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## RIGORÓZNÍ PRÁCE

# Izolace oligosacharidů z kravské syrovátky a jejich antiadhesivní účinky proti *Neisseria meningitidis*

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**2009** **Michal Kořínek**

# UNIVERSITY OF KUOPIO

## PHARMACEUTICAL FACULTY

### Department of Pharmaceutical Chemistry

## DOCTOR OF PHARMACY THESIS

# Isolation of milk whey oligosaccharides and their anti-adhesion activity against *Neisseria meningitidis*


Supervisor: Senior Scientist Carina Tikkanen-Kaukanen, Ph.D.

**2009** **Michal Kořínek**

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**“I hereby declare that I worked out this Doctor of Pharmacy thesis on my own. All cited literature and other information sources are listed in References.”**

11th August 2009

# Izolace oligosacharidů z kravské syrovátky a jejich antiadhesivní účinky proti *Neisseria meningitidis*

*Neisseria meningitidis* je specifický lidský patogen způsobující meningitidu a sepsi. Meningokokové infekce jsou vždy velmi vážné a mohou skončit úmrtím pár hodin po objevení prvních příznaků. Především děti od 6ti do 12ti měsíců věku jsou v největším ohrožení vůči propuknutí meningokokové infekce. Již dříve bylo zjištěno, že oligosacharidy lidského a kravího mléka mají inhibiční aktivitu proti mnohým patogenům včetně *Neisseria meningitidis*.

Antiadhesivní účinky kyselých oligosacharidů z kraví syrovátky proti *Neisseria meningitidis* byly zkoumány v této práci. Gelová chromatografie byla použita k frakcionaci oligosacharidů izolovaných z kraví syrovátky. Separované frakce byly analyzovány ve smyslu kyselých a neutrálních oligosacharidů změřením obsahu celkových hexóz a sialových kyselin. Inhibiční aktivita specifických frakcí kyselých oligosacharidů ze syrovátky proti navázání meningokokových pili byla studována pomocí inhibičních testů na pevné fázi *in vitro*.

Bylo zjištěno, že frakce kyselých oligosacharidů ze syrovátky inhibují adhesi proteinů typu IV pili *Neisseria meningitidis* k prasečímu thyroglobulinu, který byl použit jako referenční glykoprotein. Kyselé oligosacharidy ze syrovátky, které mají antiadhesivní aktivitu, se mohou chovat jako rozpustné analogy receptoru podobající se cukernou složkou receptoru hostitelské buňky. Proto aktivní oligosacharidy pravděpodoně mohou mít antiadhesivní aktivitu proti navázání bakterií *N. meningitidis* na povrch hostitelské buňky.

# Isolation of milk whey oligosaccharides and their anti-adhesion activity against *Neisseria meningitidis*

*Neisseria meningitidis* is a human specific pathogen causing meningitis and septis. Meningococcal diseases are always very serious and can be fatal few hours after first symptoms are observed. Infants in the age of 6 to 12 months are in the highest risk to obtain meningococcal disease. Human and bovine milk oligosaccharides have been found to have inhibitory activity against several pathogens including *Neisseria meningitidis*.

Anti-adhesive activity of acidic bovine milk whey oligosaccharides against *Neisseria meningitidis* was investigated in this study. Gel chromatography was used for fractination of the crude oligosaccharides isolated from bovine milk whey. The separated fractions were analyzed as regards acidic and neutral parts by measuring total hexoses and sialic acids content. The inhibitory activity of the specific acidic whey oligosaccharide fractions against the attachment of meningococcal pili was studied by using a solid-phase inhibition assay *in vitro.*

Fractions of acidic whey oligosaccharides were found to inhibit adhesion of *Neisseria meningitidis* type IV pili proteins to bovine thyroglobulin that was used as a reference glycoprotein. Milk whey acidic oligosaccharides that have anti-adhesive activity may act as a soluble receptor analoque resembling the carbohydrate structure of host cell receptor. Thus the active oligosaccharides may perhaps have the anti-adhesion activity against binding *N. meningitidis* bacteria to host cell surfaces.

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Thanks also and not only to my family.

August 2009 Michal Kořínek

## ABBREVIATIONS

| | |
|---|---|
| ABTS | 2, 2´-azino-di-(3-ethylbenzthiazoline sulfonate) |
| MWO | milk whey oligosaccharides |
| WAO | whey acidic oligosaccharides |
| WNO | whey neutral oligosaccharides |
| PBS | phosphate-buffered saline |
| Streptavidin-POD | streptavidin-linked $H_2O_2$ oxidoreductase |
| (HRP) | (horseradish peroxidase) |
| Hepes | *N*-2-hydroxyethylpiperazine-*N*´-2-ethanesulfonic acid |
| BBB | blood brain barrier |
| *N.* | *Neisseria* |
| GlcNAc-α1,3-Gal | *N*-acetylglucosamine-α1,3-galactose |
| Neu5Ac | *N*-acetylneuraminic acid, sialic acid |
| Neu5Gc | *N*-glycolylneuraminic acid |
| rpm | rotation per minute |
| GC | gel chromatography |
| LPS | lipopolysaccharide |
| OMP | outer membrane protein |
| OMV | outer membrane vesicle |
| Ser | Serine |
| Thr | Threonine |

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**ABBREVIATIONS**

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# 1 Introduction

*Neisseria meningitidis* is a dangerous human pathogen seriously threaten the life. This bacterium is causing septicaemia and meningitis, that can be fatal in a short time after the first symptoms occur. In this case infants in the age of 6 to 12 months are in the biggest danger. Their acquired immunity has not developed yet and maternal antibodies are decreasing especially when the infant is not breast-feeded anymore.

Meningitis should be immediately treated by antibiotics. Nowadays problem of antibiotic resistance is growing. The new ways of threatment or prevention of the disease should be found. Anti-adhesion activity, without any possible resistance, seems to be a good choice.

Recently, due to biomolecules extraordinary features compared with common pharmaceutics much research efforts have been focused on the development of biopharmaceutics. Biopharmaceutics are large complex molecules, difficult to prepare or isolated from biological sources with complicated mechanism of action, but with a huge medical potential.

Previous studies have shown a significant effect of human and also bovine milk oligosaccharides against *Neisseria meningitidis*.[1] Human oligosaccharides have been found to have anti-adhesion activity against many pathogens.[2] There is less information as regards anti-adhesion activity of bovine milk oligosaccharides. These more or less specified oligosaccharides could be used as novel antimicrobials or also as food supplements.

Bovine milk contains only minor amounts of the active compounds. Milk whey as a by-product of cheese making could be a cheap source of oligosaccharides. The use of milk whey for purification of anti-infective oligosaccharides could help to treat or prevent human infections, including meningitis and could also help to solve the ecological problem of the polluting whey.

# 2 Review of literature

## *2.1 Genus Neisseria*

There are two genetically and biochemically closely related species of Neisseria, both gram-negative bacteria. *Neisseria (N.) gonorrhoeae* causes inflammation of urogenital tract, gonorrhea. *N. meningitidis* then causes life-threatening sepsis and meningococcal meningitis. Although the genes of both bacteria are almost similar, the biological effect is very different. Possibly, the capsule that is found in the meningococcus but not in the gonococcus makes each bacteria so different.[3;4]

*N. meningitidis* often colonizes human nasopharynx as a commensal strain without any symptoms. These people spread the microbe in the way of a droplet infection to others, who cannot resist the infection. Meningococcus migrates from nasopharynx to bloodstream, causing meningococcal septicemia. Then could cross the blood-brain barrier (BBB) and invade into subarachnoid space of brain, meninges causing the most frightening meningococcal meningitis.[5]

### 2.1.1 Meningococcal diseases

MENINGOCOCCAL SEPTICEMIA is an acute infection causing death in 12 to 48 hours after the first symptoms occur. These are hypotension, purpuric rash, disseminated intravascular coagulation,[6] leading to septic shock and multiorgan failures.[7]

MENINGOCOCCAL MENINGITIS is also a very fatal infection. After crossing BBB *N. meningitidis* infects subarachnoid space of brain leading to death within a few hours after the first symptoms. The symptoms are high fever, stiff neck and headache. There is high mortality even if antibiotics are given.[6] Also patients, who survive from the meningococcal disease, suffer from permanent neurological deficits as sensorineural hearing loss or impaires vestibular functions.[8]

### 2.1.2 Immune mechanisms against *Neisseria meningitidis*

Immune responses of the infected human body are very complex, both innate and acquired imunity are playing role. Mainly there is an effect of antibodies and complement. The significant differences of immune responses can be shown depending on the age. While newborns are protected with transplacental maternal antibodies, this immunity response falls down in short time after birth. The lowest innate immunity of a baby

with the highest risk of meningococcal disease is at the age of 6 to 12 months. That time only alternative complement pathway without maternal antibodies is protecting the infant. The acquired immunity is not developed yet.[9] (Fig. 1)

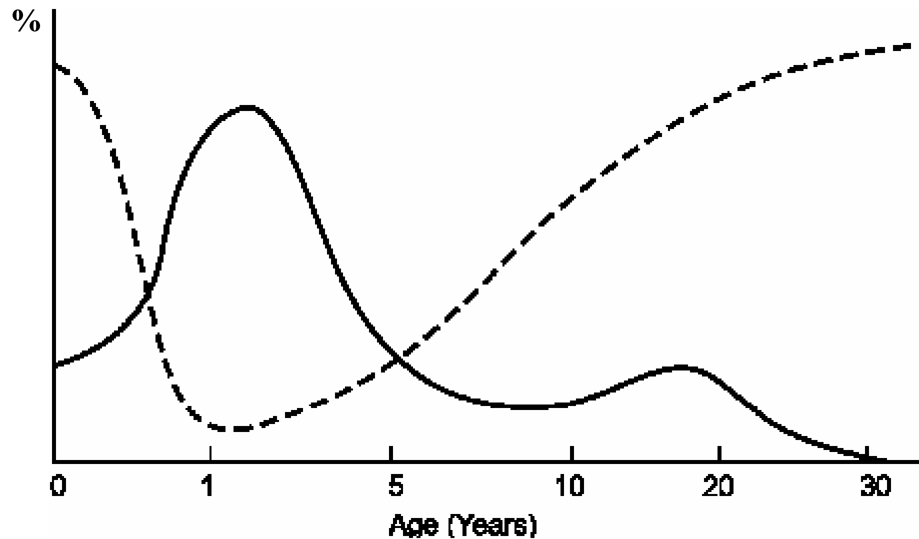


**Fig. 1 Meningococcal disease in infants depending on the age. This is to show relation of disease incidence with the amount of meningococcal antibodies in human.[10]**

