UNIVERSAL BLOOD: THE LIFE STREAM

ALL FOUR ONE AND ONE FOR ALL

AN ENZYMATIC METHODOLOGY CREATING A UNIVERSAL BLOOD SUPPLY

A Dissertation

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the Faculty of the Graduate School

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In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

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Dr. Thomas Reilly, Dissertation Supervisor

May 2012

The undersigned, appointed by the dean of the Graduate School, have examined the dissertation entitled

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presented by Thomas J. Malinski,

a candidate for the degree of Doctor of Philosophy,

and hereby certify that, in their opinion, it is worthy of acceptance.

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The journey we undertake during our life is unknown until we reach each milestone. Sometimes we have those déjà vu feelings when some of these moments occur. Until we cross that bridge or go through that door we don't know exactly what experiences we will meet. The thought of earning my doctorate has been with me for some time. The journey just took a while.

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ABBREVIATIONS

α-NAGA	α-N-acetylgalactosaminidase
α-GAL	α-galactosidase
RBCs	red blood cells
E. coli	*Escherichia coli*
S. linguale	*Spirosoma linguale*
C. perfringens	*Clostridium perfringens*
E. meningosepticum	*Elizabethkingia meningosepticum*
FDA	Food and Drug Administration
HIV	Human Immunodefficiency Virus
AIDS	Acquired Immunodefficiency Syndrome
β-Gal	β-galactosidase
GlcNAc	N-acetylglucosamine
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
CAZy	Carbohydrate-Active enZYmes
NAD^+	Nicotinamide adenine dinucleotide
Ni	Nickel
MW	Molecular Weight
mM	milli-Molar
GalNAc	N-acetylgalactosamine
kDa	kilo-Dalton
M	Molar
K_M	Michaelis constant
PBS	Phosphate Buffered Saline
μg	micro-grams

µL	micro-liter
C	Celsius
ACES	N-(2-Acetamido)-2-aminoethanesulfonic acid
mg/mL	milli-gram/milli-liter
BSA	Bovine Serum Albumin
DTT	Dithiothreitol
US	United States
UK	United Kingdom
g/dL	gram/deci-liter
DPG	Di-phosphoglycerate
COP	Colloid Osmotic Pressure
HBOC	Hemoglobin-Based Oxygen Carrier
PFC	Perfluorocarbon
PEG	Polyethylene glycol
CN	cyanuric chloride
SP	N-hydroxysuccinimidyl propionic acid
PCR	Polymerase Chain Reaction
BCA	Bicinchoninic acid
LB	Luria Broth
DNA	Deoxyribonucleic acid
RPM	Revolutions per Minute
g	Gravity
Tris-HCl	Tris(hydroxymethyl)aminomethane hydrochloride
MALDI-TOF	Matrix-assisted laser desorption/ionization Time of Flight
TEV	Tobacco Etch Virus
IPTG	Isopropyl-β-D-thio-galactoside

BCIP	5-Bromo-4-chloro-3-indolyl phosphate
UV-VIS	Ultraviolet Visble
EDTA	Ethylenediaminetetraacetic acid
Ve	Elution volume
Vo	Void volume
MS	Mass Spectrometry
ACN	Acetonitrile
TFA	Trifluoroacetic acid
V	Volts
Hz	Hertz
Nm	nano-meter
PCV	Packed cell volume
MCV	Mean corpuscular volume
MCHC	Mean corpuscular hemoglobin concentration
CHCM	Cell hemoglobin concentration mean
CH	Cellular hemoglobin content
RDW	Red cell Distribution Width
HDW	Hemoglobin Distribution Width
Min	Minimum
Max	Maximum
Std Dev	Standard Deviation
%CV	% Coefficient of Variation
ATP	Adenosine triphosphate
FITC	Fluorescein isothiocyanate
PE	Phycoerythrin
ECO	Enzyme converted

k_{cat}	Turnover rate
BLAST	Basic Local Alignment Search Tool
Cu	Copper
Zn	Zinc
	N-Acetylgalactosamine
	Galactose
	N-Acetylglucosamine
	Fucose
	N-Acetylneuraminic acid
	Mannose
	Glucose

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ABSTRACT

α-NAGA (α-N-acetylgalactosaminidase) is an exoglycosidase that cleaves a specific carbohydrate, terminal linked 1-3 α-N-acetylgalactosamine, from the A antigen. The enzymatic hydrolysis creates the H antigen. This modification transforms the immune response to the blood group from Type A to O. The product of the enzyme treatment produces Type O blood a universally transfusable product for potential medical use.

Purified recombinant α-NAGAs from *E. coli* and *S. linguale* were characterized *in vitro* for molecular mass, substrate specificity, pH and temperature optima, and product inhibition. A mutant of the *S. linguale* α-NAGA, H225A, was evaluated for activity *in vitro* and *in vivo*. *S. linguale* enzyme was characterized *in vivo* using Type A_1 and A_2 RBCs for pH, temperature, and buffer optima. *In vitro* each enzyme appeared to function as a dimer under the conditions tested, was highly active at a neutral pH, and over a range of temperatures. Turnover rate, k_{cat}, 33 s^{-1} (*E. coli*) and 173 s^{-1} (*S. linguale*) were derived from the kinetic assays. Product competitive inhibition, K_i 0.18 mM, was evident with the *S. linguale* α-NAGA while the *E. coli* α-NAGA demonstrated uncompetitive inhibition, K_i 2.6 mM. The *E. coli* α-NAGA did not appear to covert Type A RBCs. *S. linguale* α-NAGA appeared to convert Type A RBCs to blood group O with efficiency in glycine or

alanine buffers at neutral pH at 4°C or 25°C. Pretreating RBCs with glycine solution 24 hours before enzyme conversion appeared to facilitate higher enzyme efficiency. The *S. linguale* α-NAGA H225A mutant was inactive both *in vitro* and *in vivo*.

The *S. linguale* α-NAGA appeared to possess desirable attributes to enzymatically convert RBCs. The use of Type A converted RBCs by *S. linguale* α-NAGA could potentially increase the universal blood supply by 40%.

Chapter 1: Introduction

For more than 30 years the efficient capability to enzymatically convert Red Blood Cell (RBC) blood groups type A or B to type O has eluded researchers. Such a discovery would minimize or eliminate blood supply shortages. Several research groups have identified a number of enzymes that have the potential to convert RBC blood type. The enzymes targeted to amend the RBC to Type O are α-N-acetyl-galactosamindase (A→O) and α-galactosidase (B→O). Presently we possess a novel recombinant α-N-acetyl-galactosamindase from *Spirosoma linguale* that converts Type A RBCs to Type O. Currently there are two blood conversion enzyme candidates (*Elizabethkingia meningosepticum* α-NAGA and *Streptomyces griseoplanus* α-GAL) that are in development by Zymequest (Velico Medical). Dependent on the FDA review and approval, ongoing research into this field continues.

In the meantime blood shortages continue to be a common event. Instances of blood shortages around the world appear frequently. A simple query of the World Wide Web ascertains this observation in one of many articles "Blood Shortage has Red Cross Seeking Donors" [1]. Currently we have total reliance on individual donors for a steady blood supply. One may ask why blood shortages continue in spite of the daily requests for donations. A number of reasons appear to contribute to the blood shortage situation. Donated blood has a relatively short shelf life of approximately 5-6 weeks. Potential donors cite two common reasons for failing to donate: fear of needles and

simply not taking the time to donate. Additional reasons that individuals do not donate include: medical condition(s), e.g. hepatitis or HIV/AIDS; travel to an area endemic of blood-borne disease; or failure to pass additional requirements banning their availability to donate. Blood transfusions save lives! Every year in the US approximately 20 million units of blood are transfused. In 2007, the Red Cross provided over 6 million units of blood. Today, 38% of the population is eligible to donate blood, but of this, only 6% actually donate [2]. Type O blood is considered the universal blood, because it does not induce an antibody response when transfused. Although there are other considerations that need to be evaluated prior to transfusing blood, Type O blood will be utilized first when the need arises. Approximately 50% of the general population is blood type O or B and is at risk during periods of blood shortages. Depletion of these two blood types is a common occurrence because of the universal nature of O blood and the low percentage of B blood in the US. Depending on any particular blood bank's inventory, maintaining an adequate supply of each blood Type can be exigent. Day to day and/or emergency demands on a blood bank's supply have the potential to quickly deplete an existing inventory of specific blood types. These challenges become exacerbated during natural disasters and world conflict. On the national and world stage there have been a number of sounding alarms concerning the adequate supply of transfusable blood. Populations in the developed countries are aging, putting strains on existing blood supplies. It is estimated that ≥ 50% of transfusions will go to patients > 60 years of age [3]. To compound this problem the > 60 age group rarely donates blood [4]. An added problem is that many of the developing countries are in areas of endemic diseases that

limit the blood supply. The ability to respond to specific needs in the event of a regional or national emergency can be paramount to national security. In recent times natural disasters in the U.S. and other parts of the world, along with world conflicts, have provided a substantial portion of the medical demand for blood transfusions which places a significant strain on the system. Because of these issues there has been a steady desire to develop a readily available stable/alternative blood supply.

Methods to address the ability to maintain a stable blood supply or develop an alternative technique have been in the research forefront. Enzymatic conversion, perfluorocarbons, hemoglobin-based oxygen carriers, and bonding RBC with polyethylene glycol methodologies are receiving the greatest amount of attention [5-16]. Stem cell research also holds promise but less work has been pursued in this area due to the political landscape [17, 18]. Each strategy has merit but they are not without their technical challenges.

The emphasis of this research involves enzymatically converting Type A RBCs to O with a recombinant α-NAGA originating from *Escherichia coli* or *Spirosoma linguale*. α-NAGA is part of the exoglycosidase enzyme family which cleaves terminal carbohydrate groups. The catalytic activity of recombinant α-NAGA cleaves a specific carbohydrate, a terminal linked 1-3 α-N-acetylgalactosamine, from the A antigen. The cleavage of the terminal carbohydrate from the sugar moiety converts group A blood to the universally transfusable blood group O. These studies will evaluate and characterize these α-NAGAs to determine the enzyme's optimal conditions (pH, temperature, buffer effects, ionic strength, and specific activity) for cleaving the terminal α-N-acetylgalactosamine

carbohydrate from type A RBC. This work also estimated an optimal α-NAGA concentration per unit of packed RBC, evaluated the feasibility of α-NAGA for converting RBC to a universal donor state, and evaluated the effects on the RBC following the α-NAGA conversion.

Chapter 2: History and Structure of the ABO(H) Blood Groups

History

Blood is the tissue that systemically transports the essential components of life: oxygen, nutrients, hormones, immune effectors, and waste products. Blood is indispensable to these processes and to our existence. The initial discovery of the blood group ABO system was accomplished by Landsteiner in 1901 [19]. In simple experiments of mixing red blood cells and serum collected from staff scientists they discovered that some RBC samples would agglutinate when combined with another's serum, Table 2.1. Landsteiner realized the antigen-antibody relationship due to his work in immunology.

Table: 2.1 Landsteiner's Cross Testing of Sera and RBC Experiment

	Origin of RBCs					
Sera	Dr. St.	Dr. Pletschnig	Dr. Sturli	Dr. Erdh.	Zar.	Landsteiner
Dr. St	-	+	+	+	+	-
Dr. Pletschnig	-	-	+	+	-	-
Dr. Sturli	-	+	-	-	+	-
Dr. Erdh.	-	+	-	-	+	-
Zar.	-	-	+	+	-	-
Landsteiner	-	+	+	+	+	-

Adapted from Owen, R., *Karl Landsteiner and the First Human Marker Locus.* Genetics, 2000. **155**(3): p. 995-998

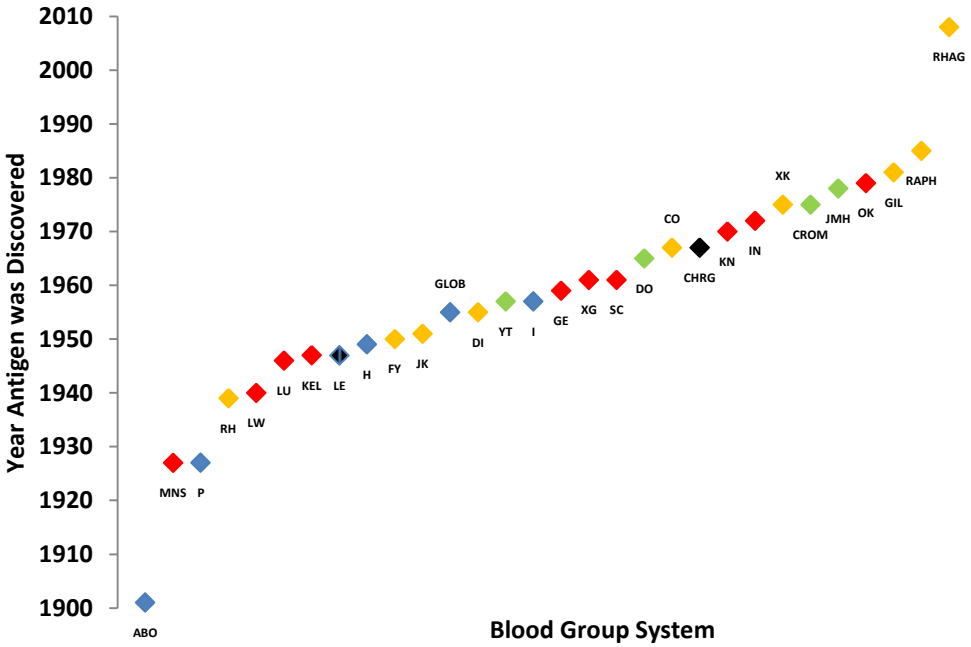
Based on this historic discovery human blood has been categorized into four different types, A, B, O, and AB, using the ABO system. The four blood types of the ABO system are due to the presence of different antigenic carbohydrate moieties on the surface of RBC. Since the ABO blood group discovery 28 additional blood group systems containing 241 antigens have been recognized, Table 2.2 [20-22]. The chronology of

discovery of blood groups is shown in Figure 2.1. The *International Society of Blood Transfusion* has also identified categories, Collections (6 blood groups) and Series (nineteen 700 series and nine 900 series antigens), identifying additional antigens but these are not classified in any of the designated System classifications because the antigens do not meet the required criteria [22]. These criteria are:

(1) An antithetical relationship between a new antigen and one already assigned to the system.
(2) Demonstration that expression of the antigen is associated with a variation in the nucleotide sequence of the gene controlling the system.
(3) Evidence, from a linkage analysis of family data, that the controlling allele is probably a newly recognized form of the pertinent gene, and supporting serological or biochemical information.
(4) Demonstration that an antigen is located on a protein or glycoprotein that carries other antigens belonging to the system. It must be remembered, however, that this could result from post-translational modification of a gene product, such as glycosylation, which would not support inclusion within the system [22].

Figure 2.1: History of Blood Group Discovery

Date of discovery or elucidation of blood group systems. Blood group systems are aligned according to the date of discovery of the first antigen in the system. The color represents the type of RBC membrane component that carries the antigens as defined in the figure.



Carbohydrate
Single-pass Membrane Protein
Multi-pass Membrane Protein
GPI-linked Protein
Adsorbed from Plasma

Adapted from Daniels, G. and M.E. Reid, *Blood groups: the past 50 years.* Transfusion. **50**(2): p. 281-289.

Table 2.2: Antigens of the blood group systems

		Antigen number																						
System		001	002	003	004	005	006	007	008	009	010	011	012	013	014	015	016	017	018	019	020	021	022	023	024
001	ABO	A	B	A,B	A1	…																			
002	MNS	M	N	S	s	U	He	Mi^a	M^c	Vw	Mur	M^g	Vr	M^e	Mt^a	St^a	Ri^a	Cl^a	Ny^a	Hut	Hil	M^v	Far	s^D	Mit
003	P	P1	…	…																					
004	RH	D	C	E	c	e	f	C^e	C^w	C^x	V	E^w	G	…	…	…	…	Hr_o	Hr	hr^S	VS	C^G	CE	D^w	…
005	LU	Lu^a	Lu^b	Lu3	Lu4	Lu5	Lu6	Lu7	Lu8	Lu9	…	Lu11	Lu12	Lu13	Lu14	…	Lu16	Lu17	Au^a	Au^b	Lu20	Lu21			
006	KEL	K	k	Kp^a	Kp^b	Ku	Js^a	Js^b	…	…	Ul^a	K11	K12	K13	K14	…	K16	K17	K18	K19	Km	Kp^c	K22	K23	K24
007	LE	Le^a	Le^b	Le^{ab}	Le^{bH}	ALe^b	BLe^b																		
008	FY	Fy^a	Fy^b	Fy3	Fy4	Fy5	Fy6																		
009	JK	Jk^a	Jk^b	Jk3																					
010	DI	Di^a	Di^b	Wr^a	Wr^b	Wd^a	Rb^a	WARR	ELO	Wu	Bp^a	Mo^a	Hg^a	Vg^a	Sw^a	BOW	NFLD	Jn^a	KREP	Tr^{a*}	Fr^a	SW1			
011	YT	Yt^a	Yt^b																						
012	XG	Xg^a	CD99																						
013	SC	Sc1	Sc2	Sc3	Rd	STAR																			
014	DO	Do^a	Do^b	Gy^a	Hy	Jo^a																			
015	CO	Co^a	Co^b	Co3																					
016	LW	…	…	…	…	LW^a	LW^{ab}	LW^b																	
017	CH/RG	Ch1	Ch2	Ch3	Ch4	Ch5	Ch6	WH				Rg1	Rg2												
018	H	H																							
019	XK	Kx																							
020	GE	…	Ge2	Ge3	Ge4	Wb	Lsa	Ana	Dha	GEIS															
021	CROM	Cr^a	Tc^a	Tc^b	Tc^c	Dr^a	Es^a	IFC	WES^a	WES^b	UMC	GUTI	SERF	ZENA											
022	KN	Kn^a	Kn^b	McC^a	Sl1	Yk^a	McC^b	Sl2	Sl3*																
023	IN	In^a	In^b																						
024	OK	Ok^a																							
025	RAPH	MER2																							
026	JMH	JMH																							
027	I	I																							
028	GLOB	P																							
029	GIL	GIL																							
		025	026	027	028	029	030	031	032	033	034	035	036	037	038	039	040	041	042	043	044	045	046		
002	MNS	Dantu	Hop	Nob	En^a	En^aKT	'N'	Or	DANE	TSEN	MINY	MUT	SAT	ERIK	Os^a	ENEP	ENEH	HAG	ENAV	MARS					
004	RH	…	c-like	cE	hr^H	Rh29	Go^a	hr^B	Rh32	Rh33	Hr^B	Rh35	Be^a	Evans	…	Rh39	Tar	Rh41	Rh42	Crawford	Nou	Riv	Sec		
006	KEL	VLAN	TOU	RAZ	VONG																				
		047	048	049	050	051	052	053	054	055	056														
004	RH	Dav	JAL	STEM	FPTT	MAR	BARC	JAHK	DAK	LOCR	CENR														

… Indicates a designation that is now obsolete: the antigen has either been transferred to another System, Collection, or removed due to inadequate documentation.

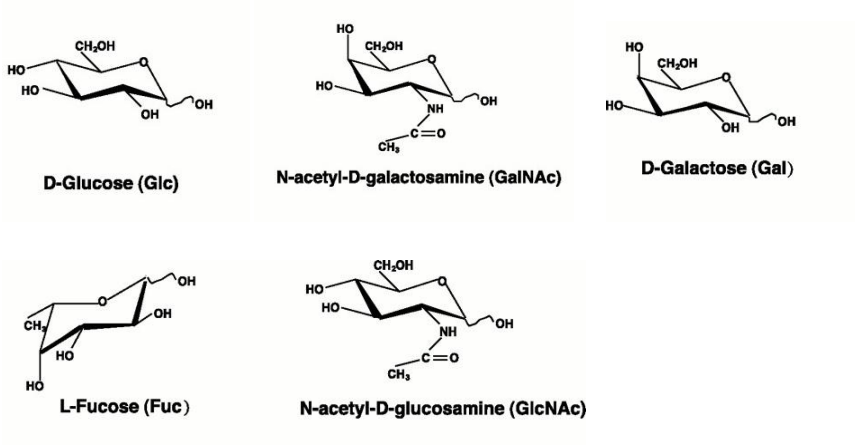
Adapted from Clausen, H. and S.-i. Hakomori, *ABH and Related Histo-Blood Group Antigens; Immunochemical Differences in Carrier Isotypes and Their Distribution1.* Vox Sanguinis, 1989. **56**(1): p. 1-20.

This text concentrates specifically on the ABO blood group system. The biochemical composition and structure of the carbohydrate antigens on RBCs were first elucidated in the 1960s [23-25]. Initial attempts to determine the structural composition were difficult due to limited functionality of analytical instrumentation available prior to this time. Because of the ABO antigens ubiquitous existence in nature, deduction of the structural composition was elucidated from tissue samples when the necessary technology emerged.

Glycoconjugates

Among the many structural details now well-known about RBC antigens, glycoconjugates are molecules consisting of sugar(s) attached to lipid (glycolipids) or protein (glycoproteins). Glycoconjugates are simple to complex substances involved with many physiological functions, e.g. cellular communication, structural integrity, and immunity [26]. Glycoproteins have similar oligosaccharide characteristics as glycosphingolipids with the exception of the oligosaccharide covalent link anchor. Synthesis of glycoconjugates occurs on the cytoplasmic face and within the endoplasmic reticulum and the Golgi apparatus. Specific glycosyltransferases add monosaccharides from nucleotide sugar donors forming the glycoconjugate [27]. The sugars forming these glycoconjugates that are associated with the human ABO blood groups are D-galactose, D-glucose, L-fucose, N-acetyl-D-glucosamine, and N-acetyl-D-galactosamine (Figure 2.2).

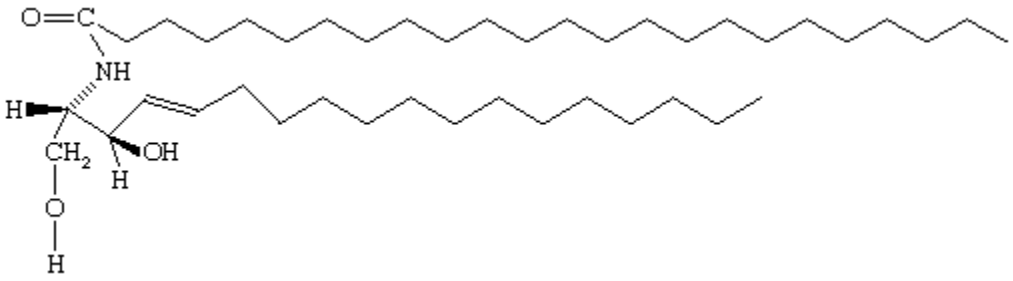
Figure 2.2: Structures of monosaccharides occurring in ABO glycoconjugates



Glycolipids

The glycolipids relating to the blood group antigens are glycosphingolipids in which the oligosaccharide chain is bound to a ceramide residue. The oligosaccharide chain is covalently linked to the hydroxyl group of carbon-1 of the sphingosine residue. Ceramides are an N-acyl complex derived from sphingosine (Figure 2.3). The associated fatty acids of the complex contain 16-26 carbon atoms and can be saturated or unsaturated molecules [26].

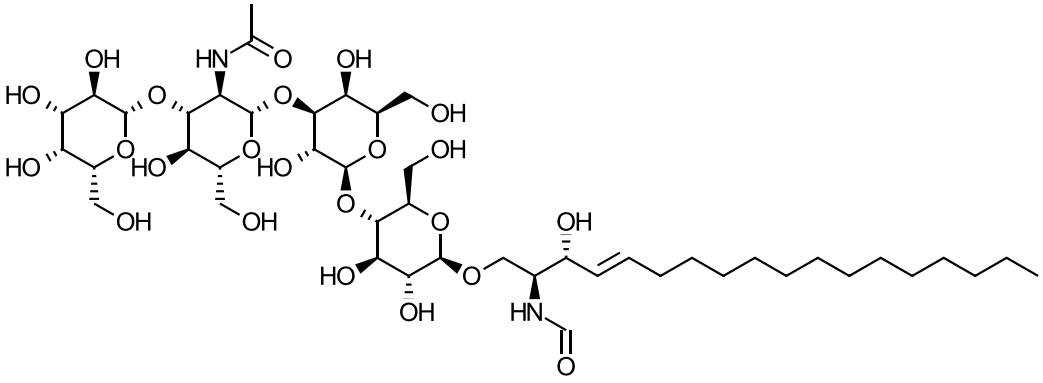
Figure 2.3: Ceramide



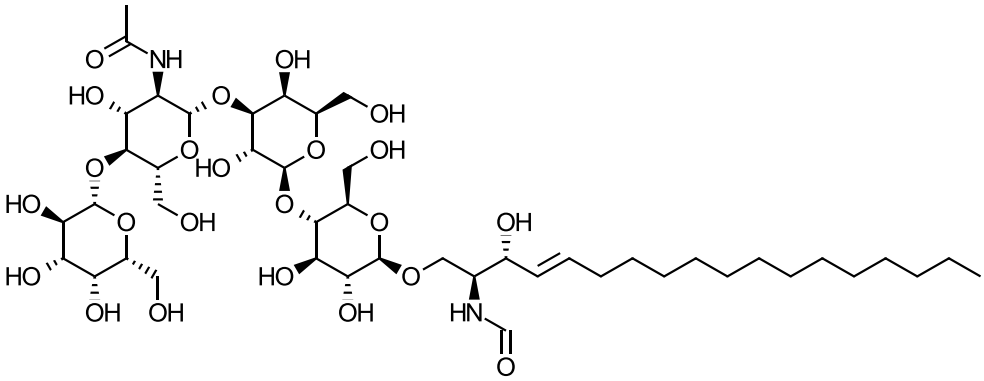
Because the sugar residues are quite variable a system based on the first 3-4 monosaccharide units was developed [28]. The ABO antigen glycolipids of the human RBC membrane incorporate glycosphingolipids from the lacto (Type 1 linkage), neolacto

(Type 2 linkage) and globo (Type 4 linkage) series (Figure 2.4). The Type 1 chain is characterized by the terminal β-Gal (1-3) GlcNAc, whereas the Type 2 chain is characterized by a β-Gal (1-4) GlcNAc chain. The Type 4 chain is characterized by an additional β-Gal (1-4) incorporated into the oligosaccharide [27, 29].

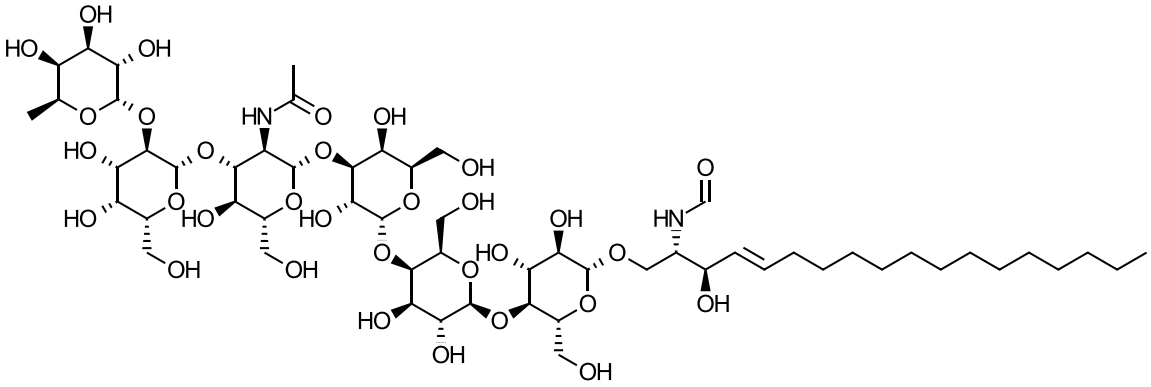
Figure 2.4: Lacto, Neolacto, and Globo Glycosphingolipids



(Lacto series) β-D-galactosyl-(1,3)-N-acetyl-β-D-glucosaminyl-(1,3)-β-D-galactosyl-(1,4)-β-D-glucosyl-(1<->1)-ceramide



(Neolacto series) β-D-Gal-(1->4)-β-D-GlcNAc-(1->3)-β-D-Gal-(1->4)-β-D-Glc-(1<->1')-Cer



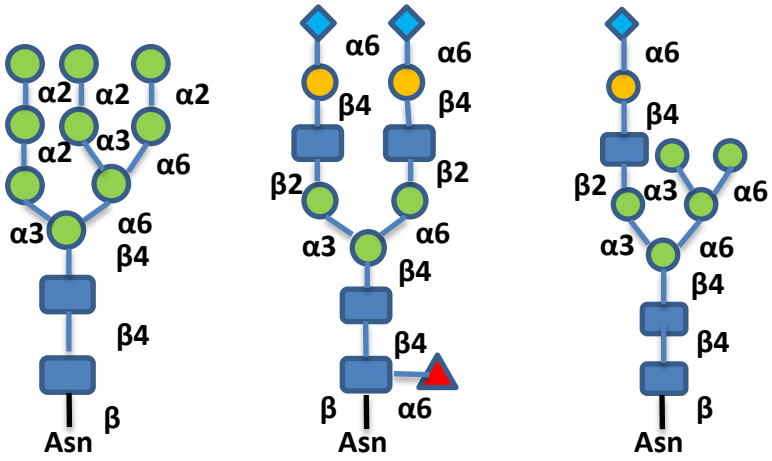
(Globo series) α-L-fucosyl-(1->2)-β-D-galactosyl-(1->3)-N-acetyl-β-D-glucosaminyl-(1->3)-α-D-galactosyl-(1->4)-β-D-galactosyl-(1->4)-β-D-glucosyl-(1->1')-ceramide

Glycoproteins

In contrast to glycolipids, glycoprotein oligosaccharide chains are N-glycosidic or O-glycosidic and linked to a protein backbone. N-glycosidic oligosaccharides are linked by an asparagine and have a high composition of mannose in the carbohydrate chain, whereas the O-glycosidic oligosaccharides are linked by a serine or threonine and are devoid of mannose (Figure 2.5). N-glycoside linked oligosaccharide entities are found primarily in plasma and cell membranes while O-glycosidic linked oligosaccharide units are primarily found from gland secretions and mucins [26].

Figure 2.5: Glycoprotein Composition

Types of N-glycans. N-glycans added to protein at Asn-X-Ser/Thr sequons are of three general types in a mature glycoprotein: oligomannose, complex, and hybrid. Each N-glycan contains the common core $Man_3GlcNAc_2Asn$.



Oligomanose Complex Hybrid

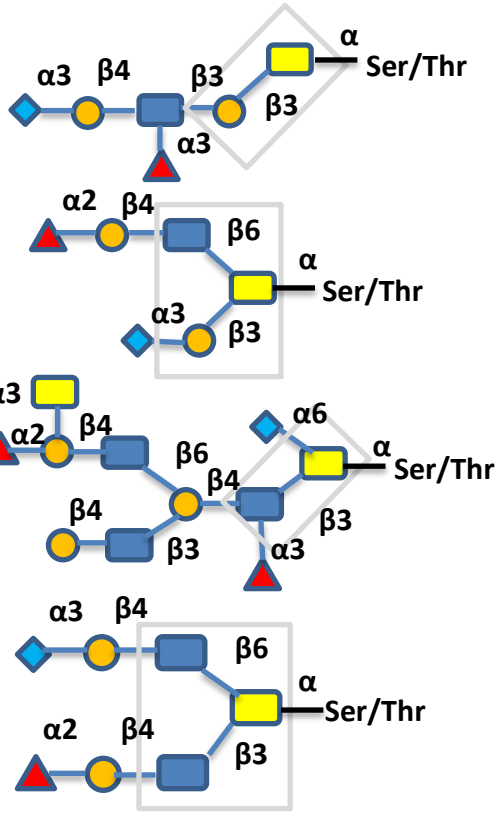
Complex O-GalNAc glycans with different core structures. Representative examples of complex O-GalNAc glycans with extended core 1, 2, or 4 structures from human respiratory mucins and an O-GalNAc glycan with an extended core 3 structure from human colonic mucins. All four core structures (in *boxes*) can be extended, branched, and terminated by fucose in various linkages, sialic acid in α2-3 linkage, or blood group antigenic determinants. Core structures 1 and 3 may also carry sialic acid α2-6-linked to the core *N*-acetylgalactosamine.

Extended Core 1

Extended Core 2

Extended Core 3

Extended Core 4



Adapted from Stanley, P., et al., *Essentials of Glycobiology.* 2nd ed, ed. A. Varki, et al. Vol. Chapter 8 and 9. 2009, Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press

Genetics and Structure of the ABO Blood Groups

The ABO(H) blood group glycoconjugate synthesis is regulated under the genetic control of three interrelated gene systems acquired through Mendelian genetics: ABO, Hh, and Sese [30, 31]. Table 2.3 shows the possible combinations of ABO blood types inherited from parents.

Table 2.3: Inheritance of the ABO genes

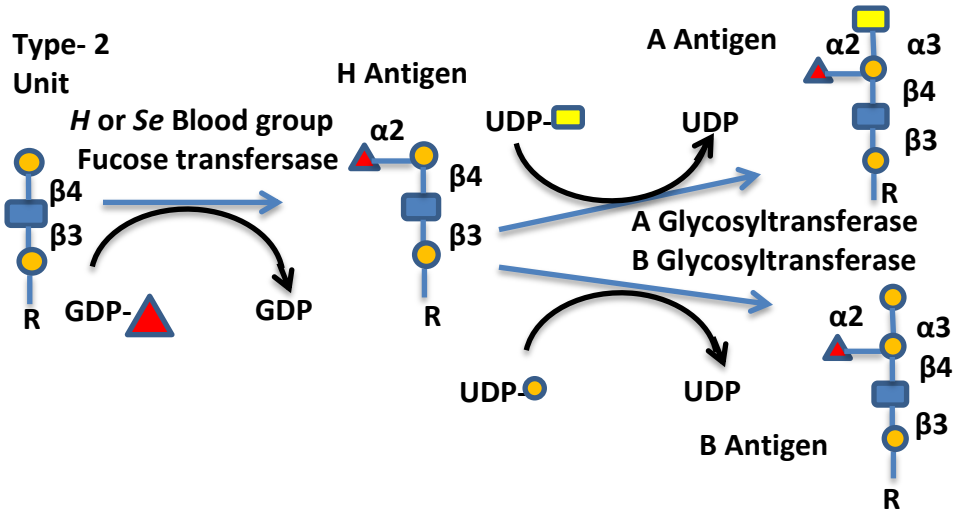
Inherited ABO Genotype in children		ABO genotype of mother		
		AA	BB	OO
ABO genotype of father	AA	AA	AB	AO
	BB	AB	BB	BO
	OO	AO	BO	OO

Adapted from Dean, L., *Blood Groups and Red Cell Antigens.* National Center Biotechnology Information (NCBI), National Library of Medicine, National Institutes of Health, 2005.

There are three alleles comprising the A, B, and O gene locus. The A and B alleles are responsible for forming the A and B terminal sugar on RBCs and in secretions. The O allele is inactive, thereby not contributing additional modification to RBCs or secretions. The H (*FUT1*) or Se (secretors, *FUT2*) allele is responsible for regulating the sugar addition characteristic of the O blood type. Each gene locus encodes for a specific glycosyltransferase that contributes to the formation of the ABO antigen. The general synthesis of antigen formation is outlined in Figure 2.6. Two separate fucosyltransferases are present in secretors, one under encoded by the H allele and the other controlled by the Se allele. The addition of fucose to galactose on the precursor

chain *N*-acetyllactosamine by the α1-2 fucosyltransferase forms the H antigen and produces blood group Type O. Additional modification of the antigen to the A or B form is accomplished by addition of N-acetylgalactosamine or galactose by the corresponding α1-3 glycosyltransferase present in those individuals with the A and/or B alleles to produce the blood groups A, B, or AB [32].

Figure 2.6: Synthesis of H (O), A, and B blood group determinants



Adapted from Stanley, P., et al., *Essentials of Glycobiology.* 2nd ed, ed. A. Varki, et al. Vol. Chapter 8 and 9. 2009, Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press

A rare genetic condition, Bombay phenotype O_h, is caused when individuals inherit the homozygote hh which lacks the formation of the H(O) antigen. The secretor gene Sese, is independent of the ABO and Hh genes. Individuals with the genetic haplotype, Sese or SeSe, are able to secrete ABH glycoconjugates in various secretions and tissue fluids. Individuals with the gene sequence sese, will not secrete ABH glycoconjugates [26]. ABO allele exon organization of the protein coding sequences is presented in Figure 2.7. The ABO alleles are located on chromosome 9, 9q34.1-q34.2, while the H and Se alleles

are located on chromosome 19, 19q13.3. There are seven nucleotide substitutions in the ABO alleles: 297A>G, 526C>G, 657C>T, 703G>A, 796C>A, 803G>C, and 930G>A. Four of these nucleotide substitutions, 526C>G, 703G>A, 796C>A, and 803G>C, relate to an amino acid translation modification. These changes correlate to the specificity of the glycosyltranserases. A single nucleotide deletion at position 261, on allele O compared to allele A, caused a frame shift resulting in inactivity of the truncated glycosyltranserases. [33]

Figure 2.7: Structure of the ABO gene locus and nucleotide sequences of A, B and O alleles.

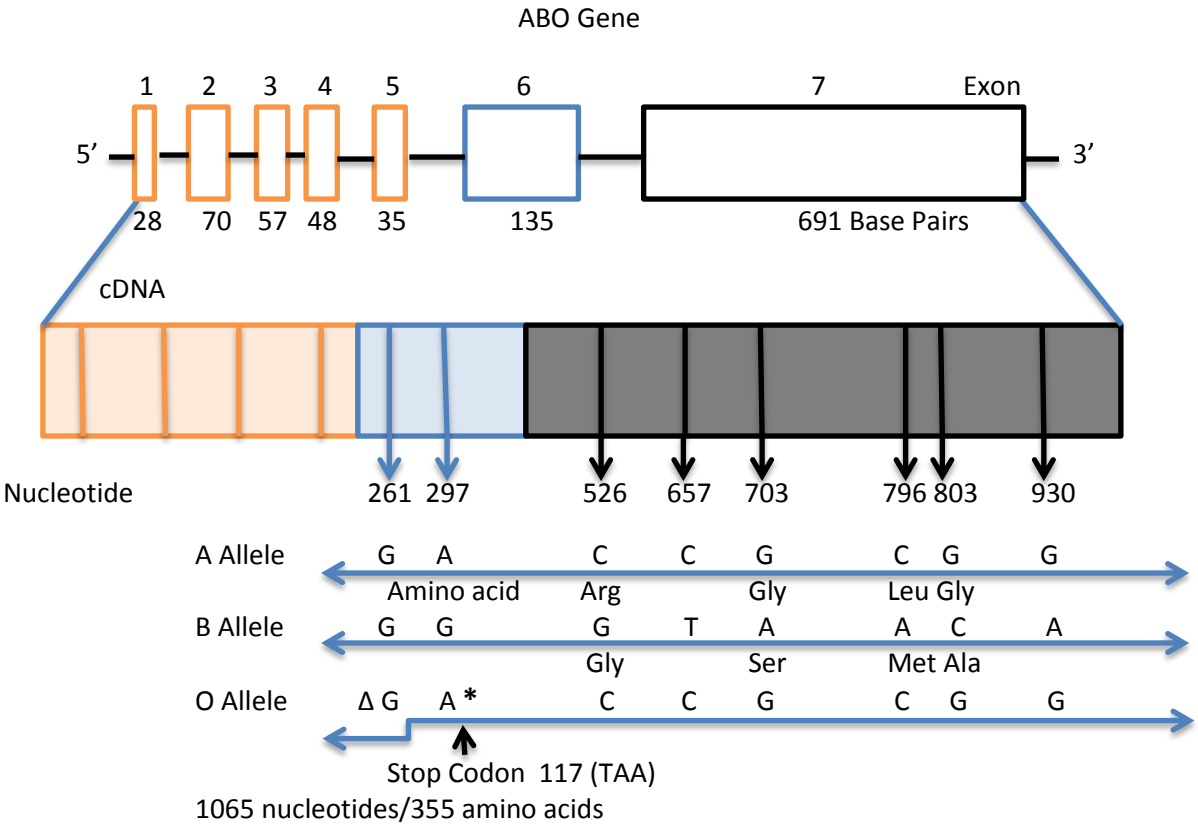


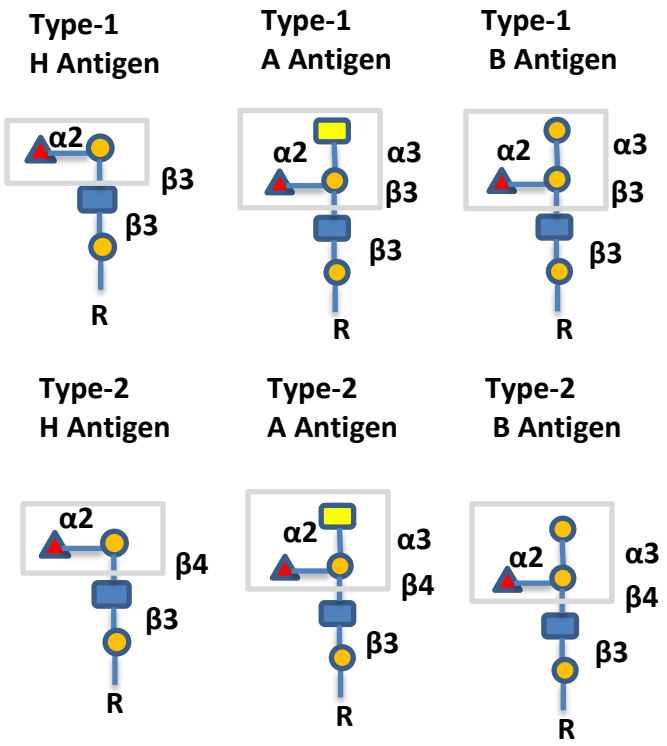
Diagram of exon organization of the protein coding sequences. *; Entirely different deduced amino acid sequence in O alleles due to frame shift caused by a single base deletion (lower).

Adapted Hosoi, E., *Biological and clinical aspects of ABO blood group system.* Journal of Medical Investigation, 2008. **55**: p. 174-182.

The ABO antigen formation is initiated through precursor complexes as indicated previously, glycoconjugates (N-linked with asparagine or O-linked with serine or threonine) and glycolipids linked with ceramide). The 4 types of glycoconjugates consist of a Type 1, 2, 3, or 4 oligosaccharide chain formation linked with a protein and/or lipid. Type 1 and 2 are associated with N- and O-linked glycoproteins and glycolipids, Type 3 with O-linked glycoproteins and glycolipids, and Type 4 with glycolipids [26, 29]. The ABO expression pathway (Figure 2.6) that occurs during antigen formation will construct each of the 4 glyconjugate types associated with RBCs (Figure 2.8). Repeats of carbohydrate entities within the saccharide chain will cause the antigen to vary in length between 15 and 150 Å from the RBC surface [34]. The ABO antigens are expressed ubiquitously on membranes of the RBC, endothelium, and epithelium. Some tissues will also secrete the ABO antigen's Type 1 chain, which can be isolated in fluids of individuals with the active Se allele [29, 32]. Type 1 chain ABO antigen expression is typical on the epithelial surface of the digestive, respiratory, urinary, and reproductive tracts in addition to the salivary and some exocrine glands. Type 2 antigens are expressed by RBCs, in addition to the epidermis, while Type 3 antigens are commonly expressed in the gastric mucosal lining and ovarian fluids. Type 4 chain antigens are only expressed on RBCs in minor quantities but are present in large quantities in the kidneys and some tumor tissues [32, 35].

Figure 2.8: Type 1, 2, 3, and 4 ABO antigens

Type-1 and -2 H, A, and B antigens that form the O (H), A, and B blood group determinants on N- and O-glycoprotein.



Type-3 H, A, and B antigens that form the O (H), A, and B blood group determinants on N- and O-glycoprotein.

