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Comparison of Lectins and their suitability in Lectin Affinity Chromatography for isolation of Glycoproteins

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Abstract

Comparison of Lectins and their suitability in Lectin Affinity Chromatography for isolation of Glycoproteins

Pontus Andersson

Virtually all extracellular proteins in humans are glycoproteins and likewise are many biopharmaceuticals. The glycosylation is directly correlated to biological function and stability of these proteins. The ability to isolate glycoproteins is thus of great importance in many applications. The most common isolation method for glycoproteins is affinity chromatography using lectins, a ubiquitous and versatile group of carbohydrate-binding proteins. The lectin Concanavalin A (ConA) has long been used for this purpose but suffers from undesired leakage into the eluate, causing an inquiry of alternative chromatography ligands or optimization of the ConA resin.
In this study, a total of 20 different lectins, including ConA, were evaluated and compared in terms of suitability as ligands in affinity chromatography for glycoprotein isolation. The lectins' binding to glycoproteins were studied, mainly through microtiter plate binding assays using a monoclonal IgG1 antibody and Conalbumin (Ovotransferrin). Further, sugar-specificities and potential eluting sugars for the lectins were examined through inhibition with eight different carbohydrates. Additionally, the glycoprotein binding and leakage of ConA columns were examined, and a potential leakage-reducing treatment of ConA resin evaluated.
ConA was found to be superior in binding to the investigated glycoproteins but exhibited a limited binding when immobilized to an agarose resin. This discrepancy is likely a consequence of structurally hidden glycans on the used glycoproteins and requirements of long residence time when used in a chromatographic setting. Binding competition with several sugars were investigated with a similar microtiter plate binding assay. This method displayed potential to predict the behaviour of sugars and their suitability as eluting agents in a chromatography column. The best eluting sugar for ConA was showed to be methylmannoside, ideally in combination with methylglucoside. Lastly, evaluation of ConA columns with a cross-linking glutaraldehyde-treatment showed that the ConA ligand leakage may be significantly reduced, although further studies and optimizations are needed.
This study thus presents a repertoire of lectins and their differences in terms of glycoprotein-binding and sugar-specificity, as well as evaluations of ConA columns' efficiency and potential leakage-prevention.

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Populärvetenskaplig sammanfattning

Inom såväl bioteknik som sjukvård är glykoproteiner av mycket stor betydelse. Glykoproteiner är proteiner till vilka sockerarter har kopplats på genom en process som benämns glykosylering. För eukaryota organismer, människor inkluderat, sker denna glykosylering som en post-translationell modifiering (PTM) för proteiner som ska lämna cellen. De adderade sockerarterna bidrar bland annat till att proteinerna får korrekt veckning och funktion. Exempel på glykoproteiner är mänskliga antikroppar, och likaså monoklonala antikroppar som ofta används inom bland annat cancerterapi. Faktum är att majoriteten av dagens biologiska läkemedel utgörs av olika typer av glykoproteiner (Hevér *et al.* 2019).

Glykoproteiner är dämed en viktig grupp av proteiner, vilket medför att effektiva metoder för att separera och rena fram glykoproteiner är nödvändigt. En vanlig metod för separation av proteiner är affinitetskromatografi, där provet passerar genom en kolonn packad med någon molekyl som känner igen och binder enbart till målproteinet i provet. Det bundna målproteiner kan därefter elueras ut med en elueringsbuffert lämplig för det aktuella proteinet. Sådana kolonner utvecklas och tillverkas till stor del av Cytiva Life Sciences (tidigare GE Healthcare Life Sciences) i Uppsala.

När det kommer till separation av just glykoproteiner, används vanligtvis kolonner packade med Concanavalin A (ConA) som främst känner igen och binder till mannos- och glukosrester som är vanligt förekommande hos glykoproteiner (O'Connor *et al.* 2017). ConA ingår i en grupp av proteiner som kallas för lektiner. Det som är gemensamt för alla lektiner är deras förmåga att känna igen och binda till sockerarter och därmed också glykoproteiner. Separation av glykoproteiner genom kromatografi med hjälp av lektiner kallas generellt för lektin-affinitetskromatografi. För att eluera ut glykoproteinerna från kolonnen efter separationen används en buffert med överflöd av fria sockermolekyler som binder till lektinen och konkurrerar ut glykoprotein-bindningen. Idag är ConA-kolonner en del av Cytiva Life Sciences produktkatalog. En nackdel med dessa kolonner är att en del av ConA-molekylerna tenderar att falla sönder under användning, vilket resulterar i ett läckage där det isolerade målproteinet kan kontamineras (Marikar *et al.* 1992). Sådan kontaminering kan vara förödande, då hög renhetsgrad av ett målprotein ofta krävs. Av denna anledning vore ett alternativ till ConA eller en stabilisering av existerande ConA-kolonner önskvärt för att undvika sådant läckage.

Syftet med denna studie var därför att undersöka alternativa lektiner och hur väl de passar i lektin-affinitetskromatografi för separation av glykoproteiner. Ett ytterligare syfte var att undersöka potentiella optimeringsmetoder för ConA-kolonner. Sammantaget var därmed studiens syfte att hitta lösningar för effektiv glykoprotein-rening där dagens läckage-problem undviks.

Detta gjordes genom att först och främst undersöka olika lektiners förmåga att binda till glykoproteiner i jämförelse med ConA. Två glykoproteiner, den monoklonala antikroppen mAb7 och äggviteproteinet Conalbumin, användes i studien. Resultat från en screening-metod och en mikrotiter-baserad metod visar på varierande bindningskapacitet men att inga av de totalt 19 undesökta lektinerna på allvar kan konkurrera med ConA vad gäller glykoprotein-bindning. Affinitetskromatografi med ConA-kolonn visade dock att ConA endast kunde binda en andel av glykoproteinerna. Särskilt dåligt var inbindningen av mAb7, då nästan 70% av den applicerade mängden åkte rakt igenom kolonnen. Detta beror sannolikt på att kolhydraterna på glykoproteinet är steriskt otillgängliga och svåra att nå då ConA är immobiliserat på en fast yta.

Lektinerna undersöktes vidare i termer av sockerspecificitet och potentiella eluerande socker genom en liknande mikrotiter-baserad metod. Detta visade att de undersökta lektinerna, som förväntat, besitter varierande sockerspecficitet. Vidare kunde potentiella eluerande socker föreslås för respektive lektin. För ConA var det exempelvis tydligt att α-metylmannosid (MeαMan) och α-metylglukosid (MeαGlc) hade störst potential. Samma resultat för ConA erhölls då olika eluerande socker användes vid affinitetskromatografi med ConA-kolumn, vilket indikerar att den använda mikrotiter-metoden ger en bra uppskattning av sockerfunktionaliteten vid kromatografi-tillämpning.

Slutligen utvärderades ConA-kolonner som behandlats med glutaraldehyd på ett sätt som potentiellt stabiliserar ConA och kan undvika dess läckage. Jämförelse av dessa behandlade kolonner med komersiellt tillgängliga ConA-kolonner visade att behandligen faktiskt tycks minska ConA-läckaget. Däremot tycks den metod som använts för stabilisering minska kolonnens bindingskapacitet något. Denna metod bör därför optimeras och studeras vidare, men kan ha potential att på sikt erbjuda en läckage-fri rening av glykoproteiner.

Denna rapport erbjuder därmed information om en reportoar av lektiner och dessas specificitet och potential för glykoprotein-rening. Den datan kan fungera som utångspunkt för framtida produktutveckling och vidare studier av lektiner. Studien visade också att den idag använda ConA har störst affinitet och bindingskapacitet för de undersökta glykoproteinerna, samt att MeαMan och MeαGlc tycks bäst för eluering från ConA-kolonner. Vidare visades att en stabiliseringsmetod av ConA-kolonner kan minska läckaget av ConA. Detta sammantaget leder till att ytterligare studier och optimering av metoder för stabilisering av ConA-kolonner rekommenderas.

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Abbreviations

Ab	Antibody
Asn	Asparagine
B_{max}	Maximal binding
ConA	Concanavalin A
ConBr	*Canavalia brasiliensis* lectin
CRD	carbohydrate recognition domain
CV	Column Volume
Cys	Cysteine
DBA	*Dolichos biflorus* agglutinin
DSL	*Datura stramonium* lectin
ECL	*Erythria cristagalli* lectin
Fab	Fragment antigen binding
Fc	Fragment crystallizable
Gal-GlcNac	*N*-acetyllactosamine
GalNAc	*N*-acetylgalactosamine
GlcNAc	*N*-acetylglucosamine
GNA	*Galanthus nivalis* agglutinin
GSL I	*Griffonia simplicifolia* lectin I
GSL II	*Griffonia simplicifolia* lectin II
HRP	horseradish peroxidase
IC_{50}	half maximal inhibition concentration
Ig	Immunoglobulin
JRL	Jacalin related lectins
LAC	Lectin-based Affinity Chromatography
LCA	*Len culinaris* lectin
LEL	*Lycopersicon esculentum* lectin
mAb	monoclonal antibody
MeαGlc	α-methylglucoside (methyl α-D-glucopyranoside)
MeαMan	α-methylmannoside (methyl α-D-mannopyranoside)
MW	Molecular weight
PA-IL	*Pseudomonas aeruginosa* lectin I
PBS	Phosphate-buffered Saline
PHA-E	*Phaseolus vulgaris* Erythroagglutinin
PHA-L	*Phaseolus vulgaris* Leucoagglutinin
PML	*Pseudomonas mandelii* lectin
PNA	Peanut Agglutinin
PSA	*Pisum sativum* agglutinin
PSL	*Polyporus squamosus* lectin
PSL	*Pseudomonas fluorescence* lectin
PTL	*Pseudomonas taiwanesis* lectin

PTM	Post-translational modification
RCA I	*Ricinus communis* agglutinin I
RPL	Recombinant Prokaryotic Lectins
SBA	Soybean agglutinin
Ser	Serine
STL	*Solanum tuberosum* lectin
sWGA	succinylated Wheat Germ agglutinin
TBS	Tris-buffered Saline
Thr	Threonine
TMB	3,3',5,5'-tetramethylbenzidine
Trp	Tryptophan
Tyr	Tyrosine
UEA I	*Ulex Europaeus* agglutinin I
VVL	*Vicia villosa* lectin
WGA	Wheat Germ agglutinin
Da	Dalton
RT	Room temperature

1 Introduction

This study is part of a master thesis project within the master's Programme in Molecular Biotechnology Engineering at Uppsala University. The master thesis project is made on behalf of Cytiva Life Sciences (formerly GE Healthcare Life Sciences) and was executed on their site in Uppsala. The aim of the study is to investigate various lectins and their suitability as ligands in affinity chromatography for isolation of glycoproteins.

Glycoproteins are proteins that contain sugar moieties covalently attached to the polypeptide sidechains. This post-translational modification (PTM) influences important features of the proteins, such as correct folding and function. In eukaryotes, virtually all secreted and extracellular proteins are glycosylated. One important group of glycoproteins is human antibodies, where deviations in the glycosylation pattern can alter its function and furthermore may cause and indicate diseases (Seeling *et al.* 2017). In the field of therapeutics, most of the biopharmaceuticals used today are glycoproteins (Hevér *et al.* 2019). The ability to isolate glycoproteins from other molecules is thus of great importance.

Lectins are a versatile group of proteins with the common property to bind carbohydrates without altering the carbohydrates' structure (Nascimento *et al.* 2012). Consequently, this property also assigns lectins the potential to recognize and bind to glycoproteins in a reversible manner. These attributes make lectins suitable for usage as ligand in affinity chromatography for separation or purification of glycoproteins. This application is henceforth referred to as lectin affinity chromatography.

In the area of lectin affinity chromatography, the most commonly used lectin is Concanavalin A (ConA) which mainly possess specificity for mannose-related sugar moieties (O'Connor *et al.* 2017). At neutral pH, ConA is a tetrameric protein containing four identical subunits (Calvete *et al.* 1999). Chromatography resins with immobilized ConA are today a renowned part of Cytiva Life Sciences product catalogue, both provided as bulk resin and as prepacked HiTrap columns. Even though ConA columns are commonly used for glycoprotein purification, ConA-tetramers tend to fall apart to monomers and thus cause leakage throughout the chromatography process (Marikar *et al.* 1992). Such leakage could, especially when occurring in the eluate, be devastating in the purification process. Furthermore, ConA and other plant lectins used for the same purpose are purified from their native source, leading to higher batch-to-batch variation and limitations in quantity, in contrast to recombinantly expressed ligands.

These grounds taken together lead to an inquiry to examine other lectins, which could serve as a complement to ConA in terms of sugar-specificity, stability and availability. Another potential way to deal with the leakage of ConA could be to optimize the resins to make the tetrameric lectin more stable.

In this study, a total of 20 different lectins, including ConA, are investigated in terms of suitability as affinity chromatography ligands for glycoprotein-isolation. This is done through studies of the lectins' ability to bind to a monoclonal IgG1 antibody (mAb7) and egg white protein Conalbumin (Ovotransferrin). Furthermore, the sugar-specificity and potential eluting sugars for the lectins are examined. Additionally, a treatment of ConA resins that constitutes a potential solution to the leakage-problem, is examined. An overview of the aim, procedures and methodology throughout the study is shown in Figure 1. The study can be divided into three main procedures: study of lectins' glycoprotein binding, study of lectins' sugar-specificities or potential eluting sugars, and lastly study of ConA resin treated with a potential leakage-reducing method. The same division is contained in the result and discussion sections of this report.

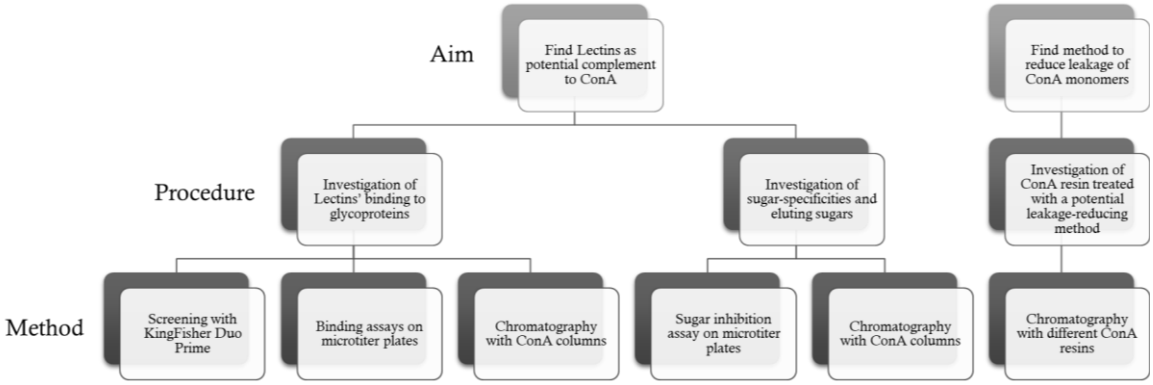


Figure 1 Overview of the study's main experiments. A brief summary of the aim of the study, as well as the main procedures and methodology used to strive towards those goals.

2 Theory

This theory section introduces glycoproteins and lectins in general, but also include subsections that goes into more detail about the proteins and lectins used in this study and other aspects that were considered interesting during the literature study. Taken all together, this gives a long theory section. Based on the readers prior knowledge and interest in the area, the subsections that are found irrelevant thus can be skipped if desired.

2.1 Glycosylation and Glycoproteins

Glycans can be defined as covalent linkages of sugars that are bound to lipids or proteins and they play an important role in many important biological processes (Ohtsubo & Marth 2006). The process where glycans are added or modified on lipids or proteins is generally called glycosylation. Glycoproteins thus are glycosylated proteins where sugar moieties are added as a post-translational modification (PTM) to regulate its folding, activity and function. Glycosylation of proteins can mainly be divided into *N*-glycosylation, *O*-glycosylation and *C*-glycosylation. The main difference between these subsets of glycosylation is where on the

peptide chain the glycans are added, as well as the typical sugar composition (Hevér *et al.* 2019).

The act of glycosylation is performed by glycosyltransferases and glycosidases in the endoplasmic reticulum (ER) and Golgi of eukaryotic cells (Hevér *et al.* 2019). Therefore, virtually all eukaryotic secreted and extracellular proteins are glycosylated. One important group of proteins that undergo glycosylation is human antibodies, where the glycosylation is crucial (Bovenkamp *et al.* 2016). Many human diseases are thought to be a consequence of abnormal glycosylation of different proteins, leading to changes in function and occasionally pathology (Corfield 2017). Additionally, most of the biopharmaceuticals used today are in fact glycoproteins (Hevér *et al.* 2019). In recent years, the field of glycoengineering has developed, allowing *in vitro* modifications of glycans to optimize glycoproteins for therapeutics in terms of functionality and homogeneity (Van Landuyt *et al.* 2019). Antibodies and other glycoproteins thus have crucial roles in biological therapeutics and diagnosis, which subsequently makes the ability to isolate and purify them properly of great interest.

2.1.1 The different types of glycosylation

The glycosylation of proteins is typically divided into C-linked glycosylation, *N*-linked glycosylation and *O*-linked glycosylation. This classification is mainly based on where on the proteins the glycans are added but the classes also differ in typical sugar composition. *C*-glycosylation, also called *C*-mannosylation, is always made on the indole side chain of tryptophan (Trp) residues and always with the sugar α-mannopyranosyl (Hevér *et al.* 2019). Both *N*-linked and *O*-linked glycosylation offer a much larger heterogeneity between glycoproteins.

N-linked glycosylation

N-linked glycans are involved in multiple regulations for the proteins. For instance, they are involved in protein folding that is performed by chaperones in ER, where it also works as a quality-control to ensure that the glycoprotein is correctly folded (Corfield 2017). *N*-linked glycosylation is always made to the amide-nitrogen of asparagine (Asn) residues of proteins. More precisely these *N*-linked glycosylation sites of a protein consist of the amino acid sequence Asn-X-Ser/Thr/Cys (Hevér *et al.* 2019). This means asparagine followed by any amino acid but proline that in turn is followed by either serine (Ser), threonine (Thr) or cysteine (Cys). The *N*-linked glycans all share a common core structure (Figure 2), consisting of the sugars mannose and *N*-acetylglucosamine (GlcNAc) (Seeling *et al.* 2017). The *N*-glycosylation as such is the formation of a *N*-glycosidic bond between the amide nitrogen of Asn and the β-GlcNAc of the glycan (Solís *et al.* 2015).

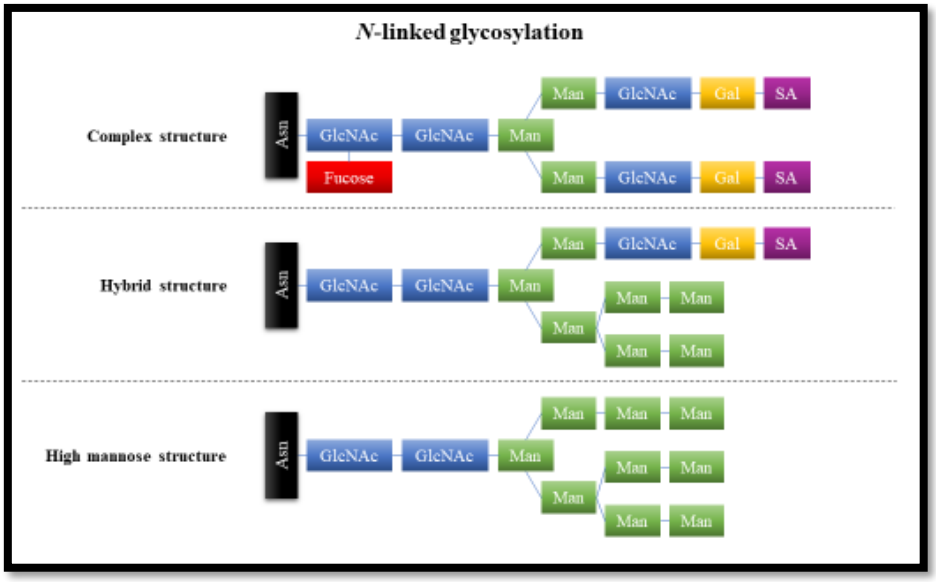


Figure 2 Illustration of possible structures for *N*-linked glycans Typified sugar composition for the different classes within *N*-linked glycans. All *N*-linked glycans are bound to Asparagine (Asn) and have a core of *N*-acetylglucosamine (GlcNAc) together with mannose (Man). Complex structures further contain a subset of additional GlcNAc, galactose (Gal), sialic acid (SA) and fucose. High mannose structures instead contain additional mannose. The hybrid structures simply are combinations of the complex- and high mannose structure.

A subdivision of the *N*-glycans is made with respect to the ingoing sugar moieties beyond the core structure. This division consists of high-mannose structures, complex structures and hybrid structures. Figure 2 shows the typical composition of these structures. During glycosylation the core glycan structure is first added to the Asn residue and is thereafter elongated by either mannose, *N*-acetyllactosamine (Gal-GlcNAc) or both, which leads to high-mannose, complex or hybrid structures, respectively (Hevér *et al.* 2019). The high mannose glycans contain, beyond the common GlcNAc and mannose core, multiple additional mannose residues. The complex glycans instead contain branches with a subset of additional GlcNAc, galactose, sialic acid and fucose (Corfield 2017). The hybrid glycans instead simply are a combination of the two structures.

O-linked glycosylation

O-linked glycosylation is performed on either Ser, Thr or tyrosine (Tyr) residues. However, no common peptide sequence for the *O*-linked glycosylation has been unambiguously determined (Hevér *et al.* 2019). The glycans neither share a common core structure. Therefore, additional terminologies are often used to further distinguish between *O*-linked glycans.

One such subcategory of *O*-linked glycosylation is the mucin-type glycosylation. This is the second most common glycosylation, after *N*-glycosylation, and is made through formation of an *O*-glycosidic bond between Ser or Thr and GalNAc (Corfield 2017). However, mucin-type glycans in turn have eight possible core structures, involving either galactose, GlcNAc or additional GalNAc residues (Corfield 2017). Other glycoproteins, mainly found in nucleus and cytosol, instead contain single *O*-linked GlcNAc on Ser and Thr residues (Corfield 2017).

Multiple other types of *O*-glycosylation have been found, including sugars as galactose, GlcNAc, GalNAc, glucose, mannose, fucose and xylose (Hevér *et al.* 2019).

2.1.2 Antibodies and their glycosylation

Antibodies, also commonly known as immunoglobulins (Ig), are an important group of proteins produced in B-cells. The production of immunoglobulins is a part of the adaptive immune system and the aim is thus to recognize foreign and harmful antigens (Zauner *et al.* 2013). The function of antibodies typically relies on two separate interactions. First, they can recognize and form complexes with antigens via the antigen-binding site of the antibody's fragment antigen binding (Fab) region. Second, the immune response related functions and other biological activities are triggered through interactions between sites on the conserved fragment crystallizable (Fc) region and the environment. Some of these Fc-interactions, such as the anti-inflammatory binding to Fc-receptors, are triggered by the antigen binding. Other Fc-interactions are independent, such as interactions for antibody transportation (Nezlin & Ghetie 2004).

In humans, there are five different classes of immunoglobulins called IgG, IgM, IgA, IgE and IgD. Although these classes somewhat share a similar structure, they differ in abundance, function and typical glycosylation. For all immunoglobulins, the glycosylation is thought to play a role in structure and function (Zauner *et al.* 2013). However, the glycosylation and its consequences are most widely studied for IgG, which also is the class that will be covered in this project.

2.1.3 Glycosylation of IgG antibodies

All human antibodies are glycoproteins, and glycans can be present in both the Fab- and Fc regions. IgG is the most common immunoglobulin in serum and consist of two Fab parts and one Fc part built up by two heavy chains and two light chains, as illustrated in Figure 3 (Zauner *et al.* 2013). When it comes to human IgG, the conserved Fc region is always glycosylated, whilst the Fab region is found to be glycosylated for 15-25% of the IgGs (Bovenkamp *et al.* 2016, Seeling *et al.* 2017). For both regions, the attached glycans are *N*-linked complex biantennary structures (Figure 2). The core of these biantennary glycans are, as all *N*-linked glycans, built up by mannose and GlcNAc. In addition to this core structure, the glycans may also contain fucose, galactose, sialic acid and additional GlcNAc. A fully processed glycan contains all these components, as the complex structure in Figure 2. High mannose glycans rarely occur in the Fab region for serum IgGs but can be more frequently occurring in monoclonal antibodies. However, high mannose structures never occur in the Fc region (Bovenkamp *et al.* 2016, Seeling *et al.* 2017).

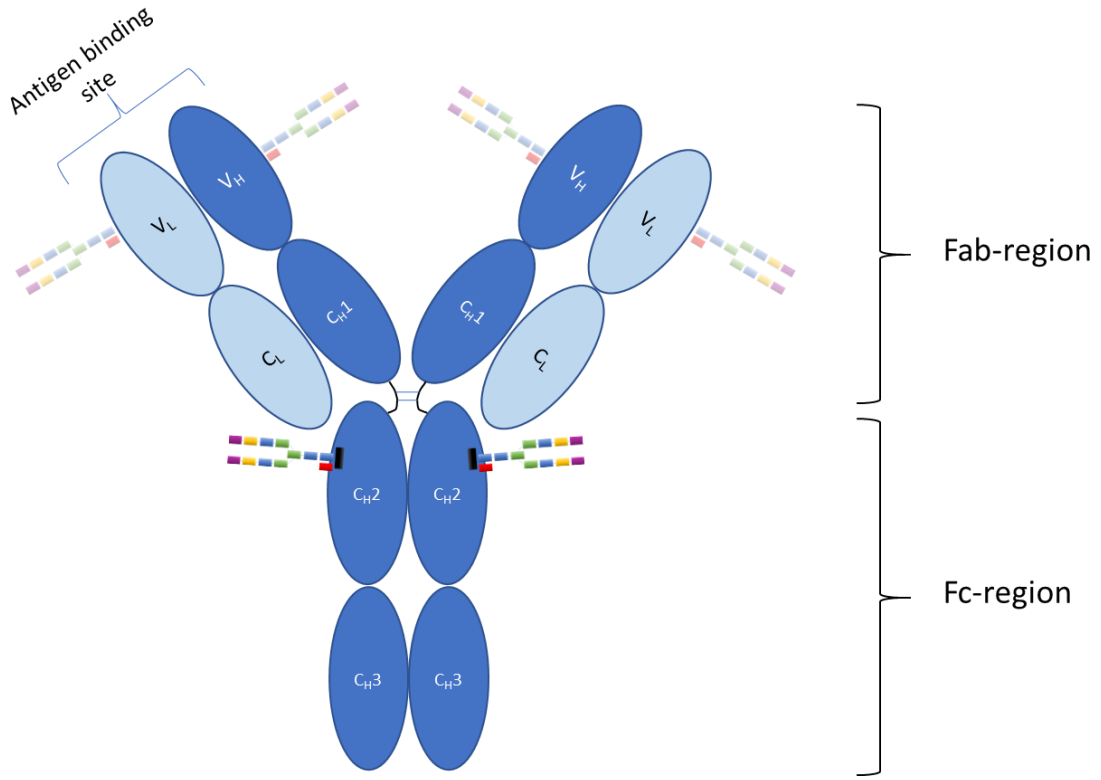


Figure 3 Schematic figure of a IgG antibody and its glycosylation The main components of a typical IgG antibody, consisting of two light chains and two heavy chains. All the glycosylation of a IgG antibody are *N*-linked and mainly complex biantennary structures. The Fc-glycans are conserved and always occur at Asn_{297} of each heavy chain, as indicated. Fab-glycans may also occur in different extent, and in that case in the variable region, indicated as semi-transparent glycans. C_H = Constant part of heavy chain, C_L = Constant part of light chain, V_H = Variable part of heavy chain, V_L = Variable part of light chain.

