Purification of *Staphylococcus aureus* Capsular Polysaccharide Serotype 5

By

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Abstract

Staphylococcus aureus continues to pose a major health threat, most prevalent in the immunocompromised, despite the development of antibiotics. Due to the increased antibiotic resistance seen with this pathogen another means of treatment is warranted. Ninety-eight percent of the clinical isolates of *S. aureus* are encapsulated, eighty percent of which are capsular polysaccharide (CP) type 5 or 8. CPs are thought to shield *S. aureus* from agglutination by antibodies to cell wall structures. For this reason, encapsulated strains are poorly phagocytosed by polymorphonuclear leukocytes. However, antibodies specific for the CP have been shown to be efficient in inducing phagocytosis of encapsulated strains. *S. aureus* remains a problem for patients with decreased immunologic function because these individuals are often unable to form immune responses to carbohydrate molecules. Preformed antibodies against the CP of *S. aureus* would aid these patients in eliminating the bacterium. Presented in this study is the first step to the production of therapeutic antibodies, the isolation and purification of *S. aureus* CP. The CP was removed from the bacterium by autoclaving the bacterial cell suspension. The CP was then purified by enzymatic digestion and anion exchange chromatography. In this study, several methods are tested for their ability to detect the CP in the anion exchange column eluate. The CP, a reducing sugar, was ultimately detected by its ability to reduce Red Tetrazolium to an intensely colored diformazan whose production was measured spectrophotometrically. The CP, which was determined to be free of teichoic acid contamination, will be used in further studies to produce preformed antibodies against *S. aureus* CPs.

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I would like to dedicate this thesis to my parents Elio and Mary, for without their love and support this project would not have been possible.

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Abbreviations

Ac	acetyl
CP	capsular polysaccharide
CTL	cytotoxic T lymphocyte
DEAE	diethylaminoethyl
DNase	deoxyribonuclease
EDTA	ethyldiaminetetraacetic acid
FucNAc	*N*-acetylfucosamine
HF	hydrogen fluoride
Ig	immunoglobulin
MAC	membrane attack complex
ManNAcA	*N*-acetylmannosaminouronic acid
MHC	major histocompatibility complex
MRSA	methicillin-resistant *Staphylococcus aureus*
NMR	nuclear magnetic resonance
PBP	penicillin binding protein
PBS	phosphate-buffered saline
PMNL	polymorphonuclear leukocytes
rEPA	recombinant *Pseudomonas aeruginosa* exoprotein A
RNase	ribonuclease
T_C	T cytotoxic cell
T_H	T helper cell
TLC	thin layer chromatography

TRIS	tris(hydroxymethyl)aminomethane
VISA	vancomycin-intermediate *Staphylococcus aureus*
VRE	vancomycin-resistant enterococcus

Introduction

Staphylococcus aureus is a faculatively anaerobic, nonmotile, coagulase positive member of the Staphylococcus genus (Davis *et al.*, 1980). A high degree of resistance to *S. aureus* is seen in healthy individuals, twenty-five percent of which asymptomatically carry *S. aureus* on their skin, nasopharynx, and intestinal surfaces but rarely have systemic infections. *S. aureus* infection occurs most often in patients with reduced resistance, causing opportunistic infections (Fattom *et al.*, 1992). It is the second most common cause of nosocomial, or hospital acquired, infections in the Unites States (Na'Was *et al.*, 1998). It is most commonly associated with infections of prosthetic devices, surgical wounds, meningitis, osteomyelitis, septic arthritis, bacteremia, endocarditis, and pneumonia in hospitalized patients (Lee, 1998). It is also an underlying cause of disease in newborns, trauma victims, burn patients, drug abusers, and individuals with decreased amounts of neutrophils (Lee, 1996). It is the most common organism causing bacteremia, or blood-borne infections, accounting for one-third of all cases in the United States (Fattom *et al.*, 1990). Once *S. aureus* has established an infection in the blood it is free to move to other organs and commonly causes an infection in the heart valves leading to endocarditis. Bacterial endocarditis can lead to septic shock, the symptoms of which are drop in blood pressure, fever, diarrhea, and widespread inappropriate blood clotting called disseminated intravascular coagulation (Hochkeppel *et al.*, 1987).

Antibiotics were introduced in the 1940s, which led to a great decrease in infection rates and death from *S. aureus* (Segal-Maurer *et al.*, 1996). It was then believed that bacterial infections, particularly nosocomial infections, were a curse of the past.

However, due to the selective pressure from antibiotic overuse, resistant strains have surfaced that are even deadlier than before. This is widely due to the overuse of antibiotics for agricultural purposes in the US and Europe. Annually, 11,200 tons of antibiotics are given to livestock in the US as feed supplements and 900 tons for veterinarian purposes. This is far greater than the annual human consumption of 1300 tons (Gorbach, 2001).

Antibiotics: Modes of Action, Mechanisms of Resistance

The more an antibiotic is used in a population, the greater the incidence of resistance. The cells that are resistant can grow and are selected for, as those that are susceptible to the antibiotic die. A mutation at a specific spot in the genetic code could be enough to confer resistance to a strain. Under long periods of antibiotic use a mutant cell appears at a rate of 1 in every 10^5 to 10^6 cells. However, mutations are not transmissible from strain to strain. Mutated strains also usually confer low levels of resistance and grow poorly. The most common way resistance occurs is through the acquisition of genes that encode resistance. These genes can be acquired from the chromosome of another cell, through a plasmid (a small piece of DNA that acts as a mini chromosome), or through transposons (linear pieces of DNA that are self transmissible between cells and are capable of integrating into plasmids or chromosomes). There are three ways these genes can be transferred: transformation (naked DNA that penetrates the cell and becomes integrated into the hosts genome), transduction (a bacteriophage injects DNA into the cell), or through conjugation (cell to cell contact where DNA is transferred) (Noble, 1997).

One of the first classes of antibiotics to be introduced were the penicillins. *S. aureus* produces four penicllin binding proteins (PBPs) that function in the biosynthesis of the bacterial cell wall (Ito and Hiramatsu, 1998). β-lactam antibiotics, such as penicillin have a strong affinity for the high molecular weight PBPs and inhibit cell wall synthesis by covalently binding to and inactivating PBPs. When PBPs are inactivated the bacterium is unable to synthesize the cell wall, resulting in the formation of holes, causing osmotic lysis and death. Due to widespread antibiotic use, by the end of the 1950s, 85% of *S. aureus* strains in the United States and Europe were resistant to penicillin. These strains produced β-lactamase, also called penicillinase, which splits the β-lactam ring of the penicillin nucleus rendering it inactive (Davis *et al.*, 1980).

In 1959 new semisynthetic penicillins, methicillin and oxacillin, which were not inactivated by penicillinase, were introduced clinically with the hopes of solving the problem of resistance. However, the first methicillin resistant strain was reported in England in 1961 just two years after its clinical introduction. Strains that are resistant to methicillin produce a low molecular weight PBP, PBP2a that is encoded by the *mecA* gene. β-lactam antibiotics have a low affinity for PBP2a and therefore PBP2a remains unbound and thus is not inactivated. PBP2a takes over the role of the high molecular weight PBPs and synthesizes the bacterial cell wall (Hartman and Thomasz, 1984). There is no homolog to the *mecA*, in strains that are susceptible to methicillin, suggesting that this is the gene responsible for resistance to methicillin, oxacillin, and all β-lactam antibiotics (Hiramatsu *et al.*, 1996).

There are three other mechanisms that are responsible for low levels of methicillin resistance. In strains that lack the *mecA* gene, an overexpression of penicillinase is

enough to confer low levels of resistance (McDougall and Thornsberry, 1986). Some strains also overproduce PBP4, a normal PBP that has a low affinity for β-lactam antibiotics. Because of its low affinity, the antibiotic does not bind all of the PBP4, and it is able to remain active to synthesize the bacterial cell wall (Henze and Berger-Bächi, 1994). There are also mutations present in some strains that lower the affinity of PBP2 for methicillin, leaving this PBP like PBP4 unbound and active to synthesize the bacterial cell wall (Michel and Gutmann, 1997).

The tetracycline class of antibiotics, which have been in use clinically for several decades, consist of eight related antibiotics which are all natural products of Streptomyces. The tetracyclines are transported into the cell and act by blocking the binding of the aminoacyl tRNA to the A site on the ribosome inhibiting protein synthesis. The combination of their broad range of activity and low level of toxicity has led to their overuse by the medical community resulting in widespread resistance. *S. aureus* has acquired a gene that increases the transport of the antibiotic out of the cell, thus reducing the inhibition on mRNA (Chopra, 1986). Glycylcyclines, semi-synthetic derivatives of tetracycline-like antibiotics are currently under development. GAR-936, which is currently undergoing clinical trials, has broad-spectrum bactericidal activity against Gram-positive, and Gram-negative aerobic and anaerobic bacteria (Sum and Petersen, 1999; Petersen *et al.*, 1999).

Fluoroquinolones, such as ciprofloxacin, perfloxacin, and ofloxacin are effective against staphylococci. Fluoroquinolones inactivate staphylococci topoisomerase IV and DNA gyrase. Point mutations in the genes that encode these enzymes are sufficient to encode resistance. Clinically high levels of resistance have been seen, especially when

this antibiotic is used alone. However this class of antibiotics may prove to be more effective when used in combination with other antibiotics. New fluoroquinolones, such as clinafloxacin and levofloxacin, with increased anti-staphylococcal activity are under development (Neu, 1988).

Rifampin blocks protein synthesis by inhibiting RNA polymerase. It penetrates very well into the tissue. However, like the fluoroquinolones, when used alone high levels of resistant *S. aureus* strains develop. A point mutation in the B-subunit of RNA polymerase is responsible for *S. aureus* resistance to this antibiotic (Chambers, 1997).

Gentamycin and netilmicin are members of the aminoglycoside class of antimicrobial agents. Again these antibiotics work best when used in combination because high levels of resistance are seen when they are used alone (Chambers, 1997). Another drawback to therapy with aminoglycoside antibiotics is their high level of toxicity. Long-term treatment has been shown to cause kidney and nerve damage.

Vancomycin, a member of the glycopeptide class of antibiotics, like the β-lactam family of antibiotics acts on peptidoglycan (PG) metabolism. It acts by binding to bacterial cell wall precursors, and interferes with the growth of PG. PG is essential for bacterial survival because it functions as an exoskeleton and provides support to the cell protecting it from internal pressure (Reynolds, 1989). It was thought that resistance to vancomycin would be unlikely because bacteria would have to drastically change their metabolic pathway to synthesize new PG precursors. Its clinical use for more than 35 years before resistance developed reinforced the idea that vancomycin resistance would never develop. In 1989 resistance of *Enterococcus faecalis* to vancomycin was first reported in London and soon became common throughout the world (Uttley *et al.*, 1989).

These resistant strains had managed what was thought to be impossible and acquired an alternative PG precursor biosynthetic pathway. Vancomycin resistant *Enterococcus faecalis* (VRE) carries a conjugative plasmid with a complex of nine genes that encode resistance allowing the bacterium to neutralize the drug's bactericidal activity (Noble, 1997; Cetinkaya *et al.*, 2000; Evers *et al.*, 1996). Coagulase negative *Staphylococcus haemolyticus* has also been shown to carry vancomycin resistance (Schwalbe *et al.*, 1987). These resistance genes have been successfully transferred *in vitro* and *in vivo* to *S. aureus* (Noble *et al.*, 1992). Researchers feel it is only a matter of time before wild-type strains of *S. aureus* gain resistance to this antibiotic. The first vancomycin intermediate *Staphylococcus aureus* (VISA) strain was discovered in Japan (Hiramatsu *et al.*, 1997). Since then it has been isolated in Europe and the United States. All strains that were intermediate for vancomycin were methicillin resistant (Marchese *et al.*, 2000). In addition to increasing resistance, there are drawbacks to vancomycin therapy. It has only moderate intravascular diffusion, a low bacteriocidal effect, is costly, has a high risk of toxicity, and is only available in an injectable form (Michel and Gutmann, 1997; Rahman, 1998).

