, rabbit and pig IgGs (Åkesson *et al.*, 1994). The gene *sph* which codes for protein H can be found in most of streptococcal strains but it is not frequently expressed. The gene is upregulated when it is injected to the mouse skin (Smith *et al.*, 2003). Protein H found to be involved in bacterial aggregation and it also helps bacteria to move from the first site of infection to the secondary site (Frick *et al.*, 2000).

Streptococcal endoglycosidases

EndoS

EndoS is an endo-β-*N*-acetylglucosaminidase from Group A streptococcus (GAS) that hydrolyzes the β1-4 linkage between the two GlcNAcs in the chitobiose core of the *N*-linked glycan on Fc region of IgG (Figure 5) (Collin & Olsén, 2001a). EndoS was found to specifically cleave *N*-glycan from IgG, but it does not affect the oligosaccharide chain on other immunoglobulins such as IgA, IgM, IgD and IgE (Collin & Olsén, 2001a). In contrast, the protease SpeB displays activity on all immunoglobulins (Collin & Olsén, 2001b). SpeB also has proteolytic activity against EndoS. (Allhorn *et al.*, 2007).

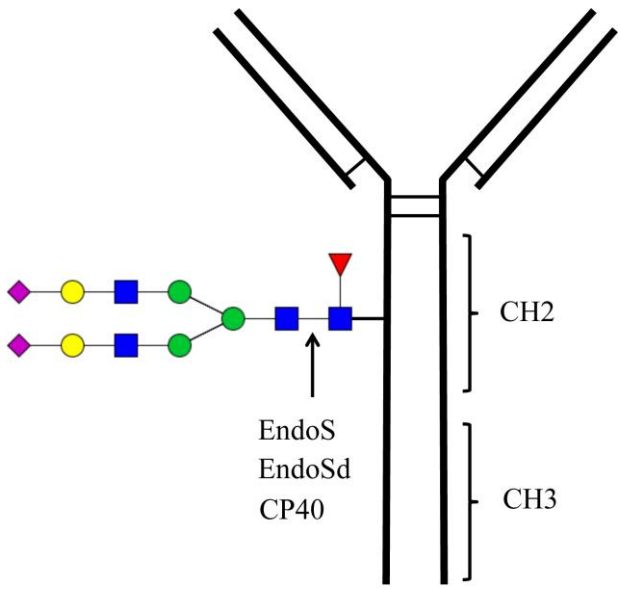

Figure 5. The structure of the *N*-linked glycan.
The *N*-glycan strucure on the CH2 domain of IgG was shown according to the Consortium for Functional Glycomics. The arrow indicates the cleavage site of EndoS, EndoSd and CP40 enzymes that is between the two GlcNAcs, in the chitobiose core of the *N*-glycan.

The Fc region of IgG is important for promoting effector function of IgG since it is interacting with FcγR on the surface of effector cells such as monocytes, macrophages, neutrophils, B cells, and also complement factor C1q (Burton, 1985). The stability of IgG is dependent to the *N*-glycan and aberrant IgG glycosylation impedes the effector function of IgG. Furthermore, alterations in IgG glycosylation has been associated with a number of autoimmune disorders, such as rheumatoid arthritis (RA) (Nandakumar *et al.*, 2007).

EndoS is able to cleave IgG glycan moiety when bacteria are grown in the presence of plasma (Collin & Olsén, 2001a). In comparison with other streptococcal IgG binding proteins such as protein G that binds to both native and deglycosylated IgG (Sjöbring *et al.*, 1991), activity of EndoS specifically cleaves the native and not denatured IgG (Collin & Olsén, 2001b). EndoS has the ability to hydrolyze *N*-glycan from all four subclasses of IgG and also when they are bound to FcγR, which causes dissociation of IgG from its receptor. EndoS behaves in a touch and go manner, however inactive EndoS with a mutated active site can bind to Fc region of IgG with relatively high affinity (Allhorn *et al.*, 2008).