The glycosylation of the Fc region always occurs on the same residue in the peptide chain, namely Asn_{297} that resides within the C_H2 domain in relative proximity to the hinge region (Bovenkamp *et al.* 2016). Since there are no glycosylation sites within the constant domains of the Fab region, all Fab-glycosylations are thought to be made in its variable region as indicated in Figure 3 (Huang *et al.* 2016). Since the Fab-glycans are exposed while the Fc-glycans are buried on the interface of the structure, the Fab-glycans can be processed and modified by glycotransferases in a larger extent than Fc-glycans. Consequently, the Fc-glycans have a great heterogeneity, while most of the Fab-glycans are fully processed (Bovenkamp *et al.* 2016).

The sugar composition of the glycans thus distinctly varies in frequency between the Fc- and Fab regions. The frequency of different components in the *N*-linked glycans of both regions have been determined through glycoproteomic analysis (Zauner *et al.* 2013). For all glycans, the core mannose and GlcNAc are always present. When it comes to Fc-glycans, the occurrence of glycoforms with one galactose is about 35%, without galactose 35% and with two galactose 16%. Furthermore, the occurrence of core fucose is 92%, bisecting GlcNAc 11% and antennary sialic acid 18%. This can be compared to the occurrence in Fab-glycans where the occurrence of fucose is 78%, bisecting GlcNAc 50% and terminal sialic acid 80% (Zauner *et al.* 2013). In other words, galactose, bisecting GlcNAc and terminal sialic acid are

much more common for Fab-glycans, which again shows that Fab-glycans more often are fully processed.

Importance of Fc- and Fab-glycosylation

Deviations in the glycosylation of the Fc region have been proved to influence antibody activity and function (Seeling *et al.* 2017). This means that the Fc-glycosylation is important for the antibody's function, but also that the glycosylation has potential as a biomarker for diseases. For example, the glycosylation of self-reactive IgG antibodies, so called autoantibodies, is altered in several autoimmune diseases. The presence of sialic acid in the glycans of these autoantibodies can prevent their pro-inflammatory activity. Moreover, high amounts of glycans with galactose and sialic acid may lead to anti-inflammatory activity (Seeling *et al.* 2017). As a result, Fc-glycans without galactose and sialic acid can indicate the disease before any symptoms are shown.

The occurrence and composition of the Fab-glycans have also been shown to vary with certain pathological or physiological states, which indicates that the Fab-glycosylation play a role in immunosuppression and can work as biomarker. The glycans coupled to the variable domain are thought to influence antigen-binding, as well as the longevity, stability and aggregation of the antibodies. (Bovenkamp *et al.* 2016)

In summary, the Fc regions are always glycosylated while Fab-glycans only sometimes occurs. However, the glycosylation of both the Fab- and Fc region of IgG antibodies can regulate its function and activity. The detection of variations in the antibodies glycans can therefore be of great importance to be able to predict, understand and possibly prevent diseases.

2.1.4 mAb7, a monoclonal IgG1 antibody

IgG1 is one subclass of human IgG, and the one most widely used as scaffold for the development of monoclonal antibodies (mAb) for therapeutic purposes (Lee & Im 2017). The monoclonal antibody mAb7 used in this study is an IgG1 antibody. In fact, it is a slightly modified variant of the clinical antibody trastuzumab, trademarked as Herceptin®. Trastuzumab is a monoclonal antibody that targets the human epidermal growth factor receptor-2 (HER-2), which is overexpressed in many cases of breast cancer (Nemeth *et al.* 2017).

The glycans of the used IgG1 antibody are *N*-linked, as for all IgGs. The most commonly occurring glycans have been found to be G0F, G1F and Man6. The annotation G0F and G1F means complex structure containing GlcNAc, mannose and core fucose, with or without a terminal galactose, respectively. Man6 instead is a high-mannose structure including two GlcNAc and six mannose-residues. These structures can be found in Figure 4.

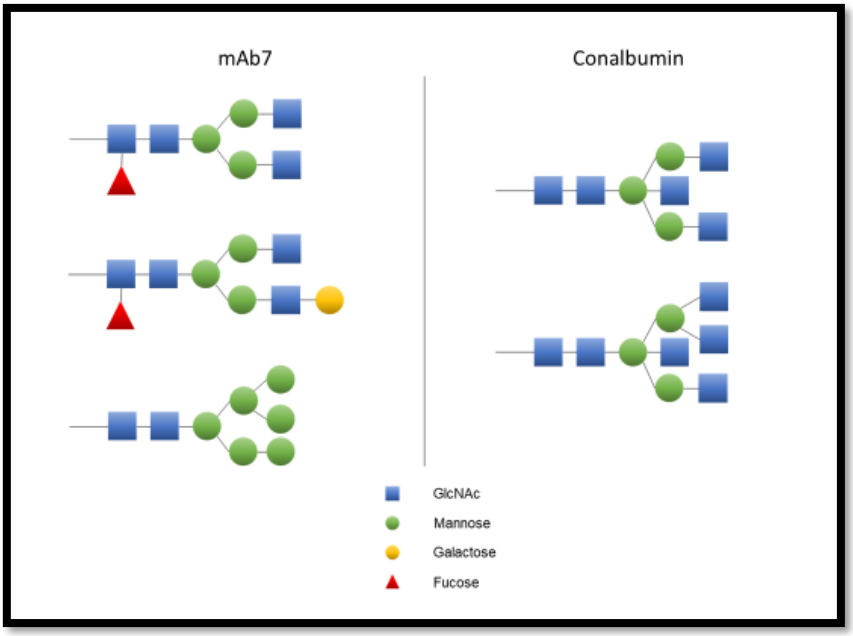


Figure 4 Common glycosylation of the glycoproteins used in this study. The most common structure of the *N*-linked glycans found on monoclonal antibody mAb7 and Conalbumin (Jiang *et al.* 2014).

2.1.5 Conalbumin, an important Glycoprotein in egg white

Conalbumin, also known as Ovotransferrin, is an iron-binding glycoprotein that make up 12-13% of egg white proteins (Jiang *et al.* 2014). Conalbumin is involved in binding and transport of iron but has also been identified as a part of inflammatory responses in chickens (Xie *et al.* 2002). In fact, Conalbumin is thought to be antibacterial, antifungal, antioxidative and antiviral (Giansanti *et al.* 2012). Furthermore, the protein has been shown to be able to regulate bone resorption (Shang & Wu 2019). These properties together make Conalbumin an interesting protein with potential in health enhancement for both animals and humans.

Conalbumin is a glycoprotein consisting of *N*-linked glycans, which are thought to be important for its function. When studying Conalbumin from both chicken and pheasant, Jiang *et al.* (2014) found that there are differences in the glycosylation pattern between the species even if the peptide sequences are the same. For chicken Conalbumin occurrence of a total of 16 different *N*-glycans were found, out of which the majority were complex and a few were hybrid or core structure glycans. Most common were biantennary and triantennary glycans, but also mono-, tetra, and pentaantennary occurred. The most commonly occurring glycoforms for chicken ovotransferrin according to their study were two complex structures of 5 GlcNAc and 3 mannoses (30.5%) as well as 6 GlcNAc and 3 mannoses (37.0%), respectively (Figure 4). Beyond these structures, other complex structures involving GlcNAc and mannose were dominant. No glycoforms included fucose nor sialic acid, while only about 5 % of the structures had a galactose-residue. (Jiang *et al.* 2014)

In this study, the Conalbumin used, originates from chicken egg white and has a molecular weight about 75 kDa (Cytiva). Ovotransferrin can also bind other metal ions than iron, including manganese Mn^{2+} ions (Giansanti *et al.* 2012).

2.2 Lectins and Lectin Affinity Chromatography

Lectins are proteins that bind to carbohydrates without altering their structure biochemically. The lectins, also called agglutinins, have at least one reversely binding carbohydrate recognition domain (CRD) with specificity for one or more sugars. Notably, enzymes that both targets and modifies carbohydrates with the same domain are thus not lectins. Apart from the presence of a CRD, lectins are a large and structurally diverse group of proteins. Lectins have been found in most organisms, but are most widely studied from plants where they often are found ubiquitously expressed in the seeds. (Nascimento *et al.* 2012)

The native function of lectins remains partly unclear, but many of them are thought to be involved in host defence mechanisms and some have shown anti-viral activity (Nascimento *et al.* 2012). For instance, a mammalian lectin called mannose-binding lectin is known to be involved in recognition of pathogens in the immune system (Morimoto & Sato 2016). Other lectins are instead involved in intracellular transport or cell growth control (Solís *et al.* 2015). Regardless of their native role, the main interest in this work lies in the lectins' specificity and affinity to carbohydrates and glycoproteins, together with other properties making them appropriate as ligands in affinity chromatography.

Since lectins bind specifically to carbohydrates or glycoproteins, without affecting their structure or function, they are suitable for Lectin Affinity Chromatography (LAC) (O'Connor *et al.* 2017). The fact that the interactions are reversible means that it is possible to elute the bound glycoprotein through competitive binding to the lectin with excess of a suitable free sugar. Another important property for a ligand used in affinity chromatography is its stability, necessary to ensure that the elution does not get contaminated by the ligand itself. However, many lectins are multimeric which can cause problems in that aspect. For some lectins, divalent metal ions such as Ca^{2+} and Mn^{2+} enhance stability and sugar binding ability (O'Connor *et al.* 2017). Lastly, a lectin used as chromatography ligand must manage immobilization to a resin without altering the functional carbohydrate binding properties.

2.2.1 Subdivision of Lectins

Since lectins are a large group of proteins, with the reversible and non-enzymatic carbohydrate recognition property in common, there are many subdivisions to be aware of. Such divisions can for instance be based on their origin, three-dimensional structure or specificity. Example of lectin family divisions based on such properties are Annexin, Chitin-binding lectins, Calcium dependent (C-type) lectins, Fucose-binding lectins, Galectin, Jacalin-related lectins (JRL), Legume lectins, Man-6Pi-binding lectins, Monocot lectins and ricin B chain-related (R-type) lectins (Hirabayashi *et al.* 2015).

In addition to these families, there are further attempts to group lectins based on their set of carbohydrate recognition domains (CRD). The three main types of lectins according to this division are Merolectins, Hololectins and Chimerolectins (Nascimento *et al.* 2012). Basically, merolectins only have one CRD, while hololectins contain at least two identical CRDs

(Nascimento *et al.* 2012). Chimerolectins are instead a subclass of lectins that have at least one CRD, but also a biologically active domain (Nascimento *et al.* 2012). One example of such a chimerolectin is PSL1a from the mushroom *Polyporus squamosus*, with a CRD that specifically recognizes sialylated glycans and another separate catalytical domain with proteolytic activity (Manna *et al.* 2017). On another note, this eukaryotic chimerolectin has in fact been recombinantly expressed in *E. coli* with maintained glycan recognition (Tateno *et al.* 2004).

2.2.2 Lectins origin and Recombinant expression

Most of the previously studied lectins are derived from plants and hence of eukaryotic origin, many of which themselves are glycoproteins. As a result, these lectins are limiting when it comes to recombinant expression in prokaryotic hosts as *Escherichia coli* because of its complexity and need for post-translational modifications (Keogh *et al.* 2014). This means that the plant-originating lectins instead usually are purified from the plant itself, with potential variation in activity and quality as a consequence, together with the limited lectin quantity that can be produced (Keogh *et al.* 2014). For example, the access to raw material of native plant lectins can be season-dependent and limited, which makes the production very expensive and ineffective (Fernandez-del-Carmen *et al.* 2013).

For a cheaper, more stable qualitative and quantitative production of lectins to be used for chromatography, recombinant expression thus could be a valuable tool. Recombinant expression also would allow modification of the gene to be expressed, which could optimize its specificity and affinity further. Several plant lectins have been recombinantly expressed in different host cells, including *E. coli* and the yeast *Pichia pastoris*. However, recombinant expression of eukaryotic lectins in prokaryotic systems often results in low yields and insoluble inclusion bodies. Lectins with prokaryotic origin show greater potential for that purpose (Keogh *et al.* 2014). In general, *E. coli* is preferred for non-glycosylated lectins while *P. pastoris* has advantages for expression of lectins that require PTMs (Oliveira *et al.* 2013). Examples of plant lectins that have been expressed recombinantly are discussed in Supplementary Theory 1.

2.2.3 Bacterial Lectins

As mentioned earlier, plant lectins are the most studied group of lectins. There are however examples of lectins with bacterial origin that have been studied. One bacterial lectin that has been investigated is PA-IL from *Pseudomonas aeruginosa* with confirmed specificity for galactose. Because this lectin is somewhat tolerant to heat, proteolysis and various pH, it has been recombinantly expressed in *E. coli* and also used as scaffold for the development of novel recombinant prokaryotic lectins (RPLs) through site-directed mutagenesis by Keogh *et al.* (2014). These RPLs were shown to be functional and with altered carbohydrate specificity and affinity than the originating PA-IL. (Keogh *et al.* 2014)

Other lectins from the *Pseudomonas* family have also been studied. For example, *Pseudomonas fluorescence* lectin (PSL), *Pseudomonas taiwanesis* lectin (PTL) and

Pseudomonas mandelii lectin (PML) were recombinantly expressed in *E. coli* and showed anti-viral activity because of their mannose-rich glycan recognition (Morimoto & Sato 2016).

2.2.4 Plant Lectins investigated in this study

In this study, 20 lectins with plant origin and different specificity and properties will be investigated (Table 1). All these lectins are extracted from their native source. One of these lectins is the commonly used Concanavalin A (ConA). Some information about these lectins are included in Table 1, while additional properties of all these lectins according to the supplier can be found in Supplementary Theory 2 (Vector Laboratories 2020).

Table 1 Plant lectins investigated in this study. The 20 lectins with plant origin that are the main subjects for investigation in this study. Included are data for sugar specificity, number of subunits and total molecular weight gathered from the supplier (Vector Laboratories 2020). Additional information about these lectins can be found in Supplementary Theory 2. Abbreviations: GlcNAc = *N*-acetylglucosamine, GalNAc = *N*-acetylgalactosamine.

Lectin	Full Lectin name	Main Sugar specificity	Subunits	MW (kDa)
ConA	Concanavalin A	Mannose	4	104
DBA	*Dolichos biflorus* agglutinin	GalNAc	4	111
DSL	*Datura stramonium* lectin	GlcNAc	1	86
ECL	*Erythria cristagalli* lectin	Galactose, GalNAc	2	54
GSL I	*Griffonia simplicifolia* lectin I	Galactose	4	114
GSL II	*Griffonia simplicifolia* lectin II	GlcNAc	2	113
Jacalin	Jacalin	Galactose	4	66
LCA	*Len culinaris* lectin	Mannose	4	50
LEL	*Lycopersicon esculentum* lectin	GlcNAc	1	71
PHA-E	*Phaseolus vulgaris* Erythroagglutinin	Galactose, Complex structures	4	126
PHA-L	*Phaseolus vulgaris* Leucoagglutinin	Galactose, Complex structures	4	126
PNA	Peanut Agglutinin	Galactose	4	110
PSA	*Pisum sativum* agglutinin	Mannose, Glucose	4	53
RCA I	*Ricinus communis* agglutinin I	Galactose, GalNAc	2	120
SBA	Soybean agglutinin	GalNAc	4	120
STL	*Solanum tuberosum* lectin	GlcNAc	2	100
sWGA	succinylated Wheat Germ agglutinin	GlcNAc	2	36
UEA I	*Ulex Europaeus* agglutinin I	Fucose	2	63
VVL	*Vicia villosa* lectin	GalNAc	4	(102-144)
WGA	Wheat Germ agglutinin	GlcNAc	2	36

As seen in Table 1, almost all these lectins are dimers or tetramers, except from the monomers *Datura stramonium* lectin (DSL) and *Lycopersicon esculentum* lectin (LEL) which are isolated from thorn apple and tomato, respectively. Notable, both PHA-E and PHA-L are included. These are different isoforms of the *Phaseolus vulgaris* lectin, which have been shown to differentiate in function (Oliveira *et al.* 2013). Notable is also that the wheat germ agglutinin (WGA) is included both in its native form and succinylated (sWGA). The succinylation considerably changes the pI of WGA, making sWGA acidic instead of basic as the native form. The succinylation also is thought to remove the specificity for sialic acid, which the native form possesses (Monsigny *et al.* 1980).

When it comes to subdivisions into families, the lectin Jacalin is included in the Jacalin-related lectin domain, which is a family based on structures similar to Jacalin. The *Ricinus communis* agglutinin I (RCA I) is instead included in Ricin-type beta trefoil lectin domain, based on its three-dimensional structure. The four lectins DSL, LEL, WGA and *Solanum tuberosum* lectin (STL) are all chitin-binding lectins, a family based on their ability to bind chitin, which consists of monomers and oligomers of *N*-acetylglucosamine (GlcNAc). The rest of the lectins in Table 1 are all included in the legume lectin domain, which is a big lectin family based on their origin from legumes or legume seeds. (Lectin Frontier DataBase 2020)

2.2.5 The Lectin ConA and its usage in chromatography

Concanavalin A (ConA) was the first structurally determined lectin and originates from jack bean seeds, where it serves as a storage- and defence protein (Nascimento *et al.* 2012). Today, ConA is a very common ligand for glycoprotein purification. The structural composition of ConA is pH-dependent, since it has been found to be a homogenous dimer at pH 5 and a tetramer at pH 7 and above (Calvete *et al.* 1999). These are built up by identical subunits of 25.5 kDa that each contains a carbohydrate recognition domain (CRD), a hydrophobic cavity and binding sites for Ca^{2+} and Mn^{2+} (Nascimento *et al.* 2012). The CRD of ConA mainly has specificity for mannose and glucose, which is reinforced in the presence of the cations Ca^{2+} and Mn^{2+} (O'Connor *et al.* 2017). An overall requirement for the sugars or glycans to be able to interact and bind to ConA was early shown to be unmodified hydroxyl groups on carbon C3, C4 and C6 of the pyranose sugar ring structure (Goldstein *et al.* 1965). The same study showed that the C2 hydroxyl group is not essential, but that the mannose-configuration of this hydroxyl group leads to tighter ConA-binding than the glucose-configuration (Goldstein *et al.* 1965).

ConA is commonly used for lectin-based affinity chromatography (LAC) for glycoproteins containing mannose- or glucose residues. Bulk ConA Sepharose 4B resin and pre-packed HiTrap ConA 4B columns are products currently available from Cytiva Life Sciences. When using ConA as ligand for LAC, the cations Ca^{2+} and Mn^{2+} should be included in the binding buffer to increase the efficiency. For elution of the bound glycoprotein, either α-methylmannoside (MeαMan), α-methylglucoside (MeαGlc) or a combination of the two are commonly used as eluting sugar (O'Connor *et al.* 2017).

The multimeric nature and rather large size of ConA complicates recombinant large-scale production, why it is purified from its native source which can cause batch-to-batch variations (O'Connor *et al.* 2017). Additionally, a big problem with ConA in chromatography purification is that it tends to fall apart during the chromatography process and hence may cause leaching which contaminate the purified glycoproteins upon elution (Marikar *et al.* 1992). In that perspective, lectins with a few or optimally just one subunit could be preferred in LAC to allow recombinant expression and prevent lectin leakage.

ConA column and antibody separation

ConA-based affinity chromatography has long been used to distinguish between symmetrically and asymmetrically glycosylated IgG antibodies (Borel *et al.* 1989). When it comes to interactions and separation of IgG antibodies using ConA, all *N*-linked glycans should in theory be able to bind to ConA because of the mannose-containing core. However, the interior glycans of the Asn_{297} residues in the Fc region have been shown to be inaccessible for ConA. As a result, only 12 % of human IgG interacts with ConA columns in its native form (Huang *et al.* 2016). For separation of IgG using ConA column, mainly Fab-glycosylated antibodies hence can be trapped into the column and subject for purification.

2.2.6 Free carbohydrates as competitive inhibitors of Lectins

For all chromatographic separation methods, the elution of the target molecule is crucial. Since lectins recognize and bind reversible to carbohydrates, the binding can be competitively inhibited through excess of a free carbohydrate with affinity for the lectin (O'Connor *et al.* 2017). Different lectins have different carbohydrate specificity, and subsequently different sugars are suitable as inhibitors. The eluting sugar should obviously ultimately be able to cause full inhibition of the particular lectins' binding. However, when it comes to large scale purification processes there are also other factors worth considering for the eluting sugar. Such considerations may be availability, cost and solubility of the sugar. The eluting sugars that will be used in this study are listed in Table 2, together with data regarding solubility and cost. For example, L-fucose costs about 100 times more than mannose, which obviously is a considerable difference.

Table 2 Carbohydrates that can be used to inhibit lectin binding. Some of the sugars that potentially can be used as eluting sugars in lectin affinity chromatography. Each sugar's molecular weight, solubility in water and approximate cost, important properties for large scale applications, are included. Solubility and cost data were received from sigmaaldrich.com, pubchem.ncbi.nlm.nih.gov and scbt.com.

Sugar	**MW [g/mol]**	**Solubility [mg/mL]**	**soluble conc. [mM]**	**Cost** *(sigma)* **[SEK/100g]**
Galactose	180,2	180 *(sigma)*	999	600 (>98%)
α-methylmannoside (MeαMan)	194,2	100 *(sigma)*	515	1 490 (>99%)
α-methylglucoside (MeαGlc)	194,2	600 *(sigma)*	3 090	545 (>99%)
Lactose	342,3	1 950 *(pubchem)*	5 700	~200
L-Fucose	164,2	50 *(sigma)*	305	27 560 (>99%)
D-Fucose	164,2	100 *(sigma)*	609	~100 000 (>98%)
N-acetylglucosamine (GlcNAc)	221,2	167 *(pubchem)*	755	1 690 (>95%)
N-acetylgalactosamine (GalNAc)	221,2	~50 *(scbt)*	226	~300 000 (~98%)
Glucose	180,2	1 200 *(pubchem)*	6 660	322 (>99,5%)
Mannose	180,2	713 *(pubchem)*	3 960	1 180 (synthetic, >99%)

3 Method

3.1 Lectins and Glycoproteins

Lectin screening kits I, II and III from Vector Laboratories, containing in total 20 biotinylated lectins with plant origin, were used in this study (Table 1). Some information about these lectins can be found in Table 1, while more detailed information about all 20 lectins provided by the supplier can be found in Supplementary Theory 2. The glycoproteins used in this study were the monoclonal antibody mAb7 (Cytiva LS038400) and Conalbumin (Cytiva).

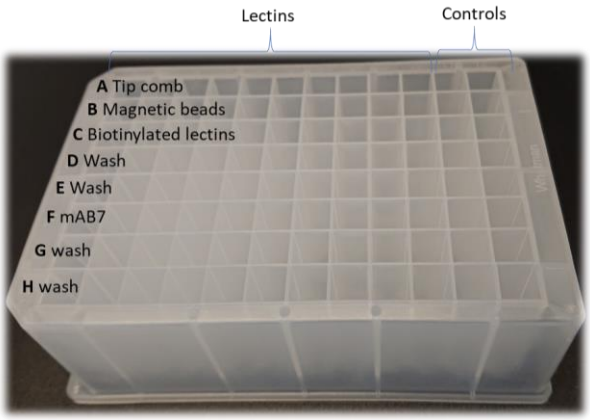
3.2 Screening with KingFisher Duo Prime

A screening of 20 biotinylated plant lectins and its ability to bind to mAb7 was performed using KingFisher Duo Prime system (Thermo Fisher Scientific) and the related BindIt software. This system makes use of a magnetic rod that can transfer magnetic beads, with immobilized components, between wells according to a programmed method. The magnetic beads used were Streptavidin Mag Sepharose (Cytiva), which are magnetic sepharose beads with attached streptavidin which allow immobilization of the biotinylated lectins.

Two 96-well plates were used, each fitting 10 of the biotinylated lectins and two controls (Figure 5). As negative control the same setup, but without any lectin, was used. As positive control another magnetic bead, containing pre-immobilized prismA (Cytiva LS027238) with known high affinity to mAb7, was used instead of lectins and Streptavidin Mag Sepharose beads. For each of the 12 columns of the plate, the rows were filled with the appropriate content, as shown in Figure 5. First, 200 µL 10% slurry of the magnetic beads was added to all wells in row B. The liquid was then removed by pipetting while the beads were held down by a magnet and the beads were washed with the washing buffer (10 mM PBS pH 7.4 with 0.1 mM $CaCl_2$ and 0.01 mM $MnCl_2$). New washing buffer was added to the solid beads to get a final volume about 500 µL. For all washing steps D, E, G and H, 500 µL of the same washing buffer was used. Each lectin were diluted in washing buffer to 200 µg/mL and 500 µL was added to well 1-10 in row C. For well 11 and 12, corresponding to controls, 500 µL washing buffer was added instead. The mAb7 was diluted to 500 µg/mL in the washing buffer and 500 µL was added to each well in row F.

The BindIt software for KingFisher instruments was programmed with the content, volumes and duration for each well according to Figure 5. The duration in the lectin-containing row C was set to 5 minutes and the duration in the mAb7 containing row F to 10 minutes. All washing steps were programmed to last two minutes each. The prepared plates were, one at a time, placed in the instrument and the programmed method executed. After the experiment, the well content in row B-G were analysed through Abs_{280} measurements with spectrophotometer SpectraMax Plus 384 (Molecular Devices), in duplicates loaded to 96-well UV-plates. The relative change in absorbance for mAb7 in comparison to the negative

control, without any lectin, was computed for each lectin. The absorbance of the subsequent washing step was also considered and subtracted since it only meant very week and probably unspecific binding. Through Abs_{280} for the lectin-containing well, also the fraction lectin bound to the magnetic beads was calculated, using absorbance measured beforehand as reference.



Well	Component	Amount	Final Volume	Duration
A	Tip comb	-	-	
B	Magnetic Beads (Streptavidin Mag Sepharose)	200 µL 10% medium slurry (20 µL beads with PBS added to final volume 500 µL)	500 µL	
C	Biotinylated lectins	100 µg (200 µg/mL)	500 µL	5 min
D	Wash (PBS buffer)	-	500 µL	2 min
E	Wash (PBS buffer)	-	500 µL	2 min
F	mAb7	250 µg (500 µg/mL)	500 µL	10 min
G	Wash (PBS buffer)	-	500 µL	2 min
H	Wash (PBS buffer)	-	500 µL	2 min

Figure 5 Plate layout and used amounts for lectin screening with KingFisher Duo Prime. The content of each well (row A-H) in the 96-well plates used for screening in KingFisher Duo Prime is shown. Different lectins were used in row C for column 1-10, while column 11 was a negative control without any lectin and column 12 was a positive control using other magnetic beads in row B and no lectin in row C. The volume and duration of each step were programmed into the BindIt software.