▪▪▪▪▪▪▪ represents Incidence of *N. meningitidis* disease

▬▬ represents Titre of *N. meningitidis* antibodies in serum

## *2.1.3* Vaccines against *N. meningitidis*

Meningococcus is a diplococcus protected by a polysaccharide capsule, that is an important virulence factor. Encapsulated forms are able to survive in the bloodstream safe from phagocytosis.[5;11] Under the capsule, there is the outer membrane containing lipopolysaccharides (LPS). There are peptidoglycans in periplasmatic space located between outer membrane and cytoplasmatic membrane.[9] (Fig. 2) The specific structure of the capsular polysaccharide characterizes each out of the 12 meningococcal serogroups: A, B, C, 25E, H, I, K, L, W135, X, Y and Z.[11] Serogroups A, B, C, W135 and Y are pathogenic.[12] Differences between these immunotypes are in the oligosaccharide part of the LPS and consist of small differences in the oligosaccharide structure, the amount and location of phosphoethanolamine groups, and the degree of *O*-acetylation of individual monosaccharides. Meningococci can endogenously sialyate their LPS that is one of the mechanisms by which *N. meningitidis* can evade the response of the human host. Therefore, incorporation of detoxified LPS or oligosaccharide components derived from the bacterial capsule is beneficial for developing

vaccine against meningococci.[13] The first purified capsular polysaccharides have been found to have limited use as vaccines owing to their low immunogenicity in infants. However, polysaccharides with covalent linkage to immunogenic carrier proteins create glycoconjugates, which elicit antibodies also in infants. These protein-polysaccharide conjugate vaccines have been developed against *N. meningitidis* serogroups A, C, W135, Y, but not against the serogroup B.[11] The main reason is the similarity between the capsular polysaccharide of serotype B and the polysialic acid in human glycoproteins as neural cell-adhesion molecule (N-CAM).[14] After all the *N. meningitidis* serogroup B genome was read,[15] five antigens were expressed in a vaccine with aluminium hydroxide as an adjuvant.[16] All the serotype B meningococcal vaccines in current research are based on the outer membrain protein (OMP) or its outer membrane vesicle (OMV). These proteins induce bactericidal serum antibodies. OMV could be presented by PorA, which probably makes the vaccine very immunogenic.[17;18] There have been several studies regarding OMP/OMV vaccines in Cuba, Norway and New Zealand. Each vaccine consists of OMP/OMV from local epidemical *N. meningitidis* strain, almost totally depleted from the lipooligosaccharides and binded to aluminium hydroxide carrier.[19]

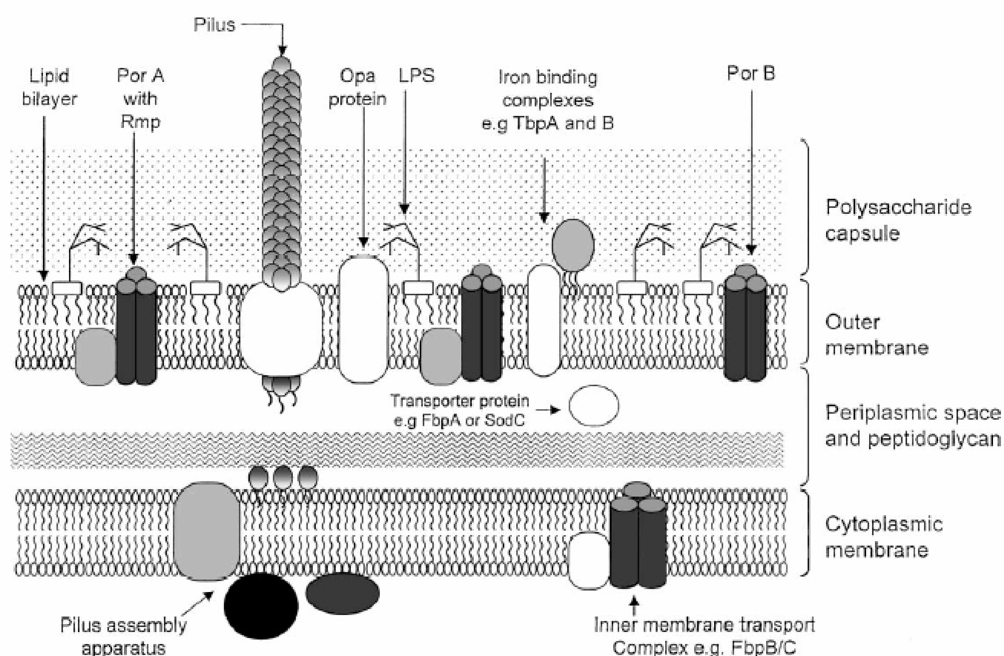


**Fig. 2 Neisseria meningitidis cell surface structure, LPS – lipopolysaccharide[9]**

### 2.1.4 *N. meningitidis* and host cell interactions

The first contact of the pathogen and the host cell is needed before microbe can colonize and infect the organism. The adhesion of bacteria is a ligand-receptor interaction, provided

by the bacterial adhesin that is binding the host cell receptor.[20;21] The oligosaccharide units of the host cell surface glycoproteins or glycolipids usually act as a receptor for the binding to other cells, hormones and other humoral effectors. But the pathogen adhesin can also bind these specific extracellular host cell receptors.[22] Binding of the pathogen to the receptor can activate complex of signal transduction cascades in the host cell that may switch on the activation of innate host defenses.[21] The binding also activates the expression of bacterial genes important for the pathogenic process. DNA microarray technology has been employed to study the first contact between the microbe and the host cell and the gene expression changes in the meningococcus after the binding. Some genome changes of *N. meningitidis* serogroup B after interaction with human epithelial cells were analyzed. This technology can be used as a complementary way besides other genome mining technics used for identifying new vaccine candidates.[23]

### 2.1.5 Type IV pilus

The surface of *N. meningitidis* carries type IV pili proteins important for adhesion and infection of the host cell.[3;24] (Fig. 2) This type of pilus occurs in all gram-negative bacteria likely *N. gonorrhoeae*, *Pseudomonas aeruginosa*, *Moraxella bovis*, *Dichelobacter nodosus*, *Vibrio cholerae* or enteropathogenic *Escherichia coli*.[25;26] Type IV pilus is a filamentous polymeric structure 1000-4000 nm long, 6 to 9 nm in diameter. It is composed of identical pilin (PilE) subunits forming right-handed helix structure. The hair-like protein structure has five pilin subunits per turn of helix.[24] Mechanical stability that type IV pilus needs is achieved by pilus central layer β-sheet hydrogen bonding.[27] The identical PilE subunits forming type IV pilus have approximately 17 to 21 kDa.[3] Type IV pili proteins from different bacterial species share common sequence (gray colour on Fig. 4) of mostly hydrophobic amino acids in their *N*-terminus, as well as a pair of cysteines in their *C*-terminus (cyan), but differ substantially beyond these sites. There are some differences in *N. meningitidis* and *N. gonorhoeae* pili amino acids sequence.[28] (Fig. 3)

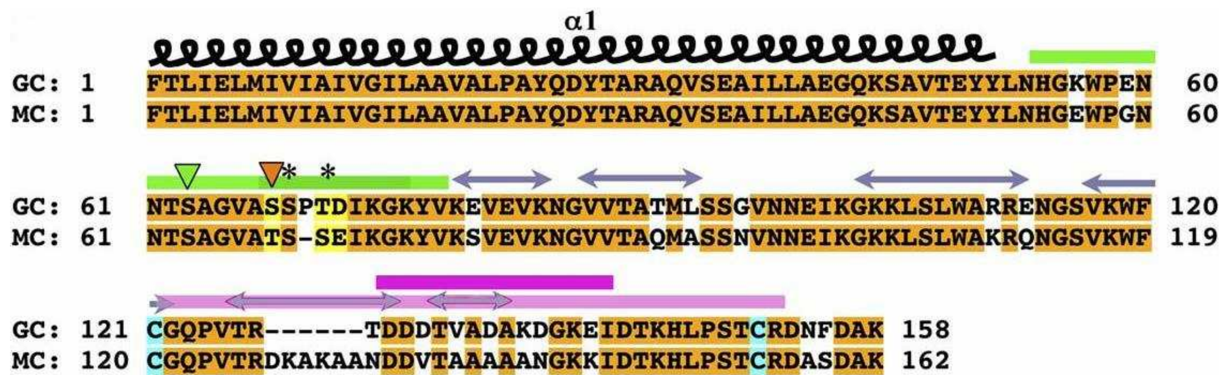


**Fig. 3 Amino acids sequence alignment of *N. gonorrhoeae* („GC“ strain C30) and *N. meningitidis* („MC“ MC58) type IV pili. Identical (orange) and conserved (yellow) residues are highlighted.[28]**

The gonococcal pilin subunit is covalently bound to disaccharide with *O*-linkage by means of 2,4-diacetamido-2,4,6-trideoxyhexose.[29] It was also found that disaccharide *N*-acetylglucosamine-α1,3-galactose (GlcNAc-α1,3-Gal) is *O*-linked to Ser 63[24] and phosphoethanolamine to Ser 68.[30] On the other hand the meningoccocal pili are post-translationally modified by addition of either an *O*-linked trisaccharide galactose-β1,4-galactose-α1,3-(2,4-diacetamido-2,4,6-trideoxyhexose) attached to Ser/Thr between residues 50 to 73[31] or an *O*-linked disaccharide GlcNAc-α1,3-Gal.[32] Another meningococcal modification is an *O*-linked α-glycerophosphate at Ser 93.[33] On type IV pili, the exposed carbohydrate may prevent antibody recognition by mimicking its carbohydrate antigens. And also variety of carbohydrates would aid immune evasion.[34] There is also a phosphocholine expressed on the surface of neisserial cell that probably serves as an „epitope“ ligand for both C-reactive protein and the receptor for platelet-activating factor in human body. The epitope is recognised by human antibodies leading to host immune responses.[29] Another unusual feature of the pilin subunits is the hypervariable loop, a segment between amino acid residues 128 and 141 that undergoes extreme sequence and also antigenic variations.[28] (purple colour on Fig. 4)

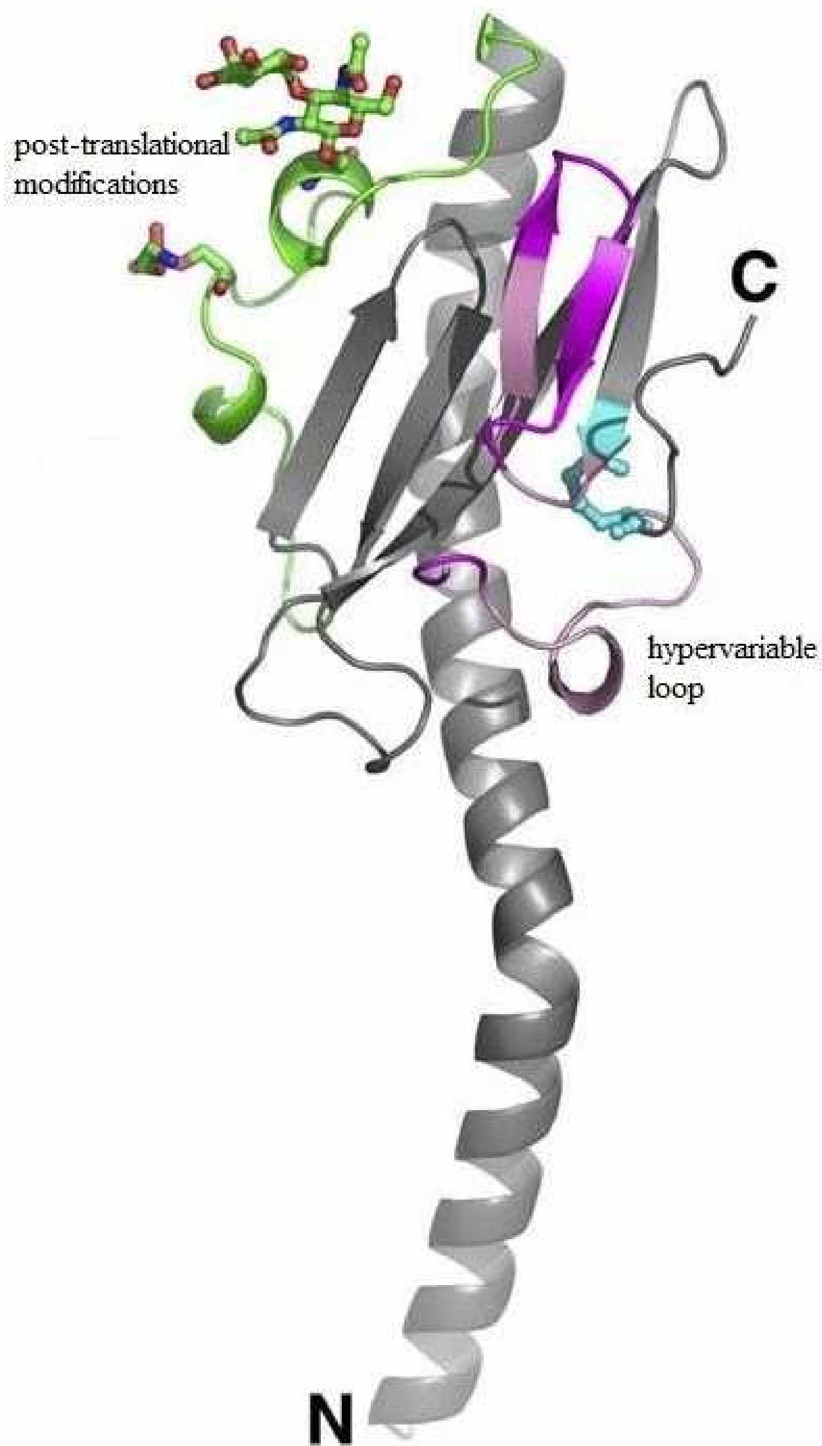


**Fig. 4 Structure of type IV pilus of *N. gonorhoeae*.**[26]

Gray refers to conserved regions.

