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Thermodynamics of metal interactions with chitin-related biopolymers by isothermal titration calorimetry and production of 2-keto-3-deoxy-D-manno-octulosonic acid from D-glucose in vivo

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LIST OF ABBREVIATIONS

Con A	Concanavalin A
DNA	Deoxyribonucleic acid
ESI-MS	Electrospray ionization mass spectrometry
G	Free energy of binding, cal/mol
Glc	Glucose
Glc-1-P	Glucose-1-phosphate
GlcNAc	*N*-Acetylglucosamine
GlcNAc-1-P	*N*-Acetylglucosamine-1-phosphate
H	Enthalpy of binding, cal/mol
ITC	Isothermal titration calorimetry
IPTG	Isopropyl β-D-1-thiogalactopyranoside
K	Kelvin
K_b	Binding constant, M^{-1}
KDO	2-keto-3-deoxy-D-manno-octulosonic acid
NMR	Nuclear magnetic resonance
PP_i	Pyrophosphate
S	Entropy of binding, cal/mol.K
T	Temperature, K
TOC	Table of contents graphics
U	Uridine
UDP	Uridine diphosphate
UDP-Glc	UDP-glucose
UDP-GlcNAc	Uridine diphosphate-*N*-acetylglucosamine
UMP	Uridine monophosphate
UTP	Uridine triphosphate

ABSTRACT

Chitin, a polymer of *N*-acetyl-D-glucosamine (GlcNAc), is used as a biomaterial and for the removal of metals in water purification. Given the role of metal binding to carbohydrates in both biological and industrial settings, isothermal titration calorimetry (ITC) was used to determine the thermodynamics of binding between chitin-related carbohydrate substrates and metal ions. The binding interactions between chitin and common water contaminants mercury, copper, iron, nickel, chromium, lead, zinc, cadmium, silver and cobalt have been studied. The strongest binding has been found towards mercury and the weakest to cobalt with a K_b of $1.16*10^5$ M^{-1} and $3.96*10^3 M^{-1}$, respectively. The formal charge of the heavy metal changed the binding strength in an inverse fashion. The chitin-heavy metal cation interactions were all determined to occur in an enthalpically driven manner. The degree of binding of a series of small chitin fragments to divalent copper ion using ITC have also been tested. The binding strength of GlcNAc has been found to be the weakest among the substrates tested with a K_b of $3.8*10^3$ M^{-1}. Penta-*N*-acetylchitopentaose $(GlcNAc)_5$ has provided the strongest metal interactions with a K_b of $22.1*10^3$ M^{-1}. All experiments afforded enthalpically driven and favorable interactions. Gibbs free energy of reaction values were all measured to be negative, which is indicative of spontaneous reactions. These results demonstrated that increasing numbers of GlcNAc units enhance the binding strength for divalent copper cation, but the magnitude of the effect points to statistical binding rather than chelation-based multivalency. KDO (2-keto-3-deoxy-D-manno-octulosonic acid) is an 8-carbon sugar that is essential for the growth of Gram-negative bacteria. The

production of reasonable quantities of KDO would allow studies to understand the chemistry and biology of this key sugar with the potential to develop anti-infective therapeutics against Gram-negative bacteria. The first synthesis of 2-keto-3-deoxy-D-manno-octulosonic acid (KDO) using glucose through pathway engineering of *Escherichia coli* was designed as a low cost strategy to obtain KDO. Although a transporter protein for the sugar is unknown, KDO could be isolated from the fermentation broth. An optimum yield of 334 mg KDO per liter of cultured cells was obtained using glucose as a carbon source.

CHAPTER 1. Introduction

Dissertation organization

This dissertation consists of five chapters. The first chapter is a general introduction presenting background information about the importance and applications of carbohydrates. Chapter 2 has been submitted to *Journal of Chemical and Engineering Data.* This chapter discusses binding interactions of a biologically important carbohydrate polymer, chitin, with common heavy metal contaminants found in industrial wastewater. The kinetic and thermodynamic binding parameters for these systems have been obtained using isothermal titration calorimetry (ITC) technique and presented in this section. Chapter 3 will be submitted to *Carbohydrate Polymers*. This chapter covers the binding experiments for a series of small chitin fragments to divalent copper. The binding strength of these substrates has been tested and determined by ITC. Chapter 4 has been submitted to *Angewandte Chemie International Edition.* This chapter discusses the design and production of a low cost protocol to obtain 2-keto-3-deoxy-D-manno-octulosonic acid (KDO) using glucose through pathway engineering of *Escherichia coli*. Chapter 5 presents conclusions and future directions.

Carbohydrates in cell recognition

Carbohydrates are essential for life and the most abundant organic compounds found in living cells (Figure 1.1). Carbohydrates are involved in many biological events. For example, they act as mediators and participate in cell-cell or cell-pathogen recognition.[1] They are also important in cell adhesion and attachment processes.

Carbohydrates are a part immune defense for living organisms and mainly act as recognition elements. The attachment of carbohydrates to proteins is known to enhance the properties of the protein by increasing their stability.[1] Glycosylation, the formation of chemical bonds by carbohydrate substrates, is important in cell growth, development and cell-matrix.[1] Carbohydrates have -OH functionalities, which allow chemical modifications for further synthetic transformations to be used in development of therapeutics.[2]

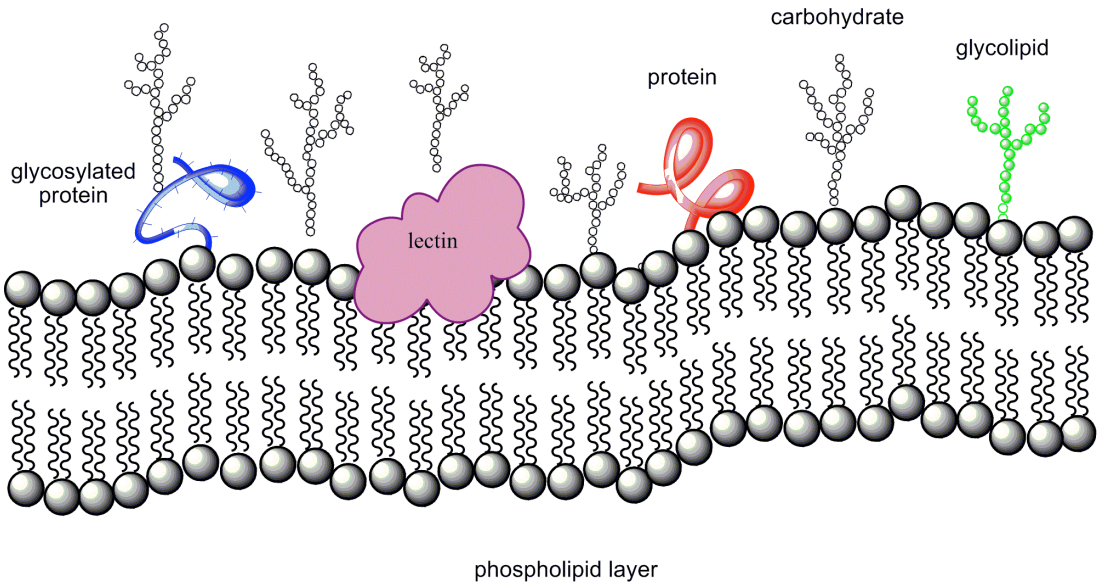

Figure 1.1. Basic structure of cell membrane

Pathogens contain different linkages and branches of carbohydrates on their cell surfaces. For this reason, the first thing that our immune system sees on a pathogen is carbohydrates. Our immune system recognizes carbohydrates as a way of identifying

pathogens; and then generates antibodies to mediate the fight with them. Pathogens contain different types of carbohydrates on their cell membranes, which are different than that of their target. That allows developing therapeutics against bacteria, viruses and parasites. Heparin is an example for medically important sugars; it is used as a blood thinner.[3] Another example would be the commercial meningococcal polysaccharide vaccines, Menactra®[4] and Menomune®[5], for meningitis groups A, C, Y and W-135.

Diversity of carbohydrates is mainly due to their ability to create numerous amounts of linkages and form different branches. For instance, blood group specificity is determined by different arrangements of four sugar molecules (L-fucose, D-galactose, *N*-Acetyl-D-galactosamine and *N*-Acetyl-D-glucosamine) to create different linkages.[6] Carbohydrate diversity differentiates them from other type of biomolecules, for example, lipids and proteins.1 This extreme diversity creates opportunities for carbohydrates to be used as targets for drug development.[2] Carbohydrates are sometimes found attached to proteins, which are called glycoproteins, as a component of cell membranes.[1-7]

Carbohydrate-protein interactions: Vaccines

Vaccines are antigenic biomedical formulations, which stimulate the immune system response to protect from certain viral or bacterial diseases. Vaccines contained killed or weakened pathogens. Administration of vaccine shots initiates the formation of antibodies against the foreign substance and causes inactivation or destruction of it.

Thus, the next time immune cells will recognize that particular pathogen and immediately destroy it and for this reason the disease would be prevented.

Vaccines have been used to stimulate immune responses against pathogens for hundreds of years. Small pox vaccines based on the live virus were first developed in the second millennium.[8] Since that time, most vaccines are still based on live or attenuated pathogens whose production must be carefully controlled to ensure safety. A great majority of current vaccines are not well-characterized. Being well-characterized is defined by the determination and control of purity, potency, identity, quality and safety of a product. Vaccine batches usually have to be tested for potency by the induction of immunity.[9] Currently, newer polysaccharide vaccines are the most well-defined and best characterized vaccines. For example, pneumococcal capsular polysaccharides such as meningococcal C conjugate (MenC) is commonly used to prevent this bacterial infection.[10] As standards for biological therapeutics become higher, the future of vaccines belongs to better-defined-perhaps wholly synthetic-constructs, which in turn requires a host of analytical techniques to ensure batch-to-batch quality and safety. Fortunately, progress in analytical methods continues to make better characterization of vaccines more tractable.

It is challenging to isolate carbohydrate antigens from natural sources as the end products might get contaminated or be heterogeneous. Synthesis of antigenic carbohydrates in the lab would be more advantageous due to quality and immunogenicity of the antigenic product and also for economical reasons.[11]

Expression levels of carbohydrates on the surface of cells alter during malignancy or disease formation.[12-13] This gives motivation to develop anti-infective reagents against diseases. The association of carbohydrates in development of tumors makes them suitable targets for vaccine design studies, because they are biocompatible, nontoxic and specific substrates.[14] Cancer vaccines are used to treat a current malignancy unlike most of the other types of vaccines, which are intended to protect form a future disease.[15]

Carbohydrate antigens, which are present on the surface of living cells, are recognized by antibodies and elicit an immune response. For this reason, vaccines could be developed for immunotherapeutic purposes. For example, a carbohydrate antigen for *Haemophilus influenzae* type b is shown to be a protective vaccine.[16,17] Development of new methods and technologies will accelerate the research towards preparing well-defined carbohydrate-based glycoconjugate vaccines.[18]

Antibodies possess protein structures and carbohydrates exhibit antigenic properties. Therefore, carbohydrate-protein interactions are often times considered as model systems for antibody-antigen interactions. Lectins are the most common proteins used in carbohydrate-protein interaction studies. Lectins are specific carbohydrate-binding proteins and they play important roles in recognition processes. Lectins are usually multivalent and they possess high specificity towards carbohydrates[19] so they are suitable candidates to study structure-function relationships.[20] Plant lectins act as antibodies[21] and take part in recognition of microorganisms and cell adhesion.

Structures of lectins are well defined and they are commercially accessible which makes them suitable candidates to be used as model systems to study antibody-antigen interactions.[21]

Concanavalin A (ConA) is the most common plant lectin utilized in carbohydrate binding studies. Con A has been the most economical commercial lectin of choice and very well characterized. In spite of their unique properties in recognition processes, unfortunately a lot of plant lectins have not been characterized. If we have enough thermodynamic data for some of the lectins we can extrapolate the binding interactions and eventually come up with energetic predictions for carbohydrate-lectin interactions based on only primary sequence data of the proteins and oligosaccharides.

Kinetic and thermodynamic characterization of carbohydrate-lectin systems is important to interpret the binding events.[22] Considering the fact that carbohydrate-lectin interactions are accepted as models for antigen-antibody interactions, characterization of these binding partners is essential to develop therapeutic reagents against pathogens. Carbohydrate-lectin systems might be useful to study for drug delivery and targeting purposes utilizing lectin functionalized therapeutics because of their binding specificities.[22] Carbohydrate-lectin interactions are reported to be moderately strong (10^3-10^4 M^{-1}).[23]

A great deal of research has been done to determine experimental binding data for computational studies. It is not feasible to do every single experiment to measure the binding interactions, for this reason, computational studies are carried out to predict these interactions. Computational chemistry and molecular modeling studies have been performed in order to model and predict thermodynamic interactions between carbohydrates and lectins. Although theoretical studies are useful for this purpose, producing reliable experimental data is essential as well.[22]

Due to numerous isomeric configurations, carbohydrates contain a lot more information compared to nucleic acids and proteins in that aspect.[22] Structural data for carbohydrate-lectin interactions can be combined with the data from obtained from quantitative techniques to predict the energetics of binding partners. Carbohydrate binding sites of lectins can be determined form computational predictions.[24] The mechanism of binding of oligosaccharides to lectins requires the knowledge of the structure of bound complex[19], which is not available for a variety of lectins so computational chemistry and molecular modeling can be utilized to solve this problem.

Simulation of carbohydrate molecules for modeling studies can be described in terms of mathematical force fields.[25] Owing to the extreme diversity in structure of carbohydrates and lack of experimental data about their energetics limits the development of force field for computational studies.[26] Isothermal Titration

Calorimetry (ITC) is a robust method to determine the contributions from enthalpy or entropy in carbohydrate-lectin interactions.[27]

As it would be advantageous to develop reliable force fields for carbohydrates for computational chemistry and molecular modeling, we need to obtain plenty of reliable kinetic and thermodynamic data for carbohydrate substrates. One of the most common techniques used in characterization of carbohydrate-protein interactions is isothermal titration microcalorimetry.

Isothermal titration microcalorimetry (ITC)

ITC is a powerful technique to determine the kinetic and thermodynamic interactions between two binding partners at constant temperature (Figure 1.2).[28-34] Characterization of biointeractions can be carried out in a label-free fashion using ITC[35] (Figure 1.3).

ITC contains two identical coin shaped cells, which are enclosed by an adiabatic jacketed outer shield (Figure 1.4). One of the cells is used as the reference cell, which is filled with deionized water and the other is sample cell that contains the sample solution. Hastelloy, which is a highly efficient thermally conducting material is used to make these ITC cells. There are highly sensitive thermocouple detectors utilized to detect the temperature difference between the reference and sample cells and also between the jacket and the cells. There is a reference heater and a sample heater located inside the instrument. In order to keep the temperature difference constant

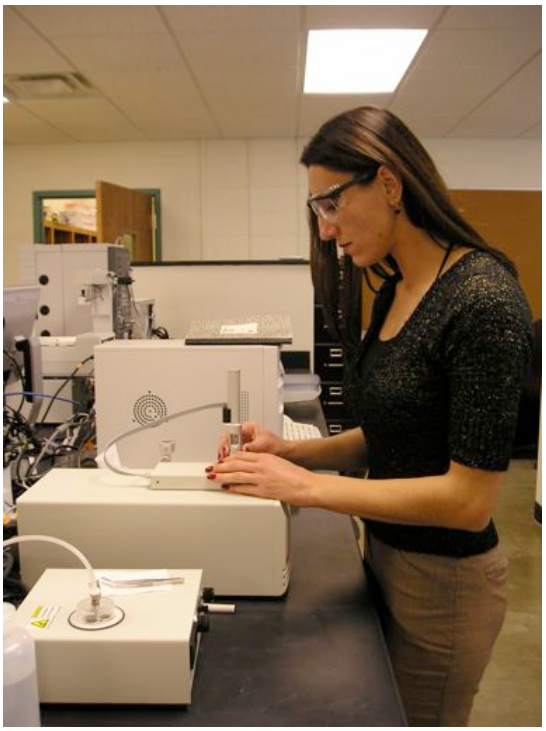

Figure 1.2. ITC instrumental set-up.