Teicoplanin, another antibiotic of the glycopeptide class is similar to vancomycin. It is not available in the United States but has been used clinically in other countries. Although it has a much longer half-life than vancomycin, it has been shown to be less effective and emergence of resistant strains to this antibiotic have already been reported (Kaatz *et al.*, 1990). Other semi-synthetic glycopeptides are currently being developed.

There are several new antibiotics currently under development. In preliminary studies L-695,256, a synthetic carbapenem, was shown to be as effective as vancomycin

on methicillin-resistant *Staphylococcus aureus* (MRSA). L-695,256 has a high affinity for PBP2a, binding to it and preventing synthesis of the bacterial cell wall.

RP59500 is a semi-synthetic injectable streptogramin, which has been shown to be active against MRSA (Chambers, 1995). RP59500 is composed of quinupristin and dalfopristin, which act together to prevent protein synthesis by binding to two different sites on the 50S subunit of the ribosome (Brumfitt *et al.*, 1992). It has also been shown to be at least as effective as vancomycin against MRSA (Entenza *et al.*, 1995). Studies on cardiac vegetations have shown that it has a good diffusion rate into the infected area (Fantin *et al.*, 1994). However, it is only recommended as a last resort against drug-resistant strains to avoid selection of strains highly resistant to RP59500. The use of a highly related compound Virginiamycin in veterinary medicine as a growth promoter might also limit the effectiveness of RP59500 as resistance is transferred from animals to humans (Gorbach, 2001).

Oxazolidinones inhibit initiation of bacterial protein synthesis at a step before the formation of the initiation complex, a feature that is different from all other antimicrobial agents that are inhibitors of protein synthesis. Oxazolidinones have been shown to have activity against *in vitro* and *in vivo* animal models of MRSA infection (Ford *et al.*, 1996). Two types of oxazolidinones, eperzolid and linezolid are currently in phase I clinical trials (Chambers, 1997). Resistance was observed first in VRE (Gonzales *et al.*, 2001) and has been reported in clinical isolates of *S. aureus* (Tsiodras *et al.*, 2001).

Two antibiotics that are currently in end stage clinical trials are Ramoplanin and Orthosomycins. Ramoplanin is a lipoglycodepsipeptide antibiotic that has been shown to inhibit the late stages of PG synthesis. It is active against both MRSA and VRE strains

(Ohare *et al.*, 1990). There have been no cases of clinical or laboratory generated resistance (Somner and Reynolds, 1990; Reynolds and Somner, 1990). Orthosomycins are oligosaccharide antibiotics that were discovered in the 1960's but not developed due to toxic effects on the kidneys (Ganguly *et al.*, 1985; Weinstein *et al.*, 1965). A less toxic form, SCH-27899 is currently being developed that inhibits protein synthesis. It binds to a different site than the other protein synthesis inhibiting antibiotics and therefore has a different form of action. It has very potent activity against both Gram-positive and Gram-negative bacteria; however resistance has already developed (Fuchs *et al.*, 1999; Urban *et al.*, 1996). The resistance gene, which is again associated with the overuse of a related antibiotic avilamycin in animals, is located on a plasmid and is readily transferable (Aarestrup, 1998, Mann *et al.*, 2001).

The trend of resistance has continued to occur. Widespread use of broad-spectrum antibiotics and lack of regulation of antibiotic use, such as over the counter availability in some countries has lead to increasing resistance to multiple antibiotics (Essawi *et al.*, 1998). Today over 90% of clinical strains produce β-lactamase and are resistant to penicillin (Na'Was *et al.*, 1998). In 1960 *S. aureus* carried its resistance genes in the form of plasmids; today it has many of these genes integrated into its chromosome. The CDC has reported that MRSA strains are becoming increasingly more resistant to other antibiotics (Hughes, 1987). Data from the National Nosocomial Infection Surveillance (NNIS) shows an increase from 2% to 29% during the last 20 years of the hospital strains that are resistant to methicillin. In hospitals with more than 500 beds, 38% of the strains are resistant (Panlilio *et al.*, 1992). The percentage of MRSA is 60% in Japan, 35% in the United States, 44% in Italy, and 30% in Spain (Inoue *et al.*, 1998). There is only a 0.6%

rate of MRSA in Holland, which has a relatively low use of antibiotics (Ayliffe, 1997). Statistics predict that one-third of patients in hospitals colonized with *S. aureus* will become infected and half of these will result in bloodstream infections or pneumonia with 50% and 33% mortality rates, respectively.

Hospital workers that are carriers of *S. aureus* serve as a reservoir for the organism. In an individual hospital setting, eradication of the carriage state by hospital workers has decreased the nosocomial infection rate. There is little inborn resistance to *S. aureus* mucosal colonization. Mupirocin is a topical antimicrobial agent also called psuedomonic acid, which interferes with protein synthesis by inhibiting isoleucyl tRNA synthetase. Mupirocin is the most effective antibiotic to control nasal carriage of *S. aureus* in the hospital setting (Chambers, 1997). However, prolonged use has seen emergence of strains with resistance. These strains have acquired a plasmid encoding a gene for an isoleucyl tRNA synthase that is not resistant to the antibiotic (Noble, 1997).

There is a high mortality rate from *S. aureus* infections despite the use of antibiotics due to strains that are resistant to multiple antibiotics (Fattom *et al.*, 1990). Isolates (40-60%) from large hospitals in the United States are resistant to multiple antibiotics including methicillin (Lee, 1998). Continued bacterial resistance has made *S. aureus* infections difficult to treat. Due to the misconception that antibiotics were infallible very few new classes of antibiotics are in the advanced stages of development (Boneca, 2003). In the preantibiotic era there was an 80% death rate associated with *S. aureus* infection (Smith and Vickers, 1960). With the current trend of antibiotic resistance we are heading back toward those infection and mortality rates. The development of another means of treating this bacterium is warranted. Passive antibody

therapy was widely used prior to the invention of antibiotics, but was widely abandoned since their development. The emergence of multi-antibiotic resistant strains has revived the search for a vaccine or antibody therapy.

Innate vs. Acquired Immunity

Innate immunity refers to the nonspecific resistance to disease with which an individual is born. It provides the first line of host defense against pathogens. Innate immune responses consist of multiple modalities that help prevent the entrance and establishment of infection. These include anatomic barriers, such as skin and mucous membranes, physiologic mechanisms such as body temperature, pH, and chemical mediators, the ability to endocytose and phagocytose foreign macromolecules or microorganisms, and an inflammatory response that leads to an influx of phagocytic cells, leading to localized tissue necrosis. *S. aureus* can enter the body via natural routes, such as the respiratory and gastrointestinal tracts, or unnatural routes such as breaks in the mucous membranes or skin. When these barriers are unsuccessful in prohibiting entrance of a pathogen, an acquired immune response is mounted.

If innate defenses are compromised, acquired immune responses become important. Acquired immune responses require recognition of an antigen, a foreign particle such as a bacterium or virus, and response to that antigen by generation of effector cells and molecules. These immune responses are specific; they are able to tell a difference as small as one amino acid in an antigen. Acquired immune responses are diverse, capable of creating billions of molecules that are able to respond to these antigens. The acquired immune system displays memory, producing a heightened response the second time it encounters the same antigen. It is also capable of

discriminating between foreign and self-antigens, allowing it to prevent potentially fatal autoimmune diseases. Generation of the acquired immune response requires antigen presenting cells and lymphocytes. There are two different types of lymphocytes, B cells and T cells.

B cells are responsible for the humoral branch of the acquired immune system. They mature in the bone marrow, where they undergo gene rearrangements and leave expressing an antibody molecule with a single antigenic specificity. They circulate throughout the blood and lymphoid organs. B cells recognize antigenic determinants or epitopes alone, such as those displayed on the surface of bacteria or virus particles, or those epitopes on soluble proteins, such as glycoproteins, lipoproteins, or polysaccharides. When a naïve B cell (first response to antigen) encounters *S. aureus* it proliferates and differentiates into either memory B cells or effector cells called plasma cells. Memory B cells express membrane bound antibody molecules of the original antigenic specificity. They have a long life span, and are responsible for the heightened response that is seen in the second encounter with the same antigen. Plasma cells have a short lifetime, living only during the course of infection, where they secrete antibodies at a rate greater than two thousand molecules per second.

The binding of antibody to the surface of *S. aureus* cell mediates activation of the complement cascade. The complement system is an important effector mechanism of the humoral immune system. The complement cascade amplifies the humoral immune response leading to an inflammatory response, opsonization of antigen, viral neutralization, and increased clearance of immune complexes. It is an enzymatic cascade that takes place via two pathways: the classical pathway and the alternative pathway, that

terminate in a common membrane attack complex (MAC). The classical pathway is specific and is initiated by the formation of antigen-antibody immune complexes. Antigen coated with complement complexes are more easily phagocytosed leading to increased antigen clearance. The alternative pathway is generated by molecules that are foreign to the host, such as cell wall constituents of bacteria. This serves as an innate nonspecific defense against invading microorganisms, which is supplemented by the more specific classical complement pathway. The MAC displaces the phospholipids in the cell membrane, forming a transmembrane pore, which disrupts the osmotic regulation of the cell, leading to cell lysis. Most Gram-positive organisms are resistant to lysis by complement because the MAC is unable to penetrate the thick PG layer in their cell wall. Complement products C3a, C4a, and C5a are anaphyltoxins that bind to receptors on mast cell cells and basophils and induce degranulation, which releases histamine and other chemicals that induce vasodilation and increased vascular permeability. This causes an influx of fluid into the affected area, which carries antibody and phagocytic cells, which then act to eliminate the bacterium. Complement products C3a, C5a, and C5b67 act together to induce monocytes to bind to the vascular endothelium and extravasate (leave the blood vessels) to the site of complement activation (Fischetti *et al.*, 2000).

T lymphocytes, the cells responsible for the cell-mediated branch of the acquired immune system, leave the bone marrow and migrate to the thymus where they mature. A mature T cell expresses an antigen binding receptor on its membrane called the T cell receptor. Unlike B cells, T cells cannot respond to antigen alone, they require the help of antigen presenting cells. Antigen presenting cells internalize antigen and present part of it complexed to cell membrane proteins on their surface known as major histocompatability

complex molecules (MHC). Antigen presenting cells include macrophages, B cells, and dendritic cells. T cells can only recognize protein epitopes bound to an MHC molecule. Like B cells, when a T cell encounters antigen, it proliferates and differentiates into memory cells and effector cells.

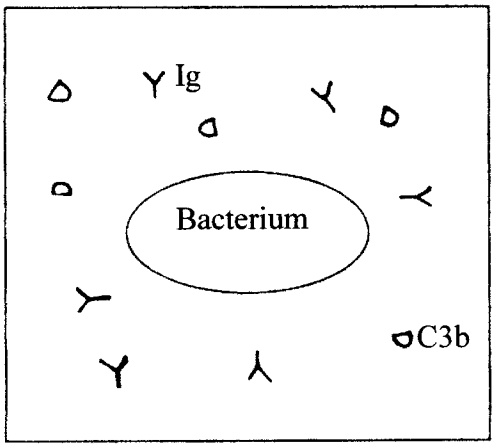
There are two types of T cells, $CD4^+$ T helper (T_H) cells and $CD8^+$ T cytotoxic (T_C) cells. A T_H cell recognizes antigen complexed to a MHC class II molecule. The T_H cell differentiates and secretes various cytokines, which activate B cells and T_C cells. T_H derived cytokines can activate phagocytic cells enabling them to engulf and eliminate pathogens more easily. T_C cells recognize antigen complexed to a MHC class I molecule and differentiate into cytotoxic lymphocytes (CTLs). CTLs kill virus-infected cells, tumor cells, and altered self-cells.