Green refers to region of post-translation modifications.

Purple refers to hypervariable region.

Cyan refers to a pair of cysteines, disulfidic bonds.

In addition to the pilin protein in the *N. meningitidis* type IV pilus the tip-located adhesin, PilC is a 110 kDa protein[35] that has a specific receptor binding activity mediating adhesion to host cell.[21;36] Human CD46 is a receptor providing *N. meningitidis* passage through BBB and access to meninges.[37] The type IV pili have more functions besides promoting the attachment to the cellular receptors during the host cell colonization.[25] The pili are also arranging twitching motility at about 1 $\mu m.s^{-1}$. Synthesis of PilT protein is required.[38] Then the bacterium is able to translocate up to 2 mm per hour.[39] Other functions provided by the type IV pili are modulation of target cell specificity and bacteriophage absorption.[21]

## 2.2 Human milk

Human milk is composed mainly of lactose (Fig. 5), galactose-β1,4-glucose disaccharide. It contains also fat, oligosaccharides, proteins and many other molecules (sorted in the way of concentration).[22]

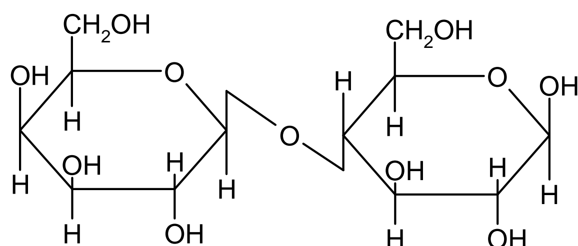


**Fig. 5 Lactose, Gal(β1→4)Glc**

### 2.2.1 Oligosaccharides

Oligosaccharides are composed of 3 to 10 carbohydrate residues. Acidic oligosaccharides contain one or more negatively charged sialic acid.[40] Mature human milk contains over 12 g/l of oligosaccharides and up to 22 g/l in early milk, colostrum.[22] Human milk is composed of five carbohydrate residues: D-glucose, D-galactose, *N*-acetylneuraminic acid, L-fucose and *N*-acetylglucosamine, that are forming almost 130 already identified different oligosaccharides.[20;41] All neutral and acidic milk oligosaccharides except for lactose are stable against hydrolysis by small intestine´s glycosidases in infants. This may result from infant´s immature small intestine.[42]

## 2.3 Bovine milk

Bovine milk is composed of fat, proteins, lactose and also vitamin, minerals.[40] Lactose forms 95% of the carbohydrate content.

### 2.3.1 Oligosaccharides

There is only a little amount of oligosaccharides including carbohydrate parts of glycoproteins and glycolipids in the bovine milk, despite of high oligosaccharide content in human milk. In human milk there are 12 -22 g/l of oligosaccharides and less then 0,1 g/l in bovine milk.[22;43] The composition of oligosaccharides varies depending on the phase of lactation and between individuals like in human milk. The total hexose concentration increases and sialic acid content decreases rapidly after parturition. High concentration of sialyloligosaccharides with other biological active components as growth factors, immune enhancers or antimicrobial compounds protect calf, whose immune system has not developed yet.[44;40] (Table 1) The chemical structure of many bovine milk oligosaccharides are similar with human milk oligosaccharides and glycoconjugates. It is believed that they could be used as bioactive anti-infection compounds.[40]

**Table 1. Composition of bovine and human milk and colostrum[45;46;47] (g/100 ml)**

| | Protein | Lactose | Oligosaccharides | Fat | Ash |
|---|---|---|---|---|---|
| Bovine milk[45] | 3,4 | 4,6 | * | 3,7 | 0,7 |
| Bovine colostrum[46] | 4,1-14,0 | 2,7-4,6 | * | 3,9-4,4 | 0,5-2 |
| Human milk[47] | 1,0 | 6,8 | 1,3 | 3,9 | 0,2 |
| Human colostrum[47] | 1,0 | 5,5 | 2,4 | 3,0 | - |

* data not reported.

Bovine milk is is composed of six carbohydrate residues: D-glucose, D-galactose, L-fucose, *N*-acetylglucosamine, *N*-acetylneuraminic acid and *N*-glycolylneuraminic acid.[48] (Fig. 6)

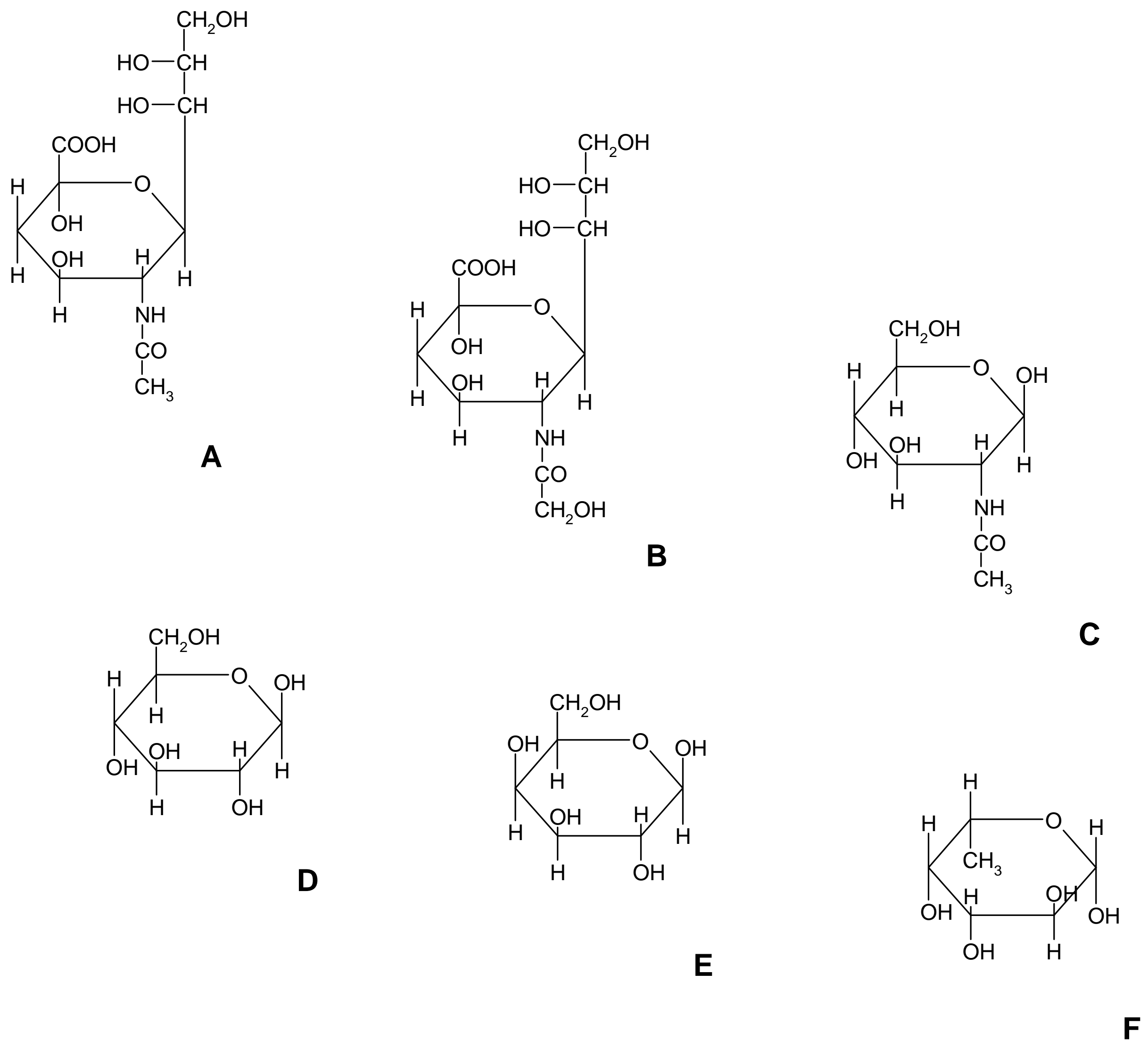


**Fig. 6 Bovine milk oligosaccharides are composed of six monosaccharide residues.[48]**

**A.** N-Acetylneuraminic acid (sialic acid), **Neu5Ac**

**B.** N-Glycolylneuraminic acid, **Neu5Gc**

**C.** N-Acetyl-β-D-Glucosamine, **GlcNAc**

**D.** β-D-Glucose, **Glc**

**E.** β-D-Galactose, **Gal**

**F.** β-L-Fucose, **Fuc**

Only 11 acidic, negatively charged, sialyl oligosaccharides and 10 neutral oligosaccharides have been found in bovine milk.[48] (Table 2) Sialyl-lactose forms more than 50% of bovine oligosaccharides. Bovine milk contains fewer components than human milk, but there are also casein, imunoglobulins, lactoglobulin, lactalbumine and lactoferrin.[49]

**Table 1 Neutral and acidic oligosaccharides of bovine milk and colostrum.[40;48]**

| ***STRUCTURE*** | ***NAME*** |
|---|---|
| Neutral | |
| Gal β(1-3) Gal β(1-4) Glc | β-3´-Galactosyl-lactose |
| Gal β(1-4) Gal β(1-4) Glc | β-4´-Galactosyl-lactose |
| Gal β(1-6) Gal β(1-4) Glc | β-6´-Galactosyl-lactose |
| Gal β(1-4) Fuc α(1-3) GlcNAc | 3-Fucosyl-*N*-acetyl-lactosamine |
| Gal β(1-4) GlcNAc | *N*-Acetyl-lactosamine |
| Gal NAcβ(1-4) Glc | *N*-Acetylgalactosaminyl-glucose |
| Gal NAcα(1-3) Gal β(1-4) Glc | α-3´-*N*-Acetylgalactosaminyl-lactose |
| Gal β(1-4) GlcNAc β(1-6) Gal β(1-3) [Gal β(1-4) Glc] | Lacto-*N*-Novopentaose I |
| Gal α(1-3) Gal β(1-4) Glc | α-3´-Galactosyl-lactose |
| Gal β(1-4) Glc-3´-$PO_4$ | Lactose-3´-*O*-Phosphate |
| Acidic | |
| Neu5Ac α(2-3) Gal | 3-Sialyl-galactose |
| Neu5Ac α(2-3) Gal β(1-4) Glc | 3´-*N*-Acetylneuraminyl-lactose (3-Sialyl-lactose) |
| Neu5Ac α(2-6) Gal β(1-4) Glc | 6-Sialyl-lactose |
| Neu5Gc α(2-6) Gal β(1-4) Glc | 6´-*N*-Glycolylneuraminyl-lactose |
| Neu5Ac α(2-6) Gal β(1-4) GlcNAc | 6-Sialyl-lactosamine |
| Neu5Gc α(2-6) Gal β(1-4) GlcNAc | 6´-*N*-Glycolylneuraminyl-lactosamine |
| Neu5Ac α(2-3) Gal β(1-3) Gal β(1-4) Glc | 3-Sialyl-galactosyl-lactose |
| Neu5Ac α(2-8) Neu5Ac α(2-3) Gal β(1-4) Glc | Disialyl-lactose |
| Neu5Ac α(2-6) Gal β(1-4) GlcNAc α-1-$PO_4$ | 6´-Sialyl-lactosamine-1-*O*-Phosphate |
| Neu5Ac α(2-6) Gal β(1-4) GlcNAc α-6-$PO_4$ | 6´-Sialyl-lactosamine-6-*O*-Phosphate |
| Neu5Gc α(2-3) Gal β(1-4) Glc | 3´-*N*-Glycolylneuraminyl-lactose |

### 2.3.2 Infant formulas

Infant formulas are based on bovine milk. From that fact are resulting the differences between nutrition of breast-fed and bottle-fed infants. There is only a little amount of sialic acid compounds in infant formulas compared to human milk. The main part of sialic acid in bovine made formulas is bound to glycoproteins, unlikely sialic acid bound to free

oligosaccharides in human milk.[50] There would be beneficial effects to apply more oligosaccharides to infant formulas, not only nutritional but also functional.