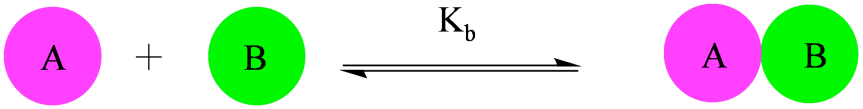

Figure 1.3. Binding of two biomolecules A and B.

between the reference and sample cells there is also a feedback heater present on the sample cell.[36]

Heat is either absorbed or given off during these injections. When there is an exothermic reaction, heat is evolved and the feedback differential power (DP) is eliminated and not required anymore. On the other hand, for an endothermic reaction

feedback DP needs to be provided in order to maintain the isothermal experiment conditions. A rotating syringe is used to inject the ligand into the macromolecule in the reaction cell. In order to degas the samples and also for cleaning purposes a degasser unit is used.

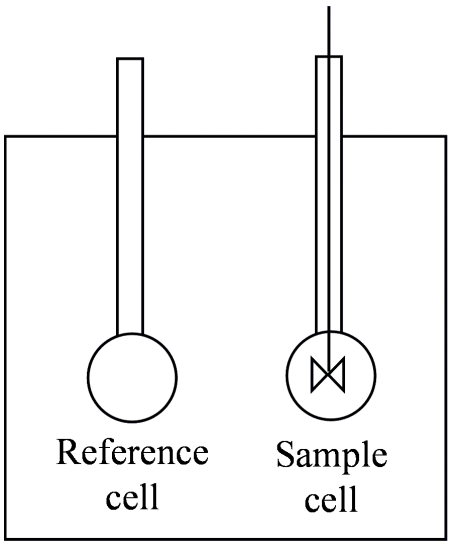

Figure 1.4. Reference and sample ITC cells.

Samples are degassed prior to use in ITC experiments as thermostatting can create bubbles. The degasser has a capacity to thermostat the samples from 0 to 80 ºC. The working volume of the sample cell and syringe are 1.4288 mL and 301 µL, respectively. ITC allows carrying out experiments between 2-80 ºC. The range of binding constants which could be determined using ITC is 10^2-10^9 M^{-1}.[36]

Given below is the set of user selectable experimental parameters for ITC (Table 1.1).

Table 1.1. Experimental ITC parameters and corresponding units.

Parameter	Unit
Temperature	ºC
Syringe concentration	mM
Cell concentration	mM
Stir speed	rpm
Injection volume	µL
Duration	Sec
Spacing	sec

In a typical ITC experiment a ligand solution in the syringe is injected into a macromolecule solution in the reaction cell (Figure 1.5).[37-39] The amount of power required to keep the temperature difference constant between a reaction cell and a reference cell is measured. Integration of the area under successive individual titration peaks over time provides the enthalpy of reaction in an ITC experiment. Heat signal typically decrease when the saturation of macromolecule by the ligand is achieved (Figure 1.6).[40]

The entire ITC operation is computer-controlled; the instrument measures enthalpy of binding directly that also provides entropy and free energy of binding values as well as binding constants.

Metal ions in biological systems

Metals play significant roles in biological processes. For example, metals interact with DNA and nucleotides, they are essential for full biological activity of enzymes and affect carcinogenesis processes. Nucleotide-metal binding events are involved in

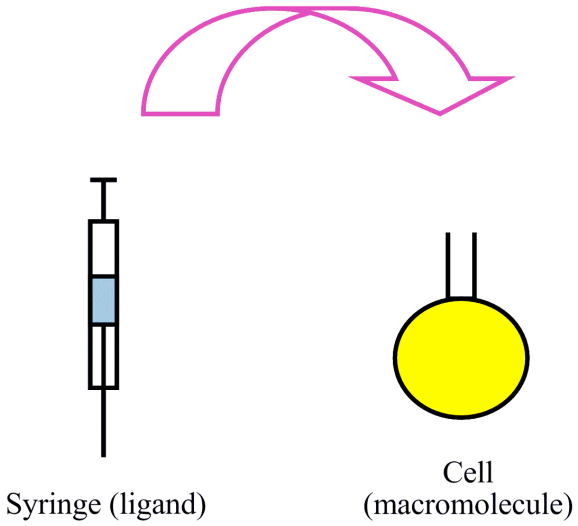

Figure 1.5. Typical ITC experiment.

some metabolic disorders like type 2 diabetes, high cholesterol and high blood pressure. These disorders are more likely to be found in people who already are hypomagnesaemia patients than others. Hypomagnesaemia is described as having lower than normal concentrations of magnesium in serum. Supplementing the patient with magnesium therapeutics perhaps arrange the magnesium-nucleotide binding equilibrium. In addition, controlling hypomagnesaemia might be related to glucose metabolism and glycogen biochemical pathway, which involves magnesium-sugar

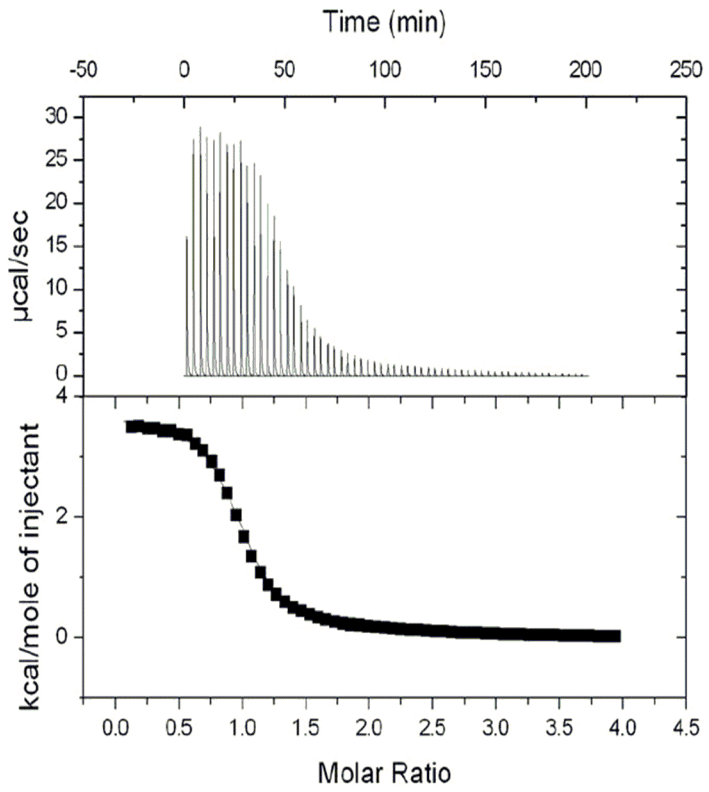

Figure 1.6. Heat signal decreases when saturation of macromolecule with ligand is achieved.[40]

substrate binding.

Metal ions are usually essential for full biological activity of enzymes. For instance, sugar nucleotidyltransferases require magnesium and type A glycosyltransferases require manganese (Figure 1.7) to exhibit their enzymatic activity. Despite its biological importance in therapeutic design, the role of metal ions in carbohydrate biocatalysis remains unclear. We have carried out a study to determine the binding affinity of magnesium and manganese to PP_i, UTP, UDP, UDP-GlcNAc, UDP-Glc,

UMP, U, GlcNAc, GlcNAc-1-P, Glc and Glc-1-P.[40] We also have determined the order of metal binding in enzymatic cycles using the data we have obtained from our

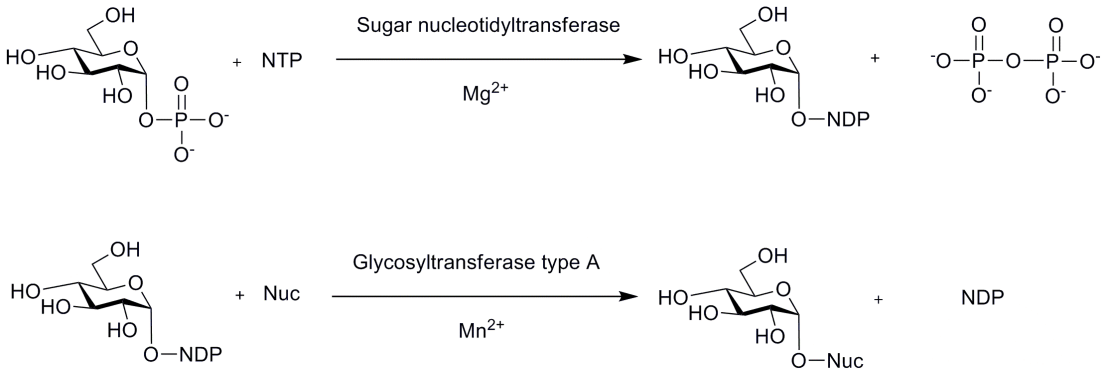

Figure 1.7. Divalent metal cations are required in efficient carbohydrate biocatalysis for complete activity of sugar nucleotidyltransferases and glycosyltransferases.

ITC experiments. Our data suggests that the metal ion binds to the nucleotide first and then leaves catalytic cycle bound to the nucleotidyldiphosphate. As the intracellular concentration of magnesium is high, there is no need for a permanent metal binding site in the sugar nucleotidyltransferase enzyme. However, intracellular concentrations of manganese is very low compared to magnesium creating a possibility for a permanent metal binding site in type A glycosyltransferases (Figure 1.8).

Binding of carbohydrate substrates to metal ions

We have reported in a previous study[40] that metal binding is proportional to the number of negatively charged phosphate groups on the carbohydrate substrates. It

would be interesting to study the behavior of metal binding versus noncharged sugars. Chitin is an example for a noncharged carbohydrate polymer and it exhibits metal chelation features.

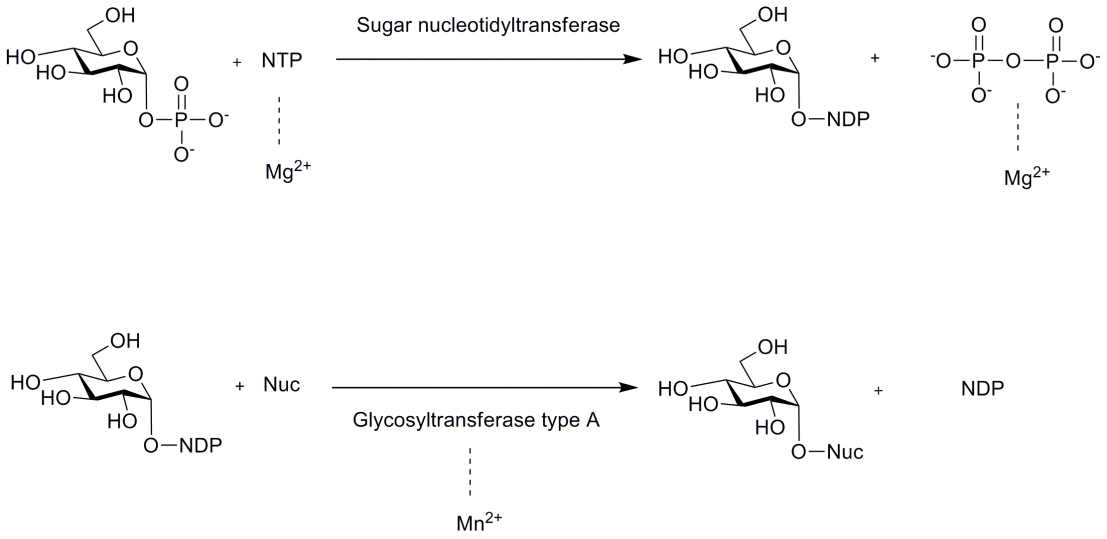

Figure 1.8. Order of metal binding for sugar nucleotidyltransferases and glycosyltransferases in carbohydrate biocatalysis.

Binding of carbohydrate polymer chitin to metal cations

People get exposed to heavy metals all the time but we cannot process them in our bodies so the accumulation in individual organs can create potential health problems.[41-44] Traditional methods to chelate heavy metals are reported in the literature.[45,46] One simple and cost efficient technique to treat wastewater is biosorption.[47,48] Chitin is the most abundant polysaccharide after cellulose in nature. Chelation ability of chitin to metal cations has been reported before.[49-51] However, the

binding affinity of chitin to heavy metal contaminants has never been quantified before which gave us the motivation to carry out a study on this subject. We have determined the binding thermodynamics of common heavy metal contaminants and chitin by ITC technique. The amount of biomaterial needed for chelation of heavy metals could be used for the selective removal of the metal cations from wastewater in a cost-effective fashion. For example, Zn/Cd battery production plants would need to remove these metals from their wastewater and it could be useful to know exactly how much of chelating agent is required. Computational modeling studies are other possible use of kinetic and thermodynamic binding data for chitin-heavy metal interactions.

Binding of chitin fragments to metal ions and multivalency effect

In addition to being a chelator for heavy metal cations, chitin is also a useful biopolymer with excellent features. For instance, it is nontoxic, biodegradable, biocompatible, and inexpensive substrate.[52-55] Chemical structure of chitin allows transformations for different applications, such as water purification, antimicrobial coatings, drug delivery agent, skin products, dietary fiber and adsorption gels.[52] Chitin is also used in neural[56], bone and cartilage tissue engineering[57-61] as a scaffold material, and in the synthesis of bioadhesive hydrogels.[62]

We have studied the kinetic and thermodynamic interactions between divalent copper ion and small chitin fragments to narrow down our findings from a previous study[63] and determined the multivalency effect of chitin in binding to metal cations.

Knowledge of binding energetics of metal ion-carbohydrate is often times required to carry out further structure-function relationship studies. In addition, such thermodynamic data is essential to develop computational models predict energy requirements for biological systems.

2-keto-3-deoxy-D-manno-octulosonic acid (KDO): a candidate vaccine component against Gram negative bacteria

KDO (2-keto-3-deoxy-D-manno-octulosonic acid) is an unusual carbohydrate being present on the cell surface of Gram-negative bacteria.[64-70] Inhibition of KDO causes the cessation of synthesis of lipopolysaccharides and eventually protein synthesis in the cell so it could be used to design antibacterial reagents against pathogens.[71] KDO is a commercial but expensive substrate. Chemical synthesis of KDO is also not cost-effective due to the requirement of expensive starting materials.[72] When single enzymes are involved to produce a desired substrate, fermentation is an alternative route.[73,74] *E. coli* is a common organism used in fermentation studies. We have carried out the synthesis of KDO using glucose as the starting material through pathway engineering of *E. coli* to provide a cost-effective procedure to produce KDO in order to develop KDO-based therapeutics against Gram-negative bacteria. We have incorporated all three genes involved in the KDO biosynthetic pathway[71,75-76] into one plasmid and used it to carry out fermentation experiments. Our optimized procedure is a cost-effective way to obtain KDO. Now that we produce KDO in reasonable quantities we can focus on modifying and incorporating KDO into larger chemical

structures as a new strategy to develop anti-infective agents Gram-negative bacteria related diseases.

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CHAPTER 2. Quantitative determination of heavy metal contaminant complexation by the carbohydrate polymer chitin

A paper submitted to *Journal of Chemical and Engineering Data*[1]

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Abstract

Chitin is a cost-efficient and nontoxic biopolymer with potential for use in heavy metal chelation from industrial wastewater. In this study we report the binding strength of chitin and the common water contaminants mercury, copper, iron, nickel, chromium, lead, zinc, cadmium, silver and cobalt. We have found the strongest binding takes place towards mercury and weakest to cobalt with binding constants of $1.16*10^5$ M^{-1} and $3.96*10^3 M^{-1}$, respectively. We observed that the formal charge state of the heavy metal inversely affects the binding strength. The divalent metal cation-chitin interactions are all enthalpically driven binding reactions. These results serve to benchmark industrial wastewater treatment by chitin chelation.

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Introduction

Chitin has been reported to be the second most abundant natural polysaccharide in nature and is commonly found in crab and shrimp shells.[1-11] Given this ubiquity, chitin is commercially accessible and inexpensive in its pure form.[1,10,12,13] Chitin has been used in the pharmaceutical, environmental, cosmetic and agricultural industries for applications that include wound dressings, contacts, lotions, package film coatings, pesticides, and wastewater treatment.[1] The biopolymer has great potential in drug delivery and tissue engineering applications.[2,9] Chitin is composed of linear repeating beta-1,4-linked *N*-acetyl-D-glucosamine monomers. A related more reactive polymer called chitosan contains the same monomer units, but with free amines on the glucosamine backbone.[1,2,7,8] Chitin is known to be less reactive towards metal chelation than chitosan; on the other hand, it is more stable than chitosan, which could make it a better choice to remove metal cations.[14] Interestingly, though, despite its many applications, the binding affinity of chitin to common metal cations found in biological systems or as heavy metal contaminants in industrial effluents has never been quantified. Quantitative data would not only provide the basis for better computational force fields related to carbohydrate-metal interactions, but also a better understanding of the molecular basis for these interactions. Herein we report the first such quantification using isothermal titration calorimetry to dissect the thermodynamics of binding of chitin to a range of metal cations.