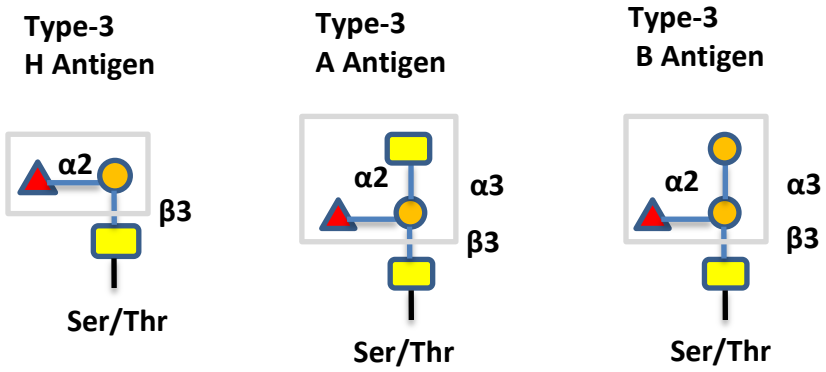
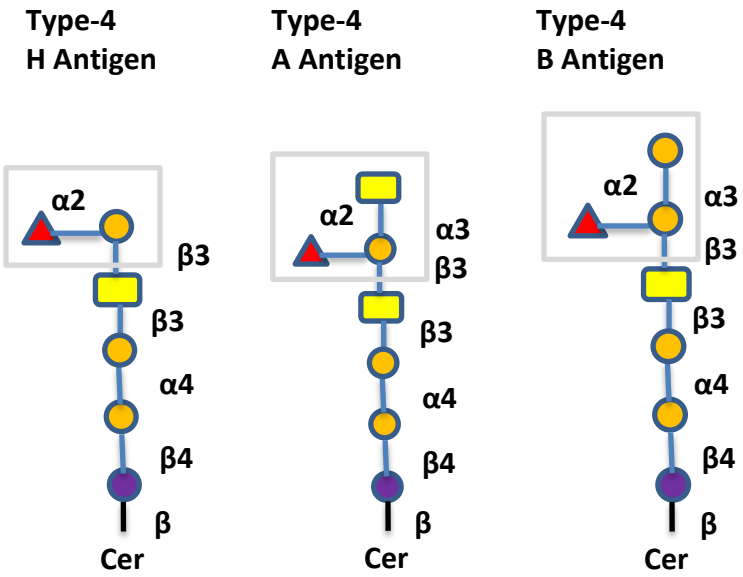


Figure 2.8 Continued: Type 1, 2, 3, and 4 ABO antigens

Type-4 H, A, and B antigens that form the O (H), A, and B blood group determinants on glycolipids.



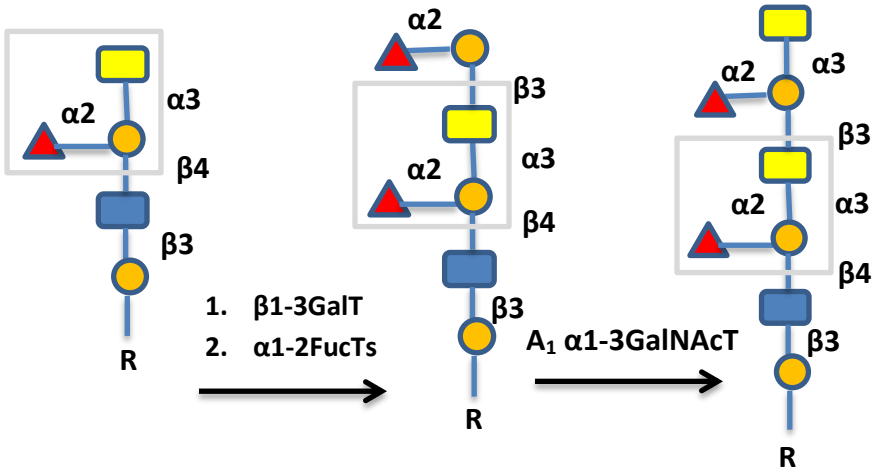
Adapted from Stanley, P., et al., *Essentials of Glycobiology.* 2nd ed, ed. A. Varki, et al. Vol. Chapter 8 and 9. 2009, Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press

The ABO antigen presence on RBCs is predominantly the Type 2 chains, while Type 3 and 4 chains are present in insignificant concentrations. Type 1 chain antigens are not endogenously produced by RBCs, but are absorbed from plasma [26]. The Type 2 chain glycosphingolipids will vary significantly from simple short chains to complex highly branched structures. The short simple chain glycosphingolipid, containing 5-15 monosaccharide units [36], is present in low concentrations compared to the complex branched structures that average 35 units, but may contain up to 60 [29]. Type 3 chain glycosphingolipids have only been isolated from the A blood group, primarily from the subgroup A_1 and only in very low amounts in the A_2 subgroup [37]. In its simplest form the Type 3 chain is an extended Type 2 chain with an additional A or H terminal group.

The H Type 3 chain occurs primarily in the A_2 subgroup but is absent from the B and O blood groups, (Figure 2.9) [38, 39]. Type 4 chain glycosphingolipids occur only with an A or H terminal group; a B terminal group has not been isolated at this time. The A or H group is linked to a terminal β-N-acetylgalactosamine of the oligosaccharide chain. The A Type 4 chain is only found within the A_1 blood group, while the H Type 4 chain is found within the O and A_2 blood groups [26]. At this time, only Type 2 chain glycoproteins have been isolated from the blood groups. The O-linked blood group glycoproteins appear to be associated with glycophorins. The N-linked glycoproteins are complex oligosaccharide chains attached to the RBC membrane. N-linked complex oligosaccharide chains that have been isolated from AB RBCs have shown that the A or B groups are located on separate chains [25, 40-42].

Figure 2.9: Type 3 Chain Comparing A_1 and A_2 blood group antigens

The type-2 A glycan is modified by a β1–3GalT and the α1–2FucT from the *H* locus to form a type-3 H determinant and the A_2 blood group. The type-3 H glycan is a substrate for the A_1 transferase to form the repeated A determinant unit proposed to be responsible for the strong serological reactivity of the A_1 phenotype. The A_2 transferase is unable to complete this last reaction efficiently. R represents glycoprotein or glycolipid. A-reactive epitopes are enclosed in boxes.



Adapted from Stanley, P., et al., *Essentials of Glycobiology.* 2nd ed, ed. A. Varki, et al. Vol. Chapter 8 and 9. 2009, Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press

The high variability of the blood group antigens create additional differences within a blood type, these include differences in the glycosyltransferase activity and efficiency and number of ABO antigen sites present. The blood groups have been separated into subgroups based on these differing phenotypic attributes. The subgroups are distinguished by genetic variations resulting in decreased amounts of A, B, or O(H) antigens present on the RBC [33]. Blood group A appears to have the highest number of subgroups. Type A_1 individuals comprise approximately 80% of the Type A blood population while the remaining 20% are made up predominantly of the A_2 subgroup plus small percentages of additional A subgroups. Genetic variation in the A allele appears to be responsible for the number of A subgroups. A single base deletion at the carboxyl end of the allele causes a frame shift resulting in the addition of 21 extra amino acids to the A_2 glycosyltransferase, (Figure 2.10) [33, 43, 44]. This addition appears to be responsible for the reduced activity of the A_2 glycosyltransferase compared with that of the A_1 group. The reduced activity of the A_2 glycosyltransferase appears to be only quantitative when compared to the A_1 transferase.

Figure 2.10: Deduced Amino Acid Sequences of C-Terminal Segment of A_1 and A_2 Alleles; - identical amino acid, * terminal codon

	327
A_1	N H Q A V R N P *
A_2	- - - - - - - - R E R L P G A L G G L P A A P S P S R P W F *

Adapted from Yamamoto, F.-i., P.D. McNeill, and S.-i. Hakomori, *Human histo-blood group A2 transferase coded by A2 allele, one of the a subtypes, is characterized by a single base deletion in the coding sequence, which results in an additional domain at the carboxyl terminal.* Biochemical and Biophysical Research Communications, 1992. **187**(1): p. 366-374.

Additional A subgroups, A_{int}, A_3, A_x, A_m, A_{end}, $A_{finn,}$ A_{bantu}, A_{el}, A_y, and A_{iae} exist and are termed “weak” due to their low agglutination ability with anti-A antibodies and A allele variants [44]. The reduced immune response of these weak A subgroups is related to the number of antigen sites per RBC, Table 2.5 [26].

Table 2.4: Number of blood group A determinants on erythrocytes from adults belonging to different A subgroups

Blood group	Number of A determinants (Range)		
A_1	810,000	-	1,170,000
A_2	160,000	-	440,000
A_3	21,300	-	187,000
A_x	7,300	-	22,500
A_{end}	0	-	137,000
A_m	100	-	1,900
A_{el}	100	-	1,400
A_Y	100	-	1,900

Adapted from Schenkel-Brunner, H., *Human Blood Groups: Chemical and Biochemical Basis of Antigen Specificity*. 2nd ed, ed. A. Salvini-Plawen2000, New York: Springer-Verlag Wein. 637.

The serological response of the A subgroups can be used to differentiate between the A blood groups (Table 2.6). The B subgroups are similarly classified as the A blood subgroups in decreasing antigen concentration starting with B, B_3, B_x, B_m, and B_{el}. The serological response of the B subgroups can also be used to differentiate between the B blood groups (Table 2.7). Subgroups exist within the AB blood type based on the heritable subgroups of the A and B groups. Blood type AB is categorized into nine subgroups (A_xB, A_1B_x, A_mB, A_1B_m, $A_{el}B$, A_1B_{el}, cisA_2B_3, cisA_2B, and cisA_1B_3) by the quantity of A and/or B antigen [33]. The cisAB subgroup is a very rare occurrence but can exist as (phenotype/*genotype*), cisA_2B_3 (*A_2B_3/O*), cisA_2B (*A_2B_3/B*), and cisA_1B_3 (*A_2B_3/A_1*).

Table 2.5: Serological characterization of A subgroups

Sub Group	Reaction of erythrocytes [a,b] with				Antibodies in serum[c]		Saliva of ABH-secretors
	anti-A	anti-(A+B)	anti-A_1[d]	anti-H	anti-A_1	anti-A	(Ratio: A:H) [e]
A_1	4	4	3	0	0[f]	0	A + H (3.1± 1.2)
A_2	3	3	0	3	w/0[g]	0	A + H (2.1± 0.5)
A_{int}	4	4	2	3			A + H
A_3	2[M]	2[M]	0	4	w/0	0	A + H (1.2± 0.5)
A_x	0/vw	w/2	0	4	w	w/vw	A_x[h] + H (0.5)
A_m	vw/0	vw	0	4	0	0	A + H (3.2± 1.0)
A_{end}	vw[M][i]	vw[M]	0	4	w/0	0	H
A_{finn}	vw[M]	vw[M]	0	4			H
A_{bantu}	w/2[M]	w/2[M]		4			H
A_{el}	0	0	0	4	2	w/0	H
A_y	0	0	0	4	0	0	(A)+ H (0.5 - 1.0)
A_{iae}	0	0	3	4			H

[M] Mixed-field agglutination (few small agglutinates in a pool of free red cells,

[a] all erythrocytes bind anti-A, no reaction with anti-B

[b] degree of agglutination ranging from 4 (strong) to w (weak) and vw (very weak); 0 (no agglutination),

[c] + antibody present, (+) reduced amount of antibody, - antibody absent, all sera contain anti B,

[d] anti-A, lectin of *Dolichos biflorus*,

[e] after Carton [53],

[f] in the case of A_1B frequently anti-H,

[g] in the case of A_2B frequently anti-A_1,

[h] A substance detected by inhibition of the donor's own red cell agglutination by anti-A sera,

[i] slow agglutination (2-3 min).

Adapted from Schenkel-Brunner, H., *Human Blood Groups: Chemical and Biochemical Basis of Antigen Specificity*. 2nd ed, ed. A. Salvini-Plawen2000, New York: Springer-Verlag Wein. 637.

Table 2.6: Serological characterization of B subgroups

Sub Group	Reaction of erythrocytes [a,b] with			Antibodies in serum[c]			Blood group substance in Saliva of ABH-secretors
	anti-B	anti-(A+B)	anti-H	anti-A_1	anti-A	anti-B	
B	4	4	0	0[f]	0	0	B + H
B_3	2[M]	2[M]	4	w/0	0	0	(B) + H
B_x	w/vw	w/2	4	w	w/vw	vw[d]	(B)[e] + H
B_m	vw/0	vw	4	0	0	0	B + (H)
B_{el}	0	0	4	2	w/0	w/0	H

[M] Mixed-field agglutination (few small agglutinates in a pool of free red cells, degree of agglutination ranging from 4 (strong) to w (weak) and vw (very weak); 0 (no agglutination),

[a]

[b] The red cells of all subgroups bind anti-**B**

[c] + antibody present, (+) reduced amount of antibody, - antibody absent

[d] anti-A, lectin of *Dolichos biflorus*,

[e] B substance detected by inhibition of the donor's own red cell agglutination by anti-B sera,

Adapted from Schenkel-Brunner, H., *Human Blood Groups: Chemical and Biochemical Basis of Antigen Specificity*. 2nd ed, ed. A. Salvini-Plawen2000, New York: Springer-Verlag Wein. 637.

There are a few O subgroups: O_h (Bombay), Parabombay (genotype **h/h Se**), Reunion phenotype, and O_{Hm} [26]. The Bombay phenotype expresses the H antigen, whereas the Parabombay individual expresses the *FUT1* glycosyltransferase. Because of this, the Parabombay subgroup does not express the H antigen on RBCs, but because they are secretors express the *FUT2* glycosyltransferase, allowing the RBCs to absorb Type 1 antigen from plasma. The Reunion phenotype has a genetic mutation that produces a functioning glycosyltransferase but with enzyme activity reduced by 90% [26]. The O_{Hm} phenotype is characterized by a weak expression of H antigen causing a lack of ABH activity [26]. Humans do not possess ABO plasma antibodies at birth, but develop these

antibodies at approximately 6 months of age. This occurs due to exposure from expressed carbohydrate moieties from foods and microorganisms that are similar or identical to the ABO antigens [45]. RBCs of infants do express blood group antigens based on heritable genetic traits but at lower concentrations than adults, Table 2.8.

Table 2.7: Expression of ABO antigens per red blood cell surface adult vs. newborn

Blood group		Number of A determinants (Range)		
A_1	Adult	810,000	-	1,170,000
A_1	Newborn	250,000	-	370,000
A_2	Adult	160,000	-	440,000
A_2	Newborn	140,000	-	
A_1B	Adult	460,000	-	850,000
A_1B	Newborn	240,000	-	290,000
A_2B		120,000	-	137,000
B	Adult	610,000	-	830,000
B	Newborn	200,000	-	320,000

Adapted Hosoi, E., *Biological and clinical aspects of ABO blood group system.* Journal of Medical Investigation, 2008. **55**: p. 174-182.

Chapter 3: α-N-Acetylgalactosaminidase

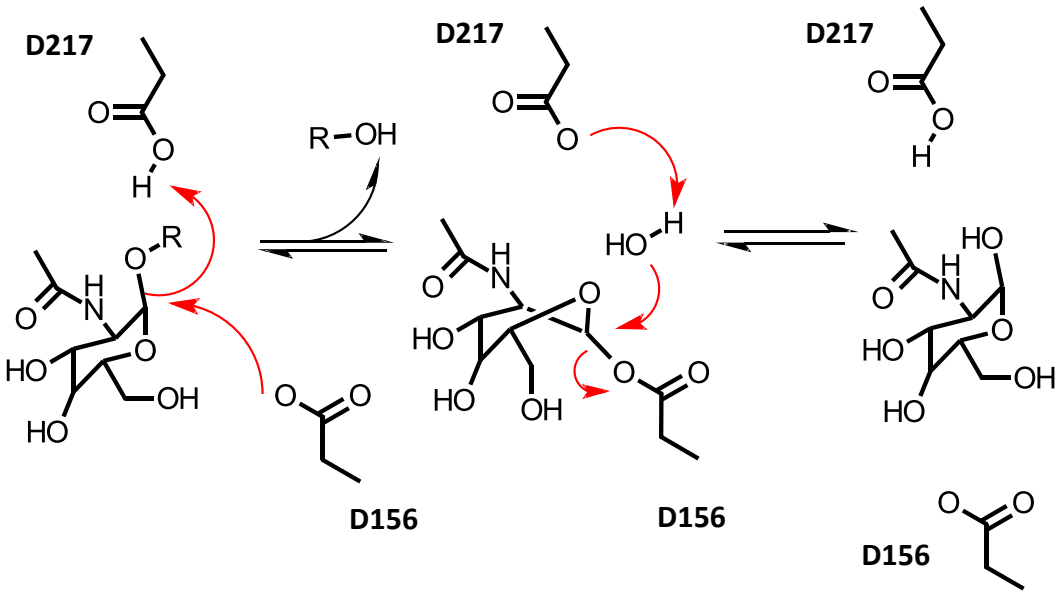
α-N-acetylgalactosaminidase is an exoglycosidase that hydrolyzes α-linked N-acetyl-D-galactosamine from the non-reducing terminal sugar of glycoconjugates. The enzyme(s) are assigned to EC 3.2.1.49, and the CAZy glycosidase hydrolase families 27, 36, or 109. Glycoconjugates are present among many tissues and fluids in humans; examples include ovarian cyst fluid, kidneys, saliva, and blood group A antigen. α-linked N-acetyl-D-galactosamine is ubiquitous in nature serving as a key to cellular signaling and communication, structural integrity, and immunity [26]. α-NAGA is an integral enzyme aiding in systemic metabolic balance. Failure to synthesize this enzyme, either due to a genetic or post translation defect to a functioning glycosidase, will lead to the disorders Schindler disease (a lysosomal storage disease); and Kanzaki disease (a neurological degenerative disease) in humans [46]. α-NAGA is under investigation as a potential therapeutic agent for lysosomal storage diseases and to enzymatically convert blood group A to O. The enzyme has been isolated from several eukaryotic and prokaryotic sources. Some of the characteristics of these enzymes are reviewed herein.

α-N-Acetylgalactosaminidase Reaction Mechanism

There are two reaction mechanisms that α-NAGA has been experimentally shown or proved to undergo during hydrolysis of α-linked N-acetyl-D-galactosamine. Glycosidase hydrolase family 27 and 36, follow a mechanism proposed by Koshland [47], involving a double-displacement reaction that hydrolyzes the terminal sugar (Figure 3.1). The

nucleophile typically involved with this reaction mechanism is an aspartate side chain under acidic conditions.

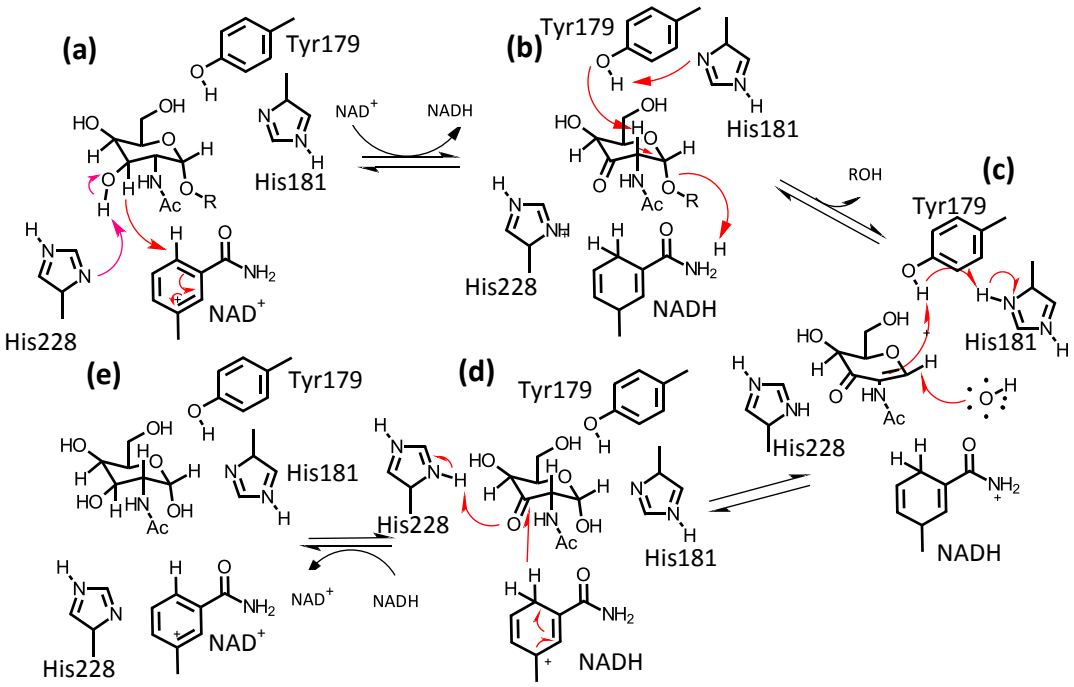
Figure 3.1: Double Displacement Reaction mechanism



The double displacement reaction mechanism in human α-NAGA. D156 acts as the nucleophile and D217 acts as the acid/base. The ligand is bent into a high energy 1S3 skew boat conformation during the reaction. Adapted from Clark, N.E. and S.C. Garman, *The 1.9 Å Structure of Human [alpha]-N-Acetylgalactosaminidase: The Molecular Basis of Schindler and Kanzaki Diseases.* Journal of Molecular Biology, 2009. **393**(2): p. 435-447.

The second reaction involving the GH109 family of enzymes hydrolyzes the terminal sugar through an NAD^+ dependent cofactor mechanism, Figure 3.2. The enzyme base typically involved with this reaction mechanism is a histidine amino acid under neutral conditions.

Figure 3.2: NAD-dependent hydrolysis



Proposed mechanism of the *E. meningosepticum* α-*N*-acetylgalactosaminidase. The mechanism involves (**a**) hydride abstraction from C3 by the NAD+ cofactor, assisted by the His228 base attack at C3-OH, oxidizing the C3-OH to a carbonyl; (**b**) proton abstraction at C2 by Tyr179, accompanied by a C1-O1 cleavage via a β-elimination, forming an α,β-unsaturated ketone intermediate; (**c**) 1,2-Michael-like addition of water across the C1-C2 double bond; (**d**) restoration of the C3-OH by the transiently formed NADH reduction of the C3 carbonyl. **(e)** The overall result is the hydrolysis of the substrate. Adapted from Liu, Q.P., et al., *Bacterial glycosidases for the production of universal red blood cells.* Nature Biotechnology, 2007. **25**(4): p. 454-464.

α-N-Acetylgalactosaminidase from Eukaryotic Sources

There have been several α-NAGAs isolated from eukaryotic and prokaryotic sources. To date, eukaryotic α-NAGAs have been characterized to various degrees from bovine, porcine, chicken, human, earthworm, snail, the gastropod *Turbo cornutus*, the limpet *Patella vulgate*, and skipjack [50]. Many of the eukaryotic α-NAGA have non-specific activity to several glycosides. The studies conducted with these enzymes are directed toward lysosomal storage diseases and conversion of group A RBC to group O. Because

of the investigation into lysosomal storage diseases, we will begin with the human enzyme isolate. In the lysosome, glycosidases degrade glycoconjugates and oligosaccharides to recycle or remove products from metabolic processes. Due to genetic mutations, many individuals are unable to reuse or clear these products, leading to systemic accumulation of sugars causing a host of diseases, e.g. Schindler's or Kanzaki's disease. The human α-NAGA has been isolated, characterized, and crystallized by Clark and Garman in 2009 [48]. The NAGA gene was cloned into a baculovirus vector and expressed in Tn5 insect cells. Several mutants were created to improve the quality of crystals and associated data sets as well as to remove glycosylation sites. Protein purification procedures included Ni-affinity and anion exchange chromatography. The enzyme was active as a homodimer with a molecular mass of 96 kD determined by gel filtration chromatography. Enzyme assays were performed in 100 mM citrate/phosphate buffer pH 4.5 with resulting Michaelis-Menten kinetic parameters (Table 3.1). As indicated in the Table the enzyme has activity with both α-GalNAc and α-Gal conjugate substrates with a high affinity for α-GalNAc.

Table 3.1: Enzyme kinetics of human α-NAGA wild type and N201Q enzymes

	*p*NP-N-Acetylgalactosamide			*p*NP-Galactose		
Enzyme	**K_M (mM)**	**k_{cat} s^{-1}**	**k_{cat}/K_M ($s^{-1}mM^{-1}$)**	**K_M (mM)**	**k_{cat} s^{-1}**	**k_{cat}/K_M ($s^{-1}mM^{-1}$)**
WT α-NAGA	0.70 ± 0.03	16.3 ± 0.1	23.3 ± 0.1	15.2 ± 1.1	8.9 ± 0.3	0.59 ± 0.08
N201Q α-NAGA	0.89 ± 0.03	17.1 ± 0.2	19.2 ± 0.1	13.9 ± 1.1	8.2 ± 0.2	0.59 ± 0.08

Summary of kinetic data on human α-NAGA. The wild type and N201Q mutant glycoproteins have similar kinetic parameters. The specificity constant kcat/K_M for each protein is 30-40 fold greater for the GalNAc substrate compared to the galactose substrate.
Adapted from Clark, N.E. and S.C. Garman, *The 1.9 Å Structure of Human [alpha]-N-Acetylgalactosaminidase: The Molecular Basis of Schindler and Kanzaki Diseases.* Journal of Molecular Biology, 2009. **393**(2): p. 435-447.

Two enzymes showing α-NAGA activity α-NAGA I and II were isolated from starfish by Harun-Or-Rashid et al. [50]. The enzymes were extracted from the digestive tract of the starfish. Protein purification was performed using cation exchange, gel filtration, and hydroxyapatite chromatography. α-NAGA I and II were active as a homopolymer and monomer respectively as assessed by gel filtration chromatography. Enzyme assays were performed in 0.5 M sodium citrate buffer pH 4.0 resulting in the Michaelis-Menten kinetic parameters shown in Table 3.2 and substrate activity is shown in Table 3.3.

Table 3.2: Characteristics of Starfish α-NAGA I and II and human placenta α-NAGA

	Starfish		Human Placenta α-NAGA
	α-NAGA I	α-NAGA II	
Molecular Mass (kDa)			
Gel Filtration	430	40	ND
SDS-PAGE	47	43	46
Optimum pH	3.5	3.0	4.2
Isoelectric Point	4.4	7.1	4.8
% Relative activity (α-GAL % relative to α-NAGA)	50	92	98
*p*NP substrate	6.74	0.29	5.26
4-MU substrate	10.69	0.63	11.70
K_M (mM)	1.04	3.97	1.79
Vmax (μmole/min/mg)	49.5	126.6	

Adapted from Harun-Or-Rashid, M., et al., *Purification and Characterization of α-N-Acetylgalactosaminidases I and II from the Starfish Asterina amurensis.* Bioscience, Biotechnology, and Biochemistry, 2009. **74**(2): p. 256-261.

Table 3.3: Starfish α-NAGA I and II and human placenta α-NAGA Substrate Activity

Substrate*	Starfish		Human placenta
	α-NAGA I	α-NAGA II	α-NAGA
*p*NP-α-N-Acetylgalactosaminide	19.6	23.2	17.7
4-MU-α-N-Acetylgalactosaminide	24.1	27.6	35.7
BGAT	0.379	0.412	0.637
N-Acetylgalactosaminide-α1-*O*-serine	0.0293	0.0295	0.0866
*p*NP-α-Galactose	1.32	0.0673	0.929
4-MU-α-Galactose	2.57	0.174	4.18
Melibiose	0.0167	ND	0.0230
Raffinose	0.0195	ND	0.0231
Methyl-α-galactose	0.0118	ND	0.0225
Stachyose	0.0142	ND	0.0208
BGBT	0.0203	ND	0.0803
Ceramide teihexoside	0.0261	ND	0.154

* µmole/min/mg enzyme product liberated from 3 replicates

ND - Not detectable

Adapted from Harun-Or-Rashid, M., et al., *Purification and Characterization of α-N-Acetylgalactosaminidases I and II from the Starfish Asterina amurensis.* Bioscience, Biotechnology, and Biochemistry, 2009. **74**(2): p. 256-261.

Uda et al. isolated an active α-NAGA from an acetone powder material from the limpet, *Patella vulgate* [51]. Purification of the protein was accomplished by ammonium sulfate precipitation and gel filtration chromatography. The enzyme was active as a tetramer, 200 kD, as determined by gel filtration chromatography. Enzyme assays were performed in 0.5 M sodium citrate buffer pH 4.0 resulting in the Michaelis-Menten kinetic parameters, K_M of 0.6 mM with *p*-nitrophenyl α-Nacetylgalactosaminide and 0.036 mM with Forssmann hapten glycolipid. Tuppy and Staudenbauer isolated an active α-NAGA from an acetone powder extract from the snail *Helix pomatia* [52]. Purification of the protein was accomplished by acetone precipitation, ammonium sulfate precipitation and gel filtration chromatography. Enzyme assays were performed

with a buffered solution at pH 5.4. Enzyme activity was evaluated with solutions of extracted glycoconjugates from ovarian cyst fluid and hog gastric mucosa. The enzyme demonstrated an 8 to 16 fold increase in blood group H activity as measured by hemagglutination tests. Kadowaki et al. isolated an active α-NAGA from the fungal group *Acremonium* [53]. Purification of the protein was accomplished by ammonium sulfate precipitation, hydroxyapatite, and gel filtration chromatography. The enzyme was active as a monomer, of 56 kD, as determined by gel filtration chromatography. Enzyme assays were performed in 50 mM sodium citrate buffer pH 4.5 resulting in the Michaelis-Menten kinetic parameter, K_M of 1.3 mM with *p*-nitrophenyl α-Nacetylgalactosaminide. Enzymatic conversion of RBCs was accomplished with a 4% RBC in PBS solution with 18 units (200 μg) of total enzyme in a 200 μL volume incubated at 37 °C for one hour. Results are shown in Table 3.4.

Table 3.4: Hemagglutination of α-N-acetylgalactosaminidase-Treated A-Type Blood Cells with Anti-A Serum and Anti-H Lectin

	Anti-A serum dilution					
	2	4	8	16	32	64
	Degree of agglutination					
No Treatment	4	3	2	1	1	0
α-NAGA treated	3	2	1	0	0	0
Trypsin Treated	4	4	3	2	1	0
Trypsin and α-NAGA Treated	2	1	0	0	0	0
	Anti-H lectin dilution					
	2	4	8	16	32	
	Degree of agglutination					
No Treatment	0	0	0	0	0	
0.5 U α-NAGA treated	2	2	1	1	1	
2 U α-NAGA treated	4	3	2	1	1	

The table represents the degree of agglutination of Type A RBCs with anti-A serum and anti-H lectin. 4 indicates strong agglutination and 0 no agglutination. Adapted from Kadowaki, S., et al., *Isolation and Characterization of a Blood Group A Substance degrading α-N-Acetylgalactosaminidase from an Acremonium sp.* Agricultural and Biological Chemistry, 1989. **53**(1): p. 111-120.

Weignerová et al. also isolated an active α-NAGA from the fungus, *Aspergillus niger* [54]. Purification of the protein was accomplished by gel filtration and ion chromatofocusing chromatography. The enzyme was active as a pentamer, 440 kD, as determined by gel filtration chromatography. Enzyme assays were performed in 50 mM citrate phosphate buffer pH 3.0 resulting in the Michaelis-Menten kinetic parameter, K_M of 0.73 mM with *o*-nitrophenyl α-Nacetylgalactosaminide.

Eukaryotic α-NAGAs have a few common attributes; the enzymes are contained within lysosomes lending to their optimal activity under acidic conditions. Inadequate substrate specificity of these enzymes places limitations for use in blood group

conversion. Each of the enzymes denoted activity against various matrices with a terminal α-GalNAc, with only one enzyme demonstrating activity in the presence of RBCs. Optimal *Acremonium* sp. enzyme concentration was not determined but the concentration utilized was elevated compared to many of the prokaryotic enzymes we will review.

α-N-acetylgalactosaminidase from Prokaryotic Sources

A concerted effort has been made to identify an α-NAGA source that exhibits the desirable characteristics, of neutral pH activity, high substrate specificity, and zero impact to RBC function, necessary to convert blood group type [55]. Hoskins et. al. isolated three isoforms of active α-NAGA from *Ruminococcus torques* strain IX-70 [56]. Purification of the protein was accomplished by ammonium sulfate precipitation, hydrophobic interaction, hydroxyapatite, and gel filtration chromatography. The enzyme isoforms were active as dimers, as determined by gel filtration chromatography (Table 3.5). Enzyme assays were performed in 20 mM ACES buffer pH 6.0 resulting in the Michaelis-Menten kinetic parameters shown in Table 3.6.

Table 3.5: *Ruminococcus torques* α-NAGA apparent molecular mass of isoforms IA, IB, and III, adapted from [56]

Molecular Mass kDa	IA	IB	III
Denaturing SDS-PAGE	158*	173*	III-PI: 201
	172	158	III-PII: 205
Non-denaturing PAGE	117	234	III-PII: 330
Average	135	229	III-PII: 295
Gel Filtration	265	417	III-PII: 530

* Dominant protein

Adapted from Hoskins, L.C., E.T. Boulding, and G. Larson, *Purification and Characterization of Blood Group A-degrading Isoforms of alpha -N-Acetylgalactosaminidase from Ruminococcus torques Strain IX-70.* J. Biol. Chem., 1997. **272**(12): p. 7932-7939.

Table 3.6: Properties of the α-NAGA isoforms;
Assayed using *p*-nitrophenyl-α-N-acetylgalactosaminide

	IA	IB	III-PII
K_M (mM)	2.2	5.8	4.8
V_{max} µmole/min/mg	78	115	97
pH optimum	5.8-6.8	5.8-6.8	5.6-6.1

Adapted from Hoskins, L.C., E.T. Boulding, and G. Larson, *Purification and Characterization of Blood Group A-degrading Isoforms of alpha -N-Acetylgalactosaminidase from Ruminococcus torques Strain IX-70.* J. Biol. Chem., 1997. **272**(12): p. 7932-7939.

Levy and Aminoff [57] and Hsieh et al. [58, 59] isolated active α-NAGA from *Clostridium perfringens*. Purification of the wild-type protein by each group was accomplished by ammonium sulfate precipitation, gel filtration chromatography, and ion chromatography. In the Levy and Aminoff publication, enzyme assays were performed in 10 mM sodium phosphate buffer pH 6.3 resulting in the Michaelis-Menten kinetic parameters shown in Table 3.7. Levy and Aminoff diluted 200 µL of packed RBC into 500 µL PBS with approximately 1.44 µg of enzyme and incubated the mixture for 30 minutes at 37 °C. Resulting in the apparent conversion of blood group A to O based on

hemagglutination data (Table 3.9). Hsieh et al. added two additional chromatographic steps during purification: hydroxyapatite and ion chromatofocusing chromatography. The NAGA gene was cloned into the pETBlue1 vector and the recombinant α-NAGA expressed in *E. coli* Tuner (DE3) pLacI [60]. The protein purification procedure was greatly simplified to two steps; ion and hydroxyapatite chromatography, resulted in expression of the recombinant enzyme. In the Hsieh et al. report enzyme assays were performed in 40 mM sodium phosphate buffer, BSA (1 mg/mL), and 1 mM DTT at pH 6.5 resulting in the Michaelis-Menten kinetic parameters shown in Table 3.8. Hsieh et al. demonstrated enzyme activity against RBC fragments of Blood Type A_2 [61].

Table 3.7: *Clostridium perfringens* Michaelis-Menten kinetic parameters

Substrate	k_M	V_{max} nmol/min
*p*NP-N-acetyl-α-D-galactosaminide	0.77	10.6
Asialo-ovine submaxillary mucin	0.44	2.9
A-active porcine submaxillary mucin	0.93	0.73
GalNAc→ Gal →GalNActitol ↑ Fuc	4.2	7.1
GalNAc→Gal→ GalNActitol ↑ ↑ Fuc NeuNGc	4.1	7.1

Adapted from Levy, G. and D. Aminoff, *Purification and properties of alpha-N-acetylgalactosaminidase from Clostridium perfringens.* J. Biol. Chem., 1980. **255**(24): p. 11737-11742.

Table 3.8: *Clostridium perfringens* α-NAGA Wild Type and Recombinant Enzyme Characteristics

	recombinant α-NAGA	native α-NAGA
K_M (mM)	1.10	1.35
Specific Activity U/mg	84.11	62.27*
pH optimum	6.5-7.0	6.5-7.0*

* Data from Levy and Aminoff

Adapted from Hsieh, H.-Y. and D. Smith, *Clostridium perfringens alpha-N-acetylgalactosaminidase blood group A2-degrading activity.* Biotechnology and Applied Biochemistry, 2003. **37**(Pt 2): p. 157-163.

Table 3.9: *Clostridium perfringens* α-NAGA Agglutination Titer Data from RBC Enzyme Conversion

RBC	Anti-Serum	Control	Enzyme Treated
A	Anti-A[1]	1:256	1:16
	Anti-H[2]	0	1:16
O	Anti-A[1]	0	0
	Anti-H[2]	1:4	1:4
B	Anti-A[1]	1:64	1:64
	Anti-H[2]	0	0

1; Human Anti-Serum

2; *Lotus tetragonolobus* lectin

Adapted from Levy, G.N. and D. Aminoff, *Purification and properties of alpha-N-acetylgalactosaminidase from Clostridium perfringens.* Journal of Biological Chemistry, 1980. **255**(24): p. 11737-11742.

Bakunina et al. isolated three forms of an active α-NAGA from the marine organism, *Arenibacter latericius* KMM 426^T [62]. Purification of the protein was accomplished by ion and gel filtration chromatography. Results from enzyme assays indicated only form IV had specificity to GalNAc substrates and were performed in 100 mM sodium phosphate buffer pH 7.2 resulting in the Michaelis-Menten kinetic parameter, K_M of 0.38 mM with *p*-nitrophenyl α-Nacetylgalactosaminide. RBCs were added to PBS pH 7.3 with

approximately 87 µg enzyme/mL of for 24 hours at 26 °C resulting in the apparent conversion of blood group A to O based on hemagglutination data (Table 3.10).

Table 3.10: Agglutination titer of donor A(II) erythrocytes before and after treatment with α-N-acetylgalactosaminidase IV

Monoclonal antibodies Solyclone-A	Agglutination titer								
	2	4	8	16	32	64	128	256	512
Anti-A + α-NAGA treated A RBCs	–	–	–	–	–	–	–	–	–
Anti-A + α-NAGA untreated A RBCs	+	+	+	+	+	+	+	+	+

Adapted from Bakunina, I.Y., et al., *α-N-Acetylgalactosaminidase from Marine Bacterium Arenibacter latericius KMM 426T Removing Blood Type Specificity of A-Erythrocytes.* Biochemistry (Moscow), 2002. **67**(6): p. 689-695.

A recent α-NAGA enzyme from *Elizabethkingia meningosepticum* by Liu et al. [49] appears to show many desirable traits for blood group conversion of RBCs. The NAGA gene was cloned into pET28 vector and the recombinant α-NAGA expressed in *E. coli* strain Rosetta (DE3) pLysS. Purification of the protein was accomplished by ion and hydrophobic interaction chromatography. Enzyme assays were performed in 100 mM $NaPO_4$, pH 6.8, 50 mM NaCl at 26 °C resulting in the Michaelis-Menten kinetic parameters shown in Table 3.11.

Table 11: Kinetic properties of the α-N-acetyl-galactosaminidase from *Elizabethkingia meningosepticum*

Substrate	*E meningosepticum* α-NAGA		
	K_M (mM)	**k_{cat} s^{-1}**	**k_{cat}/K_M ($s^{-1}mM^{-1}$)**
*p*NP-αGal	25.1 ± 0.001	0.026 ± 0.001	0.0011
*p*NP-βGal	3.6 ± 0.3	0.10 ± 0.000	0.0031
*p*NP-αGalNAc	0.077 ± 0.006	9.84 ± 0.16	127.6
*p*NP-βGalNAc	0.23 ± 0.01	0.015 ± 0.000	0.087

Data ≥ Average of 3 assays

Adapted from Liu, Q.P., et al., *Bacterial glycosidases for the production of universal red blood cells.* Nature Biotechnology, 2007. 25(4): p. 454-464.

The prokaryotic α-NAGAs listed have several common attributes; the enzymes demonstrate optimal activity under slightly acidic to neutral conditions, high degree of substrate specificity affording blood group conversion, and each of the enzymes demonstrated activity against various matrices with a terminal α-GalNAc with three of the enzymes demonstrating activity in the presence of RBCs [49, 62, 63]. Optimal *Elizabethkingia meningosepticum* enzyme concentration was determined for RBC blood group conversion based on a dose titration study, Table 3.12.

Table 3.12: Blood group typing of ECO RBCs with enzyme dose titrations from *Elizabethkingia meningosepticum*

			ABO Typing Results*							
			Ortho anti-A		ES-15		Ortho anti-B		Diagast anti-B	
RBCs	mg/mL α-NAGA treated pRBCs	mg/mL α-GAL treated pRBCs	IS	4 °C	IS	4 °C	IS	4 °C	IS	4 °C
α-NAGA treated A_1	0.73		0	0	0	0				
	0.6		0	0	0	0				
	0.5		0	0	0	0				
	0.37		0	0	0	0				
	0.23		0	0	Vw+	0				
	0.13		0	0	W+	W+				
	0.03		0	0	1+	1+				
α-NAGA/α-GAL treated A_1B	0.73	0.02	0	0	0	0	0	0	0	0
	0.6	0.01	0	0	0	0	0	0	0	0
	0.5	0.005	0	0	0	0	0	0	0	0
	0.37	0.0025	0	0	0	0	0	vw+	w+	w+
	0.23	0.0013	0	0	0	0	0	w+	w+	1+
	0.13	0.0006	0	0	vw+	w+	0	1+	1+	1+
	0.03	0.0003	1+	2+	2+	2+	vw+	2+	1+	2+

* Typing using licensed ABO typing reagents and methods using agglutination scores 0 to 4+. IS - immediate spin reactions. pRBCs - packed RBCs

Adapted from Liu, Q.P., et al., *Bacterial glycosidases for the production of universal red blood cells.* Nature Biotechnology, 2007. 25(4): p. 454-464.

Chapter 4: History and Status of Enzymatic RBC Blood Group Conversion

The history of altering the epitope landscape of an RBC can be traced back to the 1940s through the present. The historical advancements and knowledge are in approximate 20 year blocks. These blocks are comprised along the timelines of 1940-1960, 1960-1980, 1980-2000, and 2000 to the present. There were a number of discoveries during these timeframes, each having direct impact on subsequent scientists continually building upon each contribution.

The 1940-1960 period witnessed a significant amount of work on the blood groups in addition to the antigen architecture of the blood types. The sugars that encompass the blood group antigens were systematically identified by two main research groups, these of Kabat (US) and Morgan and Watkins (UK) [64, 65]. Tissues, e.g. hog stomach lining [66], and fluids, e.g. ovarian cysts [67], rich in blood group antigens were utilized to identify the specific sugar components due to difficulty to extract the antigen groups from RBCs. As technology capabilities improved, human red blood cell extractions were analyzed allowing identification of the blood group sugars [68]. The findings were that the antigen chains were identical when isolated from tissue or fluids [65]. During this same period of time, genetic factors of the blood groups were under investigation primarily evaluating inheritance of characteristics, traits, and disease [69, 70]. Several decades passed before the actual blood group genes were mapped. In 1911, the term

universal blood was conceived by Reuben Ottenberg [71] indicating that the blood Type O could be transfused into almost anyone. Experiments by Witebsky and Kleadshoj [72], mixed small volumes of Type A and/or B blood with Type O prior to transfusion to neutralize agglutination of the RBCs. This procedure was implemented with few adverse reactions. There were a number of observations of antigen degradation when extracts from micro-organisms were added to RBC, e.g. *Clostridium tertium* and *Trichomonas foetus* [73, 74]. This was the beginnings of enzyme blood conversion.

The technological advancement during the 1960-1980 period advanced the knowledge and understanding of the blood groups. With the advancements in analytical equipment the carbohydrate structure of the blood group antigens was elucidated [25, 40, 42, 65, 75-78]. Knowledge gained from these studies were used to enhance experiments with micro-organism extracts [79] followed by use of specific enzymes to convert RBC blood type [80]. The early studies involving extracts or partially purified enzymes, were used to evaluate the suppression of blood group agglutination by bacteria and/or viruses [79] or indiscriminate sugar hydrolysis by various bacterial extracts [80, 81]. An early study by Tuppy and Staudenbauer of α-NAGA from *Helix pomatia* [52] was one of the first to indicate some level of specificity towards α-linked N-acetylgalactosamine, the terminal sugar of Type A RBC. Another α-NAGA, derived from *Patella vulgata* by Uda et al. [51], also indicated activity and specificity to terminal α-linked N-acetylgalactosamine oligosaccharides. Further characterization of these two enzymes did not progress because of optimal activity at a low pH outside of the desirable RBC pH range of 6.8-7.4. Three studies by Harpaz, and Yatziv [82-84] using α-

GAL from the green coffee bean set in motion the coming research concerning conversion of blood group B to O. Huang and Aminoff [85] began work with an α-NAGA extract from *Clostridium perfringens* that would lead to additional work in his lab and others in future years.