### 2.3.3 Biological activity of oligosaccharides

Adhesion of a microbe to a host cell, that is the first step of the pathogenic infection, could be stopped by oligosaccharides. Two types of mechanism of action are thought to take part. The inhibition of the binding of the pathogen adhesin to the host cell receptor is caused by the exact carbohydrate structure of the oligosaccharide, that mimics the receptor structure. So the milk oligosaccharides possibly bind to the attachment site of the pathogen and may thus block its binding to the host cell surface receptor. Oligosaccharides may also bind to the cell surface receptor and thereby inhibit the ability of the pathogen to affect the host cell. There are several reports that human milk oligosaccharides and glycoproteins are able to inhibit bacterial adhesion to epithelial cells.[20;22] (see Table 3)

**Table 2 Human milk oligosaccharides as receptor analogues, inhibitors of pathogen adhesion.[20;51]**

| Receptor | Microbe | Disease |
|---|---|---|
| Neutral oligosaccharides | *Streptococcus pneumoniae* | pneumonia, otitis media |
| Fucosylated oligosaccharides | *Campylobacter jejuni* | gastroenteritis, diarrhea |
| Gal β(1-4) GlcNAc | *Psuedomonas aeruginosa* | various |
| Sialyl-lactose | *Helicobacter pylori* | gastritis, ulcers |
| | *Streptococcus sanguis* | endocarditis |
| | *Escherichia coli* entero- or uro-pathogenic | diarrhea or pyelonephritis, cystitis |
| | *Influenza virus* | flu |
| Sialylated glycoproteins (α2-3) | *Mycoplasma pneumoniae* | pneumonia |
| Sialylated poly-*N*-acetyllactosamine | | |

## 2.4 Milk whey

Milk whey is usually made of bovine milk and that´s why it contains most of the components of bovine milk. Whey is the liquid remaining after milk has been curdled with rennet. It is a by-product of the manufacture of cheese or casein. The composition of whey products varies according to the milk source, type of cheese and manufacturing process. Sweet whey (pH more than 5,6) is obtained during the enzyme cheese production of Cheddar, Mozzarella or Swiss cheese. Acid „sour“ whey (pH below 5,1) is a by-product of cottage or ricotta cheese production.

### 2.4.1 Whey production

Whey is manufactured mainly as a by-product of cheese making.

Milk is usually pasteurised by heating to 72-73°C for 15-20 seconds to kill the most of bacteria (coliforms) present in fresh milk. Chemicals like sodium nitrate or hydrogen peroxide can be added or mechanical means of reducing the number of microorganisms have been adopted.

There are two types of starter cultures used in cheese making: mesophilic with temperature optimum 20-40°C and thermophilic developing at up to 45°C. Single strain or mixed strain cultures can be used. These bacteria produce lactic acid from lactose, break down the proteins or produce carbon dioxide, if needed.

Lactic acid creates lower pH than needed to support contraction of the coagulum accompanied by elimination of whey. Lactic acid itself makes the milk to clot in fresh type of cheese such as cottage without any other additives. Acid whey is then produced. Most of the other types of cheese curd formation require presence of rennet. Before making the curd, other compounds can be added. Calcium chloride provides coagulation. Disodium phosphate helps low-fat cheese production. Carbon dioxide lowers pH of the milk and shortens coagulation time. Rarely sodium or potassium nitrate is added to counteract some bacteria. Also colouring agents as carotene, orleana or chlorophyll are used to avoid seasonal colour variations determined by milk fat. The active principle in rennet is chymosine. Chymosine supports transformation of casein to paracasein and helps paracasein to precipitate in the presence of calcium ions. The whole process usually takes 30 minutes at 40°C and depends on the temperature, acidity, calcium content and other factors. Rennet is extracted from stomachs of young calves or it can be substituted by coagulating enzymes

from plants or microorganisms. The very similar enzymes to those of calf rennet could by also obtained by DNA technology.[52]

### 2.4.2 Biological activity of whey compounds

Whey contains mainly proteins, but also lactose, minerals, vitamins and imunoglobulins, α-lactalbumin, β-lactoglobulin, lactoferrin, lactoperoxidase, glycomacropeptides, sphingolipids and traces of milkfat. These bioactive components are providing high nutrition effect, protection against infections and immunity enhancement. Whey products also positively affect cardiovascular system and protect against some cancers. High content of easy digested proteins without fat has made whey popular nutrition for body building and losing weight.[53;54] It has been found that whey´s leucine containing proteins could influence insulinogenic amino acids and incretin hormones and thus could increase insulin secretion in Diabetes mellitus type 2 patients.[55] Positive effects of milk whey seem to be due to high level of cysteine in proteins, that is involved in the intracellular production of glutathion antioxidant defence system.[56] Nowadays it is believed that also oligosaccharides have a role in protection against infections. Unfortunately oligosaccharides are present in bovine whey in minor. Oligosaccharides isolated from whey could be used in nutrition, infant formulas, novel dietary supplements or as drugs supporting fight against infectious diseases.[22;57]

## 2.5 Anti-adhesion therapy

Anti-adhesion therapy prevents the attachment of the bacteria to host tissue or is able to detach them from the tissue at the early stages also. That is really different mechanism from that of the current antibiotics in use. Nowadays antibiotic resistant pathogens are increasing. Oligosaccharides or other glycans blocking adhesion of pathogen can solve the problem as they are safe and non-imunogenic. They are not bactericidal, so resistence is not likely to occur. But there are still some limitations. The sensitivity of each individual is different depending on gene expression of key cell surface glycans.[58;51] Another thing is the presence of multiple adhesins of the pathogen.[2] The low afinity of free saccharides for the adhesins could be solved by attachment these saccharides to polymeric carriers. Then suitable cocktails of inhibitory glycans for the treatment of bacterial infections could be prepared, instead of the single sugars in use until now. Probably different amounts of different carbohydrate inhibitors should be used. Also the production of specific saccharides is still extremely costly. The creation of genetically modified microbes producing oligosaccharides in large scale or employing the milk whey from dairy industry promise to lower this cost.[59;58;51] It would be also a challenge to develop suitable carbohydrate analogs that are more potent inhibitors of bacterial adhesion agents than presently available saccharides.[58]

# 3 Aims of the study

Previously bovine milk acidic oligosaccharides have been shown to inhibit *Neisseria meningitidis* pili binding to thyroglobulin.[57] The rationale for the present study raised from the interest to use bovine whey as a source for inhibitory milk oligosaccharides. Being industrial by-product the use of bovine whey could be economically reasonable for large scale isolation. This gives impact also for the scientific point of view, which includes subfractionation of whey acidic oligosaccharide fractions in order to find specific oligosaccharide(s) responsible for the inhibitory activity.

The goal of this study was to analyze the inhibitory activity of whey oligosaccharides from bovine milk against *N. meningitidis* pili binding to reference glycoprotein, bovine thyroglobulin.

The first aim was to isolate and fractionate oligosaccharides from bovine whey. The second aim was to isolate and label *N. meningitidis* pili for the inhibition assays. The third aim was to measure *in vitro* the inhibitory activity of the different whey oligosaccharide fractions against binding of *Neisseria meningitidis* type IV pili to bovine thyroglobulin by using a solid phase assay.

# 4 Materials

## 4.1 Whey

Bovine whey was obtained from Lieksan Laatuherkut Oy, Lieksa, Finland.

## 4.2 Bacterial strain

*Neisseria meningitidis* serogroup C class I strain 8013 (2C4 3W) was obtained from Xavier Nassif INSERM U570, Paris, France.

## 4.3 Chemicals

| | |
|---|---|
| ABTS tablets | Roche Diagnostics, Germany 1112422 |
| Agar base | Oxoid Ltd., England, CM0367 |
| D-Biotinoyl-ε-aminocaproic acid-N-hydroxysuccinimide ester | Roche Diagnostics, Germany, 1008960 |
| $CuSO_4 \cdot 5H_2O$ | Riedel-de Haën, Germany, 31293 |
| Dimethylsulfoxide | Boehringer Mannheim, Germany, 1418165 |
| Dry milk powder, fat free | Valio Ltd., Finland |
| GCB agar | Oxoid Ltd., Basingstoke, U.K. |
| HEPES buffer, 1 mol/l | Sigma Chemicals Co., USA |
| Hydrochloric acid, 36-38% | J. T. Baker B. V., Holland |
| Phenol | Riedel-de Haën, Germany, 33517 |
| Periodic acid | Sigma Chemical Co., USA, P-7875 |
| Resorcinol | Sigma Chemical Co., USA, R-5645 |
| Sodium Chloride | J. T. Baker B. V., Holland |
| Sephadex G-25 | Sigma-Aldrich, USA |
| Streptavidin-POD conjugate | Roche Diagnostics, Germany, 1089153 |
| Sulfuric acid, 95-97% | Riedel-de Haën, Germany, 30743 |
| tert-Butanol | Merck, Schuchardt, Germany |
| Tween 20 | Fluca Chemica, 93773 |

## 4.4 *Solutions*

| | |
|---|---|
| ABTS substrate for HRP | 5 ml concentrated substrate solution, 50 ml $H_2O$ and one ABTS tablet |
| Blocking buffer | 5% (w/v) dry milk powder in 0.05% (v/v) Tween 20 in 1 x PBS, pH 7,4 |
| Kellogg´s supplement I | 400 g D-glucose, 10 g L-glutamine, 20 mg thiamin ad 1 l $H_2O$ |
| Kellogg´s supplement II | 5 g $Fe(NO_3)_3 \cdot 9H_2O$ ad 1 l $H_2O$ |
| 10 x PBS buffer | 1.36 M NaCl, 26.8 mM KCl, 123.6 mM $Na_2HPO_4 \cdot H_2O$, 14.7 mM $KH_2PO_4$, pH 7.4 |
| 6% resorcinol reagent stock | 3 g resorcinol, 30 ml of 28% HCl, 20 ml of $H_2O$, 250 µmol $CuSO_4 \cdot 5H_2O$ |
| Storage solution | 1% $NaN_3$ in $H_2O$ |
| Washing buffer | 0.05% (v/v) Tween 20 in 1 x PBS pH 7.4 |

## *4.5 Glycoprotein*

| | |
|---|---|
| Thyroglobulin bovine | Sigma Chemical Co., USA, T-1001 |

## *4.6 Equipment*

| | |
|---|---|
| Agitator | Vortex-2 Genie, Scientific Industrie, USA |
| Centrifugal filter device | Amicon Ultra Cut-off 100 kDa, Millipore, USA |
| Centrifuge | Superspeed centrifuge, Sorvall RC-5B, USA |
| Centrifuge | Sorvall TC-6, USA |
| Centrifuge | Eppendorf 5804 R, USA |
| Cuvettes | 10 x 4 x 45 mm, Sarstedt, Germany |
| Fraction collector | Frac-920, GE Healthcare, Amersham Biosciences |
| Freeze dryer (lyophilizer) | ModulyoD-230, Valu Pump VLP200, ThermoSavant, USA |
| Gel chromatography column | 106 x 1.6 cm |
| Microtiter plates | Falcon flexible plate, Becton Dickinson, Labware, USA |
| Microtiter plate measuring device | Victor$^2$ 1420 Multilabel counter, Wallac, Finland |
| PH meter | Orion 420A, USA |

| | |
|---|---|
| Pump | LKB-Bromma 2120 Varioperpex II, Sweden |
| Rotavapor | Büchi rotavapor R-114, Switzerland |
| Spectrophotometer | Hitachi U-1100 |
| Water bath | Memmert, Germany |
| Water bath | Eppendorf Thermomixer 5436, Germany |
| Water bath | Framo-Gerätetechnik M21/1, Germany |

# 5 Methods

## 5.1 Isolation of oligosaccharides

The isolation method used was modified from the method described by Kobata.[43] The isolation of oligosaccharides was processed from 250 ml of milk whey stored at –20°C. Whey was defatted by centrifugation at 3800 rpm at +4°C for 15 minutes and filtered through a glass wool to separate solidified lipids. 96% ethanol was added to filtrate to get the final concentration of ethanol to 60% and was left at +4°C overnight. The main part of proteins and lactose precipitated. Precipitate was removed by centrifugation at 15000 rpm at 0°C for 15 minutes and the remaining precipitate was washed twice with 50 ml of 67% ethanol at 0°C.