The innate and adaptive immune responses act together in the defense against *S. aureus* infections. Phagocytosis, an innate response, is the host's first line of defense against an invading pathogen (Lee, 1996). Neutrophils, macrophages, and monocytes are the cells involved in phagocytosis. The phagocytic cells are attracted to antigens by chemicals produced in the immune response. Antigens such as whole bacterial cells or viral particles adhere to the phagocyte cell membrane and are easily engulfed and killed. Encapsulated bacteria, such as S. *aureus*, adhere poorly and are less readily phagocytosed. Opsonization, or enhanced phagocytosis occurs through opsonins, which bind to the antigen and phagocytic cell membrane and enhance phagocytosis (Figure 1). There are receptors on the phagocytic cell membrane for antibody (a product of the adaptive immune response) and/or complement, both of which function as opsonins.

Figure 1: Opsonization

Infection by an extracellular pathogen, such as a bacterium induces the formation of antibody molecules, which are secreted by plasma cells (a). Antibody and C3b bind to the antigen and act as opsonins, or substances that bind to an antigen and enhance its phagocytosis (b). Phagocytic cells express a receptor, CR1, which binds to C3b. This enables them to more easily bind to and phagocytose complement-coated pathogens. Phagocytic cells also express an F_c receptor, which allows them to more readily bind pathogens coated with antibody (c). Activated phagocytic cells express an increased number of CR1 and F_c receptors. When an antigen is coated with opsonin, it binds more readily to the appropriate receptor on the phagocytic cell membrane (d) resulting in enhanced phagocytosis (e). A 4000-fold increase in the rate of phagocytosis has been shown to occur in the presence of opsonins. Abbreviation: Immunoglobulin, Ig.

a.



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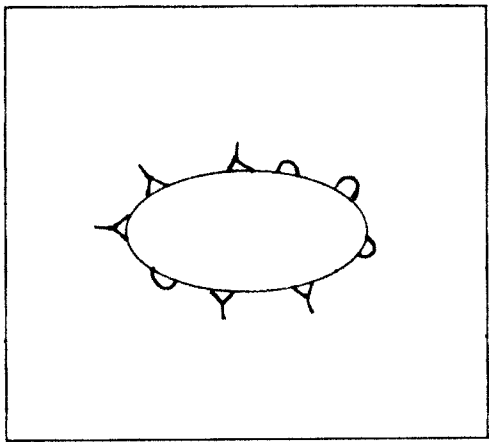

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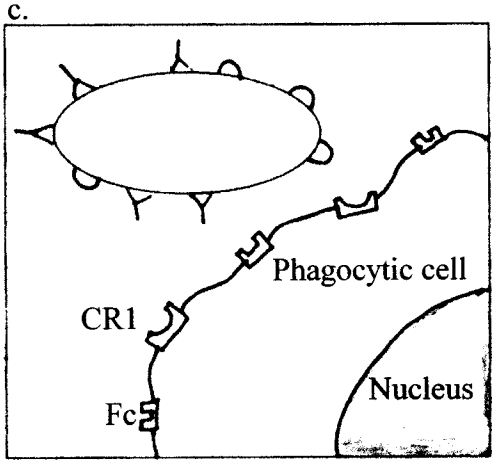

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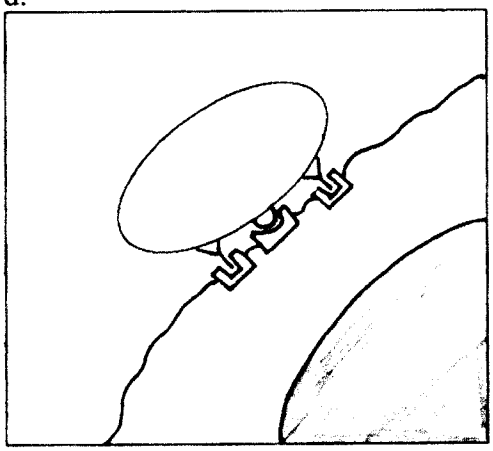
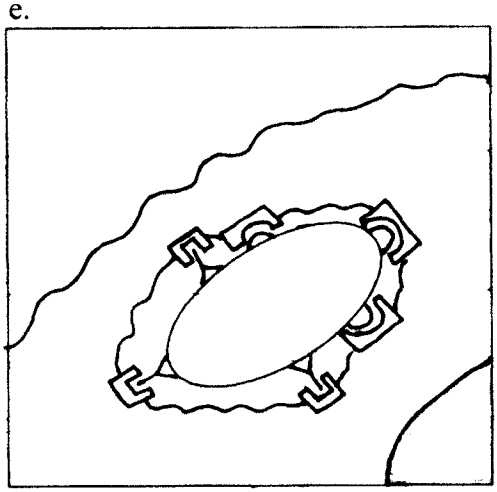


e.



C3b is the major opsonin of the complement system. Phagocytic cells express a receptor, CR1, which binds to C3b. This enables them to more easily bind and phagocytose complement-coated pathogens. Phagocytic cells also express an F_c receptor, which allows them to more readily bind pathogens coated with antibody. Activated phagocytic cells express an increased number of CR1 and F_c receptors. When an antigen is coated with the appropriate opsonin, it binds more readily to the phagocytic cell membrane resulting in enhanced phagocytosis. The rate of phagocytosis has been shown to be enhanced 4000-fold higher in the presence of opsonins (Kuby, 1997). This process is depicted in Figure 1.

Staphylococcus aureus Mechanism of Infection

Interaction of epithelial cells and bacteria are important in the initiation of invasion of the host. *S. aureus* expresses surface proteins that promote adherence to receptors on epithelial cells and components of the subepithelial matrix and allow it to infect cells (Mamo *et al.*, 1988; Lopes *et al.*, 1985; Espersen and Clemmensen, 1982). Examples of these proteins include protein A, F_c, fibronectin, fibrinogen, collagen, elastin, laminin, and, thrombospondin (Savolainen *et al.*, 2001). Implanted medical devices become coated with host proteins, which further enable *S. aureus* to adhere and initiate infection. Once *S. aureus* has established an infection and is exponentially growing it secretes alpha toxin. Alpha toxin further augments the infection by creating transmembrane pores in epithelial cells causing leakage from the cytosol and cell death. These damaged cells are more susceptible to bacterial adherence.

S. aureus mutants that have little or no capsule production have increased adherence to epithelial cells than the parent strain (Pöhlmann *et al.*, 2000). The capsule

masks the cell wall adhesins and decreases the bacterium's ability to adhere to host tissues. The ability of the microcapsules produced by *S. aureus* CPs types 5 and 8 to resist phagocytosis and killing by PMNLs is weaker than highly encapsulated strains. CP types 5 and 8 are thought to act as bacterial adhesins when infecting endothelial cells and monocytes. In a study by Soell *et al.*, purified *S. aureus* CP 5 and 8 were shown to bind human epithelial cells, endothelial cells, and monocytes in a dose dependent manner, in the absence of serum. The fact that this binding occurred in the absence of serum indicates that the CPs interact directly with cell surface receptors. Although it is proposed, there is no evidence supporting the CPs binding to identical receptors on all three cell types (Soell *et al.*, 1995).

S. aureus CPs are associated with bacteremia. Bacteremia is ranked thirteenth as a cause of death in the United States (Centers for Disease Control, 1999). Approximately ninety-eight percent of clinical isolates of *S. aureus* are capsulated (Nelles *et al.*, 1985). Ninety percent of these fall into 11 capsular types (Hanessian and Haskell, 1964). Eighty percent of the isolates from patients hospitalized with bacteremia in the United States are of CP types 5 and 8 (Arbeit *et al.*, 1984). A similar distribution of types 5 and 8 was observed in Europe (Hochkeppel *et al.*, 1987). Type 5 is the most prevalent CP causing bacteremia and wound infections (Na'Was *et al.*, 1998). Over ninety percent of MRSA strains are CP type 5 (Fournier, Bouvet *et al.*, 1987). These two CP types also account for eighty percent of the isolates from goats, sheep, and cows (Poutrel *et al.*, 1988). Type 5 and 8 CPs produce microcapsules, which are smaller and do not have the mucoid appearance usually associated with encapsulation (Karakawa *et al.*, 1982).

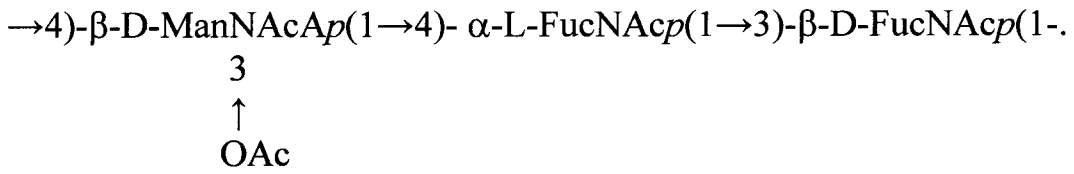
S. aureus CP types 5 and 8 are trisaccharide repeat units of identical monosaccharides: 2-acetamido-2-deoxy-D-mannuronic acid, 2-acetamido-2-deoxy-L-fucose, and 2-acetamido-2-deoxy-D-fucose (Moreau *et al.*, 1990). The structure of these CPs are depicted in Figure 2 (Fattom *et al.*, 1992; Hanessian and Haskell, 1964). These two CPs differ in their glycosidic linkages and their site of O-acetylation (Karakawa *et al.*, 1982). There is a 1→4 glycosidic linkage between the 2-acetamido-2-deoxy-D-mannuronic acid and 2-acetamido-2-deoxy-L-fucose moieties for type 5 and a 1→3 linkage for type 8 (Moreau *et al.*, 1990; Johne *et al.*, 1989). Both types are O-acetylated at the 2-acetamido-2-deoxy-D-mannuronic acid moiety, type 5 at the third carbon, and type 8 at carbon number four. Despite their similar structures, no detectable immunological cross-reactivity is observed between these two CP types (Vann *et al.*, 1988).

The CPs of *S. aureus* belong to the techuronic acid group of Gram-positive bacterial polysaccharides. These types of CPs have a uronic acid moiety and are covalently linked to the cell wall and PG (Fournier, Hannon *et al.*, 1987; Fattom *et al.*, 1990). The uronic acid moiety of *S. aureus* CP type 5 is 2-acetamido-2-deoxy-D-mannuronic acid. PG is the coarse, layered rigid meshwork that surrounds the bacterial cell membrane. It is the major component of Gram-positive cell walls, consisting of up to forty layers making up ninety percent of the cell wall. PG consists of two alternating sugar derivatives that form long chains: *N*-acetyl muramic acid and *N*-acetylglucosamine. The structure of a Gram-positive cell wall is depicted in Figure 3. PG is also cross-connected by short links of amino acids. Gram-positive cell walls also contain a polysaccharide molecule called teichoic acid. Teichoic acid is found throughout the cell

Figure 2: Structure of *Staphylococcus aureus* Capsular Polysaccharide Serotypes 5 & 8

S. aureus CPs serotypes 5 and 8 are trisaccharide repeat units of identical monosaccharides that differ in their glycosidic linkages and their point of O-acetylation. There is a 1→4 glycosidic linkage between the 2-acetamido-2-deoxy-D-mannuronic acid and 2-acetamido-2-deoxy-L-fucose moieties for type 5 and a 1→3 linkage for type 8. Both types are O-acetylated at the 2-acetamido-2-deoxy-D-mannuronic acid moiety, type 5 at the third carbon, and type 8 at carbon number four. Abbreviations: D-ManNAc – 2-acetamido-2 deoxy-D-mannuronic acid; L-FucNAc – 2-acetamido-2 deoxy L-fucose; D-FucNAc – 2-acetamido-2 deoxy D-fucose; OAc – O acetyl.