Removal of heavy metals from contaminated solutions can be carried out by different methods, such as, precipitation, oxidation or reduction, ion exchange, electrochemical

methods, filtration, coagulation, adsorption, osmosis, membrane operations, or evaporation.[10-13,15-18] Biosorption is a complementary inexpensive and easy method in such applications.[5,10,12,16,17,19] To this end, chitin has been known to chelate heavy metal ions for wastewater treatment purposes.[1,4-7,11,18,20] For instance, removal of cadmium and lead from contaminated effluents by chitin chelation has been reported.[12] In another study, vanadate and chromium cations have been adsorbed by chitin.[21] Adsorption of chromium, cadmium, copper, lead and zinc by chitin has also been shown.[22] A separate study also showed that lead as well as iron cations are adsorbed on chitin.[5] Cadmium, chromium, mercury, zinc, copper, lead, iron, silver, molybdenum and nickel are major pollutants in industrial wastewater and are of particular interest for removal given the serious problems these metals can cause in the environment.[10,22] Environmental exposure to metals is problematic as we cannot process heavy metals and metal accumulation in specific organs can cause serious health problems.[23-26]

Heavy metal chelation of chitin has been the subject of several studies.[6,7,11,18,20] Surprisingly, there have not yet been investigations that quantify the strength of metal binding to chitin. Knowledge of kinetic and thermodynamic binding data for chitin-heavy metal interactions could help with the removal of cations, such as Hg, Cd, Co, Cu, Ni, Zn, Pb, Cr, Ag and Fe from industrial wastewater in a very efficient and selective fashion. If the exact amount of biomaterial needed for chelation of heavy metals is known, then the least necessary amount to be able to get complete removal of the metal cations from industrial effluents in the least expensive manner can be

ascertained. This approach could also be used for process design. For instance, the process of zinc and cadmium removal from industrial effluents at battery production plants can be designed better by knowing exactly how much adsorbent is theoretically required. Another application of our data would be for computational modeling of such biological systems for wastewater treatment as there is currently no available quantitative data for modeling studies. To obtain such quantitative data, isothermal titration microcalorimetry (ITC) is a useful technique that can measure the interactions between two binding partners. A ligand solution is titrated into a macromolecule solution and heat absorbed or released from this chemical reaction is measured.[27-31] This computer-controlled technique measures the enthalpy of binding directly and can provide entropy and free energy of binding values as well as binding constants and does not require labeling of components.

Experimental

Lead nitrate, zinc nitrate, cadmium nitrate, cobalt nitrate, nickel nitrate, copper sulfate, iron sulfate, chromium chloride, magnesium chloride, calcium chloride and manganese chloride was purchased from Fisher Scientific (Hanover Park, IL). Chitin, mercury chloride and silver nitrate were obtained from Sigma-Aldrich (St. Louis, MO). All reagents were used as they are received; no further purifications were utilized. Nanopure Barnstead E-pure water purification system (18.1 MΩ) was used to obtain deionized water for this study.

A VP-ITC isothermal titration microcalorimeter (Northampton, MA) was used for the binding experiments. All metal solutions were prepared in deionized water at 5 mM concentrations. 0.05 mM chitin solution is prepared in water and stirred with a magnetic bar for 3 h to obtain a homogenous solution. All solutions are degassed immediately before the ITC experiments. 5 mM metal solutions were added to the instrument syringe (301 μL) and 10 μL titrations were carried out into the chitin solution in the reaction cell (1.4288 mL) at 25 °C. Subtraction of a blank experiment for each run, which does identical titrations into the reaction cell without the chitin in the solution, eliminated heat of dilution effects. Origin (7.0, OriginLab Corp., Northampton, MA) was used to analyze and fit the ITC data to obtain the kinetic and thermodynamic binding parameters: K (binding constant), enthalpy of binding (ΔH), entropy of binding (ΔS). The change in the differential heat during an experimental run is integrated over time by the software to determine the enthalpy of reaction. Successive iterations allow fitting of the binding parameters until they converge to a value. The software then calculates the entropy of binding after determining the binding constant and enthalpy of binding. Once enthalpy and entropy of binding values are obtained, the free energy of binding is calculated using the experimentally derived ΔH and ΔS values.

Results and Discussion

To quantify the strength of binding of various metals to the carbohydrate polymer chitin, fourteen different metal cations were titrated into chitin in individual experiments using ITC. Binding constants, enthalpies of binding and entropies of

binding values were then extracted from the thermograms produced from each titration after subtraction of heat of dilution effects. Free energies of each reaction could then be calculated using the enthalpy and entropy results.

Binding thermograms for mercury(II) and copper(II) are presented in Figures 2.1 and 2.2 respectively. The mercury(II)-chitin interaction gave a binding constant of $1.16*10^5$ M^{-1} with a large amount of heat released. In contrast, copper binding released much less heat to provide about a four-fold smaller binding constant for copper(II) of $2.91*10^4$ M^{-1}. This binding difference could be attributed to the larger ionic radius of mercury(II). The ionic radii of copper(II) and mercury(II) are 73 and 102 pm, respectively.[32] Given equal charges, the copper ion has a larger surface charge density; however, the larger ionic radius of mercury is perhaps providing a better fit with the metal binding areas of chitin.

The chitin-iron(II) thermogram (Figure 2.3) showed a 1.3 time decrease in the binding constant compared to copper(II). The formal charge state of iron in this experiment is 2+. We have also repeated the same experiment for iron with a formal charge of 3+ (Figure 2.4). This data showed an about two-fold decrease in the binding of iron, thereby suggesting that the formal charge state of the heavy metal is inversely proportional to the binding strength to chitin. There is approximately a six-fold difference in the chitin-nickel(II) (Figure 2.5) and chitin-cobalt(II) interaction (Figure 2.6) in terms of binding constants. Cobalt(II) gave the weakest interaction towards chitin with a binding constant of $3.96*10^3$ $M^{-1.}$ No detectable binding of

magnesium(II) with chitin (Figure 2.7) was found by ITC. We did not observe any detectable binding against manganese and calcium either. This trend is consistent with previously reported studies on the related polymer chitosan in which binding to that carbohydrate polymer followed the pattern: Cu(II)>Fe(II)>Zn(II)>Cd(II).[33]

The overall order of binding affinities towards chitin is determined to be as follows: Hg(II)>Cu(II)>Ni(II)>Fe(II)>Cr(III)>Fe(III)>Pb(II)>Zn(II)>Cd(II)>Ag(I)>Co(II). This binding trend is similar to those of chitin/cellulose composite fiber-metal cation interactions which were determined by a spectrometric method [Hg(II)>Cu(II)>Pb(II)>Ni(II)>Cd(II)].[34] In addition, the sequence of metal complex stabilities given by Irving and Williams more or less applies to our studies.[35]

The magnitude of binding of chitin to different metal cations is perhaps a result of a combination of several factors, such as, geometry of the metal complexes, ionic radii of the metal cation, valency of the metal, and also hard-soft scales. These chelation interactions are perhaps caused by electronic effects, mainly from the nitrogen and oxygen species from the sugar polymer chain as available electrons from nitrogen and oxygen have the potential to induce interactions towards positively charged metal cations.

Bar graphs for binding constants are given for comparison purposes on Figure 2.8. Also a short list of the thermodynamic parameters is given in Table 2.1. There is

about a 30-fold difference in binding interactions towards mercury(II) and cobalt(II). This could be due to the higher electronegativity of mercury(II).

All metal cation-chitin interactions showed negative enthalpy of reaction values at 25 °C in our study, implying that these interactions are all enthalpically favored. We obtained positive changes in the entropy of binding and negative changes in the free energy of reaction values in our experiments. All the studied metal chitin interactions are enthalpically driven reactions at 25 °C. Free energy of reaction values have been calculated using the enthalpy and entropy values from the ITC experiments by Eqn. 2.1 given below:

$$\Delta G=\Delta H-T\Delta S \qquad (2.1)$$

All free energy of reactions resulted in negative numbers indicating energetically favored interactions for the heavy metal cations used in this study and chitin under our experimental conditions.

Origin 7.0 software is used to evaluate the ITC data to obtain the thermodynamic parameters of the reaction systems studied. Both one-binding site and two-binding site models are utilized to determine the binding parameters. Two-site binding model did not provide a good fit of the data; on the other hand, a one-site binding model worked well for fitting of the ITC data and was therefore applied to evaluate the data

throughout. The100-iterations technique was chosen to minimize the Chi square value for our entire data set.

In summary, ITC experiments have been carried out for the first time to determine kinetic and thermodynamic parameters for chitin-metal cation chelation reactions. Mercury(II) is found to exhibit the strongest affinity towards chitin. Copper(II) is the next strongest cation followed by nickel(II) and chromium(III). Cobalt(II) is found to show the weakest measurable affinity for chitin chelation. Binding of all these cations to chitin takes place with negative enthalpy and free energy values. Binding of all the heavy metal cations to chitin is determined to be enthalpically driven with negative change in entropy values at 25 °C. From this data, chelation of copper by chitin can be more effectively carried out in comparison to nickel, iron or lead. Calcium, magnesium and manganese interactions against chitin are determined to be so weak that they cannot accurately be measured by the ITC method. Clearly, a free amine as found in chitosan is not necessary for binding to a range of metal cations. However, the exact molecular mode of binding is still unclear. Further studies are needed to determine whether carbohydrate polymers might exhibit multivalent binding to increase affinities to metals significantly over values expected for monosaccharide-metal binding.[36-40]

Abbreviations

G : Free energy of binding, cal/mol

H : Enthalpy of binding, cal/mol

K : Binding constant, M^{-1}

N: Stoichiometry of binding

S : Entropy of binding, cal/mol.K

T: Temperature, K

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Supporting Information Available: Additional ITC binding curves and complete table for thermodynamic parameters can be found in the supporting information. This material is available free of charge via the Internet at http://pubs.acs.org.

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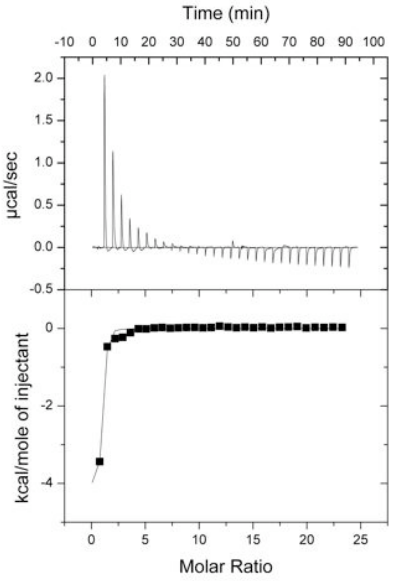


Figure 2.1. Binding thermogram and isotherm for chitin-mercury(II) interaction are given at the top and bottom, respectively.

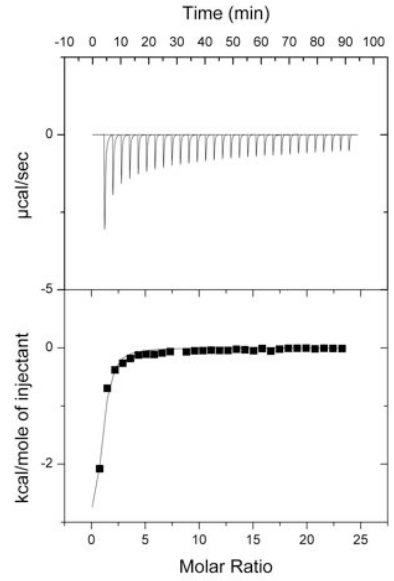


Figure 2.2. Binding thermogram and isotherm for chitin-copper(II) interaction are given at the top and bottom, respectively.

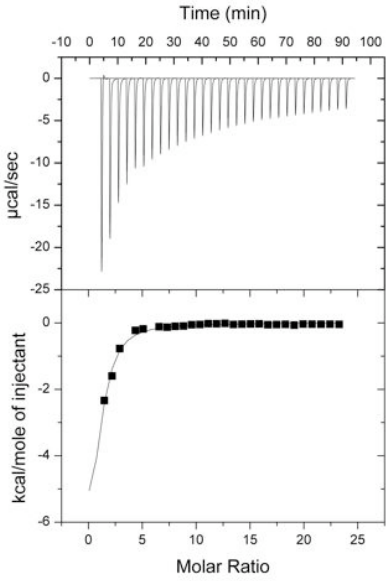


Figure 2.3. Binding thermogram and isotherm for chitin-iron(II) interaction are given at the top and bottom, respectively.

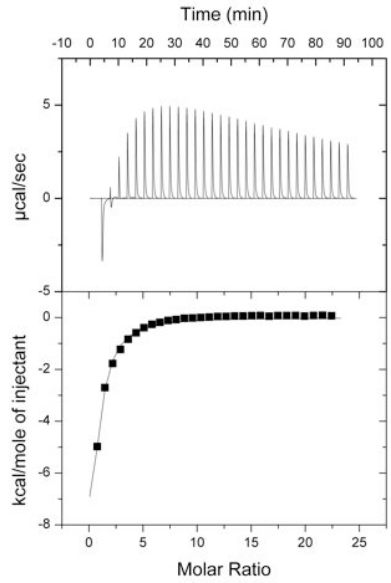


Figure 2.4. Binding thermogram and isotherm for chitin-iron(III) interaction are given at the top and bottom, respectively.

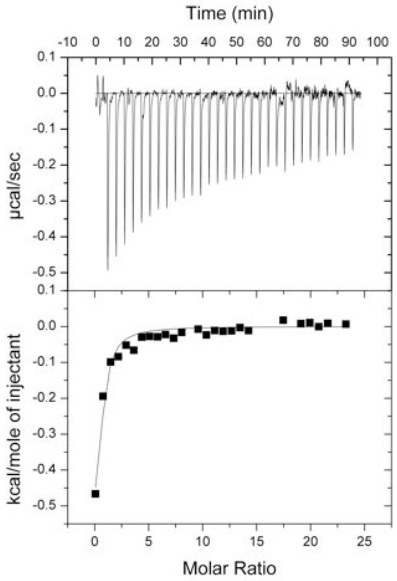


Figure 2.5. Binding thermogram and isotherm for chitin-nickel(II) interaction are given at the top and bottom, respectively.

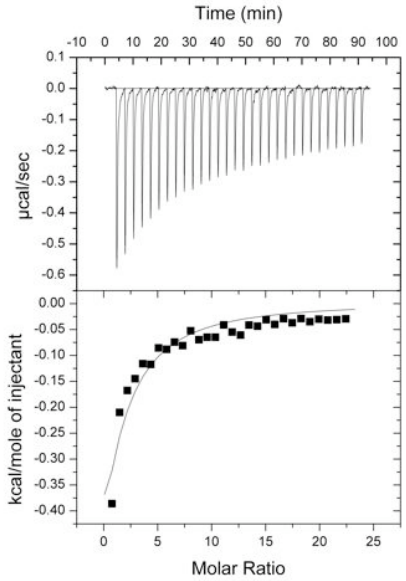


Figure 2.6. Binding thermogram and isotherm for chitin-cobalt(II) interaction are given at the top and bottom, respectively.

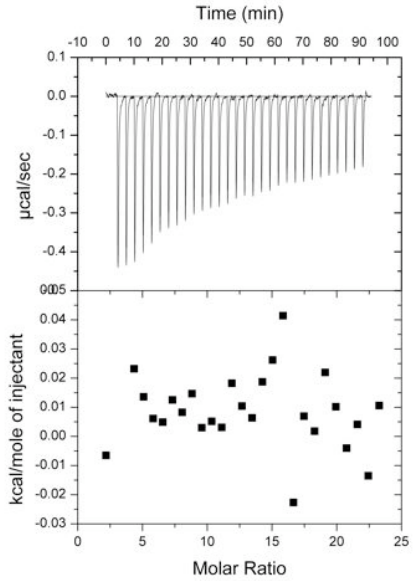


Figure 2.7. Binding thermogram and isotherm for chitin-magnesium(II) interaction are given at the top and bottom, respectively.