The interval that has had the greatest impact in this field has been the period of 1980-2000. A pioneer in enzyme blood conversion, Jack Goldstein's contributions pushed the area of RBC conversion by enzymatic means to its current juncture. Starting in the early 1980s, Goldstein's group at the New York Blood Center worked with α-GAL from green coffee beans to convert blood Type B to O [86-88]. The α-GAL conversion process, Type B to O, required a significant amount of enzyme (~ 1-2 g) per unit of blood. It was not until 1995 that the gene for α-GAL was cloned from the green coffee bean allowing for more efficient methods to obtain the recombinant enzyme [89]. The blood conversion process using this enzyme was challenging; the enzyme is optimally active at a pH of 5.7, outside of the desired pH range in addition to low enzyme efficiency. The conversion of blood group B to O RBC was conducted by adding α-GAL to RBCs in isotonic sodium phosphate/citrate/chloride buffer pH 5.6 at 26 °C for 1.5-2 hours [86, 87]. Conversion of RBCs at pH 5.7 impacts cell membrane integrity causing a reduction in long term shelf life to approximately 21 days versus 40 days for non-converted cells [90]. A 1982 study by Goldstein et al. indicated that transfused RBC survivability *in vivo* was unchanged compared to non-converted RBC [86]. Appropriate planning of enzyme converted RBCs used within days of conversion would/should alleviate the shelf life concern. Progression of the research program to convert Type B to O RBC continued. These

studies led to the successful Phase 1 and 2 research program during this time period. In 1991, Lenny et al. using α-GAL extracted from green coffee beans successfully transfused converted B to O blood [91]. In 1994 and 1995, Lenny et al., successfully transfused full unit(s) of converted O blood into volunteers [92, 93]. No clinical reactions were observed in the volunteers. Evaluation of blood samples taken from these volunteers did not indicate any abnormal laboratory test results during the study. In 2000, Kruskall et al. successfully transfused converted O blood into 21 patients [94]. No adverse reactions were noted in the patients. Red blood cell survival was comparable between patients transfused with converted versus non-converted RBC in all but two patients, one patient had gastrointestinal bleeding at the time of the transfusion and another patient's serum was incompatible with the transfused red blood cells. Post transfusion monitoring of the patients noted that 5 of 19 developed an increased anti-B titer. There were no detectable antibody titers against the recombinant enzyme. These achievements provided a step forward but also raised additional concerns. What changes to the process would be necessary to lessen hemoglobin loss during conversion? Hemoglobin concentration decreased from approximately 58.3 to 51.8 g/dL post conversion which was thought to be related to the washing steps post conversion of RBCs. Two other concerns were the anti-B immune response and the incompatibility issue with serum. Continued investigation into enzymes having properties to hydrolyze the terminal galactose from group B RBC with functional activity at a neutral pH, increased substrate specificity and efficiency pressed forward [95-98]. Three additional α-GALs, originating from *Glycine max*,

Pseudoalteromonas, and *Clostridium sporogenes* indicated specific activity with higher levels of efficiency to the terminal galactose sugar than the green coffee bean but have not been developed.

The enzymatic conversion of blood group A to O during 1980-2000 did not advance similarly. A suitable α-NAGA with optimal properties to convert RBC had not been fully identified at this time. Several eukaryotic α-NAGAs were isolated and tested for potential use to convert blood group A to O [53, 99-103]. These enzymes met with limited success and did not progress due to lack of substrate specificity and/or optimal activity at an acidic pH outside of the desirable RBC pH zone of 6.8-7.4. Likewise, several prokaryotic α-NAGAs were evaluated during this time. An α-NAGA from *Clostridium perfringens* appeared to have favorable attributes for Type A to O blood conversion. Levy and Aminoff characterized this α-NAGA [57] using the purified enzyme to convert Type A RBC to O. Under physiologic conditions, RBC in PBS incubated with enzyme at 37 °C, Type A RBC were converted to O. Advancement of this enzyme was hampered due to the difficulty of expressing adequate enzyme and removing contaminant glycosidases. One additional α-NAGA originated from *Ruminococcus torques* [56]. Three isoforms of α-NAGA were isolated with two of the isoforms showing specificity to the terminal sugar of blood group A. Isoform IA has additional specificity to galactose compared to IB. Though the enzymes were active in the pH range of 6.8-7.4, the optimal pH range for these enzymes was 5.8-6.8.

Though challenges have existed to identify a robust α-NAGA, the period of 2000-present has identified several potential α-NAGA candidates that may efficiently convert A to O

blood [49, 61, 62, 104, 105]. Recent improvements in molecular biology techniques had greatly enhanced the ability to evaluate potential enzymes. The *Clostridium perfringens* NAGA gene was cloned and the recombinant protein expressed and purified to a high level of purity. The recombinant protein was characterized by Hsieh et al. [59]. It was demonstrated the recombinant enzyme was active with high specificity to the terminal sugar of blood group A at neutral pH against blood Type A_2 RBC fragments at room temperature [61]. Additional experimentation with this enzyme with intact RBC has not been performed. Another promising enzyme investigated originated from *Elizabethkingia meningosepticum* (*E. meningosepticum*). A recombinant protein was generated by the company Zymequest following an extensive RBC conversion screening program [49]. Approximately 2500 isolates were evaluated by Zymequest for α-NAGA activity. The *E. meningosepticum* enzyme was purified and characterized with specificity and activity at a neutral pH to the terminal sugar of blood group A. Enzymatic conversion of RBCs has been demonstrated with over 200 units of blood. A Phase 1 study was completed with results pending [106]. This is a safety study to evaluate approximately 60 healthy volunteers, 18-65 years of age, which received their own red cells that were treated with α-NAGA or washed with saline. The two groups were compared for the rate and extent of any adverse reactions or unexpected laboratory test results.

Chapter 5: Blood Substitutes

Alternatives to enzymatic blood conversion exist and are actively being pursued. Blood substitutes such as perfluorocarbons, hemoglobin-based oxygen carriers, and bonding RBC with polyethylene glycol are active areas of research. The majority of research is driven by the military in pursuit of an ideal solution that can be utilized on the battlefield. This ideal solution is still being sought.

History of Blood Substitutes

Blood substitutes have long been with us almost since 1628, when William Harvey published his views about the circulation system. Some of the first blood substitutes included wine, scammony, and opium that were administered to animals by Sir Christopher Wren in 1656 [15]. The first animal transfusion was attributed to Dr. Richard Lower in 1665. The first human transfusions have been attributed to Dr. Jean Baptiste Denys in 1667. Denys successfully transfused several patients until one died causing a moratorium on transfusions and related research in Europe. In 1818, Dr. James Blundell revived interest in transfusion medicine [107]. Following this renewed interest many discoveries were necessary to successfully transfuse blood [108]. One of the first materials used as a blood substitute was milk by several physicians. This was considered a major breakthrough because of the mixed results of transfusing blood. Results of using milk were as mixed with some patients remaining unreactive while others reacted and died from the transfusion [109]. In 1883, Sydney Ringer developed Ringer's solution. This was based on experimentation in frogs which found the solution

to be close to frog plasma. Eventually lactate was added to Ringer's solution (Hartmann and Senn) to prevent alkalosis in humans. The lactate is gradually converted to sodium bicarbonate in plasma. A blood substitute that received attention during World War 1 and into the 1930s was gum saline. First developed by Karl Ludwig, gum saline held promise as a plasma extender. In animal studies the gum was found to coat RBC and promote coagulation. The aggregation of gum is dependent on its concentration, pH, salts, and temperature. Quality and consistency of gum saline was inconsistent causing variability of osmotic pressure and viscosity leading to anaphylaxis and other side effects. Plasma and serum were being investigated as early as 1871, by Bowditch as a blood substitute. It was learned with time that the proteins contained within plasma and serum maintained osmotic pressure of the blood vessels far better than saline alone. There were occasional side effects, e.g. intravascular coagulation, associated with plasma or serum transfusion that was eventually linked to RBC surface antigens. Plasma and serum were used during World War 2, with some applications still in use today, but these have been greatly reduced due to advances in whole blood collection and storage [108].

Current Status of Blood Substitutes

Crystalloid solutions are the most common blood substitute used in the U.S. Colloid solutions are commonly used in Europe. Conflicting research results from numerous studies have not provided a clear understanding which solution type is superior if any exists [110-113]. The primary difference between crystalloid and colloid solutions is the addition of protein, e.g. albumin, or hetastarch in colloid solutions to regulate osmotic

pressure when infused due to the larger molecules. These larger molecules maintain a high percentage of the colloid solution in the intravascular space. Because of this, colloids will maintain a higher vascular pressure compared to crystalloids called colloid oncotic pressure. The primary use of these solutions is for volume replacement due to hypovolaemia. A number of variations of these solutions exist, Table 5.1 [114].

Table 5.1: Properties of resuscitation fluids

Solution	Sodium Content (mmol/L)	Potassium Content (mmol//L)	Chloride Content (mmol/L)	Osmolarity (mOsmol/L)	Ideal COP (mm Hg)	Mean Molecular Mass (kDa)	Substitution	Volume Increase/Duration
Plasma	134-146	3.4-5.0	98-108	280-300	25	-	-	-
5% Albumin	150	-	130	300	20	66.5	-	100% Variable
20% Albumin	-	-	-	1500	110	66.5	-	250% Variable
Physiological Saline	154	-	154	308	-	-	-	-
Lactated Ringer's	130	4	109	273	-	-	-	-
Hartmann's	131	5	111	275	-	-	-	-
Plasmalyte 148	140	5	98	294	-	-	-	-
7.5% Sodium chloride	1283	-	1283	2566	-	-	-	-
Haemaccel	145	-	145	300	27	35	-	80% 2 Hr
Gelofusine	154	-	125	279	27	35	-	80% 2 Hr
6% Dextran 70 in Saline	154	-	154	320	70	70	-	120% 1 Hr
HaesSteril 6%	154	-	154	308	32	200	0.5	100% 4 Hr
EloHaes 6%	154	-	154	308	30	200	0.62	199% 8-12 Hr
Heapan 6%	154	-	154	310	27	450	0.7	100% 24 Hr
HaesSteril 10%	154	-	154	308	72	200	0.5	145% 2 Hr
Pentaspan 10%	154	-	154	320	50	264	0.45	145% 2 Hr

Adapted from Sutcliffe, A.J., *Crystalloids and colloids for volume replacement.* Trauma, 1999. **1**(2): p. 115-123.

When transfusing these solutions the volume of material to transfuse versus blood loss can be significant. The conventional rule for crystalloids is 3:1, but experimental data

indicate that a 3:1 ratio is too low and that ≥ 7:1 is required to restore circulatory stability [108]. Close monitoring is necessary during transfusion of these solutions due to physiological reactions of the patient. Trauma, surgery, and loss of blood can invoke an immunological response. A neutrophil burst is typically triggered during trauma with hemorrhagic shock as can be similar to an infusion of lactated Ringer's solution [108, 115]. Many of the colloid solutions containing hydroxyethyl starch do not appear to cause the same neutrophil response [115, 116]. Care not to infuse an excess of crystalloid solutions has to be considered also. Crystalloid solutions are primarily distributed in the interstitial space which can lead to edema. Pulmonary and peripheral edema can occur causing a reduction in both O_2 uptake and healing [110], while a rapid infusion of crystalloids has been associated with hypercoagulability [108]. Colloids do not appear to negatively impact hemostasis with the exception of when large doses are utilized. Bleeding time may be of longer duration but does not impact prothrombin or activated partial thrombin times. There is negative research concerning hydroxyethyl starch relating to patients with brain injuries. Two safer solutions related to hydroxyethyl starch are pentastarch and pentafraction solutions. Both penta solutions have a better molecular weight consistency and colloid oncotic pressure than hydroxyethyl starch [114].

Hemoglobin-Based Oxygen Carrier

Hemoglobin is the RBC protein component that transports O_2 and CO_2 systemically. Hemoglobin is a tetramer composed of two α and β subunits [117]. Oxygen and carbon dioxide associate with and disassociate from hemoglobin which is facilitated by a

reversible electrostatic binding of 2,3 DPG. 2,3 DPG acts as an allosteric effector such that binding to hemoglobin decreases affinity for O_2 offloading to the tissues while CO_2 binds to the globin chains and accounts for 20% of expelled CO_2 [118]. The remaining transported CO_2 is enzymatically converted to bicarbonate. Once the blood cells have returned to the lungs where local conditions include high partial pressure of O_2 and pH combined with lower temperature causes dissociation of 2,3 DPG, releasing CO_2, followed by uploading of O_2 [118]. Crystalloids and colloids can replace volumetric loss of blood to maintain systemic pressure but have no effect on O_2 needs of the biological system. Investigation into HBOCs as a blood substitute has been around for almost 100 years [13]. Because hemoglobin is intimately involved in the O_2/CO_2 cycle, HBOCs have great potential. High hurdles impeding progress in this approach include early development of side effects, abdominal pain, short intravascular half-life, and nephrotoxicity [13, 16, 119]. Four immediate challenges that had to be addressed were the high affinity of hemoglobin for nitric oxide, auto-oxidation of the heme group to undesirable metabolites, the breakdown of the tetrameric structure to αβ dimers, and stroma contaminants from RBC. Improved purification techniques have eliminated the stroma contaminant problem while various linkers have been utilized to stabilize the hemoglobin tetramer [13, 118]. The challenge to reduce HBOC affinity for nitric oxide has been more complicated. Free hemoglobin reacts with nitric oxide ultimately resulting in hypertension. Doherty et. al. demonstrated that oxy- and deoxy-hemoglobin scavenge nitric oxide from the vasculature [120]. The group substituted select hydrophobic amino acids for larger hydrophobic amino acids at the distal heme

pockets of both subunits creating mutants that significantly reduced the reaction rate with nitric oxide [120]. A potential concern that is under investigation is the auto-oxidation of hemoglobin from the active ferrous form to the inactive ferric methemoglobin state [120]. Further oxidation of methemoglobin by hydrogen peroxide to the protein radical ferryl hemoglobin has raised concerns relating to the potential use of HBOCs [117, 121]. Two mutations in the heme pocket of myoglobin appear to convey resistance to the formation of ferryl hemoglobin [117, 122]. Several HBOC configurations have been investigated, e.g. cross-linked and conjugated tetramers, polymers, and liposomes, with varying results. Several HBOCs are in various stages of development (Table 5.2) with a few products being discontinued due to adverse events [118, 123, 124]. Challenges still remain because of the potential adverse effects of free hemoglobin. Though many of the difficult challenges have been recognized and overcome, many still exist slowing the progress of using these potential products as blood substitutes.

Table 5.2: HBOCs in development as red blood cell substitutes

Product	Hb source	Technology	Developer	Status
DCL-Hb (HemAssist)	Human red cells	α-α crosslinked Hb	Baxter Healthcare (Deerfield, IL, U.S.A.)	Phase III (trauma) (suspended)
RHb1.1/1.2 (Optro)	*E. coli*	Recombinant human αα fused Hb	Baxter Healthcare (Somatogen < 1998)	Phase II (discontinued)
RHb2.0	*E. coli*	Recombinant Hb	Baxter Healthcare	Preclinical
HBOC-201 (Hemopure)	Bovine red blood cells	Gultaraldehyde polymerization	Biopure (Cambridge, MA, U.S.A.)	BLA filed (elective surgery). Approved for clinical use in S. Africa
Human POE-Hb (PHP)	Human red cells	PEG conjugation	Curacyte (Apex) (Munich, Germany)	Phase III (septic shock)
Hb-raffimer (Hemolink)	Human red cells	Oligermerization with o-raffinose	Hemosol (Toronto, Canada)	Phase III (cardiac surgery) (suspended)
Pyridoxal polyHb (PolyHeme)	Human red cells	PLP-Hb polymerized Hb with glutaraldehyde	Northfield Laboratories (Evanston, IL, U.S.A.)	Phase III (trauma) Filed BLA
Hemospan	Human red cells	Conjugated with maleiimide PEG	Sangart (San Diego, CA, U.S.A.)	Phase II (elective surgery)
HemoZyme	Human red cells	Polynitroxylated Hb	SynZyme (Irvine, CA, U.S.A.)	Preclinical
PolyHb-SOD-CAT	Bovine red cells	Hb modified with SOD and catalase	McGill University (Montreal, Canada)	Preclinical
PEG-Hb	Bovine red cellls	PEG conjugated Hb	Enzon (Piscataway, NJ, U.S.A.)	Phase Ia (discontinued)
OxyVita	Human/bovine red cells	Stabilized Hb with sebacoly diaspirin	IPBL Pharm. (Goshen, NJ)	Preclinical
HemoTech	Bovine red cells	Modified Hb with o-ATP, o-adenosine, and glutathione	HemoBioTech (Amarillo, TX, U.S.A.)	Preclinical

Adapted from Kim, H.W. and A.G. Greenburg, *Artificial Oxygen Carriers as Red Blood Cell Substitutes: A Selected Review and Current Status.* Artificial Organs, 2004. 28(9): p. 813-828.

Perfluorocarbons

Perfluorocarbons (PFCs) are linear or (poly)cyclic hydrocarbons that substitute fluorine atoms for hydrogen [125]. Most individuals will recognize two common PFCs, Teflon® and Gore-Tex® that are used extensively in cookware and clothing, respectively. Common characteristics of PFCs are their stability, inertness, in addition to their hydrophobic and lipophobic nature [126]. There are no known enzymatic processes that degrade PFCs in nature. The ability of PFCs to dissolve high concentrations of O_2 and CO_2 [126, 127] plus the inherent characteristics makes these compounds very attractive as a blood substitute. Perfluorocarbons are not miscible in plasma therefore it is necessary to emulsify PFCs prior to infusion [123]. One of the commonly used emulsifiers in PFC formulas is egg yolk phospholipids. Phospholipids, such those from egg yolk, are biocompatible with good stability characteristics having a long history of use in the pharmaceutical industry. PFCs have demonstrated value as a blood substitute but have encountered similar problems as HBOCs concerning stability issues, reactions to treatment involving flu like symptoms, and additional adverse events have hampered regulatory approval and wide spread use. Table 5.3 lists several PFCs that are under investigation or approved for use as blood substitutes. Additional investigations are ongoing in the areas of molecular imaging, ultra sound, magnetic resonance imaging, and site-directed therapeutic drug delivery [128-130].

Table 5.3: Perfluorcarbons in Development and Status

Product	Developer	Perfluoro compounds	Status
Fluosol-DA	Green Cross Corp. (Osaka, Japan)	Perfluorodecalin, Perfluoropropylamine	Approved for clinical use for perfusate for angioplasty in 1989. Withdrawn from market in 1994.
Oxygent	Alliance Corp. (San Diego, CA, U.S.A.)	Perfluorooctyl bromide (perflubron)	Phase II/III (suspended)
S-9156	Sonus Corp. (Seattle, WA, U.S.A.)	Dodecafluoropentane (DDFP)	Preclinical. Stabilized microbubbles for ultra small volume resuscitation
PHER-O2	Sanguine Corp. (Pasadena, CA, U.S.A.)	Similar to Fluosol-DA?	Preclinical?
Perftoran	Perftoran (St. Petersburg, Russia)	Perfluorodecalin, Perfluoromethyl-cyclohexylpiperidin	Approved by Russian Ministry of Health for clinical use (1999)
Oxycyte PFC	Synthetic Blood International (Kettering, OH, U.S.A.)	N/A	Phase I
Oxyflour	HemaGen (St. Louis, MO, U.S.A.)	Perfluorodichloro octane	Phase II

Adapted from Cohn, C.S. and M.M. Cushing, *Oxygen Therapeutics: Perfluorocarbons and Blood Substitute Safety.* Critical care clinics, 2009. 25(2): p. 399-414 and Lowe, K.C., *Blood substitutes: from chemistry to clinic.* Journal of Materials Chemistry, 2006. 16(43): p. 4189-4196.

PEG Coated RBC

Bound RBC with PEG is a strategy to mask the surface antigens from immune system recognition. PEG is a polymer of ethylene oxide composed structurally of $HO\text{-}CH_2\text{-}(CH_2\text{-}O\text{-}CH_2\text{-})_n\text{-}CH_2\text{-}OH$ with a molecular weight range of 100 to 10,000,000 g/mole in linear, branched, or star configurations [131, 132]. Early work in the 1970s demonstrated that PEG 1900 or 5000 bound to bovine liver catalase did not trigger an immunological response [133]. This eventually led to Jeong and Byun's demonstration that RBCs bound to PEG 5000 significantly reduced antibody response to RBC surface antigens [134]. Hortin et. al. corroborated Jeong and Byun's work using RBCs bound to PEG 3350 demonstrating reduced immune reaction to treated RBCs [135]. These initial projects demonstrated the concept that pegylated RBCs could cloak the cells from an immunological attack by the recipient. The two linking reagents used to bond PEG and RBCs in these projects were cyanuric chloride (CN) and N-hydroxysuccinimidyl ester of methoxypoly (ethylene glycol) propionic acid (SP). Three issues arose from these studies; the amount and formulation of PEG needed to adequately mask the RBCs, stability of the pegylated RBCs and decreasing pegylated RBC immunogenicity. To increase stability of pegylated RBCs Huang et al. bound RBCs to SP-PEG 20,000 cross-linked with albumin followed by additional binding with CN-PEG 5,000. This process increased RBC stability from hours to days [8]. Second generation PEG formulations [136], e.g maleimidophenyl-PEG in the presence of 2-iminothiolane [12], appear to have succeeded in identifying a process to mask RBC and to negate RBC immunogenicity *in vivo*. As these challenges seem mastered, a new concern has become evident whereby

detection of PEG antibodies are raising questions [136-138]. Środa et al. in 2005, demonstrated induced PEG antibody generation within 2 weeks in a rabbit model following weekly injections for 6 weeks [139]. Armstrong et al. demonstrated PEG antibody generation in human studies while testing PEG-RBC conjugates, > 25% occurrence in healthy blood donors, and PEG-asparaginase cancer therapies [137, 138]. The clinical relevance of these PEG-induced antibodies has yet to be determined. One of the potential promises of PEG-RBCs involved patients that receive blood transfusions at a frequent interval that have developed allosensitivity [140]. If some of the concerns related to PEG-masked RBCs can be overcome there exists a potential niche for use of this technology.

Blood substitutes do have potential value when circumstances require their use. Currently approved blood substitutes are limited in number. Safety concerns are slowing progress on HBOCs, perfluorcarbons, and PEG-masked RBCs. Some of these concerns may be overcome at some point in time but until then alternatives such as enzyme-converted RBCs have great potential.

Chapter 6: Materials and Methods

Materials

The *Escherichia coli* strain CFT073 was obtained from Michael Donnenberg, University of Maryland at Baltimore. *Spirosoma linguale* #33905, was purchased from the American Type Culture Collection, Rockville, Maryland. Chromatography columns (HisTrap, Q Sepharose, and Zorbax GF-250), SDS-PAGE materials (gels, buffers, and protein markers), QuickChange® Site-Directed Mutagenesis Kit (Stratagene), Western Blot materials (Nitrocellulose Bio Trace™ Pall Life Science, buffers, and developing reagent), BCA and Bradford protein determination solutions, BSA standards, restriction enzymes, the competent cells *E. coli* CD41 (DE3), TEV protease, the pET22b vector, fluorescent tagged antibodies, Hampton Research crystallography screens were obtained from Fisher Scientific, Waltham, Massachusetts. Chromatography and assay reagents, and microbiological media were obtained from Fisher Scientific, Waltham, Massachusetts or VWR Scientific, Radnor, Pennsylvania. Glycosidase conjugates and gel filtration molecular weight markers were obtained from Sigma-Aldrich Company, St. Louis, Missouri. DNA primers were obtained from Integrated DNA Technologies, Coralville, Iowa. The pCR®-Blunt vector and the competent cells *E. coli* DH5α and BL21 (DE3), and gel chromatography column were obtained from Invitrogen, Carlsbad, California. The pPAL7 vector and gel chromatography standard were obtained from Bio-Rad, Hercules, California. The pKA8H vector was obtained from Jack Tanner, University of Missouri.

Gene amplification was performed using an Eppendorf Mastercycler® pro thermal cycler, Hauppauge, New York. Protein purification was accomplished with an ÄKTAprime™ plus chromatography system from GE Healthcare, Piscataway, New Jersey. Packed RBCs were obtained from Biological Specialty Corporation, Colmar, Pennsylvania. Gel chromatography was performed on a Shimadzu LC-10 system, Columbia, Maryland. Protein and enzyme assays were performed on a FLUOstar Omega plate reader from BMG LABTECH, Cary, North Carolina. Flow cell cytometry was performed on an Attune® Acoustic Focusing Cytometer, Carlsbad, California.

Methods

Cloning

***E. coli* strain CFT073 α-NAGA**

The cloned *E. coli* (Ec) NAG gene comprised 1821 of the 1998 nucleotides from the locus c1174. DNA primers were designed to incorporate *Nco*I and *Xho*I or *Nde*I and *Bam*HI restriction enzyme sites for subcloning of the target gene.

E. coli α-NAGA Primers

pET22b Forward Primer
*Nco*I Restriction site (GTGTCTCATAGTGCAA**CCATGG**ATAACTATACGTTCAATAAC)

pET22b Reverse Primer
*Xho*I Restriction site (CCCGGG**CTCGAG**GTTAATGGTCACCGCCTCAAACACC)

pKA8H Forward Primer
*Nde*I Restriction site (GTGTCTCATAGTGCA**CATATG**GATAACTATACGTTCAATAAC)

pKA8H Reverse Primer
*Bam*HI Restriction site (CCCGGG**GGATCC**TTAGTTAATGGTCACCGCCTCAAACAC)

The α-NAGA gene was amplified by PCR with the following cycling parameters 94 °C for 60 seconds, 52 °C for 60 seconds, 72 °C for 60 seconds increasing 10 seconds per cycle for 35 cycles then maintained at 4 °C at completion. The amplified gene products were initially ligated into pCR®-Blunt vector overnight in a water bath incubated at 16 °C. The ligation product was transformed into *E. coli* DH5α and plated on LB Agar containing kanamycin (40 μg/mL) then incubated overnight at 37 °C. Following incubation, a single colony was picked and grown in LB medium supplemented with kanamycin (40 μg/mL). These cultures were incubated at 37 °C overnight and shaken at 250 RPM in an incubator-shaker for further plasmid preparation. A double restriction digest of the plasmid with the appropriate enzymes incubated at 37 °C for approximately 2 hours was performed to extract the target gene construct and was gel purified using a 0.8% (w/v) agarose gel. The *Nco*I/*Xho*I purified construct was ligated into the vector pET22b as similarly described and transformed into the *E. coli* strain DH5α to amplify the plasmid number and plated on LB Agar containing ampicillin (50 μg/mL) then incubated overnight at 37 °C. Following incubation, a single colony was picked and grown in LB medium supplemented with ampicillin (50 μg/mL). The *Ec* α-NAGA pET22b plasmid was extracted and the cloned gene was confirmed by sequence analysis by the University of Missouri DNA Core Facility. The *Ec* α-NAGA pET22b plasmid was transformed into the expression strain *E. coli* BL21(DE3) or *E. coli* CD41(DE3) and plated on LB Agar containing ampicillin (50 μg/mL) then incubated overnight at 37 °C. The insert containing pET22b vector was used to produce a soluble protein whereby the 59 N terminal residues were replaced by the *pelB* leader sequence from *Erwinia chrysanthemi* so that the

recombinant protein expressed in *E. coli* is exported to the periplasmic space. This leader sequence is removed by a signal peptidase once the protein occupies the periplasmic space. Therefore the predicted first six residues of the mature recombinant protein are NYTFNN. The *Nde*I/*Bam*HI purified construct was ligated into the vector pKA8H following the same process as described. The pKA8H vector codes for an N-terminal 8×His affinity tag and a tobacco etch virus protease site. The *Ec* α-NAGA pKA8H plasmid was transformed into the expression strain *E. coli* BL21(DE3) or *E. coli* CD41(DE3) and plated on LB Agar containing ampicillin (50 µg/mL) then incubated overnight at 37 °C. To evaluate enzyme activity presence singly picked colonies were placed in 10 mL of LB broth and 50 µg/mL ampicillin then incubated at 37 °C with continuous shaking at 200 RPM overnight. Two mL samples were removed and the cells concentrated into a pellet. The cells were resuspended with 500 µL PBS then 10 µL was removed to a 96 well plate containing 180 µL PBS. Ten µL of 100 mM *p*NP-α-N-Acetylgalactosaminide was added then incubated up to 30 min. Periodic visual observations were made to determine the presence of enzyme activity demonstrated by the colorimetric change to yellow. The addition of 100 µL 500 mM glycine solution pH 10 was added to stop the reaction and enhance the colorimetric appearance.

***S. linguale* α-NAGA**

The ATCC lyophilized *S. linguale* sample was resuspended in 1 mL sterile water. 10 mL of *Spirosoma* medium (equal parts glucose, tryptone, and yeast) was inoculated and incubated at 25 °C with continuous shaking at 200 RPM overnight. Genomic DNA was extracted with the Promega Wizard Genomic DNA Purification Kit. The entire coding

sequence of locus Slin 6637 a predicted α-N-Acetylgalactosaminidase was cloned from *S. linguale*. DNA primers were designed to incorporate *Nde*I and *Bam*HI restriction enzyme sites for amplification of the target gene as described above.

S. linguale α-NAGA Primers

pKA8H Forward Primer
*Nde*I Restriction site (CCCGGG**CATATG**CCTTCGCTGTACACGACCGCTCAG)

pKA8H Reverse Primer
*Bam*HI Restriction site (CCCGGG**GGATCC**TCAGTACACGTCGGTAAGGCCAAAGATGGG)

The *Nde*I/*Bam*HI purified construct was ligated into the vector pKA8H following the same process as described earlier. The *Sl* α-NAGA pKA8H plasmid was transformed into the expression strain *E. coli* BL21(DE3) or CD41(DE3) and plated on LB Agar containing ampicillin (50 μg/mL) then incubated overnight at 37 °C as described. In addition a site-directed mutant of *S. linguale* α-NAGA gene in which the purported residue that protonates the leaving group in conjunction with NAD^+, His225, was changed to Alanine (H225A) designed for the purpose of evaluating enzyme activity.

S. linguale α-NAGA H225A Mutation Primers

Forward Primer
GATCTCTACCCCACG**GCC**GGTCTGGGGCCGGTG

Reverse Primer
CACCGGCCCCAGACC**GGC**CGTGGGGTAGAGATC

The α-NAGA gene mutantion was amplified by PCR using the following cycling parameters 94 °C for 30 seconds, 55 °C for 60 seconds, and 68 °C for 7.5 minutes for 20 cycles then maintained at 4 °C at completion. The mutation was introduced into the aforementioned plasmid using the QuickChange® Site-Directed Mutagenesis Kit. The

cloned genes were confirmed by sequence analysis by the University of Missouri DNA Core Facility.

Protein Expression and Purification

The α-NAGA proteins were expressed using the auto-induction methodology of Studier [141]. Single colonies of the transformants were picked to inoculate four 1000 mL culture made of 1% (w/v) tryptone, 0.5% (w/v) yeast extract, 50 mM phosphate, 50 mM ammonium chloride, 5 mM sodium sulfate, 2 mM magnesium sulfate, 1x trace minerals, 0.5% (v/v) glycerol, 0.05% (w/v) glucose, 0.2% (w/v) lactose, and ampicillin (50 µg/mL). The culture was incubated at 37 °C with constant shaking at 300 RPM, overnight. Cells were harvested approximately 20 hours later by centrifugation at 4 °C and 2100 x *g* and resuspended in 20 mM Tris 150 mM NaCl pH 7.5 or 20 mM sodium phosphate 0.5 M NaCl pH 7.0. The cell pellet was frozen at -20 °C for later use. Frozen cells were thawed at 4 °C followed by disruption using sonication. Unbroken cells and debris were removed by centrifugation at 4 °C for 60 min at 31,000 x *g*. Additional cellular debris was removed by ultra-centrifugation at 4 °C for 60 min at 183,960 x *g*. Purification of the pET22b plasmid expressed protein was accomplished through nickel-chelate chromatography (HisTrap) using a loading buffer of 20 mM sodium phosphate 0.5 M NaCl pH 7.0. Elution of the protein was achieved using a linear gradient up to 2 M imidazole equilibrium buffer. Fractions were collected and the most enzymatically active samples combined and dialyzed in a 50 mM Tris-HCl pH 8.5 buffer. The dialyzed sample was further purified by Q Sepharose anion exchange chromatography using a loading buffer of 50 mM Tris-HCl pH 8.5. Elution of the protein was achieved using a

linear gradient up to 1 M NaCl equilibrium buffer. Fractions were collected and the most active samples combined and dialyzed in a 20 mM sodium phosphate pH 7.0 buffer. Purity of the protein was assessed by SDS-PAGE, gel filtration chromatography, MALDI-TOF mass spectrometry by the Proteomics Core University of Missouri, and protein crystals. Purification of the pKA8H plasmid expressed proteins was accomplished through nickel-chelate chromatography (HisTrap) using a loading buffer of 20 mM Tris 0.15 M NaCl pH 7.5. Elution of the protein was achieved using a linear gradient up to 1 M imidazole equilibrium buffer. Fractions were collected and the most enzymatically active samples combined with 1,000 units of TEV protease and dialyzed in a 20 mM Tris 0.15 M NaCl pH 7.5 buffer. The dialyzed sample was loaded into a nickel-chelate chromatography (HisTrap) column using a buffer of 20 mM Tris 0.15 M NaCl pH 7.5. Fractions were immediately collected and combined. The purified protein was dialyzed into 20 mM Tris pH 7.5. The respective protein was aliquoted into thin-walled PCR tubes, quick-frozen in liquid nitrogen, and stored at -70 °C. Purity of the protein was assessed by SDS-PAGE, gel filtration chromatography, MALDI-TOF mass spectrometry by the Proteomics Core University of Missouri, and protein crystals.

Enzyme assays

A fixed enzyme dilution with a substrate concentration range approximately ±5 (K_M) was employed to determine the Michaelis-Menten parameters. A BMG FLUOStar Optima plate reader equipped with a 410 nm reference filter was used to collect data. VisualEnzymics 2010 software was used to model the Michaelis-Menten factors. An

assay of 80 µL of 20 mM sodium phosphate pH 7.0, 10 µL each of eleven substrates (10 mM), and 10 µL of dilute α-NAGA (10 µg) were tested for relative activity;

4-Nitrophenyl N-acetyl-α-D-galactosaminide,

4-Nitrophenyl β-D-glucopyranoside,

4-Nitrophenyl α-D-galactopyranoside,

4-Nitrophenyl α-L-arabinopyranoside,

4-Nitrophenyl N-acetyl-α-D-glucosamine,

4-Nitrophenyl N-acetyl-β-D-galactosaminide,

4-Nitrophenyl α-D-glucopyranoside,

4-Nitrophenyl α-D-mannopyranoside,

4-Nitrophenyl β-D-galactopyranoside,

4-Nitrophenyl α-L-fucopyranoside,

4-Nitrophenyl N-acetyl-β-D-glucosamine.

Determination of the Michaelis-Menten constants was completed with 4-Nitrophenyl N-acetyl-α-D-galactosaminide. A fixed amount of diluted enzyme per substrate concentration assay was performed using a continuous UV-VIS detection method. The assay utilized 80 µL of 20 mM sodium phosphate pH 7.0 or 7.4, 10 µL of the substrate dilution and the automated addition of 10 µL of dilute enzyme (10 µg) maintained at room (~ 22 °C) or 37 °C temperature. Enzyme activity related to solution effect was evaluated. Sodium phosphate, sodium acetate, bicine, sodium citrate, phosphate buffered saline, Tris buffered saline, EDTA, glycine, and citrate phosphate dextrose solutions were used in the enzymatic assay as described. Assays to determine the pH

optimum of the enzyme for 4-Nitrophenyl N-acetyl-α-D-galactosaminide were performed in 20 mM sodium phosphate in the pH range 6.0-8.0, 50 mM sodium acetate in the pH range 3.5-6.0, or 100 mM bicine pH 9.0. The assays were performed as previously described using the appropriate buffer solution, substrate and enzyme dilution. Assays to determine temperature response of the enzyme for 4-Nitrophenyl N-acetyl-α-D-galactosaminide were performed in 20 mM sodium phosphate pH 7.4 using a temperature range 20-45 °C in increments of 5 °C. The assays were performed as previously described using the buffer and substrate solution at the desired temperature with the automated addition of dilute enzyme. Product inhibition assays were performed to determine the effect of N-acetyl-galactosamine accumulation. The assays were performed at room temperature (~22 °C) in 70 µL of 20 mM sodium phosphate pH 7.0 buffer, 10 µL of the substrate dilution, 10 µL of N-acetyl-galactosamine at concentrations of 0.05, 0.1, 2.5, 5.0, or 10.0 mM, and the automated addition of 10 µL diluted enzyme.

Protein Determination

Protein concentrations were determined using bichinchoninic acid (BCA Protein Assay Reagent), ThermoFisher (Rockford, IL) [142] or the Bradford assay (Bradford Ultra™ Coomassie-based protein quantitation method), Expedeon (Harston, Cambridgeshire) [143]. Diluted BSA standards ranging from 12.5 – 2000 µg/mL and unknowns were setup on a 96 well plate as described in the assay pamphlets. Samples were analyzed on a FLUOstar Omega plate reader using UV-VIS reference filters 562 and 595 nm respectively.

Crystallization of CFT073 α-NAGA

Alpha NAGA was expressed and purified as previously described. Sample concentration was assessed with the bicinchoninic acid (BCA) method and further concentration was done by using a 50 kDa cutoff amicon devices. Typically protein concentration 5-6 mg/mL was used for setting up the crystallization trays. Various Hampton Research screens, Index, PEG/Ion, Detergent, and Silver Bullet, were used for setting up crystallization experiments. Initial crystallization trials included sitting drop vapor diffusion method by mixing 2 μL of protein solution and an equal amount of reservoir solution. Satisfactory crystallization conditions found in solutions containing 0.2 M ammonium sulfate, 0.1 M Bis-Tris pH 6.5 and 20% (w/v) PEG 3350. This condition was optimized and diffraction quality crystals were obtained in 0.1-0.2 M ammonium sulfate, 0.1 M Bis-Tris pH 6.0-6.5 and 18-23.5 (w/v) PEG 3350. Note that this condition was obtained when the protein was dialyzed overnight in 20 mM phosphate buffer, pH 7.0. Crystals of α-NAGA were cryoprotected using 23-28 % (w/v) PEG 3350, 0.1-0.2 M ammonium sulfate, 0.1 M Bis-Tris pH 6.0-6.5 and 25% PEG 200 and plunged into liquid N_2 for low temperature data collection.

Chromatography Methods

Sample analysis by gel filtration chromatography was done as follows: approximately 500 μL purified protein sample was pipetted into a chromatography vial. The chromatography vials were loaded onto a sample tray and placed in the auto injector. The sequence used for data acquisition included a mixture of five proteins (standard) plus samples from each recombinant protein. The proteins in the standard solution

included β-amylase, alcohol dehydrogenase, albumin, carbonic anhydrase, and cytochrome C. A Shimadzu LC-10A system was used perform the protein analysis. The system was equipped with a Zorbax GF-250, 4 micron particle size, 4.6 X 250 mm (internal diameter X length) column for assays. The column was maintained at a temperature of 30 °C. A TBS (50 mM Tris 150 mM NaCl) pH 7.4, mobile phase flowing at 1.0 mL per minute was used during the analyses. A UV detector set at 280 nm wavelength was used to detect the proteins. Chromatographic data were collected with TotalChrom software version 6.2.1.0.104.0104. The molecular mass determination of unknown proteins was made by comparing the ratio of Ve/Vo for the protein in question to the Ve/Vo of protein standards of known molecular mass (Ve, elution volume and Vo, void volume). A calibration curve was generated by plotting the logarithms of the known molecular mass of each protein standard versus their respective Ve/Vo value. The Proteomics Center at the University of Missouri-Columbia performed mass spectrometry analysis of the protein samples. Sample analysis by MALDI TOF MS chromatography was done as follows: approximately 500 μL each of purified protein sample and Sinapinic acid matrix solution (20 mg/mL 500/470/30 by volume ACN/water/10% TFA) was pipetted into a polished stainless steel target. The sample was stirred with a pipette tip until crystals appeared. The crystals which formed were washed twice with 2-3 μL 0.1% (v/v) aqueous trifluoroacetic acid (4 °C). The crystals were allowed to dry between washings. MALDI TOF MS spectra were acquired from an AB Sciex Voyager DE Pro system. The system was operated in the positive ion, linear, delayed-extraction mode at 25,000 V accelerating potential with a 91% extraction

grid voltage. Extraction delay times were adjusted to optimize the resolving power (extraction delay time was 550 nsec and 900 nsec for the mass ranges 20,000-65,000 and 30,000-85,000 respectively) for the ions of interest. The spectra results were summed/averaged of the number of 512-laser shot spectra for each sample. Samples were desorbed/ionized with a 20-Hz 337-nm nitrogen laser. An external calibration was performed which bracketed the expected mass of the unknowns. Enolase I (monomer $[M+H]^+$ 46,671.9 Da) and alpha-chymotrypsinogen (dimer $[M+H]^+$ 51,313.2 Da) were used to calibrate at the 20,000-65,000 mass range while an alpha-chymotrypsinogen and mixed dimer of enolase I and alpha-chymotrypsinogen ($[M+H]^+$ 72,328.0 Da) were used to calibrate the system at the 30,000-85,000 mass range. Spectra were analyzed with Data Explorer software version 4.0.0.0.

Protein Immunological Methods

Rabbit Anti-rEc α-NAGA antiserum was obtained using standard immunological methods. Laboratory animals were utilized with approval of the University of Missouri Animal Care and Use Committee in accordance with the *Guide for the Care and Use of Laboratory Animals* [144]. Purified recombinant *E. coli* α-NAGA (~250 µg) was dialyzed against 0.9% (w/v) NaCl, filter-sterilized, and emulsified in Freund's complete adjuvant. The immunogen was then injected subcutaneously at multiple sites into a New Zealand white rabbit. Approximately twenty-four days after primary immunization, the response was boosted by single subcutaneous injection in Freund's incomplete adjuvant. Serum was collected by ear puncture fourteen days following secondary immunization. The observed rabbit anti-rEc α-NAGA serum IgG titers, were assessed with an ELISA assay

described previously [145]. For Western blot detection, rEc α-NAGA was first separated by SDS-PAGE (4-12% Gradient Gel) and transferred to a Nitrocellulose Bio Trace™ membrane in an Owl Bandit Blot System (100 mA for 6 Hr) [146]. Rabbit anti- rEc α-NAGA IgG polyclonal antibodies were used as primary antibodies and detected with goat anti-rabbit IgG (H+L) conjugated to alkaline phosphatase.

RBC Enzyme Conversion Methods

The initial proof of concept experiment to evaluate enzyme activity *in vivo* was set up as follows: 1 mL of human RBCs in glycine buffer (200 mM pH 6.8) [49] with 100 µg of enzyme maintained at room temperature (~22 °C). These samples were evaluated with standard ABO typing solution. Conversion of RBC followed many of the experiments that were performed during the *in vitro* characterization of the enzyme(s). *In vivo* evaluation of the enzyme(s) included enzyme dose titration, kinetic activity, buffer system, PCV, pH, temperature, product inhibition, and reverse enzyme activity. Blood group A_1 and A_2 RBC were used in the enzyme dose titration and temperature experiments the remaining experiments; were completed with A_2 RBC. With the exception of the PCV experiment, each test was conducted with 20-30% PCV. All experiments were completed with 0.5 or 1 mL of sample. All samples (except kinetic activity samples) were inverted to mix every 15 minutes to minimize RBC aggregation with 10 µL aliquots removed at 1 and 2 hours and placed in 200 µL of PBS to discontinue enzyme activity. Samples were inverted to mix prior to each aliquot collection for the kinetic activity experiment. Several buffer solutions were evaluated: glycine, alanine, lysine, and PBS. Glycine, alanine, and lysine were tested at 150-300 mM concentrations

with 50 mM increments. Phosphate buffer was evaluated at 5 mM in addition phosphate buffer was evaluated with added NaCl concentrations (ionic strength) ranging from 0-20 mM NaCl concentrations in 5 mM increments. Each assay used 100 µg of enzyme maintained at room temperature. A 250 mM glycine solution was used to evaluate enzyme activity at various pHs. The solutions were pH adjusted and ranged from 5.5 – 8.0 in 0.5 pH increments. Each assay used 100 µg of enzyme maintained at room temperature. Samples containing 25, 50, 75, and 100 percent PCV were tested to evaluate the impact of red cell concentration on enzyme activity. Each assay used 100 µg of enzyme maintained at room temperature. An enzyme kinetic experiment composed of samples collected at 0, 1, 5, 10, 15, 30, 45, 60, 75, 90, 105, and 120 minutes post addition of α-NAGA was completed with 100 µg of enzyme. Enzyme dose titration experiments were completed with varying α-NAGA concentrations at both refrigerated (~ 4 °C) and room temperature (~ 22 °C) conditions. Enzyme concentrations tested were 0, 1, 5, 10, 20, 25, 30, 40, 50, 60, 70, 75, 80, 90, 100, 250, 300, 350, 400, 450, 500, or 1000 µg. The product, N-acetyl-galactosamine, was utilized at concentrations of 10, 50, or 100 mM and was tested with Blood group O to evaluate reverse enzyme activity using 250 µg of protein. Inhibition of enzyme activity was evaluated with blood group A_1 RBC infused with 500 µg α-NAGA. The experiment included a sample under the conditions outlined, a sample that at 1 hr was centrifuged and the supernatant removed then replaced with fresh buffer, and a sample that had 1X additional buffer added after 1 hr. All samples were evaluated by Flow Cytometry.