Type 5


→4)-β-D-ManNAcA*p*(1→4)- α-L-FucNAc*p*(1→3)-β-D-FucNAc*p*(1-.
3
↑
OAc

Type 8

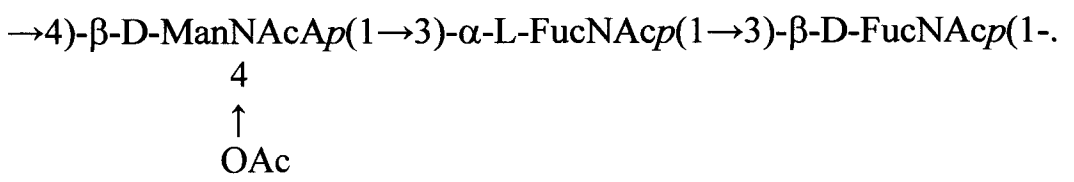
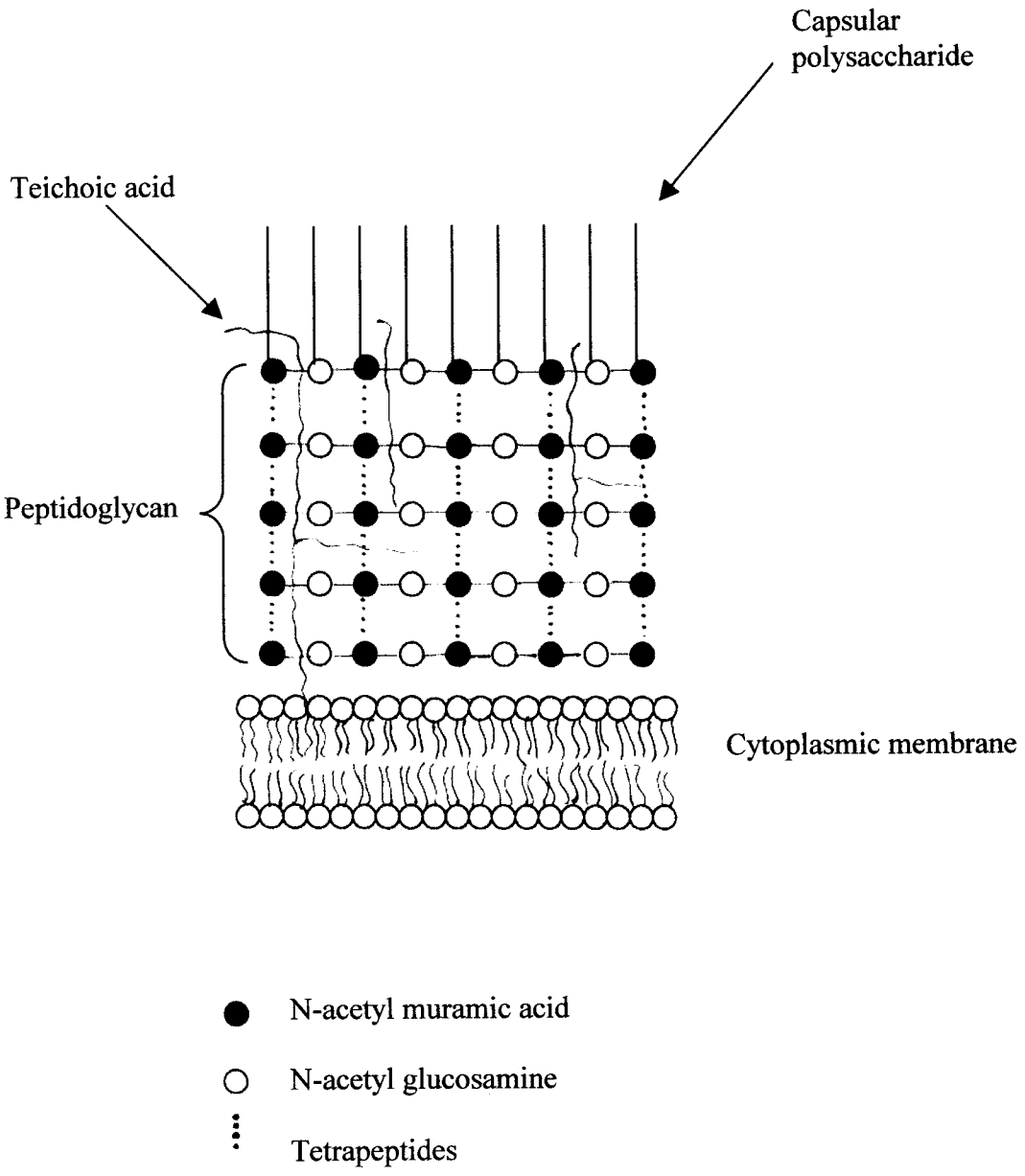

→4)-β-D-ManNAcA*p*(1→3)-α-L-FucNAc*p*(1→3)-β-D-FucNAc*p*(1-.
4
↑
OAc

Figure 3: Structure of Gram-Positive Bacterial Cell Wall

Peptidoglycan is a major constituent of Gram-positive bacterial cell wall, consisting of up to ninety percent of the bacterial cell wall. It consists of two alternating sugar derivatives that form long chains: *N*-acetyl muramic acid and *N*-acetylglucosamine. The CP is linked to the surface of the peptidoglycan. Gram-positive cell walls also contain polysaccharide molecules called teichoic acid, composed of repeating carbohydrate-phosphate polymers. Teichoic acid is found throughout the cell wall and can be attached either to peptidoglycan or to the cell membrane.


Capsular
polysaccharide
Teichoic acid
Peptidoglycan
Cytoplasmic membrane
N-acetyl muramic acid
N-acetyl glucosamine
Tetrapeptides

wall and can be attached either to PG or to the cell membrane (Jensen and Wright, 1993). The mode of attachment of the capsule to Gram-positive bacteria is unknown. In *S. aureus*, this link is strong and is not disrupted by washing in ionic detergents (Wilkinson *et al.*, 1979).

In unencapsulated bacteria the PG is sufficiently exposed to the surface to interact in the immune response. Antibodies to PG are found regularly in the sera of animals and humans. This is thought to contribute to the natural immunity to Gram-positive bacteria (Krause, 1975). Studies by Wilkinson and his coworkers have shown that PG particles isolated from *S. aureus* were readily opsonized by normal human serum and phagocytosed by PMNLs. However, PG particles that retained a portion of the capsule were opsonized less efficiently (Wilkinson *et al.*, 1979). The role that phagocytosis plays in the defense against invading pathogens is clearly illustrated by the fact that individuals with abnormal phagocyte function have a high incidence of bacterial infection. A mutant that was deficient in teichoic acid was opsonized at the same rate as the wild-type that contains teichoic acid. Therefore teichoic acid neither hinders nor increases the phagocytosis of *S. aureus* (Peterson *et al.*, 1979).

Capsulated strains of *S. aureus* are more resistant to phagocytosis than nonencapsulated strains (Nilsson *et al.*, 1997). Nonencapsulated *S. aureus* cells are agglutinated by antibodies to cell wall structures. Cell wall structures are not shielded by a capsule and are easily recognized and phagocytosed and killed by polymorphonuclear leukocytes (PMNL). Nonencapsulated strains were shown to be opsonized by complement alone (Thakker *et al.*, 1998). Capsulated strains of *S. aureus* types 5 and 8 are poorly phagocytosed by PMNLs and can infect the blood and tissues. The capsules of

S. aureus are negatively charged acidic molecules. Physiochemically a hydrophilic and negatively charged molecule may inhibit phagocytosis. A repulsive force may occur between the negatively charged capsular surface and the negatively charged surface of the phagocytic cell (Fattom, 1995). Capsulated strains were also not agglutinated by anti-teichoic acid, an antibody specific for the bacterial cell wall (Karakawa *et al.*, 1985). This suggests that the capsule masks the cell wall teichoic acid, which is the major antigen recognized by PMNLs when phagocytosing nonencapulated *S. aureus* cells. CPs also shield *S. aureus* cells from serum complement. Capsulated and nonencapsulated *S. aureus* both stained positive with anti-C3b (complement-derived opsinin) antibodies, indicating that C3b is bound to the cell surface. Scanning electron microscopy of highly encapsulated *S. aureus* strain Reynolds shows C3b bound to the cell membrane is physically separated from complement receptors on the PMNL membrane (Verbrugh *et al.*, 1982). Acidic CPs have also been shown to limit complement activation through the alternative pathway (Joiner, 1988).

Summary

There is a high mortality rate from *S. aureus* infections despite the use of antibiotics due to its widespread antibiotic resistant strains (Fattom *et al.*, 1990). Isolates (40-60%) from large hospitals in the United States are resistant to multiple antibiotics including methicillin (Lee, 1998). Continued bacterial resistance has made *S. aureus* infections difficult to treat. Due to the misconception that antibiotics were infallible very few new classes of antibiotics are in the advanced stages of development (Boneca, 2003). In the pre-antibiotic era there was an 80% death rate associated with *S. aureus* infection (Smith and Vickers, 1960). With the current trend of antibiotic resistance we are heading

back toward those infection and mortality rates. The development of another means of treating this bacterium is warranted. Passive antibody therapy was widely used prior to the invention of antibiotics, but was widely abandoned since their development. The emergence of multi-antibiotic resistant strains has revived the search for a vaccine or antibody therapy. In this study, we present the first step in vaccine development or production of monoclonal antibodies against *S. aureus* CP serotype 5. The CP was isolated, purified and chemically broken into mono- and oligosaccharide units that will be further characterized and utilized in further steps of vaccine development. Several biochemical methods for detecting the CP after purification are presented.

Materials

Sodium chloride, diethylaminoethyl (DEAE) Sephacel, sodium carbonate, sodium hydroxide and p-dimethylaminobenzaldehyde were purchased from Fischer Scientific (Fair Lawn, NJ). Columbia broth was purchased from Becton Dickinson (Sparks, MD). Sodium acetate, lysostaphin, Deoxyribonuclease I, Ribonuclease A, dialysis tubing, glycerol, crystal violet, sodium bicarbonate, Gram's iodine, safranin, nigrosin, methylene blue, acetone, magnesium sulfate, Tris(hydroxymethyl)aminomethane (TRIS), proteinase K, ethylenediaminetetraacetic acid (EDTA), anhydrous monobasic sodium phosphate, anhydrous dibasic sodium phosphate, ethanol, *N*-acetyl D-glucosamine, Congo red, and 2,3,5 triphenyltetrazolium chloride (Tetrazolium red) were purchased from SIGMA Chemical Company (St. Louis, MO). *S. aureus,* subspecies Rosenbach, strain Lowenstein, serotype 5 was purchased from American Type Culture Collection (Rockville, MD). Nutrient broth, nutrient agar, concentrated hydrochloric acid, and concentrated sulfuric acid were purchased from VWR Scientific Company (West Chester, PA). Sodium molybdate 4-hydrate and ascorbic acid were purchased from J. T. Baker, Inc. (Philipsburg, NJ).