3.3 Binding assays

During this study, many microtiter plate binding assays were performed. For all the binding assays, 96 well microtiter plates (Thermo Scientific) were used. For absorbance measurements the spectrophotometer SpectraMax Plus 384 (Molecular Devices) with microplate reader was used.

3.3.1 Binding assays with various Lectin-concentrations

Binding assays for the biotinylated lectins were performed according to a created protocol for Binding assay (Supplementary Method 1), modified from Lundbäck *et al.* (2016). Briefly, microtiter plates were coated with 100 µL of 100 ng/mL mAb7, or 50 ng/mL Conalbumin, diluted in PBS buffer. After being incubated at room temperature (RT) overnight, the plates were washed with washing buffer (0.1% TBS-T: 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Tween20). Each of the biotinylated lectins were diluted in binding buffer (1 mM Tris pH 7.5, 1 mM $CaCl_2$, 1 mM $MnCl_2$, 0.1% Tween20) through twofold dilution series leading to appropriate concentration gradients. 100 µL of each lectin concentration were then added to the plate in duplicates and incubated for 1 h in 37°C. As reporter, the enzyme horseradish peroxidase (HRP) coupled to Streptavidin was used, diluted in washing buffer. Through interaction between streptavidin and the biotin attached to the lectins, the amount HRP became directly proportional to the lectin concentration. The plate was incubated with 100 µL diluted Streptavidin-HRP in RT for 20 minutes. The HRP enzyme was then allowed to react with 100 µL 3,3',5,5'-tetramethylbenzidine (TMB) for 25 minutes, creating a blue colour.

Through addition of 50 µL 2N sulfuric acid, the reaction was stopped and a yellow colour detectable at 450 nm was produced, which could be measured in a spectrophotometer.

Equation 1 Saturation, One site – Specific binding. The equation used for nonlinear regression of the binding assay data in GraphPad Prism. Y is the response signal for each X which is the ligand concentration, B_{max} is the maximal binding signal and K_d is the concentration giving $B_{max}/2$.

$$Y = B_{max} \cdot \frac{X}{K_d + X}$$

The GraphPad Prism 8 software was used to plot the absorbance against concentration. With this software, a nonlinear regression was made for each graph, coupling the data to the equation for saturation curve *One site – Specific binding*. This equation is based on the relationship in Equation 1, and the regression thus makes it possible to estimate maximal binding B_{max} and affinity K_d. For several lectins, the binding assay was redone multiple times in order to optimize the concentration gradient so that the resulting saturation curve could be clearer.

3.3.2 Binding assays with inhibiting sugars

For 12 of the previously studied lectins, new binding assays were made according to a created protocol for sugar inhibition binding assay (Supplementary Method 2). This assay was similar to the previous binding assay, with the exception that the lectins were mixed with a carbohydrate prior to their addition to the mAb7-coated microtiter plates. A fixed lectin concentration was used for each lectin, corresponding to their K_d value estimated from the previous binding assays. The sugar was diluted to 200 mM in binding buffer (1 mM Tris pH 7.5, 1 mM $CaCl_2$, 1 mM $MnCl_2$, 0.1% Tween20), and then diluted further in a tenfold dilution series leading to eight different sugar concentrations between 0 and 200 mM. Each lectin were then diluted to its estimated K_d in each of the sugar concentrations, in duplicates. After the addition of lectins diluted in sugar-solution to the plates they were incubated for 1 h at 37°C. The rest of the assay was made identical as for the previous binding assays. The sugar inhibition assay was made for the sugars α-methylmannoside (MeαMan), α-methylglucoside (MeαGlc), *N*-acetylglucosamine (GlcNAc), *N*-acetylgalactosamine (GalNAc), lactose, galactose, L-fucose, D-fucose, glucose and mannose. For all these sugars, a stock solution of 200 mM was prepared through dissolving the sugar in the binding buffer.

Equation 2 Inhibition, [Inhibitor] vs response – variable slope. The equation used for nonlinear regression of the inhibition by sugars made in GraphPad Prism. Y is the response signal for each inhibitor-concentration X, Y_{max} and Y_{min} are the inhibition curves top and bottom plateau, respectively, IC_{50} is the inhibitor-concentration needed to achieve half the inhibition $(Y_{max}-Y_{min})/2$. Hillslope is the slope factor, which is estimated based on the values.

$$Y = Y_{min} + \frac{Y_{max} - Y_{min}}{1 + \left(\frac{IC_{50}}{X}\right)^{HillSlope}}$$

For analysis of the data, the GraphPad Prism 8 software was used. The absorbance was plotted against the sugar concentration and a nonlinear regression was made using the equation *[Inhibitor] vs. response – variable slope (four parameters)* and least square

regression as fitting method. This regression is made assuming the response follow the behaviour in Equation 2, making it possible to estimate a maximal inhibition and the half-inhibiting concentration IC_{50}.

3.4 Lectin Affinity Chromatography and SDS-PAGE

Several experiments using affinity chromatography and analysis through SDS-PAGE were performed. For all these experiments, the ConA columns and methodology as described below, were used.

3.4.1 ConA columns and resins

For the study, three different ConA Sepharose 4B columns were used.

1. A standard 1 mL HiTrap Con A 4B (Cytiva) pre-packed column.
2. Cross-linked Con A Sepharose 4B, modified from ConA 4B resin (Cytiva). This resin was treated with glutaraldehyde to create a cross-linking between ConA and the Sepharose resin, in accordance to the method proposed by Kowal & Parsons (1980) using 25% glutaraldehyde solution.
3. Reference Con A Sepharose 4B, which underwent the same treatment as the Cross-linked ConA resin, but without the addition of glutaraldehyde solution.

While the first one is a pre-packed column, the last two columns were packed in HiTrap 1 mL columns at the Cytiva R&D department.

3.4.2 Affinity Chromatography

Con A Sepharose 4B purification was performed on a ÄKTA pure system (Cytiva) together with the associated Unicorn 7.5 software for control and data analysis. The method and parameters programmed into Unicorn were modified from documents related to the HiTrap ConA 4B columns (GE Healthcare 2014, GE Healthcare 2016). As glycoprotein samples mAb7 (diluted) and Conalbumin (dissolved from powder) were mixed in binding buffer (20mM Tris-HCl, 500mM NaCl, 1mM $MnCl_2$, 1mM $CaCl_2$, pH 7.4). For mAb7 and Conalbumin a concentration of 0.5 mg/mL and 1 mg/mL were used, respectively.

For all experiments, the column was washed and equilibrated with 8 column volumes (CV) binding buffer (20mM Tris-HCl, 500mM NaCl, 1mM $MnCl_2$, 1mM $CaCl_2$, pH 7.4). The glycoprotein sample was applied with a flow rate of 0.2 mL/min, followed by 7 CV washing with binding buffer. For the elution a flow rate of 0.5 mL/min with elution buffer (20mM Tris-HCl, 500mM NaCl, pH 7.4) and a 10 CV linear gradient of the eluting sugar between 0 and 200 mM were used. The eluate was collected in 1mL fractions. The flow-through from column wash, sample application and wash of unbound protein were all collected in Falcon tubes. As eluting sugar, MeαMan was mostly used, dissolved in the elution buffer. The binding buffer as well as all sugar-solutions were filtered through a 0.22 µm filter prior to use. After usage, the column was washed with 5 CV cleaning buffer 1 (500 mM NaCl, 20 mM

Tris-HCl, pH 8.5) followed by 5 CV cleaning buffer 2 (500 mM NaCl, 20 mM acetate, pH 4.5), repeated three times. Lastly, 5 CV of storage buffer (20% ethanol in 100 mM acetate, 1 M NaCl, 1 mM $MnCl_2$, 1mM $CaCl_2$, 1 mM $MgCl_2$, pH 6.0) was added to the column which then was stored at 4°C.

3.4.3 Electrophoresis and SDS-PAGE

SDS-PAGE gel electrophoresis was performed using the Multiphor II Electrophoresis System (Cytiva) with ExcelGel SDS Gradient 8-18 (Cytiva). To concentrate samples, Vivospin 6 10 kDa MVCO (Cytiva) was used together with centrifugation at 4000xg. Each sample were mixed 1:1 with reducing sample buffer (50 mM Tris-Acetate pH 7.5, 1% SDS, 0.01% Bromophenol blue, 100 mM DTT) and heated in 95°C for 5 minutes prior to application to the gel. As molecule weight reference a LMW standard (10 mM Tris pH 8.0, 1 mM DTT, 2% SDS) is mixed with 50 µL of the reducing sample buffer. The electrophoresis was run (600V, 50mA, 30W) for about 90 minutes. The gel was stained by Coomassie Brilliant Blue with shaking for 1h and destained by destaining solution (25% Ethanol, 8% acetic acid) with shaking overnight. The gel was lastly scanned with Amersham Imager 600 (Cytiva).

4 Results

4.1 Binding between Lectins and Glycoproteins

The aim of the experiments included in this section was to examine 20 different lectins with plant origin (Table 1) and their ability to bind to glycoproteins. The two glycoproteins used for this purpose were the monoclonal antibody mAb7 and the egg white protein Conalbumin. Important for the lectin-glycoprotein interactions are both the maximal binding and the kinetic affinity leading up to that binding. An initial screening of the 20 lectins and their binding to mAb7 was made using a rapid screening method. The lectins binding capacity and affinity to both mAb7 and Conalbumin were further investigated using microtiter plate binding assays. Both glycoproteins were also examined as samples in affinity chromatography using ConA columns.

4.1.1 Lectin screening to investigate binding to mAb7

To get an image of the 20 plant-lectins (Table 1) and their ability to bind glycoproteins, a screening against the monoclonal antibody mAb7 was performed. The screening was made using KingFisher Duo Prime (Thermo Fisher Scientific) together with the associated BindIt software, as described in Figure 5. The setup was aimed to first couple the biotinylated lectins to magnetic Streptavidin-coated sepharose beads. The lectin-coated beads could then be transferred between wells by the instruments magnetic rod to let the lectins interact with mAb7 in one of the wells. Through Abs_{280} measurements for each lectin and the negative control, the changes in mAb7 concentration could be calculated for each lectin (Figure 6).

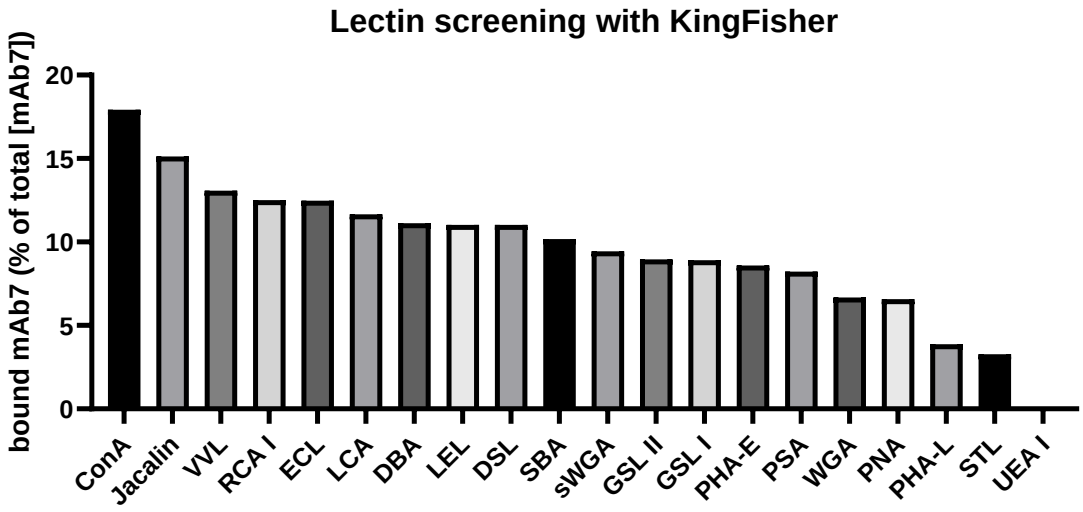


Figure 6 Screening of 20 plant lectins reveal their binding to the monoclonal antibody mAb7. Fraction of mAb7 bound to each lectin, after 10 minutes interaction through KingFisher Duo Prime. The fraction of mAb7 bound to lectins is based on the difference in absorbance between each lectin and the negative control. The subsequent washing step is also considered to take away the very loosely bound mAb7 from the result.

Figure 6 shows that ConA bound the most mAb7 of the lectins, through biding of 17% of the initial amount of mAb7. Notably, that binding is only a fraction of the positive control prismA, which showed 90.5% binding (not shown). However, they cannot really be compared since the number of prismA molecules on each bead likely is significantly higher than the number of lectins on each bead in the samples. Moreover, the binding capacity of prismA is not dependent on the accessibility of the glycans. The positive control thus functions as a proof that the method works, rather than a direct reference for comparison.

The percentage of bound mAb7, of the initial 250 µg, to ConA (17.1%) corresponds to 43 µg bound mAb7 which is about 0.29 nmol (MW ~150 kDa). The relation between number of bound mAb7-molecules and the number of ConA molecules however is dependent of how much biotinylated ConA that bound to the streptavidin-beads. A rough estimation based on the difference in ConA-absorbance before and after the reaction (not shown) indicates that about 80% of starting material had bound to the beads, that is about 80 µg, which corresponds to 0.77 nmol (MW ~104 kDa). With this assumption, that 0.77 nmol of ConA molecules bound 0.29 nmol of mAb7, about 38% of the ConA molecules bound mAb7 molecules. Only about two fifths of the ConA molecules hence bind mAb7, even if the mAb7 material certainly is enough for more binding. However, longer residence time than the 10 minutes used here may improve binding.

Compared to ConA binding (17.1%), several other lectins show good potential, not least Jacalin (15.1%), VVL (13.1%), RCA I (12.5%), ECL (12.5%), LCA (11.7%), DBA (11.1%), LEL (11.0%) and DSL (11.0%). Once again, a direct comparison between the lectins is based on the assumption that the molar number of lectin-molecules bound to the magnetic beads is constant for all lectins. However, even if ConA shows the best binding to mAb7, several other of the lectins thus are interesting to investigate further.

4.1.2 Binding assay to investigate Lectins' binding to mAb7 and Conalbumin

To further investigate the interaction between the lectins and glycoproteins, microtiter plate binding assays were performed according to a created protocol (Supplementary Method 1). This was done with mAb7 for all 20 lectins and with Conalbumin for 12 of the lectins. Each well of the microtiter plates were coated with 100 µL of 100 ng/mL mAb7 or 50 ng/mL Conalbumin, respectively, which both corresponds to about 0.67 nM glycoprotein. To these glycoprotein-coated plates, the biotinylated lectins were added in different concentrations adjusted to give a reliable saturation curve. Streptavidin-HRP was used as a reporter and left to react with TMB to produce colour. The reactions were then stopped with 2N sulfuric acid that also produced a colour detectable at 450 nm which was measured. The GraphPad Prism 8 software was then used to interpret a saturation curve for each lectin through a nonlinear regression (Equation 1).

The saturation curves for ConA and GSL II and their binding to mAb7 and Conalbumin can be seen in Figure 7. From the interpreted saturation curves, a saturated maximal signal B_{max} could be estimated. Based on the lectin-concentration needed to reach half of that maximal signal, also a rough estimation of the affinity K_d could be made (Table 3). The values from the lectins that gave unreliable graphs for mAb7 were excluded in Table 3, that is PHA-E, PHA-L, PNA and succinylated WGA. The saturation curves as well as estimated K_d and B_{max} from all the lectins' binding assays can be found in Supplementary Result 2.

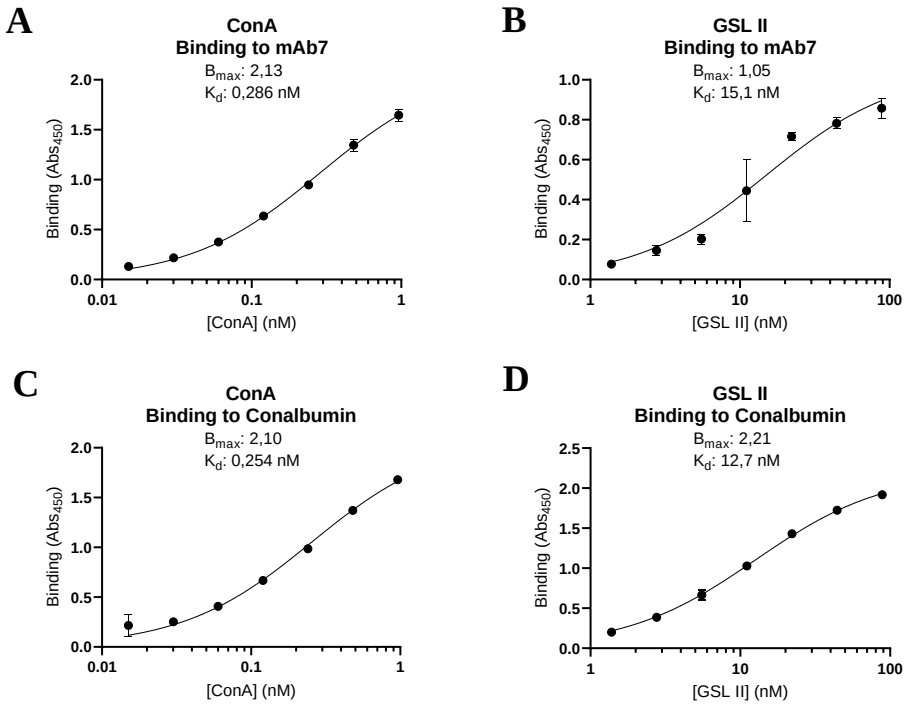


Figure 7 Binding between lectins and mAb7 respectively Conalbumin for different lectin-concentrations. After microtiter plate binding assay with different lectin concentration and a nonlinear regression of a saturation curve, an estimation of maximal signal B_{max} and affinity K_d could be made. The Absorbance at 450 nm for each lectin-concentration as well as the fitted saturation curve are shown **A** for ConA against mAb7, **B** for GSL II against mAb7, **C** for ConA against Conalbumin and **D** for GSL II against Conalbumin. The corresponding graphs for all the studied lectins can be found in Supplementary Result 2.

Since a low K_d corresponds to high affinity, the lectins in Table 3 are sorted from low to high estimated molar K_d against mAb7. Table 3 shows that ConA by far has the lowest estimated K_d against both mAb7 (0.286 nM) and Conalbumin (0.254 nM), indicating the highest affinity. Additionally, ConA gives a high maximal signal B_{max} which indicates higher maximal binding than the other lectins. Notable is that ConA show almost identical estimations of K_d and B_{max} for both mAb7 and Conalbumin. GSL II also show similar K_d for mAb7 (15.1 nM) and Conalbumin (12.7 nM), but a higher maximal binding for Conalbumin.

As Figure 7 shows, both ConA and GSL II resulted in sound saturation curves for both glycoproteins, which indicates that these results are reliable. LCA also showed a low estimated K_d and reliable saturation for mAb7 (9.67 nM), while its binding to Conalbumin did not show a perfect saturation-behaviour at all, why it is excluded from the table. However, LCA's maximal binding for Conalbumin seems to be much lower than its binding to mAb7 (Supplementary Result 2) Many of the other lectins that were tested for both glycoproteins showed significantly lower K_d and B_{max} for Conalbumin. This indicates a faster binding but lower total binding capacity and hence lower specificity for the glycans of Conalbumin. Especially big decrease in maximal binding to Conalbumin compared to mAb7-binding is shown for ECL, SBA, UEA I, RCA I and Jacalin. On the other hand, GSL II and STL show higher maximal binding for Conalbumin than for mAb7. Other lectins, such as DSL, show similar maximal binding for both glycoproteins.

Table 3 Affinity and maximal signal for each lectins' binding to mAb7 and Conalbumin, respectively. The estimated affinity K_d and maximal Abs_{450} signal B_{max} after a nonlinear regression of a saturation curve for different lectin-concentrations against mAb7 and Conalbumin, respectively. The lectins that did not show a typical saturation-curve behaviour against mAb7 are excluded. The lectins that were not tested against Conalbumin are marked '-' while the lectins that did not show a reliable saturation-curve for Conalbumin are marked '-*'.

Lectin	mAb7 K_d (nM)	mAb7 B_{max}	Conalbumin K_d (nM)	Conalbumin B_{max}
ConA	0.286	2.13	0.254	2.10
LCA	9.67	1.24	-*	-*
GSL II	15.1	1.05	12.7	2.21
DBA	21.2	1.42	-*	-*
ECL	28.5	1.02	2.54	0.09
SBA	29.2	0.61	2.15	0.19
UEA I	34.2	0.52	37.2	0.10
STL	51.9	0.36	68.6	1.17
LEL	56.5	0.70	12.5	0.22
DSL	69.6	0.72	26.2	0.50
RCA I	77.8	1.08	1.39	0.11
Jacalin	85.0	1.81	17.6	0.35
GSL I	223	0.30	-	-
VVL	258	0.76	-	-
WGA	261	1.05	-	-
PSA	265	0.48	-	-

The approximate K_d for ConA against both Conalbumin (0.254 nM) and mAb7 (0.286 nM) is about two orders of magnitude lower than the other lectins affinity for the same glycoproteins. This is also the same order of magnitude as the applied concentration of glycoproteins (0.667

nM). Looking at the ConA concentration at full saturation in Figure 7A and 7C, it is about 1 nM. A similar magnitude in the concentration of ConA and the glycoprotein thus is enough to obtain full saturation. On the contrary, all the other examined lectins require hundredfold higher concentration in relation to the glycoprotein concentration to reach saturation, which indicate low affinity.

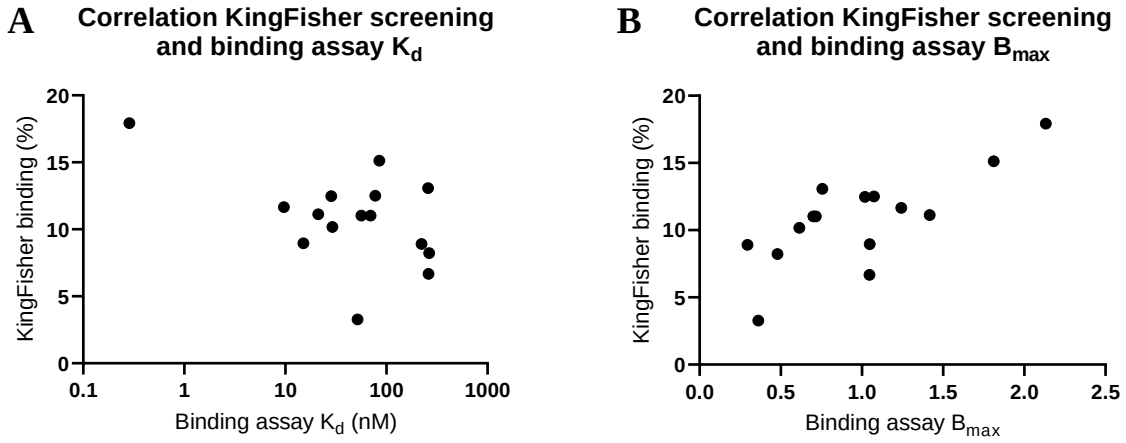
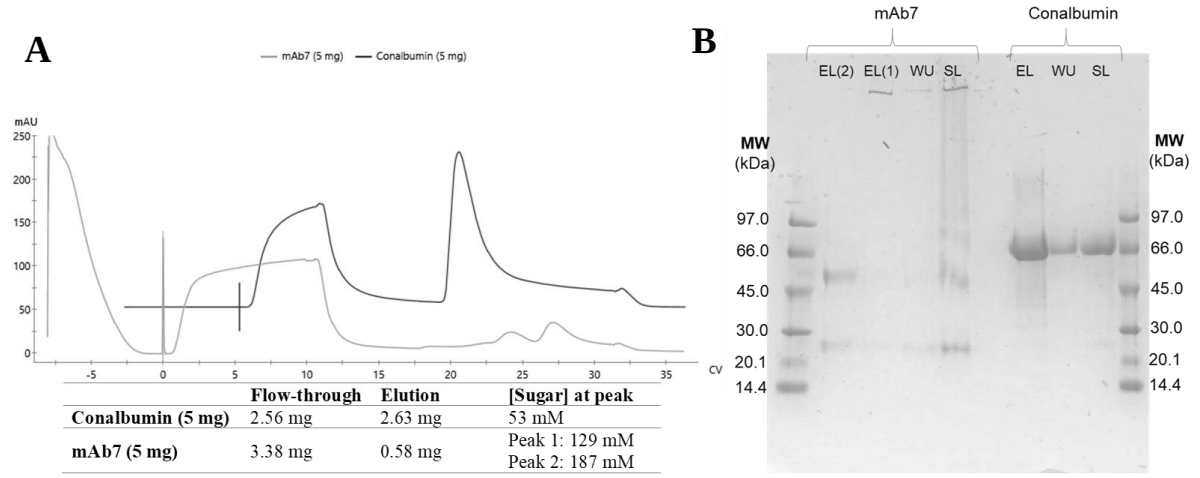


Figure 8 Correlation between the methods used for investigation of lectin-glycoprotein binding. A comparison of the results from binding between each lectin and mAb7 with two different methods. For each lectin, the percentage mAb7-binding from the KingFisher screening is plotted against **A** the estimated affinity K_d from the binding assay **B** the estimated maximal Abs_{450} signal B_{max} from the binding assay.

To relate the results from the binding assays (Table 3) with the previous screening of lectins using KingFisher Duo Prime (Figure 6), an investigation of the correlation between the result for each lectin was made (Figure 8). This shows that there is not an obvious correlation between the increasing binding according to KingFisher experiment and the decreasing affinity according to the binding assay (Figure 8A). A better correlation, however, seems to occur between the KingFisher binding and the binding assay maximal signal B_{max} (Figure 8B). Both the binding from KingFisher and the B_{max} corresponds to the maximal binding between the lectins and mAb7, meaning that the data somewhat supports each other. The affinity K_d on the other hand, does not necessarily correspond directly to the maximal binding.

4.1.3 Lectin Affinity Chromatography with ConA Sepharose 4B columns to study interactions with mAb7 and Conalbumin

To study the glycoprotein-binding ability of ConA resins, affinity chromatography was performed using 1 mL HiTrap ConA 4B columns (Cytiva). A method including 8 column volumes (CV) equilibration, 5 mg sample load, 7 CV wash of unbound sample and 15 CV elution with a linearly increased gradient of MeαMan was used with mAb7 and Conalbumin, respectively, as samples (Figure 9A). To ensure that the Abs_{280} in the chromatogram answered to the expected protein, each step of the chromatography were collected and analysed through SDS-PAGE (Figure 9B).



	Flow-through	Elution	[Sugar] at peak
Conalbumin (5 mg)	2.56 mg	2.63 mg	53 mM
mAb7 (5 mg)	3.38 mg	0.58 mg	Peak 1: 129 mM Peak 2: 187 mM

Figure 9 ConA 4B column's binding to Conalbumin and mAb7. Executing the same affinity chromatography method on HiTrap ConA 4B columns using mAb7 and Conalbumin as sample, respectively, and MeαMan as eluting sugar. **A** The resulting chromatograms for Conalbumin (top) and mAb7 (bottom). The amount protein during flow-through and elution was calculated with the extinction coefficients $\boldsymbol{\varepsilon_{280\,nm}^{0.1\%}}$ 1.48 for mAb7 and 1.16 for Conalbumin. **B** Analysis of the content from sample load (SL), wash of unbound sample (WU) and elution (EL) through SDS-PAGE to identify the expected proteins mAb7 (MW ~150 kDa; 23 kDa light chain, 50 kDa heavy chain) and Conalbumin (MW ~ 75 kDa), respectively.