The supernatants were combined and concentrated under reduced pressure by rotary evaporation. The syrup was diluted to 25 ml by $H_2O$ and centrifuged at 3800 rpm for 15 minutes at +4°C to separate insoluble material. The supernatant was divided to 5 ml aliquots stored at –20°C before gel chromatography. The procedure was employed in every three isolations carried out in this study (isolations I, II, III).

## 5.2 Gel chromatography

Gel filtration chromatography is a method of separation proteins, peptides, oligonucleotides and other macromolecules according to the differences in sizes of their molecules. Molecules move through a bed of porous beads (stationary phase) in a solution - aqueous buffered solution (mobile phase), more or less diffusing into the beads. Smaller molecules diffuse further into the pores of the beads, stuck there, and therefore move through the bed more slowly. Larger molecules cannot enter the pores at all and thus move more quickly. Degree of retention depends on both molecular weight and three-dimensional shape. (Fig. 7) Gel filtration media features influence the separation process.[60]

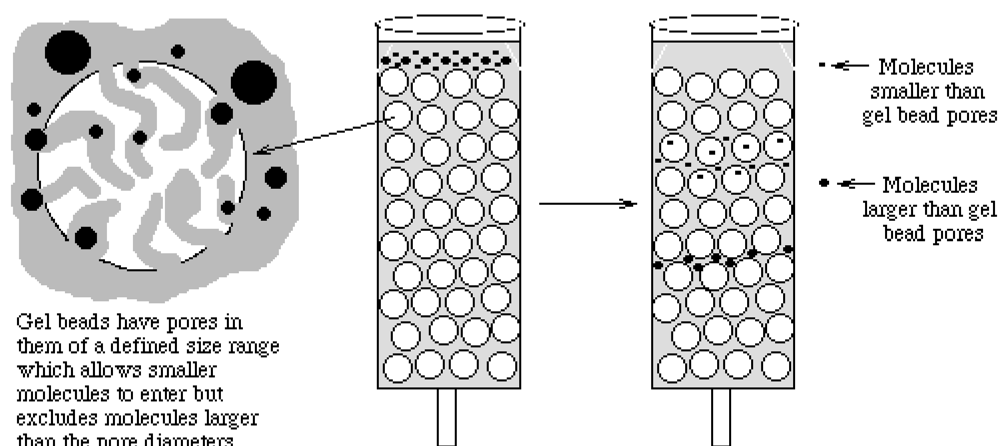


**Fig. 7 Scheme of gel filtration chromatography.[61]**

There are different materials used in gel filtration. Each is suitable for different molecular weight compounds separation. (Fig. 8) Sephadex is a cross-linked dextran (polysaccharide polymer), which forms spherical beads. Because of its high content of hydroxyl groups, it has great affinity for water or electrolyte solutions. Bio-Gel series is based on cross-linked polyacrylamide gel. It is also suitable for gel filtration in aqueous media. Sepharose or Biogel A is a cross-linked agarose, a neutral polymer derived from agar. It is used for the fractionation of high molecular weight substances as certain polysaccharides, proteins or nucleic acids. Special modified media is a product derived from the dextran-based gels by reacting the hydroxyl groups with a reagent to render them hydrophobic. The modified gel particles swell in non-aqueous solvents. For example Sephadex LH-20 has some of the hydroxyl groups of the dextran gel alkylated so the gel will swell in polar organic solvents, water or mixtures of both.[62]

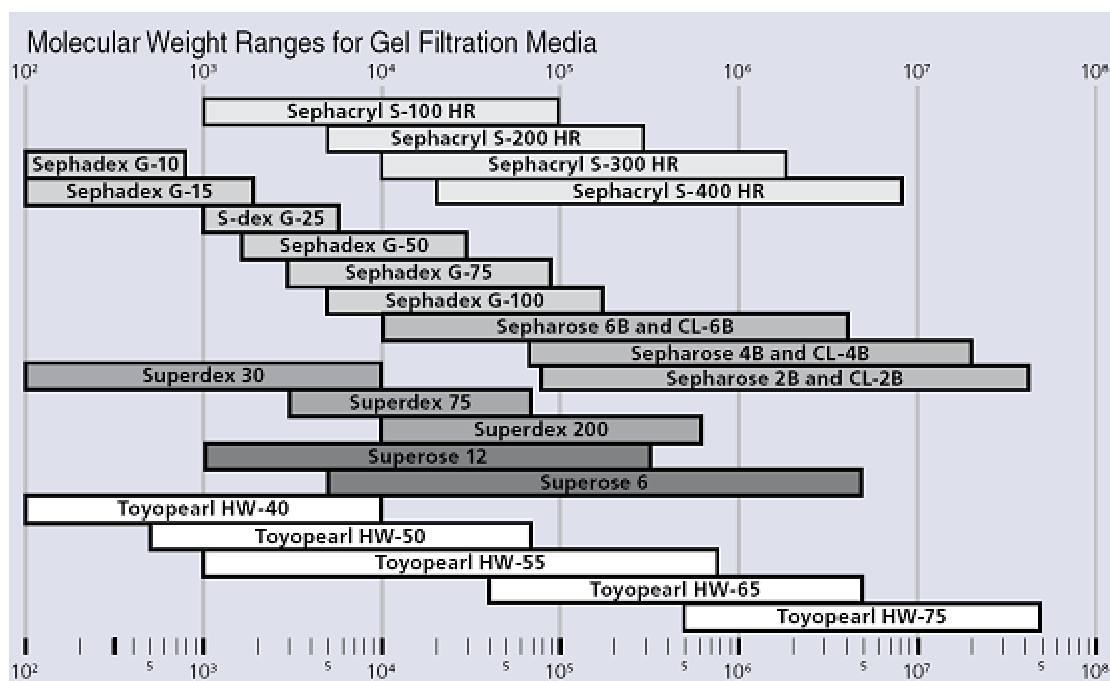


**Fig. 8 Molecular weight ranges of gel filtration media** [63]

Gel chromatography employed in the study was modified from the Kobata´s method as well.[43] It was performed on a column (106 x 1.6 cm) using Sephadex G-25 media. Column void volume was 76 ml. 5 ml of crude oligosaccharide solution was loaded on the top surface of the Sephadex G-25 column and the oligosaccharides were eluted with $H_2O$. After the void volume the fractions of about 3 ml were collected. The crude milk whey oligosaccharides were analysed from collected eluate for total hexose using phenol-sulfuric acid method and for sialic acids using periodate-resorcinol method after gel filtration (mostly at speed 0,14 ml/min). All together 15 gel chromatography separations were done from the material obtained from the isolations I, II and III.

## *5.3 Analysis of total hexose and sialic acids*

<u>Total hexose</u>

Phenol-sulfuric acid method for total hexose content was performed by the method of Kobata.[43] Aliquots of 50 µl were taken from every second gel chromatography fraction and 200 µl $H_2O$ was added. Then 200 µl 5% aqueous phenol and 1000 µl concentrated sulfuric acid were added. The tubes were incubated in water bath at +37°C for 30 minutes. Absorbances were measured at 490 nm using spectrophotometer.

Sialic acids

To measure sialic acids content periodate-resorcinol method was used (Jourdian et al).[64] Samples of 250 µl were taken from every second gel chromatography fraction. 50 µl of 0.004 M periodic acid was added. Tubes were shaken and incubated on ice for 20 minutes. 625 µl of 0.6% resorcinol reagent (6% resorcinol reagent stock was diluted 1:10) was added. Solutions were mixed and incubated on ice for 5 minutes. Tubes were then heated to +95°C for 15 minutes and cooled down in tap water. Finally 625 µl of *tert*-butanol was added. The tubes were placed in a 37°C water bath for 3 minutes to stabilize color. The absorbances were measured at 630 nm.

Isolated acidic oligosaccharides (WAO, WAO 1, WAO 2, WAO 3, WAO 4) and neutral oligosaccharides (WNO) were combined and lyophilized.

All together 15 gel chromathography separations and total hexose and sialic acids analysis were carried out to get enough material of oligosaccharides for testing anti-adhesion activity.