Reagents and Solutions

10X Phosphate Buffered Saline (PBS)

Anhydrous monobasic sodium phosphate (2.23 g) and anhydrous dibasic sodium phosphate (11.92 g) were mixed with 800 ml Milli-Q water. The pH of the solution was adjusted to between 7.2 – 7.4. Sodium chloride (87.66 g) was added and the final volume brought to 1 L with Milli-Q water.

Preparation of Dialysis Tubing

Dialysis tubing was cut into 20 cm pieces and boiled for 10 minutes in 2% sodium bicarbonate containing 1 mM EDTA. The tubing was rinsed in Milli-Q water and boiled for 10 minutes in 1 mM EDTA. The tubing was cooled and stored submersed in 1 mM EDTA at 4°C (Maniatis *et al.*, 1982).

0.05 M sodium acetate, 0.1 M NaCl

Sodium acetate (4.11 g) and sodium chloride (5.84 g) were mixed with Milli-Q water and diluted to 1 L. The pH of the solution was adjusted to 6.0.

0.05 M sodium acetate, 0.15 M NaCl

Sodium acetate (4.11 g) and sodium chloride (8.77 g) were mixed with Milli-Q water and diluted to 1 L. The pH of the solution was adjusted to 6.0.

0.05 M sodium acetate, 0.20 M NaCl

Sodium acetate (4.01 g) and sodium chloride (14.61g) were mixed with Milli-Q water and diluted to 1L. The pH of the solution was adjusted to 6.0.

Preparation of DEAE Sephacel column

DEAE Sephacel stored in ethanol was placed in a graduated cylinder with 500 ml of 0.05 M sodium acetate, 0.10 M NaCl buffer, pH 6.0 and swirled. Once the mixture had settled (30 min) the buffer was aspirated off. This washing process was repeated 5 times. The DEAE Sephacel was then placed into a sealed Erlenmeyer flask with 250 ml of 0.05 M sodium acetate, 0.10 M NaCl buffer, pH 6.0 and a vacuum applied to remove gas from the matrix. The DEAE Sephacel buffer slurry was then poured into a closed 2.0 x 50.0 cm chromatography column and the adsorbent was allowed to settle by gravity. Once a bed had formed, the stopcock was opened and the excess liquid was allowed to enter the matrix until it was approximately 1 cm above the bed surface. The stopcock was then closed and the top 2-3 cm of the matrix was resuspended by stirring. Additional slurry was added and the process repeated until the bed surface was 5 cm from the top of the column (Boyer, 1986).

Ehrlich's Reagent

Dimethylaminobenzaldehyde (1.60 g), concentrated HCl (30 ml), and 95% ethanol (30 ml) were mixed. The solution was stored at room temperature in a chemical hood.

Sodium hydroxide, 10%

Sodium hydroxide (10.00 g) was mixed with Milli-Q water and diluted to 100 ml. The solution was stored at 4°C.

Tetrazolium red, 0.5%

2,3,5 Triphenyltetrazolium chloride (Tetrazolium red) (0.50 g) was dissolved in

Milli-Q water and diluted to 100 ml. Tetrazolium red was stored protected from light, and a fresh solution was prepared at each use.

Reagent C

One volume of 6N sulfuric acid was mixed with 2 volumes of Milli-Q water and 1 volume of 2.5% ammonium molybdate. One volume of 10% ascorbic acid was then added and mixed. Reagent C is unstable, and was prepared fresh for each test.

Ascorbic acid, 10%

Ascorbic acid (10.00 g) was dissolved in Milli-Q water and diluted to 100 ml. The solution was stored under refrigeration at 4°C. The solution is stable for 7 wks.

Ammonium molybdate, 2.5%

Ammonium molybdate tetrahydrate (2.66 g) was dissolved in 100 ml of Milli-Q water. The solution was stored at room temperature.

Columbia Broth/2% NaCl Media Preparation

Columbia broth (35.00 g) and NaCl (20.00 g) were dissolved into 1000 ml of Milli-Q water. The pH of the media was adjusted to 7.5. Media (250 ml) was placed in a 1 L Erlenmeyer flask and autoclaved at 121°C for 15 minutes.

Methods

Staphylococcus aureus stock Preparation

S. aureus, subspecies Rosenbach, strain Lowenstein, serotype 5 lyophilized powder was brought up in 1 ml of nutrient broth. A nutrient agar plate was inoculated with the bacterial mixture and incubated overnight. This plate was sealed with tape and stored at 4°C for up to one month. Columbia broth/2% NaCl (30 ml) was inoculated and incubated while shaking at 200-250 cycles per minute for 3 hours at 37°C. A 10 ml aliquot was transferred to a conical tube and centrifuged at 9,000 x *g* for 5 minutes at 4°C. The supernatant was discarded. The cell pellet was resuspended in 2 ml of a sterile 10% glycerol/nutrient broth mixture, transferred to a cryogenic vial, and stored in a sealed styrofoam container at –70°C for 24 hours. The cells were then transferred to liquid nitrogen for permanent storage (Maniatis *et al.*, 1982). The remaining bacterial broth was stored at 4°C for up to one month.

Bacterial Capsule Demonstration

One loopful of Congo red dye (25 mg/ml) was placed on a grease free glass slide and mixed with one loopful *S. aureus*. The mixture was spread to form a thin film, air-dried, heat fixed, and cooled. The slide was flooded with 0.15 M hydrochloric acid, blotted dry, and was warmed to eliminate excess acid. The slide was then stained with carbol fuchsin for 15 seconds. The excess dye was poured off without rinsing. The slide was blotted dry and examined at 1,000X magnification (Okeke & Lamikanra, 1995).

Gram Stain

S. aureus, subspecies Rosenbach, strain Lowenstein, serotype 5 was mixed with water on a glass slide. The smear was air-dried and heat fixed. The slide was flooded with

crystal violet and the crystal violet was mixed with 5 drops of sodium bicarbonate for 10 seconds. The remaining crystal violet was rinsed off the slide with iodine, and additional iodine was added for 10 seconds. The slide was rinsed with acetone until the acetone ran colorless from the slide. The acetone was allowed to air dry and the slide was then flooded with safranin for 10 seconds, rinsed with water, and examined at 1,000X magnification (Harley and Prescott, 1999).

Bacterial Culture

Columbia broth was used to cultivate *S. aureus* as it has a low phosphate concentration, which has been shown to increase CP production (Karakawa *et al.*, 1985). Columbia broth containing 2% NaCl (250 ml) in a 1 L Erlenmyer flask was inoculated with 1 ml of *S. aureus* bacterial stock. Maximal CP production relative to cell mass in broth culture was shown to occur during exponential (18-24 h) growth phase (Poutrel *et al.*, 1995). Therefore, the flasks were incubated at 37°C in a Lab-Line Environ-Shaker (Dubuque, IA) for 18-24 hours at 200-250 cycles per minute. The bacterial culture was centrifuged at 17,000 x *g* for 30 min at 4°C. The cell pellet was collected and stored at 4°C (Fattom *et al.*, 1990).

Carbohydrate Preparation

S. aureus cell pellet was suspended at a concentration of 0.5 g/ml in 0.05 M TRIS containing 2 mM $MgSO_4$, pH 7.5 (Fattom *et al.*, 1990). The cells were autoclaved at 121°C for 15 minutes (Gilbert *et al.*, 1994). Lysostaphin (100 μg/ml) was added and the mixture was incubated at 37°C for 4 hours while mixing (Fattom *et al.*, 1990). The bacterial suspension was centrifuged at 17,000 x *g* for 30 minutes and the cell pellet was

discarded. The supernatant was dialyzed against Milli-Q water overnight and lyophilized. The lyophilized sample was resuspended at a concentration of 20 mg/ml in PBS. DNase and RNase (100 μg/ml each) were added and the mixture was incubated for 24 hours at 37°C while mixing. Proteinase K (100 μg/ml) was added and the incubation was continued for 24 hours. The sample was then dialyzed against Milli-Q water overnight and lyophilized (Gilbert *et al.*, 1994).

Dialyzing Samples

Dialysis tubing stored in 1 mM EDTA was rinsed with Milli-Q water. Two knots were tied at one end of the tubing. Sample was added to the dialysis tubing. The tubing was sealed with two knots and placed in 1 L of Milli-Q water at 4°C for 24 hours while stirring (Cooper, 1977).

Lyophilization of Samples

Dialyzed samples were transferred under sterile conditions to 50 ml conical tubes. A Kim wipe was attached to the top of the conical tube using a rubber band. The tubes were frozen in a dry ice/acetone bath. The tubes were then transferred to the Lyph-Lock-6 Freeze Dry System (Labconco, Ramsey, NJ) and lyophilized for 48 hours.

Purification of Carbohydrate

The DEAE Sephacel column was equilibrated with two column volumes (250 ml) of equilibrating buffer (0.05 M sodium acetate, 0.1 M NaCl, pH 6.0). The lyophilized crude CP sample was suspended in 0.05 M sodium acetate, 0.1 M NaCl, pH 6.0 and applied to the column. The column was then washed with 5 column volumes (625 ml) of the equilibrating buffer. The CP was eluted with 2 column volumes of elution buffer

(0.05 M sodium acetate, 0.15 M NaCl, pH 6.0) (Fattom *et al.*, 1990). Fractions (6 ml) were collected using a FC-80K fractionator (Gilson Medical Electronics, Middleton, WI). Teichoic acid was eluted with two column volumes (250 ml) of 0.05 M sodium acetate, 0.20 M NaCl, pH 6.0 (Moreau *et al.*, 1990). Fractions (6 ml) were collected using a FC-80K fractionator. Fractions were tested for the presence of CP using ultraviolet absorbance at 206 nm, the Morgan-Elson test for *N*-acetyl 2-amino sugars, and the Red Tetrazolium test. Fractions positive for CP were dialyzed against Milli-Q water overnight at 4°C and lyophilized.

The Morgan-Elson Test for *N*-acetyl 2-amino Sugars

The Morgan-Elson test was used to test fractions for the presence of *N*-acetyl 2-amino sugars and thus the CP. The Ehrlich's reagent, which is responsible for developing the red color change, was added after brief heating in alkaline solution. The fraction to be tested (500 µl) and 1.25 M sodium carbonate (500 µl) were mixed in a test tube, heated to 95°C for 5 minutes, and cooled. The fractions (25 µl) were transferred to a 96 well plate and the Ehrlich's reagent, (200 µl) was added to the wells simultaneously using a multichannel pipette. A red color change indicates the fraction was positive for an *N*-acetyl 2-amino sugar (Robyt & White, 1987). The absorbance of the samples at 490 nm was determined immediately using a Microplate autoreader (Bio-Tek Instruments, Melrose Park, IL) as the positive test result fades quickly. *N*-acetyl D-glucosamine was used as a positive control. Equilibrating buffer (0.05 M sodium acetate, 0.1 M NaCl, pH 6.0) and elution buffer (0.05 M sodium acetate, 0.15 M NaCl, pH 6.0) were used as negative controls.

Red Tetrazolium Test

The Red Tetrazolium test is a highly sensitive test for detecting reducing sugars. Red Tetrazolium is a water-soluble colorless substance that oxidizes the sugar, and thereby is reduced forming a water-insoluble, red colored substance called red tetrazolium-diformazan that falls out of solution as a red precipitate. The fraction to be tested (25 µl), 0.5% aqueous solution of tetrazolium red (1 ml), and 10% sodium hydroxide (25 µl) were placed in a test tube. The tube was placed in a beaker of hot water for 5 minutes. A red color change indicated a positive result and thus the presence of the CP (Fieser & Williamson, 1987). The absorbance was determined at 490 nm using a Microplate Autoreader. *N*-acetyl D-glucosamine was used as a positive control. Negative controls were the buffer present in the fraction being tested, either equilibrating buffer (0.05 M sodium acetate, 0.1 M NaCl, pH 6.0), or elution buffer (0.05 M sodium acetate, 0.15 M NaCl, pH 6.0).