Comparing the binding of 5 mg mAb7 and 5 mg Conalbumin to the ConA column (Figure 9A), there is a significant difference. Studying the mAb7 peak area together with the mAb7 extinction coefficient $\boldsymbol{\varepsilon_{280\,nm}^{0.1\%}}$ of 1.48 reveals that of the 5 mg applied sample, about 3.4 mg came out in the flow-through and only about 0.6 mg came out during the elution. This indicates that about 1 mg of mAb7 was still bound to the resin. This was also confirmed when the column was cleaned after usage with a pH 8.5 Tris-buffer and a pH 4.5 acetate buffer, causing additional protein to elute (not shown). Additionally, the chromatogram of mAb7 shows two peaks in the elution, one when the concentration MeαMan is about 130 mM and the other about 190 mM. Comparing this to Conalbumins chromatogram, one clear elution peak occurs and reaching maximum elution when the MeαMan concentration is about 50 mM. Calculation of the peak areas together with the Conalbumin extinction coefficient $\boldsymbol{\varepsilon_{280\,nm}^{0.1\%}}$ of 1.16 shows that of the applied 5 mg, about half comes out in the flow-through while the other half ends up in the eluate.

Figure 9B shows that the proteins present in each collected fraction of the experiments are the expected glycoproteins. For mAb7 bands are present at about 25 kDa as well as 50 kDa, answering to the light chain (~23 kDa) and heavy chain (~50 kDa) of the antibody. For the Conalbumin fractionation, clear bands are present about 70 kDa which is expected for Conalbumin (~75 kDa). Only by looking at the strength of the band on the gel answering to the elution peaks of mAb7 and Conalbumin, it appears clear that the eluted Conalbumin concentration is indeed greater. It also clearly shows that for both samples, a lot of protein present in the flow-through (sample load and wash unbound in Figure 9B).

Even if the binding of Conalbumin to ConA column is better than for mAb7, about half of the applied sample does not bind at all. To investigate if this was due to overload of the column or that 0.2 mL/min was to high application flow for sufficient binding, experiments with

lower amount sample and lower application flow rate were performed (Supplementary Result 5) However, decreasing the sample application to 1 mg did not increase the proportion of bound protein, and neither did reduction of the sample application flow to 0.1 mL/min.

4.1.4 Examination of whether Mg^{2+} ions are required to enhance Lectin binding

In a previous study that used similar microtiter plate binding assays to investigate the lectin LCA and its interaction with an antibody, the three metal ions Ca^{2+}, Mn^{2+} and Mg^{2+} were used in the binding buffer (Lundbäck *et al.*, 2016). Since other literature suggest that only Ca^{2+} and Mn^{2+} are essential to enhance binding for all lectins investigated in this study, including LCA, the dependence of Mg^{2+} was tested. To investigate whether magnesium ions are essential or not, a binding assay was made for LCA using a twofold dilution series concentration gradient of mAb7 between 0 and 1 µg/mL and a fixed concentration LCA of 1 µg/mL. The assay was performed according to the binding assay protocol (Supplementary Method 1), with the exception that $MgCl_2$ was added to the binding buffer (1 mM Tris pH 7.5, 1 mM $CaCl_2$, 1 mM $MnCl_2$, 0.1% Tween20) for some of the LCA samples. This gave a comparison between LCA's binding to mAb7 with and without present Mg^{2+} ions (Figure 10).

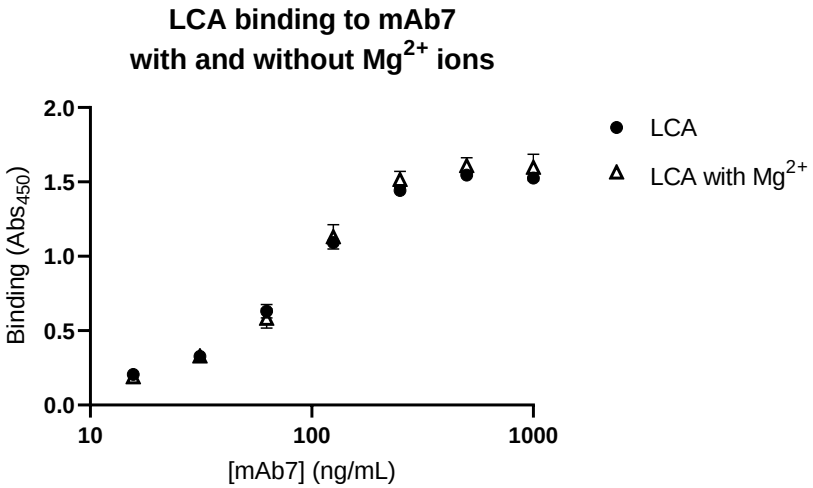


Figure 10 Binding between LCA and mAb7 in presence or absence of Mg^{2+} ions. Binding between LCA and mAb7 for different mAb7-concentrations, using a binding buffer with or without 1 mM $MgCl_2$. The binding signal on the Y-axis is the absorbance at 450 nm produced after the reaction between HRP and TMB, and hence give a relative comparison of binding, not an exact number of bound molecules.

As Figure 10 shows, the Abs_{450} for the samples are next to identical for all different concentrations of mAb7 regardless of the binding buffer containing $MgCl_2$ or not. This means that the presence of Mg^{2+} ions is dispensable in binding of LCA to mAb7. The same is assumed valid for all the lectins studied, meaning that only Ca^{2+} and Mn^{2+} ions are needed in the binding buffer for all experiments. For all the experiments in this study, Mg^{2+} ions are thus not used in any binding buffers.

4.2 Eluting sugars for different Lectins

The aim of all experiments in this section was to investigate possible eluting sugars for different lectins, which are important for the lectins' suitability as chromatography ligands. Most important is the sugars' maximal inhibition capacity of the lectins' binding to other compounds, as well as the sugar-concentration needed to reach inhibition. In other words, how much of a glycoprotein that potentially can be eluted by the sugar and how fast the elution can occur. To explore potential eluting sugars for 12 different lectins, microtiter plate sugar inhibition binding assays were made. The suitability of the identified competitive sugars as an eluting agent in lectin affinity chromatography was further evaluated through chromatography with ConA columns.

4.2.1 Identification of potential eluting sugars through inhibition of Lectin-mAb7 binding

Based on the result from the previous binding assay (Table 3), twelve of the lectins were included in the sugar inhibition studies. To investigate different carbohydrates and their inhibitory activity for each of these lectins, the binding assay was repeated almost identically. As before, microtiter plates were coated with 100 ng/mL mAb7. This time, however, the lectins were added to sugar solutions of eight different concentrations prior to their addition to the mAb7-coated microtiter plates, as described in competitive sugar binding assay protocol (Supplementary Method 2). For each lectin, one fixed concentration was used that corresponded to their estimated affinity K_d against mAb7 found in Table 3. The resulting absorbance at 450 nm after the binding assay was plotted against the sugar concentration in the software GraphPad Prism 8. For the graphs that showed reduced lectin-mAb7 binding as a result of increased sugar concentration, a nonlinear fit to an inhibition curve was made (Equation 2). A maximum inhibition I_{max}, as well as the sugar concentration needed to achieve half of that inhibition IC_{50}, was therefrom estimated for the lectin-sugar combinations that showed inhibition of the binding to mAb7.

To give an overview of the different sugars influence on each lectin, the estimated I_{max} was plotted against the estimated IC_{50} value. These summary graphs for the lectins ConA and GSL II are found in Figure 11. In Table 4 the IC_{50} concentration for the sugars causing at least 70% inhibition of the lectin-mAb7 binding are presented for each lectin. The rest of the summary graphs as well as the inhibition curves with maximal inhibition and IC_{50} values for all 12 lectins by the eight different sugars can be found in Supplementary Result 3.

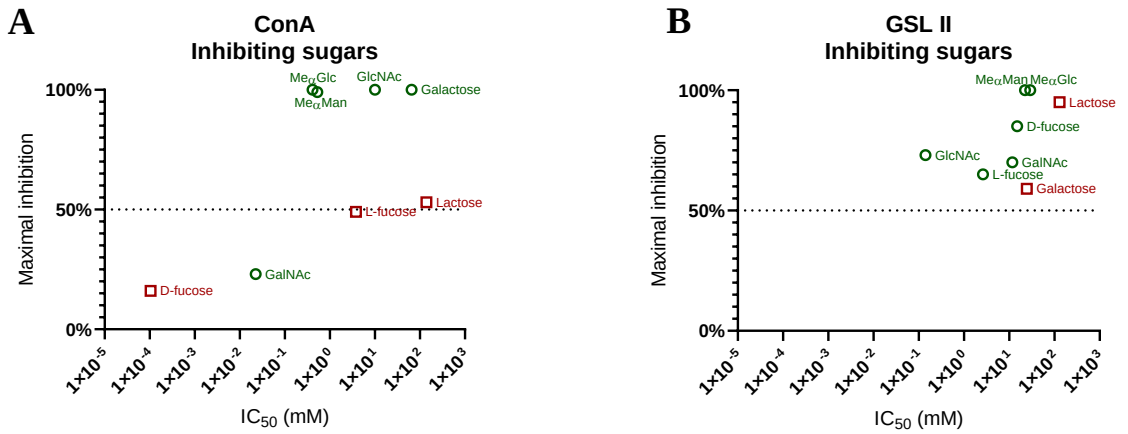


Figure 11 Maximal inhibition and inhibiting concentration by different sugars for binding between lectins and mAb7. Summarizing sugar-inhibition graphs for ConA (**A**) and GSL II (**B**), indicating the maximal inhibition between lectin and mAb7 on the y-axis as well as the inhibiting concentration IC_{50} logarithmically on the x-axis. The red squares indicate that the inhibiting curves (Supplementary Result 3) does not align perfectly and hence those values are somewhat uncertain. Sugars that did not show any inhibition at all are excluded.

When studying the sugar summary graphs in Figure 11, the optimal position for an elution sugar would be high up along the y-axis, and to the left along the x-axis. That means a high maximal inhibition and simultaneously a low required inhibiting concentration. When looking at ConA in that perspective, full inhibition seems to be possible by both MeαMan, MeαGlc, GlcNAc and galactose. However, the inhibiting concentrations for MeαMan and MeαGlc are about one order of magnitude lower than GlcNAc, that in turn is about one order of magnitude lower than galactose. For ConA, the greatest potential as eluting sugar thus are MeαMan and MeαGlc, even if GlcNAc and galactose also hold potential. An important note is that the inhibition behaviour can vary depending on glycoprotein, currently only demonstrated for the IgG1 mAb7.

Table 4 Summary of sugars causing at least 70% inhibition of lectins binding to mAb7. An overview of the twelve lectins and the eight different inhibiting sugars used, showing the estimated IC_{50} in mM, for the sugars estimated to achieve at least 70% inhibition. '*' indicates that the graph leading up to the estimation is not following a typical inhibition curve, although inhibition obviously occurs, which means that the estimated maximal inhibition and IC_{50} are not necessarily definite.

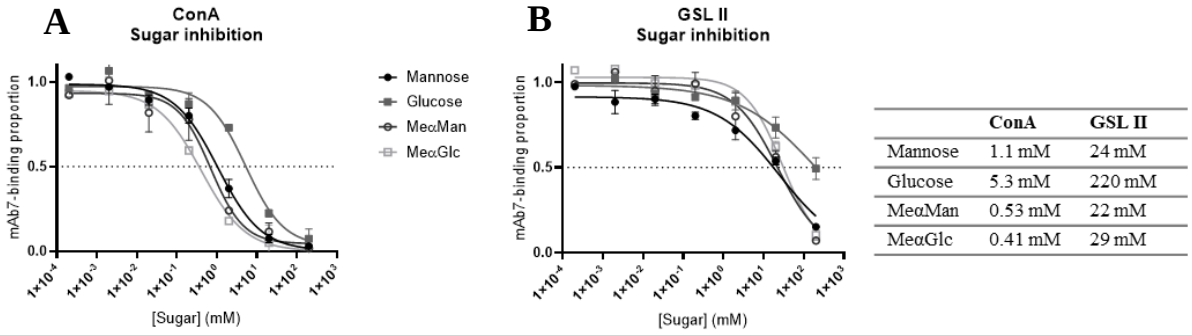
	MeαMan	MeαGlc	GlcNAc	GalNAc	Galactose	Lactose	D-fucose	L-fucose
ConA	0.53	0.41	10		66			
LCA	1.5×10^{-3}	12	190*	1.6*	92*	200*		
GSL II	22	29	0.14	12		130*	15	
DBA	12			10	1.5			
ECL				90	56		23	
SBA	150*		6.7×10^{-2}*		4.4×10^{-2}		1.2×10^{-5}	
UEA I							1.4×10^{-2}	
STL								
LEL					220*	200*		
DSL								
RCA I					0.31		5.5×10^{-2}*	
Jacalin				140*	150			

When studying the rest of the lectins in Table 4 it is clear that the different lectins have different preferred inhibiting sugars. Since all the values in Table 4 are for sugars causing at

least 70% inhibition, the sugar-concentration needed to reach half the maximal inhibition can be the compared. In that regard LCA prefer MeαMan; DBA galactose; ECL D-fucose; SBA both GlcNAc, galactose and D-fucose; UEA I D-fucose; RCA I galactose or D-fucose, Jacalin GalNAc or galactose. LEL show inhibition for galactose and lactose, but need higher concentration than used in this experiment, which also makes that result uncertain. Neither STL nor DSL show 70% inhibition for any of the examined sugars. Notable is also that L-fucose does not cause 70% inhibition for any lectin at all. Another trend is that lactose tend to inhibit lectin-glycoprotein binding, but only at very high sugar-concentrations.

4.2.2 Comparison of Mannose and Glucose with MeαMan and MeαGlc

In the literature, when eluting sugars are used for lectins with mannose- or glucose-specificity, the methylated versions α-methylmannoside (MeαMan) and α-methylglucoside (MeαGlc) most often are recommended without stating any clear explanation. The difference in inhibition of lectin-mAb7 binding between these sugars was therefore investigated in this study. For the three lectins that showed the greatest influence of MeαMan and MeαGlc in the previous sugar inhibition binding assay (Table 4, Figure 11 and Supplementary Result 3), that is ConA, LCA and GSL II, an identical sugar inhibition binding assay was performed using mannose and glucose as inhibiting sugar. The lectin-mAb7 binding was normalized to the maximal binding for each sugar to give comparable graphs (Figure 12). The corresponding comparison for LCA is excluded, since all sugar inhibitions did not give reliable inhibition curves. The separate inhibition graphs for ConA, GSL II and LCA by the four sugars can be found in Supplementary Result 4.



	ConA	GSL II
Mannose	1.1 mM	24 mM
Glucose	5.3 mM	220 mM
MeαMan	0.53 mM	22 mM
MeαGlc	0.41 mM	29 mM

Figure 12 Comparison of inhibition by methylated and unmethylated mannose or glucose. Normalized binding between mAb7 and **A** ConA **B** GSL II, using different sugar concentrations. To each result, a nonlinear regression is made to simulate an inhibition curve. The estimated IC_{50} values, that is the sugar concentrations needed to reach half of the inhibition, according to these inhibition curves are shown for each sugar.

Comparing the inhibition caused by the methylated and unmethylated forms of mannose and glucose, they all show potential to give full inhibition of both ConA and GSL II. To do this, however, mannose and glucose seems to require higher concentrations than its methylated forms. For instance, as shown in the table included in Figure 12, the estimated IC_{50} for mannose (1.1 mM) is about twice as high as the IC_{50} for MeαMan (0.53 mM), although they both can achieve full inhibition of ConA's binding to mAb7. The IC_{50} for glucose (5.3 mM) instead is one order of magnitude higher than the one for MeαGlc (0.41 mM). The proportion

is similar for GSL II when it comes to glucose (220 mM) compared to MeαGlc (29 mM). For mannose and MeαMan, however, the concentration to achieve inhibition of GSL II is almost equal around 20 mM. The methylated version of mannose and glucose thus require similar or lower concentration to reach full inhibition.

4.2.3 Study of eluting sugars in ConA columns

The sugars that showed potential as eluting sugars for ConA in the previous sugar inhibition binding assay (Figure 11, Table 4) were mainly MeαMan, MeαGlc, GlcNAc and galactose. Furthermore, the inhibition efficiency from mannose and glucose showed to be close to MeαMan and MeαGlc (Figure 12). These six sugars were therefore tested as eluting sugars in ConA columns during affinity chromatography. Additionally, a mixture of MeαMan and MeαGlc was tested to investigate if the elution could be increased by combining these sugars.

A 1 mL HiTrap ConA 4B column (Cytiva) was used and the method included 8 CV equilibration, 5 mg sample application, 7 CV wash of unbound sample and 15 CV elution with a linearly increased gradient up to 200 mM of the sugar was used. The sample used for all sugars was Conalbumin in a concentration of 1 mg/mL, and an application flow of 0.2 mL/min. The same column was used for all eluting sugars, but the column was washed with cleaning buffers (Tris-buffer pH 8.5 and acetate-buffer pH 4.5) between runs. The chromatograms obtained from each experiment are shown in Figure 13. From each peak area corresponding to flow-through and elution, respectively, the amount Conalbumin ($\boldsymbol{\varepsilon}_{\mathbf{280\,nm}}^{\mathbf{0.1\%}}$ = 1.16) was calculated as well as the present sugar-concentration at the elution maximum (Table 5).

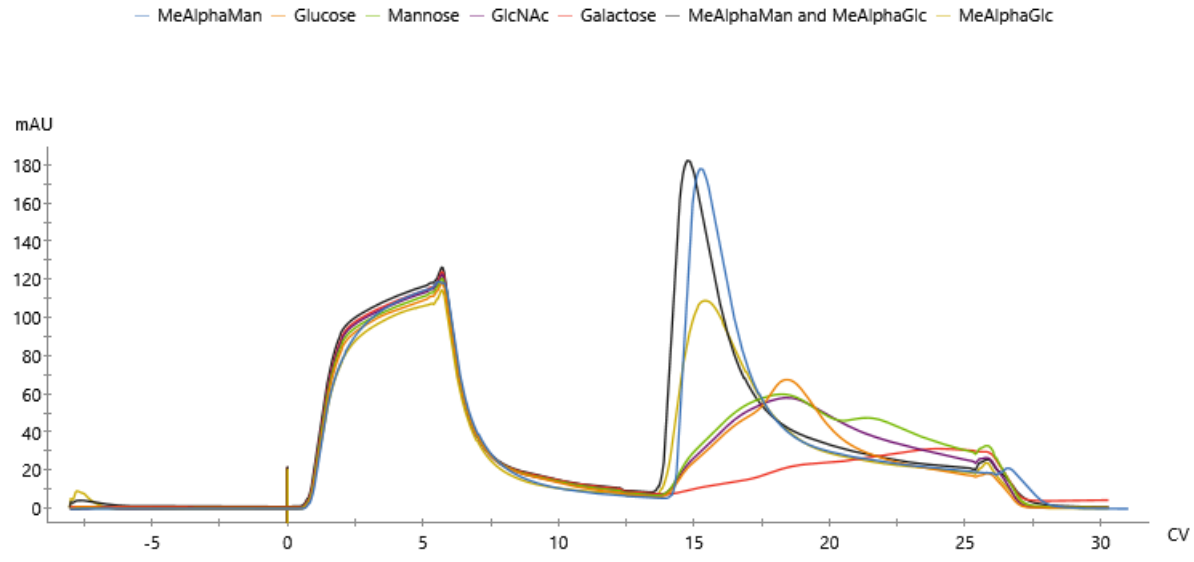


Figure 13 Eluting sugars ability to elute Conalbumin from ConA 4B column. Elution of Conalbumin from a HiTrap ConA 4B column using seven different eluting sugars under identical conditions. The first peak corresponds to sample application and the second to elution. Elution was made with a 10 CV long linear sugar-concentration gradient between 0 and 200 mM. The highest and narrowest elution top hence indicates the best elution and lowest sugar-concentration needed. Further comparison between the sugars can be found in Table 5.

The resulting chromatograms in Figure 13, together with the calculated amount in Table 5, shows that the flow-through is similar for all runs, indicating that the capacity is the same and the elution-behaviour should be comparable. When studying the elution peaks for each eluting sugar in Figure 13 the best candidates for elution from ConA columns are MeαMan, MeαGlc and a combination of the two. When comparing these two sugars separately, MeαMan elute a total of 2.6 mg Conalbumin while MeαGlc elute 2.2 mg. Furthermore, the peak occurs at 53 mM MeαMan, and at 64 mM MeαGlc. MeαMan thus achieves the highest elution of Conalbumin and moreover at the lowest concentration. Slightly better elution, however, seems to be achieved when a mixture of MeαMan and MeαGlc are used, reaching eluting maximum at a concentration of 52 mM MeαMan and 52 mM MeαGlc.

Table 5 Amounts of Conalbumin in flow-through and elution, as well as concentration of eluting sugar needed for maximal elution. Calculated amounts of Conalbumin leaving the ConA column during sample application (flow-through) and elution, respectively, using the peak area and extinction coefficient for Conalbumin $\boldsymbol{\varepsilon_{280\,nm}^{0.1\%}}$ = 1.16. The sugar-concentration when the elution-peak was reached is also indicated.

Sugar	Flow-through	Elution	[Sugar] at peak top
MeαMan	2.56 mg	2.63 mg	53 mM
MeαGlc	2.41 mg	2.24 mg	64 mM
Mannose	2.61 mg	2.19 mg	121 mM
Glucose	2.57 mg	1.74 mg	125 mM
GlcNAc	2.65 mg	1.97 mg	125 mM
Galactose	2.67 mg	1.26 mg	200 mM
MeαMan/MeαGlc	2.70 mg	2.80 mg	52 mM/52 mM

When comparing the other sugars, the elution peak is not at all as narrow and high as for MeαMan. When looking at the total eluted amount by mannose, glucose and GlcNAc it is not a huge difference, and if the elution proceeds long enough all of the protein would possibly be eluted. The big difference to MeαMan and MeαGlc instead is the retention time and sugar-concentration needed to achieve elution. Both mannose, glucose and GlcNAc reach the elution top at about 120 mM, that is about double the concentration needed for elution with MeαMan and MeαGlc. For galactose, even higher concentration is needed to reach maximum elution. In the chromatogram, maximum elution seems to be at 200 mM, but since the concentration gradient ends at 200 mM and only a fraction of the bound amount is eluted it is reasonable to assume that even higher concentration is needed to achieve full elution. These results together support MeαMan as the best eluting sugar, ultimately combined with MeαGlc. Moreover, the sugar-inhibition show the same trend as the microtiter plate binding assays.

4.3 Possible prevention of ConA leakage

Since the previous results indicates that ConA by far is the best alternative for binding to both mAb7 and Conalbumin, the ability to optimize the existing ConA resins was investigated. As discussed in the introduction and theory of the report, one of the main drawbacks with ConA as chromatography ligands, and the rationale to this project, is its tendency to fall apart and leach into the eluate which can cause contamination of the isolated sample. A potential solution could be a stabilizing cross-linking between monomers and between ConA and the

resin, preventing the subunits to fall apart. Such a cross-linking has been made on ConA 4B resin, through treatment with 25% glutaraldehyde solution, based on a method suggested by Kowal & Parsons (1980).

To investigate if this treatment affected the ConA leakage, as well as if the binding capacity of the resin was impaired, the Cross-linked ConA resin was compared to the common HiTrap ConA 4B column. The Cross-linked resin was first packed into 1 mL columns. The same was done for a Reference resin, which came from the same lot and went through the same treatment as the Cross-linked resin, but without addition of the cross-linking agent glutaraldehyde. For all three columns (1 mL HiTrap ConA 4B, 1 mL packed Cross-linked resin, 1 mL packed Reference resin), the same chromatography method was executed. The method included 8 CV equilibration, 5 mg sample application, 7 CV wash of unbound sample and 15 CV elution with a linearly increased gradient up to 200 mM of MeαMan. The sample used was Conalbumin in a concentration of 1 mg/mL, and an application flow rate of 0.2 mL/min. The results were thus comparable chromatograms (Figure 14A). The flow-through from all the parts of the method was collected and analysed through SDS-PAGE (Figure 14B).

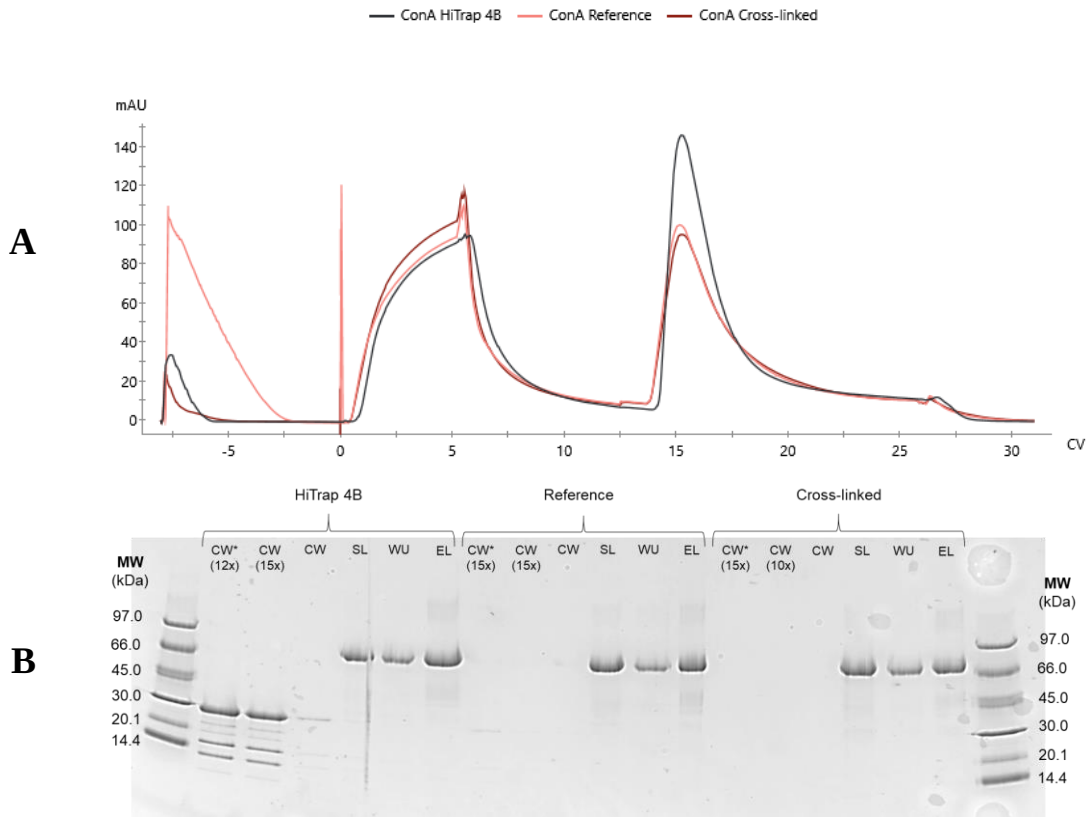


Figure 14 Comparison of ConA 4B columns with and without cross-linking. Comparison of 1 mL HiTrap ConA 4B column, Cross-linked ConA resin as well as a Reference ConA resin which gone through the same washing steps as the cross-linked one. **A** Chromatogram illustrating the difference in Abs_{280} for the different columns during column wash, Conalbumin sample load and unbound wash, gradient elution with MeαMan and lastly equilibration. **B** SDS-PAGE analysis of collected fractions for the different steps of the chromatography-method for each column. CW = first column wash, CW* = first column wash for another identical column, SL = sample load, WU = wash unbound, EL = elution. The CW fractions were concentrated 10-15 times, as indicated in parenthesis. The comparison is made in terms of leakage of ConA monomers (MW ~25 kDa) in column wash (CW) fraction. The rest of the fractions (SL, WU, EL) instead aims to assure that the protein is Conalbumin (MW ~75 kDa) as expected.