RBC Stability

Enzyme converted RBCs were compared to non-converted RBCs to evaluate their long term stability. RBCs were converted as described using 100 µg of enzyme with one group at room temperature and a second group refrigerated. The cells were washed with PBS then aliquoted into separate tubes for analysis on Days 0, 1, 7, 14, 21, 28, 35, 42, 49, and 56. Non-converted cells in PBS were aliquoted similarly. At each time point the samples were analyzed using a Siemens Advia 120 hematology system. Controls were analyzed before and after sample analysis to establish the instrument was operating within specifications. A complete RBC profile was collected which included RBC count, hemoglobin, hematocrit, MCV, MCHC, CHCM, CH, RDW, and HDW.

Flow Cytometry Method

To determine antigenic conversion of RBC each sample was evaluated by Flow Cytometry after enzymatic conversion. Samples were stained with mouse Anti-H FITC, mouse Anti-A, and goat Anti-mouse PE monoclonal antibodies. The initial staining included 1 µL Anti-H FITC and 1 µL Anti-A with 10 µL RBC and 50 µL PBS. The samples were mixed and incubated for 20 minutes in the dark at room temperature. An additional 200 µL PBS was added at the end of the incubation period and the samples centrifuged for 5 minutes at 550 x *g*. The supernatant was removed and the pellet was washed 2 additional times with 200 µL PBS. 1 µL of the PE antibody was added to each sample with 50 µL PBS mixed and incubated in the dark for 20 minutes at room temperature. The samples were washed as described with a final volume of 200 µL PBS added. 150 µL of each sample was diluted into 3 mL of PBS followed by analysis; 10,000

events were collected at a flow rate of 100 µL per minute, on the Attune® Acoustic Focusing Cytometer. Unstained, FITC, and PE stained compensation samples were analyzed prior to RBC analysis.

RBC Immunological Methods

Nine New Zealand White rabbits were challenged with Type A, O, or ECO-A RBC. Laboratory animals were utilized with approval of the Merial Institutional Animal Care and Use Committee, APS 11-99M-09/12. On Day 0 and 21 each rabbit was challenged as indicated in Table 6.1. Preparation of challenge material was performed the day of challenge, Day 0 treatments were emulsified in Freund's adjuvant while Day 21 was emulsified in Incomplete Freund's.

Table 6.1: Rabbit Challenge Treatment Schedule

Group	Vaccine	Dose Volume	Route of Administration	Frequency of Administration	No. of Animals
1	Type A_1 blood group	500,000 RBC/~1mL	Subcutaneous	2 doses, Day 0 and 21	3
2	Type O blood group	500,000 RBC/~1mL	Subcutaneous	2 doses, Day 0 and 21	3
3	Type A_1-ECO blood group	500,000 RBC/~1mL	Subcutaneous	2 doses, Day 0 and 21	3

On Day 0, rabbits were bled for baseline antibodies and immunized with the test antigens in CFA. The rabbits were observed for adverse reactions to the challenge. The rabbits were boosted with the test antigens in IFA on Day 21. Blood samples were collected from the ear on Day 28. All rabbits were anesthetized with acepromazine prior to blood collections. Blood samples were collected into serum tubes, processed into serum, and kept frozen at ≤20 °C. Samples were analyzed against blood groups A

and O for reactivity to antibodies by Flow Cytometry. Undiluted serum samples were also analyzed for induced antibody response to *S. lingulae* α-NAGA by Western Blot.

Chapter 7: Results

Identification of α-NAGA

A BLAST sequence search against the *C. perfringens* encoded α-NAGA identified several potential enzymes that may have activity to alpha linked N-acetylgalactosamine. Within the identified sources a uropathogenic *E*. *coli* strain indicated the presence of an unidentified encoded protein with potential α-NAGA activity. An analysis of uropathogen CFT073 *E. coli* c1174 and the deduced amino acid product indicated an open reading frame encoding a protein with similarity to the α-NAGA from *C. perfringens*.

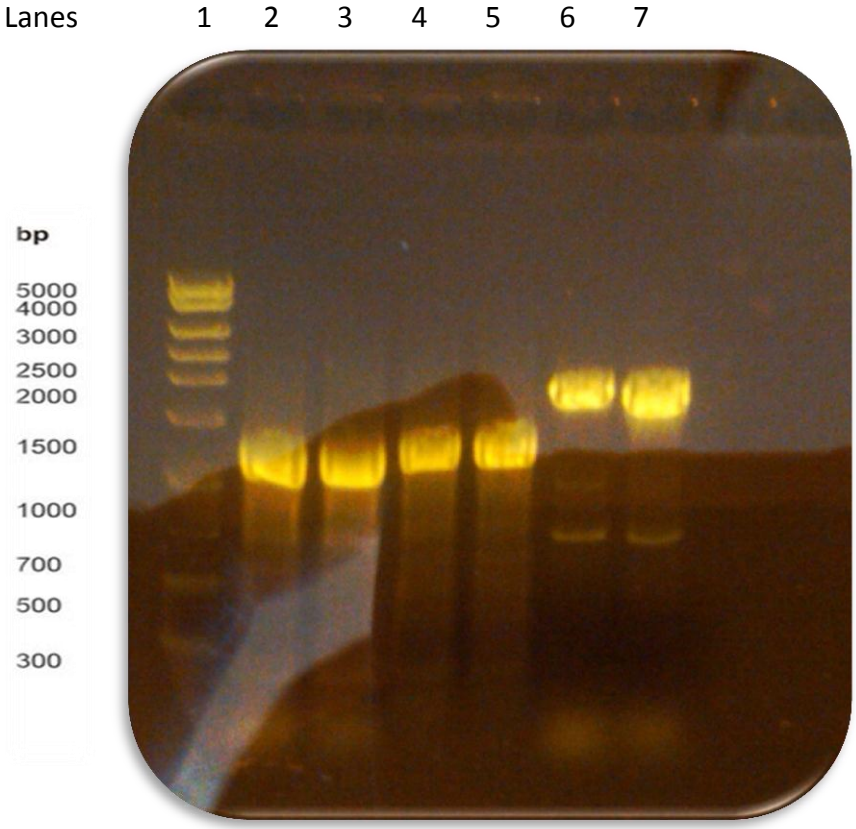
A BLAST sequence search against the *E. meningosepticum* encoded α-NAGA identified several potential enzymes that may have activity to alpha linked N-acetylgalactosamine. Within the identified sources an environmental organism *S*. *linguale* indicated the presence of a potential α-NAGA sequence. An analysis of *S*. *linguale* Slin 6637 and the deduced amino acid product from *S*. *linguale* strain ATCC 33905 indicated an open reading frame encoding a protein with similarity to the α-NAGA from *E. meningosepticum*.

Cloning of α-NAGA

The cloned *E. coli* α-NAGA gene comprised 1821 of the 1998 nucleotides from the locus c1174. The entire coding sequence of locus Slin 6637 was cloned from *S. linguale*. Amplification of the target NAGA gene from *E. coli* and *S. linguale* appear as indicated in

Figure 7.1. Bands in lanes 2-5 are consistent with NAGA gene (1305 bases) from *S. linguale* while bands in lanes 6 and 7 are consistent for the NAGA gene (1821 bases) from *E. coli*. To transform the amplified constructs into an *E. coli* expression stain a two-step procedure as outlined in the Methods section was followed whereby the constructs were ligated into pCR®-Blunt vector and transformed into the *E. coli* strain DH5α. The pCR®-Blunt plasmid was purified from an overnight culture followed by a double restriction digest, gel purification, and ligation into the vector pKA8H which were transformed into the *E. coli* expression strain BL21(DE3). Resulting transformants tested positive for α-N-acetylgalactosaminidase activity using a *p*NP conjugate as described in the Methods.

Figure 7.1: Amplification of NAGA Gene

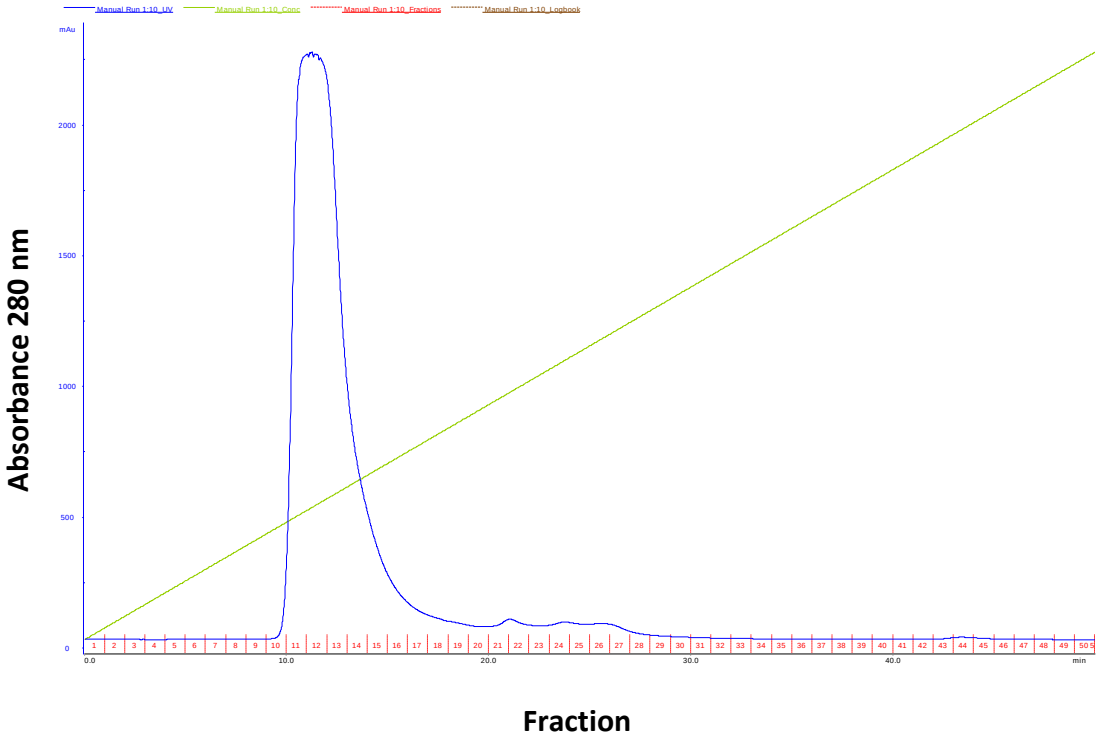


Agarose gel electrophoresis of PCR amplified α-NAGA gene: Lane 1 DNA ladder, Lanes 2-5 *S. linguale* NAG gene, Lanes 6-7 *E. coli* NAG gene

Protein Expression and Purification

Expression of the proteins was achieved using the complex auto-inducing media ZYM-5052 method by Studier [141] as described. Harvested cells were ruptured by sonication releasing the expressed recombinant protein followed by centrifugation at 31,000 x *g* resulting in the collection of the protein rich supernatant. The supernatant was loaded onto a chromatography column extracting the target protein by nickel-chelate column chromatography. The target protein was eluted using an imidazole gradient, 2 mL fractions were collected (Figure 7.2).

Figure 7.2: Nickel-chelate Column Protein Purification Chromatogram



The fractions containing the protein of interest, 10-18, were pooled and dialyzed in TBS. The expressed protein included a TEV protease cleavage site to facilitate removal of the histidine tag. The protein was incubated with TEV protease and gently rocked overnight to remove the tag. The protein was again loaded onto the chromatography column and fractions 3-11 collected (Figure 7.3). Protein purity was accessed by SDS-PAGE (Figure 7.4). Table 7.1 summarizes the protein purification steps, activity, and yield of the enzymes.

Figure 7.3: TEV Purification Chromatogram

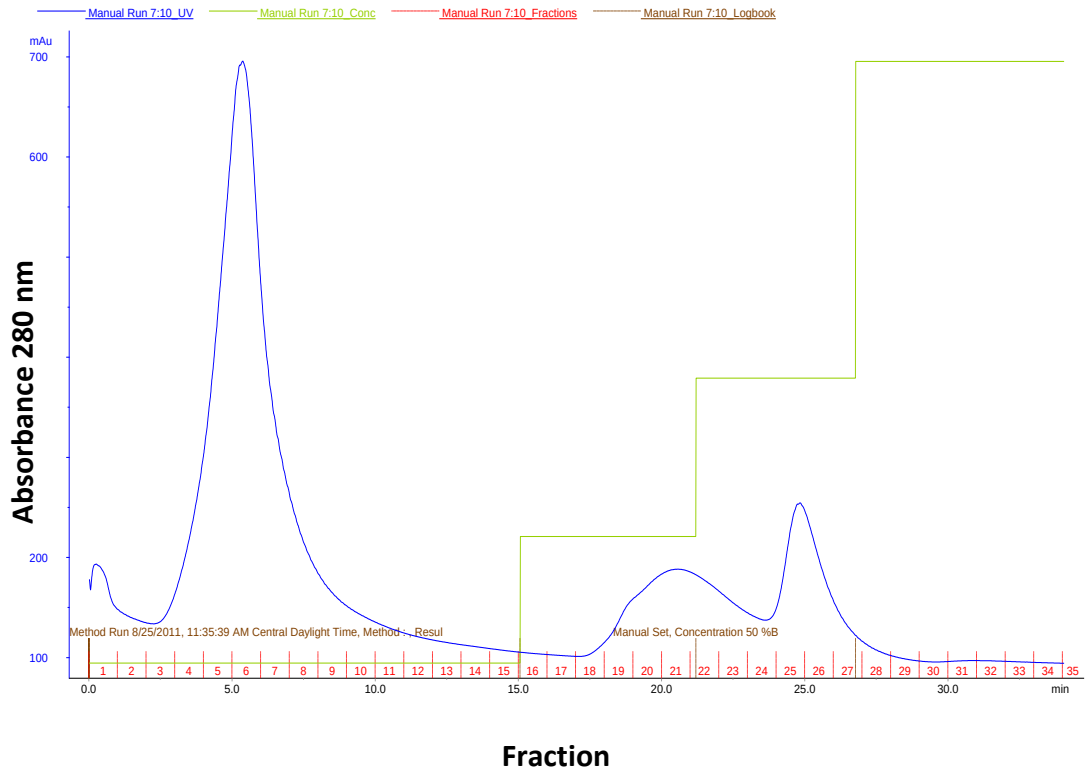
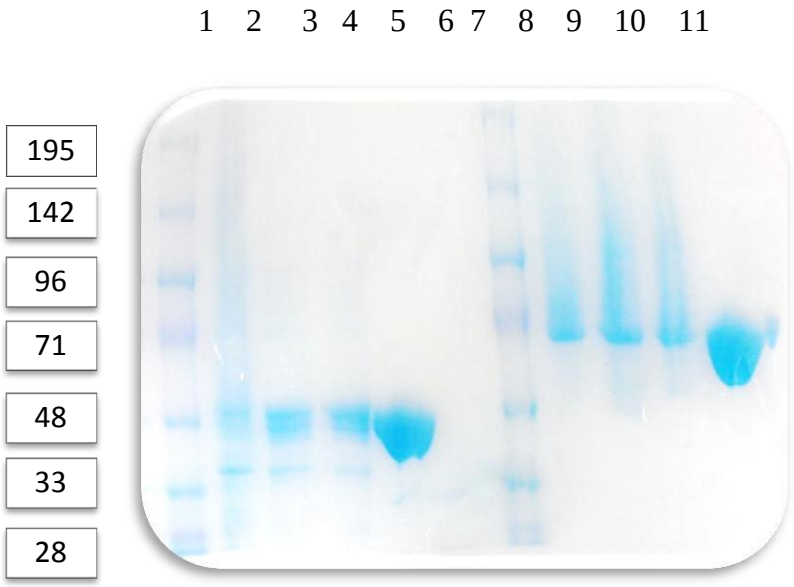


Figure 7.4: SDS-PAGE Gel of *E. coli* and *S. linguale* α-NAGA



SDS-PAGE gel electrophoresis: Lane 1,7 Protein standard markers (kDa), Lanes 2-5 *S. linguale* α-NAGA Lanes 8-11 *E. coli* α-NAGA (Post cell Disruption, Low Speed Centrifugation, High Speed Centrifugation, purified protein, respectively per enzyme)

Table 7.1: Summary of α-NAGA Purification

***S. linguale* α-NAGA**

	Volume (mL)	Total Protein (mg)	Activity µmole N-Acetylgalactosamine Liberated/s	Total Activity µmole N-Acetylgalactosamine Liberated/s	Specific Activity µmole N-Acetylgalactosamine /s	Fold Purification	% Yield
Post Sonication	60	6515	66	3987	0.612	1.00	100
Low Speed Centrifugation Supernatant	50	2285	73	3656	1.60	2.61	92
High Speed Centrifugation Supernatant	45	1785	76	3440	1.93	3.15	86
HisTrap Chromatography	11	57	84	882	16	25	22

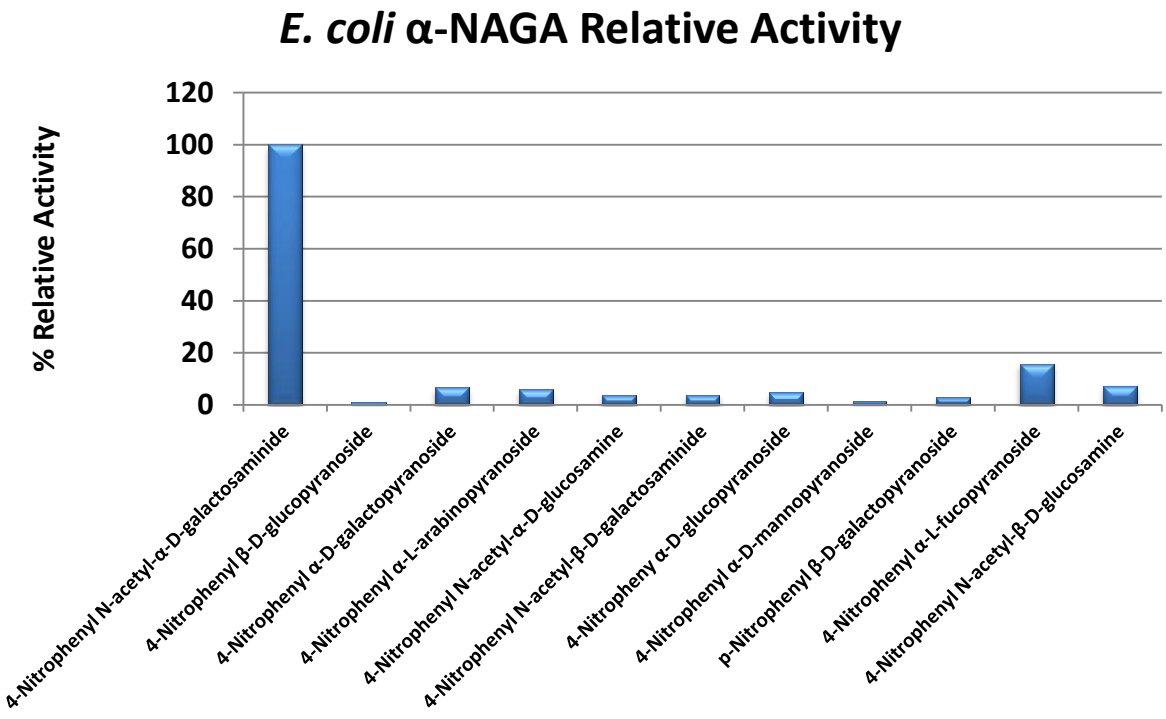
***E. coli* α-NAGA**

	Volume (mL)	Total Protein (mg)	Activity µmole N-Acetylgalactosamine Liberated/s	Total Activity µmole N-Acetylgalactosamine Liberated/s	Specific Activity µmole N-Acetylgalactosamine /s	Fold Purification	% Yield
Post Sonication	75	8753	9	687	0.079	1.00	100
Low Speed Centrifugation Supernatant	55	2262	10	571	0.253	3.22	83
High Speed Centrifugation Supernatant	40	2105	13	538	0.256	3.26	78
HisTrap Chromatography	15	87	26	397	4.6	58	58

Enzyme Characterization

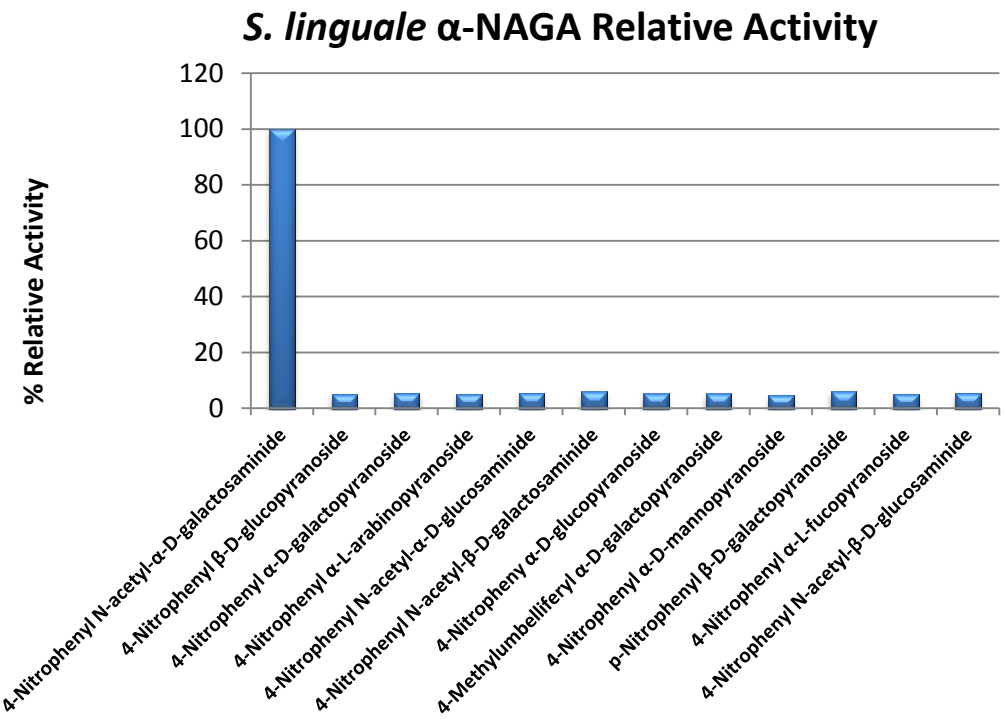
The enzyme assays characterizing α-NAGA proteins from *E. coli* and *S. linguale* included demonstration of substrate specificity and steady state kinetics with variable substrate concentrations, pH, temperatures, and buffers. The assays were performed with 80 µL buffer, 10 µL conjugate substrate, and 10 µL enzyme in 96 well plates consisting 100 µL total volume per well. Activity of the enzyme was demonstrated with *p*NP-O-R' conjugated sugars, R' representing the respective sugar tested. A BMG FLUOStar Optima plate reader with a 410 nm reference filter was used to determine absorbance data. VisualEnzymics 2010 and/or Origin Pro v8 software was used to calculate the Michaelis-Menten factors. Relative enzyme activity based on absorbance response of α-NAGA showed the highest activity for 4-Nitrophenyl N-acetyl-α-D-galactosaminide (Figure 7.5 and 7.5) compared to ten additional substrates tested for enzyme activity. Using Nonlinear regression analysis no relevant measurable enzymatic activity was demonstrated for the following substrates: 4-Nitrophenyl β-D-glucopyranoside, 4-Nitrophenyl α-D-galactopyranoside, 4-Nitrophenyl α-L-arabinopyranoside, 4-Nitrophenyl N-acetyl-α-D-glucosamine, 4-Nitrophenyl N-acetyl-β-D-galactosaminide, 4-Nitrophenyl α-D-glucopyranoside, 4-Nitrophenyl α-D-mannopyranoside, p-Nitrophenyl β-D-galactopyranoside, 4-Nitrophenyl α-L-fucopyranoside, and 4-Nitrophenyl N-acetyl-β-D-glucosamine.

Figure 7.5: Relative *E. coli* α-NAGA Activity on Carbohydrate Substrates



Substrate specificity of *E. coli* α-NAGA. Recombinant enzyme was incubated with each the indicated substrates and assessed for glycosidase activity by measuring the amount of glycoside produced as described under Materials and methods. 4-Nitrophenyl N-acetyl-α-D-galactosaminide, 4-Nitrophenyl β-D-glucopyranoside, 4-Nitrophenyl α-D-galactopyranoside, 4-Nitrophenyl α-L-arabinopyranoside, 4-Nitrophenyl N-acetyl-α-D-glucosamine, 4-Nitrophenyl N-acetyl-β-D-galactosaminide, 4-Nitrophenyl α-D-glucopyranoside, 4-Nitrophenyl α-D-mannopyranoside, p-Nitrophenyl β-D-galactopyranoside, 4-Nitrophenyl α-L-fucopyranoside, and 4-Nitrophenyl N-acetyl-β-D-glucosamine. Results are presented as percent activity relative to the amount of glycoside released from the glycoside-catalyzed hydrolysis of *p*NP-glycoside.

Figure 7.6: Relative *S.linguale* α-NAGA Activity on Carbohydrate Substrates



Substrate specificity of *S. linguale* α-NAGA. Recombinant enzyme was incubated with each the indicated substrates and assessed for glycosidase activity by measuring the amount of glycoside produced as described under Materials and methods. 4-Nitrophenyl N-acetyl-α-D-galactosaminide, 4-Nitrophenyl β-D-glucopyranoside, 4-Nitrophenyl α-D-galactopyranoside, 4-Nitrophenyl α-L-arabinopyranoside, 4-Nitrophenyl N-acetyl-α-D-glucosamine, 4-Nitrophenyl N-acetyl-β-D-galactosaminide, 4-Nitrophenyl α-D-glucopyranoside, 4-Nitrophenyl α-D-mannopyranoside, p-Nitrophenyl β-D-galactopyranoside, 4-Nitrophenyl α-L-fucopyranoside, and 4-Nitrophenyl N-acetyl-β-D-glucosamine. Results are presented as percent activity relative to the amount of glycoside released from the glycoside-catalyzed hydrolysis of *p*NP-glycoside.

Several pH adjusted solutions were tested to determine enzyme activity efficiency (Figure 7.7 and 7.8). Each solution tested was close to neutral pH 7.0, though some solutions were outside of their buffering capacity. Enzyme activity was highest in phosphate solutions with the exception of *E. coli* α-NAGA activity in bicine solution.

Figure 7.7: Relative *E. coli* α-NAGA Activity vs. Solution

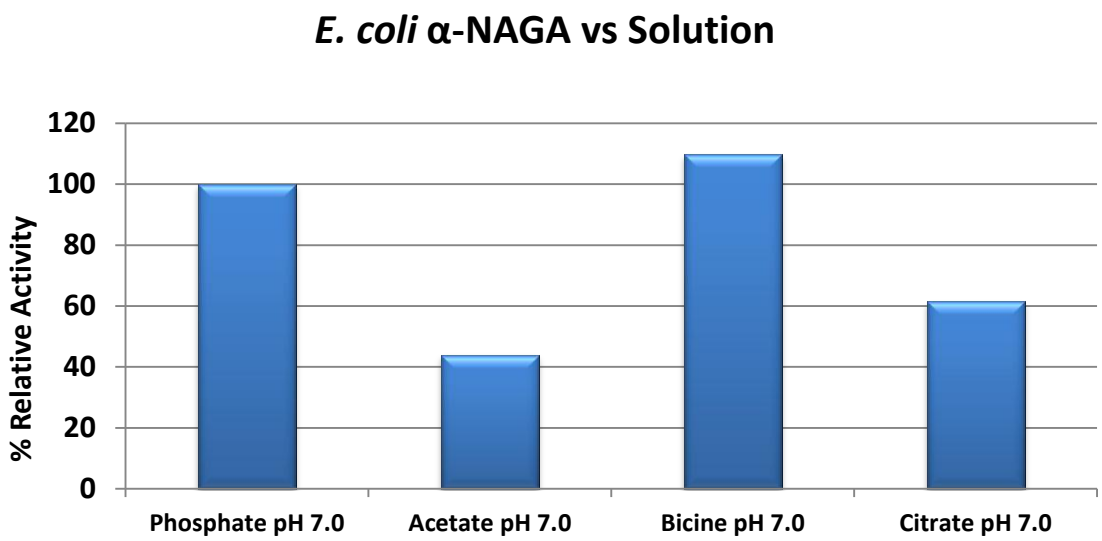
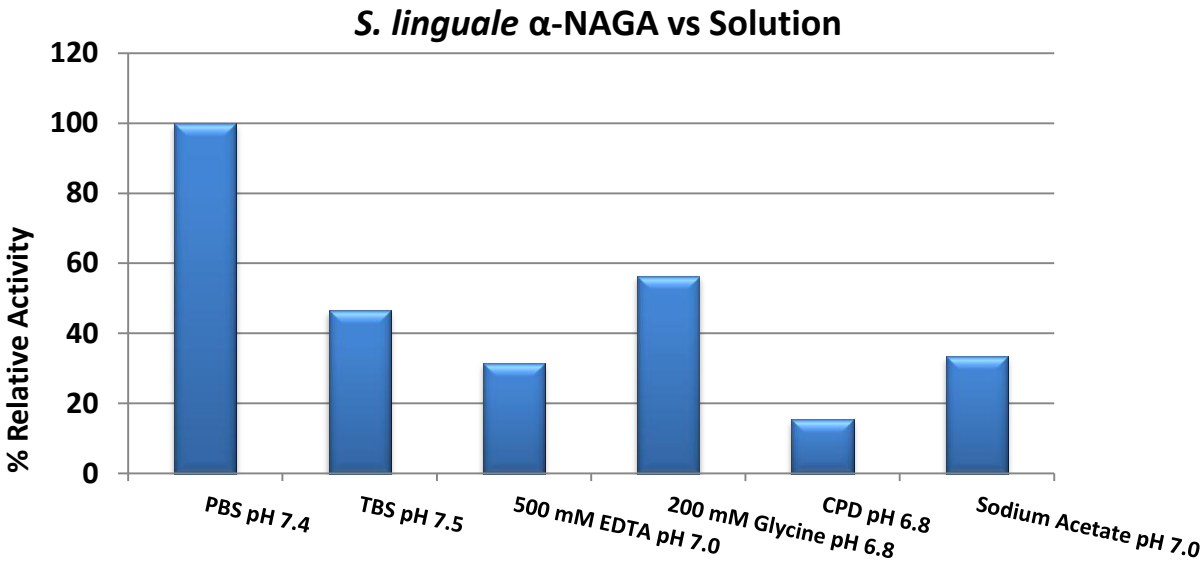


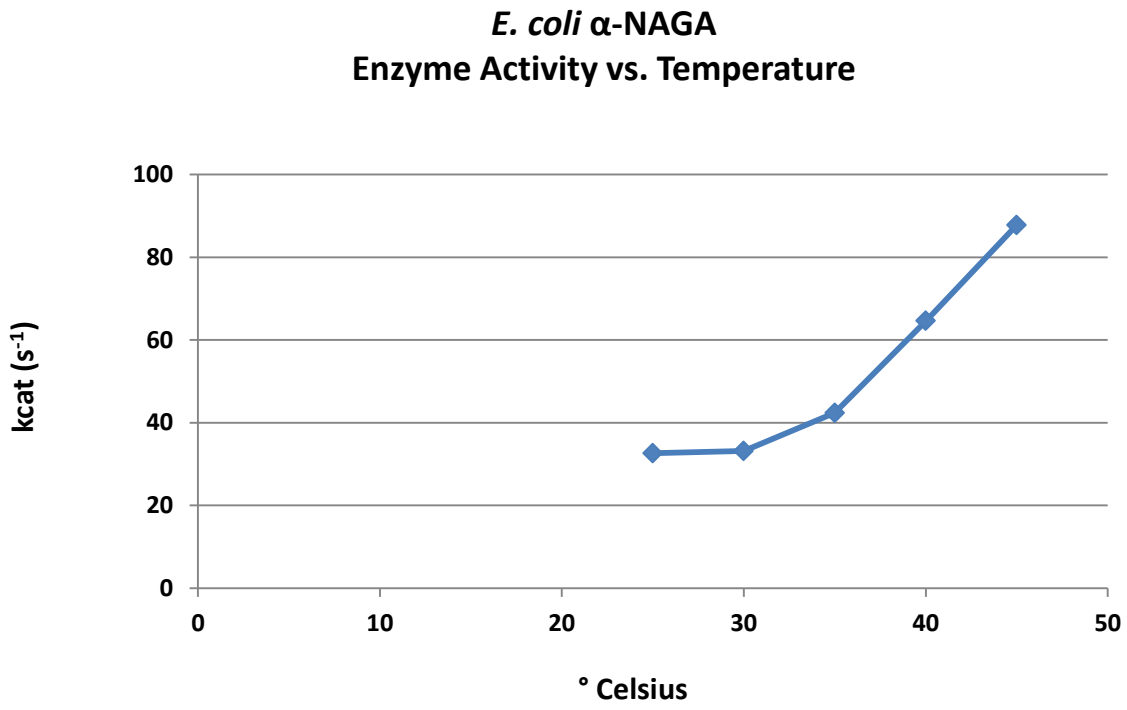
Figure 7.8: Relative *S. linguale* α-NAGA Activity vs. Solution



For both purified recombinant proteins enzyme activity responded to temperature variances as shown in Figures 7.9-7.13. As the temperature was increased the *E. coli* enzyme activity increased rapidly (Figure 7.9). In contrast, the *S. linguale* enzyme activity decreased with increased temperature (Figure 7.10). Eyring plots show both

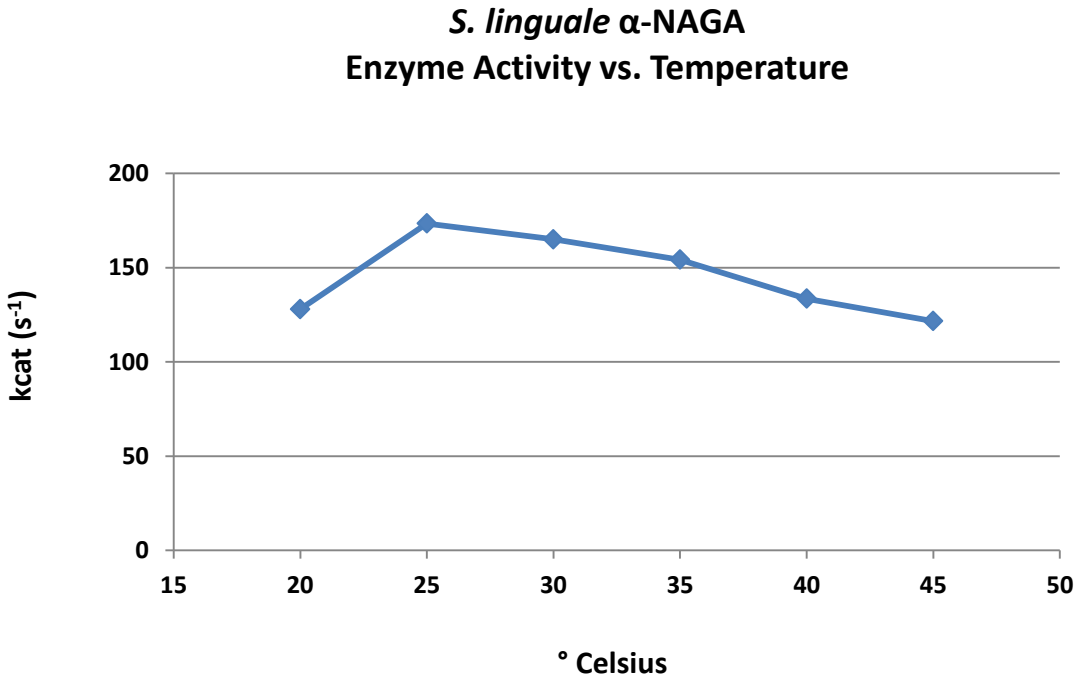
enzymes had a linear response to temperature change in opposite directions (Figure 7.11 and 7.12). The Eyring equation is a theoretical model based on the transition state that describes the temperature dependence of the reaction rate [147]. *S. linguale* enzyme activity had a linear response in the direction of lower temperature. To evaluate this response the enzyme activity was compared at 4 °C and 25 °C. Enzyme activity was approximately 25% lower at 4 °C (Figure 7.13).

Figure 7.9: *E. coli* α-NAGA Activity vs Temperature



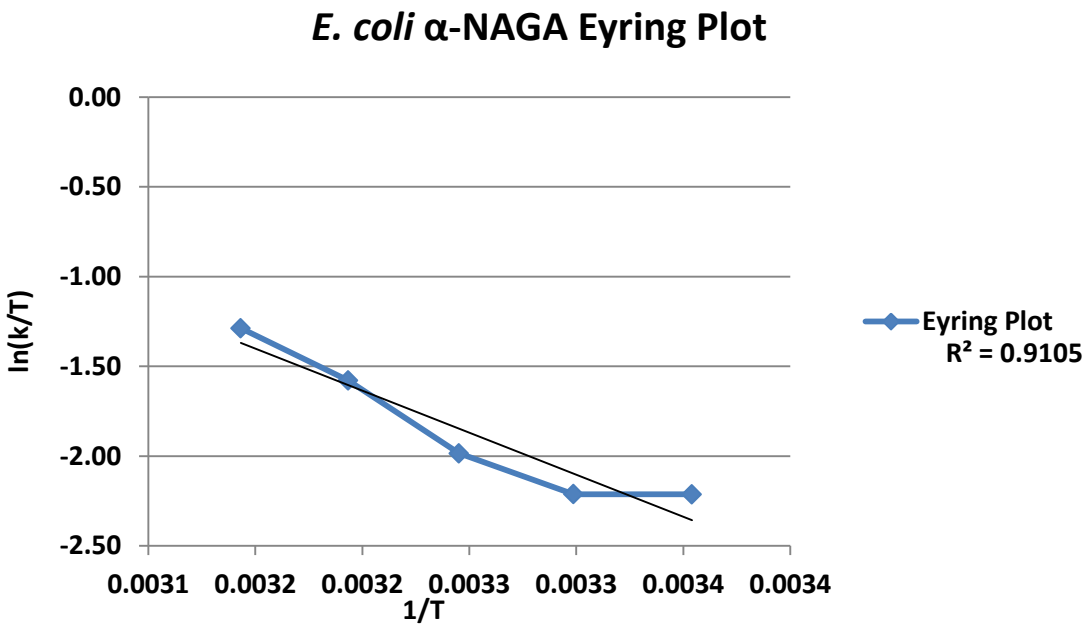
Influence of temperature on the activity of the *E. coli* recombinant enzyme preparation. For the temperature profile, activity was measured in 20 mM sodium phosphate buffer (pH 7) at different temperatures.

Figure 7.10: ***S. linguale*** **α-NAGA Activity vs Temperature**



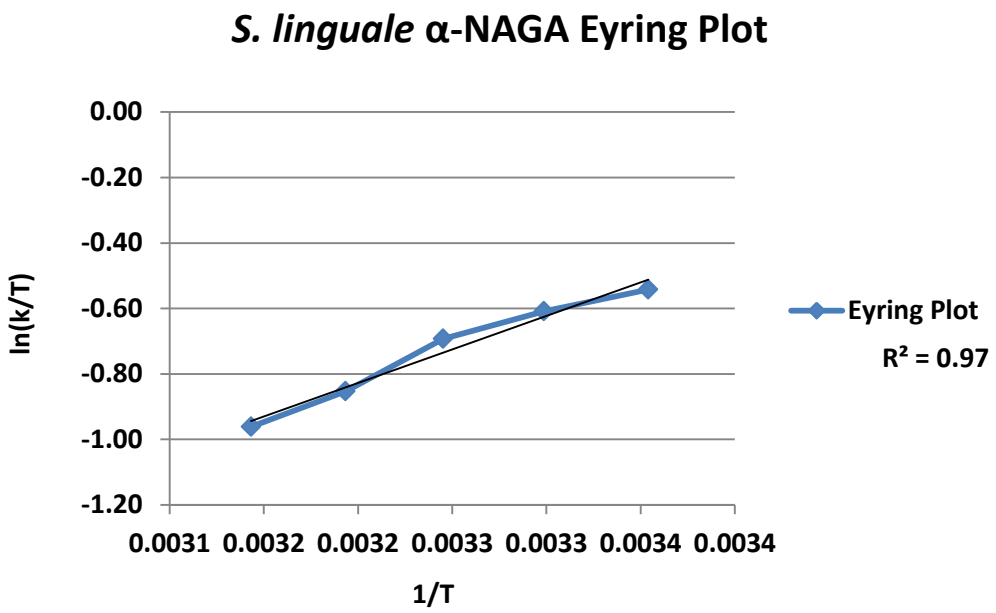
Influence of temperature on the activity of the *S. linguale* recombinant enzyme preparation. For the temperature profile, activity was measured in 20 mM sodium phosphate buffer (pH 7) at different temperatures.

Figure 7.11: Eyring Plot *E. coli* α-NAGA Activity vs Temperature



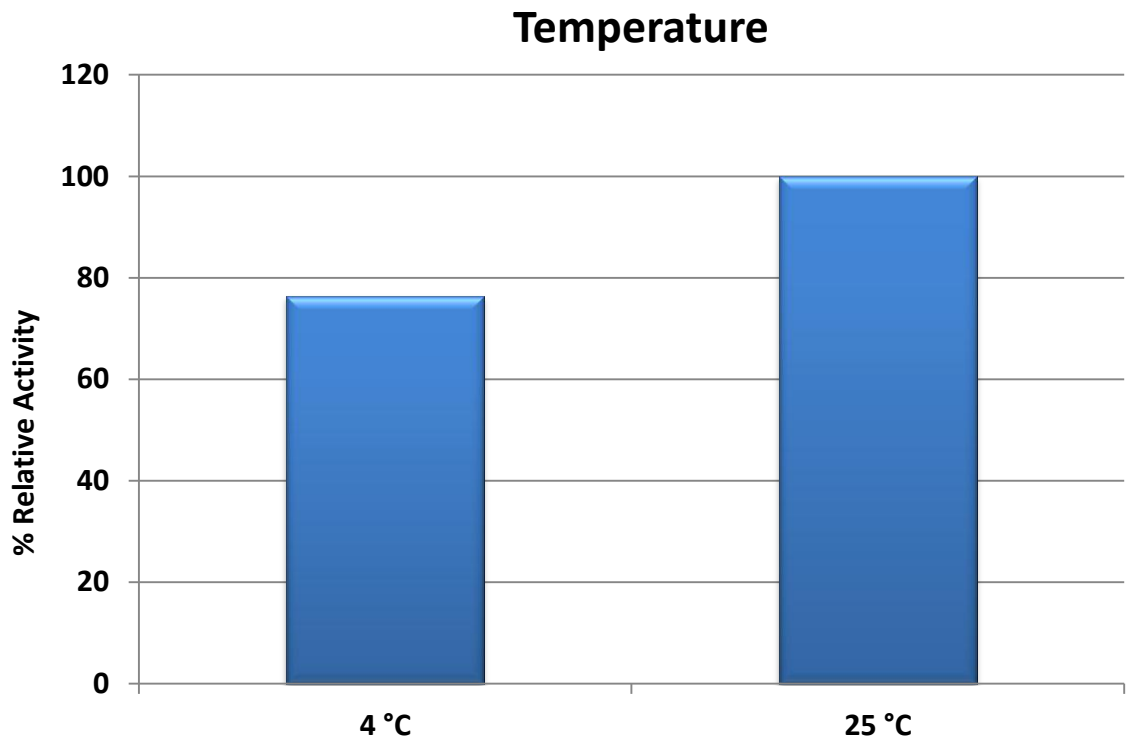
Eyring plot of *E. coli* α-NAGA modeling the transition state of enzyme catalysis indicating the temperature (1/T) dependence of the reaction rate (ln of rate constant of the reaction/T)

Figure 7.12: Eyring Plot *S. linguale* α-NAGA Activity vs Temperature



Eyring plot of *S. linguale* α-NAGA modeling the transition state of enzyme catalysis indicating the temperature (1/T) dependence of the reaction rate (ln of rate constant of the reaction/T)

Figure 7.13: *S. linguale* α-NAGA Activity 4 °C vs 25 °C



Relevant effect of temperature of *S. linguale* α-NAGA. Recombinant enzyme was incubated with *p*NP N-acetyl-α-D-galactosaminide substrate and assessed for glycosidase activity by measuring the amount of N-acetyl-α-D-galactosaminide produced. Results are presented as percent activity relative to the amount of N-acetyl-α-D-galactosaminide released from the glycoside-catalyzed hydrolysis of *p*NP N-acetyl-α-D-galactosaminide.