Test for Phosphate

The fractions that tested positive for carbohydrate were also tested for phosphate to determine the presence of the cell wall contaminant, teichoic acid. The carbohydrate sample (1 mg) was dissolved into 0.5 ml HCl (2M) and placed in a boiling water bath for 30 minutes. The carbohydrate sample was transferred to a new tube and the volume was adjusted to 4 ml with Milli-Q water. Reagent C (4 ml; see reagents and solutions) was added, each tube was capped with paraffin and mixed. Tubes were placed in a 37°C oven for 1.5 - 2 hours and observed for color change. The absorbance of each sample was determined at 820 nm using a Beckman DU-40 spectrophotometer (Chen, Jr. *et al.*,

1956). PBS was used as a positive control. Elution buffer (0.05 M sodium acetate, 0.15 M NaCl) was used as a negative control.

Solvolysis of the Fractions Containing Capsular Polysaccharide

The carbohydrate samples that tested negative for teichoic acid were sent to CS Bio Company (San Carlos, CA) for solvolysis. The samples were treated with 0.5 ml of 70% aqueous hydrogen fluoride (HF), prepared by distillation of anhydrous HF onto a known volume of ice. The HF was removed from the solution after 3 hours at 4°C by evaporation under a stream of N_2 (Moreau *et al.*, 1990).

Results

In order to determine the integrity of the *S. aureus* subspecies Rosenbach, strain Lowenstein purchased from ATCC, a Gram Stain was performed (Figure 4). As anticipated the *S. aureus* sample stained blue/purple, indicating that it is Gram-positive. A capsule stain was then performed to ensure that this *S. aureus* strain produces a capsule (Figure 5). The capsule is observed as a clear area surrounding the red stained cell in a blue-green background.

The type 5 CP was released into the supernatant by autoclaving and lysostaphin treatment. DNase, RNase, and proteinase K were added to further digest bacterial cell components. Ion-exchange chromatography was used to purify the negatively charged CP and also remove the negatively charged cell wall contaminate, teichoic acid. The extracted CP was adsorbed onto a DEAE Sephacel anion exchange chromatography column. The unadsorbed molecules were eluted with starting buffer (0.05 M sodium acetate, 0.10 M NaCl, pH 6.0). The carbohydrate was eluted with 0.05 M sodium acetate, 0.15 M sodium chloride elution buffer. Fractions (6 ml) obtained from the elution were tested with the Morgan-Elson test for *N*-acetyl 2-amino sugars (Figure 6). Absorbance was read at 480 nm using a spectrophotometer. A red color change indicated the presence of an *N*-acetyl 2-amino sugar and thus the CP. *N*-acetyl D-glucosamine was used as a positive control. The formation of a red color was observed only with the positive control and not with any of the fractions. However the color change produced with this test faded too quickly for absorbance values to be accurately read using a spectrophotometer. A fraction that may have produced a faint color change after addition of the Ehrlich's reagent could have faded before it was read on the spectrophotometer. The fractions were

Figure 4: Gram Stain

Photomicrograph of a Gram stain of *S. aureus* subspecies Rosenbach, strain Lowenstein, serotype 5.

Figure 5: Capsule Stain

Photomicrograph of a capsule stain of *S. aureus* subspecies Rosenbach, strain Lowenstein, serotype 5.

Figure 6: Spectrophotometric Absorbance of Fractions Tested with the Morgan-Elson Test for *N*-acetyl 2-amino Sugars After Elution from DEAE Sephacel Column

The crude CP was absorbed onto a DEAE Sephacel column that was subsequently washed with equilibrating buffer, and eluted. Eluent was collected in 6 ml fractions and tested for the presence of CP using the Morgan-Elson test for *N*-acetyl 2-amino sugars. A red color indicates a positive result. The absorbance of the fractions was read using a spectrophotometer at 480 nm. *N*-acetyl D-glucosamine, used as a positive control had an absorbance value of 0.7190. Elution buffer tested with the Morgan Elson test was used as a negative control and its absorbance value was subtracted from each fraction.

Absorbance at 480 nm
-0.0500
0.0000
0.0500
0.1000
0.1500
0.2000
0.2500
0.3000
0.3500
0.4000
Fractions
1
4
7
10
13
16
19
22
25
28
31
34
37
40
43
46
49
52
55
58
61
64
67
70
73
76

retested in a manner that allowed for all fractions to be read immediately following addition of the Ehrlich's reagent. A sample of each fraction was heated briefly in alkaline solution, allowed to cool, and then added to a well of a 96 well plate. The Ehrlich's reagent, which is responsible for developing the color change, was added to each sample simultaneously and the absorbance was read immediately at 490 nm using a Microplate autoreader (Figure 7). None of the fractions tested positive. The high absorbance values or peaks absorbed were due to precipitation and not to the formation of a positive red color change (fractions 68, 69, 71, 75, 76).

The precipitation seen in fractions 68, 69, 71, 75, and 76 was thought to be from the sodium acetate in the elution buffer interfering with the Morgan-Elson test. To test this hypothesis, every five fractions were pooled (30 ml) and dialyzed to remove the salt from the elution buffer. CP absorbs at 206 nm so the absorbance of each set of pooled fractions was taken at 206 nm on a spectrophotometer (Figure 8). No observable peak was detected. Milli-Q water was used as a negative control. The pooled fractions were thought to be too dilute to accurately test for the presence of polysaccharide, so they were again dialyzed against Milli-Q water, and then concentrated by lyophilization. The lyophilized pellets were brought up in 5 ml Milli-Q water and absorbance was again taken at 206 nm to test for the presence of the capsule (Figure 9). Two sharp peaks and one broad peak were observed.

It was then determined that the elution buffer used to elute the polysaccharide was not at the correct pH. The DEAE Sephacel column was reeluted with 0.05 M sodium acetate, 0.15 M NaCl at pH 6.0. Fractions were tested at 206 nm for the presence of carbohydrate (Figure 10). A definite peak was observed between fractions 9-13 and a

Figure 7: Absorbance of Fractions Tested with the Morgan-Elson Test for *N*-acetyl 2-amino Sugars After Elution from DEAE Sephacel Column

Fractions eluted from the DEAE Sephacel column with elution buffer were re-tested with the Morgan-Elson test for *N*-acetyl 2-amino sugars. A Microplate autoreader was used to read absorbance at 490 nm due to the quick fading of a positive color change. Samples to be tested were placed in a 96 well plate and the Ehrlich's reagent, which is responsible for developing the color change, was added to all samples simultaneously. The absorbance was read immediately. *N*-acetyl D-glucosamine, used as a positive control had an absorbance value of 1.724. Elution buffer tested with the Morgan Elson test was used as a negative control and its absorbance value was subtracted from each fraction.

Absorbance at 490 nm
0.000
0.100
0.200
0.300
0.400
0.500
0.600
0.700
0.800
0.900
1.000
Fractions
1
4
7
10
13
16
19
22
25
28
31
34
37
40
43
46
49
52
55
58
61
64
67
70
73
76

Figure 8: UV Absorbance of Pooled Fractions Eluted from DEAE Sephacel column

Every five fractions eluted from the DEAE Sephacel column were pooled and dialyzed. Absorbance was read at 206 nm to detect the presence of CP. Milli-Q water was used as a negative control and its absorbance value was subtracted from each sample.

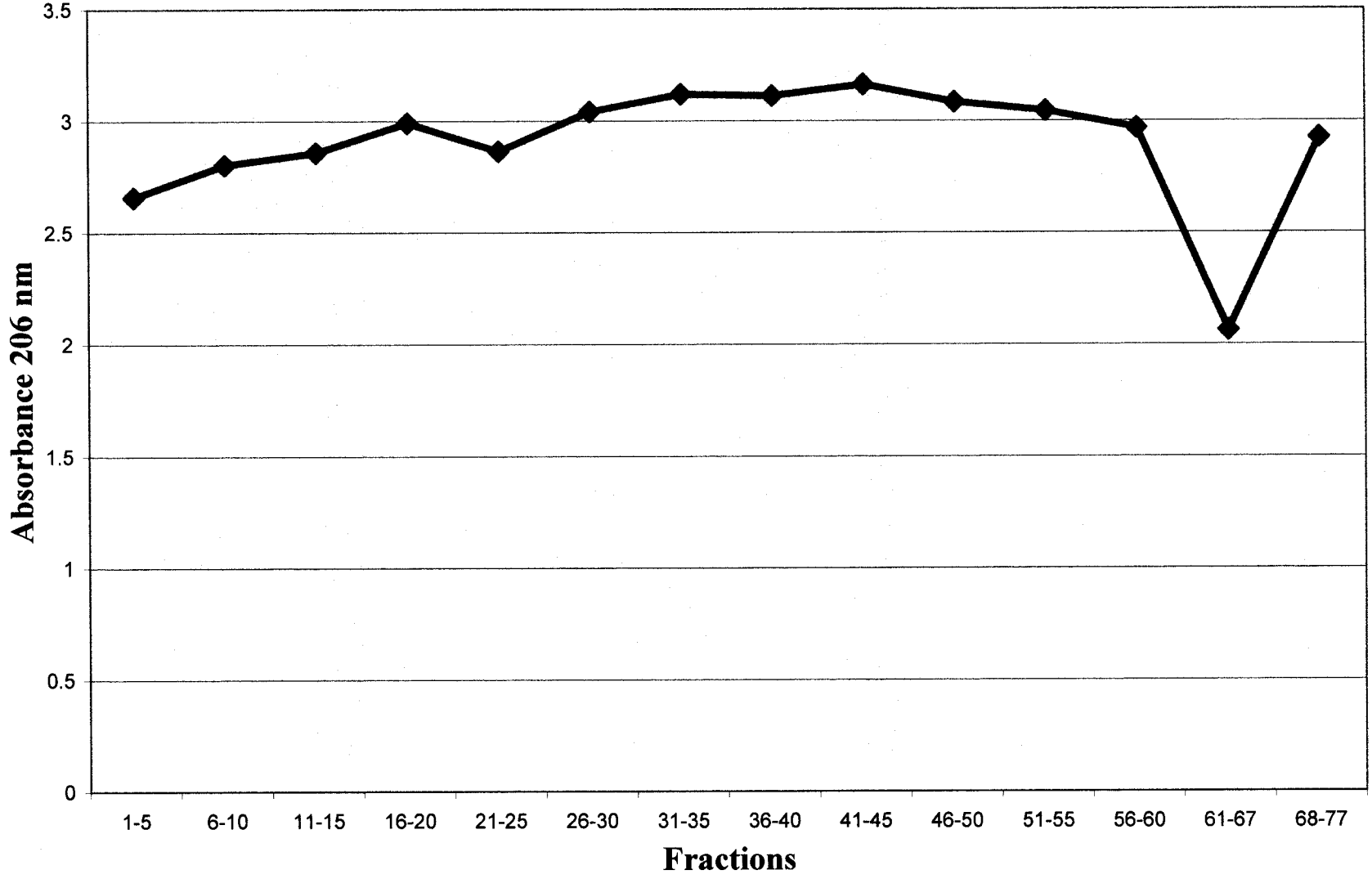

3.5
3
2.5
2
1.5
1
0.5
0
Absorbance 206 nm
1-5
6-10
11-15
16-20
21-25
26-30
31-35
36-40
41-45
46-50
51-55
56-60
61-67
68-77
Fractions

Figure 9: UV Absorbance of Pooled Fractions (After Concentration) Eluted from DEAE Sephacel Column

Pooled fractions were dialyzed, lyophilized and reconstituted in 5 ml Milli-Q water. Absorbance was determined at 206 nm to detect the presence of CP. Milli-Q water was used as a negative control and its absorbance value was subtracted from each sample.