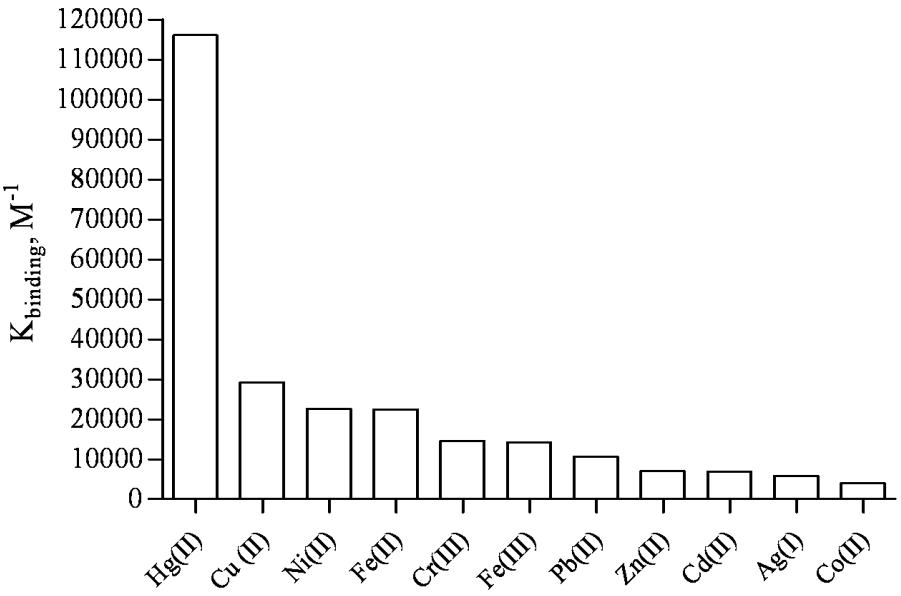
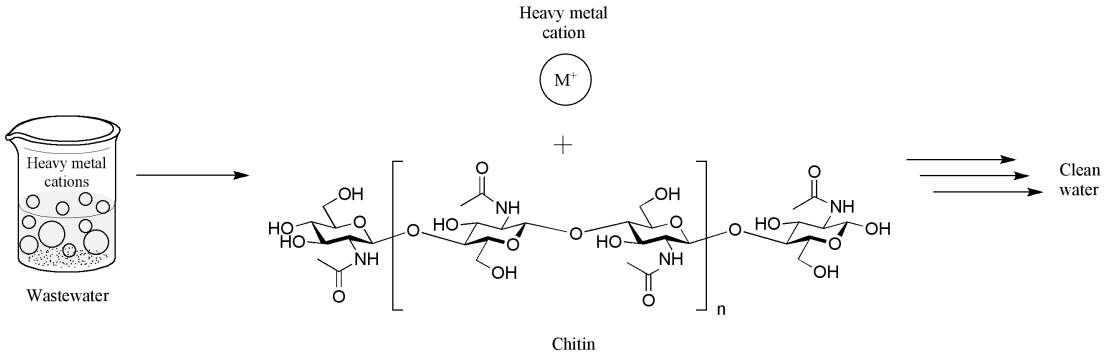


Figure 2.8. Comparison of binding constants for chitin-metal cation interactions (all interactions are included).

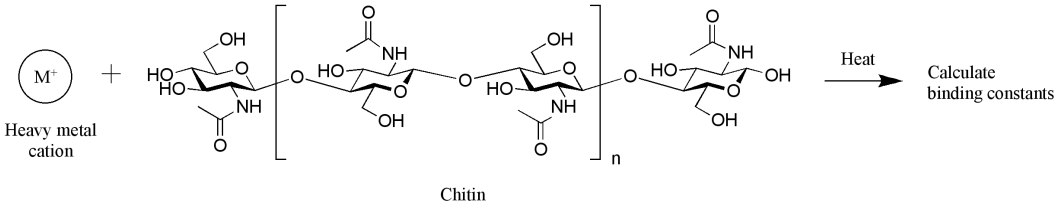


Scheme 2.1. Biosorption of heavy metals by chitin.

Table 2.1. Thermodynamic binding parameters for chitin-metal cation interaction.

Metal cation	$K*10^{-3}$, M^{-1}	ΔH, kcal/mol	ΔG, kcal/mol	
Hg(II)	116±12	-41.6±0.5	-6.98	High K_b
Cu(II)	29.1±1.6	-34.0±0.7	-6.08	
Ni(II)	22.5±3.0	-9.00±0.9	-5.95	
Fe(II)	22.4±3.5	-105±3.8	-5.82	
Cr(III)	14.5±1.4	-59.6±2.9	-5.63	
Fe(III)	14.1±0.9	-119±3.7	-5.80	
Pb(II)	10.6±0.8	-32.6±1.4	-5.49	
Zn(II)	6.90±0.5	-20.8±0.9	-5.23	
Cd(II)	6.81±0.7	-9.94±0.6	-5.23	
Ag(I)	5.77±0.4	-55.7±1.4	-4.98	
Co(II)	3.96±0.5	-19.8±1.4	-4.92	Low K_b

SYNOPSIS TOC 2.1.



Appendix

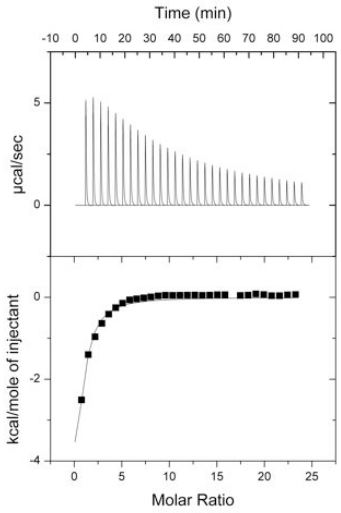


Figure 2.9. Binding thermogram and isotherm for chitin-chromium(III) interaction are given at the top and bottom, respectively.

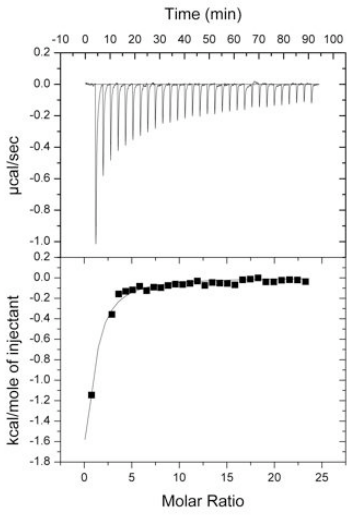


Figure 2.10. Binding thermogram and isotherm for chitin-lead(II) interaction are given at the top and bottom, respectively.

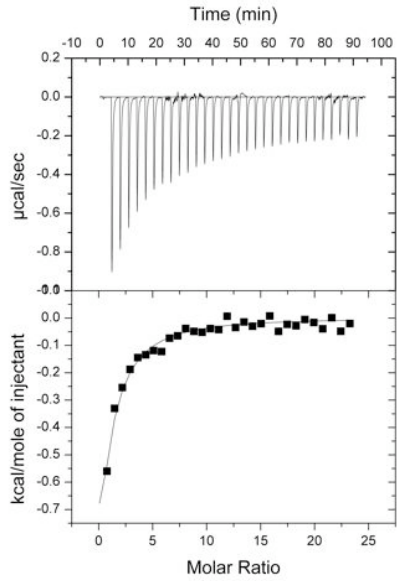


Figure 2.11. Binding thermogram and isotherm for chitin-zinc(II) interaction are given at the top and bottom, respectively.

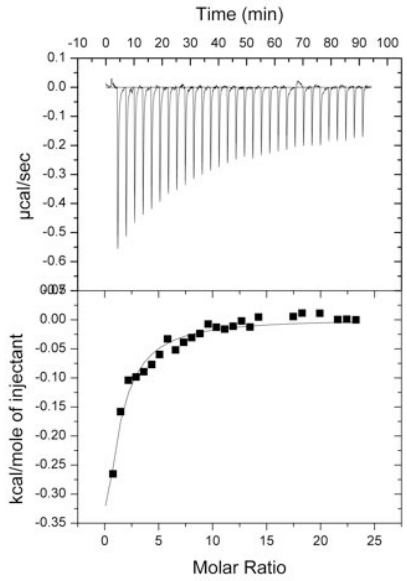


Figure 2.12. Binding thermogram and isotherm for chitin-cadmium(II) interaction are given at the top and bottom, respectively.

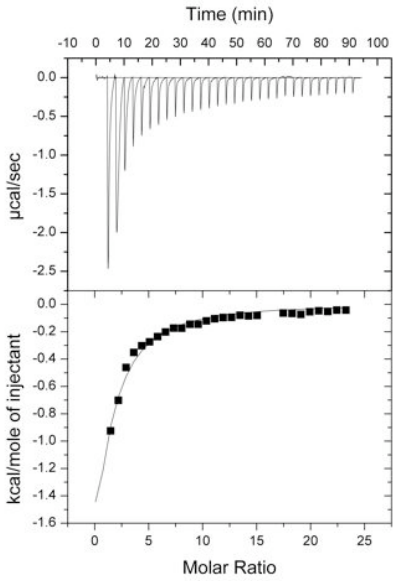


Figure 2.13. Binding thermogram and isotherm for chitin-silver(I) interaction are given at the top and bottom, respectively.

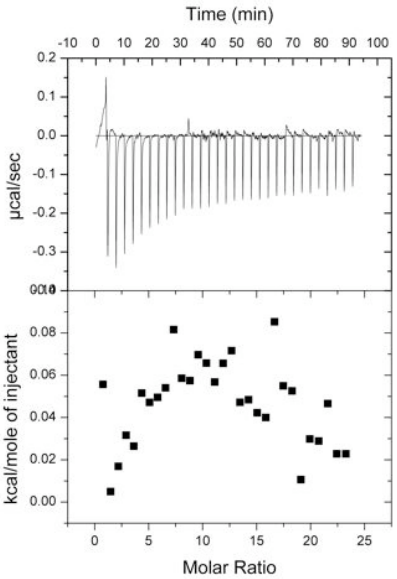


Figure 2.14. Binding thermogram and isotherm for chitin-calcium(II) interaction are given at the top and bottom, respectively.

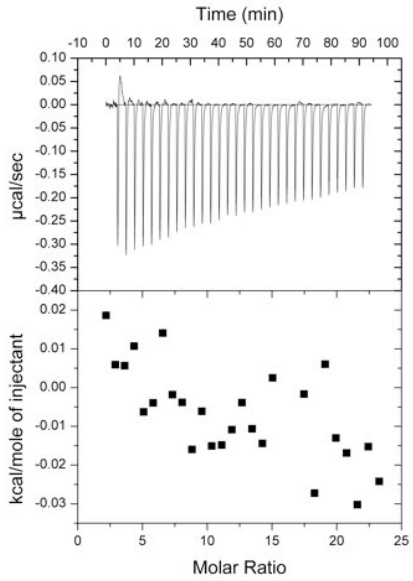


Figure 2.15. Binding thermogram and isotherm for chitin-manganese(II) interaction are given at the top and bottom, respectively.

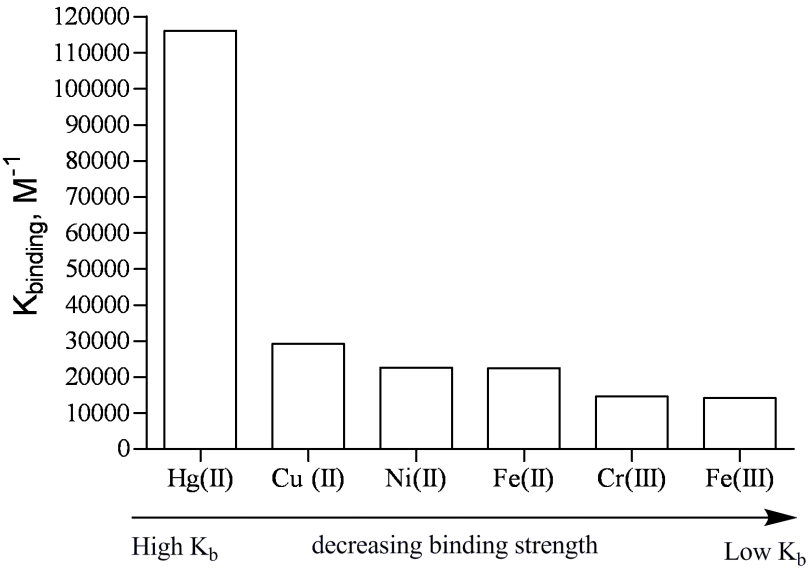


Figure 2.16. Comparison of binding constants for stronger chitin-metal cation interactions.

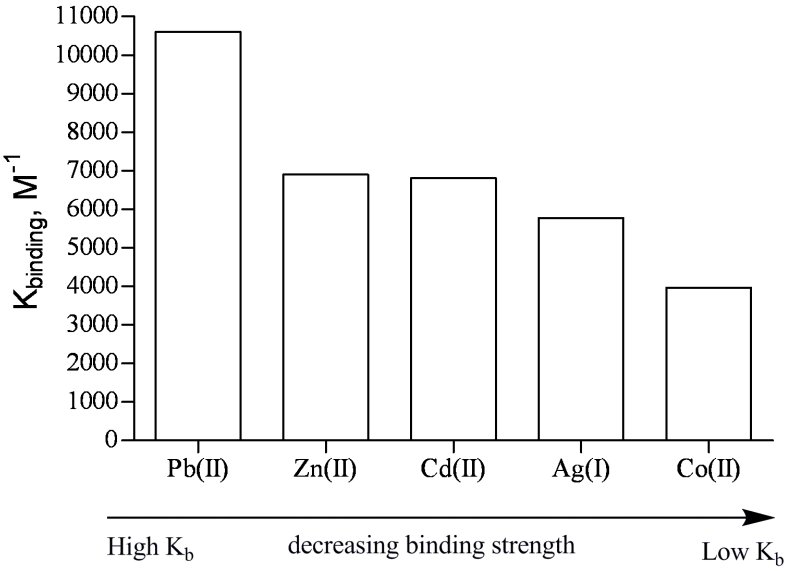


Figure 2.17. Comparison of binding constants for weaker chitin-metal cation interactions.

Table 2.2. Complete list of the thermodynamic binding parameters for chitin-metal cation interaction.

Metal cation	$K*10^{-3}$, M^{-1}	ΔH, kcal/mol	ΔS, cal/mol*K	ΔG, kcal/mol
Hg(II)	116±12	-41.6±0.5	-116	-6.98
Cu(II)	29.1±1.6	-34.0±0.7	-93.5	-6.08
Ni(II)	22.5±3.0	-9.00±0.9	-10.2	-5.95
Fe(II)	22.4±3.5	-105±3.8	-333	-5.82
Cr(III)	14.5±1.4	-59.6±2.9	-181	-5.63
Fe(III)	14.1±0.9	-119±3.7	-381	-5.80
Pb(II)	10.6±0.8	-32.6±1.4	-90.8	-5.49
Zn(II)	6.90±0.5	-20.8±0.9	-52.1	-5.23
Cd(II)	6.81±0.7	-9.94±0.6	-15.8	-5.23
Ag(I)	5.77±0.4	-55.7±1.4	-170	-4.98
Co(II)	3.96±0.5	-19.8±1.4	-49.8	-4.92

CHAPTER 3. Thermodynamics of binding interactions between divalent copper and chitin fragments by isothermal titration calorimetry (ITC)

A paper to be submitted to be submitted to *Carbohydrate Polymers*[1]

Gulden Camci-Unal[2,3] and Nicola L. B. Pohl[2,4]

Abstract

Herein we report the degree of binding of chitin fragments to divalent copper cation using isothermal titration calorimetry (ITC). In a previous study we have reported the kinetic and thermodynamic binding constants for chitin polymer interactions with a range of metal cations including copper (Camci-Unal & Pohl, 2009). In order to better determine the molecular basis for metal binding to these carbohydrate polymers, a series of chitin fragments were tested for their ability to bind to divalent copper. Based on ITC data, the binding strength of *N*-acetyl-D-glucosamine (GlcNAc) is weakest among the tested substrates with a binding constant of $3.8*10^3$ M^{-1}. *N,N*'-diacetylchitobiose $(GlcNAc)_2$ and *N,N',N*''-triacetylchitotriose $(GlcNAc)_3$ provided binding constants of $5.1*10^3$ M^{-1} and $13.3*10^3$ M^{-1}, respectively. Penta-*N*-acetylchitopentaose $(GlcNAc)_5$ demonstrated the strongest metal interactions with a binding constant of $22.1*10^3$ M^{-1}. For comparison, a binding value of $24.8*10^3$ M^{-1} was found for D-glucosamine, which is the deacetylated analog of GlcNAc, with the divalent copper. All experiments showed enthalpically driven interactions. Free energy of reaction values are all determined to be negative indicating spontaneous

reactions. Our results indicate that increasing numbers of GlcNAc residues increase the binding strength towards divalent copper cation. However, the effect of adding sugars to the polymer chain only modestly increases the binding affinity, thereby ruling out any chelation multivalency effects.