It was shown that EndoS can hydrolyze bisecting GlcNAc but slower than biantennary complex glycans (Dixon *et al.*, 2014).

Streptococcus equi subsp. equi expresses a homolog of EndoS named EndoSe (Flock *et al.*, 2012). EndoSe shows 70% identity to the EndoS. EndoS2 is another endoglycosidase that is expressed by serotype 49 GAS strains with 37% identity with EndoS. EndoS2 showed endo-β-*N*-acetylglucosaminidase activity on native IgG and also α_1-acid glycoprotein (AGP) (Sjögren *et al.*, 2013). All of theses enzymes belong to the 18 glycoside hydrolase family (GH18) (CAZy, 2016; http://www.cazy.org). All members of this family have the conserved motif DXXDXDXE (The X represents any amino acid). The glutamic acid in this motif is important for the glycosidase activity of EndoS. This glutamic acid is in the position 235 in EndoS (Allhorn *et al.*, 2007).

X-ray crystal structure of EndoS indicates that this enzyme is composed of five distinct protein domains, including glycosidase, leucine-rich repeat, hybrid Ig, carbohydrate binding module (CBM), and three-helix bundle domains in a V-shaped conformation (Figure 6) (Trastoy *et al.*, 2014). The chitinase domain of EndoS is located at the N-terminal of the protein where as LRR and CBM are located downstream of chitinase motif. Although the chitinase motif is important for the activity of the enzyme, C-terminal truncation can impair this activity. So the C-terminal region of EndoS contributes to its activity (Dixon *et al.*, 2014). EndoS is able to hydrolyze glycans from IgG fragments, so it seems that this enzyme can continue its activity on the IgG fragments after IgG cleavage with SpeB and IdeS (Dixon *et al.*, 2014).

There are a lot of studies indicating the potential therapeutic effect of EndoS on inhibiting antibody mediated diseases. For example it was shown that EndoS in rabbits can cleave *N*-glycan from the whole IgG pool even in the presence of anti-EndoS antibodies without any effect on the health of the animals (Collin *et al.*, 2008). EndoS can deactivate antibodies against red blood cells (RBCs) in autoimmune hemolytic anemia, so aggregation of these cells and complement-mediated hemolysis cannot occur in the presence of EndoS. *In vivo* study in a

mouse model indicated that the development of anemia in mice caused by anti-RBCs and complement activation was reduced by EndoS (Allhorn *et al.*, 2010).

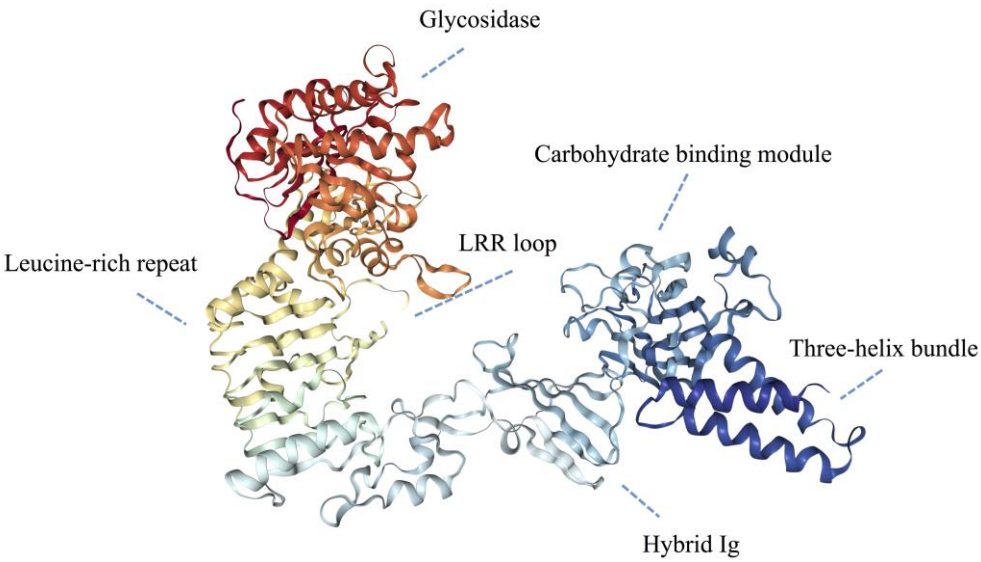

Figure 6. The structure of EndoS.
The two dimentional structure of EndoS and its different domains is presented. The dmains are arrenged in a V-shaped conformation. The crystal structure was obtained from Protein Data Bank (PDB) under 4NUY PDB ID.