When the chromatograms (Figure 14A) are compared, the HiTrap column shows a better yield than both the Cross-linked column and the Reference, since the flow-through peak is slightly smaller and the elution peak higher. However, the yield compared between the Cross-linked resin and the Reference is close to identical, suggesting that the cross-linking itself does not impair the resin's capacity.

To further investigate if the difference in obtained yield between HiTrap and the Reference is due to lot-to-lot variations, a totally untreated resin from the same lot as the Cross-linked and Reference resins, was packed. The same chromatography method was executed for this column leading to a chromatogram (Supplementary Result 6) with elution peak almost identical to the HiTrap column, suggesting that lot-to-lot variation is not the answer. To instead investigate the influence of washing of resin, a comparison was made of an unused HiTrap column and a HiTrap column from the same lot but that had been used multiple times, with cleaning steps in-between each usage. The resulting chromatogram (Supplementary Result 6) was almost identical, suggesting that the HiTrap columns' yield do not decrease despite multiple usages and washings of the packed resin.

When looking at the Abs_{280} signal during the first column wash, which answers to potential ConA leakage, the HiTrap column shows a clearer peak than the cross-linked one. The signal from the Reference resin, however, is unreasonably big, why this comparison is not reliable. Instead, the SDS-PAGE analysis of the collected fractions (Figure 14B) can be studied. To be able to determine whether leakage occurred or not during the first column wash, the collected fraction of 7 CV was concentrated around 15 times. Furthermore, column wash samples from two identical columns of each type were used to get sample duplicates.

The resulting SDS-PAGE gel in Figure 14B first of all show that Conalbumin (MW ~75 kDa) as expected occur in the collected flow-through and elution fractions. Furthermore, the gel shows that the column wash of the HiTrap column resulted in leakage of ConA-monomers (MW ~25 kDa), even visible without concentrating the fraction. For the Reference column, weak bands around 25 kDa for the concentrated fractions indicates that some leakage occured. The Cross-linked column, however, did not result in any bands at all, neither for the concentrated fractions. This indicates that less leakage occurred for the glutaraldehyde-treated Cross-linked ConA 4B column, even if more accurate methods are required to confirm that claim.

5 Discussion

5.1 Binding between Lectins and Glycoproteins

To study the binding between different lectins and glycoproteins, several experiments were executed. First, a screening of 20 lectins (Table 1) and their interaction with mAb7 was made, using KingFisher Duo Prime. Second, microtiter plate binding assays were made to study interactions between lectins and mAb7 or Conalbumin. Lastly, both mAb7 and Conalbumin were also investigated as samples in affinity chromatography with ConA columns.

5.1.1 Comments on screening results

When calculating the ratio between lectins and mAb7 in the screening, it seems like only a fraction of the available lectins bind to mAb7. For ConA, for instance, only about 40% of the available ConA (~0.77 nmol) bound mAb7 (~0.29 nmol). Additionally, ConA consists of four identical subunits, each with a carbohydrate recognition site (CRD) which means that even more binding should be possible (Nascimento *et al.* 2012). However, during the screening the biotinylated lectins were immobilized to the streptavidin-coated magnetic beads, which probably caused a sterically hindrance for some of the subunits. In fact, the lectins immobilized on the beads might be too tightly packed, meaning that there is not enough room for all lectin molecules to interact with a glycoprotein. In addition, the degree of biotinylation may affect the activity of the lectins if the biotin molecules are conjugated to amino acids in a way that interferes with the lectins carboydrate interaction.

Another possible explanation could be that only a fraction of the mAb7 includes Fab-glycosylation. The Fc-glycosylation, which always occurs for IgG antibodies, is buried on the inside of the structure which usually makes it unavailable for binding (Huang *et al.* 2016). The mAb7-molecules that only contains Fc-glycosylation hence most likely are unavailable for lectin-binding. An additional potential explanation to why all lectins do not bind to the glycoprotein might simply be that the time-duration for lectin-mAb7 binding in this screening method (10 minutes) is not long enough for sufficient binding.

Regardless of the relationship between amount lectin and glycoprotein, the lectin screening shows that ConA is superior to the other lectins with regards to mAb7 binding. Furthermore, most of the lectins seems to achieve decent binding to mAb7. However, the lectin UEA I in this screening did not show any binding. A poor binding could be explained by factors as inaccessibility of the core fucose of the mAb7 glycans, since UEA I has documented specificity for fucose (Vector Laboratories 2020). However, the calculations in fact indicates that UEA I achieved less binding than the unspecific binding of the negative control (Supplementary Result 1). This is probably not accurate, but rather caused by a source of error.

In this work, the KingFisher reaction was only made once for each lectin. The results are thus not based on duplicates which obviously affect the reliability. The absorbance measurements

after the reaction were made in duplicates to ensure correct values. The binding results are also based on the difference in absorbance in the well containing mAb7, together with the absorbance in the following washing step. A more correct analysis would probably be to elute the bound mAb7 in a well and measure its absorbance to determine the actual binding. The number of wells on one plate was not enough to include such a step, which meant that the binding rather was based on the disappearance of mAb7. A future improvement could thus be to immobilize the lectins to the magnetic Sepharose beads beforehand, to give enough wells on the plate to include an elution step. However, such a elution step would also take longer time and require optimization for each lectin to ensure that all the bound glycoprotein gets eluted. Another improvement could be to perform each reaction in duplicates to give more reliable data.

5.1.2 Notes on Binding assay results

When studying the molar binding and affinity of each lectin, it is important to note that the lectins have different number of subunits and hence different binding sites which affects the binding capacity. Tetramers can thus be expected to have around four times higher molar binding than a monomer with the same affinity per binding site. With this in mind, the K_d values can be slightly misleading since the calculations were made in the same way for all lectins regardless of the number of binding sites.

Some variations can also occur for the maximal signal B_{max} between the lectins binding curves, since the magnitude of the signal, aside from the number of bound molecules, also is dependent on the incubation time after TMB addition. That is, the longer time the enzyme HRP has to react with the substrate TMB, the higher the signal will be. The relation between signals of different lectins with identical incubation time thus should indicate the relative maximal binding. However, since the lectins binding assays were made at different times and on different microtiter plates, the magnitude of the signal can vary somewhat between experiments and hence between the lectins. However, a lectins B_{max} value should give a good indication of the maximal binding in relation to the other lectins, even if an exact comparison cannot be done.

Additionally, both the K_d value and B_{max} used for each lectin were estimated through the nonlinear regression made based on the signal. For some of the lectins, the concentration interval did not allow the interaction to reach full saturation (Supplementary Result 2). This means that the maximal saturation signal B_{max} is a estimation assuming that the saturation-curves behaviour continues as in the concentration interval. Since B_{max} for each lectin is a more or less rough estimation, also the subsequent K_d values used are rough estimations. These estimated values should therefore not be interpreted as exact values but rather a source for comparison between the lectins. Another important factor, that was not examined in this study, is the time dependence of the binding. In the binding assays all lectin-glycoprotein interactions lasted for one hour, and hence does not guarantee that equilibrium is reached for all binding events.

5.1.3 Evaluation of the Lectins' binding to Glycoproteins in Binding assays

In total, lectin binding assays against mAb7 were performed for 20 lectins. For 12 of these lectins, who showed the lowest molar K_d for mAb7, the same binding assay was performed against Conalbumin. As Figure 7 shows, the binding for both these glycoproteins is similar for ConA in terms of both K_d and maximal binding. Comparing the other lectins in Table 3, the maximal binding seems to be much bigger for mAb7 than for Conalbumin when it comes to the lectins ECL, SBA, UEA I, RCA I and Jacalin. The opposite trend is witnessed for GSL II and STL.

The different glycoforms found associated to Conalbumin, do not at all include core fucose (Jiang *et al.* 2014). On the other hand, the glycoforms associated with mAb7, most often contain core fucose. Consequently, the lectins with specificity for fucose should reasonably show inferior binding for Conalbumin then for mAb7. The only lectin that previously has been reported to have specificity for fucose is UEA I (Vector Laboratories 2020). In accordance to this hypothesis, UEA I showed significantly lower B_{max} for Conalbumin (0.10) than for mAb7 (0.52) while showing a similar K_d for both (~35 nM).

In a similar way, galactose occur frequently in mAb7 glycoforms, while hardly any galactose-containing glycoforms at all are found for Conalbumin (Jiang *et al.* 2014). Lectins with specificity for galactose or similar sugars should hence show greater affinity for mAb7 than for Conalbumin. In fact, the remaining lectins that showed lower maximal binding for Conalbumin (ECL, SBA, RCA I, Jacalin) have recorded specificity for galactose or related sugar moieties (Vector Laboratories 2020). The galactose-specificity reported for these lectins is thus supported by this study.

Sialic acid does not seem to bre present in any of the investigated glycoproteins (Jiang *et al.* 2014). The only mutual constituents of all glycans, for both glycoproteins, thus are GlcNAc and mannose. This explains why ConA, with a recorded specificity for both mannose and glucose, show a very similar binding and K_d for both mAb7 and Conalbumin. The lectins that showed reinforced binding to Conalbumin (GSL II and STL), both have recorded specificity for GlcNAc (Vector Laboratories 2020). The two monomers LEL and DSL show a comparable binding capacity for both mAb7 and Conalbumin. In fact, also these lectins have previously recorded specificity for GlcNAc (Vector Laboratories 2020). In other words, the lectins that exhibit good binding against Conalbumin have also earlier shown specificity for mannose and GlcNAc. The result of the binding assay does thus support the glycoforms illustrated in Figure 4.

When looking at the results of the binding assay (Table 3), regardless of the glycosylation of the two investigated glycoproteins, superior binding capacity and affinity are exhibited by ConA. The ConA concentration needed to reach full saturation is around the used concentration of glycoprotein and is about hundredfold lower than the other lectins. Notable is that even if the maximal binding between ConA and glycoprotein occur at a low concentration, it is not possible to determine how much of the ConA molecules that have

actually bound to the glycoprotein. So even if the glycoprotein-concentration and the ConA-concentration are similar at full saturation, it does not necessarily mean that all these molecules bind to each other. However, since the resulting maximal signal B_{max} from ConA binding to both glycoproteins is higher than for the other lectins, the number of lectin-glycoprotein interactions do not seem to be beaten by any other. In other words, when it comes to separation of glycoproteins in general, the capacity of ConA seems to be hard to overrule.

5.1.4 Comparison of the methods used to study Lectins' binding

The screening with the KingFisher instrument allowed a screening of all 20 plant lectins and their binding against mAb7 within one hour. In other words, this method is an effective and fast way to get a picture of the binding capacity of all lectins. The method is fully automated once the plate is loaded and the software is programmed, except from the analysis part. Because of the rapid screening and short binding time (10 minutes), the result subsequently only shows the maximal binding after 10 minutes. The result hence does not necessarily visualize which lectins that have the highest affinity, but rather which lectins that can bind the most during the used binding time. Since a substrate-target binding in the end is an equilibrium between on- and off-rate, a fast binding does not mean a stable binding. Although, it is important to keep in mind that this work investigate the lectins potential as substrate in chromatography which means that a rather fast binding is critical to pick up the flow of target molecule. A stable binding is however still necessary to maintain the target molecule in the chromatography resin until eluted.

The screening through microtiter plate binding assays took considerably longer time to perform, since the protocol for one plate took about five hours to perform. For all lectins, eight different concentrations were used, and all lectins were studied in duplicates. This allowed six different lectins per plate, which means that the screening of all 20 lectins took some time. It also meant that the lectins were incubated with mAb7 for at least one hour and that the result showed lectin-mAb7 binding for different lectin concentrations. Consequently, these results not only gave a picture of the lectins binding to mAb7, but also a picture of the affinity. Since all reactions were made in duplicates, the data are also more reliable.

To summarize, the methods give slightly different information – while KingFisher screening reveal fast binding to mAb7, the binding assay rather briefly reveal the affinity and kinetics of the interaction. When it comes to the application as ligand in lectin-based chromatography, both of those factors are important to consider. There is also a difference in the capacity and time consumption for the two methods – while KingFisher screening allow screening of 20 lectins with one concentration within one hour, the binding assay allow much more data points for each lectin but in total take days to perform. However, the correlation between the methods (Figure 8) indicates that the maximal binding of the binding assay somewhat correlates to the binding from the KingFisher screening. When only comparing the maximal binding capacity of different lectins, both methods thus seems to be valid. Additionally, that

indicates that the maximal binding behaviour is similar after 10 minutes and after 60 minutes, possibly because equilibrium is reached.

5.1.5 ConA columns' ability to bind Glycoproteins

Affinity chromatography with HiTrap ConA 4B columns was performed for both mAb7 and Conalbumin. Something that becomes obvious in the comparison of these two glycoproteins in chromatography is that Conalbumin binds to the ConA column significantly better than mAb7, which is illustrated in Figure 9. However, Conalbumin did not show complete binding to the ConA column as about half of the applied Conalbumin was detected in the flow through. The capacity of the 1 mL HiTrap ConA 4B column used should be around 20-45 mg porcine thyroglobulin (GE Healthcare 2014). Thyroglobulin is a large protein of 670 kDa, and hence about four times larger than mAb7 and nine times larger than Conalbumin. If the capacity is correlated to moles (Molecular Weight taken into consideration), the maximal capacity of the used glycoproteins thus should be around 5-10 mg mAb7 and 2-5 mg Conalbumin. In other words, the column should not be fully saturated by the applied amount of mAb7 (5 mg). This is however expected based on the data obtained from KingFisher screening, For Conalbumin on the other hand the applied amount (5 mg) could potentially overload the column. However, the same behaviour for Conalbumins binding is shown when using 1 mg sample (Supplementary Result 5), which should not be enough to overload the column. Instead, the reason to the limited binding must be due to limited accessability of the glycans.

ConA showed very poor binding to mAb7 (Figure 9). Out of 5 mg applied sample, about 3.5 mg was detected in the flow-through and hence did not bind to ConA at all. As mentioned above, the capacity of the columns should in theory be enough to bind 5 mg mAb7. Moreover, the HiTrap ConA column consist of 10-15 mg ConA (GE Healthcare 2014). Converted to moles (MW ~104 kDa) that corresponds to 96-144 nmol. The applied 5 mg mAb7 converted to moles (MW ~150 kDa) corresponds to 33 nmol. Assuming that 1.5 mg of the mAb7 bound to the ConA column, that represent 10 nmol. This means that approximately 10 nmol mAb7 molecules have bound to 100 nmol ConA molecules and hence only 10% of the ConA molecules should be occupied. This can be compared to the findings related to the screening with KingFisher Duo Prime, discussed in section 4.1.1, where approximately 38% of the present ConA managed to bind mAb7 molecules. Obviously, the circumstances are different during these two methods, as well as the applied sample. However, both these findings indicate that only a fraction of the ConA molecules binds to mAb7, at least when ConA is immobilized to a solid support. Such poor binding cannot simply be because of the crowding of ConA molecules hindering each other from binding. Instead, the lack of flexibility when immobilized onto a solid surface is a possible explanation, together with the short residence time.

As mentioned earlier, Fc-glycans of IgG antibodies have been shown not to interact with ConA columns during affinity chromatography, since they are buried on the inside of the antibody's structure (Huang *et al.* 2016). The very poor binding of mAb7 to ConA column

hence indicates that mAb7 rarely has Fab-glycosylation. If all mAb7 molecules would have Fab-glycosylation, the ConA column reasonably would bind much more protein. Since all *N*-linked glycans, including those identified for mAb7, contains mannose in some extent (Figure 2), ConA in theory somewhat should possess specificity for all glycans. However, around 30% of the applied mAb7 molecules bind to the column, which indicate that Fab-glycosylation does occur in some extent. Otherwise, some of the Fc-glycans find it way to the ConA CRDs even if they are structurally buried at the interior. However, high-mannose glycans have previously been found to occur for a fraction of mAb7. High-mannose structures should never occur in antibodies' Fc region (Seeling *et al.* 2017). In other words, the mAb7 proteins that contain high-mannose structures can be assumed to have Fab-glycosylation. If the complex structures instead are assumed to be Fc-glycans, the hypothesis of differences in occurrence of Fab-glycosylation within the mAb7 sample is a reasonable explanation for the poor binding to ConA columns.

As illustrated in Figure 9, only about half the applied amount of Conalbumin binds to the ConA column. Even if the binding-capacity is much better than the binding to mAb7, the flow-through during sample application is surprisingly high. Making the same molar conversion as for mAb7 above, the applied 5 mg of Conalbumin (MW ~75 kDa) corresponds to approximately 67 nmol. Assuming that half the applied sample bind hence means that around 33 nmol bind to the ConA column, containing at least 100 nmol ConA molecules. This means that 2/3 of the ConA molecules remain free and unbound. This could simply be because of the crowding of ConA molecules hindering each other from binding, but once again, the column should not be saturated from neither the mAb7- nor Conalbumin sample. The ratio for Conalbumin is significantly better than the corresponding ratio for mAb7, but still is notably low. Conalbumin only contain *N*-linked glycans (Jiang *et al.* 2014). Since *N*-linked glycans always contain mannose in some extent, ConA should in theory be able to bind more than half of the applied Conalbumin sample.

Conalbumin is an iron-binding protein, and hence contains a Fe binding site (Xie *et al.* 2002). This Fe-binding site can however also bind other metal ions, such as Mn^{2+} (Giansanti *et al.* 2012). During sample application, the Conalbumin therefore can bind some of the Manganese ions in the binding buffer. This could potentially somewhat impair the binding capacity of ConA, which otherwise is enhanced by presence of Mn^{2+} ions (O'Connor *et al.* 2017). However, the Mn^{2+} ions in the used buffer are in excess, which means that this phenomena should not affect the binding significantly. Another explanation to the poor Conalbumin binding, is that the Conalbumin sample used potentially varies in present glycoforms or occupied glycosylation sites. Suppose that a part of the Conalbumin molecules only contain glycosylation on the interior of the structure, while the rest contain exposed glycans. Then the affinity chromatography would only bind the available glycans while the rest ends up in the flow-through. When it comes to Conalbumin glycosylation, GlcNAc residues usually are the terminal sugars of the glycans (Jiang *et al.* 2014). If these residues block the availability to the mannose-residues within it could impair the potential of ConA's binding to these glycans.

Notwithstanding this, ConA should have enough specificity against Conalbumin glycans to bind half of the applied Conalbumin sample. To study the hypothesis of different glycoforms in the sample, mass spectrometry could be a good way to compare the mass of the glycoprotein in the flow-through and eluate, respectively.

Especially evident for mAb7, all bound glycoprotein was not eluted from the column with MeαMan as eluting sugar. A solution could be to decrease the pH during elution to achieve a better elution. However, a low pH should be avoided since the lectin ConA has been found to prefer a dimer stage at pH below 5 (Calvete *et al.* 1999). Too low pH could consequently lead to increased ConA leakage and would likely negatively affect the column performance. Additionally, too low pH during the elution, could affect the eluate proteins negatively depending on its pH stability. A better solution would thus be to try to increase the eluting capacity through optimization of the eluting sugar.

5.2 Eluting sugars for different Lectins

To examine potential eluting sugars for 12 of the lectins, microtiter plate sugar inhibition binding assays were performed with eight different sugars for each lectin. Furthermore, the most promising sugars for ConA were tested as eluting sugars during affinity chromatography with ConA columns.

5.2.1 Notes on Sugar inhibition assay

As shown in Figure 11, Table 4 and Supplementary Result 3 the choice of eluting sugar is important. For many lectins, several sugars demonstrate significant inhibition and could be considered as eluting sugars in a chromatography setting. However, the sugar-concentration needed to achieve that sufficient inhibition varies widely. For LCA, for example, MeαMan shows good inhibition and an IC_{50} of 1.5×10^{-3} mM while lactose also seems to achieve high maximum inhibition but with a IC_{50} of 200 mM. Notable, such high IC_{50} should be considered critically since the highest sugar-concentration used in the assay was 200 mM. This means that it is hard to secure a good inhibition-curve behaviour for sugars that require such high concentration for inhibition. Furthermore, when applying this to a chromatography methodology, too high sugar-concentrations are not preferable because of solubility and cost considerations.

A source of error in the methodology leading up to the results is that especially two of the sugars, L-fucose and GlcNAc probably were not fully solubilized upon usage. For GlcNAc this was displayed through the fact that too high concentrations of GlcNAc in the assay led to decreased inhibition for many lectins. These values were excluded when making the linear regression of an inhibition curve, which means that several of the GlcNAc results only includes a concentration gradient up to 2 mM. For L-fucose, none of the lectins showed any significant inhibition. Since L-fucose has low solubility (Table 2), an explanation would hence be that the sugar-solution was not solubilized enough.

Notably, the inhibition was only studied for lectins' interaction with the glycoprotein mAb7. The inhibiting capacity for the sugars hence may be somewhat different for other glycoproteins. Ideally, the inhibition should hence be studied for multiple glycoproteins to be able to find the optimal sugar that inhibits binding of each lectin.

5.2.2 Evaluation of inhibiting sugar for each Lectin

The obtained sugar-inhibition results for each lectin (Table 4) can be compared to the previously reported eluting sugars, which are listed in Supplementary Theory 2 (Vector Laboratories 2020). Furthermore, the results can be examined in terms of cost, according to Table 2. When, in this discussion, using the term "good inhibition" it refers to inhibition above 70%.

ConA showed a high inhibition at low sugar-concentrations of both MeαMan and MeαGlc. This match the recommended eluting sugar solution which consist of a combination of MeαMan and MeαGlc (Vector Laboratories 2020). Decent inhibition was also achieved by GlcNAc and galactose, even if higher concentrations are needed. For ConA, also unmethylated mannose and glucose were tested (Figure 12) to investigate their inhibition compared to MeαMan and MeαGlc. These results indicate that mannose and glucose also accomplish full inhibition, but at significantly higher sugar-concentrations compared to their methylated analogoues. This further confirms that the suggested MeαMan and MeαGlc are superior as eluting sugars. When looking at the price for these two sugars (Table 2), MeαGlc is about three times cheaper than MeαMan.

For **LCA**, MeαMan seems to be preferred based on this study. MeαMan is recommended as eluting sugar, but in combination with MeαGlc (Vector Laboratories 2020). MeαGlc also showed good inhibition in this study, but whether a mixture of the two would enhance inhibition or not cannot be determined by the data. Further, GlcNAc, GalNAc, galactose and lactose also inhibited binding to LCA but at higher IC_{50} values. Unmethylated mannose and glucose were also tested for LCA (Supplementary Result 4). These showed high inhibition, with comparable IC_{50} as MeαGlc. However, MeαMan by far has the lowest IC_{50} against LCA and therefore appears as the best choice as eluting sugar.

For **GSL II**, GlcNAc is recommended as eluting sugar (Vector Laboratories 2020). In this study, GlcNAc exhibited the lowest IC_{50} concentration, but a higher maximal inhibition was shown with MeαMan and MeαGlc. Also, GalNAc showed decent inhibition. Further, mannose and glucose were tested (Figure 12). They both showed full inhibition and mannose had an inhibiting concentration similar to MeαMan and MeαGlc. Glucose instead had an inhibiting concentration tenfold higher. Thus, either GlcNAc, MeαMan, mannose or MeαGlc is the best eluting sugar candidates for GSL II. From a economical point of view, MeαGlc is about three times cheaper than the other three (Table 2).

DBA has GalNAc as suggested eluting sugar (Vector Laboratories 2020). Good inhibition was found in this study for GalNAc. However, galactose showed greater potential, since

lower concentration was needed. Additionally, MeαMan seems to be promising as inhibitor for DBA-binding. The sugar to recommend based on this study thus is galactose, since it has the lowest inhibiting concentration. Additionally, according to the costs listed in Table 2, galactose is about 500 times cheaper than GalNAc, making it an obvious choice for large scale usage.

For **ECL**, lactose is recommended as eluting sugar, while galactose and GalNAc also are listed as the lectin's sugar-specificity (Vector Laboratories 2020). This study indicates that galactose and GalNAc cause high inhibition, while lactose does not obtain more than 50% inhibition. Interesting, however, is that good inhibition and the lowest IC_{50} for ECL were demonstrated by D-fucose. This suggests that ECL, apart from galactose- and GalNAc-specificity, also has high specificity for D-fucose. However, since both D-fucose and GalNAc are hundredfold more expensive than galactose (Table 2), the latter may be considered for large scale usage.

SBA has GalNAc listed as suggested eluting sugar (Vector Laboratories 2020). In this study the best inhibition was achieved by galactose, GlcNAc and D-fucose. However, GalNAc also showed a decent inhibition of 68%, but at a higher sugar-concentration than the others. When comparing D-fucose with galactose, D-fucose exbibited 1000 times lower inhibiting concentration and is about 500 times more expensive (Table 2) than galactose. With these circumstances, D-fucose thus seems to be the best candidate as eluting sugar, both from a functional and an economical view.

UEA I is the only one of the lectins, that has reported specificity for fucose (Vector Laboratories 2020). More precisely, L-fucose is suggested as the eluting sugar. In this study, however, the greatest inhibition of UEA I was achieved by D-fucose, while L-fucose did not show inhibition at all. L-fucose did in fact not achieve good inhibition of any of the lectins. This could be due to the poor solubility of L-fucose of 50 mg/mL (Table 2) which potentially interrupted the experiments. Hence, L-fucose and its inhibition of UEA I could be studied further. With that said, this study reveals D-fucose as a promising eluting sugar candidate for UEA I.

RCA I has galactose or lactose recommended as eluting sugars (Vector Laboratories 2020). This study showed that galactose indeed is a good inhibitor of RCA I binding. Also, D-fucose seems to achieve high inhibition of RCA I and at an even lower concentration than galactose. Lactose on the other hand only achieved around 50% inhibition. The best candidate as eluting sugar thus seems to be D-fucose. Considering how much lower inhibiting concentration that was required for D-fucose it is also beneficial from a economical point of view even if D-fucose is much more expensive than galactose (Table 2).

For **Jacalin**, a high concentration of galactose is recommended (Vector Laboratories 2020). This study indicates that Jacalin is inhibited by both galactose and GalNAc. Further, the required concentration of both these sugars were quite high, as expected. In that aspect,

galactose may be the best candidate since it is cheaper than GalNAc. Note that also melibiose is suggested as eluting sugar but was not examined in this study.