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[1]A paper to be submitted to *Carbohydrate Polymers*, Unpublished work.
[2]Graduate student and Professor, respectively, Department of Chemistry, Iowa State University.
[3]Primary researcher and an author.
[4]Corresponding author.

Introduction

Chitin, obtained primarily from seashells, is known to be the second most abundant natural polysaccharide after cellulose (Cohen-Kupiec & Chet, 1998; McAfee et al., 2001; Min et al., 2004; Shahidi et al., 1999; Tudor et al., 2006). This oligosaccharide contains linear beta-1,4-linked *N*-acetyl-D-glucosamine monomer repeating units. Chitosan is a partially deacetylated analog of chitin containing beta-1,4-linked D-glucosamine repeating units (Agboh & Qin, 1997; Illium, 1998; Ravi Kumar et al., 2004; Singla & Chawla, 2001). Chitin or chitosan can be used to remove heavy metal cations from industrial wastewater by a cost-effective technique called biosorption (Amuda et al., 2007; Kratochvil & Volesky, 1998; Veglio & Beolchini, 1997; Volesky & Holan, 2008; Zhou et al., 2004). Chitin in particular is reported to chelate heavy metal cations and thereby is useful to treat industrial effluents (Benguella & Benaissa, 2002; Hoshi et al., 1988). It is an inexpensive substrate and commercially accessible. Chitin is also a nontoxic biomaterial which finds use in biomedical applications, especially in tissue engineering. For instance, it is used to deliver drugs, in wound dressings, or as bone substitutes (Khor, 2001; Khor, 2002; Khor & Lim, 2003; Shigemasa, 1996).

Metal cations are involved in numerous biological processes. For example, they act as catalysts in chemical and enzymatic reactions, mediate the oxidation of proteins, interact with nucleotides and DNA, have the potential to induce pathogenesis and carcinogenesis (Kroneck, 2005), are used as chelators in protein purifications or affinity separations (Arnold, 1991), are helpful for targeting and probing the

variations on DNA (Barton, 1986), hydrolysis of RNA or enzymes (Dupureur, 2008; Hampel & Cowan, 1997), are present as electrolytes in animals to help osmoregulation (Geldmacher-von Mallinckrodt & Meissner, 1994) and are involved in some metabolic disorders (Volpe, 2008). Given the many processes in which metals are involved, thermodynamic data for a systematic set of metal-ligand interactions is important to predict the binding energetics and thereby help the study of metal cation functions in a range of systems.

Binding interactions of biomolecules can be determined by a powerful technique called isothermal titration calorimetry (ITC) (Cooper, 2003; Dam & Brewer, 2002; Doyle, 1997; Jelesarov & Bosshard, 1999; Ladbury & Chowdhry, 1996; Leavitt & Freire, 2000). This technique requires no substrate immobilization or labeling and allows detection of binding interactions in a computer-controlled fashion at a constant temperature. The injection syringe stirs and titrates the ligand of interest into the macromolecule substrate in the instrument cell. As this is a chemical reaction, heat is either absorbed or evolved during the successive injections. At the end of the experimental run, software is used to integrate the area under these individual titrations to provide the heat of reaction value. One of the powerful aspects of ITC instrument is that it measures the enthalpy of binding directly and provides the binding constant and entropy of binding values as well. The kinetic and thermodynamic binding parameters are calculated by a single ITC experiment along with a reference titration.

In a previous study we have determined the kinetics and thermodynamics of polymeric chitin binding to a range of metal cations (Camci-Unal & Pohl, 2009). In that study, the strength of copper(II) to chitin binding was measured as being $2.91*10^4$ M^{-1}; this magnitude of binding is often considered to be a moderate-strength binding. Copper(II)-chitin interactions are enthalpically favored and enthalpically driven thermodynamically spontaneous binding reactions. It was unclear from these results, however, if chitin contained specific binding sites in which multiple ligands might chelate the cation and thereby show enhanced metal affinities. The presence of multiple hydroxyl groups and sometimes other functional groups such amines and amides on carbohydrates create multiple potential metal chelation sites (Zheng et al., 1997). Although ITC studies cannot provide direct structural data, such chelation-based multivalency effects should show significantly enhanced binding in a one-site binding model than binding that does not involve multiple chelation sites or enhanced binding as a result of only statistical multivalency effects (Pieters 2009). In this study we report the first kinetic and thermodynamic binding results for divalent copper with small chitin fragments, namely GlcNAc, $(GlcNAc)_2$, $(GlcNAc)_3$, and $(GlcNAc)_5$ along with D-glucosamine for comparison. This data allows us to draw some conclusions about the nature of the metal-sugar interaction that should aid future computational modeling studies.

Experimental

General methods

N-acetyl-D-glucosamine (GlcNAc), *N,N*'-diacetylchitobiose $(GlcNAc)_2$, *N,N',N*''-triacetylchitotriose $(GlcNAc)_3$, penta-*N*-acetylchitopentaose $(GlcNAc)_5$ were obtained from Sigma-Aldrich (St. Louis, MO). D-glucosamine hydrochloride was purchased from Ferro Pfanstiehl Laboratories, Inc. (Cleveland, OH). Copper sulfate was obtained from Fisher Scientific (Hanover Park, IL). No further purifications have been utilized for these reagents; they were used as they are received. Nanopure Barnstead E-pure water purification system (18.1 MΩ) was used to provide deionized water for this study.

Isothermal titration calorimetry (ITC)

A VP-ITC isothermal titration microcalorimeter (Northampton, MA) was used for thermodynamic binding experiments. 10 mM copper sulfate and 0.05 mM or 1 mM range of solutions of small chitin fragments were prepared to be used as the ligand and macromolecules in ITC experiments. All solutions were degassed right before the experimental runs. Copper solutions were added by the ITC syringe (301 uL) and 10 uL titrations were performed into the chitin fragments in the reaction cell (1.4288 mL) at 25 °C and at 180 sec intervals utilizing a stir speed of 310 rpm. Blank ITC experiments were done to correct heat of dilution effects. Origin 7.0 (OriginLab Corp., Northampton, MA) was used to analyze the ITC data to determine the binding constant (K) and enthalpy of binding (ΔH) directly from the binding thermograms.

From this, the entropy of binding (ΔS) and free energy of binding values are calculated.

Results and discussion

Binding results

Titration of divalent copper into chitin fragments yielded the parameters for binding thermodynamics. In order to evaluate our experimental data, we have used the Origin 7.0. 100-iterations technique to fit the data into a one-site binding model provided with the software. A two-site binding model was also investigated, but was found to not fit any of the acquired data. The software calculated from the binding curves and amount of heat generated the binding constants and enthalpies of the reaction. From that information, the entropies and free energies of binding were extracted.

The binding thermogram and isotherm for the smallest chitin fragment, GlcNAc, are shown in Figure 3.1. The binding constant of divalent copper to GlcNAc is determined to be relatively small with a value of only $3.8*10^3$ M^{-1}. This value is still large enough to be reliably measured using ITC, however (Ladbury & Chowdhry, 1996). When there are two GlcNAc residues, binding increases about 1.5 times giving a value of $5.1*10^3$ M^{-1}. The trimeric sugar $(GlcNAc)_3$ afforded a binding constant of $13.3*10^3$ M^{-1} indicating an approximately 2.5 fold increase. Overall, increasing the number of GlcNAc residues showed increasing strength in binding interactions with divalent copper cation. $(GlcNAc)_5$ provided the tightest binding among the chitin fragments investigated with a K_b of $22.1*10^3$ M^{-1}. D-glucosamine, the deacetylated

form of GlcNAc that forms part of the related polymer chitosan, gave a binding constant of $24.8*10^3$ M^{-1}.

Thermodynamic interpretation

All of the experiments gave negative values of enthalpy of reaction; binding of divalent copper to chitin fragments is enthalpically favored. In contrast, all reactions afforded negative values of entropy of reaction. This data suggests that divalent cation-chitin fragment interactions are enthalpically driven at 25 °C. The negative free energy of reaction values show that these binding interactions are all spontaneous reactions. We observed a higher value of enthalpy of reaction for D-glucosamine compared to the other substrates used in this study. Clearly the amine is important for binding to the copper given the approximately six-fold increase in copper binding in the change from the monosaccharide *N*-acetylglucosamine to glucosamine.

Multivalency effect

Oligosaccharides possess multiple potential binding sites that can lead to multivalency effects that strengthen their binding affinities. However, in this study we did not observe a significant increase in binding toward copper(II) with the addition of additional GlcNAc units to the small chitin polymer fragments. From these experimental ITC binding results, we conclude that multivalency chelation effects are not dominant in copper(II)-chitin fragment interactions. The essentially additive binding affinity increases seen as individual GlcNAc residues are added to the growing chain are only indicative of statistical effects. In other words, the effective

local concentration of the metal binding site is increased with the addition of each additional sugar residue, but the addition does not help create a new tighter binding site for the metal cation.

GlcNAc-lectin binding comparison

We would expect a lower binding interaction when a single GlcNAc molecule binds to a plant lectin with hydrogen bonds instead of to a divalent metal cation through an electrostatic interaction. This hypothesis is indeed supported by the data reported by Baines et al. (1992). These workers titrated GlcNAc into the plant lectin wheat germ agglutinin (WGA) using ITC and obtained a binding constant of $4*10^2$ M^{-1}. In comparison to our divalent copper-GlcNAc binding data, this number is 9.5 fold smaller—a difference that supports the idea of the reduction in binding strength with a lectin binding partner for Glc*N*Ac.

Implications

Determination of thermodynamic parameters is crucial to explain the energetics of reactions where biosubstrates are involved. This also sheds light in the studies regarding structure-function relationships. That information could possibly allow us to carry out computational modeling studies to provide reliable predictions for carbohydrate substrates. In addition, knowledge of the affinity of metal cations toward biosubstrate systems could aid designing therapeutics as metals act as catalysts in many biological processes.

Conclusion

Chitin is known to have metal binding ability and therefore can be used as a chelating agent for industrial wastewater (Barrida et al., 2007; Benguella & Benaissa, 2002; Niu & Volesky, 2006; Yang & Zall, 1984). In addition, it is a valuable biopolymer to be used in drug delivery and tissue engineering research (Synowiecki & Al-Khateeb, 2003; Tharanathan & Kittur, 2003). We previously reported the degree of binding of polymeric chitin to divalent copper cation (Camci-Unal & Pohl, 2009) and in this study report binding interactions of much smaller fragments of the polymer with the metal to get some sense of the nature of the metal binding interaction. Enthalpy of reaction, entropy of reaction and free energy of reaction values are all determined to be negative. These interactions are all enthalpically favored, enthalpically driven and spontaneous reactions. The minimum sugar motif for metal binding is found to be only one GlcNAc residue. The number of GlcNAc residues appears to boost the degree binding to divalent copper. However, the addition of GlcNAc molecules to the small chitin substrates resulted in only a moderate amount of increase in the binding strength, an increase that points to statistical but not chelation-based multivalency effects. This data should serve as a good basis for the development of better computational models of metal binding to carbohydrate substrates and thereby better predicative powers for the design of metal-carbohydrate-based materials and binding partners.

Abbreviations

G : Free energy of binding, cal/mol

H : Enthalpy of binding, cal/mol

K : Binding constant, M^{-1}

S : Entropy of binding, cal/mol.K

T: Temperature, K

Acknowledgements

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Appendix

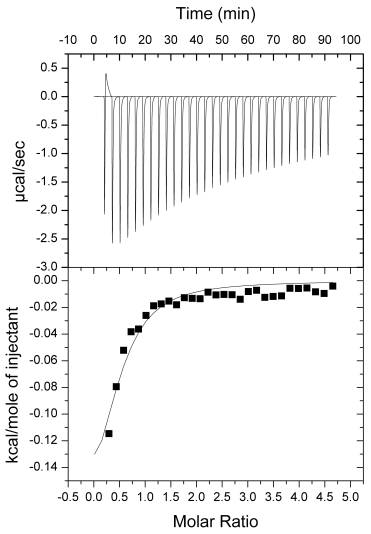


Figure 3.1. Binding thermogram and isotherm for *N*-acetyl-D-glucosamine-copper(II) interaction are given at the top and bottom, respectively.

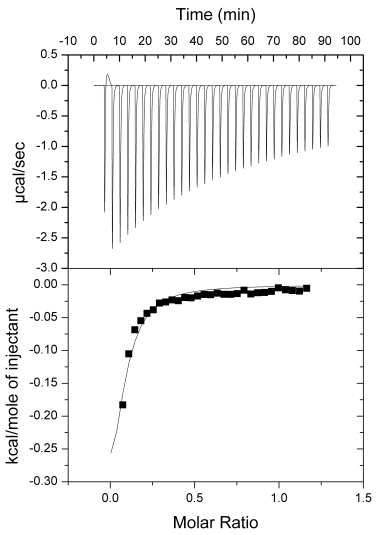


Figure 3.2. Binding thermogram and isotherm for *N,N*'-diacetylchitobiose-copper(II) interaction are given at the top and bottom, respectively.

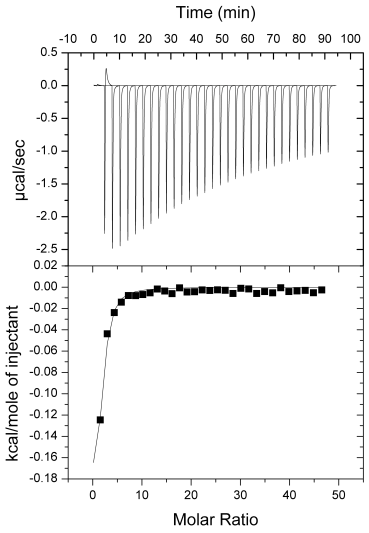


Figure 3.3. Binding thermogram and isotherm for *N,N',N''*-triacetylchitotriose-copper(II) interaction are given at the top and bottom, respectively.

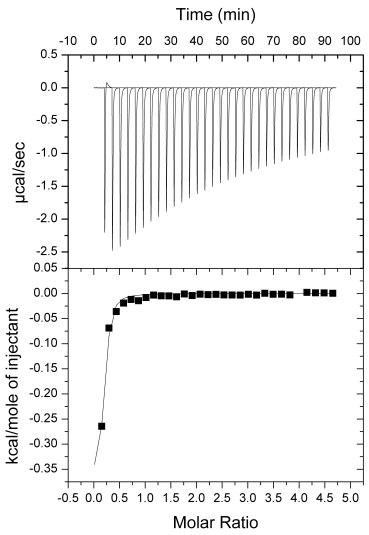


Figure 3.4. Binding thermogram and isotherm for penta-*N*-acetylchitopentaose -copper(II) interaction are given at the top and bottom, respectively.

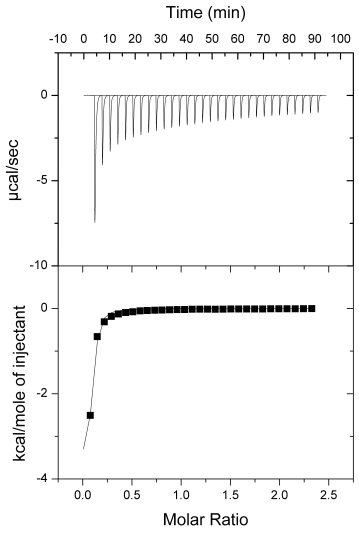


Figure 3.5. Binding thermogram and isotherm for D-glucosamine-copper(II) interaction are given at the top and bottom, respectively.