IgG glycan hydrolysis by EndoS can inhibit progression of chronic neuroinflammatory demyelinating disorders. For example in multiple sclerosis (MS), intrathecal IgG synthesis and accumulating of B cells in MS lesions, indicates the important role of IgG in demyelinating and MS plaques (Breij *et al.*, 2007). Presence of deposited IgG around demyelinating MS plaques is observable in mice. IgG deglycosylation by EndoS in this model reduced the inflammatory lesions and demyelination and also serum complement consumption in the animals (Benkhoucha *et al.*, 2012). EndoS was also used to protect mice from immune thrombocytopenic purpura (ITP), that is an autoimmune disease with antibodies against platelets (Collin *et al.*, 2008).

EndoS2

As it was previously described EndoS2 is a unique endoglycosidase expressed by serotype 49 GAS. EndoS2 is a secreted enzyme and expression of that is dependent of the presence of sucrose in the culture medium. This enzyme has broader substrate profile compared with EndoS (Sjögren *et al.*, 2013). EndoS2 shows endo-β-*N*-acetylglucosaminidase activity on IgG and can hydrolyze

biantennary and sialylated *N*-glycans of AGP (α1-acid glycoprotein) (Sjögren *et al.*, 2013).

EndoS2 hydrolyzes glycans on therapeutic antibodies more rapidly than EndoS. This enzyme also hydrolyzes hybrid and high-mannose-type glycans of Fc region of IgG in addition to complex glycans. So EndoS2 can be used for rapid quantification of high mannose content of therapeutic antibodies (Sjögren *et al.*, 2015).

Corynebacteria

Corynebacterium is a genus of gram positive, bacilliform bacteria. Many of them can be found in normal flora of human and animals, however some of them are pathogenic. The most important human pathogen in this genus is *Corynebacterium diphtheriae,* whereas *Corynebacterium pseudotuberculosis* can cause disease in animals. *C. pseudotuberculosis* is the causative agent of caseous lymphadenitis (CLA, or cheesy gland) mainly in sheep and goat.

The bacteria are non-capsulated and non-mobile organisms (Dorella *et al.*, 2006). Since the bacteria are facultative intracellular pathogen they must be able to uptake iron in the poor environments. They have four genes, *fagA, B, C* and *D* that encode for proteins involved in iron acquisition. Proteins encoded by this gene cluster are involved in bacterial virulence (de Mattos Guaraldi *et al.*, 2013). This facultative anaerobe spreads within the herd mainly via contamination of superficial wounds, which can appear during shearing, castration and ear tagging, or by other traumatic events (Dorella *et al.*, 2006). Although *C. pseudotuberculosis* is an animal pathogen, some cases of lymphadenitis due to infection with these bacteria were reported in humans who occupationally were exposed to sheep (Peel *et al.*, 1997).

One of the most important virulence factors in these bacteria is phospholipase D (PLD), which is a potent exotoxin. PLD promotes the hydrolysis of ester bonds in sphingomyelin in mammalian cell membranes and facilitates bacterial spread from the initial site of the infection to other organs in the host. This is a permeability factor that raises dermonecrotic lesions (Songer, 1997). CLA is a chronic bacterial infection that cause abscess formation in lymph nodes and in organs such as lungs, liver and kidneys, liver and spleen (Seyffert *et al.*, 2010).