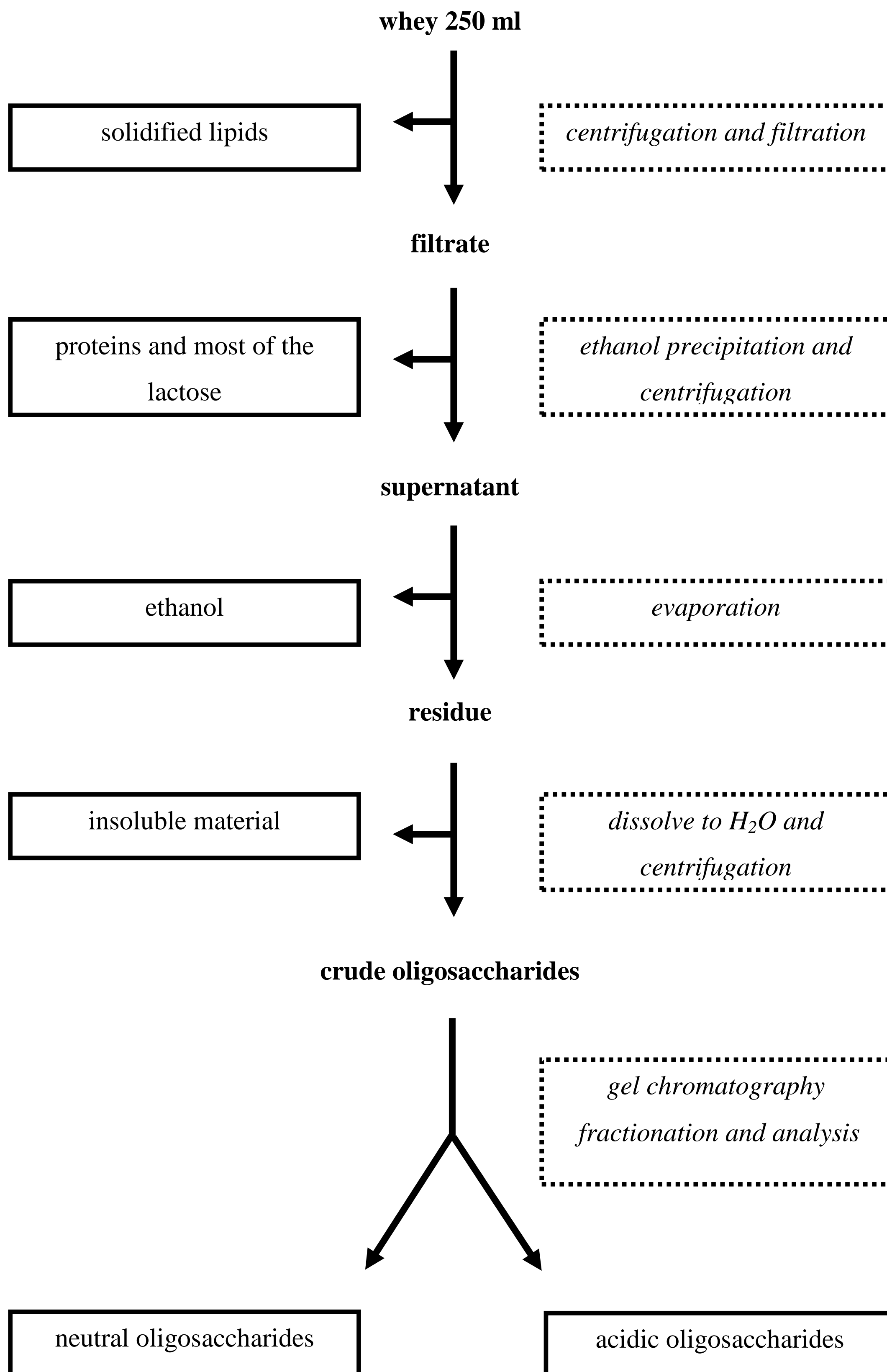


**Fig. 9 Diagram of isolation of oligosaccharides**

## 5.4 Cultivation of Neisseria meningitidis and isolation of pili

Isolation of pili was carried out as described before (Hakkarainen et al., 2005).[57] Strain 8013 *N. meningitidis* serogroup C class 1 (2C4 3W) bacterium stock was stored at –80°C. Bacterium stock was transfered to –20°C before cultivation on GCB agar plates containing Kellogg´s supplement I and II. The bacterial material was spread under laminar flow to the plates. Plates were incubated at +37°C in $CO_2$ atmosphere for 18 hours.

Isolation of the pili was carried out at 0°C under laminar flow. Each five plates of cultivated bacterium were harvested and suspended in 20 ml of sterile 10 mM Hepes solution (1 M Hepes diluted 1:100). The tubes were vortexed vigorously for exactly 30 seconds. The mixture was centrifuged at 8000g at +4°C for 20 minutes. 16 ml of the supernatant was loaded to a centrifugal filter device of 100 kDa cutt-off and centrifuged at 1000g at +4°C to a volume of 1 ml. The concentrated solution was washed twice with 15 ml of 10 mM Hepes buffer and centrifuged to 1 ml again as written above. Isolated pili were stored at +4°C.

## 5.5 Biotin labeling of pili

Meningococcal pili were biotinylated as described before (Hakkarainen et al., 2005).[57] First 2.2 mg of *D*-Biotinoyl-*ε*-aminocaproic acid-*N*-hydroxysuccinimide ester was diluted by adding 110 µl of dimethylsulfoxide and mixed vigorously in order to get the biotinylation reagent. Isolated pili were diluted 1:4 by phosphate-buffered saline (1 x PBS: 10 x PBS diluted 1:10 by $H_2O$) to get 1 ml solution. 50 µl of the prepared biotinylation reagent and 1 ml 1:4 diluted pili was incubated and gently mixed on the rocking platform at room temperature. Afterwards the mixture was loaded to the 100 kDa cut-off centrifugal filter device, filled up with 1 x PBS and centrifuged at 1000g at +4°C to a volume of 1 ml. Concentrated solution was washed three times by 1 x PBS, centrifuged at 1000g at +4°C to a final volume of 1 ml as described above. Biotin labeled pili were stored at +4°C.

## 5.6 Inhibition assay with gradually decreased oligosaccharide content

Bovine thyroglobulin was chosen as a reference glycoprotein for inhibition assays based on earlier results (Hakkarainen et al., 2005).[57] Thyroglobulin was diluted with 1 x PBS to get a solution of 0.1 mg/ml concentration. The thyroglobulin solution was immobilized to the microtiter plate wells by adding 100 µl of the solution to each well and by incubating

overnight at +4°C. Then the wells were washed five times with the washing buffer and dried. In the glycoprotein coated wells additional binding sites were blocked by adding 250 µl of the blocking buffer per well and the plates were incubated for 60 minutes at room temperature. The wells were washed five times with the washing buffer and dried. Inhibition of the binding of *N. meningitidis* to the glycoprotein was carried out by first preincubating the biotin labeled pili with different concentrations of milk whey oligosaccharide fractions. The lyophilized fractions of WAO (WAO, WAO 1, WAO 2, WAO 3, WAO 4) or WNO were dissolved into 1 x PBS in order to make 20 mg/ml solution. The solution was diluted 1:1 by 1 x PBS to get desired decreasing oligosaccharide concentrations: 20, 10, 5, 2.5, 1.25, 0.625, 0.313, 0.156, 0.078, 0.039, 0.02 and 0.01 mg/ml. The biotin labeled pili were diluted 1:4 with 1 x PBS. The solutions of biotin labeled pili and different oligosaccharide concentrations of different fractions were mixed 1:1 and preincubated for 60 minutes at room temperature gently rocking on the rocking platform. Control biotin labeled pili were diluted 1:8 with 1 x PBS and treated identically. After preincubation 100 µl of the 1:1 mixed solutions and the 1:8 diluted control biotin labeled pili were added to thyroglobulin coated microtiter plate wells per well and incubated for 60 minutes at room temperature. The control wells were prepared as shown in Fig. 10. 1 x PBS was used as a control for pili (P- control) and pipetted to the wells according to schema (Fig. 10). The wells were washed five times with the washing buffer and dried. Streptavidin-POD conjugate was diluted 1:4000 into the blocking buffer and added 100 µl into the wells. The blocking buffer was used as a control for Streptavidin-POD and added to the wells (S- control, Fig. 10). The wells were incubated for 60 minutes at room temperature, washed five times with the washing buffer and dried. 100 µl of ABTS substrate for HRP was added to each well for the enzyme reaction and colour development. After various times of incubation (20 – 60 minutes), absorbances were measured at 405 nm on Victor$^2$ Multilabel counter. Each determination was carried out in triplicate.

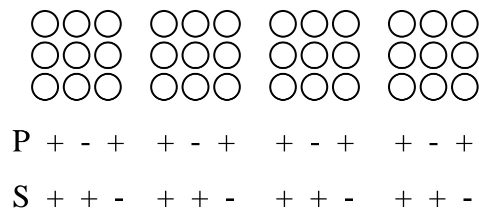


**Fig. 10 Inhibition assay microtiter plate wells pipetting schema. Each group of 9 wells refers to a different concentration of oligosaccharides or control pili.**

**P+** represents wells containing pili.

**P-** represents control wells not containing pili, only 1 x PBS.

**S+** represents wells containing streptavidin-POD conjugate.

**S-** represents control wells without streptavidin-POD conjugate.

At least one inhibition assay from each isolation of milk whey acidic oligosaccharide fractions (WAO 1, WAO 2, WAO 3, WAO 4) was performed. Also WAO fraction was tested in three inhibition assays. Whey neutral oligosaccharide fraction was tested only once as a control as that fraction was supposed to be non-active.[1]

# 6 Results

## 6.1 Gel chromatography and fractionation

The determination was made from an aliquot of every odd fraction (approximately 3 ml). Sialic acid fractions were eluted just after the void volume (76 ml) and were divided into two main peaks (Fig. 11). Neutral oligosaccharides were eluted as one broad peak (Fig. 11). Whey oligosaccharides obtained from isolations I, II and III were used. In all the isolations the separation profile was equal (Fig. 11-13). Altogether 15 fractionations were carried out during the study.

The first fractination was performed to separate WAO containing fractions from the fractions containing WNO. Tubes number 5 to 23 were collected as WAO fraction. WNO then from tubes 24 to 37 (Fig. 11).

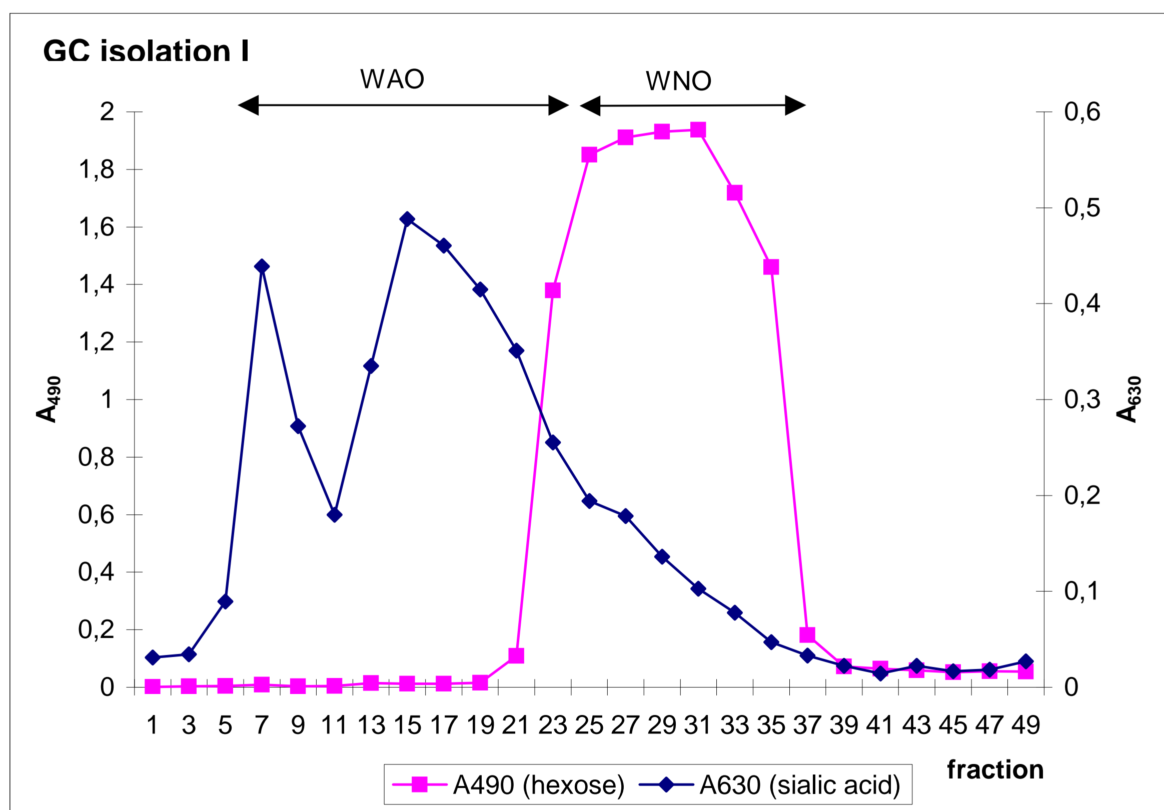


**Fig. 11 Gel chromatography separation of acidic whey oligosaccharides after analysis for total hexose and silic acids content. Speed 0,7 ml/min. Fractions WAO (tubes 5 to 23) and WNO (tubes 24 to 37).**

Further isolation, analysis and fractionation were attempted by collecting the sialic acid peaks as following. WAO 1: tubes number 6 to 10, WAO 2: tubes 11 to 17, WAO 3: tubes 18 to 30, WAO 4: tubes 31 to 34. WNO then tubes 35 to 48. (Fig. 12)