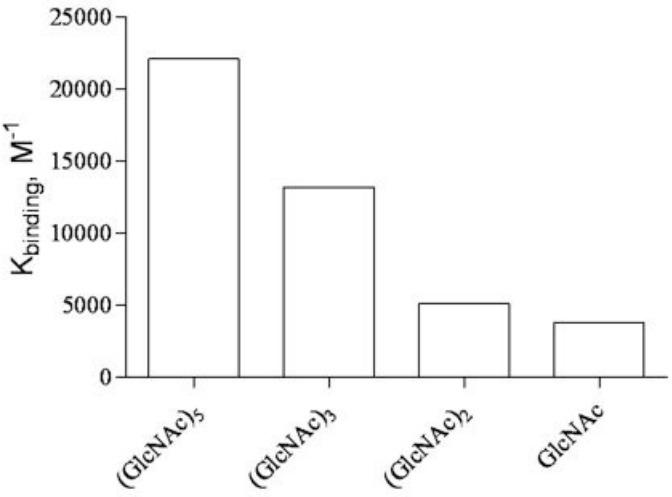
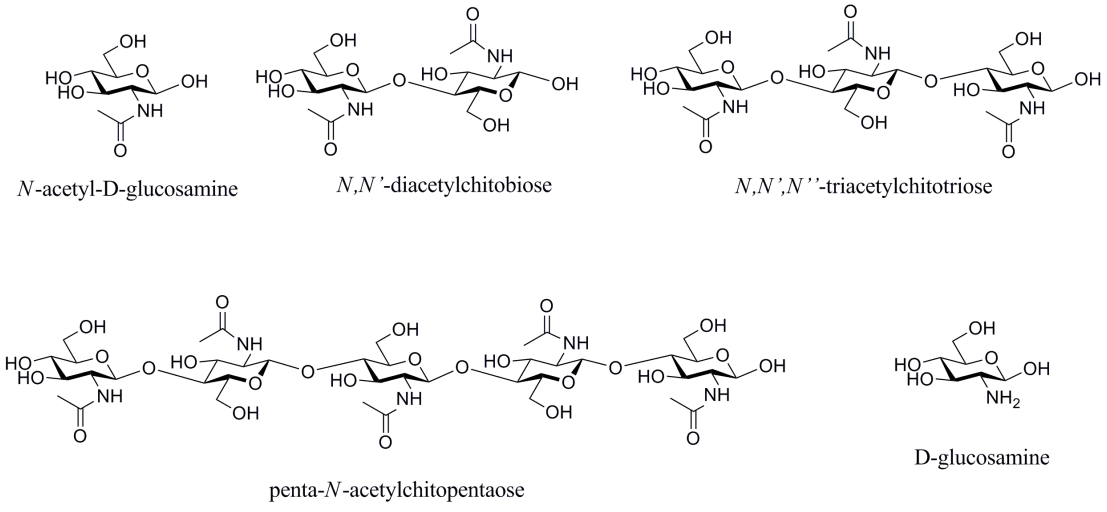


Figure 3.6. Comparison of binding constants for chitin fragments-divalent copper interactions.

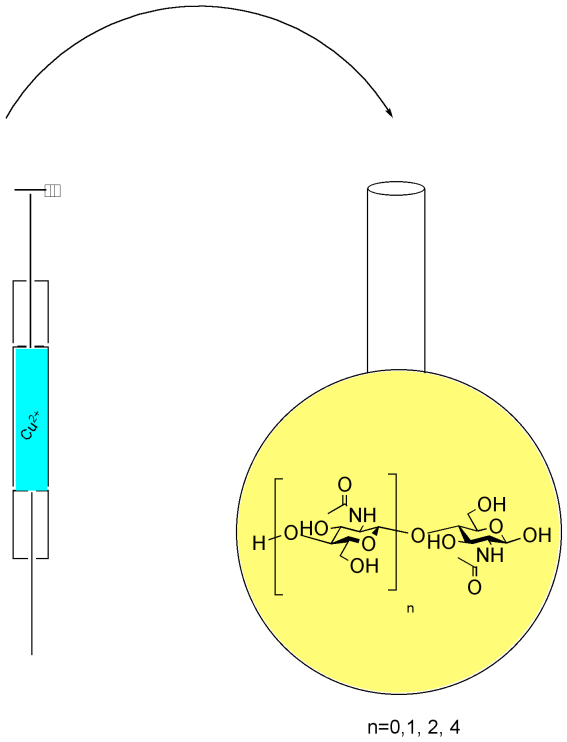
Table 3.1. Complete list of the thermodynamic binding parameters for chitin-metal cation interactions.

Substrate	$K*10^{-3}$, M^{-1}	ΔH, kcal/mol	ΔS, cal/mol*K	ΔG, kcal/mol
N-acetyl-D-glucosamine	3.8±0.4	-6.0±0.2	-3.8	-4.87
N,N'-diacetylchitobiose	5.1±0.6	-8.4±0.2	-11.3	-5.03
N,N',N''-triacetylchitotriose	13.3±1.1	-13.9±0.5	-27.7	-5.64
Penta-*N*-acetylchitopentaose	22.1±0.9	-15.0±0.2	-30.4	-5.94
D-glucosamine	24.8±0.3	-30.2±0.1	-81.2	-5.99

Scheme 3.1. Chitin-based substrates used in the ITC binding experiments.



SYNOPSIS TOC 3.1



CHAPTER 4. Pathway engineering for the synthesis of 2-keto-3-deoxy-D-manno-octulosonic acid (KDO) from D-glucose in vivo

A portion of this chapter has been submitted to *Angewandte Chemie International Edition*[1]

Gulden Camci-Unal[2,3], Mizanur M. Rahman[2,4], Yonghai Chai[2,5] and Nicola L. B. Pohl[2,6]

Abstract

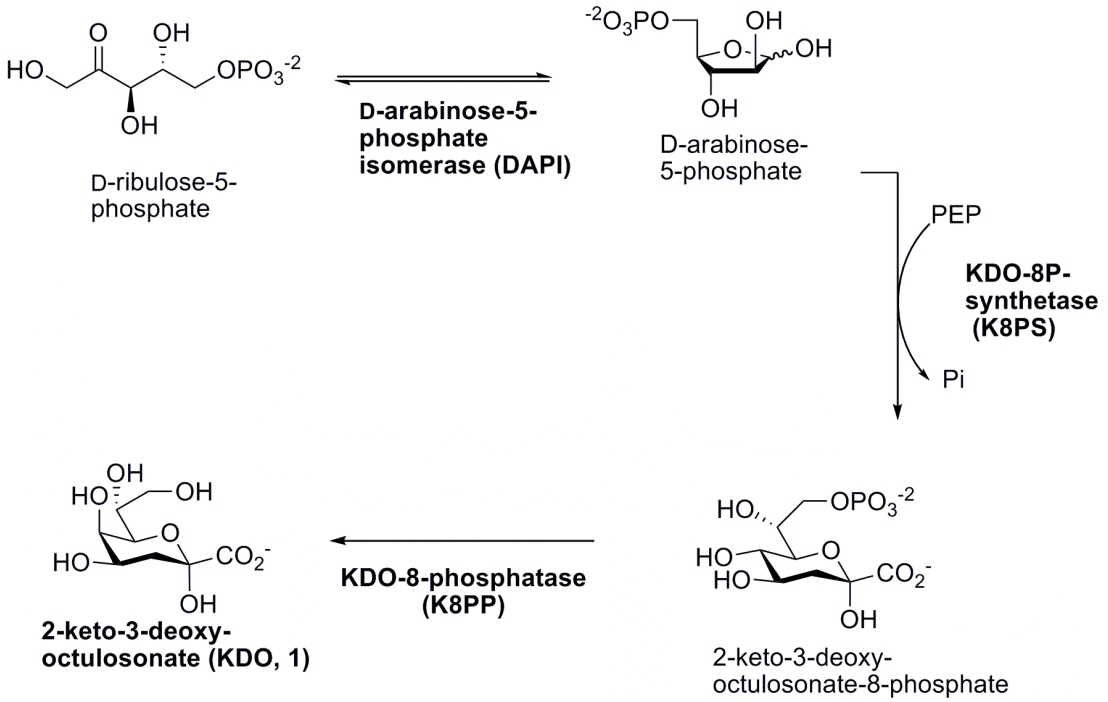
Herein we report the first synthesis of 3-Deoxy-D-manno-octulosonic acid (KDO) starting from glucose through pathway engineering of *E. coli* to provide a relatively low cost method to produce KDO as a starting point to develop KDO-based antibiotics and the chemistry and biology of this ubiquitous sugar.

[1]A paper submitted to *Angewandte Chemie International Edition*, Unpublished work.
[2]Graduate student, Post-doctoral Fellow, Post-doctoral Fellow and Professor, respectively, Department of Chemistry, Iowa State University.
[3]Primary researcher and an author: Carried out the fermentation, isolation and purification experiments, identified the final product and wrote the manuscript
[4]An author: Incorporated KDO genes into *E.coli* and wrote this procedure for the experimental part of the manuscript.
[5]An author: Acetylated and methylated the free fermentation product KDO and wrote this part in the manuscript.
[6]Corresponding author: Conceived of the study, participated in coordination and contributed to drafting the manuscript.

Introduction

KDO (2-keto-3-deoxy-D-manno-octulosonic acid) is an unusual 8-carbon acidic sugar that is essential for the growth of many pathogenic bacteria affecting plants and animals.[1] For example, in Gram-negative bacteria such as *Brucella*—which causes severe infections in humans and lifestock—KDO is a crucial part of the lipid A-based lipopolysaccharides.[1] KDO has also been discovered in plants and green algae, although its biological roles in these organisms are still unclear.[2] Despite its biological ubiquity, synthetic efforts to incorporate KDO into larger structures to explore structure/function relationships have been limited.[3] KDO is commercially available, but is over 2 million times more expensive than glucose per mole. Given this expense, although no enzymatic or biosynthetic approaches have been reported, multiple chemical syntheses of KDO have been developed over several decades.[4] The only route that has proven amenable to producing the 8-carbon sugar in sufficient quantities for further synthetic transformations relies on the Cornforth procedure[5] for aldol condensation of D-arabinose and oxaloacetic acid followed by decarboxylation.[6] Although the 66 % yield of this procedure is moderate, the starting materials are also expensive, thereby limiting full exploration of the chemistry and biology of KDO and development of possible therapeutics based on KDO. Herein we report the first synthesis of KDO starting from glucose through pathway engineering of *Escherichia coli* to provide a relatively low cost method to produce KDO. Our purpose is not to alter the scaffold and make analogs of KDO, we would like to incorporate it into combinatorial oligosaccharide synthesis.

E. coli is a commonly used host for chemical production through fermentation and conveniently already makes KDO for its own membranes.[7] The enzymes for KDO biosynthesis are known (Scheme 4.1, 4.2) as they have already attracted interest as potential targets for antibacterial compounds against Gram-negative bacteria.[8]

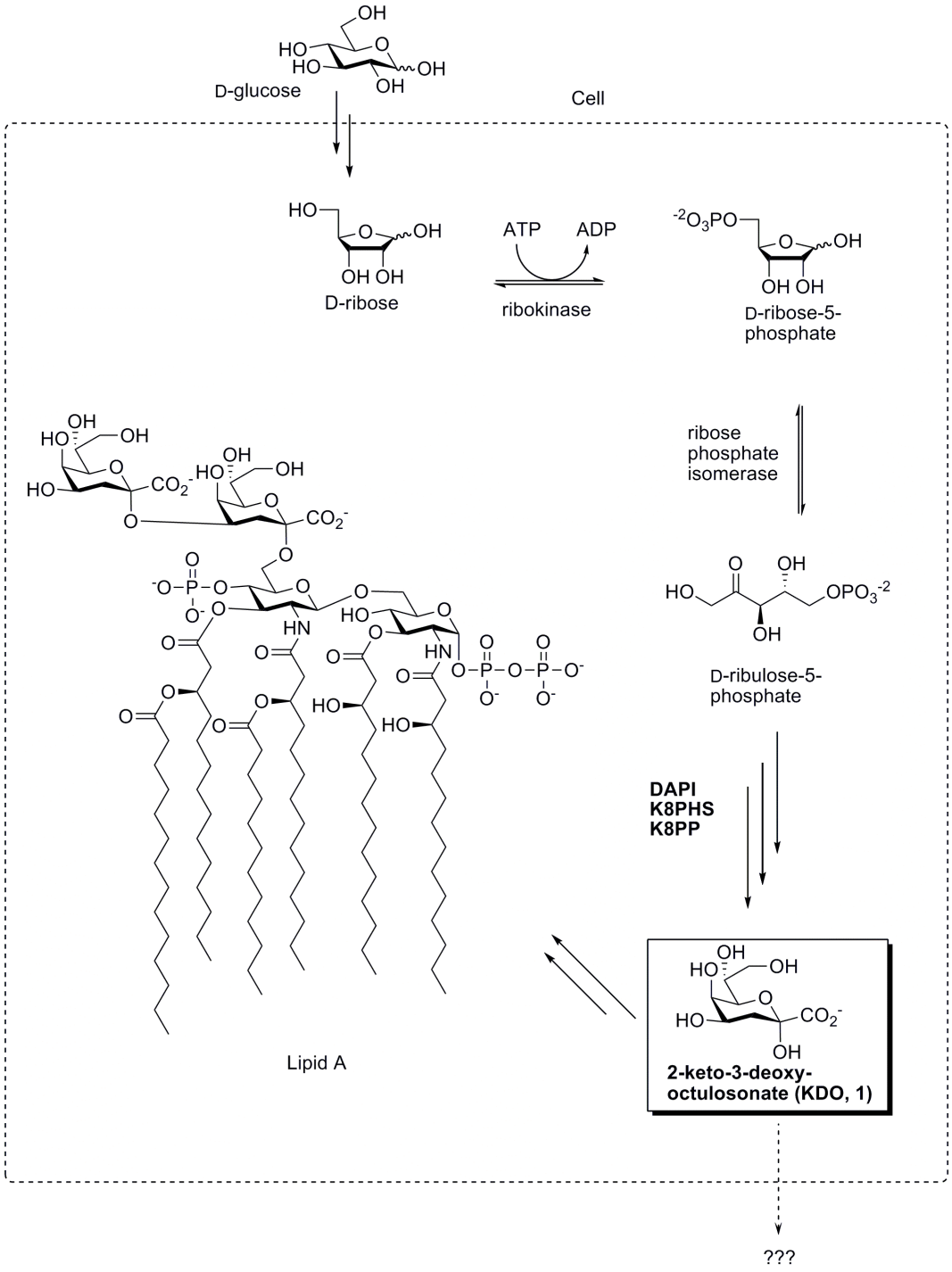


Scheme 4.1. Enzymes that were overexpressed in the current work.

When KDO synthesis stops, lipopolysaccharide synthesis stops and this event eventually leads to cell growth arrest.[9] Because *E. coli* normally makes KDO for direct incorporation into its LPS and not as a free sugar, it was uncertain at the outset if overproduction of this carbohydrate would be toxic to the cultured cells. There is no known KDO exporter protein and therefore the sugar could potentially build up

inside the cell and serve to inhibit the biosynthetic pathways related to KDO. Also, ideally KDO would be secreted into the media for ease of isolation. Fortunately, related work in the production of sialic acid in *E. coli*[10] showed the production of that carbohydrate in the media despite lack of a known export protein. Finally, overexpression of the genes should only produce KDO itself and not related analogs from nonstandard cellular substrates for a cost-effective purification.

To test if overexpression of KDO biosynthetic genes would lead to the production of secreted KDO, a plasmid was designed to encode three *E. coli* genes which are necessary for KDO biosynthesis from ribose: D-arabinose 5-phosphate isomerase (API, yrbH),[11] KDO-8-phosphate synthetase (KPS, kdsA),[12] and KDO-8-phosphatase (KPP, yrbI)[13] (Scheme 4.1). The plasmid containing the three KDO biosynthesis genes for IPTG induced expression of recombinant genes for the fermentative production of KDO is given in Scheme 4.3. The first enzyme interconverts D-ribulose-5-phosphate and D-arabinose-5-phosphate. The resulting D-arabinose-5-phosphate is converted into 2-keto-3-deoxy-octulonate-8-phosphate by the addition of KDO-8-phosphate synthetase and phosphoenolpyruvate (PEP) yielding the product and inorganic phosphate (Pi) Finally, an enzyme is included to cleave the phosphate to render the substrate less charged and therefore more likely to be transported to the extracellular media (Scheme 4.1). The new plasmid was transformed into an *E. coli* strain for IPTG-induction of expression of the three genes. Fortunately, SDS-PAGE analysis confirmed expression of all three expected enzymes (See supporting information).