CLA is of commercial interest due to reduced production of milk, meat, and wool of the infected animals. Infected animals also show very low reproductive efficiencies. In this disease viable bacteria are protected inside the abscesses and treatment and controlling the infection with antibiotics is problematic. Furthermore, the disease is spreading quickly among the animals (Arsenault *et al.*,

2003). Various vaccine strategies have been tested, such as vaccines based on attenuated strains but the protection against the CLA has not been satisfactory. Search for finding cost effective strategies with the aim of obtaining long-lasting protection with few side effects lead researchers to use recombinant proteins to produce vaccines against CLA (Silva *et al.*, 2014).

CP40 is a *C. pseudotuberculosis* protein that has been shown to have protective effect against CLA when used as a vaccine. This secreted protein is one of the earliest antigens which antibodies against can be detected in the serum of infected sheep (Wilson *et al.*, 1995). Using CP40 as a vaccine shows 82% reduction in the proportion of infected animals (Walker *et al.*, 1994). Vaccine formulations using the recombinant CP40 provides a satisfactory survival percentages and antibody production in the murine model (Silva *et al.*, 2014). There are some reports about CLA infection in human, so vaccine against *C. pseudotuberculosis* in sheep could lower the number of human incidence of this zoonotic disease (Peel *et al.*, 1997).

C. pseudotuberculosis and *C. diphtheriae* are both toxin producing species. Although the major virulence factor in *C. pseudotuberculosis* is the exotoxin PLD, these bacteria can also produce diphtheria toxins (Selim *et al.*, 2016). *C. diphtheriae* is the causative agent of the toxemic disease diphtheria. In this acute disease, bacteria are localized to the upper respiratory tract, and ulcerates the mucosa through pseudomembrane formation (Hadfield *et al.*, 2000). In both *C. diphtheriae* and *C. pseudotuberculosis*, surface expressed pili are very important adhesion factors that aids bacteria to bind to epithelial cells (Tauch & Burkovski, 2015).

C. diphtheriae is transmitted by direct contact, and from the first site of the disease the toxin enters the circulation and damages other organs. It can cause systemic lesions of the heart, nerves and also skin (Hadfield *et al.*, 2000). The *tox* gene is encoding diphtheria toxin (DT) carried by corynebacteriophages (β tox+, γ tox+, ω tox+) that have the ability to integrate into the bacterial chromosome. The DT is a polypeptide composed of two subunits, the enzymatic active fragment A, and the cell receptor binding B. These subunits are linked together by disulfide bonds. The B subunit is not toxic but it is essential for penetration into the cell cytoplasm. Only one molecule of fragment A is enough to kill a cell by cleaving chromosomal DNA and also inhibiting protein synthesis (Nakamura & Wisnieski, 1990). There are nontoxigenic strains that can become toxigenic strains by lysogenic conversion (Anderson & Penfold, 1973).

C. diphtheriae present different carbohydrate residues such as sialic acids on its surface. Sialic acid molecules aid bacterial immune invasion via adherence and maintenance on the host mucous surface (Mattos-Guaraldi *et al.*, 2000). The bacteria contain a trans-sialidase mechanism that is also involved in immune invasion via increasing adherence to the host. Bacteria scavenge the sialic acid

from the glycoconjugates of host cells and use trans-sialidase or sialidase to decorate their surface with sialic acid, in order to evade host's immune system (Mattos-Guaraldi *et al.*, 2000). *C. diphtheriae* secretes NanH that is a sialidase. This enzyme cleaves α-linked sialic acid and also transfers the sialic acid from α-sialosides to galactose residue on glycan chains (Kim *et al.*, 2010a, b). This enzyme is cell wall-anchored and a virulence factor. The bacteria also produce the enzyme NanI, that in some strains is an intracellular sialidase without a signal peptide (Kim *et al.*, 2010a).