Michaelis-Menten steady state parameters were calculated to evaluate temperature enzyme response characteristics (Table 7.2). Assays to determine temperature response of each enzyme for 4-Nitrophenyl N-acetyl-α-D-galactosaminide were performed in 20 mM sodium phosphate pH 7.4 using a temperature range 20-45 °C in increments of 5 °C. The turnover rate, k_{cat}, of the *E. coli* enzyme increased by approximately 3 fold over the temperature range tested. At the same time substrate affinity, K_M, decreased approximately 2 fold though the catalytic efficiency, k_{cat}/K_M,

remained somewhat constant. *S. linguale* enzyme activity over a similar temperature range differed from the *E. coli* enzyme. The *S. linguale* enzyme turnover rate and substrate affinity was highest at 25 °C, whereas the enzyme's catalytic efficiency was at the polar ends of the tested temperatures.

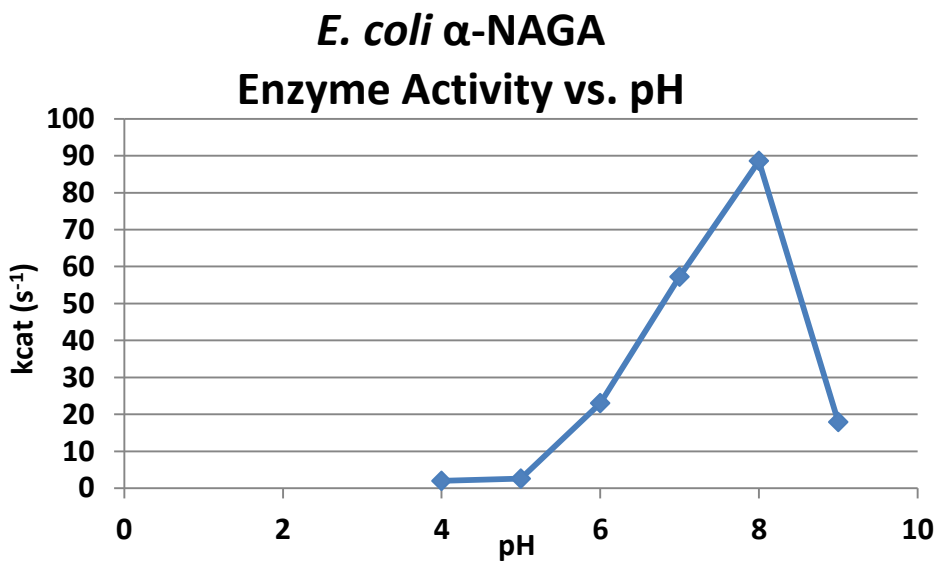
Table 7.2: Temperature Michaelis-Menten steady state kinetics

Enzyme	Temperature °C	µmole N-Acetyl-galactosamine/s	K_M (mM)	k_{cat} s^{-1}	k_{cat}/K_M ($s^{-1}mM^{-1}$)
E. coli α-NAGA	25	0.48 ± 0.018	3.4 ± 0.28	33	10
	30	0.49 ± 0.014	3.0 ± 0.20	33	11
	35	0.63 ± 0.043	3.5 ± 0.44	42	12
	40	0.96 ± 0.20	4.9 ± 1.5	65	13
	45	1.30 ± 0.18	6.1 ± 1.3	88	14

Enzyme	Temperature °C	µmole N-Acetyl-galactosamine/s	K_M (mM)	k_{cat} s^{-1}	k_{cat}/K_M ($s^{-1}mM^{-1}$)
S. linguale α-NAGA	20	0.14 ± 0.005	0.63 ± 0.05	128	203
	25	0.19 ± 0.007	1.07 ± 0.07	173	163
	30	0.18 ± 0.012	1.09 ± 0.12	165	152
	35	0.17 ± 0.014	0.94 ± 0.15	154	163
	40	0.14 ± 0.012	0.67 ± 0.12	133	198
	45	0.13 ± 0.006	0.79 ± 0.08	122	154

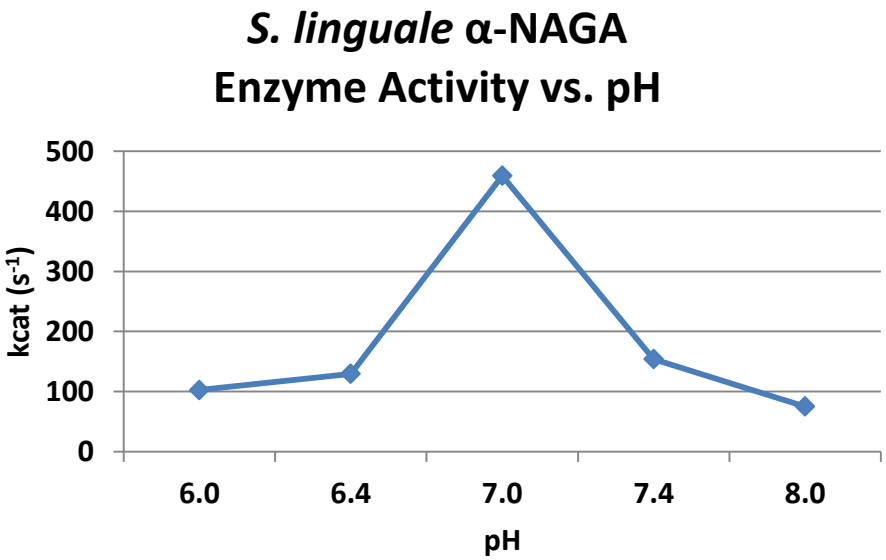
Enzyme activity responded to pH variances for both proteins. Assays to determine the pH optimum of the enzyme for 4-Nitrophenyl N-acetyl-α-D-galactosaminide were performed in 20 mM sodium phosphate in the pH range 6.0-8.0, 50 mM sodium acetate in the pH range 3.0-6.0, or 100 mM bicine pH 9.0. Enzyme activity was decreased at acidic pH then peaked at close to neutral pH and again decreased as pH became alkaline. Enzyme activity of *E. coli* was highest approximately pH 8.0 while peak enzyme activity of *S. linguale* was approximately pH 7.0 (Figure 7.14 and 7.15).

Figure 7.14: *E. coli* α-NAGA Activity vs pH



Effect of pH on the activity of the recombinant *E. coli* enzyme preparation. For the pH profile, activity was measured at 25 °C in buffers of different pH values.

Figure 7.15: *S. linguale* α-NAGA Activity vs pH



Effect of pH on the activity of the *S. linguale* recombinant enzyme preparation. For the pH profile, activity was measured at 25 °C in buffers of different pH values.

Michaelis-Menten steady state parameters were calculated to evaluate pH enzyme response characteristics (Table 7.3). Both enzymes exhibited increased turnover rates as the optimal pH was achieved, with lower rates in the acidic or alkaline range. The *E. coli* enzyme had a strong affinity for the substrate at lower pH, but this was offset by a low turnover rate, whereas at a higher pH the affinity for substrate decreased, but turnover rate increased. The catalytic efficiency remained stable through the pH range with a significant drop at pH 9.0. *S. linguale* α-NAGA substrate affinity increased as the pH was increased, with turnover rate and catalytic efficiency at the highest levels close to neutral pH. Van Etten has expressed concerns of evaluating pH optimum with conjugated substrates due to pKa effects of the leaving group [148]. The pKa of the leaving group of 4-Nitrophenyl N-acetyl-α-D-galactosaminide is 7.6. With the leaving group pKa within the pH range of the optimum of each enzyme the value of the conjugate data is tentative.

Table 7.3: pH Michaelis-Menten steady state kinetics

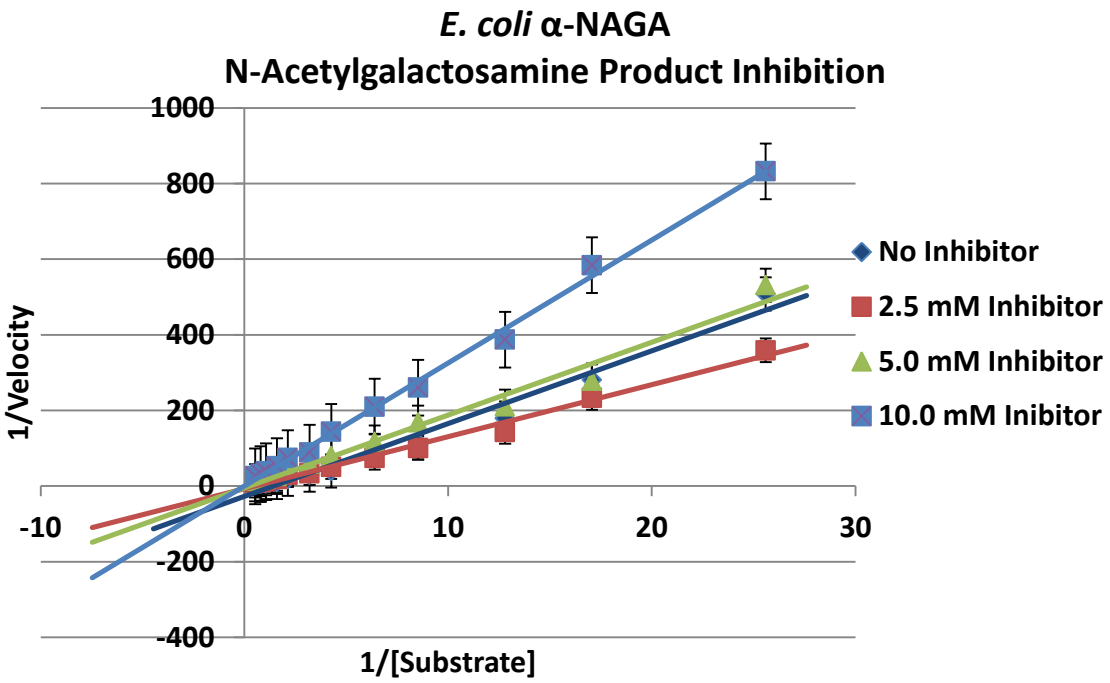
Enzyme	pH	µmole N-Acetyl-galactosamine/s	K_M (mM)	k_{cat} s^{-1}	k_{cat}/K_M ($s^{-1}mM^{-1}$)
E. coli α-NAGA	4.0	0.029 ± 0.001	0.13 ± 0.04	2	15
	5.0	0.038 ± 0.002	0.27 ± 0.07	3	10
	6.0	0.34 ± 0.028	2.8 ± 0.63	23	8
	7.0	0.85 ± 0.088	4.4 ± 0.86	57	13
	8.0	1.31 ± 0.28	9.1 ± 2.6	89	10
	9.0	0.27 ± 0.020	4.7 ± 0.67	18	4

Enzyme	pH	µmole N-Acetyl-galactosamine/s	K_M (mM)	k_{cat} s^{-1}	k_{cat}/K_M ($s^{-1}mM^{-1}$)
S. linguale α-NAGA	6.0	**0.11 ± 0.01**	0.50 ± 0.08	103	206
	6.4	0.14 ± 0.02	0.89 ± 0.18	130	145
	7.0	0.50 ± 0.05	0.67 ± 0.12	459	686
	7.4	0.17 ± 0.01	0.20 ± 0.04	154	765
	8.0	0.08 ± 0.01	0.13 ± 0.03	75	579

Inhibition analyses have been reported on similar α-NAGA proteins. Liu et. al. showed inhibition of *E. meningoseticum* enzyme activity with several divalent metals, Cu^{2+}, Ni^{2+}, and Zn^{2+}, at concentrations of 1 or 10 mM, but EDTA did not impact enzyme activity at these concentrations [49]. Undilute Adsol solution tested against the *C. perfringens* enzyme was the only solution to lower activity as demonstrated by Hsieh and Smith [104]. Product inhibition was not evaluated. Since the goal is to use these enzymes to convert RBCs, enzyme activity brings about accumulation of product which may impact complete conversion of RBCs. Approximately 0.4 µM/L of N-acetylgalactosamine is present on RBCs. The product, N-acetylgalactosamine, was used to test if increasing concentrations would inhibit enzyme activity. The inhibition assays were performed at room temperature (~22 °C) in 70 µL of 20 mM sodium phosphate pH 7.0 buffer, 10 µL of the substrate dilution, 10 µL of N-acetyl-galactosamine at concentrations of 0.05, 0.1,

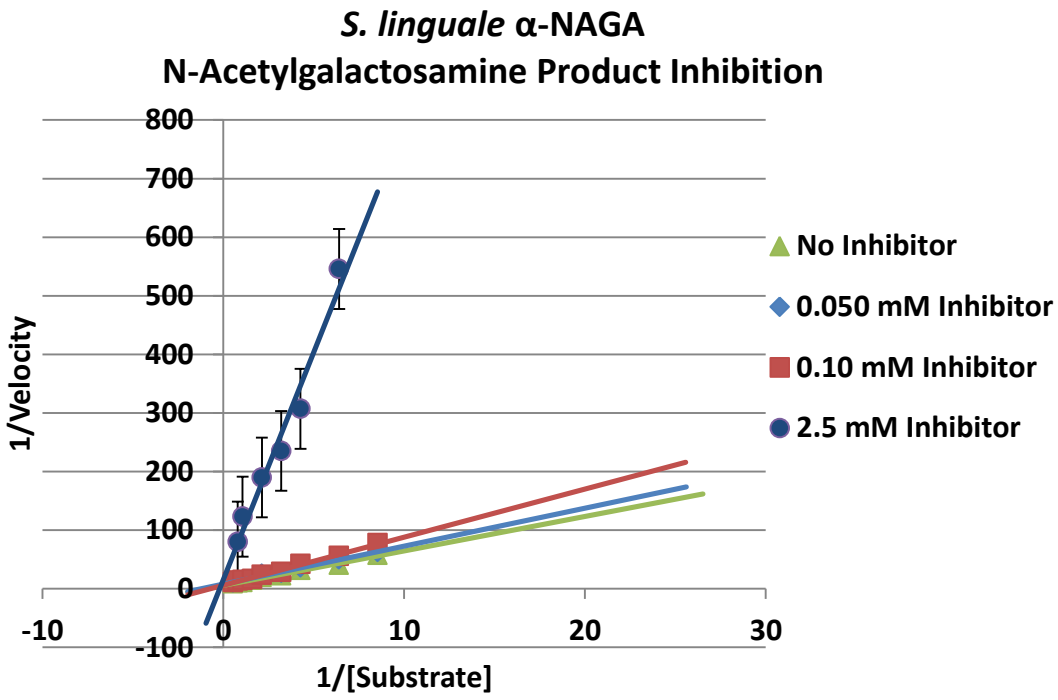
2.5, 5.0, or 10.0 mM, and the automated addition of 10 µL diluted enzyme. *E. coli* α-NAGA was affected 10 fold less than *S. linguale* α-NAGA to product generation. The Lineweaver-Burke plots (Figure 7.16 and 7.17), provide an overview of product inhibition. The *E. coli* enzyme actually had increased activity in the presence of 2.5 mM N-acetylgalactosamine before activity decreased at the 5.0 and 10.0 mM concentrations. In contrast, the *S. linguale* enzyme appeared to be sensitive to N-acetylgalactosamine concentrations as low as 50 µM. Three inhibition kinetic models, competitive, non-competitive, and uncompetitive, were evaluated using VisualEnzymics 2010 to determine the appropriate model to calculate the K_i. An uncompetitive model was used to calculate the Michaelis-Menten inhibition parameters of *E. coli* α-NAGA. The calculated parameters, k_{cat} and k_{cat}/K_M, suggested that enzyme activity was superior in turnover rate and equally efficient catalytically (Table 7.4) compared to the temperature and pH data. A competitive model was used to calculate the Michaelis-Menten inhibition parameters of *S. linguale* α-NAGA. The calculated parameters, k_{cat} and k_{cat}/K_M, indicated a marked difference compared to pH enzyme activity but to a lesser degree of the temperature data.

Figure 7.16: Product Inhibition of *E. coli* α-NAGA Activity



Lineweaver-Burke plot of the uncompetitive result of 2.5 mM, 5.0 mM, and 10.0 mM N-Acetylgalactosamine on the hydrolysis of *p*NP N-acetyl-α-D-galactosaminide by *E. coli* α-NAGA.

Figure 7.17: Product Inhibition of *S. linguale* α-NAGA Activity



Lineweaver-Burke plot of the competitive result of 0.050 mM, 0.10 mM, and 2.5 mM N-Acetylgalactosamine on the hydrolysis of *p*NP N-acetyl-α-D-galactosaminide by *S. linguale* α-NAGA.

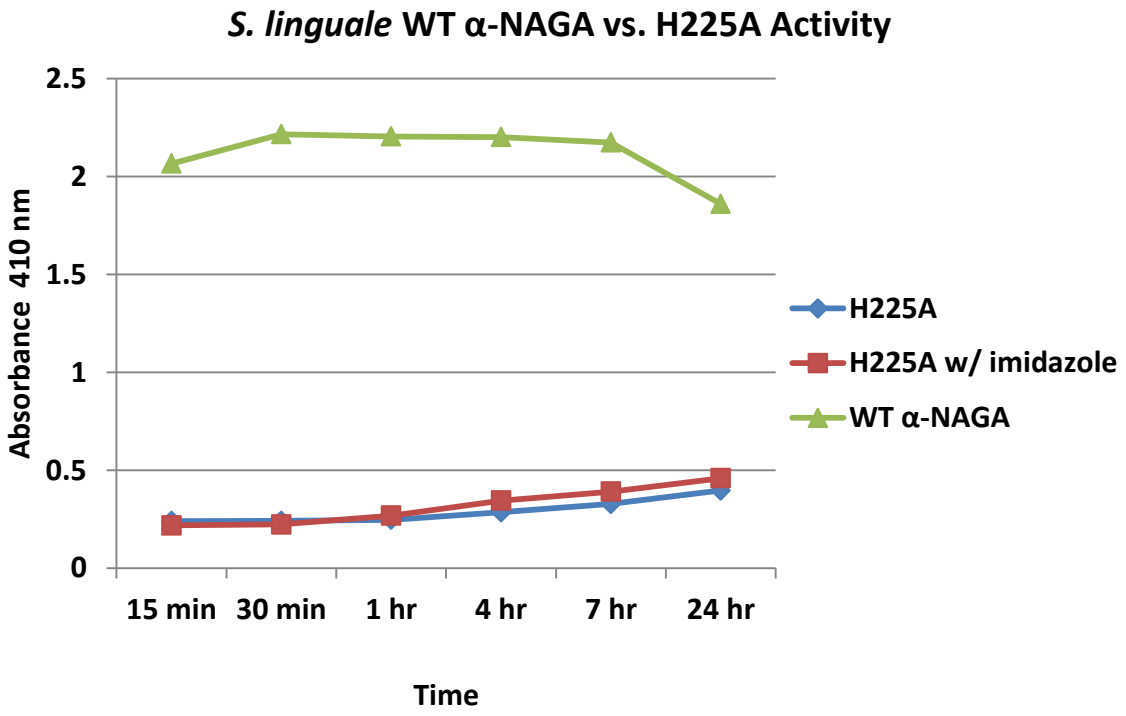
Table 7.4: Michaelis-Menten single substrate inhibition kinetics

Enzyme	µmole N-Acetyl-galactosamine/s	K_M (µM)	K_i (mM)	k_{cat} s^{-1}	k_{cat}/K_M ($s^{-1}mM^{-1}$)
E. coli α-NAGA	1.37 ± 0.37	8.7 ± 2.7	2.6 ± 0.14	92	11
S. linguale α-NAGA	0.15 ± 0.006	0.80 ± 0.069	0.18 ± 0.024	138	173

A genetic mutation of the purported histidine cofactor, amino acid 225, to alanine was introduced to evaluate *S. linguale* α-NAGA activity. Histidine 225 acts in concert with a tightly integrated NAD^+ cofactor during the hydrolysis reaction of the N-

acetylgalactosamine molecule. The mutation H225A eliminated enzymatic activity, disrupting the ability of the enzyme to catalyze hydrolysis of N-acetylgalactosamine. Enzymatic activity was monitored at selected intervals comparing the wild-type enzyme to the H225A mutant protein with or without supplemental imidazole (Figure 7.18). Imidazole supplemented with wild type enzyme was not evaluated because during purification steps it was visually noted that enzyme activity was impeded.

Figure 7.18: *S. linguale* Wild Type vs H225A α-NAGA Activity



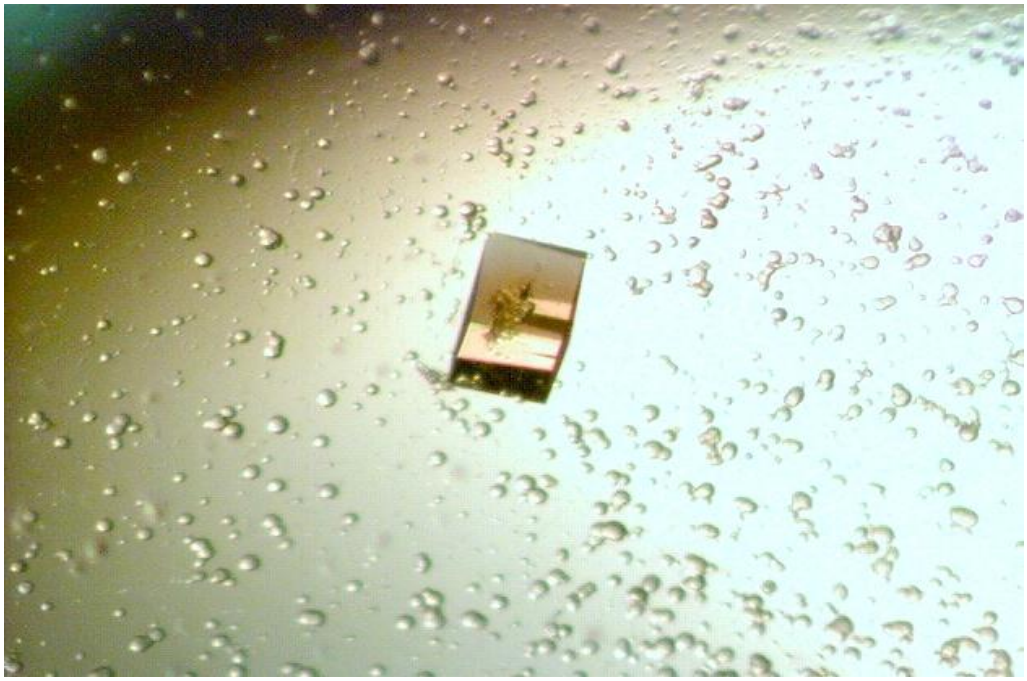
Relevant activity of WT versus H225A *S. linguale* α-NAGA. Each enzyme was incubated with *p*NP N-acetyl-α-D-galactosaminide substrate and assessed for glycosidase activity. Results are absorbance at 410 nm relative to the amount of glycoside released from the glycoside-catalyzed hydrolysis of *p*NP N-acetyl-α-D-galactosaminide.

Crystallization of *E. coli* CFT073 α-NAGA

Alpha NAGA was expressed and purified as described. Sample concentration was assessed with the bicinchoninic acid (BCA) method and further concentration was achieved by using an amicon device with a 50 kDa cutoff. A 5-6 mg/mL protein concentration was used for setting up the crystallization trays. Various Hampton Research screens, Index, PEG/Ion, Detergent, and Silver Bullet, were used for setting up crystallization experiments. Initial crystallization trials included sitting drop vapor diffusion method by mixing 2 µL of protein solution and an equal amount of reservoir

solution. Initial crystal formation involved 0.2 M ammonium sulfate, 0.1 M Bis-Tris pH 6.5 and 20% (w/v) PEG 3350. This condition was optimized and diffraction quality crystals (Figure 7.19) was obtained in 0.1-0.2 M ammonium sulfate, 0.1 M Bis-Tris pH 6.0-6.5 and 18-23.5 (w/v) PEG 3350.

Figure 7.19: Crystal Image of *E. coli* α-NAGA



Crystals of α-NAGA were cryoprotected using 23-28 % (w/v) PEG 3350, 0.1-0.2 M ammonium sulfate, 0.1 M Bis-Tris pH 6.0-6.5 and 25% (w/v) PEG 200 and plunged into liquid N_2 for low temperature data collection. Dr. Harkewal Singh collected a 2.2 Å data set at ALS 4.2.2. Data processing statistics are listed in Table 7.5.

Table 7.5: *E. coli* α-NAGA Crystallography Data Processing Statistics

•Spacegroup		P222
• Unit cell dimensions Å	a =	74.98 b = 138.41 c = 139.80
• Mosaicity		0.44
•Resolution range (Å)		47.96 - 2.20 (2.28 - 2.20)
•Total number of observations		545493
•Number of unique reflections		74608
•Average redundancy		7.31(6.88)
• % completeness		100.0(99.9)
•Rmerge (*I*)		0.128(0.410)
•Reduced ChiSquared		0.99(1.58)
•Output <I/sigI>		8.7(4.1)

Attempts to determine the structure by molecular replacement were unsuccessful due to the lack of a reasonable search model in the Protein Data Bank (PDB). Several attempts to determine the structure of α-NAGA by heavy atom derivitization have been unsuccessful, which was due to the unstable crystal form. Attempts to solve the structure by using SAD (single-wavelength anomalous dispersion) phasing have not been successful. Although a selenomethionine derivative was expressed and purified the inherent low number of methionines in the native protein has impeded the crystal structure determination.

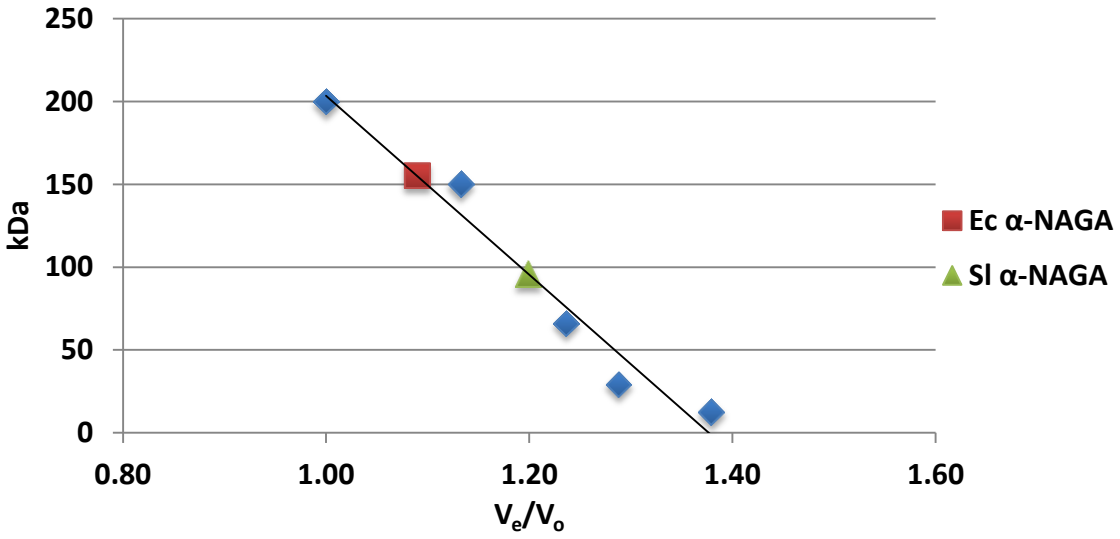
Chromatography Results

The estimated molecular mass of each protein was determined by gel filtration chromatography; *E. coli* 155,000 Da and *S. linguale* 96,000 Da (Table 7.6 and Figure 7.20). Both proteins appear to act as dimers under the conditions tested.

Table 7.6: Gel Filtration Chromatography Molecular Mass Estimation

***E. coli* α-NAGA**						
	(Da)	V_e	V_e/V_o			
β-Amylase	200000	24.51	1.00			
Alcohol Dehydrogenase	150000	27.76	1.13			
Albumin	66000	30.30	1.24			
Carbonic Anhydrase	29000	31.57	1.29			
Cytochrome C	12400	33.81	1.38		Monomer	Dimer
				Est (Da)	Calculated (Da)	Calculated (Da)
***E. coli* α-NAGA**		26.71	1.09	155000	74863	149726
***S. linguale* α-NAGA**						
	(Da)	V_e	V_e/V_o			
β-Amylase	200000	24.51	1.00			
Alcohol Dehydrogenase	150000	27.76	1.13			
Albumin	66000	30.30	1.24			
Carbonic Anhydrase	29000	31.57	1.29			
Cytochrome C	12400	33.81	1.38		Monomer	Dimer
				Est (Da)	Calculated (Da)	Calculated (Da)
***S. linguale* α-NAGA**		29.39	1.20	96000	48215	96430

Figure 7.20: Gel Filtration Chromatography Molecular Mass Estimation

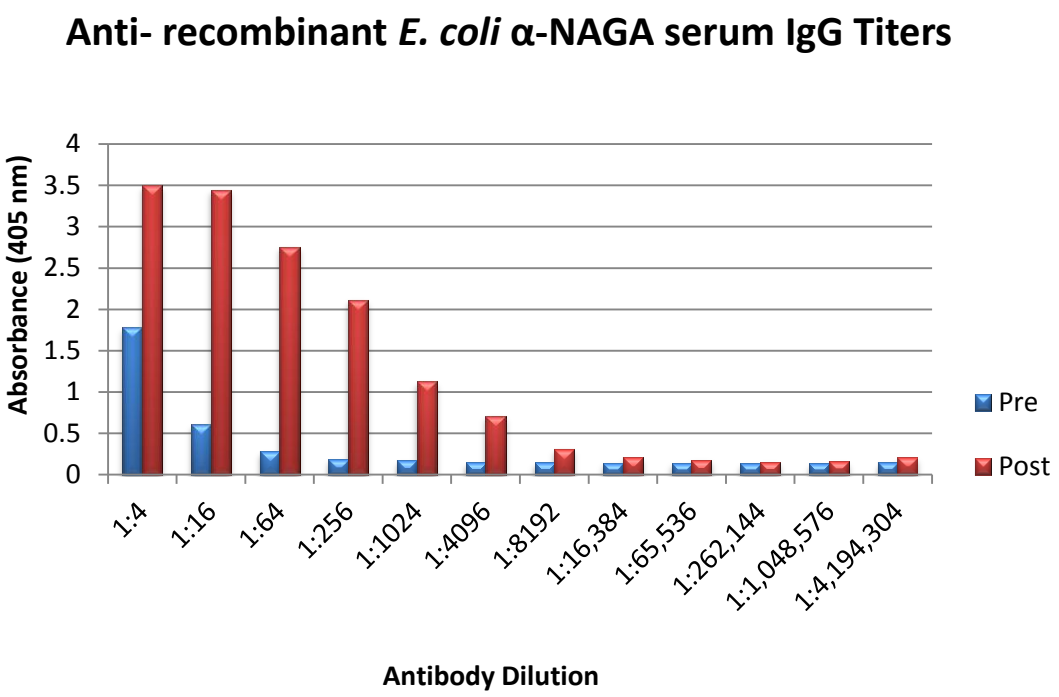


Calculation of Ve/Vo for *S. linguale* and *E. coli* α-NAGA to determine molecular mass (kDa) against the standard curve composed of β-amylase, alcohol dehydrogenase, albumin, carbonic anhydrase, and cytochrome C.

Protein Immunological Results

Immunological response of rabbits to *E. coli* α-NAGA was induced in a rabbit model following a subcutaneous injection challenge. Figure 7.21 illustrates the induced antibody response post inoculation as assessed by ELISA methodology. A titer response of 1:4,096 was induced in the rabbit model. A tentative titer below 0.5 was considered a negative response to the protein.

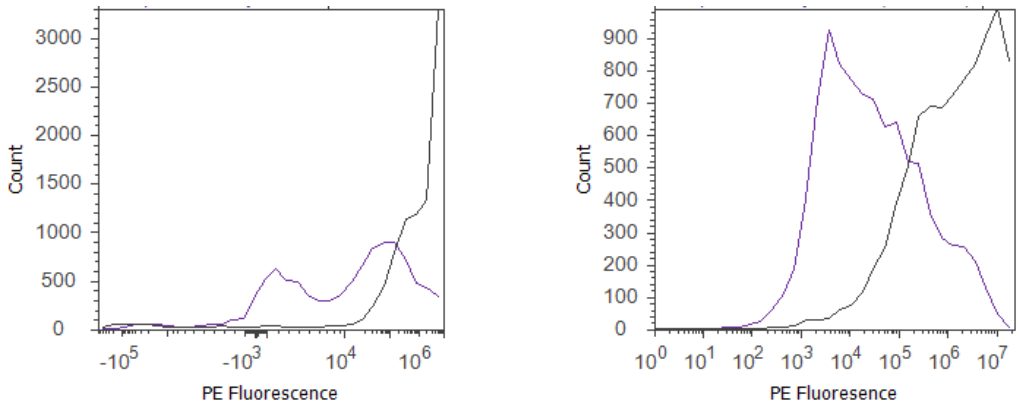
Figure 7.21: Titer Response Post Injection of the *E.coli* α-NAGA in a Rabbit Model



RBC Enzyme Conversion Results

RBCs were stained with a fluorescent fluorophore, phycoethyrin (PE) and fluorescein isothiocyanate (FITC), to determine blood group status pre and/or post enzymatic conversion. Blood group A is composed of the sub-groups A_1 and A_2, each of which generates a distinct PE flow cytometric histogram (Figure 7.22).

Figure 7.22: Flow Cytometry Profiles of PE Stained Blood Sub-group A_1 and A_2

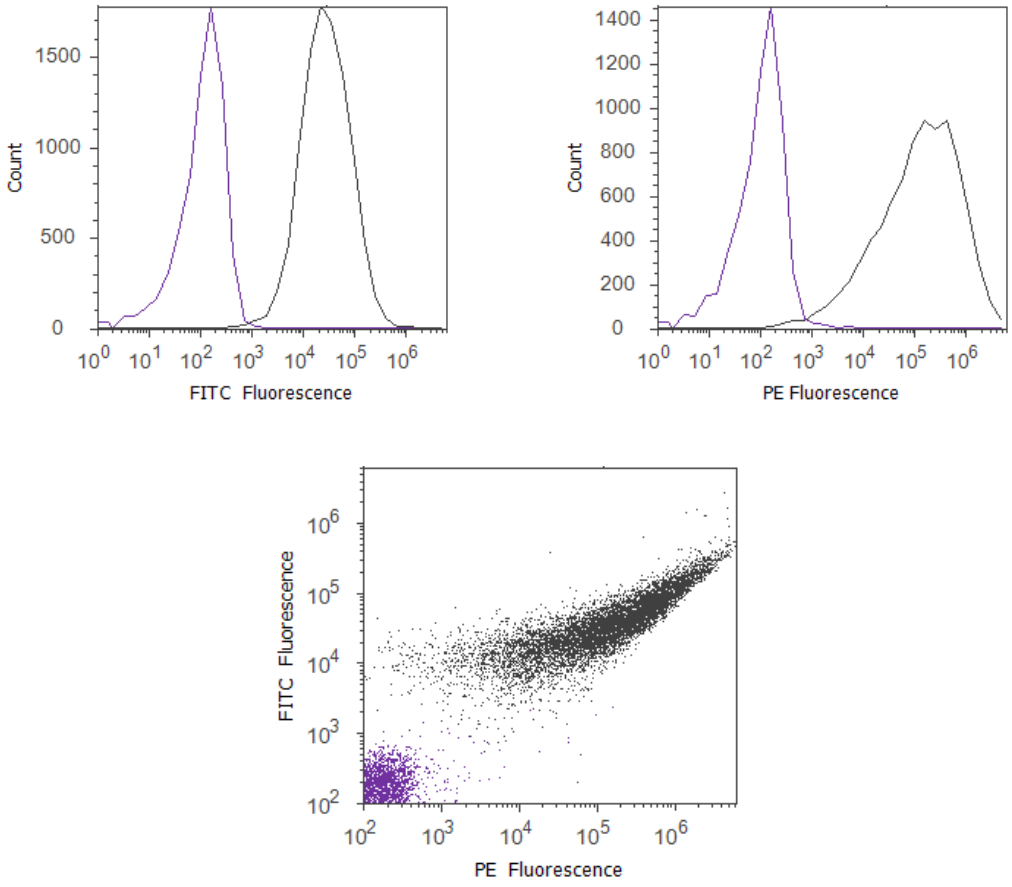


PE stained Fluorescence response of Blood Sub-group A_1 and Blood Sub-group A_2. The left Figure X-axis is a linear log scale view and the Figure on the right is a log scale view.

RBCs were subjected to either *E. coli* or *S. linguale* α-NAGA to determine the conversion of Blood group A to O under several conditions, e.g. solutions, pH, PCV, temperature, and enzyme amount (µg/mL). Four solutions were tested, glycine, alanine, lysine, and phosphate with varying amounts of NaCl, to determine an optimal conversion solution. Glycine, alanine, and lysine solutions were tested at 150, 200, 250, 300, and 350 mM pH 7.0. The phosphate buffer solution was tested at 5 mM phosphate pH 7.0, with 0, 5, 10, 15, 20, or 25 mM NaCl. The RBC conversion solutions glycine, alanine, and lysine data were obtained in 250 mM solutions. *E. coli* α-NAGA was evaluated under these conditions (Figure 7.23 A-D). It did not appear that conversion of blood group A to O occurred in any of the solutions after 4 hours incubation at room temperature. *S. linguale* α-NAGA was also evaluated under these conditions (Figure 7.24 A-D). It appeared that conversion of blood group A to O occurred in the glycine and alanine solutions, but did not occur in the lysine or phosphate buffer solutions following 1 hour incubation at room temperature.

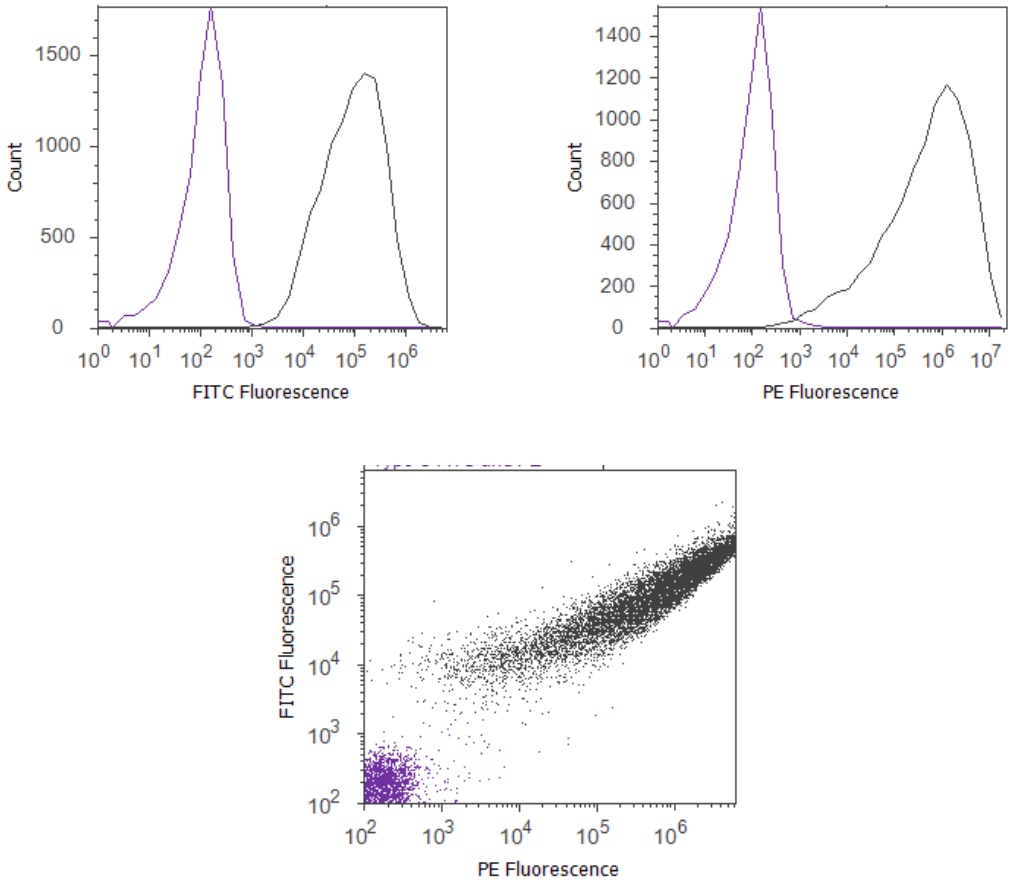
Figure 7.23: ***E. coli*** **α-NAGA Blood Group Conversion by Solution**

A. Glycine Solution (Type O RBC and Enzyme Treated Type A_2 RBC), FITC and PE Fluorescence Histograms and Scatter gram



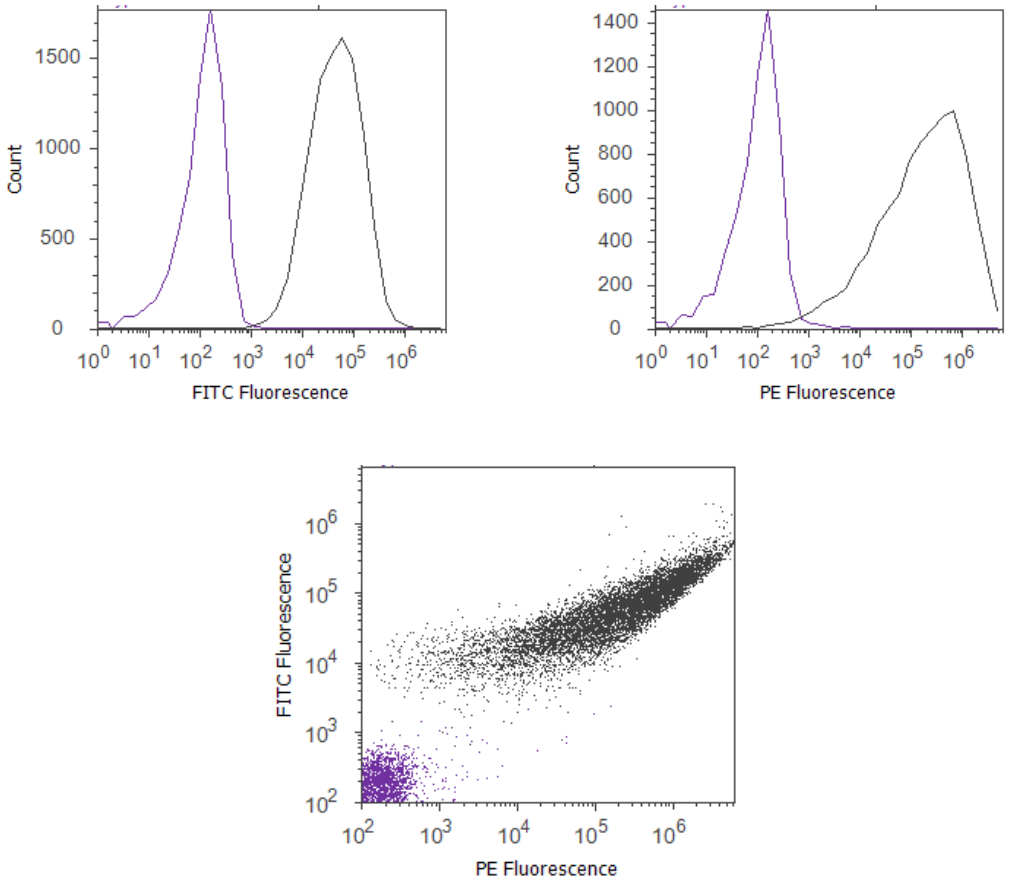
4 hour incubation of Type A_2 RBCs with *E. coli* α-NAGA in 250 mM glycine pH 7.0. RBCs stained with FITC and PE, Figures as labeled. Type O fluorescence response is in purple and Type A_2 fluorescence response is in black. Histograms X-axis Log scale view

B. Alanine Solution (Type O RBC and Enzyme Treated Type A_2 RBC), FITC and PE Fluorescence Histograms and Scatter gram



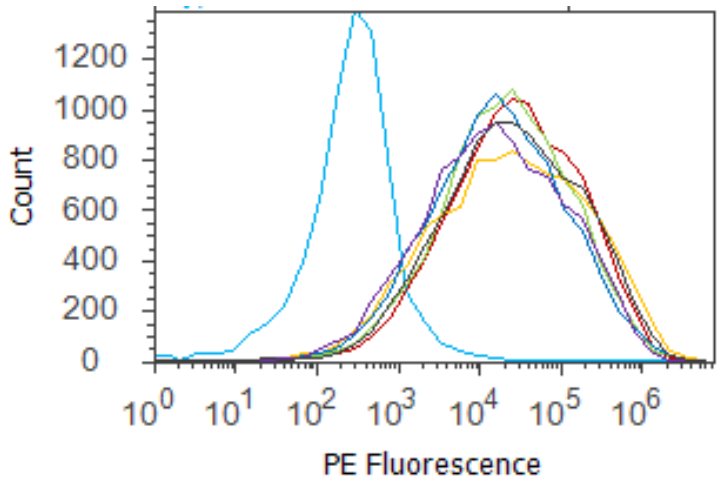
4 hour incubation of Type A_2 RBCs with *E. coli* α-NAGA in 250 mM alanine pH 7.0. RBCs stained with FITC and PE, Figures as labeled. Type O fluorescence response is in purple and Type A_2 fluorescence response is in black. Histograms X-axis Log scale view