Scheme 4.2. Pathway for the biosynthesis of KDO.

Given that the proteins were expressed, the production of KDO itself was probed next. Gratifyingly, initial experiments using D-ribose as the carbon source in shake-flask experiments using the newly engineered bacteria clearly showed the presence of KDO in the fermentation media. Apparently, the sugar could be removed from the inside of the bacterial cell. The approximate amount of KDO in the supernatant solution was determined by the known thiobarbituric acid assay.[14] Given this success, we next tried to replace D-ribose with the 100-fold cheaper carbon source D-glucose. Apparently, conversion of the glucose into the required KDO precursors is not yield limiting as glucose worked as efficiently as D-ribose in the production of KDO. The fermentation experiments were repeated for *E. coli* containing the carbenecillin resistance gene but without the KDO-related genes (pUC 19) as a control.

Table 4.1. Production of KDO, mg/L.

	t=1 h	t=48 h	t=96 h
E.coli with KDO gene, IPTG induced	ND	122±10	334±57
E.coli with KDO gene, no IPTG induction	ND	51±16	101±17
E.coli without KDO gene (pUC 19), IPTG induced	ND	ND	ND
E.coli with DAPI gene, IPTG induced	ND	ND	ND
E.coli with K8PHS gene, IPTG induced	ND	ND	ND
E.coli with (KPHS+K8PP) gene, IPTG induced	ND	ND	ND

ND: not detectable.

The results indicated no evidence of KDO production in the fermentation media without the presence of the plasmid containing the three KDO biosynthetic genes (Table 4.1).

In addition, we investigated the effect of IPTG induction on the production of KDO given the possibility of leaky protein expression from the T7 promoter. Although the bacteria still produces KDO to an extent without IPTG induction, yields are about 3-fold less than that of IPTG-induced cells. With IPTG induction, an optimized procedure yielded 334 mg of KDO per liter of culture—yields that are comparable to those found for the recent production of sialic acid in *E. coli.*[10] Moreover, we have done fermentation experiments using the *E.coli* which contain DAPI, K8PHS and (K8PHS+K8PP) genes separately. KDO production is found to be non-detectable in all of the cases implying we need all these three KDO related genes in the *E.coli* to be able to overproduce KDO.

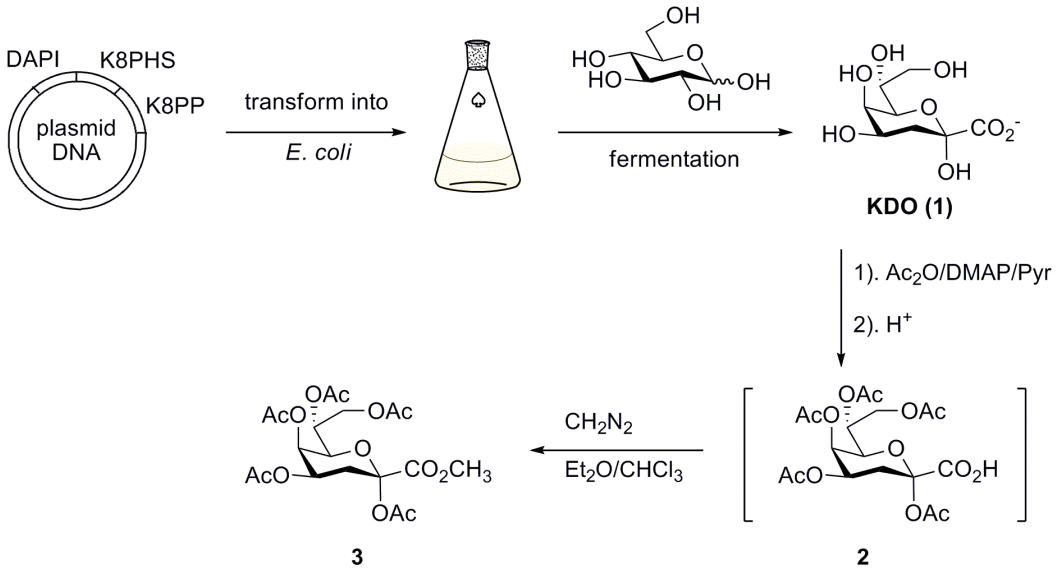
The cost of fermentation experiments is still lower than the oxaloacetic acid procedure reported by Ogura et al.[6] as our minimal fermentation medium includes just basic salts for bacterial growth and our carbon source—glucose—is also very inexpensive. These findings are in line with other results in that the most cost-effective method to synthesize such molecules is fermentation as abundant, inexpensive, and renewable starting materials can be used.[15] Furthermore, the oxaloacetic acid method uses $NiCl_2$ which is a toxic heavy metal and known to be

harmful to both humans and the environment.[16] The fermentation method for KDO production does not involve any toxic heavy metals in any stage of the production.

To confirm the identity and yield of the products and to demonstrate that the fermentation product could be readily transformed into the known protected form of KDO **3** for further synthetic manipulations, the product was freeze-dried and subjected to anion exchange chromatography. The resulting product co-migrated on a thin layer silica gel chromatography plate with an authentic KDO sample and showed a proton NMR spectrum consistent with the known product. High-resolution quadruple time-of-flight (QTOF) electrospray ionization mass spectrometry of the final KDO sample gave the expected $[H-1]^-$ peak in the (-) ion mode. Subjection of the crude fermentation mixture to acetylation followed by methyl esterification conditions (Scheme 4.3) resulted in the isolation of the known peracetylated KDO methyl ester **3** despite the presence of salts in the original starting material to confirm the yield of KDO in the fermentation. Proton and carbon NMR as well as HRMS and optical rotation of this product indeed confirmed the identity of the fermentation product. Fortunately, no evidence was found for the production of any KDO-like analogs that would have significantly complicated the purification efforts.

In summary, production of KDO in fermentation media could be attained with *E. coli* cells engineered to overproduce the three enzymes involved in the biosynthetic production of this 8-carbon sugar. The 334 mg KDO yields per liter of culture using glucose as a carbon source should substantially reduce the cost of obtaining KDO and

therefore finally spur the development of the chemistry and biology of this ubiquitous sugar.



Scheme 4.3. Production and protection of KDO.

In addition, work can now also commence on modification of KDO for the design of inhibitors of the KDO biosynthetic pathway as a new antibiotic strategy against multidrug resistant Gram-negative bacteria.[17] Future work will include incorporation of KDO in combinatorial oligosaccharide synthesis. In addition, the lectin binding ability of this unique 8-C sugar can now be systematically investigated.

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Appendix

Materials and equipment

Enzymes and reagents used for the molecular biology procedures, DNA ladders and deoxynucleotide triphosphates (dNTPs) were purchased from Promega (Madison, WI) or New England Biolabs (Beverly, MA). Oligonucleotides for DNA amplification were synthesized by Integrated DNA Technologies (Coralville, IA). Protein molecular weight standards were obtained from BioRad (Hercules, CA). The QIAQuick gel extraction kit was obtained from Qiagen (Valencia, CA), and the Zero blunt PCR cloning kit was purchased from Invitrogen (Carlsbad, CA).

Periodic acid (H_5IO_6), thiobarbituric acid (TBA), sodium meta arsenite ($NaAsO_2$) and the ammonium salt of 2-keto-3-deoxyoctonate (KDO) were purchased from Sigma (St. Louis, MO). Sulfuric acid (H_2SO_4), hydrochloric acid (HCl), potassium phosphate dibasic (K_2HPO_4), potassium dihydrogen phosphate (KH_2PO_4), ammonium sulfate ($(NH_4)_2SO_4$), magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$), dimethyl sulfoxide (C_2H_6OS), sodium hydroxide (NaOH) and D-Glucose were obtained from Fisher (Pittsburgh, PA). Isopropyl-beta-D-thiogalactopyranoside (IPTG) and carbenecillin were purchased from Lab Scientific (Livingston, NJ). Bio-Gel® P-2 Gel (45-90 um) and AG®1-X8 resin (100-200 mesh) formate form were purchased from Bio-Rad Laboratories, Inc (Hercules, CA). Ammonium formate (NH_4COOH) was obtained from Fluka (St. Louis, MO). All other chemicals were received from Aldrich (Milwaukee, WI). All chemicals were used without further purification.

A Buchi rotary evaporator (West Chester, PA) was used to remove water from the fermentation medium. Thermo IEC Centra CL3R and Thermo IEC Micromax RF (Needham Heights, MA) were used for centrifugation purposes. VWR Orbital Incubator Shaker Model 1575R (West Chester, PA) was used to grow cells. A Fisher (Pittsburgh, PA) brand heat block was used to boil samples for the TBA assay. A Spectronic 20 Genesys UV Spectrophotometer (Waltham, MA) was used to take optical density measurements at 548 nm and 600 nm. Deionized water (18.0 MHz) was obtained from a Barnstead/Thermolyne Water Purification system (Dubuque, IA).

General methods

Analytical thin layer chromatography (TLC) was performed using Sorbent Technologies 0.25 mm glass-backed silca gel HL TLC plates with UV254. Visualization was accomplished with UV light and staining with a 10% sulfuric acid in ethanol solution dip followed by heating. Flash chromatography was performed using 230–400 mesh silica gel (Type 60A) purchased from Sorbent Technologies. NMR spectroscopy (^{1}H, ^{13}C) was conducted using a Bruker DRX400 spectrometer at 400 MHz and 100 MHz respectively. Chemical shifts are reported in ppm relative to CDCl3 (^{1}H = 7.27 ppm, ^{13}C = 77.23 ppm) or to D_2O (^{1}H = 4.63 ppm) as an internal reference and coupling constants are reported in Hz. Mass spectrometry was performed by the W. M. Keck Metabolomics Research Laboratory at Iowa State University using an Applied Biosytems QSTAR® XL Hybrid LC/MS/MS System.

Melting points were recorded on a MEL-TEMP® capillary melting point apparatus without correction. The optical rotation mesurement was taken on a JASCO Digital polarimeter DIP-370 at 598 nm at 20 °C, L= 0.5 dm.

Standard procedures to manipulate DNA, including plasmid DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, DNA ligation and transformation of *E. coli*, were performed by conventional methods (Sambrook et al. 1989). The PCR was carried out in an Eppendorf Mastercycler gradient thermocycler (Eppendorf Scientific Inc. Westbury, NY). Protein was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, Tris-HCl 10-20% gradients, Bio-Rad Laboratories, Hercules, CA). The gels were stained with Coomassie brilliant blue. Protein concentrations were determined with the Bio-Rad protein assay kit according to the method of Bradford (Bradford 1976) using bovine serum albumin as the standard.

Bacterial strains and growth conditions

Escherichia coli K12 was used to isolate chromosomal DNA. *E. coli* BL21 (DE3) was used to create a D-ribose constitutive mutant (EDR001) which was then used for expression of recombinant genes after introducing T7 RNA polymerase to create the strain EDR002. Oneshot Top10 competent cells (Invitrogen, Carlsbad, CA), *Escherichia coli* XL-10Blue (Stratagene, La Jolla, CA) and PCR Blunt vectors (Invitrogen) were used for direct cloning of PCR products. *Escherichia coli* cells

were grown on Luria Bertani (Sigma) medium at 37 °C in an incubator shaker at 225 rpm. When required, the antibiotics carbenicillin or kanamycin was added at 50 µg ml^{-1} to make the selective media.

PCR amplification and construction of expression vector

The primers were designed to construct the D-arabinose 5-phosphate isomerase (*yrb*H), KDO 8-phosphate synthase (*kds*A) and KDO 8-phosphate phosphatase (*yrb*I) expression plasmids. The lists of primers and oligonucleotides used in this study are given in Table S1. The chromosomal DNA of *E.coli* K12 was amplified in three separate reactions using the forward and reverse primers designed for *yrbH*, *kdsA* and *yrbI* genes. The amplification reaction mixtures contained standard *Pfu* DNA polymerase buffer, 375 µM of dNTPs, 3 ng of each primer, 4 ng of total genomic DNA and 2.5 units of *Pfu* DNA polymerase. The cycling parameters were as follows 94 °C for 2 min 40 s followed by 30 cycles of 94 °C for 30 s, 58 °C for 45 s and 72 °C for 2 min 15 s, with a final elongation step of 72 °C for 15 min. The amplified DNA from each reaction was checked by agarose gel electrophoresis and purified using gel extraction kit before cloned into zero-blunt vector and was transformed into OneShot Top10 and *E. coli* XL10 competent cells to check the correct insert. The resulting constructs were digested with appropriate restriction enzymes for cloning into an *Avr*II introduced modified pET21a vector. The *Avr*II restriction site was introduced into the pET21a vector right after the *EcoR*I site using the synthetic oligonucleotides (*Avr*IIF and *Avr*IIR) listed in Table 1. The two complementary

oligonucleotides were mixed in T-4 ligase buffer in equal molar concentration and heated at 95 °C for 2 min before incubating at room temperature for 40 min for annealing. The annealed dinucleotide was then ligated into an *EcoR*I-*Xho*I digested pET21a vector which was named pMNP26. Each gene was individually cloned into pMNP26 to generate plasmids pMNP21 (pMNP26-*yrb*H), pMNP22 (pMNP26-*kds*A) and pMNP23 (pMNP26-*yrb*I) and were subsequently subcloned under the T7-promoter of the vector pMNP26 to generate the plasmids pMNP24 (pMNP26-*kds*A*yrb*I) and pMNP25 (pMNP26-*kds*A*yrb*I*yrb*H) using standard molecular cloning techniques.

Construction of metabolically engineered *E. coli* strain

A D-ribose constitutive mutant of E. coli BL21 (DE3) was created by repeated growing of cells in MOPS minimal medium (Teknova, Hollister, CA) supplemented with 0.2% D-ribose as the sole carbon source and was named as *E. coli* BL21 (DE3) DR001 (EDR001). A control was run in parallel using 0.2% D-glucose as the sole carbon source. The D-ribose mutant was then modified further to allow for gene expression from plasmids containing T7 promoter. In order to achieve a T7 promoter compatible mutant a T7 RNA polymerase gene under a lac-promoter was inserted into the chromosome of EDR001 using the lDE3 lysogenization kit (Novagen, San Diego, California) according to the manufacturer's instruction to generate the strain *E. coli* BL21 (DE3) DR002 (EDR002). The chemically competent EDR002 was then transformed with the plasmid pMNP 25 for IPTG induced expression of recombinant

genes for the fermentative production of KDO. Transformants were selected at 37 °C and grown on LB medium supplemented with carbenicillin.

Table 4.2. Oligonucleotides used in this study.

Oligonucleotide	Description
yrbH-fwd	5’-aaacatatgtcgcacgtagagttacaaccggg -3’
yrbH-rev	5’-aaagaattcttacactacgcctgcacgcagtaaatcatgcatgtgtaacacacc-
kdsA-fwd	3’
kdsA-rev	5’- aaacatatgaaacaaaaagtggttagcattggc g -3’
yrbI-fwd	5’- aaagaattcttacttgctggtatccagttcttcgaaacc -3’
yrbI-rev	5’ -aaacatatgagcaaagcaggtgcgtcgcttgcgacc -3’
AvrII-fwd	5 -aaagaattctcatatcgattgccctttggcttcatccagtttgc -3’
AvrII-rev	5’-aattcctaggacagc-3’
	5’-tcgagctgtcctagg-3’

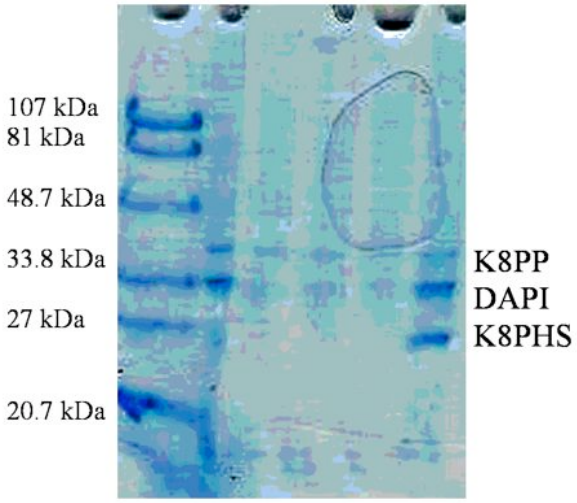


Figure 4.1. SDS-PAGE for expression of 3 KDO genes.