Important bacterial glycosidases that cleave *N*-glycans

Glycosidases play important roles for bacterial survival through immune modulation, adherence, and acquisition of nutrients. Bacteria express extracellular glycosidases that modify carbohydrate moieties of immune system glycoproteins and aid bacteria to evade host immune system. In pharmaceutical industry glycosidases can be used for glycoprotein modulation, glycoengineering and glycan analysis. In the previous part important glycosidases from *Streptococcus* and *Corynebacterium* have been discussed. Here are some examples of other bacterial glycosidases that can modulate *N*-linked glycan from host glycoproteins:

PNGaseF and EndoF

Elizabethkingia meningoseptica (formerly *Flavobacterium meningosepticum*) is a Gram-negative rod-shaped bacterium that is present in the human normal flora, but is also associated with infections in humans. It has recently been isolated from hospitalized patients and is an emerging hospital acquired pathogen in patients on hemodialysis. It can also cause meningitis, bacteremia and pneumonia in the newborns (Ratnamani & Rao, 2013).

PNGase (peptide-*N*-glycosidase) is not a glycosidase but an amidase from *Elizabethkingia meningoseptica* hydrolyzing the amide linkage between GlcNAc and asparagine in *N*-glycans. This enzyme hydrolyzes all classes of *N*-linked glycans such as high mannose, hybrid, complex and also bi-, tri- and triantennary type glycans. PNGaseF can hydrolyze glycan chains both on native and denatured glycoproteins, however high concentrations of this enzyme is required for complete or nearly complete release of glycans from native glycoproteins (Tarentino *et al.*, 1985). PNGaseF nowadays is used for glycan analysis and glycoengineering. Although his enzyme is an important enzyme in glycobiology and biotherapeutic industry, but there have been no investigations about the role for this enzyme in pathogenicity of the bacteria.

These bacteria produce three endoglycosidase named $EndoF_1$, $EndoF_2$, $EndoF_3$. The enzyme $EndoF_1$ hydrolyzes the linkage between two GlcNAc in the chitobiose

core of high-mannose and biantennary hybrid-type but not complex oligosaccharides. It leaves a single GlcNAc on the asparagine attached to the backbone of the protein. EndoF_2 hydrolyzes both high mannose and biantennary glycans (Tarentino *et al.*, 1985). EndoF_3 can hydrolyze high mannose and also bi- and triantennary glycans (Plummer & Tarentino, 1991).

EndoH

Endoglycosidase H is a β-*N*-acetylglucosaminidase from *Streptomyces plicatus* that hydrolyzes the linkage between two GlcNAc in the chitobiose core of *N*-glycans (Robbins *et al.*, 1984). This enzyme can cleave the bond between two GlcNAc in high mannose and hybrid glycans, but it does not affect biantennary complex glycans (Trimble & Tarentino, 1991). It hydrolyses the only *N*-glycan on the human glycoprotein RNaseB (Trimble *et al.*, 1978). Since all glycans are derived from the precursor of high mannose structure, this enzyme is used to study biosynthesis process of glycans and *in vitro* glycan synthesis.

NanA, BgaA, StrH

Streptococcus pneumoniae is an important human pathogen causing infections in the respiratory tract. These bacteria cause sepsis, meningitis, and pneumonia in humans (Bogaert *et al.*, 2004). *S. pneumoniae* expresses three surface associated exoglycosidases that act on *N*-glycan one after the other: NanA is a neuraminidase, BgaA a β-galactosidase and StrH a β-*N*-acetylglucosaminidase. NanA cleaves the terminal sialic acids from the glycan chains and aids bacteria in adherence to the epithelial surfaces. NanA, BgaA, StrH sequentially hydrolyze sialic acid, galactose and *N*-acetylglucosamine from oligosaccharide chain on human glycoproteins (King *et al.*, 2006). Association of these three enzymes to hydrolyze carbohydrate moiety of human glycoconjugates in the airway, reduce deposition of complement component C3 on the pneumococcal surface, makes bacteria resistant to opsonophagocytotic effect of neutrophils subsequent and contribute to bacterial survival (Dalia *et al.*, 2010).