For both **STL**, **LEL** and **DSL**, the suggested eluting sugar is chitin hydrolysate, which basically is a concentrated mixture of GlcNAc monomers and oligomers (Vector Laboratories 2020). However, neither of the lectins showed significant inhibition by GlcNAc in this study. This might be due to poor solubility of the GlcNAc-solution used in the experiment. It could also be that chitin hydrolysate is required to achieve the inhibiting property. Instead, LEL showed inhibition by lactose and galactose, but at very high concentrations. DSL and STL did not show good inhibition for any sugar at all. Consequently, further studies would be necessary to find suitable eluting sugars for these lectins, using both chitin hydrolysate and fully dissolved GlcNAc.

5.2.3 Eluting sugars' efficiency in ConA columns

To apply the results from the sugar inhibition binding assay discussed above, to chromatography, it is obviously important to relate the inhibition on the microtiter plates to the elution in chromatography resins. Since different eluting sugars were used for ConA columns (Figure 13), based on the sugars that showed good inhibiting potential in the microtiter assay, such a comparison can be made. A difference between the methods, however, is that mAb7 was used for all sugar inhibition binding assays while Conalbumin was used as sample for the chromatography eluting comparison.

Figure 13 show that good-looking elution peaks were obtained for MeαMan, MeαGlc and a combination of the two. The peaks for glucose, mannose, GlcNAc and galactose on the other hand, are wider and thus clearly require higher sugar-concentrations. This is especially the case for galactose which does not really lead to any peak at all. When looking at the estimated amounts in Table 5 it can be concluded that the total eluted amounts for MeαGlc, mannose and GlcNAc are not significantly lower than for MeαMan. Glucose and galactose however show a significantly lower elution. However, also these sugars would probably achieve similar amounts eluate if the sugar-concentration was high enough. So, the concentration needed to achieve the elution top is similar for MeαMan (53 mM) and MeαGlc (64 mM), about twice as high for mannose (121 mM), glucose (125 mM) and GlcNAc (125 mM) and even higher for galactose.

Coupling this to the results from the sugar inhibition assay (Figure 11, Figure 12), it reveals the same behaviour; Low IC_{50} concentration for MeαMan (0.53 mM) and MeαGlc (0.41 mM), somewhat higher for mannose (1.1 mM), glucose (5.3 mM) and GlcNAc (10 mM) and highest for galactose (66 mM). Additionally, all these sugars showed close to full inhibition of ConA also in the binding assay.

Noteworthy is that the glycoprotein used in chromatography for this experiment was Conalbumin, while mAb7 was used in the microtiter sugar inhibition assay. The choice of glycoprotein investigated will likely have big influence on the inhibiting capacity of different

sugars. Depending on lectins' affinity to the glycoprotein, the interaction will be more or less challenging to inhibit. To study potential eluting sugars on microtiter plates, the glycoprotein that will be used in chromatography also optimally should be the one studied on plates. However, the glycosylation of both mAb7 and Conalbumin contain mannose, which means that for the mannose-recognizing ConA studied here, the behaviour should be similar for both glycoproteins.

With this in mind, the results discussed here indicate that the initial sugar inhibition screening in microtiter plates serve as a good approximation of the eluting sugars suitability in a chromatography setting and require less material. Furthermore, it confirms that MeαMan, MeαGlc or ultimately a combination of the two are the preferred as eluting sugars for ConA resins.

5.3 Possible prevention of ConA leakage

To study the leakage of ConA columns, as well as evaluate a potential cross-linking method to prevent leakage, chromatography was performed for treated and untreated resins. The cross-linking treatment had been made beforehand in accordance to a method proposed by Kowal & Parsons (1980) using 25% glutaraldehyde solution.

5.3.1 Evaluation of Glutaraldehyde-treated ConA resin

The results indicate that the cross-linked ConA resin does not suffer from leakage, which HiTrap ConA 4B column obviously do. Important to note is that the main comparison is between the Reference (Ref) column and the Cross-linked column, which both were packed in HiTrap columns under similar conditions as compared to the HiTrap ConA 4B column. The Ref resin was treated in the same way as the Cross-linked resin but without the presence of the cross-linking agent glutaraldehyde. In other words, the Ref resin and Cross-linked resin have gone through the same amount of washing steps. This means that if the Cross-linking would not work, the same amount of leakage could be expected from the Cross-linked column as for the Ref column.

As Figure 14B shows, the column wash of the Ref column leads to leakage as expected, even if 15x concentrated fractions were required to give a visible bond on the SDS-PAGE gel. The leakage hence is significantly lower for the Ref column than for the HiTrap column, which is expected because of the washing steps experienced by the Ref resin. The column wash fraction from the Cross-linked column does not show any band at all, even when concentrated 15x, which indicates that no leakage, or at least less than for the Ref column, occur.

The chromatograms from each column (Figure 14A) show an unreasonably high absorbance peak for the Ref-column in the first column-wash section. This must be due to error in the UV-sensor or the storage solution that makes an unexpected impact on the sensor. The Abs_{280} for the column wash of each column in the chromatograms are therefore not considered as comparable data. Instead the main source of comparison of the columns is the SDS-PAGE

gel. However, to be able to unambiguously ensure that no leakage occur for the Cross-linked column, further and more accurate methods should be performed to compare the column wash flow-through from the Cross-linked and Ref column. The indication from this study, however, should motivate further investigation of the cross-linking method.

The important thing to conclude when studying the chromatograms for each column (Figure 14A) is that the Cross-linked resin and the Ref resin seems to have identical behaviour and yield. This indicates that the cross-linking as such, or the cross-linking agent glutaraldehyde, does not impair the resins function.

However, both the Ref- and Cross-linked column show decreased capacity compared to the HiTrap ConA column. One considered reason for such a difference was the fact that the HiTrap ConA columns are pre-packed while the Ref- and Cross-linked resin were packed prior to usage in this study. Slightly different column-packing methods could thus lead to differences in capacity. However, when a totally untreated version of the Reference resin (uRef) was packed in a identical way as Ref and Cross-linked column, it showed that uRef had similar capacity as the HiTrap column (Supplementary Result 6). That result hence exclude the column packing as source of the capacity difference.

Instead, the additional washing steps of the Ref and Cross-linked resin is a potential reason for their impaired yield relatively to the untreated resins. When comparing HiTrap columns with different number of usages and washing steps, however, no difference was found (Supplementary Result 6). In other words, multiple washes of a packed column should not affect the yield significantly. However, the additional washing steps of the Ref- and Cross-linked resin were made on unpacked resin. The explanation hence could be that the washing procedure of unpacked resin tends to wash away ConA molecules more extensively than the washing of packed resin. Since the molecules in unpacked resin are not as tightly and rigidly packed together, such an event is not impossible. This should be studied further, by for example washing the unpacked uRef resin and compare its capacity with the other columns. If this is the case, the process in which the cross-linking is performed has room for improvement. Optimally, the cross-linking steps thus could be executed on packed resin. However, the glutaraldehyde reactions causing the cross-linking may not be sufficiently efficient in that condition of the resin.

Another way to decrease the leakage of ConA columns, could simply be to wash the columns with a high number of column volumes binding buffer or other solution prior to use. This would decrease leakage since most of the leakage appears to take place in the first usage of the column. Furthermore, the washing of packed resin has in this study been shown to not impair its capacity (Supplementary Result 6) which means that it would be a very simple way to reduce leakage without affecting the binding capacity significantly. The manual coming with the HiTrap ConA 4B column hence could be extended with a section advising the user to wash the column a number of times before usage.

6 Conclusions and Future perspecitves

The following is a summary of the discussion above, pointing out the main conclusions to be drawn from each part of this study. Furthermore, future perspectives for each block are suggested.

6.1.1 Binding between Lectins and Glycoproteins

To summarize, what can be said about the lectins and their ability to bind to the glycoproteins mAb7 and Conalbumin is the following: First, Mg^{2+} ions do not seem to reinforce binding to glycoproteins. Secondly, there seems to be a correlation between the measured maximal binding through the microtiter plate binding assay and the KingFisher screening, meaning that the KingFisher screening could be a fast way to get a quantitative comparison between several lectins. Third, ConA unambiguously exhibits the best binding and affinity to both mAb7 and Conalbumin according to both used methods in the study. ConA, however, is less efficient in binding to mAb7 when immobilized on a solid surface, probably due to limited Fab-glycosylation of mAb7 and limited residence time. Conalbumin binds significantly better to ConA columns but still exhibit a significant amount of Conalbumin in the flow-through, which could be due to glycosylation heterogeneity in the sample. Fourth, when generalizing the binding of different lectins, the lectins with expected fucose or galactose-specificity seems to prefer mAb7 over Conalbumin. Simultaneously, lectins with mannose or GlcNAc specificity shows a more comparable binding to mAb7 and Conalbumin. This collectively supports the expected glycosylation pattern shown in Figure 4. On that note, lectins with specificity for mannose and/or glucose could be preferred for glycoproteins in general since those sugars are present in all *N*-linked glycosylation. Lectins with other specificities may however also be of interest and potentially enable separation of different glycoforms. For instance, a lectin with high galactose-specificity could be used to separate proteins with complex glycans from proteins with high-mannose glycans.

For future work on lectins, the comparison between lectins and their binding to glycoproteins from this study can be used as starting point. Further investigation of these lectins could be made with additional glycoproteins to compare the affinity to different glycans. Other methods could be used, such as surface plasmon resonance biosensors to more accurately be able to determine actual affinity, as well as methods to study the time-dependence of the interactions to assure equilibrium. Most interesting may be further studies of DSL and LEL, whose monomeric nature could avoid leakage from the column and additionally make them more suitable for recombinant expression. If other lectins are investigated, the methodology for comparison used in this study otherwise could be helpful. For the binding to ConA column, the heterogeneity in glycoforms for mAb7 and Conalbumin could be examined. This could for instance be made through mass spectrometry of the flow-through and eluate, respectively. This could verify that the relatively poor binding to ConA columns is because the glycans of some glycoforms are structurally hidden for the immobilized ConA proteins.

6.1.2 Eluting sugars for different Lectins

When summarizing the conclusions from the study of sugar-specificity and eluting sugars, the following can be highlighted: First, potential eluting sugars for 12 different lectins are suggested (Table 4 and section 5.2.2), based on their inhibiting power and required concentration, also indicating the lectins' sugar specificities. These suggestions are somewhat different than the eluting sugars suggested by literature (section 5.2.2) and could thus be valuable for applications with the studied lectins. Secondly, the application to ConA columns indicates that the used sugar inhibition binding assay could be a good approximation of the sugars' suitability as eluting sugars in lectin affinity chromatography. Third, when it comes to eluting sugars for ConA MeαMan, MeαGlc or ultimately a combination of the two seems to be preferred as eluting sugar for the used glycoproteins. Lastly, the comparison to mannose and glucose shows that their methylated versions binds similarly well or better to lectins with mannose or glucose specificity, why MeαMan and MeαGlc in general should be recommended over mannose and glucose, respectively.

For future work, if any of the lectins studied are considered as ligand for affinity chromatography, the suggested eluting sugars from this study could be used as starting point for both the lectins specificity and to optimize elution. Further studies should however be made for the inhibition by L-fucose and GlcNAc which worked poorly, probably due to insufficient solubilization. Additionally, studies of sugars inhibition of the lectins' interactions with other glycoproteins should be studied to consolidate the suggestions in this study.

6.1.3 Possible prevention of ConA leakage

To summarize what can be said about ConA leakage from the results in this study is, first, that distinct leakage of prepacked ConA HiTrap 4B columns is observed upon first usage but seems to decrease along the number of usages. Second, the cross-linking treatment with glutaraldehyde potentially improve the ConA stability and reduce leakage, although more confirmatory studies are needed. Third, multiple washes of resins packed into columns do not seem to affect the glycoprotein yield significantly, while washing of unpacked resin does.

Recommendation for future work in the subject of ConA leakage is, first, to use a more sensitive method to further analyse if any leakage at all occurs for the Cross-linked column. Second, further investigation of the impact on washing of unpacked resin could be made to ensure that this is the reason for the impaired yield. If that results matches the results of this study, optimizations of the cross-linking method could be made by reducing the washing steps or perform it on packed resin if possible. A first, simple way to reduce ConA leakage for the customers without impair the capacity could however be to wash the column before usage. The instruction manual supplied with the HiTrap ConA 4B column could thus be updated with a exhortation of such a washing step with binding buffer or deionized water.

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Supplementary Theory 1 – Recombinantly expressed plant Lectins

Several plant lectins have been recombinantly expressed in different host cells, including *E. coli* and the yeast *Pichia pastoris*. In general, *E. coli* is preferred for non-glycosylated lectins while *P. pastoris* has advantages for expression of lectins that require PTMs. The aim of the recombinant expression varies, but several of the following lectins are expressed for applications in the biomedical field and studied in terms of cancer or viral targeting. (Oliveira *et al.* 2013)

Recombinant expression in *Pichia pastoris*
The plant lectin *Nicotiana tobacum* lectin has been recombinantly expressed in the yeast *P. pastoris* with binding properties like the native lectin. Normally this lectin is a non-glycosylated homodimer expressed in the leaves of tobacco plants, only upon treatment with the plant hormones jasmonates, and specificity has been proven to *N*-acetylglucosamine oligomers and high-mannose glycans. Previously, other plant lectins such as *Phaseolus vulgaris* agglutinin (PHA), *Galanthus nivalis* agglutinin (GNA) and *Canavalia brasiliensis* lectin (ConBr) have been expressed in *Pichia*. (Lannoo *et al.* 2007)

Recombinant expression in *Escherichia coli*
A mannose binding Jacalin-related lectin (JRL) that has been expressed in *E. coli* is Helja, a lectin extracted from sunflower seedlings. This lectin does not need PTMs in its native form, which allow the bacterial expression. The aim of the study was to investigate its potential for neuroblastoma cancer treatment. (Pinedo *et al.* 2017)

Frutalin is a partly glycosylated, homotetrameric lectin with galactose-specificity originating from *Artocarpuc incisa* (breadfruit). This lectin has been recombinantly expressed in both *P. pastoris* and *E. coli*. The recombinant lectin's carbohydrate affinity was inferior than the native one but showed great potential as biomarker for prostate cancer and also as apoptosis-inducer. (Oliveira *et al.* 2014)

The gene for *Erythrina cristagalli* lectin (ECL) has also been isolated, recombinantly expressed and solubilized from inclusion bodies in *E. coli* with maintained functionality compared to the native lectin. In the end 870 mg/L biologically active lectin was obtained, but this required inclusion body recovery and refolding after expression. (Stancombe *et al.* 2003)

An additional lectin that has been expressed in *E. coli* is the tetramer *Dolichos biflorus* agglutinin (DBA), originating from horse gram seeds. About 20% of the expressed lectin was soluble and the rest was solubilized, with the same function and properties. The recombinant lectin showed a slightly lower affinity than the native protein. (Chao *et al.* 1994)

Other plant lectins that have been expressed in *E. coli* with varying yields are for example ASAL, CRAMOLL I, GNA, MAH mutants, PTA, PCL and Aviscumine (Oliveira *et al.* 2013).

Recombinant expression in other hosts

The two JRLs Bana lectin and Jacalin have successfully been recombinantly expressed in a plant-based platform, namely through the tobacco-relative plant *Nicotiana benthamiana*. Banana lectin is a mannose-binding JRL and has previously been expressed in yeast and *E. coli*, but since Jacalin and other galactose-binding JRL have more complex structure and need of glycosylation it seems to need a eukaryotic plant-host to maintain function and specificity. (Fernandez-del-Carmen *et al.* 2013)

The Soybean agglutinin (SBA) has also been expressed in the plant *N. benthamiana* as well as the potato tubers *Solanum tuberosum*, resulting in a recombinant lectin with similar glycosylation and specificity as the native form (Oliveira *et al.* 2013).

Another eukaryotic platform that has been used as a tool for recombinant lectin expression is insect cells. For example, Peanut agglutinin (PNA) has been recombinantly expressed in this way, resulting in PNA yield of 9,8 mg/L medium and with maintained specificity as compared to the native lectin (Oliveira *et al.* 2013).

Supplementary Theory 2 – Lectin properties

Table ST2.1 Lectin properties of the used lectins. The biotinylated plant lectins (Vector Laboratories) used in this study, together with information about each lectin according to Vector Laboratories documentation (Vector Laboratories 2020).

Name	Full Lectin name	Sugar specificity	Source	Sub unit	MW (kDa)	Present Metal ions	Proposed eluting sugar	pI	glycoprotein	$\varepsilon^{0.1\%}_{280nm}$
			KIT I							
ConA	Concanavalin A	Mannose	*Canavalia ensiformis* (Jack bean) seeds	4	104	Ca^{2+}, Mn^{2+}	200 mM α-methylmannoside/200 mM α-methylglucoside	6.3-7	No	1.20
DBA	Dolichos biflorus agglutinin	*N*-Acetylgalactosamine	*Dolicos biflorus* (Horse Gram) seeds	4	111	Ca^{2+}, Mn^{2+}, Mg^{2+} Zn^{2+}	200 mM N-acetylgalactosamine	4.6-5	Yes	1.22
PNA	Peanut Agglutinin	Galactose	*Arachis hypogaea* peanuts	4	110	Ca^{2+}, Mg^{2+}	200 mM galactose	5.5-6.5	No	0.89
RCA I	Ricinus communis agglutinin I	Galactose, *N*-Acetylgalactosamine	*Ricinus communis* (Castor Bean) seeds	2	120	no	200 mM galactose or lactose	7.8	Yes	1.17
SBA	Soybean agglutinin	*N*-Acetylgalactosamine (also partly galactose)	*Glycine max* (soybean) seeds	4	120	Ca^{2+}, Mn^{2+}	200 mM *N*-Acetylgalactosamine	5.8-6	Yes	1.33
UEA I	Ulex Europaeus agglutinin I	Fucose	*Ulex europaeus* (Furze Gorse) seeds	2	63	Ca^{2+}, Mn^{2+}, Zn^{2+}	50-100 mM L-fucose	4.5-5.1	Yes	1.30
WGA	Wheat Germ agglutinin	*N*-Acetylglucosamine	*Triticum vulgaris* (wheat germ)	2	36	Ca^{2+}	Chitin Hydrolysate or 500 mM *N*-Acetylglucoseamine	> 9	No	1.46
			KIT II							
GSL I	Griffonia simplicifolia lectin I	Galactose	*Griffonia simplicifolia* seeds	4	114	Ca^{2+}, Mn^{2+}	200 mM galactose/200 mM *N*-Acetylgalactosamine	5-6.5	Yes	1.40
LCA	Len culinaris lectin	Mannose	*Lens culinaris* (lentil) seeds	4	50	Ca^{2+}, Mn^{2+}	200 mM α-methylmannoside/200 mM α-methylglucoside	7.6-8-4	No	1.25
PHA-E	Phaseolus vulgaris Erythroagglutinin	Galactose, Complex structures	*Phaseolus vulgaris* (Red Kidney Bean) seeds	4	126	Ca^{2+}, Mn^{2+}	100 mM acetic acid	6-8	Yes	1.16
PHA-L	Phaseolus vulgaris Leucoagglutinin	Galactose, Complex structures	*Phaseolus vulgaris* (Red Kidney Bean) seeds	4	126	Ca^{2+}, Mn^{2+}	100 mM acetic acid	4.2-4.8	Yes	1.16
PSA	Pisum sativum agglutinin	Mannose, Glucose	*Pisum sativum* (Pea) seeds	4	53	Ca^{2+}, Mn^{2+}	200 mM α-methylmannoside/200 mM α-methylglucoside	6.0-6.7	trace	1.20
sWGA	Wheat Germ agglutinin, succinylated	*N*-Acetylglucosamine	*Triticum vulgaris* (wheat germ)	2	36	Ca^{2+}	Chitin Hydrolysate or 500 mM *N*-Acetylglucoseamine	< 3	No	1.46
			KIT III							
DSL	Datura stramonium lectin	*N*-Acetylglucosamine	*Datura stramonium* (Thorn Apple, Jimson Weed) seeds	1	86	no	Chitin Hydrolysate	> 9	No	0.80
ECL	Erythria cristagalli lectin	Galactose, *N*-Acetylgalactosamine, Lactose	*Erythrina cristagalli* (Coral Tree) seeds	2	54	Ca^{2+}, Mn^{2+}, Zn^{2+}	200 mM lactose	6.3-6.5	Yes	1.30
GSL II	Griffonia simplicifolia lectin II	*N*-Acetylglucosamine	*Griffonia simplicifolia* seeds	2	113	Ca^{2+}, Mn^{2+}	Chitin Hydrolysate or 200 mM *N*-Acetylglucoseamine	5-6	Yes	1.25
Jacalin	Jacalin	Galactose	*Artocarpus integrifolia* (Jackfruit) seeds	4	66	no	800 mM galactose or 100 mM melibiose	7.8	Yes	1.50
LEL	Lycopersicon esculentum lectin	*N*-Acetylglucosamine	*Lycopersicon esculentum* (tomato) fruit	1	71	--	Chitin Hydrolysate	> 9	Yes	0.76
STL	Solanum tuberosum lectin	*N*-Acetylglucoseamine	*Solanum tuberosum*, (potato) tubers	2	100	no	Chitin Hydrolysate	> 9	Yes	0.80
VVL	Vicia villosa lectin	*N*-Acetylgalactosamine	*Vicia villosa* (Hairy Vetch) seeds	4	(102-144)	Ca^{2+}, Mn^{2+}	200 mM *N*-Acetylgalactosamine	5.5-6.2	Yes	0.78

Supplementary Method 1 – Protocol for Binding assay

This protocol is modified from Lundbäck *et al.* (2016) and was followed to perform a binding assay to investigate the binding between the monoclonal antibody mAb7 and a biotinylated lectin.

Washing buffer: 0.1% TBS-T
(50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Tween20)

Binding buffer
(1 mM Tris pH 7.5, 1 mM $CaCl_2$, 1 mM $MnCl_2$, 0.1% Tween20)

Method

4. Dilute mAb7 in PBS buffer to desired concentration
5. Coat microtiter plate with 100 µL diluted mAb7 and incubate at RT overnight
6. Wash plate 5 times with washing buffer and let stand in washing buffer in RT for 1h (300 µL/well)
7. Equilibrate plate through 5 washes with Binding buffer (300 µL/well)
8. Dilute biotinylated lectins to desired concentration in Binding buffer
9. Add 100 µL lectins to each well and incubate in 37°C for 1h
10. Wash plate 5 times with washing buffer (300 µL/well)
11. Dilute Streptavidin-HRP according to the bottle in washing buffer, add 100 µL to each well and incubate for 20 min in RT
12. Wash plate 5 times with washing buffer (300 µL/well)
13. Add 100 µL TMB substrate solution to start reaction, incubate 25-30 min in RT, protected from light
14. Add 50 µL 2N sulfuric acid to stop reaction
15. Measure Absorbance at 450 nm

Supplementary Method 2 – Protocol for Sugar inhibition binding assay

This protocol is modified from Lundbäck *et al.* (2016) and was followed to perform a binding assay to investigate the influence of different sugar concentrations on the binding between the monoclonal antibody mAb7 and a biotinylated lectin.

Washing buffer: 0.1% TBS-T
(50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Tween20)

Binding buffer
(1 mM Tris pH 7.5, 1 mM $CaCl_2$, 1 mM $MnCl_2$, 0.1% Tween20)

Assay

1. Coat microtiter plate with 100 µL of 100 ng/mL mAb7 in each well and incubate at RT overnight
2. Wash plate 5 times with washing buffer and let stand in washing buffer in RT for 1h (300 µL/well)
3. Equilibrate plate through 5 washes with Binding buffer
4. Dilute sugar in Binding buffer, making tenfold dilution series
5. Dilute biotinylated lectins to concentration around estimated K_d with each sugar concentration
6. Add 100 µL lectin+sugar to each well and incubate in 37°C for 1h
7. Wash plate 5 times with washing buffer (300 µL/well)
8. Dilute Streptavidin-HRP (according to bottle) in washing buffer, add 100 µL to each well and incubate for 20 min in RT
9. Wash plate 5 times with washing buffer (300 µL/well)
10. Add 100 µL TMB substrate solution to start reaction, incubate 25 min in RT (dark)
11. Add 50 µL 2N sulfuric acid to stop reaction
12. Measure Absorbance at 450 nm

Supplementary Result 1 – Binding according to Lectin screening

Table SR1.1 Screening of 20 plant lectins reveal their binding to mAb7. Calculated binding of lectins to mAb7 according to Abs_{280} after execution of the KingFisher instrument. The fraction of mAb7 bound to lectins is based on the difference in absorbance between each lectin and the negative control. Also, the following washing step is considered to take away the very loosely bound mAb7.

Lectin	Bound mAb7
UEA I	-7.6%
STL	3.3%
PHA-L	3.9%
PNA	6.6%
WGA	6.7%
PSA	8.2%
PHA-E	8.6%
GSL I	8.9%
GSL II	9.0%
sWGA	9.4%
SBA	10.2%
DSL	11.0%
LEL	11.0%
DBA	11.1%
LCA	11.7%
ECL	12.5%
RCA I	12.5%
VVL	13.1%
Jacalin	15.1%
ConA	17.9%
prismA (ctrl)	90.5%

Supplementary Result 2 – Saturation curves from Binding assays

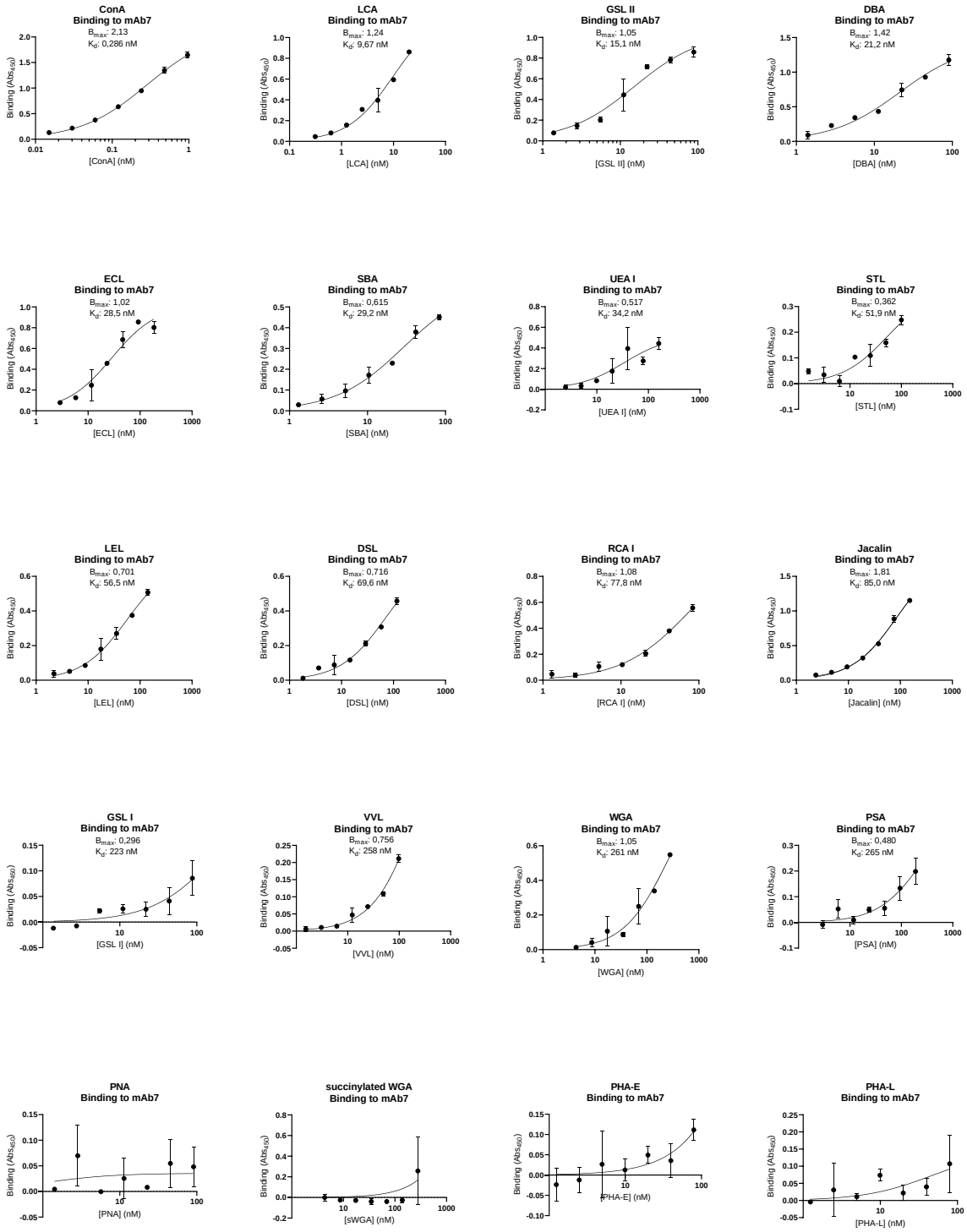

Figure SR2.1 Binding between 20 lectins and mAb7. Abs_{450} measured for each lectin after binding assay using mAb7 and different lectin-concentrations. A nonlinear regression is made to fit a saturation curve to the concentration-dependence, from which a maximal signal B_{max} and affinity K_d were estimated.