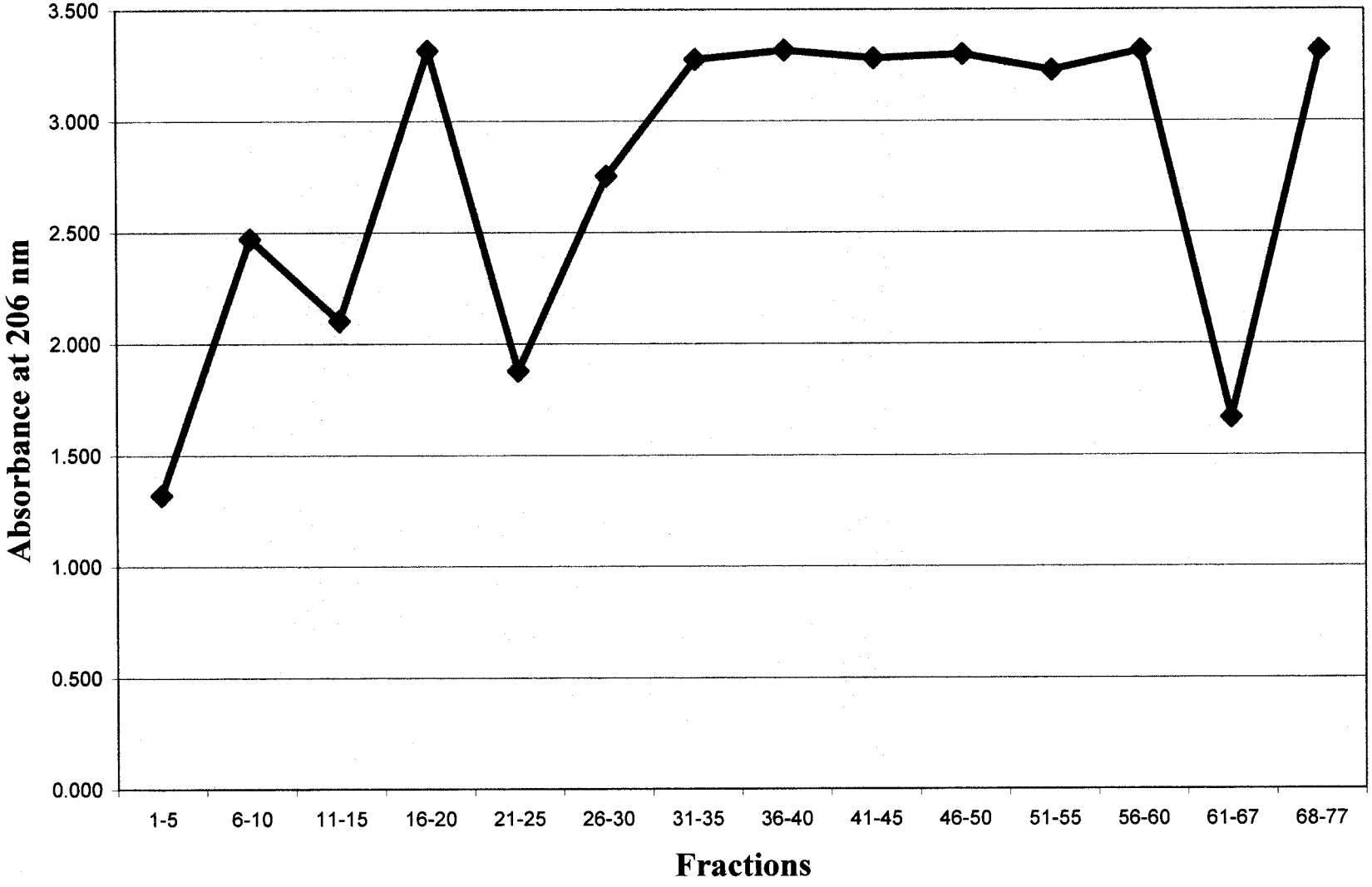

3.500
3.000
2.500
2.000
1.500
1.000
0.500
0.000
Absorbance at 206 nm
1-5
6-10
11-15
16-20
21-25
26-30
31-35
36-40
41-45
46-50
51-55
56-60
61-67
68-77
Fractions

Figure 10: Fractions Reeluted from DEAE Sephacel Column with 0.05 M Sodium Acetate, 0.15 M NaCl, pH 6.0 Observed at 206 nm to Test for Presence of Capsular Polysaccharide

The DEAE Sephacel column was reeluted with elution buffer at the pH 6. Eluent was collected in 6 ml fractions. The absorbance of the fractions was read at 206 nm to test for the presence of CP. Elution buffer was used as a negative control and its absorbance value was subtracted from each sample.

Absorbance at 206 nm
0.000
0.050
0.100
0.150
0.200
0.250
0.300
0.350
Fractions
1
4
7
10
13
16
19
22
25
28
31
34
37
40
43
46
49
52
55
58
61
64
67
70
73
76
79

possible peak at fractions 25-40. The fractions were then tested with the Morgan-Elson test for *N*-acetyl 2-amino sugars (Figure 11). Many peaks were obtained (fractions 4, 9, 11-13, 32, 33, 39, 56, 59, 66) including a peak at fractions 9-13 that was similar to that obtained when these same fractions were tested with the Morgan-Elson test. Again high absorbance readings were from precipitation and not from the formation of a red color. Fractions 9-13 were pooled, dialyzed against Milli-Q water, lyophilized, resuspended in 5 ml Milli-Q water and tested with the Morgan-Elson test for *N*-acetyl 2-amino sugars (Figure 12). No significant difference was observed between the absorbance of pooled fractions 9-13, elution buffer, or Milli-Q water. These fractions were thus determined to be free of CP and were discarded.

Due to the fact that some fractions when tested with the Morgan-Elson test for *N*-acetyl 2-amino sugars have a precipitate that interferes with the absorbance readings giving a false positive result, a new means for testing for the presence of polysaccharide was sought. The Red Tetrazolium test is a highly sensitive test for reducing sugars. Red Tetrazolium is a water-soluble colorless substance that oxidizes the sugar, thereby forming a water-insoluble, red colored substance called Red Tetrazolium-Diformazan that falls out of solution as a red precipitate. The sensitivities of the two tests were evaluated using known sugar *N*-acetyl D-glucosamine (Figure 13). The Morgan-Elson test was able to detect the reducing sugar at concentrations as low as 3.13×10^{-3} g/ml, while the Red Tetrazolium test was only able to do so at concentrations of 1.25×10^{-1} g/ml or higher. Hence, the Morgan-Elson test is more sensitive than the Red Tetrazolium test for detecting the presence of the known reducing sugar. No precipitation was observed with the Morgan-Elson test when testing *N*-acetyl D-glucosamine. A new batch

Figure 11: Fractions Eluted from DEAE Sephacel Column with 0.05 M Sodium Acetate, 0.15 M NaCl, pH 6.0 Tested with the Morgan-Elson Test for *N*-acetyl 2-amino Sugars

Fractions were tested with the Morgan-Elson test for *N*-acetyl 2-amino sugars and absorbance was read at 490 nm using a Microplate Autoreader. *N*-acetyl D-glucosamine, used as a positive control, had an absorbance value of 0.764. Elution buffer tested with the Morgan Elson test was used as a negative control and its absorbance value was subtracted from each sample.

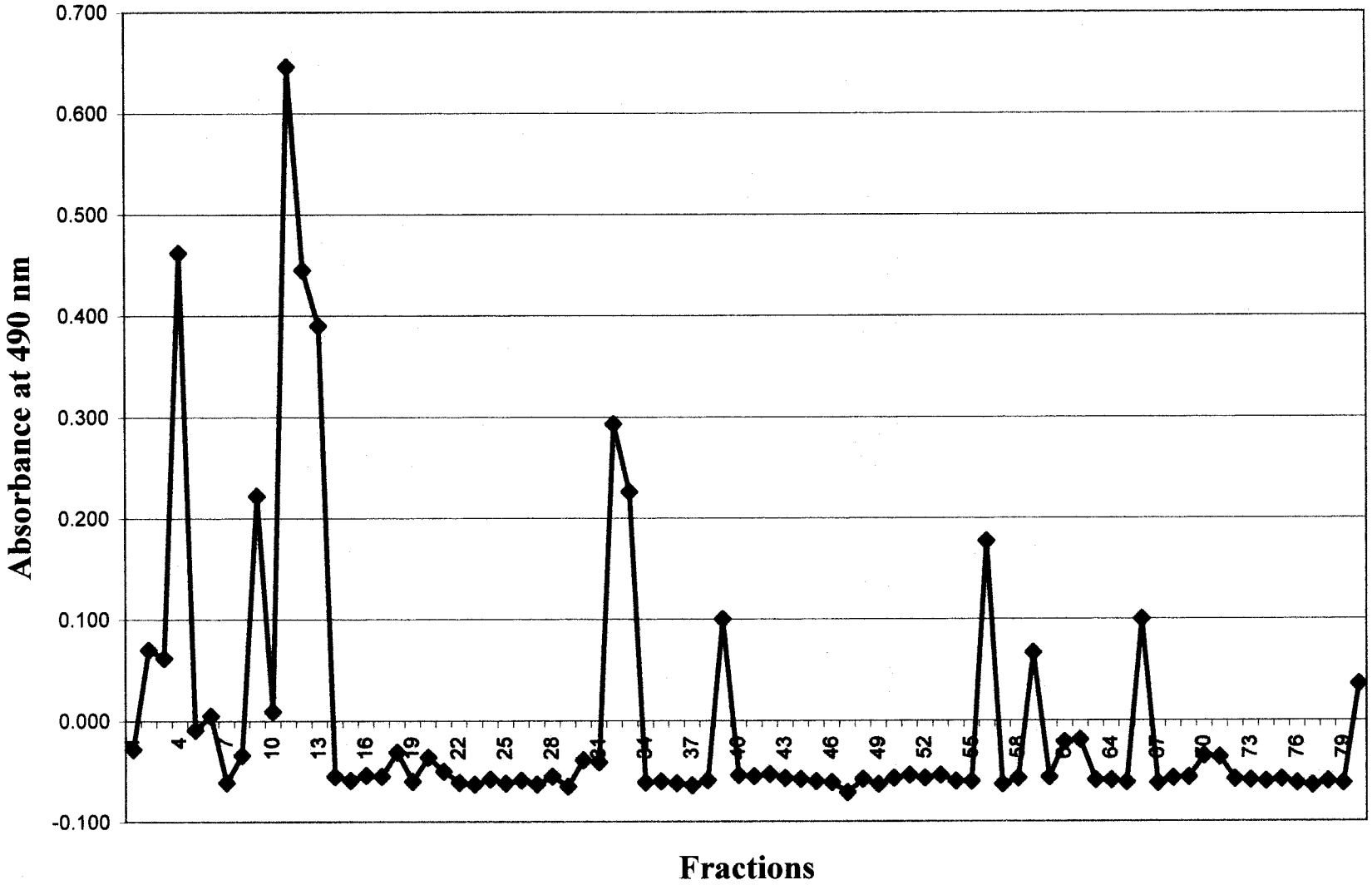

Absorbance at 490 nm
0.700
0.600
0.500
0.400
0.300
0.200
0.100
0.000
-0.100
4
7
10
13
16
19
22
25
28
31
34
37
40
43
46
49
52
55
58
61
64
67
70
73
76
79
Fractions

Figure 12: Absorbance of Samples Tested with the Morgan-Elson Test for *N*-acetyl 2-amino Sugars

Fractions 9-13 were pooled, dialyzed, lyophilized, reconstituted in 5 ml of Milli-Q water and tested with the Morgan-Elson test for *N*-acetyl 2-amino sugars. Elution buffer and Milli-Q water were both tested and used as negative controls. No significant differences were observed between the negative controls and the pooled fractions. *N*-acetyl D-glucosamine was used as a positive control.