EndoE

Enterococcus faecalis is a Gram-positive bacterium in the normal flora of the oral cavity and gastrointestinal tract. However some cases of pathogenicity of these bacteria were reported such as urinary tract infection, bacteremia and sepsis (Fisher & Phillips, 2009). These bacteria secrete the endoglycosidase EndoE that hydrolyses *N*-glycan on IgG and human lactoferrin (hLF). EndoE also can hydrolyze *N*-glycan from RNaseB, a human glycoprotein with predominantly high mannose glycans (Roberts *et al.*, 2000). The hLF is able to inhibit biofilm formation, but EndoE can reduce the biofilm inhibiting property of hLF by deglycosylating this glycoprotein (Garbe *et al.*, 2014).

It was shown that carbohydrate moiety from glycoproteins can be served as nutrition for bacteria in nutrient limited medium (Roberts *et al.*, 2000). *E. faecalis* uses the released glycan from hLF and RNaseB as nutrition during growth to survive in the lack of nutrition (Garbe *et al.*, 2014; Roberts *et al.*, 2000).

EndoE contains two enzymatic domains: α domain that contains a family 18 glycosyl hydrolase (GH18) motif and the β domain, which contains a family 20 glycosyl hydrolase (GH20) motif. These two domains give a unique feature to the enzyme (Collin & Fischetti, 2004). The β domain of EndoE is similar to the StrH from *S. pneumoniae* that contains two glycoside hydrolase family 20 catalytic domains (Pluvinage *et al.*, 2011).

Present investigation

Paper I

EndoS is a secreted endoglycosidase from *S. pyogenes* with the ability to cleave *N*-glycan from human IgG (Collin & Olsén, 2001a). Since EndoS affects IgG specifically, this enzyme is used as a tool for modulating IgG glycan in glycoengineering (Sjögren & Collin, 2013). We have investigated if the related bacterium *S. dysgalactiae* subsp. *dysgalactiae* (SDSD), expresses EndoS-like activity.

We found that in the genome of *S. dysgalactiae* there is an *ndoSd* gene exactly in the same genomic context as *ndoS* in *S. pyogenes*. The *ndoS* gene is located between *scrA* and *scrK* genes that are part of a phosphotransferase system (Collin & Olsén, 2001a). In two strains of *S. dysgalactiae* there was instead a putative *ndoS2d* gene in the same genomic context.

We found that EndoSd is a secreted endo-β-*N*-acetylglucosaminidase that cleaves the β1-4 linkage between the two GlcNAcs in the chitobiose core of the *N*-linked glycan of IgG. The chitinase motif of EndoSd is very similar to EndoS, however it has an extended C-terminal. EndoSd seems specific for IgG and hydrolyzes *N*-glycan from human and animal IgGs. Although the activity of EndoSd is very similar to EndoS, it can only hydrolyze biantennary and not glycans with bisecting GlcNAc. The SDSD expression of EndoSd is dependent on carbohydrate availability in the culture medium.

Paper II

EndoS hydrolyzes the carbohydrate moiety specifically on native IgG and not on other glycoproteins. The specificity of EndoS motivated us to test if inactive enzyme could be used to purify IgG, and compare the activity with the golden standard of IgG purification, protein G.

We have shown that a mutated EndoS with a deletion in the glutamic acid in the active site (EndoS(del235)) can bind to human and animal IgGs. Binding of EndoS(del235) to IgG can inhibit the binding of other IgG binding proteins to IgG. EndoS(del235) binds only native and glycosylated IgG, in contrast to protein G that binds irrespectively of denaturation and glycosylation status. We show that native IgG can be purified by EndoS(del235) using affinity chromatography.

We conclude that inactive EndoS is a very useful tool for purification native IgG, where biological activity and natural structure of IgG is essential. This enzyme could also be helpful in determination of glycosylation and denaturation status of IgG preparations.

Paper III

C. pseudotuberculosis is the causative agent of CLA, an infectious and contagious disease in sheep and goats. This disease can cause an economical loss due to reduction in meat, wool, and milk production (Walker *et al.*, 1994).