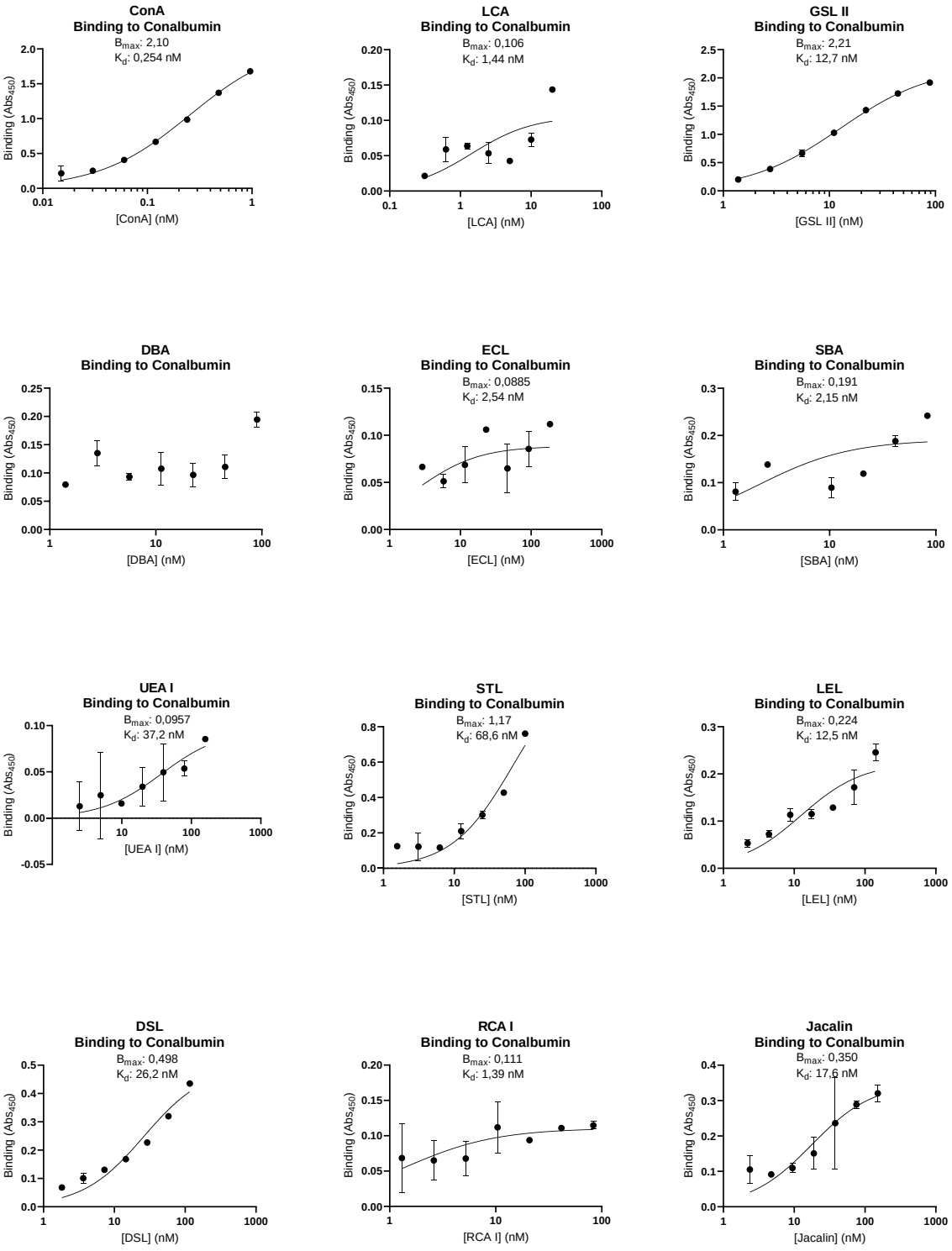

Figure SR2.2 Binding between 12 lectins and Conalbumin. Abs_{450} measured for each lectin after binding assay using Conalbumin and different lectin-concentrations. A nonlinear regression is made to fit a saturation curve to the concentration-dependence, from which a maximal signal B_{max} and affinity K_d were estimated.

Supplementary Result 3 – Inhibition curves for all Lectins and sugars

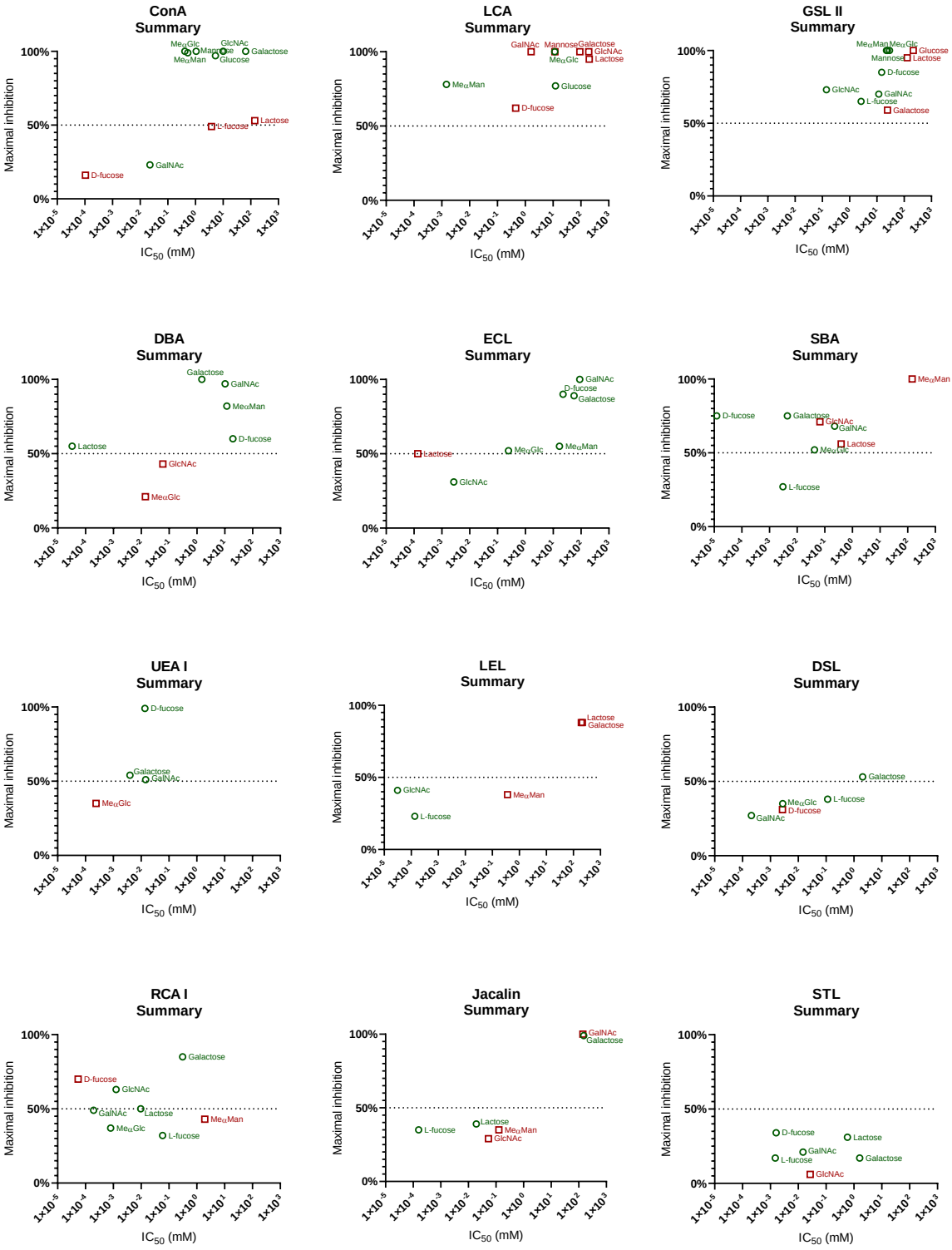

Figure SR3.1 Summary of inhibiting properties of different sugars for each lectin.

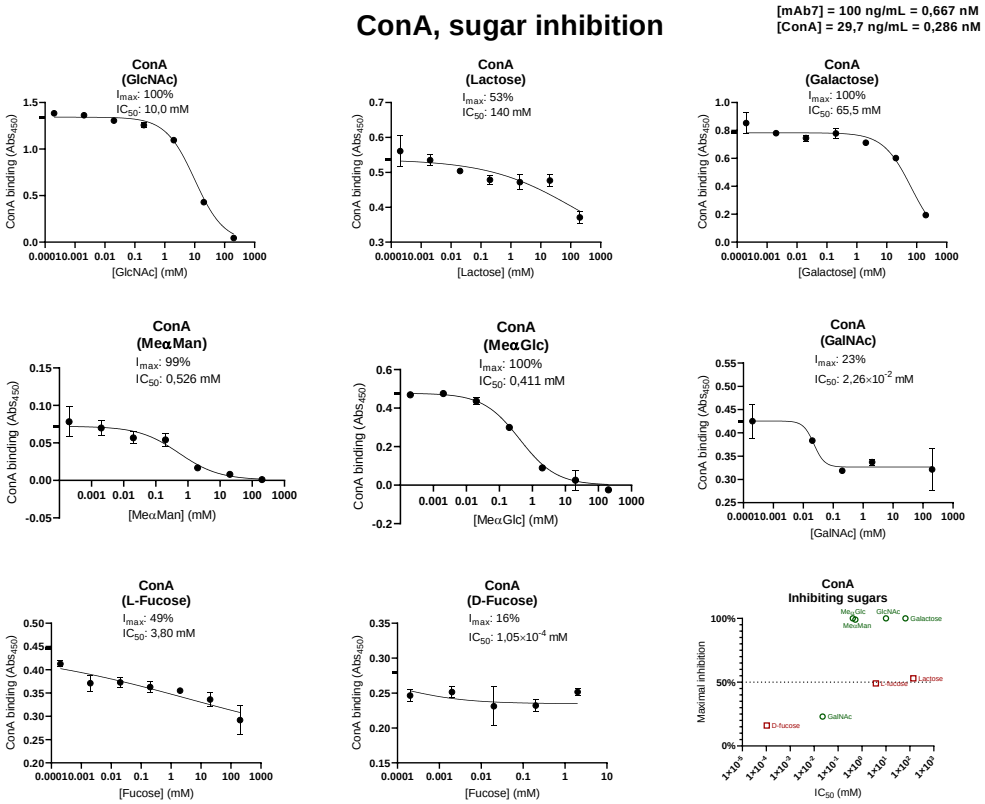

Figure SR3.2 Different sugars' influence on binding between ConA and mAb7.

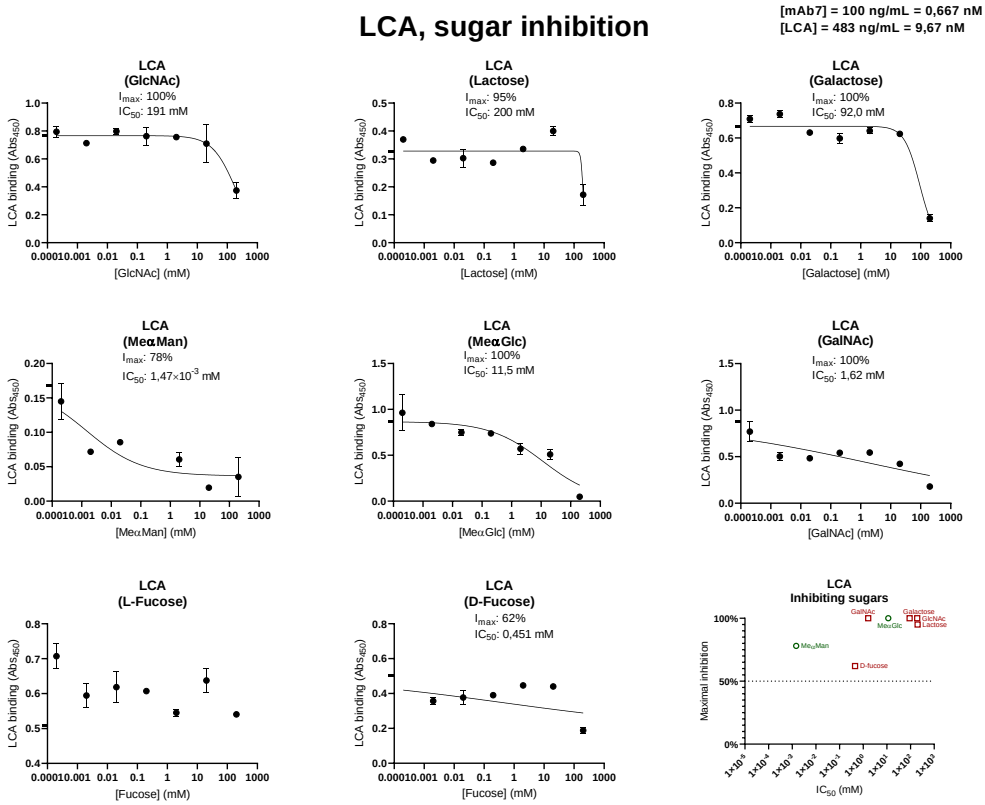

Figure SR3.3 Different sugars' influence on binding between LCA and mAb7.

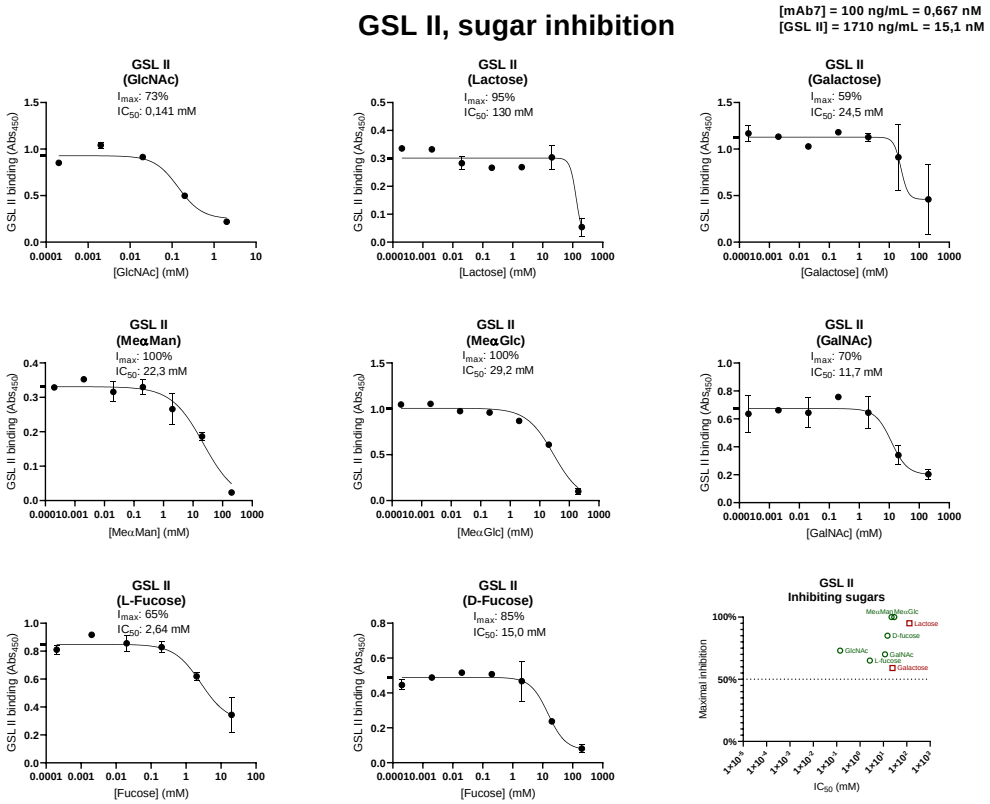

Figure SR3.4 Different sugars' influence on binding between GSL II and mAb7.

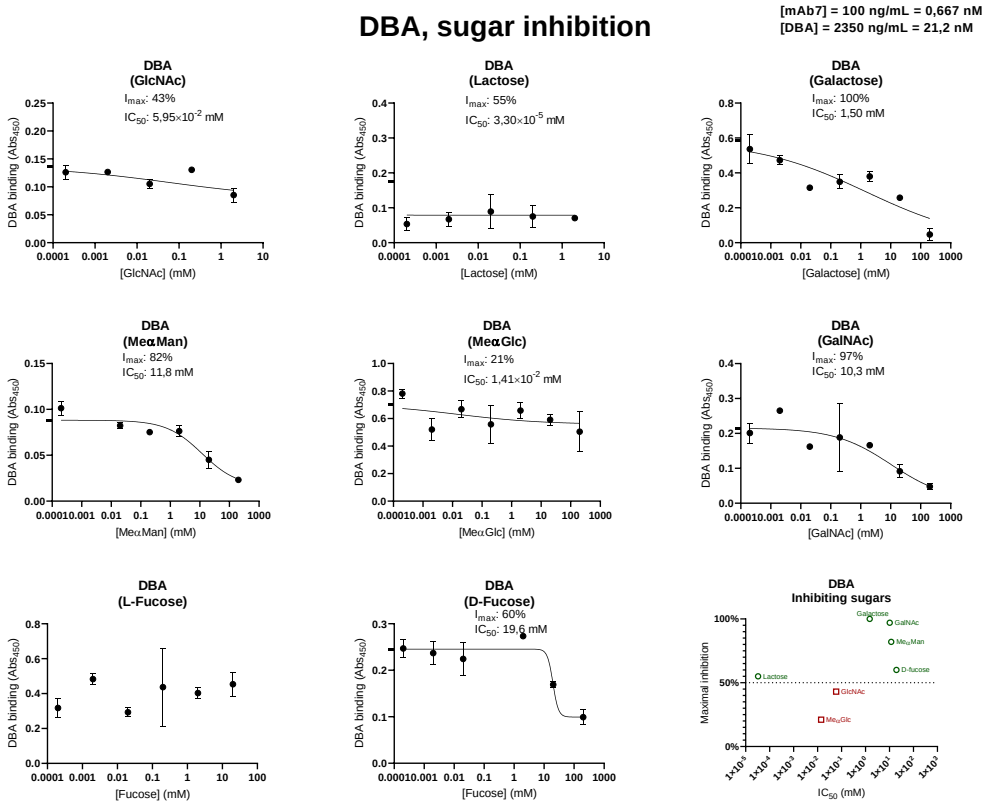

Figure SR3.5 Different sugars' influence on binding between DBA and mAb7.

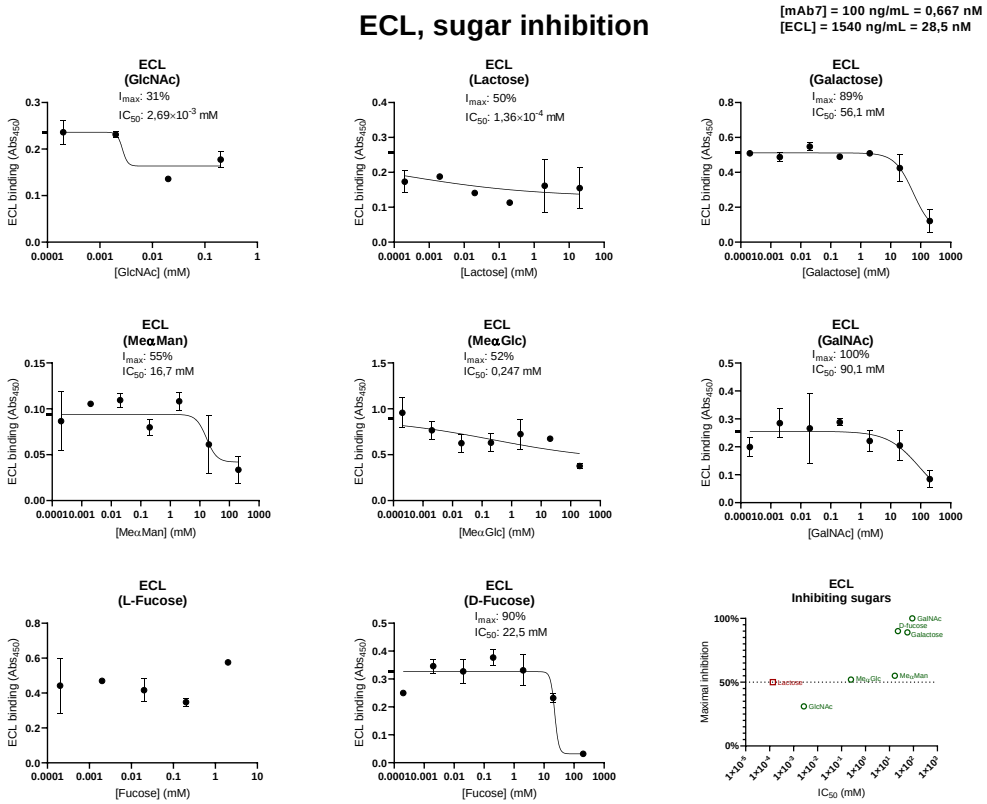

Figure SR3.6 Different sugars' influence on binding between ECL and mAb7.

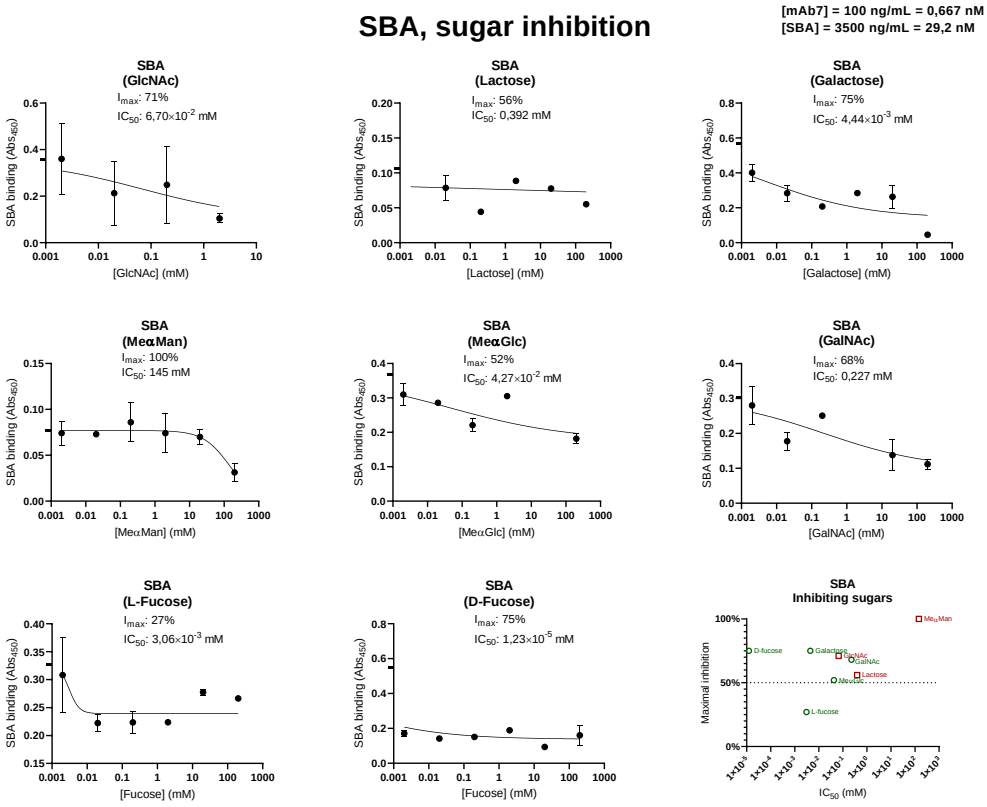

Figure SR3.7 Different sugars' influence on binding between SBA and mAb7.

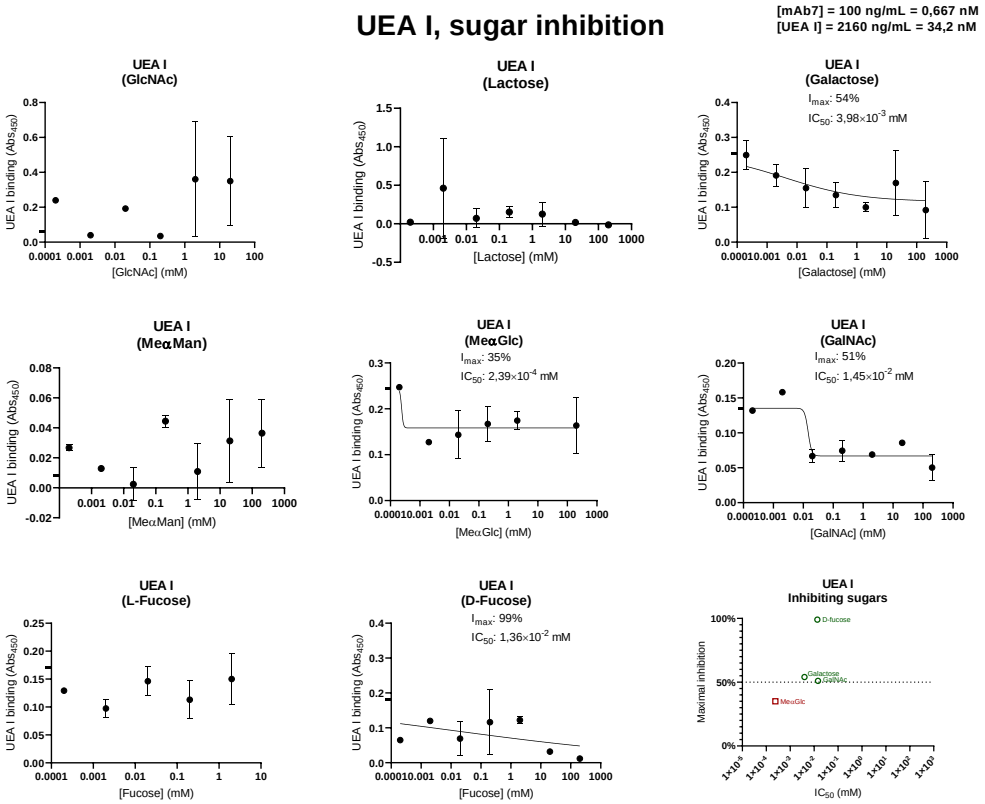

Figure SR3.8 Different sugars' influence on binding between UEA I and mAb7.