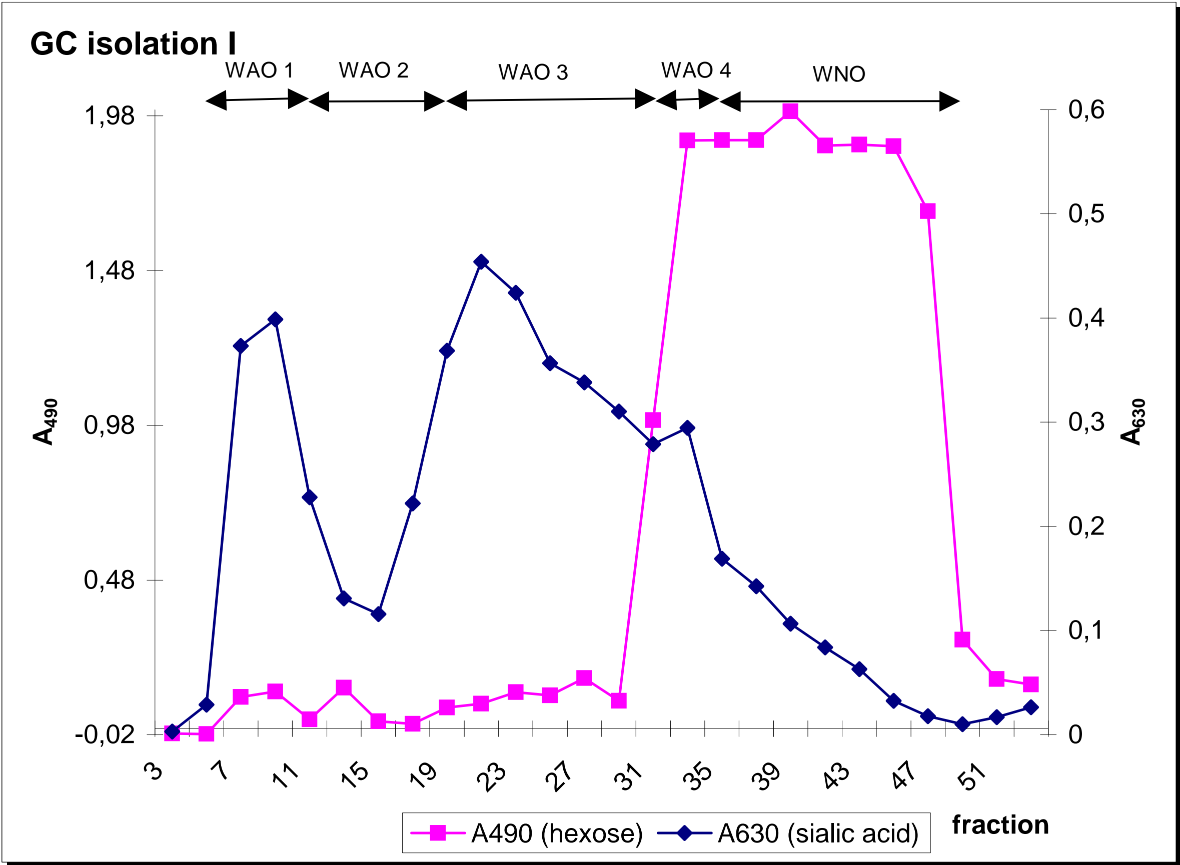


**Fig. 12 Gel chromatography separation of acidic whey oligosaccharides after analysis for total hexose and silic acids content. Speed 0,48 ml/min. Fractions WAO 1 (tubes 6 to 10), WAO 2 (11 to 17), WAO 3 (18 to 30), WAO 4 (31 to 34) and WNO (35 to 48).**

All other fractinations from isolation I, II and III were performed in the same way as described above. Isolation III fractionation: WAO 1: tubes 3 to 7, WAO 2: 8 to 15, WAO 3: 16 to 20, WAO 4: 21 to 25, WNO: tubes 26 to 32. (see Fig. 13)

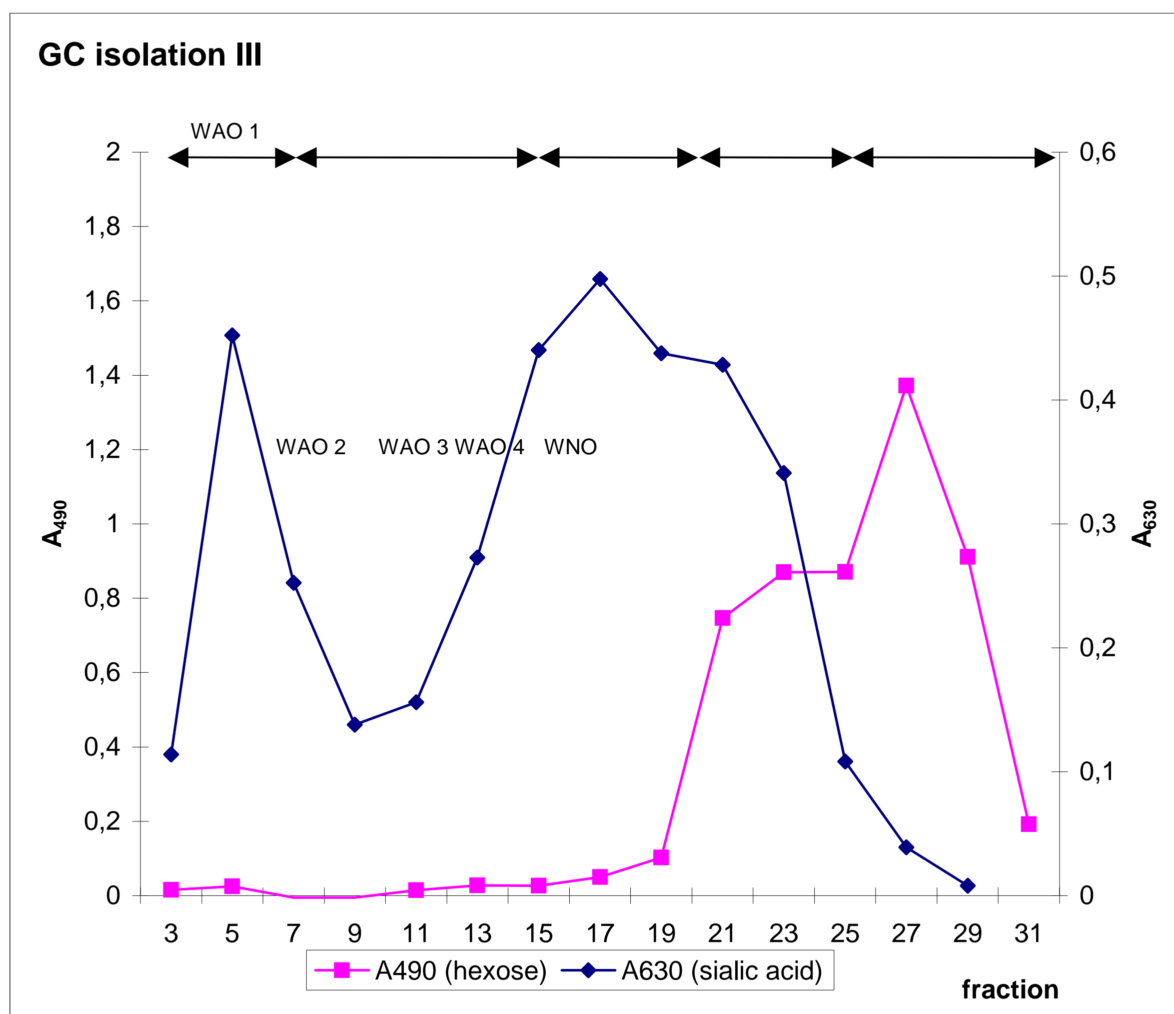


**Fig. 13 Gel chromatography separation of acidic whey oligosaccharides after analysis for total hexose and silic acids content. Speed 0,14 ml/min. Fractions WAO 1 (tubes 3 to 7), WAO 2 (8 to 15), WAO 3 (16 to 20), WAO 4 (21 to 25) and WNO (26 to 32).**

## 6.2 Inhibition assays with gradually decreasing oligosaccharide content

The binding of pili to bovine thyroglobulin was inhibited by 12 different decreasing concentrations of milk whey oligosaccharides of each separated fraction. The results are compared to the binding of pili without oligosaccharide inhibition and data are shown as percent inhibition of binding of pili to thyroglobulin. Each fraction (WAO 1, WAO 2, WAO 3, WAO 4) was tested separately. From each isolation (I, II and III) at least one inhibition assay was carried out. Only the results from the inhibition assays made from isolations I and III show relevant inhibition of pili binding to thyroglobulin. Results from other assays are ignored, because they represent inhibition of the binding to the background (milk powder). In these experiments binding of the pili to the thyroglobulin was on the background level.

### 6.2.1 WAO 1

All acidic whey oligosaccharides were separated into four parts according to Fig. 12. The inhibitory activity of the WAO 1 was tested from each isolation (I, II and III). Only the results of the inhibition assay from the isolation I showed relevant inhibition of pili binding to thyroglobulin (Fig. 14). The inhibition assay shows high anti-adhesion activity: inhibition reached 90 % at the concentration of 5 mg/ml and over 50 % inhibition at the concentration of 0,02 mg/ml. 20 % inhibitory activity was achieved at the concentration of 0,01 mg/ml.

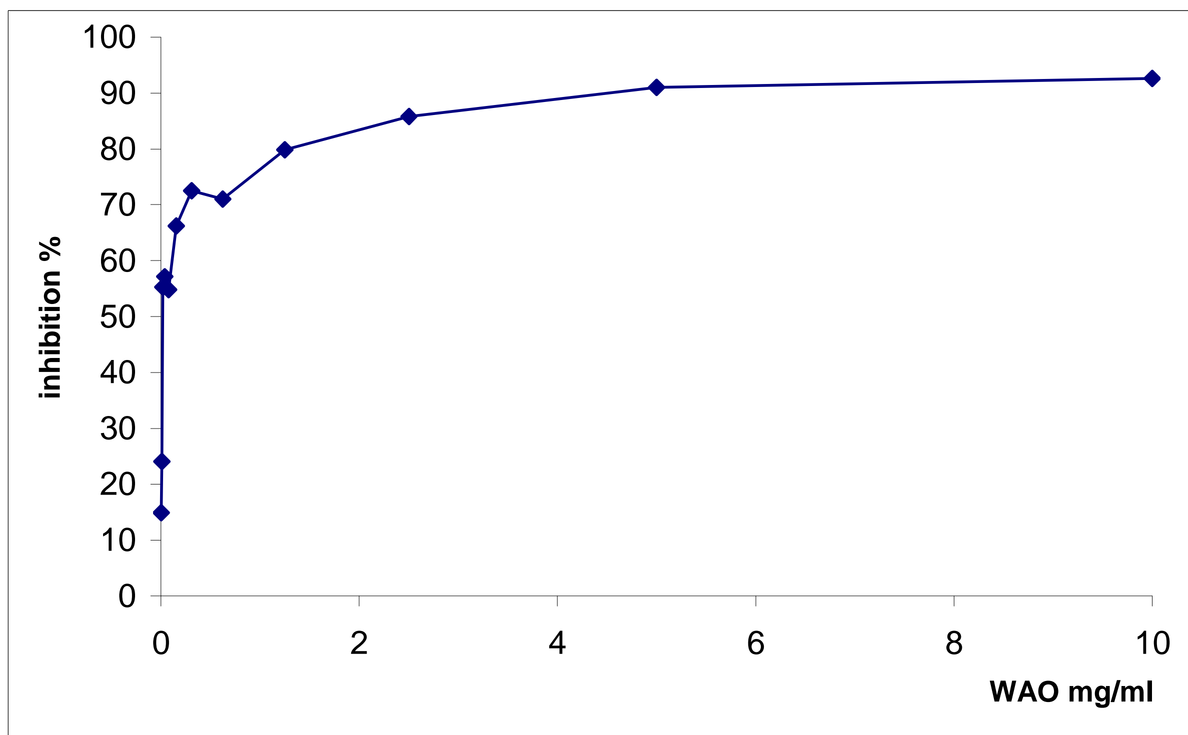


**Fig. 14 Inhibition assay of binding pili to thyroglobulin. Inhibition with gradually decreased concentration of WAO 1. Data from one assay,**

### 6.2.2 WAO 2

The inhibitory activity of the WAO 2 was tested from every three isolations (I, II and III). According to the pili binding to thyroglobulin, only the results of the assays achived from the isolations I and III were used (Fig. 15). The data show that the anti-adhesion activity was reached at higher concentrations compared to WAO 1. Inhibition was 75 % at the concentration of 5 mg/ml, 50 % at the concentration of 2 mg/ml and 20 % inhibition was achieved at the concentration of 0,078 mg/ml.