CP40 is a 40 kDa secreted enzyme from *C. pseudotuberculosis*. This enzyme was known as a serine protease, however it contains a putative chitinase motif similar to EndoS in *S. pyogenes*. Even the glutamic acid that is known to be important for the activity of enzyme is conserved in CP40 (Allhorn *et al.*, 2007). We therefore hypothesized that CP40 is an endoglycosidase with similar activity as EndoS.

Lectin blot shows endoglycosidase activity of CP40 on human, horse and sheep IgGs. Performing HPLC indicated the released glycans from IgG and revealed that glycosidase activity of CP40 is more similar to EndoSd a homolog of EndoS from *S. dysgalactiae*, since it cannot hydrolyze bisecting GlcNAc (Shadnezhad *et al.*, 2016b). Chitinase assays demonstrated that CP40 is not a general chitinase.

Gelatin zymography assay was performed to check the protease activity of CP40. It indicated that CP40 does not have any protease activity on gelatin. Based on the results of zymography, lectin blot and HPLC, it was obvious that CP40 is an endo-β-*N*-acetylglucosaminidase. Based on the protein sequence and endoglycosidase activity of CP40 on IgG, we believe that this enzyme has been wrongly annotated as a serine protease. Rather CP40 belongs to GH18 endoglycosidase and can potentially be used as a tool in glycobiology to remove *N*-glycosylation from IgG.

Using CP40 as a vaccine candidate in sheep has shown promising results (Silva *et al.*, 2014). Therefore, characterization of CP40 as a glycosidase may aid in the continued development of the enzyme as a vaccine and deepen the understanding *C. pseudotuberculosis* pathogenicity.

Paper IV

CP40 is an endo-β-*N*-acetylglucosaminidase from *C. pseudotuberculosis* (Shadnezhad *et al.*, 2016a). *C. diphtheriae* is the causative agent of the dreaded disease diphtheria and is closely related to *C. pseudotuberculosis.* We aimed to identify of CP40-like proteins in *C. diphtheriae*. Putative genes encoding proteins similar to CP40 was found in 22 different *C. diphtheriae* strains, however the gene was smaller that *cp40* in *C. pseudotuberculosis.* The gene was denoted *ndoCd* and the encoded protein EndoCd for endoglycosidase of *C. diphtheriae*.

A putative chitinase motif was found in all of the examined strains, however in the NCTC11397 strain the aspartic acid was replaced by the amino acid valine. There is no predicted signal peptide in EndoCd indicating that this enzyme could be an intracellular enzyme. Alignment of EndoCd sequence with a number of endoglycosidases and reconstruction of a phylogenetic tree indicated that EndoCd is closely related to EndoE from *Enterococcus faecalis*.

EndoCd could be over-expressed in *Escherichia coli*, however the protein formed inclusion bodies. The protein was solubilized and refolding was attempted. However, the protein preparations did not show any activity on a number of glycoproteins. At present we do not know if this is due to complete inactivity of the enzyme, or that we have not identified the correct substrate.

Further studies will show if EndoCd has activity on human glycoproteins and if the naturally occurring alleles with an incomplete chitinase motif are enzymatically active or not.

Conclusions

I. EndoSd is an endo-β-*N*-acetylglucosaminidase from *S. dysgalactiae* that hydrolyzes biantennary complex glycan from Fc region on human and animal IgGs. It is not a general chitinase and EndoSd expression is dependent on carbohydrate availability in the medium.

II. EndoS(del235) is a potent tool for purification of native and glycosylated IgG.

III. CP40 is an endo-β-*N*-acetylglucosaminidase from *C. pseudotuberculosis* that can hydrolyze biantennary glycans from horse, sheep and human IgGs.