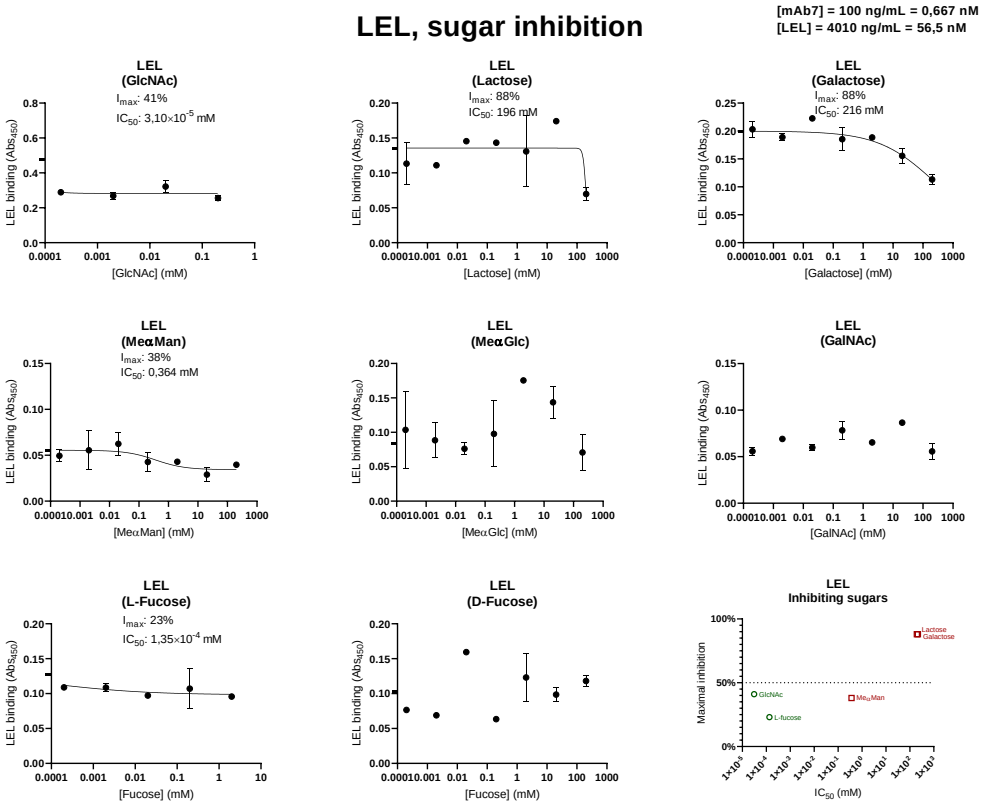

Figure SR3.9 Different sugars' influence on binding between LEL and mAb7.

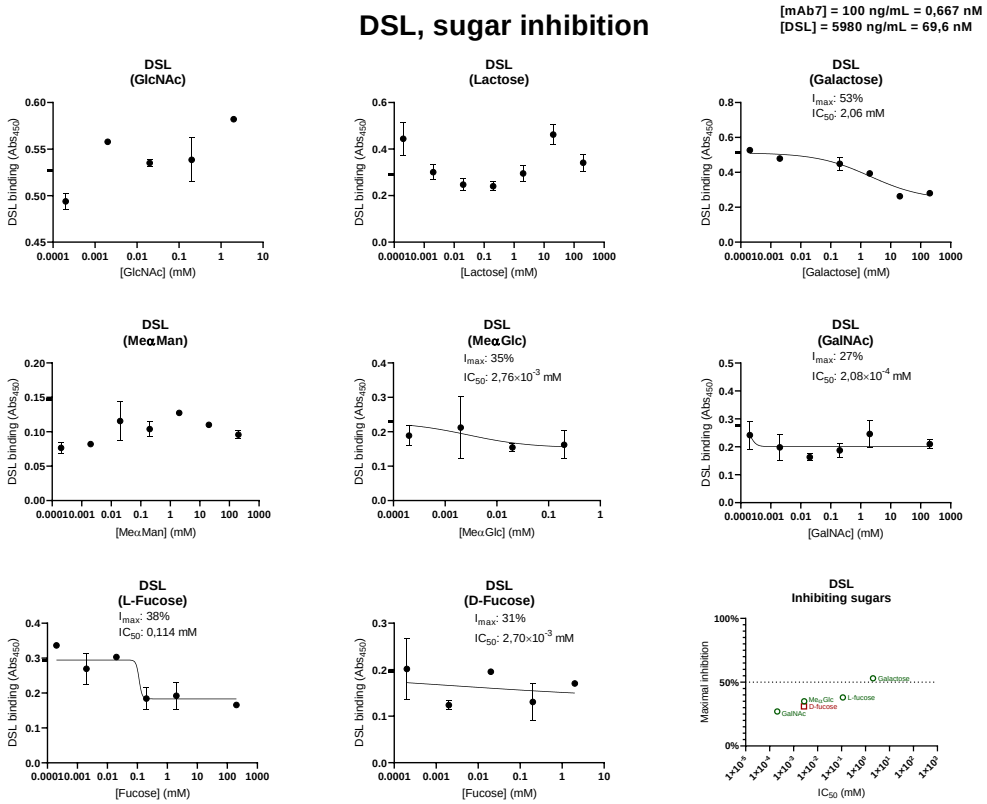

Figure SR3.10 Different sugars' influence on binding between DSL and mAb7.

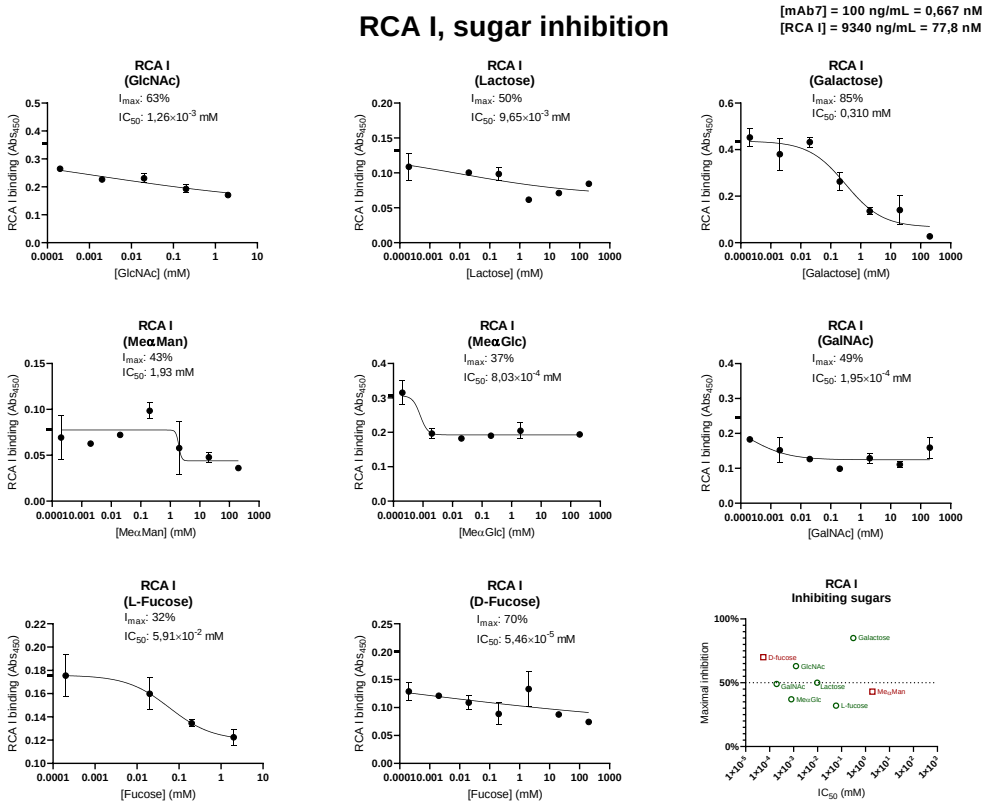

Figure SR3.11 Different sugars' influence on binding between RCA I and mAb7.

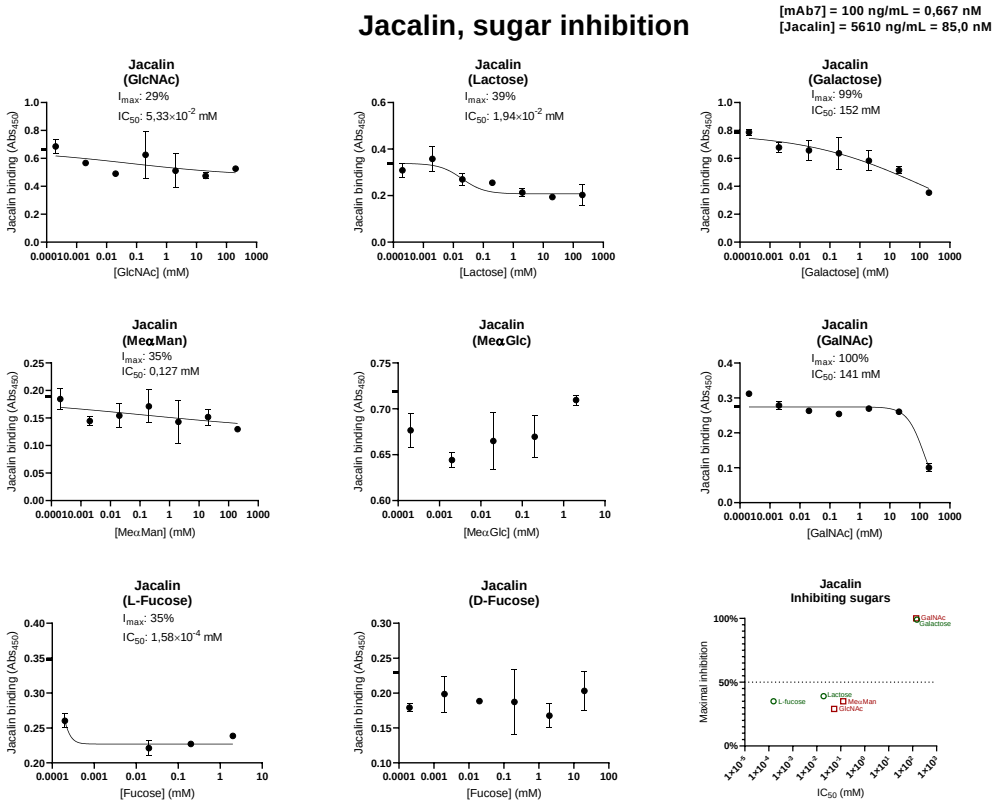

Figure SR3.12 Different sugars' influence on binding between Jacalin and mAb7.

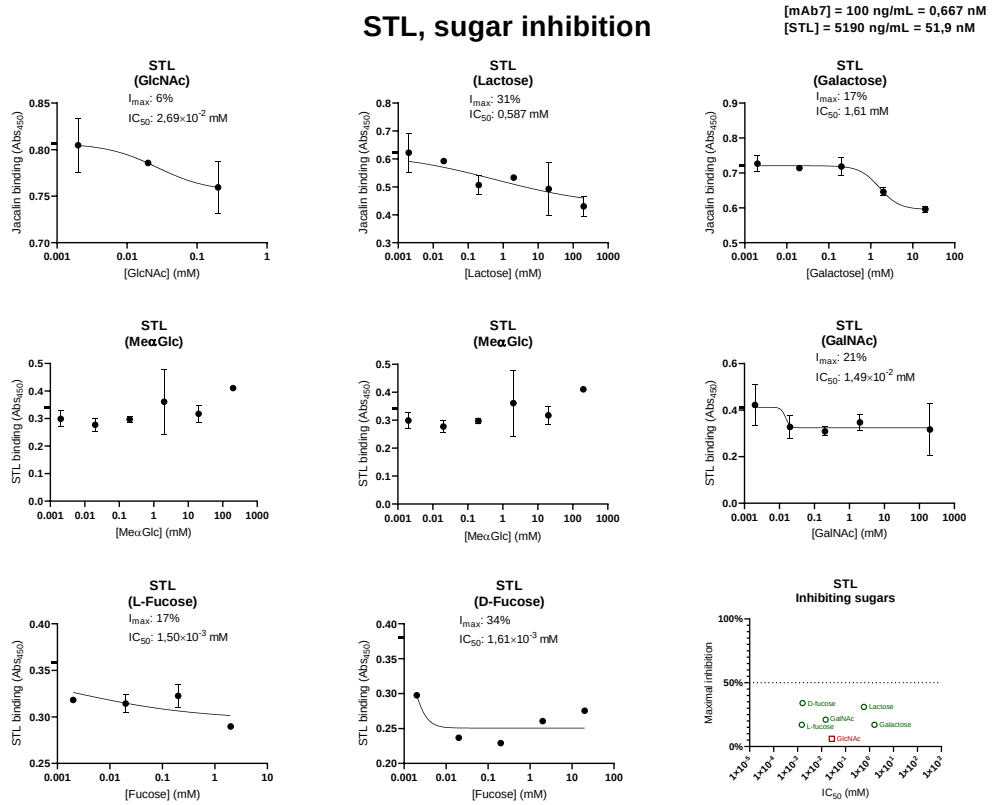

Figure SR3.13 Different sugars' influence on binding between ConA and STL.

Supplementary Result 4 – Comparison of methylated and unmethylated sugars

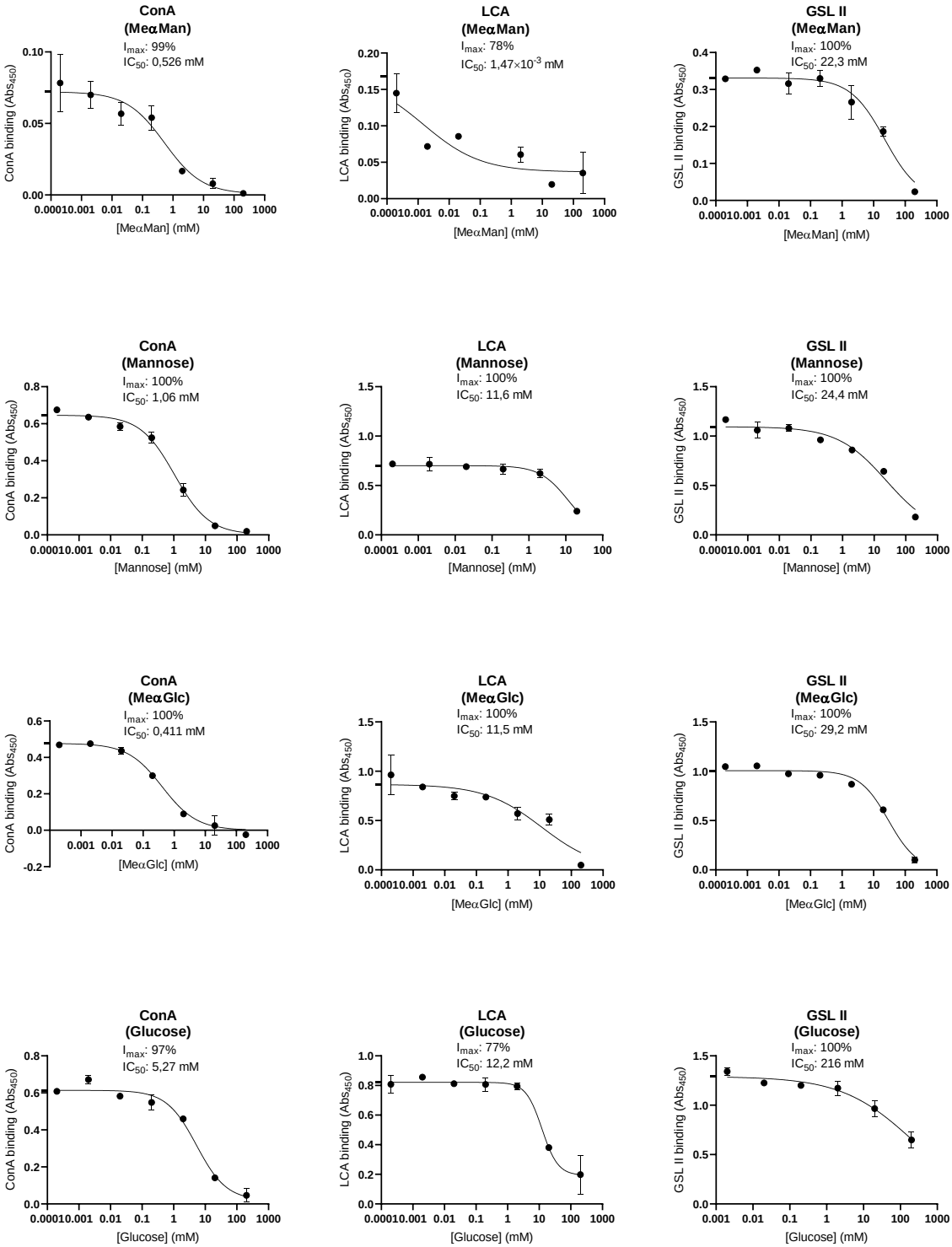

Figure SR4.1 Influence by mannose, glucose, MeαMan and MeαGlc on ConA, LCA and GSL II, respectively.

Supplementary Result 5 – Chromatograms related to ConA-Glycoprotein interactions

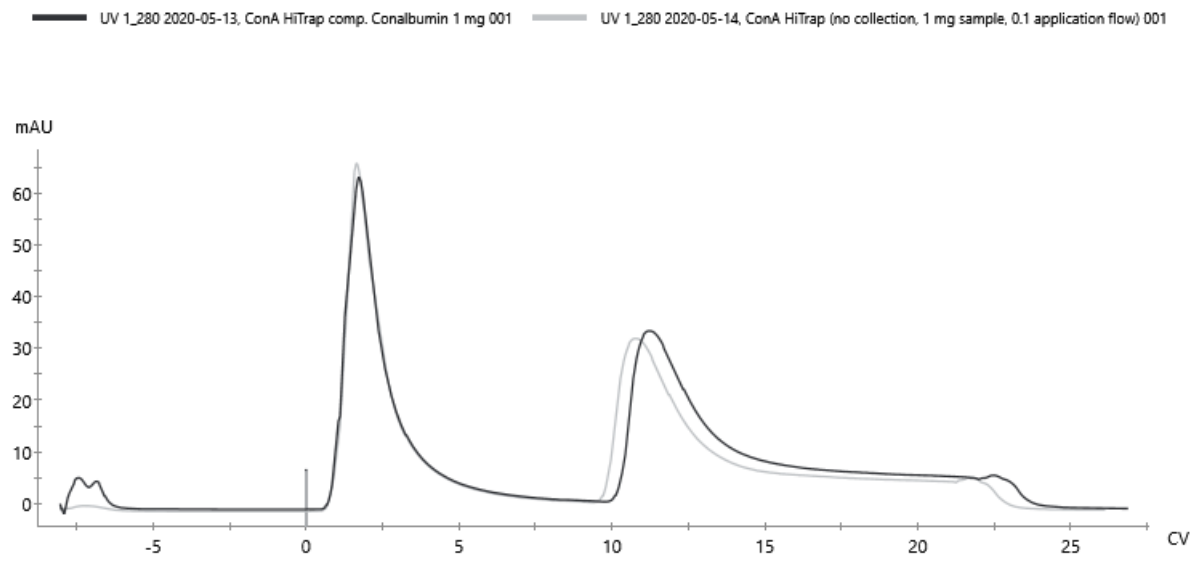

Figure SR5.1 Chromatogram comparing sample application flow 0.2 mL/min (black) and 0.1 mL/min (grey).

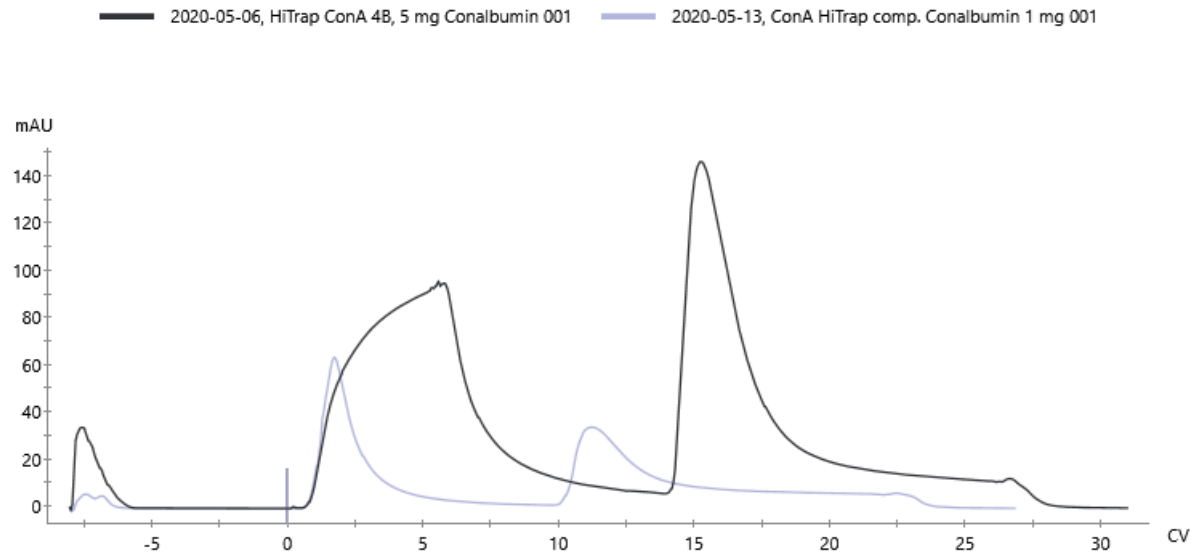

Figure SR5.2 Chromatogram comparing 5 mg Conalbumin (black) with 1 mg Conalbumin (grey) as sample.

Supplementary Result 6 – Chromatograms related to Glutaraldehyde-treated ConA columns

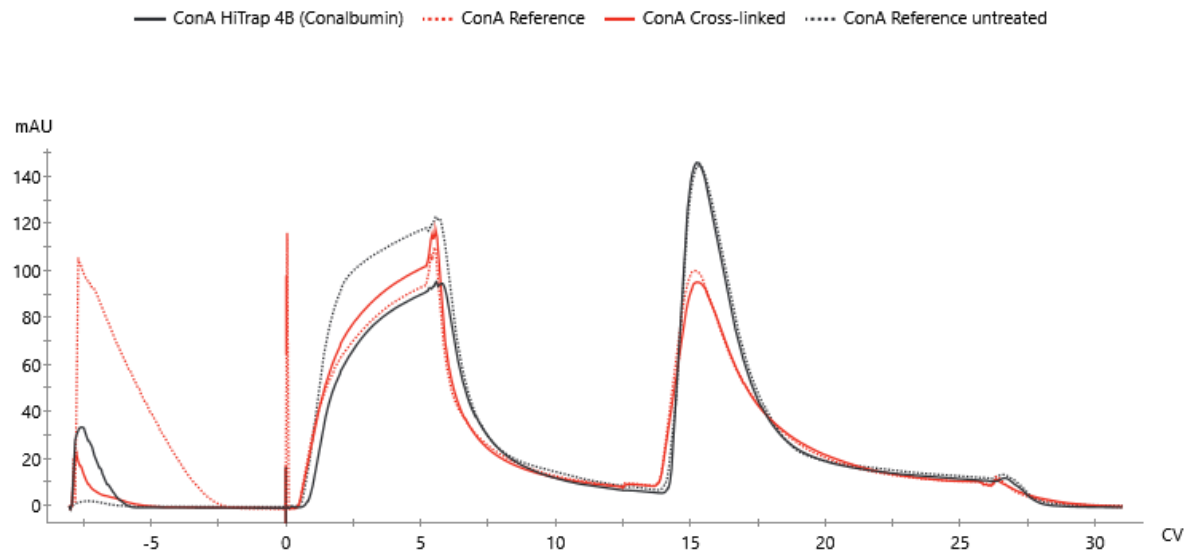

Figure SR6.1 Chromatogram comparing HiTrap ConA 4B (black), Cross-linked (red) and Ref column (red dashed) with a untreated Ref (black dashed).

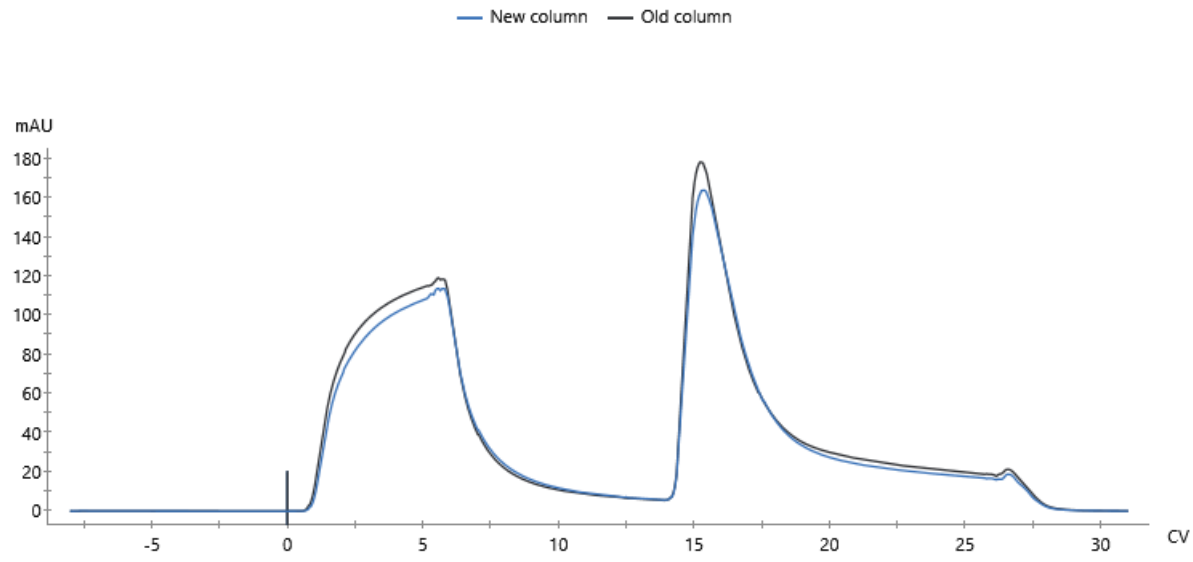

Figure SR6.2 Chromatogram comparing a new HiTrap ConA 4B column (blue) with a well-used and washed HiTrap ConA column (black).