Cell growth and fermentation

A number of different conditions have been experimented for the shake flask experiments. For example, experiments have been carried out using different carbon sources (D-glucose or D-ribose), shaking speeds (170 rpm or 240 rpm), temperatures (30 °C or 37 °C), minimal fermentation mediums (MOPS or phosphate buffer). The procedure for the most effective fermentation conditions is given below:

E. coli cells harboring the recombinant KDO biosynthetic genes were grown overnight at 37 °C, 240 rpm in LB Broth. Cells were then harvested by centrifuging the overnight culture at 10,000 rpm for 10 min. The minimum fermentation medium contained 49.5 mL autoclaved mixture of potassium hydrogen phosphate (0.331 g),

potassium dihydrogen phosphate (0.15 g), magnesium sulfate (0.0085 g) and ammonium sulfate (0.2 g), 50 µL of 50 mg/mL carbenecillin, 0.5 mL 15% (w/v) sterile D-ribose or D-glucose solution and cells from a 15 mL overnight culture. Total volume of 50 mL fermentation was incubated at 37 °C, 240 rpm. 20µL of 1 M IPTG was added once OD_{600} reached 0.6-0.8. The fermentation experiment was stopped after 96 h. 0.5 mL samples were taken and 0.5 mL of 15% D-ribose or D-glucose was added to the flask each day. Cells were then centrifuged at 3600 rpm for 40 min and discarded; the supernatant contained the KDO. The KDO in the final solution was determined by the thiobarbituric acid method (Karkhanis 1978). TLC of the fermentation product showed consistent results as that of the commercial KDO sample. TLC of the final medium was run in *n*-butanol/acetic acid/ water (2/1/1) and stained with 10% sulfuric acid in ethanol. Finally, the product was freeze-dried to obtain the solid KDO product. ^{1}H NMR of the solid residue in D_2O is consistent with an authentic sample from Sigma.

TBA Assay

The calibration curve was obtained for known concentrations of KDO using the previously reported thiobarbituric acid assay (Karkhanis 1978). Concentrations of D-ribose and D-glucose measured using the TBA assay are shown on the curve for comparison. The OD value from the assay is converted into an approximate amount of KDO present in the media using the calibration curve below.

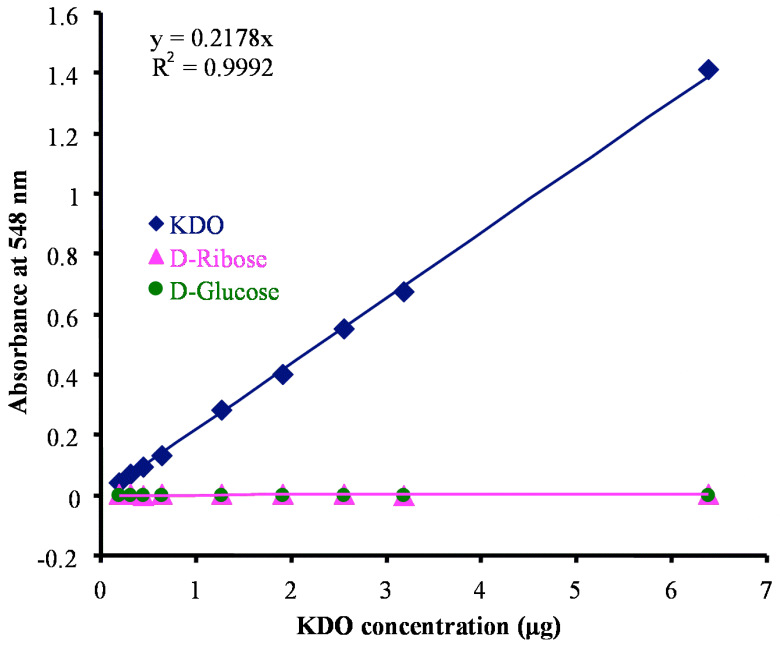
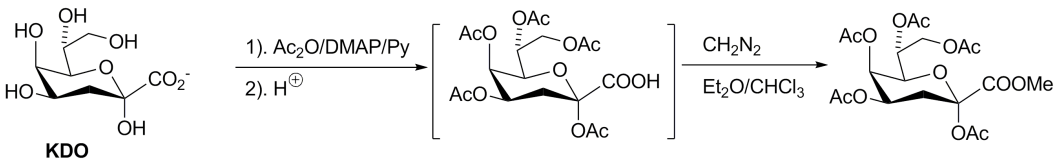


Figure 4.2. KDO Calibration curve.

Purification of KDO

The lyophilized solid mixture containing KDO is partially purified using the formate form of strong anion exchange resin Bio-Rad AG®1-X8. KDO is eluted from the column by 1 M ammonium formate. The fractions containing KDO are combined and lyophilized from dI H_2O to afford a solid residue. This residue is then passed through a desalting column, Bio-Gel® P-2. The fractions containing KDO are lyophilized. ^1H NMR in D_2O matches that of an authentic sample. QTOF electrospray ionization mass spectrometry of the final sample gave the expected $[H-1]^-$ peak in the (-) ion mode.

HRMS (+ TOF) calcd for (M+-H) $C_8H_{13}O_8^-$, 237.18; found 237.07



Scheme 4.4. Preparation of the acetylated KDO methyl ester **3.**

Methyl 4,5,7,8-tetra-O-acetyl-3-deoxyl-alpha-D-manno-2-octulopyranosonate (**3**) (Unger 1980, Charon 1979, Smith 1990)

To a suspension of the crude KDO (400 mg, after desalting column described above) in pyridine/acetic anhydride (v/v = 1:1, 5 mL) was added 4-dimethylamino pyridine (DMAP, 20 mg, 0.16 mmol). The reaction mixture was allowed to stir at room temperature under nitrogen atmosphere. After 8 h, pyridine/acetic anhydride was removed under reduced pressure to afford a brown oily residue (2.2 g). $CHCl_3$ (10 mL) and H_2O (5 mL) were added to the residue. The mixture was transferred into a separatory funnel and washed with 1N cold sulfuric acid aqueous solution (10 mL). The organic layer was separated and the aqueous layer was extracted with $CHCl_3$ (2 x 10 mL). The combined organic layers were dried over anhydrous $MgSO_4$. The solid was removed by filtration and the solvent was removed under reduced pressure to provide a yellow syrup (50 mg). The yellow syrup was dried under high vacuum for 20 h and then directly used for the following methyl esterification. To a solution of the syrup in $CHCl_3$ (10 mL) was slowly added a solution of diazomethane (Anzalone 1985) in Et_2O at room temperature until yellow color persisted and no gas was

produced. The solvent was removed under reduced pressure and the residue was purified by flash column chromatography (Et_2O/cyclohexane, 1:1, v/v) to provide a white solid (26 mg).

m.p. 156-158 °C

^{1}H NMR (400 MHz, $CDCl_3$): d 5.38 (s, 1H), 5.33-5.30 (m, 1H), 5.24-5.19 (m, 1H), 4.48 (dd, 1H, J = 2, 12 Hz), 4.17 (d, 1H, J = 9.6 Hz), 4.11 (dd, 1H, J = 4, 12 Hz), 3.80 (s, 3H), 2.31-2.17 (m, 2H), 2.14 (s, 3H), 2.11 (s, 3H), 2.04 (s, 3H), 1.99 (s, 6H).

^{13}C NMR (100 MHz, $CDCl_3$): d 170.6, 170.5, 170.2, 169.7, 168.1, 166.9, 97.6, 69.8, 67.4, 66.1, 64.1, 62.3, 53.4, 31.1, 20.9, 20.8

HRMS (+ TOF) calcd for (M^++Na) $C_{19}H_{26}NaO_{13}$, 485.1271; found 485.1033

Optical rotation: $[a]_D^{20}$ = + 81.9 ^{o} (*c* 0.035, chloroform)

Previously reported (Unger 1980): $[a]_D^{20}$ = + 87.1 ^{o} (*c* 0.81, chloroform)

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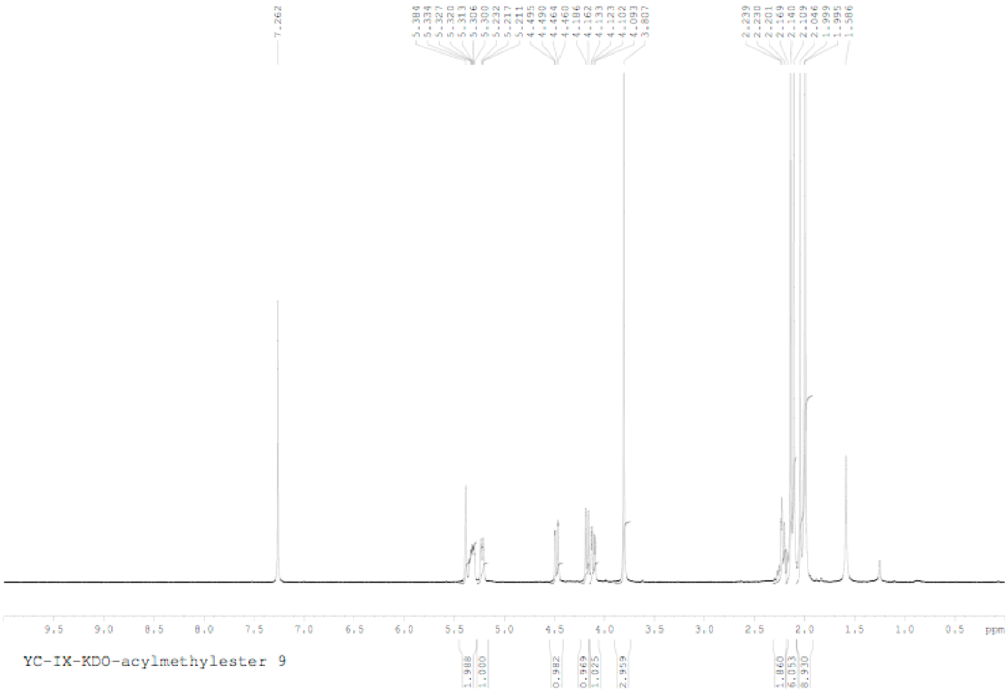

Figure 4.3. ^{1}H NMR spectra of KDO methyl ester **3.**

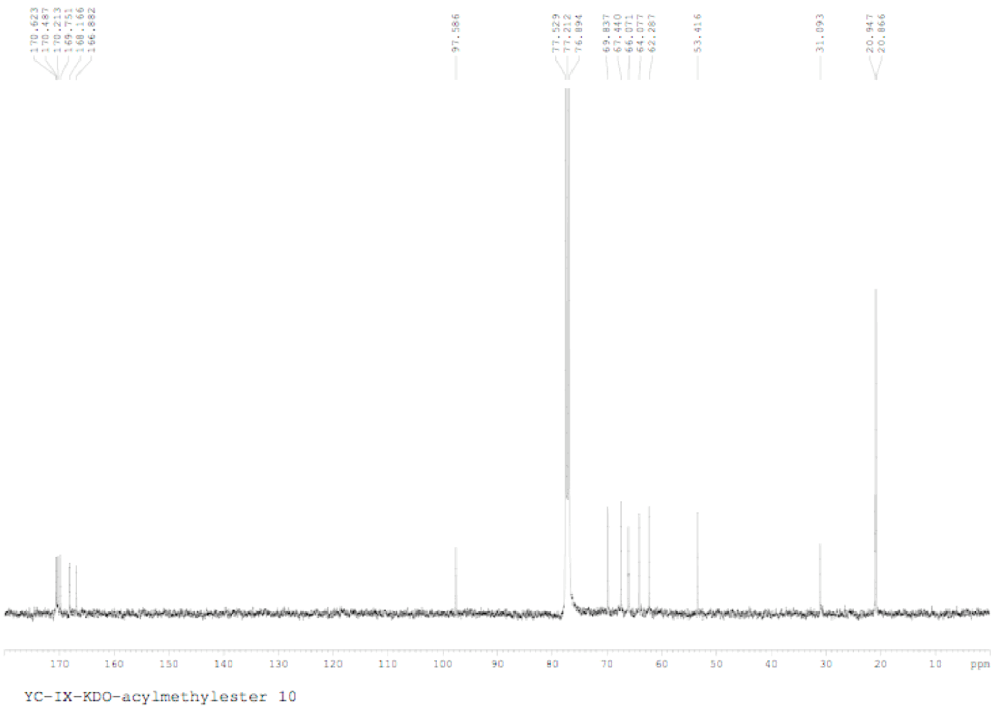

Figure 4.4. ^{13}C NMR spectra of KDO methyl ester **3.**

CHAPTER 5. Conclusion and future directions

Work done

We have performed binding studies between carbohydrate substrates and metal cations to determine kinetic and thermodynamic parameters.

We have determined the binding constants and thermodynamic binding parameters for the interactions between heavy metal cations and carbohydrate polymer chitin. From our ITC experiments we found out that formal charge state of the heavy metal inversely affects the binding strength. Divalent metal cation-chitin interactions all turned out to be enthalpically driven and spontaneous reactions. We have obtained all the enthalpy, entropy and Gibbs free energy of reaction values as negative. Our data is useful in process optimizations for wastewater treatment to carry out the chelation in the least expensive fashion as we have provided metal selectivity data. In addition, our binding data can be used to develop computational models and force fields for carbohydrate-metal cation systems.

We have also measured the degree of binding of small chitin fragments to divalent copper cation by ITC. The minimum sugar-binding motif for Cu^{2+}-chitin fragment binding is found to be one GlcNAc residue. We have found out that increasing numbers of GlcNAc residues enhances the binding strength against divalent copper. Addition of more GlcNAc residues to the chitin polymer chain though slightly increased the binding constant. Therefore, we have reported that multivalency effects are not dominant in copper(II)-small chitin fragment interactions. These interactions

were all determined to be enthalpically favored, enthalpically driven and spontaneous reactions. The knowledge of binding strength of metal cations against carbohydrates might be used to design therapeutics.

We have carried out the first one-step biosynthesis of KDO with yield of 334 mg per liter of fermentation medium. We have utilized metabolically engineered *E. coli* containing the genes, which are essential for KDO biosynthesis. Our experiments showed that overexpression of KDO genes did not shut down the biosynthetic pathway in the cell. *E.coli* produced the KDO and released it to the fermentation medium from where it was then isolated and further purified. We also have optimized the production protocol trying different experimental conditions. Production cost of this method turned out to be considerably cheaper than the commercially available KDO from Sigma. The reason for this is, we were able to use a very cheap carbon source, D-glucose, as the starting material for our fermentation experiments.

Future directions

Although we have characterized binding interactions between chitin substrates and some of the heavy metal contaminants, there is still a need to extend it to other possible metal cations. Computational models require many experimental data points, for this reason, we could perform more ITC studies to model other wastewater contaminants as well. Reliable experimental data will be useful in the development of force fields for carbohydrates in computational studies. In a future study, it could also be intriguing to investigate the effect of counter ion for heavy metal binding to chitin.

The results from the chitin fragment-divalent copper study could be used in structure-function relationships studies in future. It is also possible to perform computational studies to obtain reliable energetic predictions for carbohydrates in similar systems.

As we have developed a cost-effective optimum procedure to produce KDO, now we can incorporate this unusual sugar into larger structure to mimic the sugars related to gram-negative bacteria related infections, for instance, brucella. It would be possible to develop therapeutic agents following this strategy. Besides, KDO can be incorporated into automated oligosaccharide synthesis as we produce it in reasonable quantities for further studies. This efficient KDO production protocol could also be extended to develop procedures to obtain other unusual sugar substrates in future. We could also use this information to design inhibitors for KDO metabolism to prevent Gram-negative bacteria related infections. One other research area that we can utilize KDO would be in plant lectin recognition studies.

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