IV. EndoCd is a CP40-like enzyme in *C. diphtheriae* with a putative a chitinase motif.

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Printed by Media Tryck, Lund University 2017 Nordic Ecolabel 3041 0903

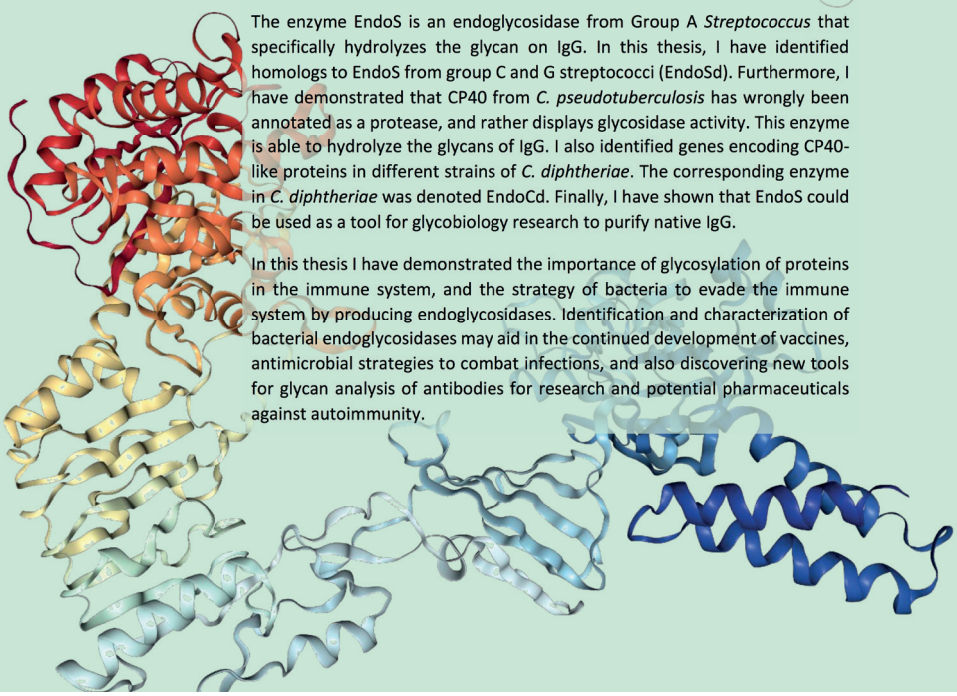
Bacterial IgG Modulating Endoglycosidases

Azadeh Shadnezhad

IgG is the most abundant immunoglobulin in the blood and an important factor for combatting bacterial infections. Like many plasma proteins, IgG is glycosylated. Alterations of these carbohydrates significantly affect its effector function and impede binding of IgG to its receptor on the surface of leukocytes. Many pathogenic bacteria produce glycosidases that hydrolyze these *N*-linked glycans on IgG and thus inhibit the effector function of IgG as an opsonizing molecule.

The enzyme EndoS is an endoglycosidase from Group A *Streptococcus* that specifically hydrolyzes the glycan on IgG. In this thesis, I have identified homologs to EndoS from group C and G streptococci (EndoSd). Furthermore, I have demonstrated that CP40 from *C. pseudotuberculosis* has wrongly been annotated as a protease, and rather displays glycosidase activity. This enzyme is able to hydrolyze the glycans of IgG. I also identified genes encoding CP40-like proteins in different strains of *C. diphtheriae*. The corresponding enzyme in *C. diphtheriae* was denoted EndoCd. Finally, I have shown that EndoS could be used as a tool for glycobiology research to purify native IgG.

In this thesis I have demonstrated the importance of glycosylation of proteins in the immune system, and the strategy of bacteria to evade the immune system by producing endoglycosidases. Identification and characterization of bacterial endoglycosidases may aid in the continued development of vaccines, antimicrobial strategies to combat infections, and also discovering new tools for glycan analysis of antibodies for research and potential pharmaceuticals against autoimmunity.



LUND UNIVERSITY
Faculty of Medicine

Lund University, Faculty of Medicine
Department of Clinical Sciences
ISBN 978-91-7619-401-0
ISSN 1652-8220