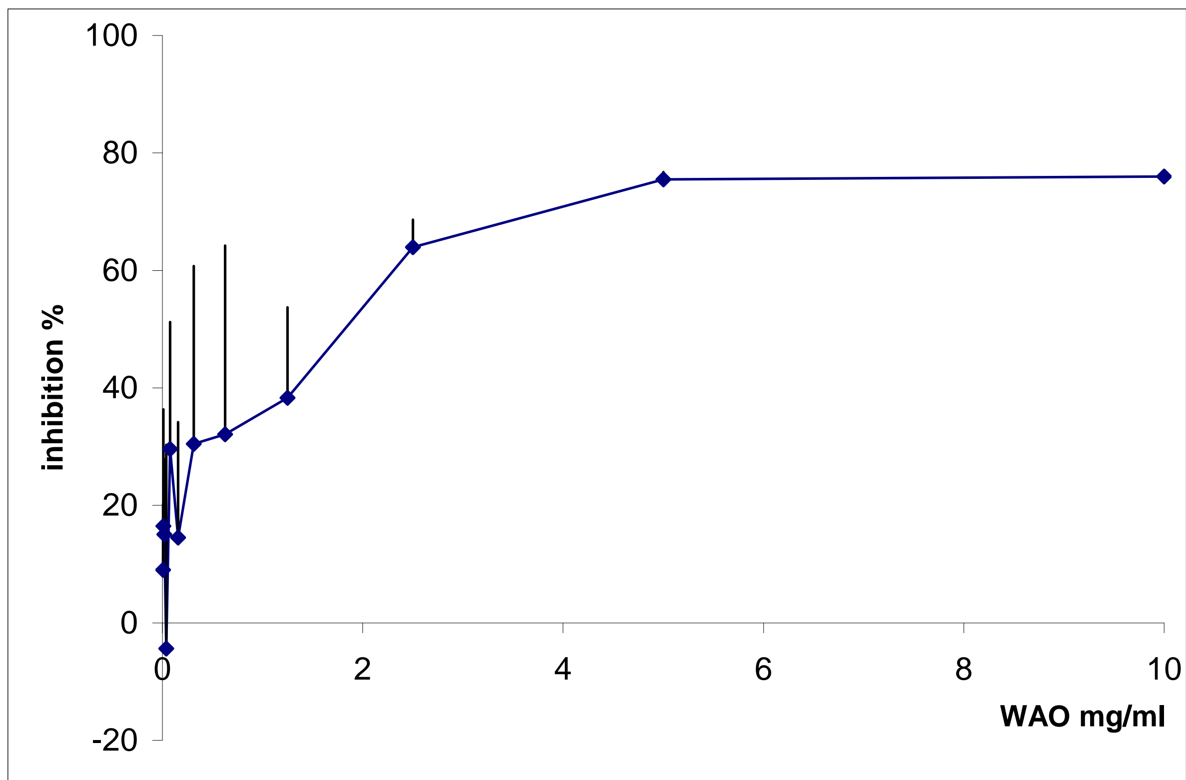


**Fig. 15 Inhibition assay of binding pili to thyroglobulin. Inhibition with gradually decreased concentration of WAO 2. Data from three assays.**

### 6.2.3 WAO 3

Only the results from the inhibition assays with WAO 3 material from the isolations I and III were accepted due to the binding activity of pili to thyroglobulin (Fig. 16). Inhibition level is lower compared to WAO 1 and WAO 2, but the activity is evident. Inhibition reached 65 % at the concentration of 10 mg/ml, 50 % at the concentration of 5 mg/ml and almost 20 % inhibition at the concentration of 0,078 mg/ml.

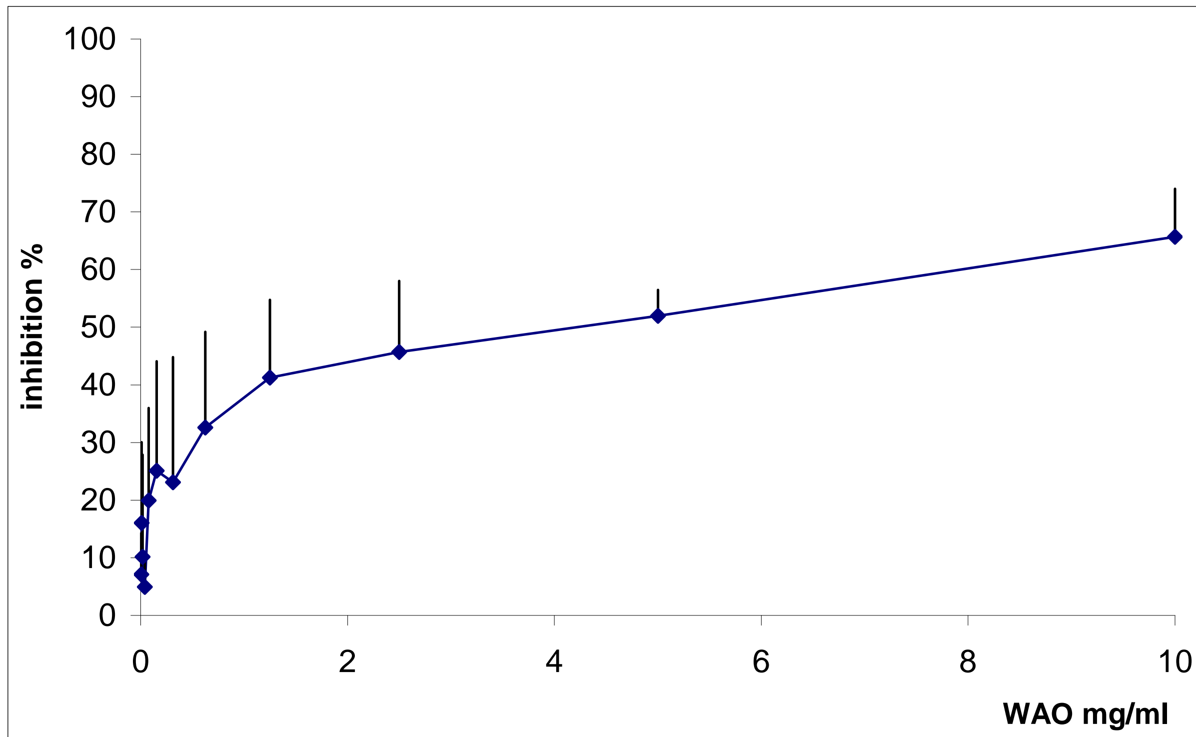


**Fig. 16 Inhibition assay of binding pili to thyroglobulin. Inhibition with gradually decreased concentration of WAO 3. Data from three assays.**

# 7 Discussion

Antibacterial activity of carbohydrates is under studies all over the world. There are already several studies concerning anti-adhesive human milk oligosaccharide activity against various pathogens[51] and studies dealing with bovine milk oligosaccharides and their activity against *Neisseria meningitidis*.[1;57] These studies and human antibiotic resistance are asking for more detailed research of bovine active oligosaccharides.[57] This study is directed to anti-adhesion activity of acidic oligosaccharides isolated from bovine milk whey against *Neisseria meningitidis*.

Four fractions of whey acidic oligosaccharides were isolated by gel chromatography method: WAO 1, WAO 2, WAO 3 and WAO 4 (Fig. 12). The anti-adhesion activity of these fractions was tested by inhibition assays against *N. meningitidis* pili binding to bovine thyroglobulin.[57] The inhibition assays with gradually decreased oligosaccharide content were carried out. These assays showed inhibitory activity of all acidic fractions except of WAO 4. The inhibition was dose dependent with the higher concentration of oligosaccharides the higher inhibition was achieved. The best inhibition against attachment of pili to thyroglobulin was provided by WAO 1 fraction with 90 % inhibitory activity at 5 mg/ml of WAO 1 (Fig. 14). This is a result from only one experiment and has to be repeated. The other inhibitory experiments carried out with WAO 1 were not included in the results, because there was unfortunately no binding to the thyroglobulin. However, the result here supports the previous activity achieved with bovine milk oligosaccharides.[57] The positivve result could be due to the bigger molecules of one or more acidic oligosaccharides eluted in the first fraction. Specific receptor binding sites in the pili could have been recognised by more specific receptor oligosaccharide as well. Thus more specific acidic oligosaccharide structure in the WAO 1 could have induced high inhibitory activity at very low oligosaccharide concentration. When acidic oligosaccharides were isolated from bovine milk the acidic oligosaccharides achieved 80 % inhibition with 10 mg/ml of the fraction.[57]

WAO 2 and WAO 3 fractions showed good inhibitory activity, reaching 80 % and 65 %, respectively. The active oligosaccharides were associated to sialic acid containing oligosaccharides as in bovine milk[57] and the active component was mainly eluted in the first peak (e.g. tubes 1-11 in Fig. 12) but was still present in the second peak (e.g. tubes 12-21 in Fig. 12). This gives a clue that active component is bigger oligosaccharide associated with some smaller ones.

The exact structures of the anti-adhesive oligosaccharide(s) should be found and further studies will be needed. The method of isolation should be improved to get enough material for the future studies. In the present study approximately 100 mg of each WAO fraction was isolated from 250 ml of whey.

Binding of *N. meningitidis* pili to thyroglobulin varied and therefore several experiments were not successful and were ignored. In previous studies different glycoproteins have been tested for *N. meningitidis* binding activity. Binding to thyroglobulin has been found to be the highest and most constant.[57] The variation of the binding may occur from sequence variation on the binding site of the pili. Pilin protein, of which neisserial pili is composed, has highly variable area, the hypervariable loop, in its amino acid sequence.[28] The adhesion of *N. meningitidis* has been suggested to be mediated by pilin or pilC1.[65]

This study was focused on isolation and testing of whey acidic oligosaccharides. Compared to the previously found activity of bovine milk acidic oligosaccharides, whey acidic oligosaccharides seem to have the same tendency in inhibiting the attachment of bacteria to bovine thyroglobulin *in vitro*. These oligosaccharides could be applied e.g. in infant formulas or used in army supplements for risk groups of acute meningococcal diseases. These compounds would not increase alarming antibiotic resistance as they are not bactericidal. Milk whey would be a perfect source of oligosaccharides giving one solution also to the ecological whey problem.

# 8 Conclusion

In this study I found that bovine milk whey acidic oligosaccharides inhibit binding of *N. meningitidis* pili *in vitro* as previous studies with bovine milk oligosaccharides predicted. Fractions of whey oligosaccharides were isolated by gel chromatography and tested on solid-phase inhibition assays for anti-adhesion activity with thyroglobulin as a reference glycoprotein. The activity depends on the exact isolated fraction according to the results. Milk whey thus could be promising and economically reasonable source for anti-infective compounds to be used in anti-adhesion therapy. The exact structure of these anti-adhesive oligosaccharides should be found and other studies are needed in the future as well, which include inhibition assays in cell culture and clinical trials.

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