C. Lysine Solution (Type O RBC and Enzyme Treated Type A_2 RBC), FITC and PE Fluorescence Histograms and Scattergram



4 hour incubation of Type A_2 RBCs with *E. coli* α-NAGA in 250 mM lysine pH 7.0. RBCs stained with FITC and PE, Figures as labeled. Type O fluorescence response is in purple and Type A_2 fluorescence response is in black. Histograms X-axis Log scale view

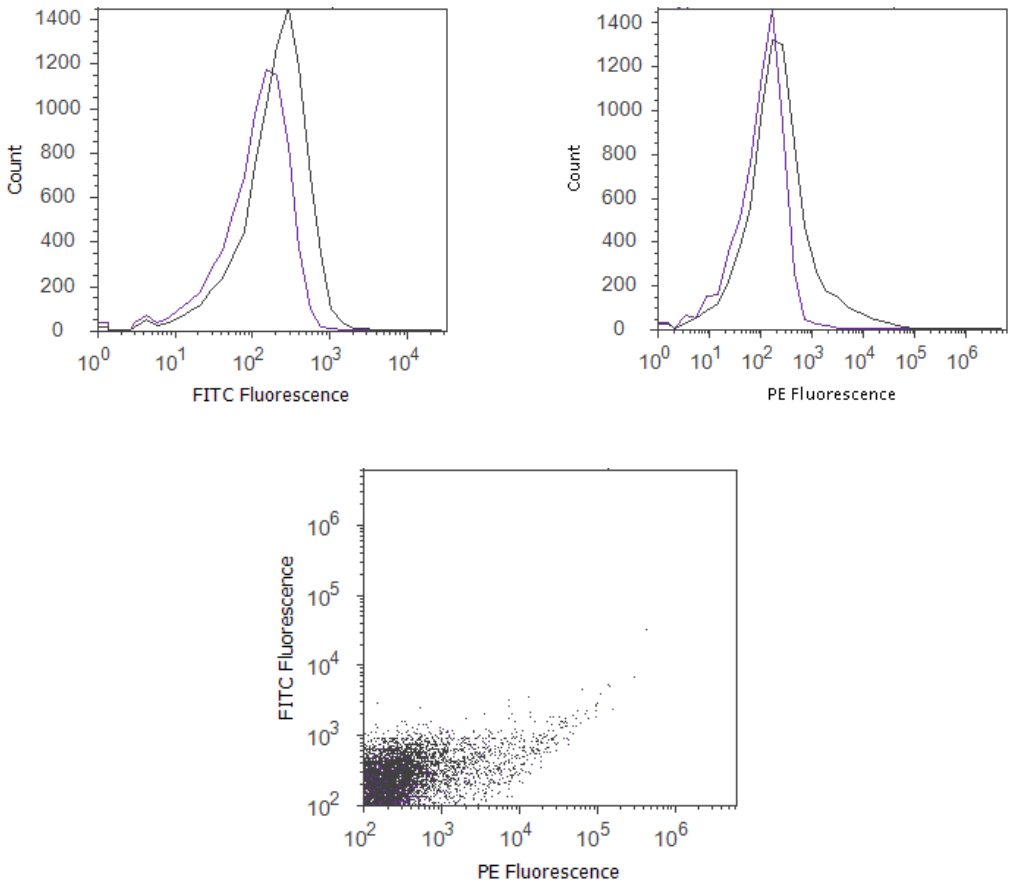
D. Phosphate Solution (Type O RBC and Enzyme Treated Type A_2 RBC), PE Fluorescence Histogram (0, 5, 10, 15, 20, 25 mM NaCl)



2 hour incubation of Type A_2 RBCs with *E. coli* α-NAGA in 5 mM phosphate pH 7.0 with increasing ionic strength by addition of NaCl. RBCs stained with PE, Figure as labeled. Type O fluorescence response is in light blue and Type A_2 fluorescence response is in 0mM NaCl black, 5mM NaCl purple, 10 mM NaCl dark blue, 15 mM NaCl orange, 20 mM NaCl green, and 25 mM NaCl red. Histogram X-axis Log scale view

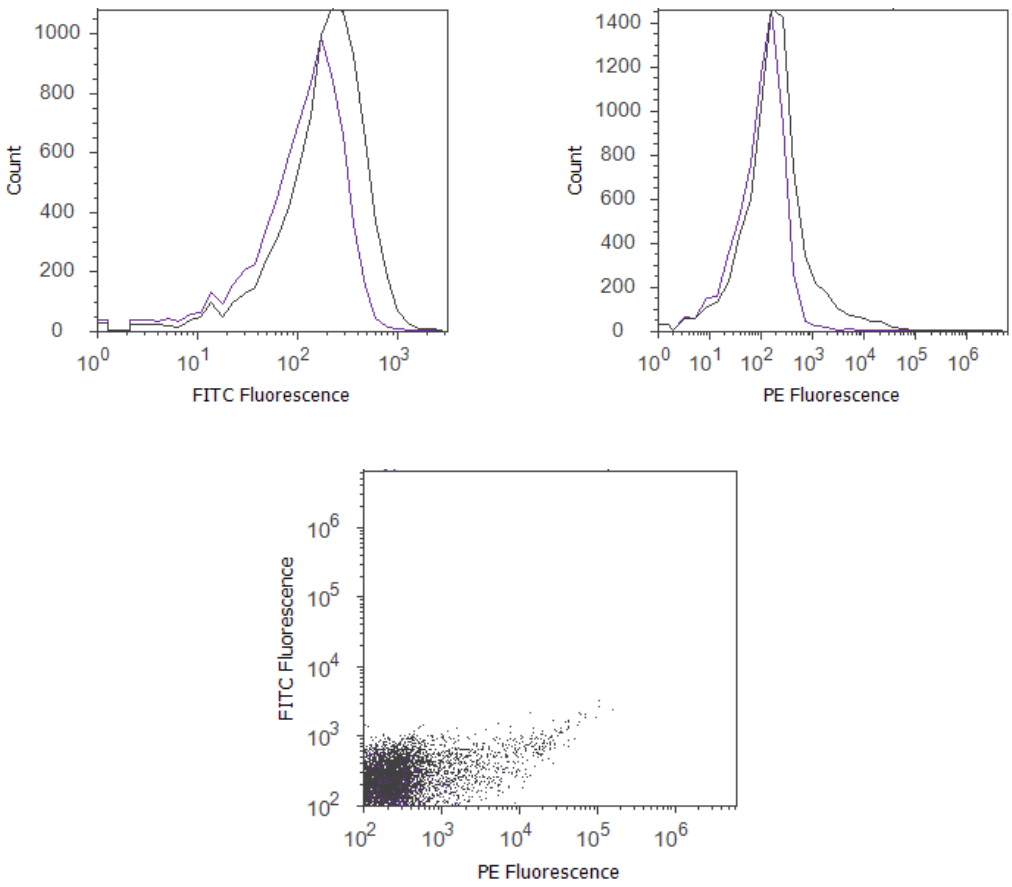
Figure 7.24: ***S. linguale*** **α-NAGA Blood Group Conversion by Solution**

A. Glycine Solution (Type O RBC and Enzyme Treated Type A_2 RBC), FITC and PE Fluorescence Histograms and Scatter gram



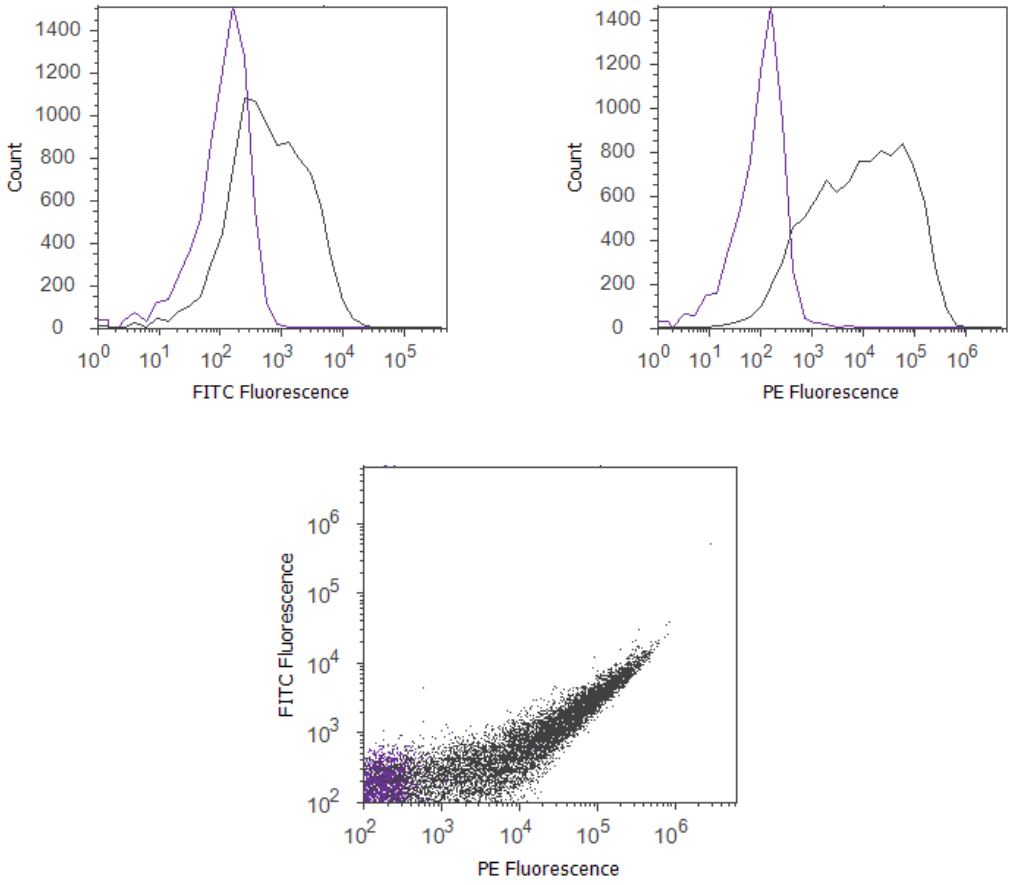
1 hour incubation of Type A_2 RBCs with *S. linguale* α-NAGA in 250 mM glycine pH 7.0. RBCs stained with FITC and PE, Figures as labeled. Type O fluorescence response is in purple and Type A_2 fluorescence response is in black. Histograms X-axis Log scale view

B. Alanine Solution (Type O RBC and Enzyme Treated Type A_2 RBC), FITC and PE Fluorescence Histograms and Scatter gram



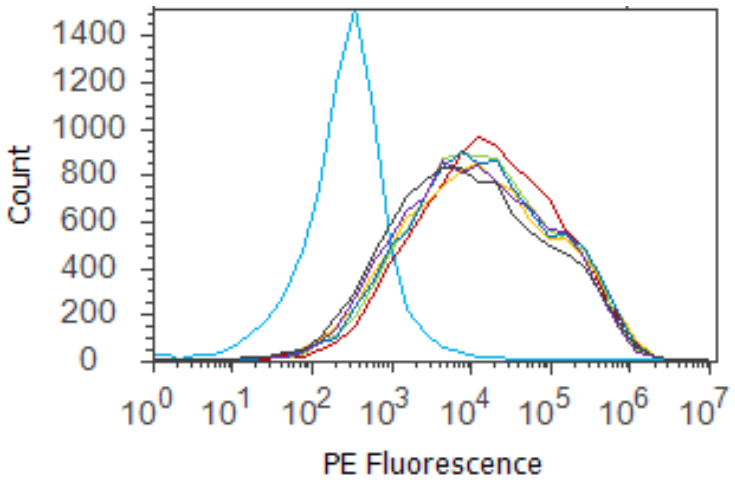
1 hour incubation of Type A_2 RBCs with *S. linguale* α-NAGA in 250 mM alanine pH 7.0. RBCs stained with FITC and PE, Figures as labeled. Type O fluorescence response is in purple and Type A_2 fluorescence response is in black. Histograms X-axis Log scale view

C. Lysine Solution (Type O RBC and Enzyme Treated Type A_2 RBC), FITC and PE Fluorescence Histograms and Scattergram



1 hour incubation of Type A_2 RBCs with *S. linguale* α-NAGA in 250 mM lysine pH 7.0. RBCs stained with FITC and PE, Figures as labeled. Type O fluorescence response is in purple and Type A_2 fluorescence response is in black. Histograms X-axis Log scale view

D. Phosphate Buffer (Type O RBC and Enzyme Treated Type A_2 RBC), PE Fluorescence Histogram (0, 5, 10, 15, 20, 25 mM NaCl)

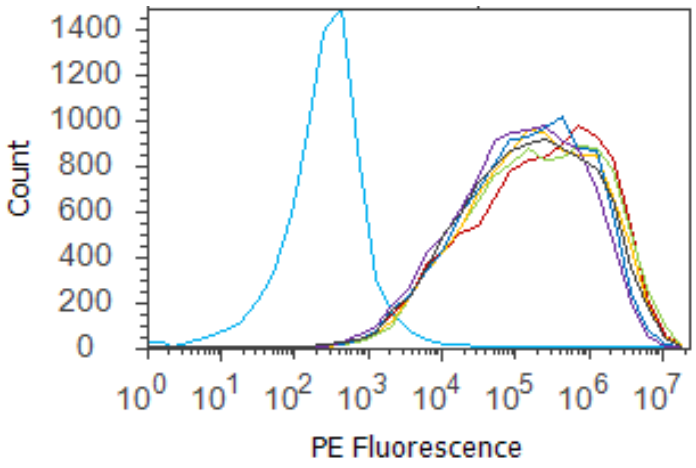


1 hour incubation of Type A_2 RBCs with *S. linguale* α-NAGA in 5 mM phosphate pH 7.0 with increasing ionic strength by addition of NaCl. RBCs stained with PE, Figure as labeled. Type O fluorescence response in light blue and Type A_2 fluorescence response in 0mM NaCl black, 5mM NaCl purple, 10 mM NaCl dark blue, 15 mM NaCl orange, 20 mM NaCl green, and 25 mM NaCl red. Histogram X-axis Log scale view

Enzymatic activity was determined in glycine and alanine solutions. Optimal pH enzyme activity was tested in 250 mM glycine solution pH 5.5, 6.0, 6.5, 7.5, or 8.0. RBCs were incubated for 2 hours with the *E. coli* α-NAGA or with the *S, linguale* α-NAGA for 1 hour at room temperature (Figure 7.25 A and B).

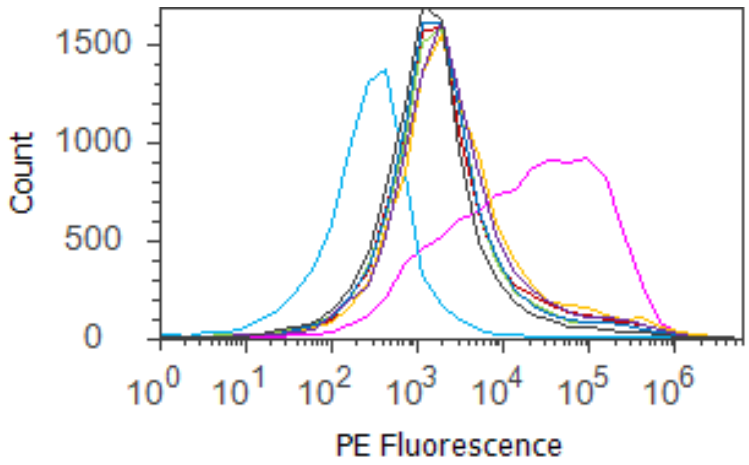
Figure 7.25: 250 mM Glycine Solution (Type O RBC, Type A_2 RBC, and Enzyme Treated Type A_2 RBC), PE Fluorescence Histogram (5.5, 6.0, 6.5, 7.0, 7.5, 8.0 pH)

A. *E. coli* α-NAGA



2 hour incubation of Type A_2 RBCs with *E. coli* α-NAGA in 250 mM glycine pH adjusted range of 5.5-8.0. RBCs stained with PE, Figure as labeled. Type O fluorescence response is in light blue and Type A_2 fluorescence response is in 5.5 black, 6.0 purple, 6.5 dark blue, 7.0 orange, 7.5 green, and 8.0 red. Untreated Type A_2 RBCs is in magenta. Histogram X-axis Log scale view

B. *S. linguale* α-NAGA

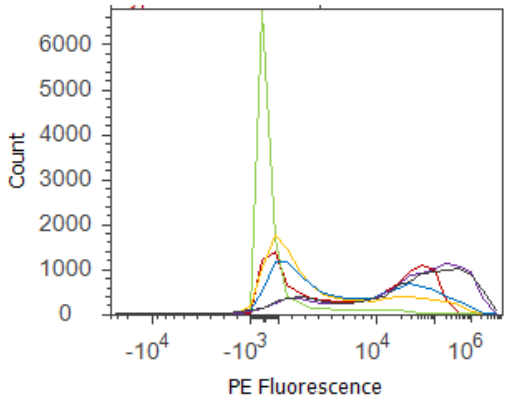


1 hour incubation of Type A_2 RBCs with *S. linguale* α-NAGA in 250 mM glycine pH adjusted range of 5.5-8.0. RBCs stained with PE, Figure as labeled. Type O fluorescence response is in light blue and Type A_2 fluorescence response is in 5.5 black, 6.0 purple, 6.5 dark blue, 7.0 orange, 7.5 green, and 8.0 red. Untreated Type A_2 RBCs is in magenta. Histogram X-axis Log scale view

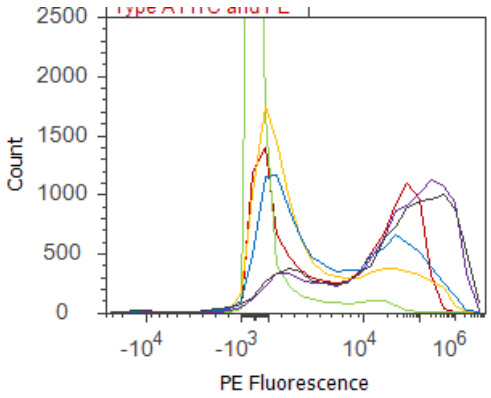
The effects of packed cell volume, PCV, related to enzyme activity were tested with *S. linguale* α-NAGA in 250 mM glycine pH 7.0, at room temperature with 25, 50, or 75 percent PCV (Figure 7.26 A and B). No additional solution was added to the 100% PCV sample.

Figure 7.26: 250 mM Glycine Buffer (Type O RBC, Type A_2 RBC, and Enzyme Treated Type A_2 RBC), PE Fluorescence Histogram (100, 75, 50, 25 PCV)

A. Full Scale



B. Magnified



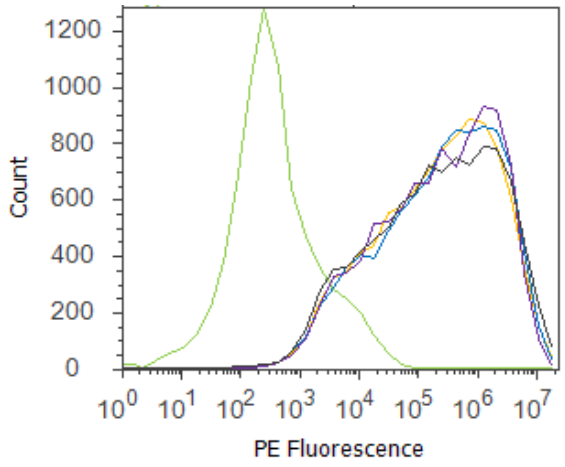
2 hour incubation of Type A_2 RBCs packed cell volume ranging 25-100% with *S. linguale* α-NAGA in 250 mM glycine pH 7.0. RBCs stained with PE, Figure as labeled. Type O fluorescence response is in green and Type A_2 fluorescence response is in 100 black, 75 purple, 50 dark blue, and 25 orange. Untreated Type A_2 RBCs is in red. Histograms X-axis Linear Log scale view

Various amounts of enzyme were used to determine the optimal level to convert RBCs in solution. The amount of enzyme ranged from 1 - 1,000 µg/mL. A 250 mM glycine pH 7.2, solution at room or refrigerated temperature was used to evaluate enzyme activity. Each Figure (7.27 – 7.32) indicates the enzyme amounts and the testing conditions. *E. coli* α-NAGA was tested with 100 – 1,000 µg/mL of enzyme, with no apparent conversion of Type A_2 RBCs (Figure 7.27). *S. linguale* α-NAGA was tested at several enzyme concentrations ranging from 1 – 100 µg/mL of enzyme at room temperature, and 100 – 500 µg/mL of enzyme at refrigerated temperature of treated Type A_2 RBCs. The initial test performed at room temperature with 10 – 100 µg/mL of enzyme indicated conversion of RBCs without any apparent difference in enzyme concentration required (Figure 7.28). Additional testing with an enzyme concentration range of 1 – 100 µg/mL of enzyme provided definitive results. At 1 hour of incubation apparent conversion of RBCs resulted with between 50 and 100 µg/mL of enzyme (Figure 7.29). Allowing 2 hours of incubation resulted in conversion of RBCs with between 25 and 50 µg/mL of enzyme (Figure 7.30). At refrigerated temperatures approximately 8 fold additional enzyme, 400 µg/mL, is required to achieve conversion of RBCs (Figure 7.31). Type A_1 RBCs have approximately 6 fold more antigen sites than A_2 RBCs, 1.5 vs. 0.25 million respectively. Initial titration of Type A_1 RBCs with 100 – 500 µg/mL of enzyme at refrigerated temperature did not convert the RBCs to Type O. At room temperature this enzyme titration range yielded mixed results at the 500 µg/mL enzyme concentration (Figure 7.32). Several of the samples have the Type A_2 Flow Cytometric signature

indicating enzymatic conversion is occurring but is still incomplete. Based on the *in vitro* data *S. linguale* α-NAGA was shown to be sensitive to product accumulation. An experiment to evaluate this potential event compared as follows:

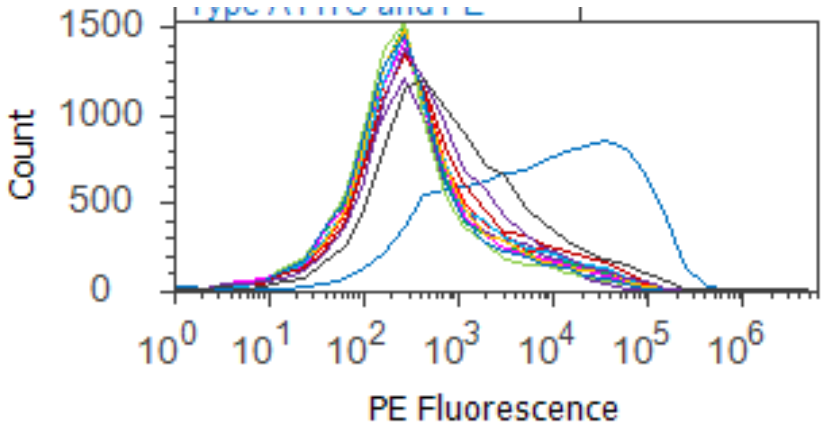
3 samples were set up as described; 3 samples were set up where after 1 hour incubation the samples were centrifuged with the subsequent supernatant removed and replaced with fresh solution; and 3 samples were set up where after 1 hour incubation additional solution was added effectively diluting the sample by two (Figure 7.33). No differences were noted from these two additional treatments. While evaluating the data an unexpected result occurred. On the day glycine buffer was added to the Type A_1 RBCs excess material was prepared, saved, and used on two consecutive days. This generated the results noted in Figure 7.32 and 7.33. These data were compared the original data collected on Day 0 (Figure 7.34 A) and the data collected on Day 1 overlayed with Day 0 data (Figure 7.34 B). This data suggests that pretreatment with the conversion solution approximately 24 hours prior to blood conversion enhances enzymatic activity.

Figure 7.27: *E. coli* α-NAGA at Room Temperature (Type O RBC, and Enzyme Treated Type A_2 RBC), PE Fluorescence Histogram (100, 250, 500, 1,000 µg/mL enzyme)



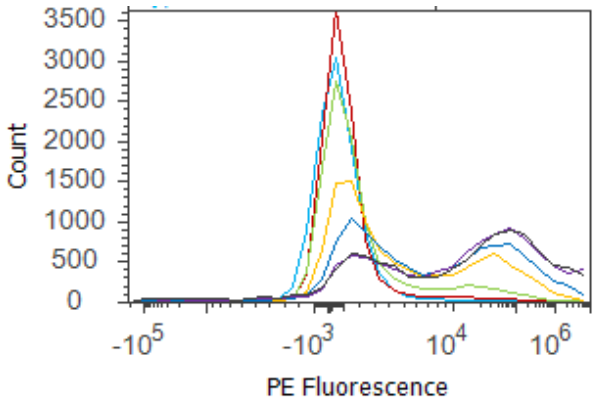
2 hour incubation of Type A_2 RBCs with *E. coli* α-NAGA ranging 100-1,000 µg/mL in 250 mM glycine pH 7.0. RBCs stained with PE, Figure as labeled. Type O fluorescence response is in green and Type A_2 fluorescence response is in 100 black, 250 purple, 500 dark blue, and 1,000 orange. Histogram X-axis Log scale view

Figure 7.28: *S. linguale* α-NAGA at Room Temperature (Type O RBC, Type A_2 RBC, and Enzyme Treated Type A_2 RBC), PE Fluorescence Histogram (10, 20, 30, 40, 50, 60, 70, 80, 90, 100 µg/mL enzyme)



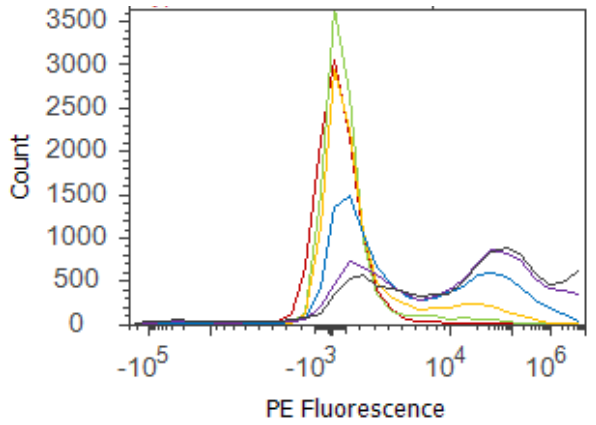
1 hour incubation of Type A_2 RBCs with *S. linguale* α-NAGA ranging 10-100 µg/mL in 250 mM glycine pH 7.0. RBCs stained with PE, Figure as labeled. Type O fluorescence response is in light purple and Type A_2 fluorescence response is in 10 black, 20 purple, 30 dark blue, 40 orange, 50 green, 60 red, 70 light blue, 80 magenta, 90 bright green, and 100 dark orange. Histogram X-axis Log scale view

Figure 7.29: *S. linguale* α-NAGA at Room Temperature 1 Hour Incubation (Type O RBC, and Enzyme Treated Type A_2 RBC), PE Fluorescence Histogram (0, 1, 10, 25, 50, 100 µg/mL enzyme)



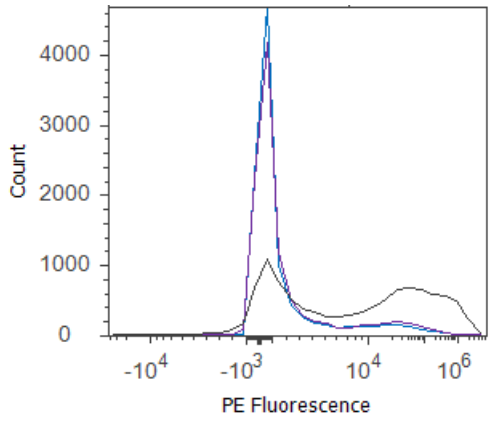
1 hour incubation of Type A_2 RBCs with *S. linguale* α-NAGA ranging 0-100 µg/mL in 250 mM glycine pH 7.0. RBCs stained with PE, Figure as labeled. Type O fluorescence response is in light blue and Type A_2 fluorescence response is in 0 black, 1 purple, 10 dark blue, 25 orange, 50 green, and 100 red. Histogram X-axis Linear Log scale view

Figure 7.30: *S. linguale* α-NAGA at Room Temperature 2 Hour Incubation (Type O RBC, and Enzyme Treated Type A_2 RBC), PE Fluorescence Histogram (0, 1, 10, 25, 50, 100 µg/mL enzyme)



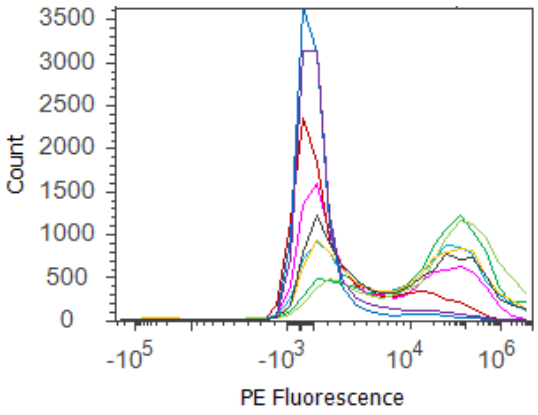
2 hour incubation of Type A_2 RBCs with *S. linguale* α-NAGA ranging 0-100 µg/mL in 250 mM glycine pH 7.0. RBCs stained with PE, Figure as labeled. Type O fluorescence response is in light blue and Type A_2 fluorescence response is in 0 black, 1 purple, 10 dark blue, 25 orange, 50 green, and 100 red. Histogram X-axis Linear Log scale view

Figure 7.31: *S. linguale* α-NAGA at Refrigerated Temperature 2 Hour Incubation (Type O RBC, and Enzyme Treated Type A_2 RBC), PE Fluorescence Histogram (350 and 400 µg/mL enzyme)



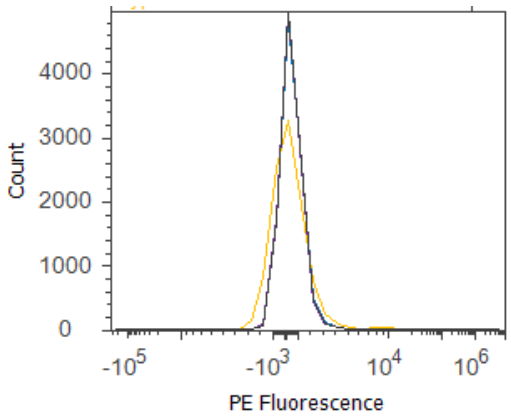
2 hour refrigerated, 4 °C, incubation of Type A_2 RBCs with *S. linguale* α-NAGA 350 and 400 µg/mL in 250 mM glycine pH 7.0. RBCs stained with PE, Figure as labeled. Type O fluorescence response is in light blue and Type A_2 fluorescence response is in 3500 black and 400 purple. Histogram X-axis Linear Log scale view

Figure 7.32: *S. linguale* α-NAGA at Room Temperature 2 Hour Incubation (Enzyme Treated Type A_1 RBC), PE Fluorescence Histogram (500 µg/mL enzyme)



2 hour incubation of 9 Type A_1 RBC samples with *S. linguale* α-NAGA 500 µg/mL in 250 mM glycine pH 7.0. RBCs stained with PE, Figure as labeled. Type A_1 fluorescence response is in each of the colors. Histogram X-axis Linear Log scale view

Figure 7.33: *S. linguale* α-NAGA at Room Temperature 2 Hour Incubation (Enzyme Treated Type A_1 RBC), PE Fluorescence Histogram (500 µg/mL enzyme). Enzyme conversion without additional steps, enzyme conversion with solution replacement, enzyme conversion with additional solution added, and Type O RBC.

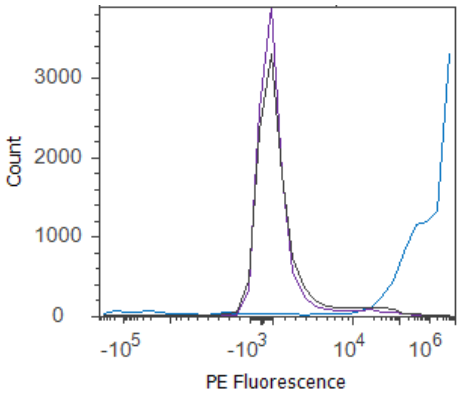


2 hour incubation of 3 Type A_1 RBC samples with *S. linguale* α-NAGA 500 µg/mL in 250 mM glycine pH 7.0. RBCs stained with PE, Figure as labeled. Type O fluorescence response is in (orange) and Type A_1 fluorescence response is in conversion reaction without additional steps (black), conversion reaction with solution replacement (purple), and conversion with additional solution added (blue). Histogram X-axis Linear Log scale view

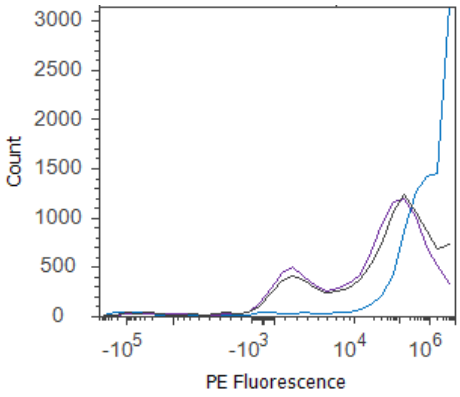
Figure 7.34: ***S. linguale*** **α-NAGA at Room Temperature (Enzyme Treated Type A_1 RBC), PE Fluorescence Histogram (500 µg/mL enzyme)**

A. Day 0 Sample Preparation (Results 1 Hour, Results 2 Hour, Control)

1. Sample converted Day 0

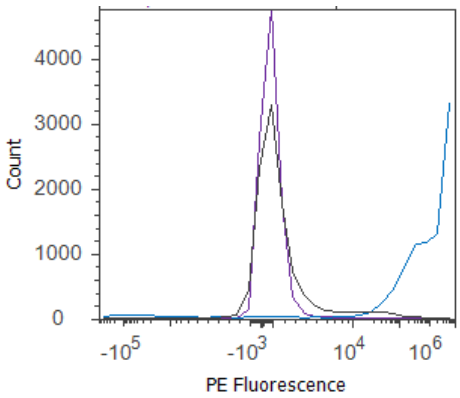


2. Sample incomplete conversion Day 0

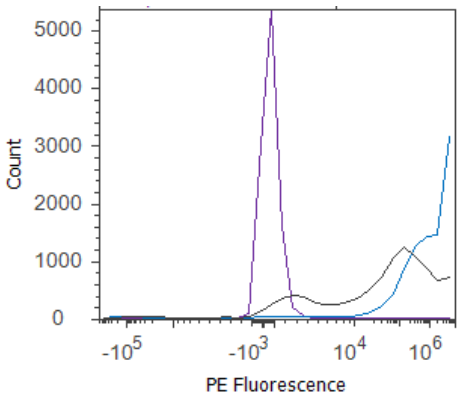


B. Day 0 vs. Day 1 Comparison (Results Day 0 (1 Hour), Results Day 1 (1 Hour), Control)

1. Sample converted Day 0



2. Sample incomplete conversion Day 0

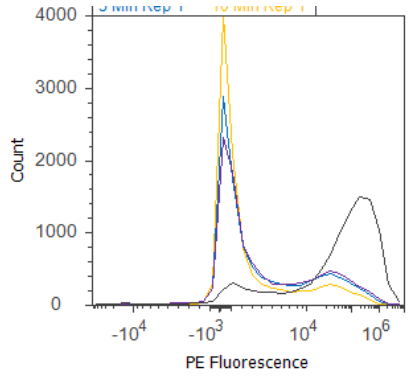


Packed Type A_1 RBC samples (2) diluted with 250 mM glycine pH 7.0 on Day 0. *S. linguale* α-NAGA 500 µg/mL tested concentration. RBCs stained with PE, Figure as labeled. **A.** Day 0 Fluorescence response of untreated (control) Type A_1 (blue), 1 hour conversion (black), and 2 hour conversion (purple). **B.** Fluorescence response of untreated (control) Type A_1 (blue), Day 0 (1 hour) conversion (black), and Day 1 (1 hour) conversion (purple). Histograms X-axis Linear Log scale view

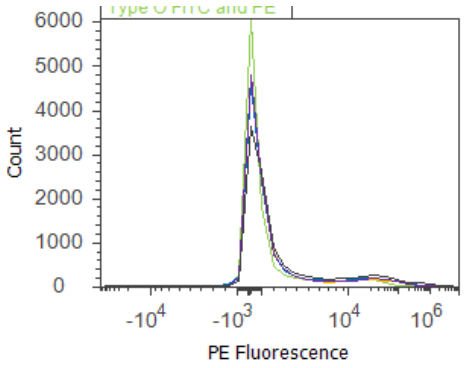
In vivo kinetics of the *S. linguale* α-NAGA were evaluated with Type A_2 RBCs. The experiment used 250 mM glycine solution pH 7.0, with RBCs at approximately 30% PCV and 100 µg/mL enzyme. Samples were collected at pre-enzyme treatment (0 min), and 1, 5, 10, 15, 30, 45, 60, 75, 90, 105, and 120 minutes post treatment. Conversion of the Type A_2 RBCs appears to occur at approximately 15 minutes post enzyme addition (Figure 7.35 A-C).

Figure 7.35: *S. linguale* α-NAGA at Room Temperature (Enzyme Treated Type A_2 RBC), PE Fluorescence Histogram (100 µg/mL enzyme)

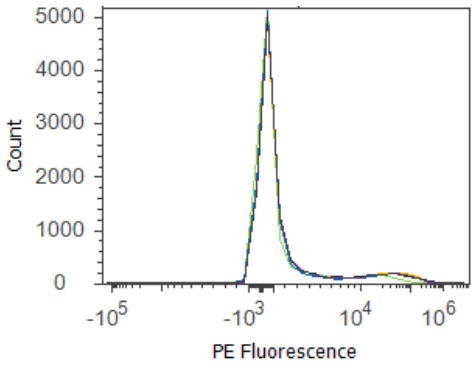
A. 0 – 10 Minutes (0, 1, 5, 10 minutes)



B. 15 – 60 Minutes (15, 30, 45, 60 minutes and Type O RBC)



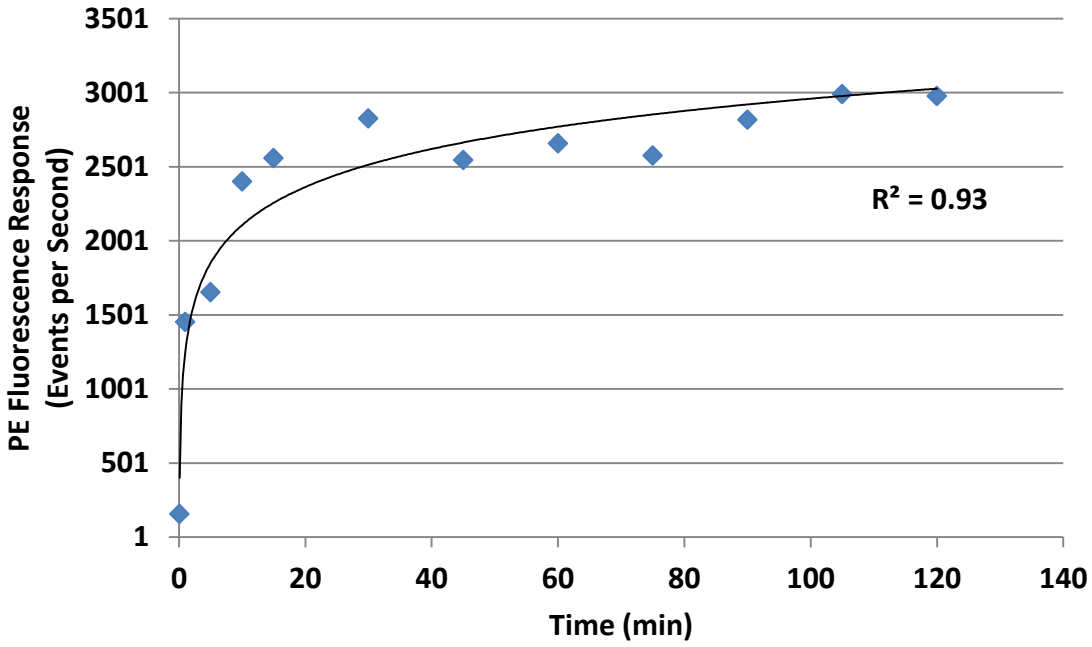
C. 75 – 120 Minutes (75, 90, 105, 120 minutes and Type O RBC)



Timed (minutes) incubation of Type A_2 RBCs with *S. linguale* α-NAGA 100 µg/mL in 250 mM glycine pH 7.0. RBCs stained with PE, Figure as labeled. **A.** Type A_2 fluorescence responses are in 0 (black), 1 (purple), 5 (blue), and 10 (orange). **B.** Type A_2 fluorescence responses are in 15 (black), 30 (purple), 45 (blue), and 60 (orange). Type O fluorescence response is in (light green). **C.** Type A_2 fluorescence responses are in 75 (black), 90 (purple), 105 (blue), and 120 (orange). Type O fluorescence response is in (light green). Histograms X-axis Linear Log scale view

Plotting the fluorescence response (events per second) over time indicates saturation of the enzyme activity during liberation of the α-N-acetylgalactosamine sugar from the A antigen (Figure 7.36).

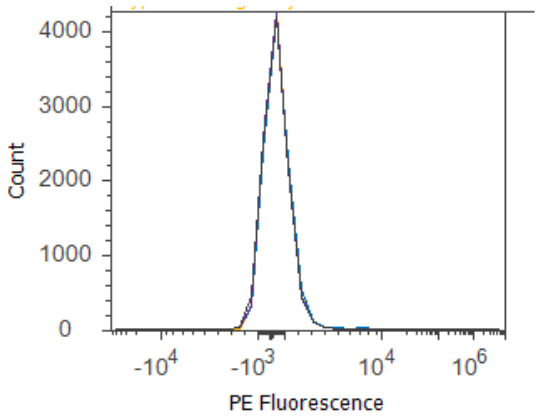
Figure 7.36: *S. linguale* α-NAGA Enzyme Conversion of Treated Type A_2 RBCs over Time



Timed (minutes) incubation of Type A_2 RBCs with *S. linguale* α-NAGA 100 µg/mL in 250 mM glycine pH 7.0. PE fluorescence events per second response plotted against time. Logarithmic function trend line plotted to conversion responses.

Reverse enzyme activity was tested to determine any residual sugar addition to the antigen complex. Type O RBCs were combined with enzyme, 250 mM glycine solution pH 7.2, and 0, 10, 50, or 100 mM N-acetylgalactosamine then incubated for 2 hours at room temperature. Conversion to Type A RBCs does not appear to occur in the presence of N-acetylgalactosamine (Figure 7.37).

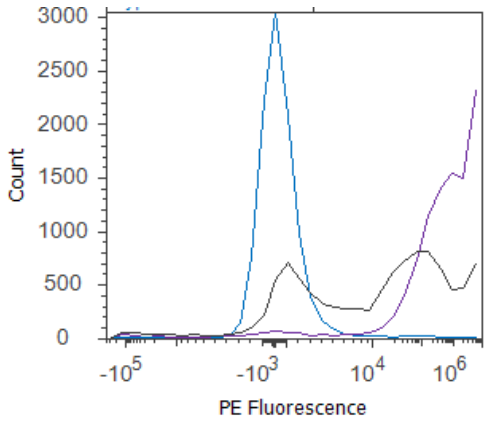
Figure 7.37: Reverse Enzyme Activity of *S. linguale* α-NAGA at Room Temperature (Enzyme Treated Type O RBC), PE Fluorescence Histogram (250 µg/mL enzyme), 2 Hour Incubation, Control 0 mM substrate, 10 mM Substrate, 50 mM Substrate, 100 mM Substrate



Evaluations of *S. linguale* α-NAGA reverse activity with addition of various amounts of N-acetylgalactosamine. 2 hour incubation of Type O RBC samples with *S. linguale* α-NAGA 250 µg/mL in 250 mM glycine pH 7.0. RBCs stained with PE, Figure as labeled. Type O fluorescence response is in 0 mM (black), 10 mM (purple), 50 mM (blue), and 100 mM (orange). Histogram X-axis Linear Log scale view

In vitro, the *S. linguale* H225A mutant protein did not exhibit enzyme activity. The H225A mutant was also tested *in vivo* with Type A_1 and A_2 RBCs for the presence of enzyme activity. Type A_1 and A_2 RBCs were combined with H225A enzyme and 250 mM glycine solution pH 7.2, then incubated for 2 hours at room temperature. The H225A mutant protein did not appear to convert Type A_1 or A_2 RBCs to the Type O blood group (Figure 7.38).

Figure 7.38: *S. linguale* H225A α-NAGA at Room Temperature (Enzyme Treated Type A_1 and A_2 RBC), PE Fluorescence Histogram (250 µg/mL enzyme), 2 Hour Incubation, Type A_2 RBC, Type A_1 RBC, Type O RBC



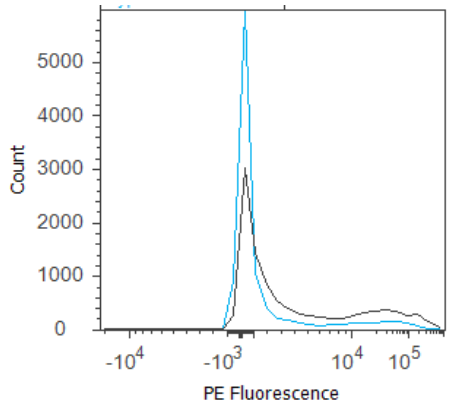
Evaluation of H225A *S. linguale* α-NAGA activity with Type A_1 and A_2 RBCs for 2 hour incubation 250 µg/mL in 250 mM glycine pH 7.0. RBCs stained with PE, Figure as labeled. Fluorescence responses of RBCs; Type A_1 (black), Type A_2 (purple), and Type O (blue). Histogram X-axis Linear Log scale view

RBC Stability

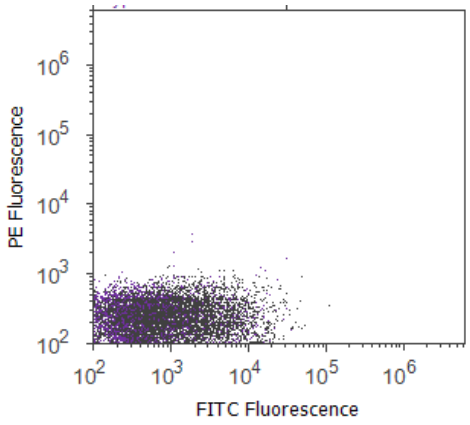
RBC stability was evaluated comparing enzyme converted versus non-treated RBCs. Enzymatic conversion of Type A_2 RBCs was completed at room (~22 °C) and refrigerated (4 °C) temperature in 250 mM glycine solution pH 7.0 using 100 µg/mL of enzyme. Enzymatic conversion of the room and refrigerated treated A_2 RBCs is in Figure 7.39 and 7.40. The cells were washed with PBS then aliquoted into separate tubes for analysis on Day 0, 1, 7, 14, 21, 28, 35, 42, 49, and 56. Non-converted Type A cells in PBS were aliquoted similarly.

Figure 7.39: *S. linguale* α-NAGA at Refrigerated Temperature (Enzyme Treated Type A_2 RBC), 100 µg/mL enzyme, 2 Hour Incubation

A. PE Fluorescence Histogram; Type A_2 RBC and Type O RBC



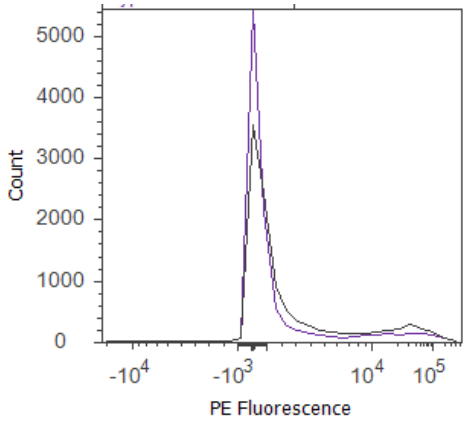
B. FITC/PE Fluorescence Scattergram; Type A_2 RBC and Type O RBC



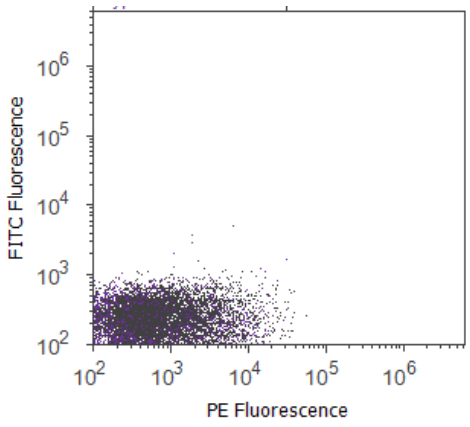
Confirmation of *S. linguale* α-NAGA RBC conversion with Type A_2 RBCs for 2 hour incubation (4 °C) 100 µg/mL in 250 mM glycine pH 7.0. RBCs stained with PE, Figure as labeled. Fluorescence responses of RBCs; Type A_2 (black) and Type O (blue). Histogram X-axis Linear Log scale view

Figure 7.40: *S. linguale* α-NAGA at Room Temperature (Enzyme Treated Type A_2 RBC), 100 µg/mL enzyme, 2 Hour Incubation

A. PE Fluorescence Histogram; Type A_2 RBC and Type O RBC



B. FITC/PE Fluorescence Scattergram; Type A_2 RBC and Type O RBC



Confirmation of *S. linguale* α-NAGA RBC conversion with Type A_2 RBCs for 2 hour incubation (22 ° C) 100 µg/mL in 250 mM glycine pH 7.0. RBCs stained with PE, Figure as labeled. Fluorescence responses of RBCs; Type A_2 (black) and Type O (blue). Histogram X-axis Linear Log scale view

Complete RBC profiles were determined on a Siemens Advia 120 hematology analyzer. Data collected included RBC count, hemoglobin, hematocrit, MCV, MCHC, CHCM, CH, RDW, and HDW. Hemolysis was observed in the enzyme converted cell samples. Analyzed RBC indices, Min, Max, Average, Std Dev, and %CV of the Type A and enzyme converted RBCs are shown in Table 7.7. Increased relative variability, %CV, in virtually all of the indices was observed in the enzyme converted cells compared to the Type A RBCs. An ANOVA of the three directly measured RBC indices, RBC count, hemoglobin, and hematocrit, indicated a significant difference between the three groups (Table 7.8). Clinically there was no difference between the three treatment groups. Graphs of RBC counts, hemoglobin concentration, % hematocrit, and cellular hemoglobin are displayed in Figures 7.41 through 7.44, respectively, show overlapping values and trends occurring with the RBC samples.

Table 7.7: RBC Indices and Stability for 56 Day Period

Type A	Day	0	1	7	14	21	28	35	42	49	56	Min	Max	Average	Std Dev	%CV
	RBC 10^6/µL	4.31	4.29	4.21	4.33	4.27	4.24	4.20	3.84	3.96	3.82	3.82	4.33	4.15	0.1964	4.7
	HGB g/dL	10.0	10.1	10.1	10.2	10.1	10.1	10.1	10.0	10.1	10.1	10.0	10.2	10.1	0.0568	0.6
	HCT %	35.7	35.9	35.8	36.8	37.4	37.4	37.2	34.8	36.3	35.3	34.8	37.4	36.3	0.9119	2.5
	MCV fL	82.8	83.7	84.9	85.0	87.7	88.2	88.6	90.7	91.6	92.5	82.8	92.5	87.6	3.3876	3.9
	MCH pg	23.3	23.6	23.9	23.5	23.8	23.8	24.0	26.1	25.5	26.6	23.3	26.6	24.4	1.1893	4.9
	MCHC g/dL	28.1	28.2	28.2	27.7	27.1	26.9	27.1	28.8	27.8	28.7	26.9	28.8	27.9	0.6653	2.4
	CHCM g/dL	26.9	26.8	26.2	26.3	25.0	24.8	24.7	23.8	23.4	22.9	22.9	26.9	25.1	1.4320	5.7
	CH pg	22.1	22.2	22.0	22.1	21.7	21.7	21.8	21.4	21.3	21.0	21.0	22.2	21.7	0.3945	1.8
	RDW	15.0	15.3	15.0	14.8	14.5	14.0	13.7	13.9	13.4	13.2	13.2	15.3	14.3	0.7376	5.2
	HDW	2.44	2.53	2.51	2.7	2.29	2.26	2.46	2.31	2.28	2.12	2.12	2.72	2.39	0.1727	7.2
ECO 4 °C	Day	0	1	7	14	21	28	35	42	49	56	Min	Max	Average	Std Dev	%CV
	RBC 10^6/µL	4.47	4.46	4.40	4.39	3.90	3.93	3.93	3.81	3.84	3.78	3.78	4.47	4.09	0.2966	7.2
	HGB g/dL	10.3	10.5	10.3	10.4	10.2	10.2	10.2	10.1	10.0	10.1	10.0	10.5	10.2	0.1494	1.5
	HCT %	35.6	35.7	36.3	36.6	33.7	34.2	34.4	34.0	34.8	34.4	33.7	36.6	35.0	1.0100	2.9
	MCV fL	79.5	80.0	82.5	83.5	86.3	86.9	87.6	89.2	90.7	91.0	79.5	91.0	85.7	4.1734	4.9
	MCH pg	23.1	23.4	23.3	23.7	26.1	26.0	25.9	26.5	26.1	26.7	23.1	26.7	25.1	1.4928	6.0
	MCHC g/dL	29.0	29.3	28.3	28.4	30.2	29.9	29.6	29.7	28.7	29.4	28.3	30.2	29.3	0.6381	2.2
	CHCM g/dL	29.3	28.7	27.4	26.8	25.5	25.1	25.0	23.9	23.3	22.9	22.9	29.3	25.8	2.2038	8.5
	CH pg	22.9	22.6	22.4	22.2	21.8	21.6	21.7	21.1	20.9	20.6	20.6	22.9	21.8	0.7569	3.5
	RDW	17.7	17.2	15.9	15.3	14.9	14.8	14.6	14.8	14.7	14.6	14.6	17.7	15.5	1.1306	7.3
	HDW	4.36	3.82	3.26	3.03	2.76	2.74	2.89	2.60	2.48	2.25	2.25	4.36	3.02	0.6427	21.3
ECO 22 °C	Day	0	1	7	14	21	28	35	42	49	56	Min	Max	Average	Std Dev	%CV
	RBC 10^6/µL	4.84	4.86	4.76	4.51	4.24	4.29	4.00	4.14	4.27	4.23	4.00	4.86	4.41	0.3084	7.0
	HGB g/dL	11.2	11.2	11.2	11.3	10.9	10.7	10.5	10.5	11.0	10.9	10.5	11.3	10.9	0.2951	2.7
	HCT %	36.7	38.6	41.6	40.7	38.7	39.6	37.1	39.2	40.3	40.1	36.7	41.6	39.3	1.5429	3.9
	MCV fL	75.7	79.4	87.3	90.1	91.1	92.3	92.8	94.5	94.5	94.8	75.7	94.8	89.3	6.6366	7.4
	MCH pg	23.2	23.1	23.5	25.1	25.6	25.0	26.3	25.3	25.8	25.8	23.1	26.3	24.9	1.1719	4.7
	MCHC g/dL	30.6	29.0	26.9	27.8	28.1	27.1	28.4	26.8	27.3	27.2	26.8	30.6	27.9	1.1783	4.2
	CHCM g/dL	31.4	28.9	25.5	24.4	23.6	23.1	22.6	22.3	22.1	21.8	21.8	31.4	24.6	3.2007	13.0
	CH pg	23.5	22.7	21.9	21.6	21.1	21.0	20.7	20.8	20.6	20.5	20.5	23.5	21.4	0.9958	4.6
	RDW	15.0	15.3	16.8	17.2	17.2	16.9	16.4	15.9	16.0	14.8	14.8	17.2	16.2	0.8947	5.5
	HDW	3.66	2.91	3.39	3.62	3.24	3.06	2.80	2.63	2.43	2.13	2.13	3.66	2.99	0.5054	16.9

Table 7.8: ANOVA of RBC, HGB, and HCT

RBC Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Type A	10	41.47	4.147	0.038578889
ECO 4 °C	10	40.91	4.091	0.087965556
ECO 22 °C	10	44.14	4.414	0.095115556

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.595846667	2	0.297923333	4.032166381	0.029357935	3.354130829
Within Groups	1.99494	27	0.073886667			
Total	2.590786667	29				

HGB Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Type A	10	100.9	10.09	0.003222222
ECO 4 °C	10	102.3	10.23	0.022333333
ECO 22 °C	10	109.4	10.94	0.087111111

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	4.154	2	2.077	55.30473373	2.82917E-10	3.354130829
Within Groups	1.014	27	0.037555556			
Total	5.168	29				

HCT Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Type A	10	362.6	36.26	0.831555556
ECO 4 °C	10	349.7	34.97	1.020111111
ECO 22 °C	10	392.6	39.26	2.380444444

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	96.894	2	48.447	34.34243482	3.81968E-08	3.354130829
Within Groups	38.089	27	1.410703704			
Total	134.983	29				

Figure 7.41: Stability of RBC count 10^6/µL

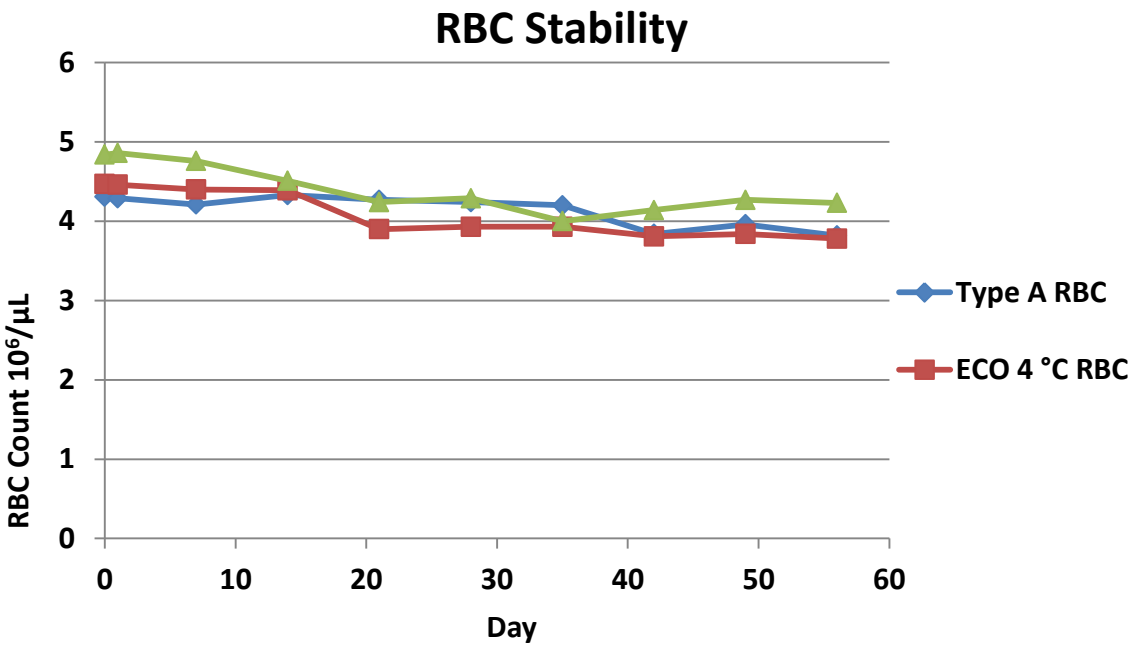


Figure 7.42: Hemoglobin Concentration g/dL

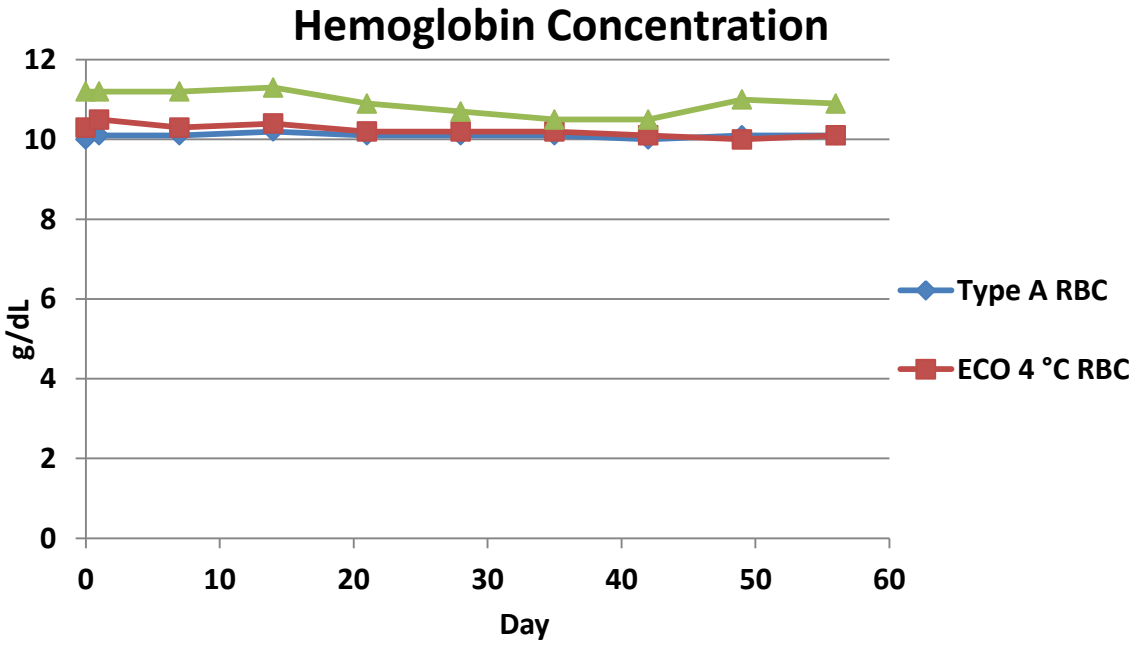


Figure 7.43: % Hematocrit (PCV)

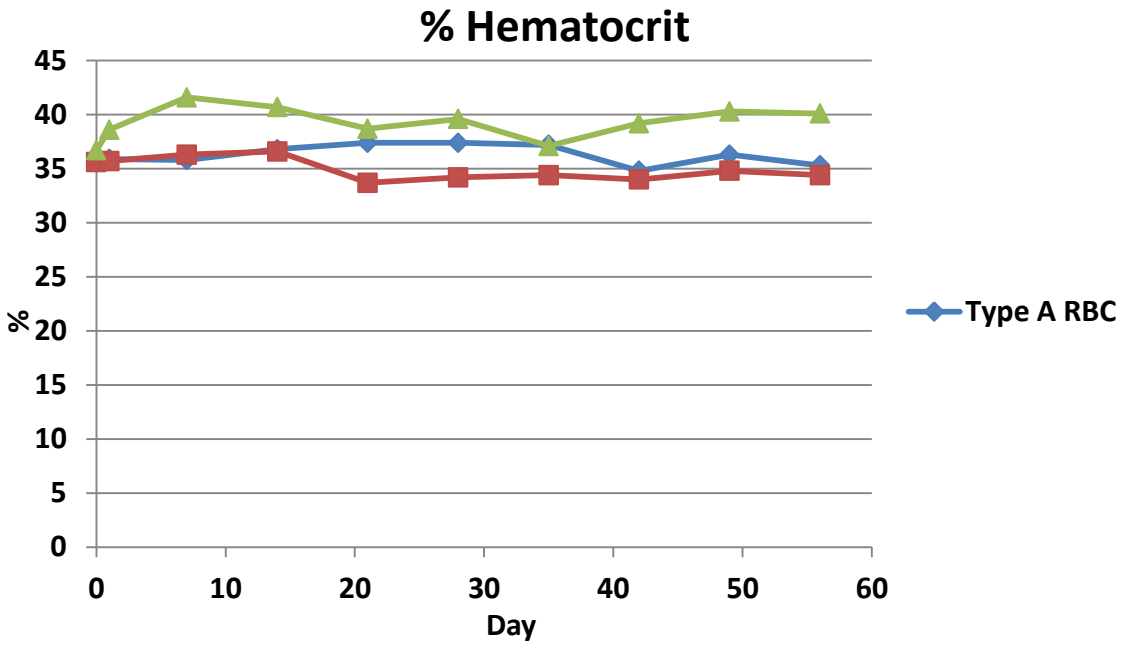
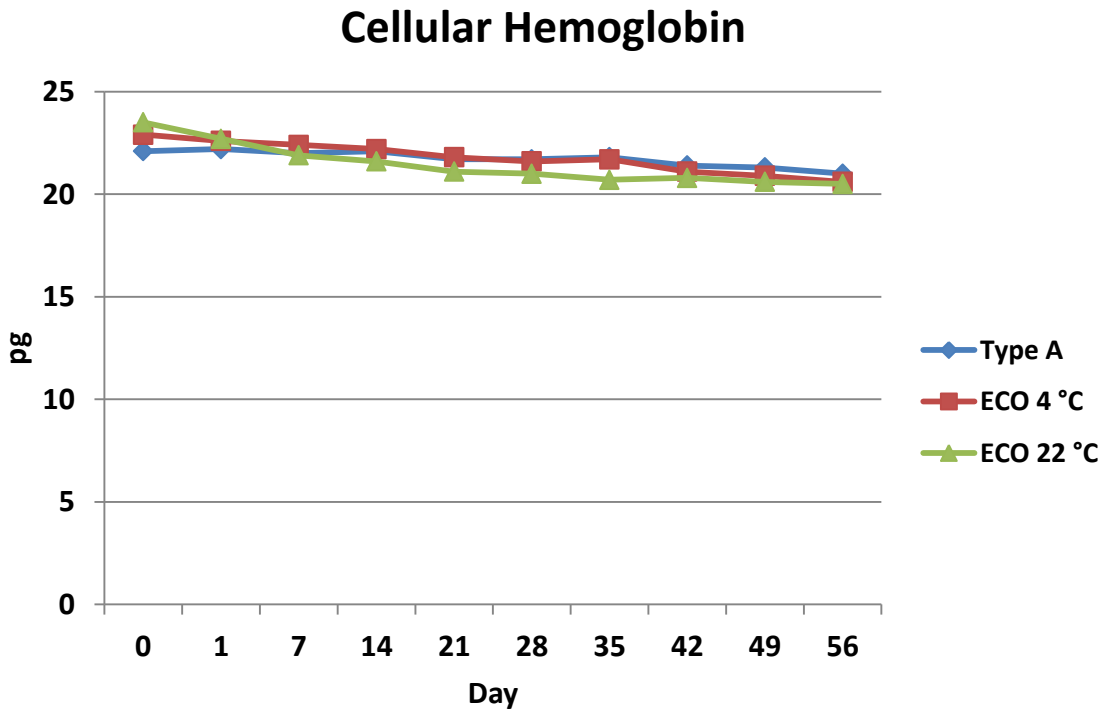


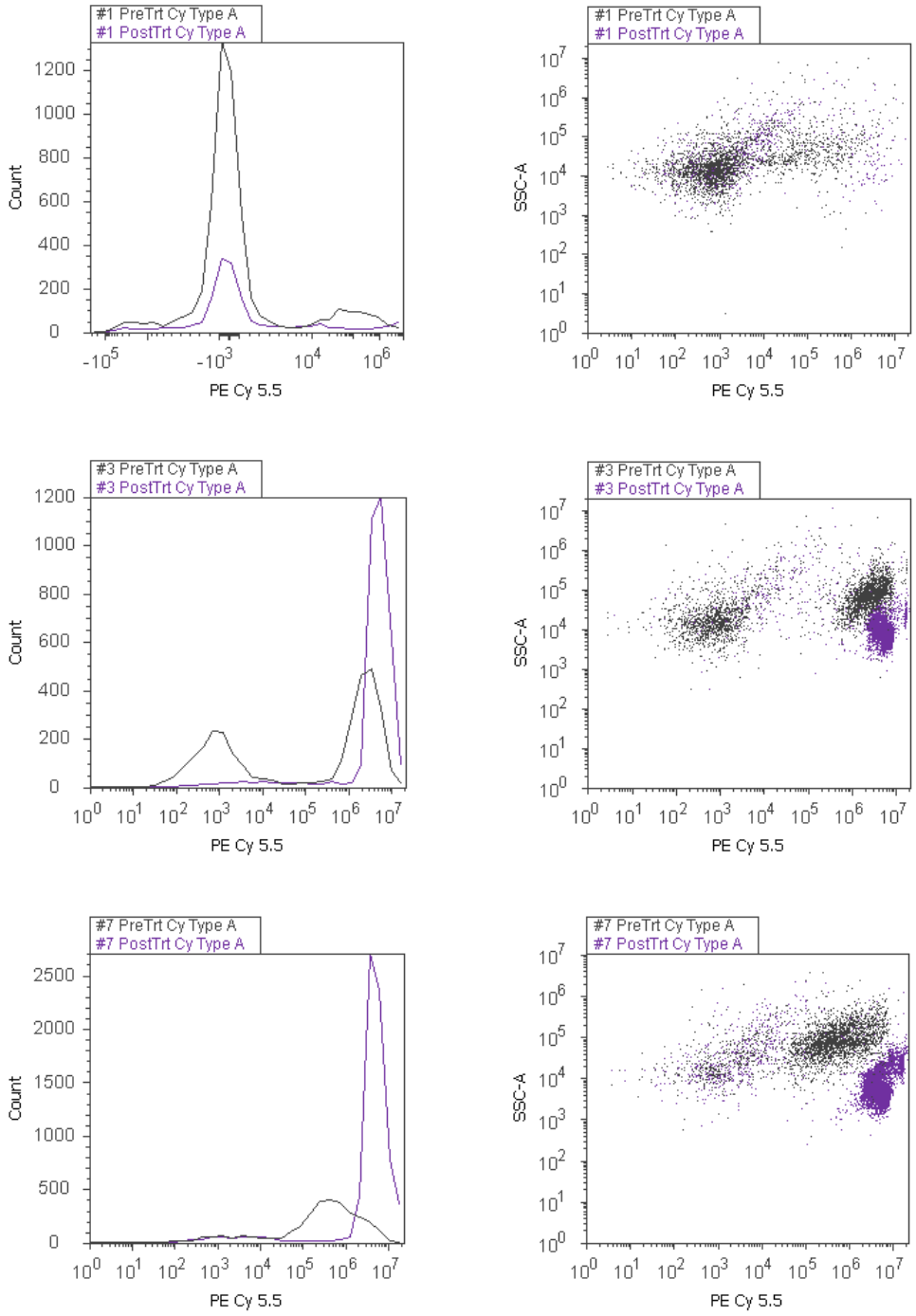
Figure 7.44: Cellular Hemoglobin pg



RBC Immunological Results

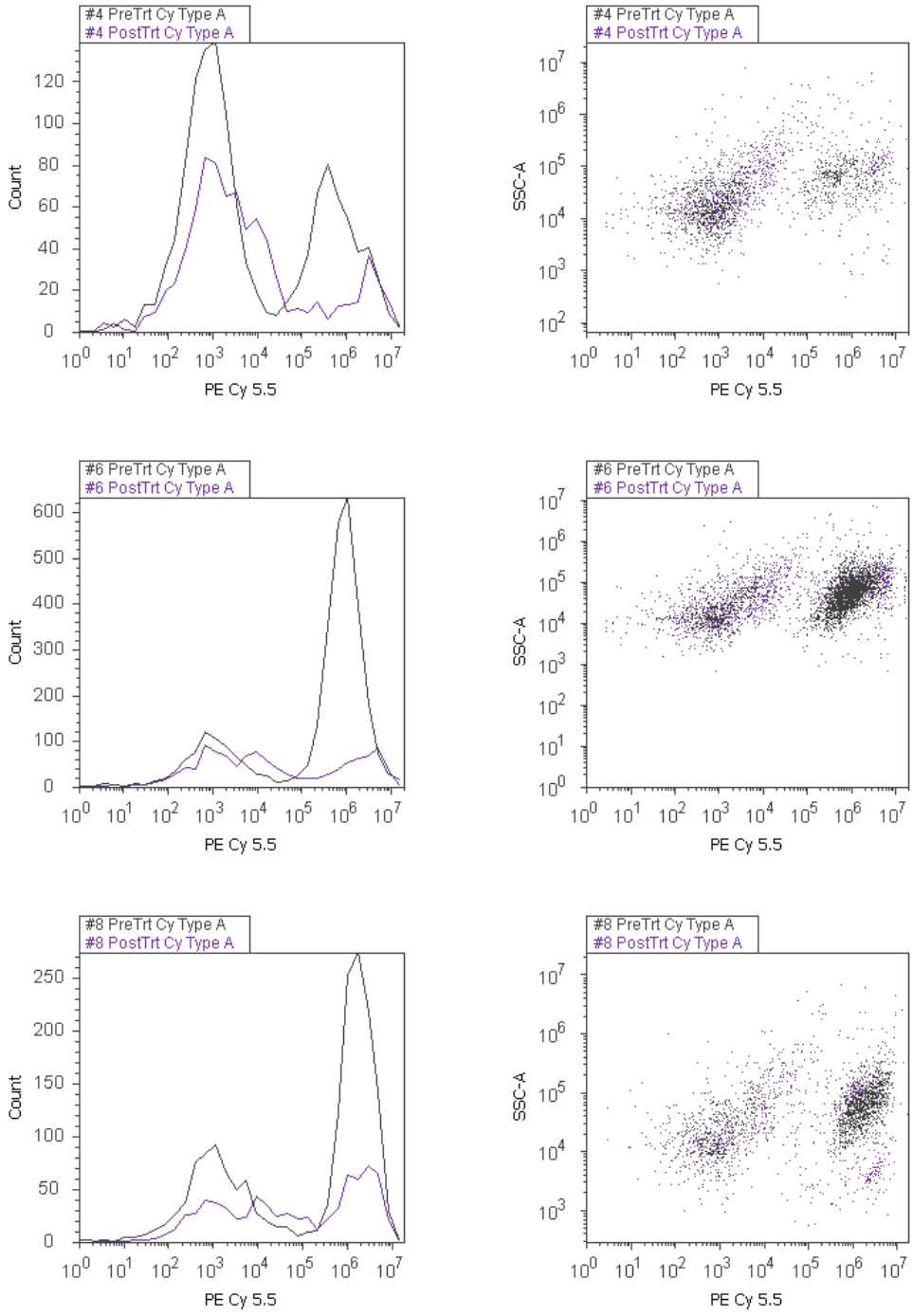
Nine New Zealand White rabbits were challenged with Type A, O, or ECO-A RBCs. On Day 0 and 21 each rabbit was challenged as indicated in Table 6.1 of Materials and Methods. No adverse reactions to the challenges were observed in the rabbits. Blood samples collected were analyzed against blood group A tetrasaccharide and H trisaccharide substrates for reactivity to antibodies by flow cytometry (Figures 7.45 and 7.46). Two of three rabbits challenged with Type A RBCs responded strongly to the A antigen tetrasaccharide, but also responded moderately to the H antigen trisaccharide. The rabbits challenged with Type O RBCs did not appear to have an induced response to either antigen. Two of the ECO-A challenged rabbits had a weak to low antibody response to the A antigen substrate and all three rabbits responded similarly to the H antigen as the rabbits challenged with Type O RBCs. Serum samples from the ECO-A challenged rabbits were also analyzed for induced antibody response to *S. linguale* α-NAGA by Western blot (Figure 7.47). There was no apparent induced antibody response to *S. linguale* α-NAGA by the ECO-A challenged rabbits.

Figure 7.45: Rabbit Antibody Response to Type A Tetrasaccharide
Group 1: Rabbits Challenged with Type A RBCs



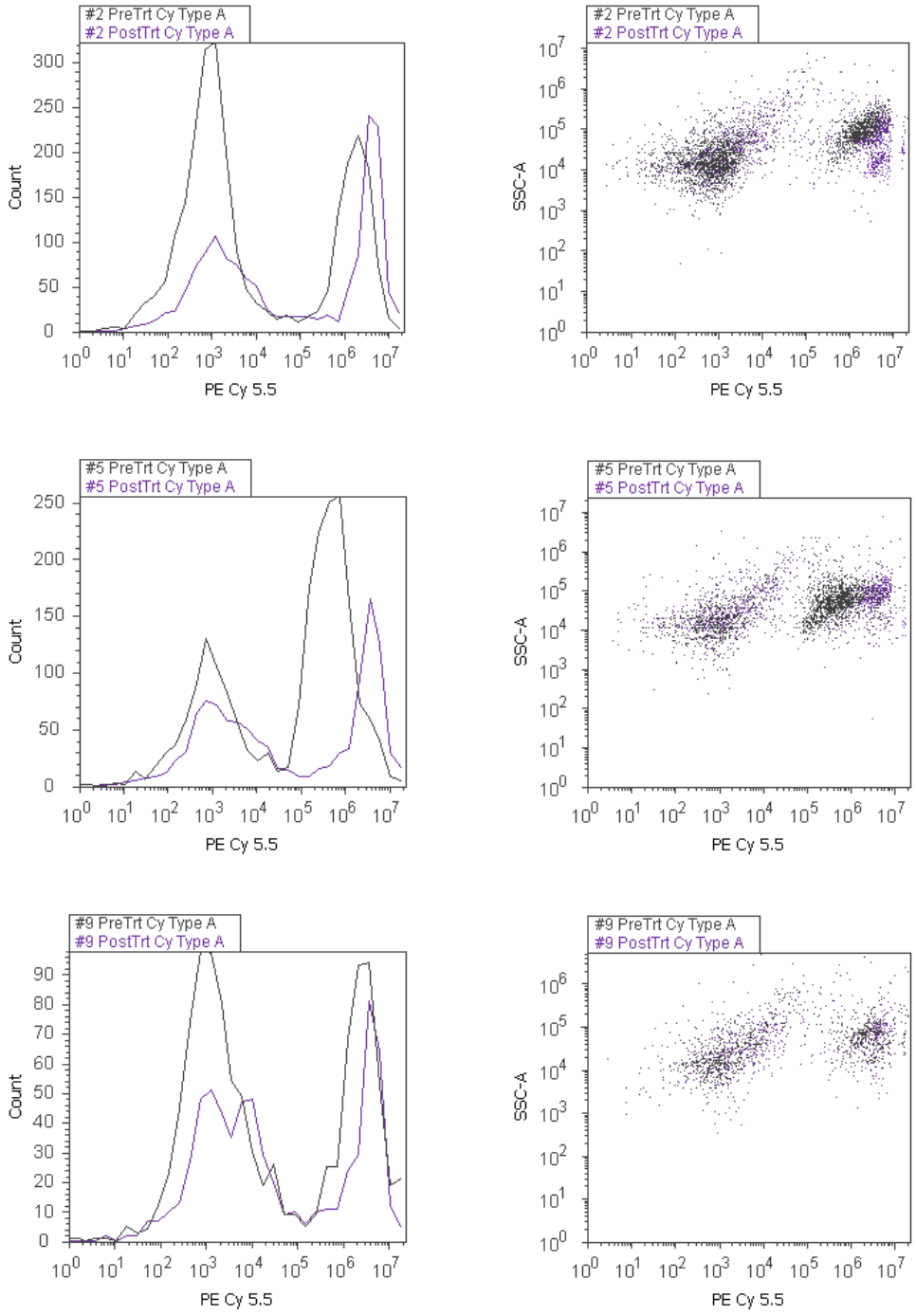
Rabbit antibody response to RBC challenge. Rabbit serum was exposed to Type A terminal tetrasaccharide sugars then stained with PE Cy 5.5 fluorophore conjugate. Fluorescent response of antibody adherence to Type A epitope pre-challenge (black), post-challenge (purple). Histograms X-axis Linear Log scale view

Group 2: Rabbits Challenged with Type O RBCs



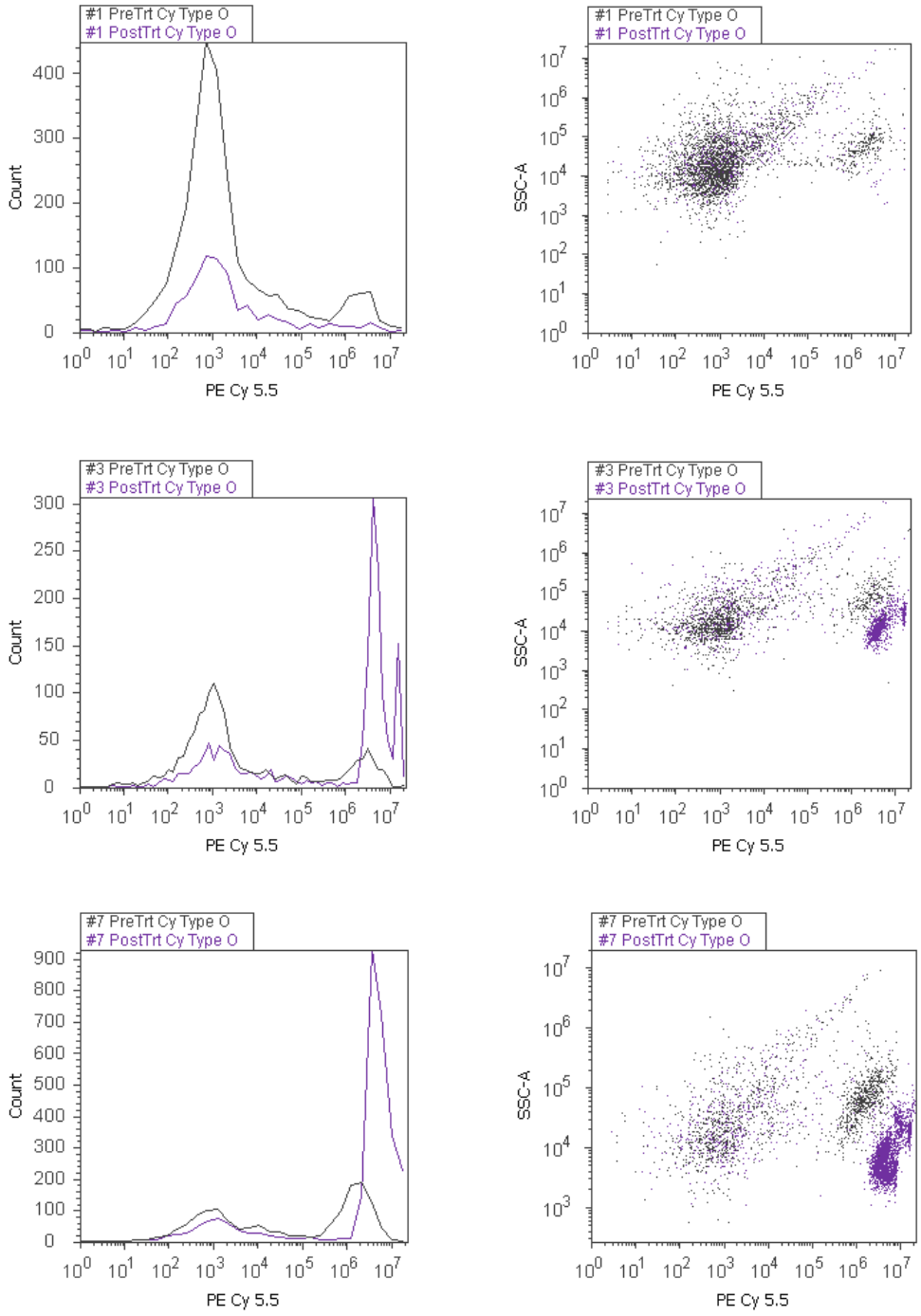
Rabbit antibody response to RBC challenge. Rabbit serum was exposed to Type A terminal tetrasaccharide sugars then stained with PE Cy 5.5 fluorophore conjugate. Fluorescent response of antibody adherence to Type A epitope pre-challenge (black), post-challenge (purple). Histograms X-axis Linear Log scale view

Group 3: Rabbits Challenged with Type ECO RBCs



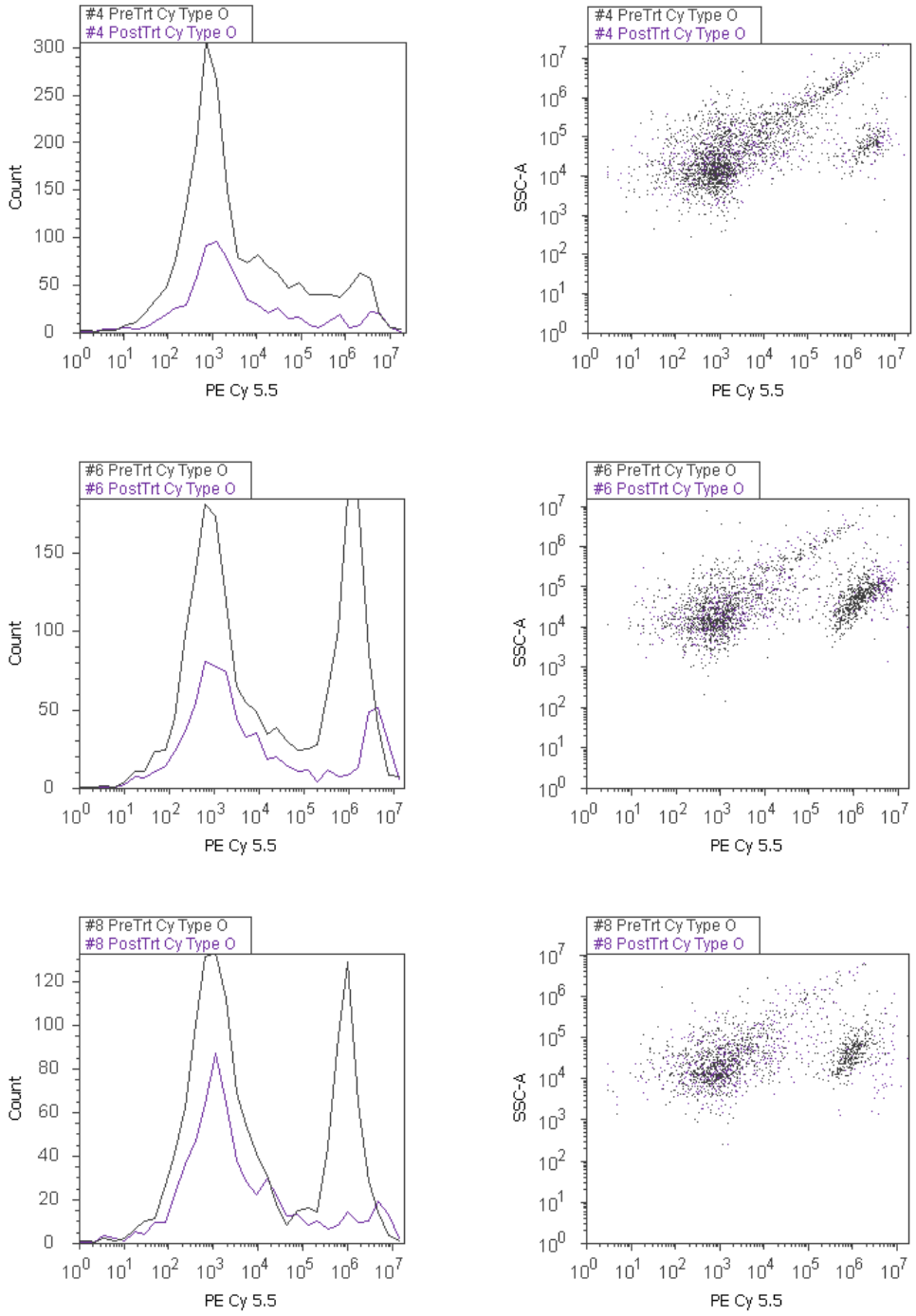
Rabbit antibody response to RBC challenge. Rabbit serum was exposed to Type A terminal tetrasaccharide sugars then stained with PE Cy 5.5 fluorophore conjugate. Fluorescent response of antibody adherence to Type A epitope pre-challenge (black), post-challenge (purple). Histograms X-axis Linear Log scale view

Figure 7.46: Rabbit Antibody Response to Type H Antigen Trisaccharide
Group 1: Rabbits Challenged with Type A RBCs



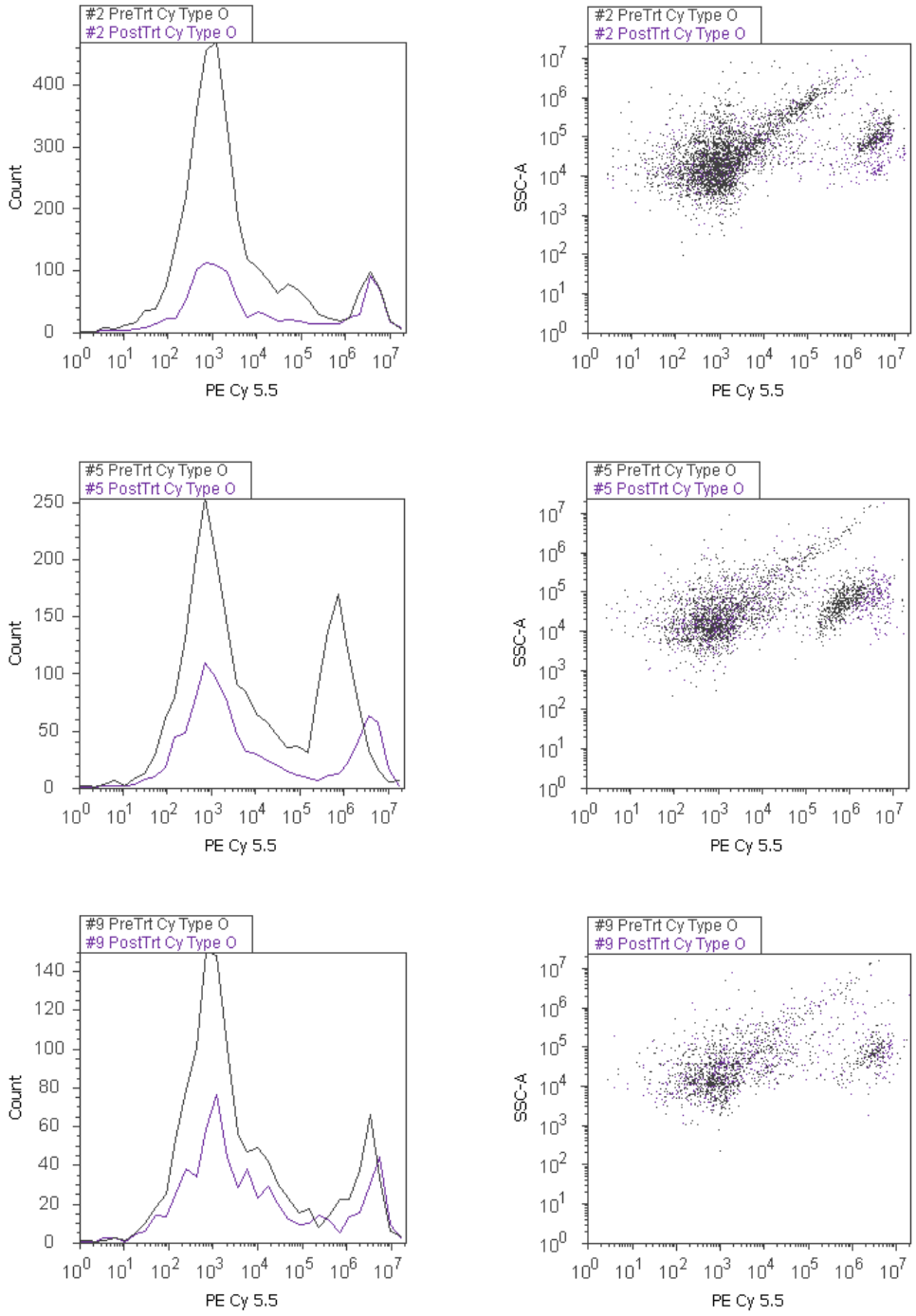
Rabbit antibody response to RBC challenge. Rabbit serum was exposed to Type O (H antigen) terminal trisaccharide sugars then stained with PE Cy 5.5 fluorophore conjugate. Fluorescent response of antibody adherence to Type O epitope pre-challenge (black), post-challenge (purple). Histograms X-axis Linear Log scale view

Rabbit Antibody Response to Type O RBCs
Group 2: Rabbits Challenged with Type O RBCs



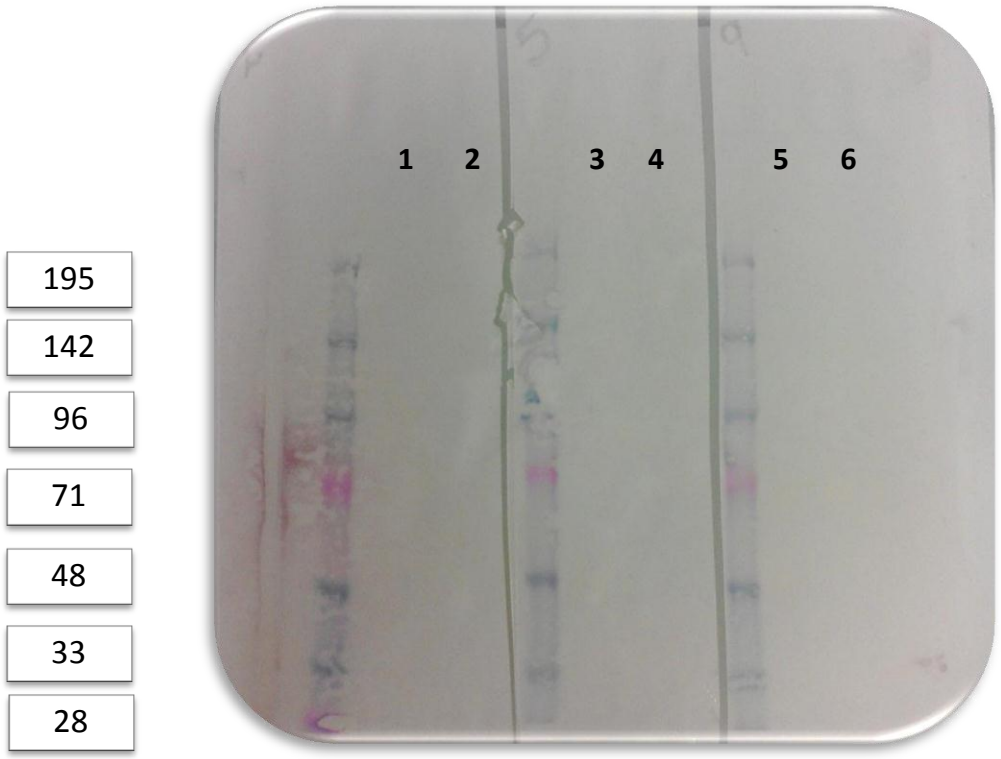
Rabbit antibody response to RBC challenge. Rabbit serum was exposed to Type O (H antigen) terminal trisaccharide sugars then stained with PE Cy 5.5 fluorophore conjugate. Fluorescent response of antibody adherence to Type O epitope pre-challenge (black), post-challenge (purple). Histograms X-axis Linear Log scale view

Rabbit Antibody Response to Type O RBCs
Group 3: Rabbits Challenged with Type ECO RBCs



Rabbit antibody response to RBC challenge. Rabbit serum was exposed to Type O (H antigen) terminal trisaccharide sugars then stained with PE Cy 5.5 fluorophore conjugate. Fluorescent response of antibody adherence to Type O epitope pre-challenge (black), post-challenge (purple). Histograms X-axis Linear Log scale view

Figure 7.47: Western Blot of the *S. linguale* α-NAGA ECO-A challenged rabbits



Lane 1,3,5 Protein standard markers (kDa), Lane 2 Rabbit #2, Lane 4 Rabbit #5, and Lane 6 Rabbit #9. Antibody response to residual *S. linguale* α-NAGA from rabbits subcutaneously challenged with ECO RBCs.

Chapter 8: Discussion

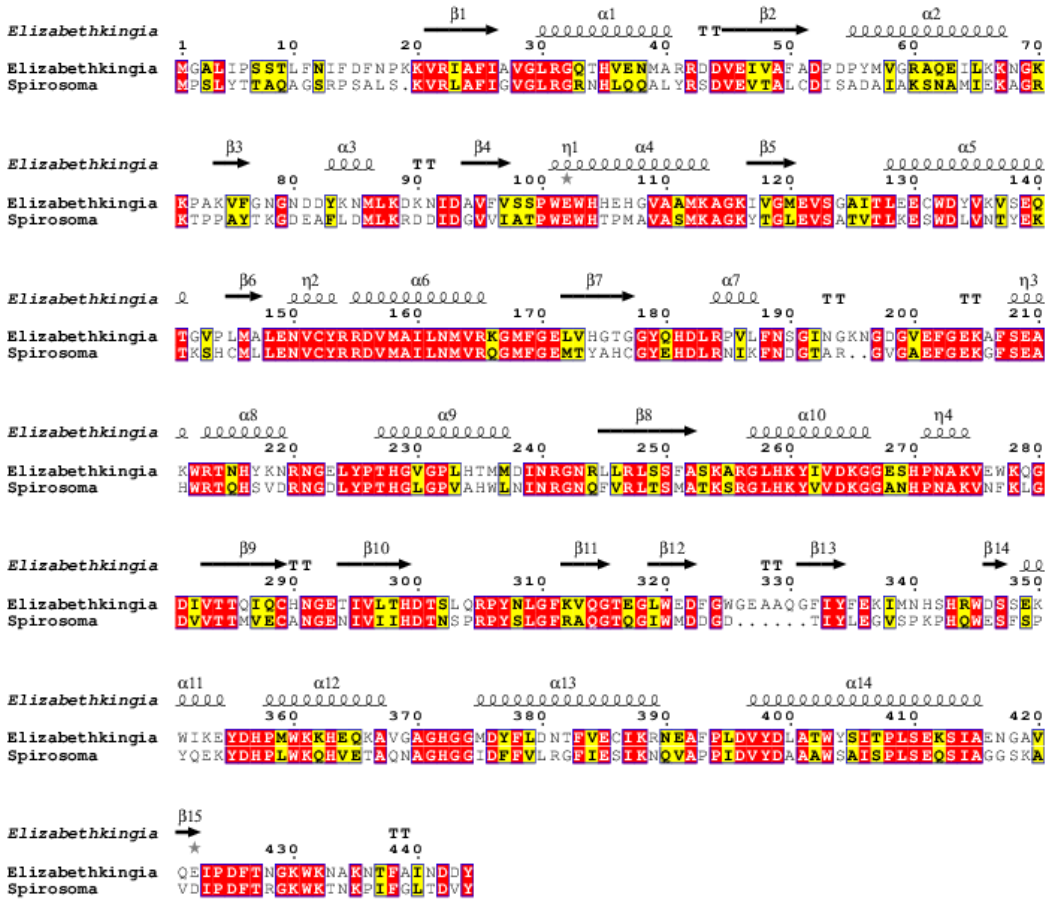
The goal of this research is to enzymatically treat RBCs to generate a universal blood supply. Enzymatic conversion of one blood group to another has been sought for over three decades. A number of enzymes have been identified that have desirable attributes and that convert RBC blood types A, B, and AB to O [50, 53, 56, 62, 63, 86, 89, 90, 95, 98, 99, 104, 149]. Two α-NAGAs that demonstrated conversion of Type A_2 RBCs to blood group O originated from chicken [150] and *C. perfringens* [57]. The enzymatic conversion conditions were not optimal, acidic conditions are required for the chicken enzyme and the *C. perfringens* enzyme preparation included additional glycosidases. Both of these enzymes have not been able to demonstrate the enzymatic conversion of Type A_1 RBCs. To date, conversion of Type A_1 RBCs to O has been the most challenging due to the added complexity of the antigen group [55]. Currently there are relatively few enzymes identified with the desired characteristics of highly efficient and specific, the activity within the blood pH range, and easily generated [49]. The results from the current project are consistent with the identification of an enzyme from *S. linguale* that has excellent properties to convert blood Type A to O. We also identified an enzyme from *E. coli* that has the potential to convert blood Type A to O, but under the experimental conditions tested was found to lack the desired activity. This study suggests the *S. linguale* enzyme has potential use as a tool to convert type specific RBCs to universal RBCs. This has been demonstrated by the specificity of the enzyme for the

target substrate, high enzyme activity using various buffers, temperatures, and optimal activity at a neutral pH.

NAGA Sequence

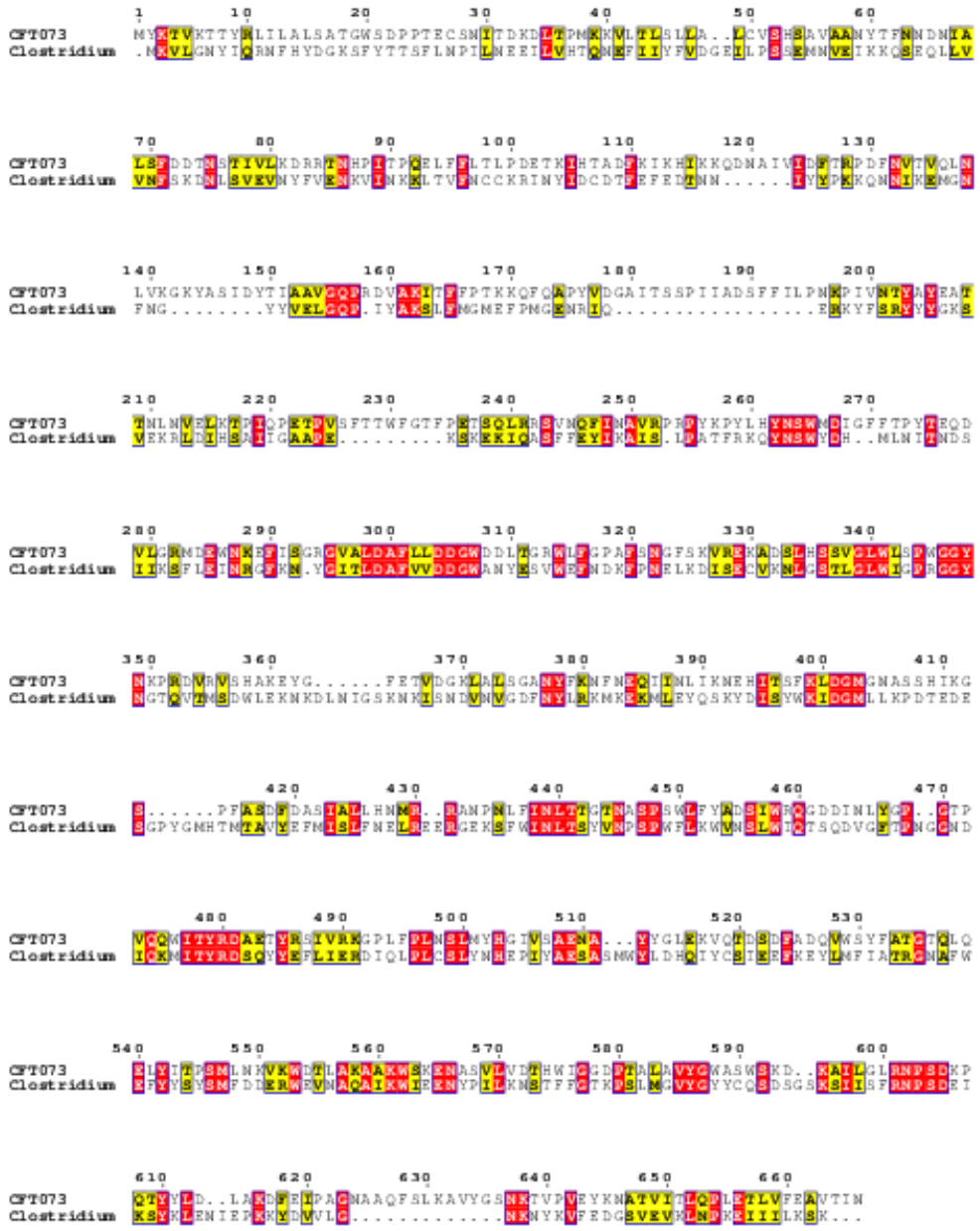
The gene encoding α-NAGA was cloned into an expression vector and transformed into an *E. coli* expression strain for each enzyme. The cloned DNA was sequenced and were confirmed to be identical to the genomic sequence by the University of Missouri DNA Core. Sequence alignment shows that *S. linguale* α-NAGA has 52% identity to the *E. meningosepticum* α-NAGA (Figure 8.1), while the *E. coli* α-NAGA has 27% identity to the *C. perfringens* α-NAGA (Figure 8.2).

Figure 8.1: α-NAGA Sequence Alignment *S. linguale* and *E. meningosepticum*



The amino acid sequence alignment of *S. linguale* and *E. meningosepticum* α-NAGA proteins. The upper protein of the alignment with black numbers designating residues and the secondary structure is for *E. meningosepticum* α-NAGA. Residues identically conserved in the sequences are denoted by white letters on a red background. Other regions of high sequence conservation are indicated by black letters on a yellow background [151, 152].

Figure 8.2: α-NAGA Sequence Alignment *E. coli* and *C. perfringens*



The amino acid sequence alignment of *E. coli* and C*. perfringens* α-NAGA proteins. The upper protein of the alignment with black numbers designating residues is for *E. coli* strain CFT073 α-NAGA. Residues identically conserved in the sequences are denoted by white letters on a red background. Other regions of high sequence conservation are indicated by black letters on a yellow background [151, 152].

As determined by the relative identity and protein sequence of the investigated α-NAGA, the *S. linguale* enzyme falls within the GH109 family classification, whereas the *E. coli* enzyme is within the GH36 family classification as indicated in the carbohydrate-active enzyme database http://www.cazy.org/. CAZy is a database classifying glycoside hydrolases based on analysis of genomic, structural and biochemical information on Carbohydrate-Active Enzymes (CAZymes). The classification of a glycosidic hydrolase can be derived from the hydrolytic reaction, endo or exo, Enzyme Commission number, or the mechanism or sequence classification [153]. Experimental data for GH36 family enzymes suggest that the nucleophile involved in the hydrolysis of the α-N-acetylgalactosamide is an aspartate residue [154]. The *E. coli* α-NAGA nucleophile has not been identified. The GH109 family involves a NAD^+ dependent hydrolysis mechanism of α-N-acetylgalactosamide as described by Koshland [47]. The glycoside hydrolase family 109 uses a mechanism that requires an NAD^+ cofactor, which remains tightly bound throughout catalysis. The reaction begins with an anionic transition state with elimination and redox steps. There is an initial oxidation of the hydroxyl group attached to carbon 3 by the enzyme-bound NAD^+ cofactor. This causes the acidity of carbon 2 to increase whereby the enzymatic base, e.g. histidine 225, removes a proton. An α,β-unsaturated intermediate is formed followed by the addition of water at the anomeric carbon. The ketone at C3 is reduced generating the hydrolyzed sugar resetting the enzyme. This reaction sequence was determined by NMR studies, kinetic isotope properties, equilibrium studies, X-ray crystallography and UV/Vis spectrophotometry [142, 144]. The *S. linguale* α-NAGA purported hydrolytic reaction

involves histidine 225 in conjunction of the NAD^+ cofactor as the mutation H225A abrogates enzymatic activity *in vitro* on conjugated substrates, and *in vivo*, on RBCs.

Protein Expression

The protein expression auto-induction methods developed by Studier have been used successfully for 20+ years. The methods work similarly to the IPTG induction system in that protein synthesis is under the control of the T7*lac* promoter, but does not require monitoring the density of the culture timing the induction of protein synthesis. Several advantages exist with the auto-induction vs. IPTG methodology;

1. Allows achievement of higher bacterial density increasing protein yield
2. Efficient inhibition of protein synthesis during log phase growth because of the non-inducing medium environment
3. Increased aeration of the system inhibits induction of protein by low levels of lactose
4. Increased retention of plasmids increasing protein synthesis and yield
5. Auto-induces protein synthesis
6. True walk away methodology

Expression of the proteins was efficiently achieved at approximately 14.25 mg/L (*S. linguale*) and 21.75 mg/L (*E. coli*) using Studier's auto-induction methods [141]. α-NAGA from *S. linguale* was purified approximately 25 fold while α-NAGA from *E. coli* was purified approximately 58 fold. Each enzyme was determined to be homogenous by SDS-PAGE, crystallography, gel filtration and MALDI-TOF chromatography. For the purpose of this research the protein yield was adequate, but future efforts will require substantially increased quantities of protein acquired with greater efficiency. Protein

requirements under the current expression conditions to convert one unit of Type A_2 RBCs would consume a significant percentage of expressed enzyme, but would place constraints converting Type A_1 RBCs. Improved techniques based on modifications to Studier's methodology have been shown to yield the same amount of protein in a 50 mL culture [155, 156]. These techniques have developed a step wise selection process whereby bacterial colonies are selected from the highest protein synthesizing hardy organisms from each generation. The selection process leads to the most efficient producing and robust organisms to express the target protein.

Enzyme Characteristics

Initial physicochemical substrate specificity of both enzymes with the reagent α-N-acetylgalactosamide was highly selective compared to eleven additional substrates. Similar specific activity was demonstrated by *E. meningosepticum* [49, 105] and *C. perfringens* [59] α-NAGA to α-N-acetylgalactosamide substrate. Optimal enzyme activity was demonstrated by both *S. linguale* and *E. coli* in the pH range 6.0 - 8.0, well within the range RBCs are stored. Comparison of Michaelis-Menten kinetic parameters in Table 8.1, illustrate the diverse activity and substrate affinity of this enzyme class. The catalytic efficiency of the *S. linguale* α-NAGA signifies approximately a 25% greater efficiency than the *E. meningosepticum* α-NAGAs, approximately 50% greater efficiency than the *C. perfringens* α-NAGA, and approximately 90% greater efficiency than the *E. coli* α-NAGA. This may provide some insight as to the lack of enzymatic conversion of RBCs by the *E. coli* α-NAGA. This variability of catalytic efficiency may relate to the amino acids at the active site. The *S. linguale* and *E. meningosepticum* enzymes involve

a histidine residue behaving as a base in conjunction with NAD^+ to hydrolyze the N-Acetylgalactosamine sugar, whereas the *E. coli* and *C. perfringens* enzymes appear to employ an aspartate residue, as the nucleophile, initiating the sugar hydrolysis. At the reaction pH and the enzyme architecture of the enzymes the histidine may be superior compared to the aspartate under these conditions. Another factor that may play a role is both the *E. coli* and *C. perfringens* enzymes are approximately a third larger by mass than the *S. linguale* and *E. meningosepticum* enzymes. Steric hindrance may play a key reason between the catalytic efficiencies of the enzymes.

Table 8.1: α-NAGA Michaelis-Menten Kinetic Factors

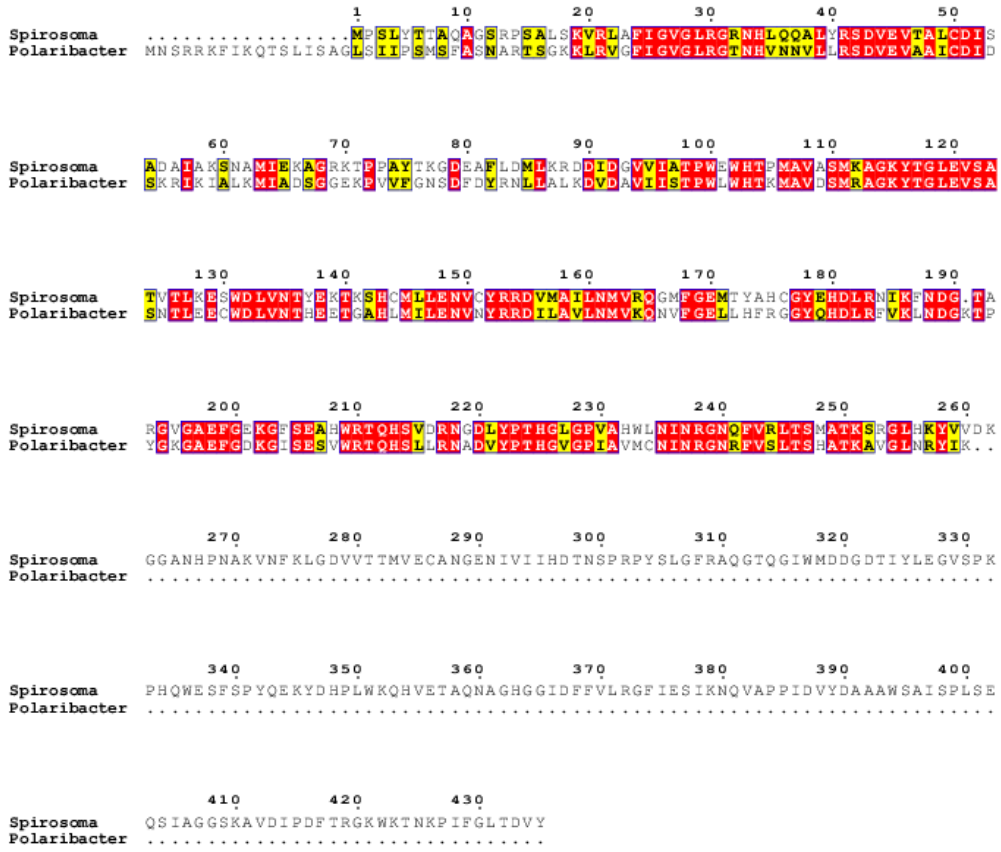
α-NAGA Source	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M ($s^{-1}mM^{-1}$)	% Difference k_{cat}/K_M*
Escherichia coli	3.4	33	10	6
Clostridium perfringens [157]	1.1	101	92	56
Elizabethkingia meningosepticum [49]	0.077	9.8	128	78
Elizabethkingia meningosepticum [105]	0.063	7.5	119	73
Spirosoma linguale	1.1	173	163	100

*Relative to *S. linguale*

Each α-NAGA enzyme, *E. coli* and *S. linguale*, exhibited different linear responses to temperature. Both enzymes originate from mesophilic organisms where the response of *E. coli* α-NAGA activity increased with temperature while *S. linguale* α-NAGA activity moderately decreased with increasing temperature. *E. coli* α-NAGA gave indication of continued elevation of activity at 45 °C in contrast *S. linguale* α-NAGA demonstrated optimal enzyme activity at 25 °C. When assessed at 4 °C the *S. linguale* enzyme activity decreased approximately 25% with Type A_2 RBCs. Since RBCs are stored at refrigerated temperatures there are advantages to maintaining the cells in this environment.

Removal of RBCs from the stored environment to convert the cells then returned to the refrigerated temperature may add stress to the cells. Identifying an enzyme that has increased efficiency at refrigerated temperatures compared to *S. linguale* α-NAGA would allow for conversion of RBCs without changing the environmental conditions to which the cells are acclimated to. An α-NAGA originating from a psychrophilic organism may harbor optimal activity at the 4 °C range. A BLAST search against the *S. linguale* α-NAGA sequence identified an enzyme from *Polaribacter* sp. [158], sharing 73% identity to identified residues, which may exhibit this quality (Figure 8.3). Additionally the *S. linguale* NAGA sequence could be mutated to the alignment in Figure 8.3 to determine potential benefits of enzymatic conversion at 4 °C.

Figure 8.3: Sequence Alignment *S. linguale* and *Polaribacter* sp.



The amino acid sequence alignment of *S. linguale* and *Polaribacter* sp. α-NAGA proteins. The upper protein of the alignment with black numbers designating residues is for *S. linguale* α-NAGA. Residues identically conserved in the sequences are denoted by white letters on a red background. Other regions of high sequence conservation are indicated by black letters on a yellow background [151, 152].

Using a phosphate buffer pH 7.0, as a base to relate the effect on enzyme activity, solutions composed of 50 mM acetate, 10 mM citrate, 10 mM tris, and 500 mM EDTA appeared to reduce relative enzyme activity of *S. linguale* α-NAGA. A glycine solution, used in studies by Liu et al. [49] and Yu et al. [105], appeared to reduce enzyme activity *in vitro* compared to the phosphate solution. Liu et al. showed inhibition of *E.*

meningoseticum enzyme activity with several divalent metals, including Cu^{2+}, Ni^{2+}, and Zn^{2+}, at concentrations of 1 or 10 mM but EDTA did not impact enzyme activity at these concentrations [49]. Undiluted Adsol solution tested against the *C. perfringens* enzyme was the only solution found to lower activity, as demonstrated by Hsieh and Smith [104]. Product accumulation was tested for enzyme inhibition during this research project. The *E. coli* α-NAGA activity was shown to have uncompetitive inhibition from N-Acetylgalctosamine accumulation during enzyme activity. On the other hand, *S. linguale* α-NAGA produced a concerning level of competitive inhibition during product accumulation. The reaction mechanism proposed by Liu et al. [49] outlined that H228 was involved with the NAD^+ cofactor during the enzyme hydrolysis reaction. The H228, as aligned with the *S. linguale* sequence is equivalent to H225. Histidine 225 was changed to an alanine to assess enzyme activity. The H225A mutation resulted in the production of an inactive enzyme when compared to the wild-type recombinant α-NAGA.

RBC Enzyme Conversion

Conversion of Type A_1 RBCs to blood group O has proved to be difficult. The ability to convert Type A_2 and B RBCs has been known for over two decades. This is not to say the enzymes and technology used during the past two decades were optimal. As technology has improved and the choice of enzyme(s) has increased, the ability to select the optimal conditions has been enhanced. Two decades into the future we may reflect back and have a similar conclusion.

The difficulty with Type A_1 RBCs stands out because of the number of antigen sites, the linkage type of the carbohydrate chains, and the number of carbohydrate repeats of the antigen of this blood group compared to Type A_2 or B RBCs. Table 8.2, provides data of the difference between Type A_1 and A_2. The difference illustrated is only of the approximate antigen number of the two Type A sub-groups. Using the *in vitro* turnover rate, k_{cat} (s^{-1}), of the two enzymes of this research and two closely related enzymes the significant difference to convert Type A RBCs to blood group O is astounding.

Table 8.2: α-NAGA Conversion Rate Relating Antigen Number to Turnover Rate

	Blood Type	Antigen number	k_{cat} (s^{-1})	Conversion Rate (Hr)
E. coli α-NAGA	A_1	1.50E+06	34	13
	A_2	250000	34	2.1
C. perfringens α-NAGA	A_1	1.50E+06	101	4.2
	A_2	250000	101	0.7
E. meningosepticum α-NAGA	A_1	1.50E+06	10	42
	A_2	250000	10	6.9
S. linguale α-NAGA	A_1	1.50E+06	173	2.4
	A_2	250000	173	0.4

Of the enzymes listed in Table 8.2, the *E. coli* α-NAGA did not demonstrate the ability to convert RBCs during this research effort, the *C. perfringens* α-NAGA did demonstrate the ability to convert RBCs but with known glycosidase contaminants in the enzyme preparation [63]. The *C. perfringens* enzyme also demonstrated conversion of RBC Type A_2 extracted antigen adhered to a 96 well plate [157]. Under similar experimental circumstances as the *C. perfringens* α-NAGA the *E. coli* α-NAGA may have demonstrated this similar ability. The organisms from where the two enzymes originate are of pathogenic character [159, 160]. Both organisms are hemolytic and this lytic function to

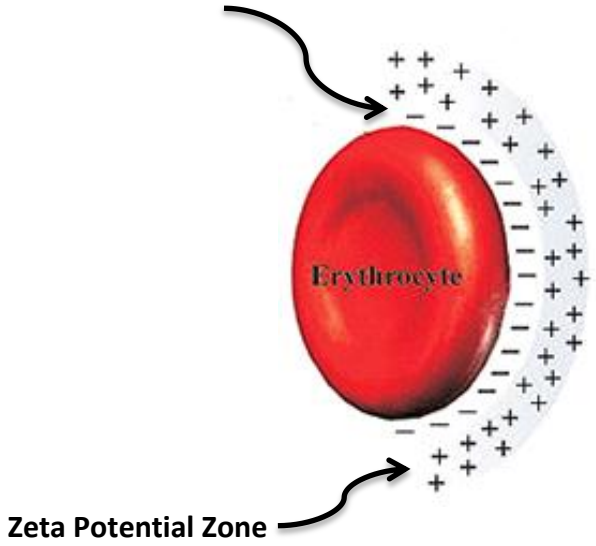
fragment the RBC may be important to the activity of these enzymes. This theory has not been demonstrated at this time.

The α-NAGAs from *S. linguale* and *E. meningosepticum* have both been demonstrated to convert Type A RBC to blood group O in this research, and by two groups Liu et al. [49] and Yu et al. [105], respectively. The *E. meningosepticum* enzymes had very similar steady state Michaelis-Menten kinetic parameters. An alignment of the protein sequences is identical with the exception of four amino acids at positions 160G>V, 174H>R, 426F>S, and 434V>A. The major variance between the Liu and Yu work is the enzyme amount reported to convert RBCs. To convert 200 mL of packed RBCs Liu, reported that the enzyme required for Type A_1 is 60 mg and for A_2 15 mg, on the other hand, Yu reported the enzyme requirements as 3.0 mg for A_1 and 0.8 mg for A_2, a 95% difference. The estimated concentration of *S. linguale* α-NAGA required to convert 200 mL of packed Type A_1 RBCs is 80 mg and for A_2 20 mg. There may be an explanation for the difference between the data of Liu and Yu, but it is not evident.

As indicated the *E. coli* α-NAGA did not appear to successfully convert Type A RBCs compared to *S. linguale* α-NAGA. For the later, four solutions were evaluated demonstrating that glycine or alanine could equally serve as buffer of choice to convert RBCs. Phosphate buffer with varied ionic strength was an unsuitable buffer selection to convert RBCs for either enzyme. Smith and Walker [161] showed that a glycine solution was a superior selection to enzymatically convert RBCs. Glycine and alanine are known to disrupt the negative charged environment around the red cell membrane [162]. Figure 8.4 provides an illustration of the charges associated with RBCs. It is prerequisite

that these external charges be displaced for the enzyme to gain access to the antigens on the RBC surface.

Figure 8.4: Illustration of the Zeta Potential of RBCs

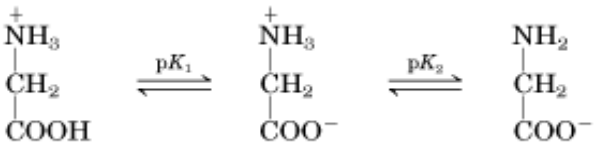


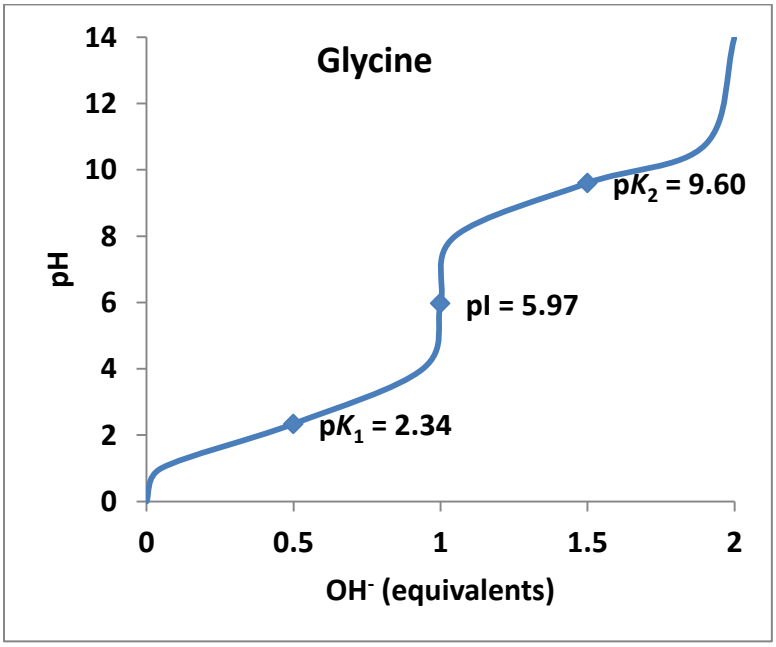
Erythrocyte (negative charges) in suspension causes a rearrangement of charges through the formation of two ionic layers that generate an electrical potential difference between them and their environment called the Zeta potential. Adapted from Fernandes, H.P., C.L. Cesar, and M.d.L. Barjas-Castro, *Electrical properties of the red blood cell membrane and immunohematological investigation.* Revista Brasileira de Hematologia e Hemoterapia, 2011. **33**: p. 297-301.

Glycine and alanine both serve as potentiators reducing the zeta potential around the RBC, enhancing enzyme activity. The titration of glycine provides an understanding why it serves as a potentiator (Figure 8.5). Since glycine and alanine are zwitterions within the pH range of 4-8, they have a net zero charge. This characteristic allows both amino acids to neutralize both positive and negative charges. For this same reason, lysine and phosphate solutions have either a positive or negative overall charge, and thus, because

of the inability to reduce the zeta potential did not create an environment for the enzyme to interact with RBCs.

Figure 8.5: Titration of Glycine





Adapted from Nelson, D. and M. Cox, *Lehninger Principles of Biochemistry 4th Edition*. 4 ed 2004 Freeman, W.H. & Company.

This modified environment improves the α-NAGA RBC interface leading to the hydrolysis of α-N-acetylgalactosamine. An observation made during the conversion of RBCs involved pretreating cells in glycine buffer the day prior to adding enzyme. This led to additional enzyme efficiency, specifically for Type A_1 RBC conversion. Due to the reduced zeta potential by glycine, additional care of the blood sample is required when using these potentiators, because agglutination of RBCs is augmented. Jan and Chien

showed the effect of altering the charged environment around the RBC impacted aggregation [163]. Due to the effects of glycine, *S. linguale* α-NAGA exhibited significant enzyme activity *in vivo* across a broad pH range, 5.5-8.0. The enzyme demonstrated efficient activity at both room and refrigerated temperatures but requires approximately 10X more enzyme when used at 4 °C. Packed cell volume approaching 50% has a negative impact on enzyme activity. A packed cell volume range used in this research was 20-35%. *In vitro* testing suggested that product accumulation would be competitive during enzyme activity. Competitive inhibition due to product accrual was not noted during any experiment. Experimental data suggested the potential for reverse enzyme activity when evaluating flow cytometry results collected at 1 and 2 hour. Testing with Type O RBCs and α-N-acetylgalactosamine indicated that conversion to Type A RBC did not occur. *In vivo* testing with the mutant H225A, did not account for any conversion of Type A RBCs.

RBC Stability

Stability of the RBCs after enzymatic conversion is paramount if it is to advance beyond the research laboratory environment. On the surface the conversion process of RBCs seems minimal cleaving the terminal α-N-acetylgalactosamine. Nevertheless this is a multiple-step procedure that may place stress on the RBC. This research project inspected the stability of enzyme converted RBCs. RBC count, hemoglobin concentration, and % hematocrit showed statistical significance between Type A RBCs and enzyme converted cells. Visual hemolysis was observed in the enzyme converted RBC samples. The primary cause of hemolysis appears to be linked to the centrifugation

steps to where the RBCs were subjected. The Type A unconverted cells did not undergo centrifugation when prepared. Graphically the data from the three groups, Type A and cells converted at room or refrigerated temperature, appear similar. Clinically the RBC samples may behave no differently. Goldstein et al. showed that enzyme converted Type B cells behaved and survived similarly to unconverted RBCs [86]. Vosnidou et al. incurred similar results as this research when comparing converted and non-converted RBCs [95]. Kruskall et al. noted hemoglobin concentration decreased from approximately 7.4 g/dL post enzyme conversion which was thought to be related to the washing steps following conversion of RBC [94]. Within the same study RBC survival was found to be similar comparing converted and non-converted blood. Additional studies should be conducted to fully evaluate the presence or absence of cellular stress to converted RBCs.

Protein and RBC Immunology

Typically foreign host proteins induce an immune response when injected into the system. An immune response, 1:4096 titer, was induced in a rabbit challenge model with *E. coli* α-NAGA. The *S. linguale* α-NAGA did not appear to induce an immunological reaction in the rabbit model when challenged with enzyme converted RBCs. A follow-up blood transfusion study will need to be accomplished to provide a definitive indication whether *S. linguale* α-NAGA has immunological properties. Two studies using α–galactosidase from coffee beans triggered an immune response post-transfusion of enzyme converted blood in Rhesus monkeys and humans [90, 94]. Each of the studies did not observe any adverse events relating to this antibody response. The two studies

involved single transfused units of blood. Transfusion of multiple units of blood over time has not been investigated in subjects to evaluate for a potential increased immune response and/or reaction.

A report of infusing larger amounts of the enzymes to evaluate immunological/toxicological concerns is currently sparse in the literature. A 150 µg/kg level of α–galactosidase from coffee beans has been demonstrated to be safe in Rhesus monkeys [90]. Zhang determined this concentration represented approximately 2500X by body weight (µg/kg) safe concentrations of enzyme. The Zhang study indicated that approximately 10 ng/mL of enzyme remained after conversion and washing of the RBCs. An approximate 1X dose of enzyme based on the Zhang study would equate to 60 ng/kg of α-NAGA. Notwithstanding the immune response to residual enzyme following conversion of the RBCs the response of blood transfusion reactions begets a higher level of concern to the recipient.

Nine New Zealand White rabbits were challenged with Type A, O, or ECO-A RBCs. Each rabbit was challenged on Day 0 and 21 according to group assignment. Baseline and post challenge blood samples were collected to determine induced antibody response. The samples were analyzed against blood group A tetrasaccharide and H trisaccharide substrates for reactivity to ABO blood antibodies by Flow Cytometry. As indicated in the results section two of the rabbits challenged with Type A RBCs responded strongly to the A antigen tetrasaccharide but also responded moderately to the H antigen trisaccharide, while rabbits challenged with Type O RBCs did not appear to have an induced response to either antigen and two of the ECO-A rabbits had a weak to low

induced antibody response to the A antigen substrate and all three rabbits responded similarly to the H antigen as the rabbits challenged with Type O RBCs. This initial experiment provided valuable mixed data but additional studies to evaluate transfused enzyme converted blood to test subjects need to be completed to determine the extent of an antibody response. There has been limited experimental work to determine the effects of enzyme converted transfused blood.

From 1991 throuh 1995, Lenny et al. successfully transfused blood converted to O using a α-galactosidase from green coffee beans [91, 92, 93]. No clinical reactions were observed in the volunteers. Nor were there abnormal laboratory test results from blood samples taken during these studies. In 2000, Kruskall et al. successfully transfused converted B to O blood into 21 patients [94]. Likewise no adverse reactions were noted in any of the patients. Red blood cell survival appears comparable between patients transfused with converted vs. non-converted RBCs in these studies with rare exception. Two patients indicated reduced RBC survival that was related to specific reasons, one patient had gastrointestinal bleeding at the time of the transfusion and another patient's serum was incompatible with the transfused red blood cells. Also determined post transfusion monitoring were 5 of 19 patients developed increased anti-B titers. These achievements have provided a step forward but also raised additional questions. Better understanding of the anti-B immune response and the cross-matching compatibility issue with serum or plasma are pivotal to this aim. Currently additional immunological data that may exist is not available regarding enzymes under investigation.

Summary

This research program characterized the physico-chemical attributes of *E. coli* and *S. linguale* α-NAGA. The experimental potential to convert RBCs to a universal donor state by the *E. coli* α-NAGA was not demonstrated. The initial process to convert RBCs with the *S. linguale* α-NAGA was established. The experimental testing provided confirmation of the conversion of RBCs. Quality and storage stability of the converted RBCs was exhibited. Initial data were produced to evaluate the immunological properties of *S. linguale* α-NAGA. The concept and ground work has been established to convert RBCs with *S. linguale* α-NAGA.

Chapter 9: Future Direction

In the research completed to date *S. linguale* α-NAGA has demonstrated the potential as a robust candidate to develop for use as an enzyme to convert RBCs from blood group A to O. Additional experimental work should be conducted to further refine the methodology used to convert RBCs. This would include:

- Enzyme expression efficiency
- evaluating other zwitterion solutions
- evaluate the zeta potential of RBCs to optimize enzyme requirements
- pretreatment of RBCs
- enzyme requirement studies
- alternative enzyme(s)
- converted RBC functionality
- RBC fragility
- RBC storage stability
- development of RBC conversion device

Initial blood transfusion studies in animals would need to be investigated for transfusion tolerance (single and multiple), RBC stability, safety, enzyme immunology and toxicology. Transfusion tolerance and immunology studies of converted RBCs has been accomplished in some Phase I studies [91-94]. The majority of this work has involved the conversion of Type B to blood group O. This research evaluated storage of converted RBCs with additional investigation follow up needed to understand the

stability of the cells. Vosnidou et al. evaluated RBCs converted with *Glycine max* α-galactosidase and noted significant differences in the RBC indices similar to this research [95]. Stability of enzyme converted RBCs has been investigated in rabbits and humans by Lenny and Goldstein et al. [86, 164]. Zhang et al. examined the toxicity of α-galactosidase in rhesus monkeys for side effects [90]. These studies have all been evaluated for the α-galactosidase from green coffee bean. A study by Gunter has evaluated the *E. meningosepticum* α-NAGA converted RBCs in autologous transfusions for reactions [106]. Similar requisite studies testing *S. linguale* α-NAGA and enzyme converted cells would be initiated.

Tissues likewise possess ABO antigens. Tissue antigens are an extremely important consideration when contemplating transplant surgery. Typing of tissues is as important as blood type cross matching involved for blood transfusions. The assessment of *S. linguale* α-NAGA to seroconvert ABO tissue antigens for transplantation has consequence to tissue rejection. Review of this potential enzyme use has merit and should be evaluated.

The direction forward for the α-NAGA from *E. coli* as a tool to convert RBCs is unpromising. A possible avenue for the enzyme is evaluation as a vaccine candidate for urinary tract infections or as a therapeutic to treat diseases such as Schindler's and Kanzaki's.

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VITA

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