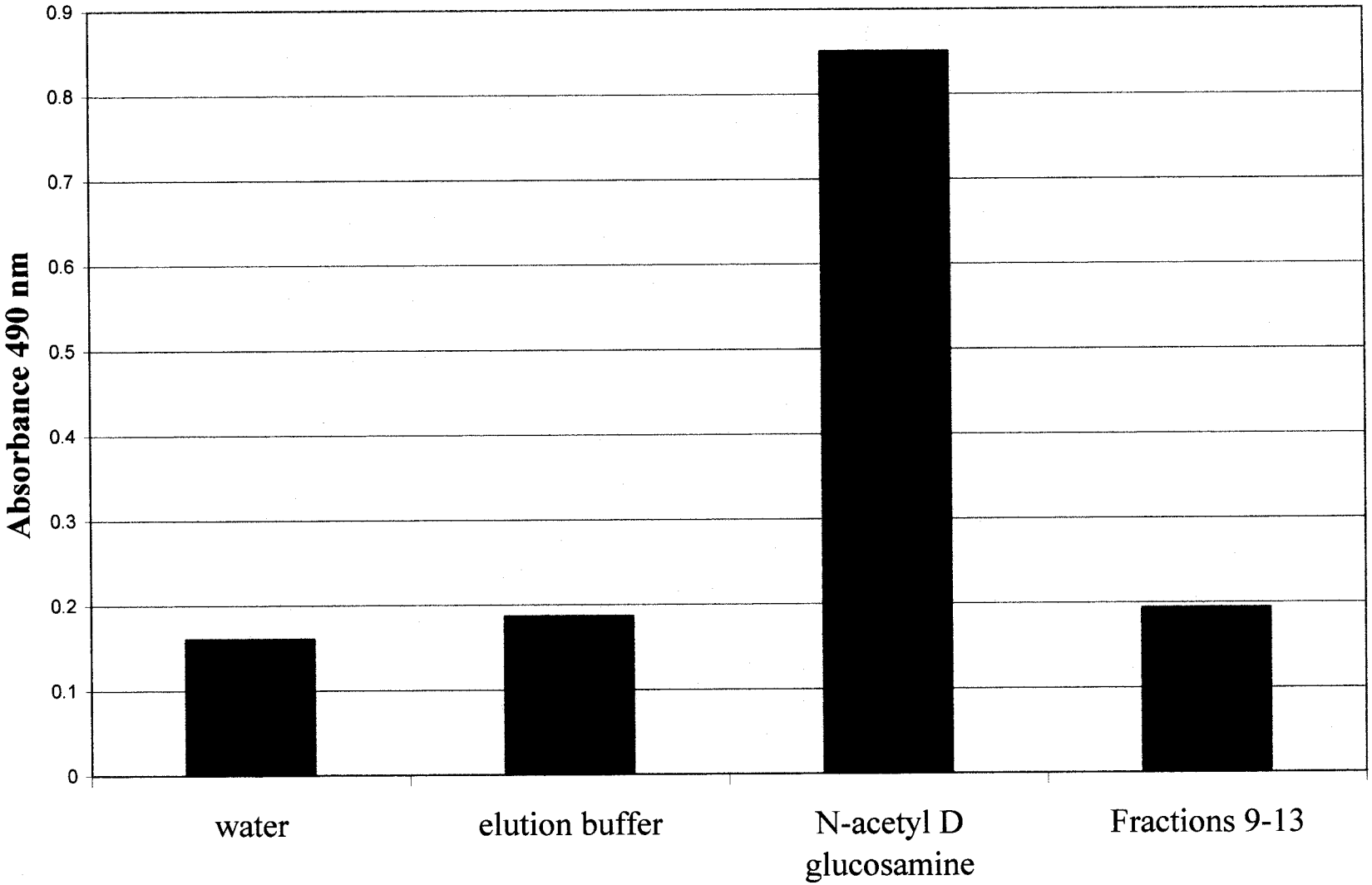
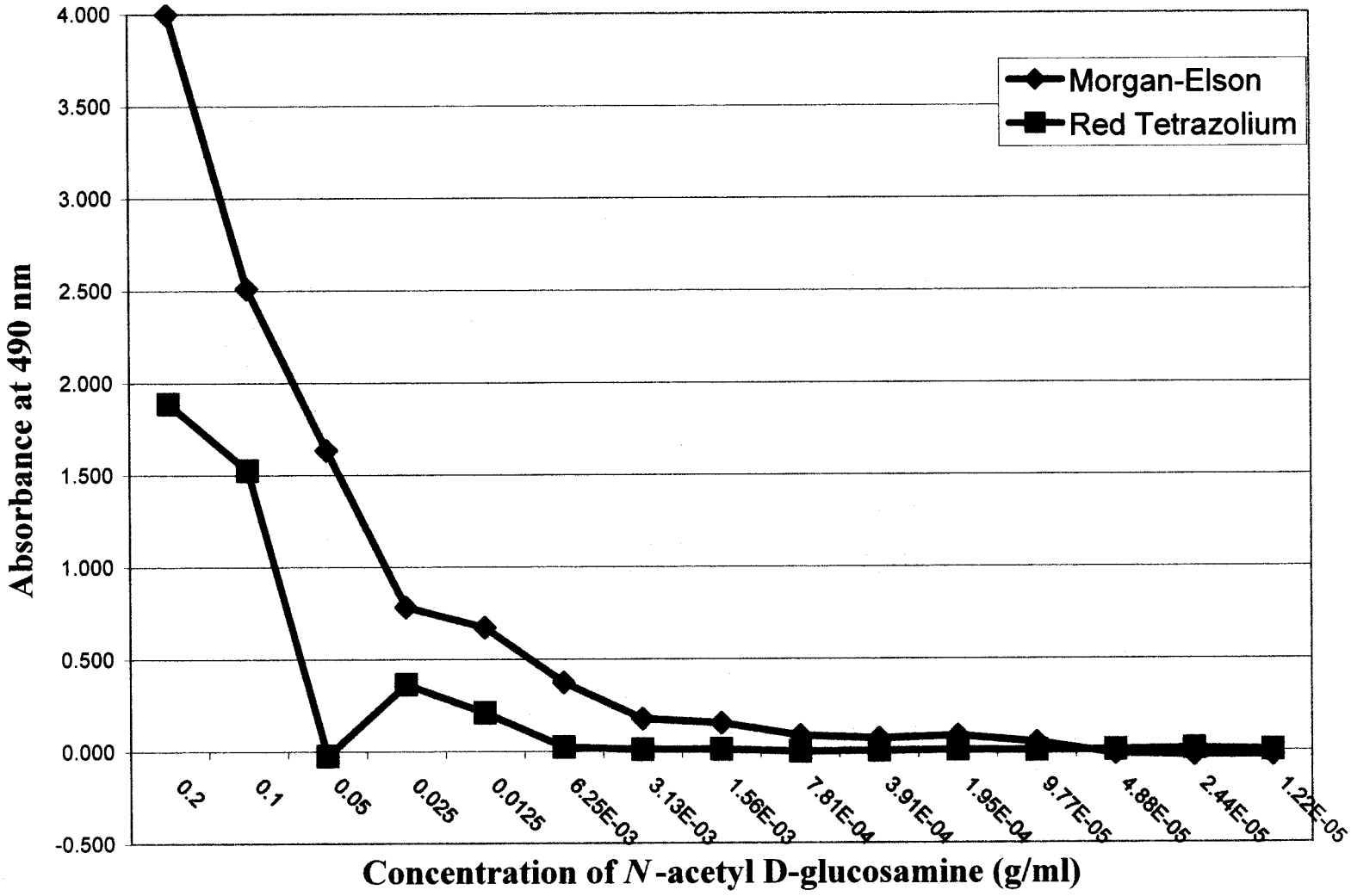

Absorbance 490 nm
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
water
elution buffer
N-acetyl D glucosamine
Fractions 9-13

Figure 13: Comparison of the Sensitivities of the Morgan-Elson Test for *N*-acetyl 2-amino Sugars and the Red Tetrazolium Test on *N*-acetyl D-glucosamine

The sensitivity of the Morgan-Elson test for *N*-acetyl 2-amino sugars was compared to the Red Tetrazolium test using known positive sugar *N*-acetyl D-glucosamine. Two fold serial dilutions of *N*-acetyl D-glucosamine were tested by both methods and absorbance values were measured at 490 nm.


Morgan-Elson
Red Tetrazolium
Absorbance at 490 nm
4.000
3.500
3.000
2.500
2.000
1.500
1.000
0.500
0.000
-0.500
0.2
0.1
0.05
0.025
0.0125
6.25E-03
3.13E-03
1.56E-03
7.81E-04
3.91E-04
1.95E-04
9.77E-05
4.88E-05
2.44E-05
1.22E-05
Concentration of N-acetyl D-glucosamine (g/ml)

of crude CP was extracted and was used to test the sensitivities of both the Morgan-Elson and the Red Tetrazolium tests (Figure 14). Although the Morgan-Elson test was determined to be more sensitive for reducing sugars when using the positive control, when it was used to test the crude polysaccharide a precipitate was again observed which interfered with the absorbance results. It was therefore determined that the precipitate was not a component of the elution buffer because the crude polysaccharide tested had not yet been placed over the DEAE Sephacel column. The crude CP had also been repeatedly dialyzed following enzymatic digestion. The Red Tetrazolium test was sensitive to a concentration of crude polysaccharide of 3.50×10^{-3} g/ml. The Morgan-Elson test was therefore abandoned and the Red Tetrazolium test was used to determine the presence of CP in all remaining experiments.

New CP was prepared and adsorbed onto a DEAE Sephacel column that had been equilibrated with starting buffer. The column was then washed with five column volumes of equilibrating buffer. After approximately one column volume of equilibrating buffer had passed through the column, the eluent started to become cloudy so fraction collection was begun and fractions were collected throughout the entire equilibrating wash. The fractions were tested with the Red Tetrazolium test (Figure 15). A peak was observed in fractions 1-9. These fractions were pooled and saved for further analysis. Elution buffer was then run through the column and fractions were collected and tested with the Red Tetrazolium test for the presence of CP (Figure 16). A peak was observed at fractions 15-22. These fractions were pooled and saved for further analysis. A broad peak was also observed near the end of the elution (fractions 45-47) with elution buffer so the column was reeluted with a higher NaCl concentration (0.2M) to remove any remaining

Figure 14: Comparison of the Sensitivities of the Morgan-Elson Test for *N*-acetyl 2-amino Sugars and the Red Tetrazolium Test on *Staphylococcus aureus* Capsular Polysaccharide Type 5

The sensitivity of the Morgan-Elson test for *N*-acetyl 2-amino sugars was compared to the Red Tetrazolium test using crude *S. aureus* CP type 5. Two fold serial dilutions of the crude CP were tested by both methods and the absorbance of each was read at 490 nm.

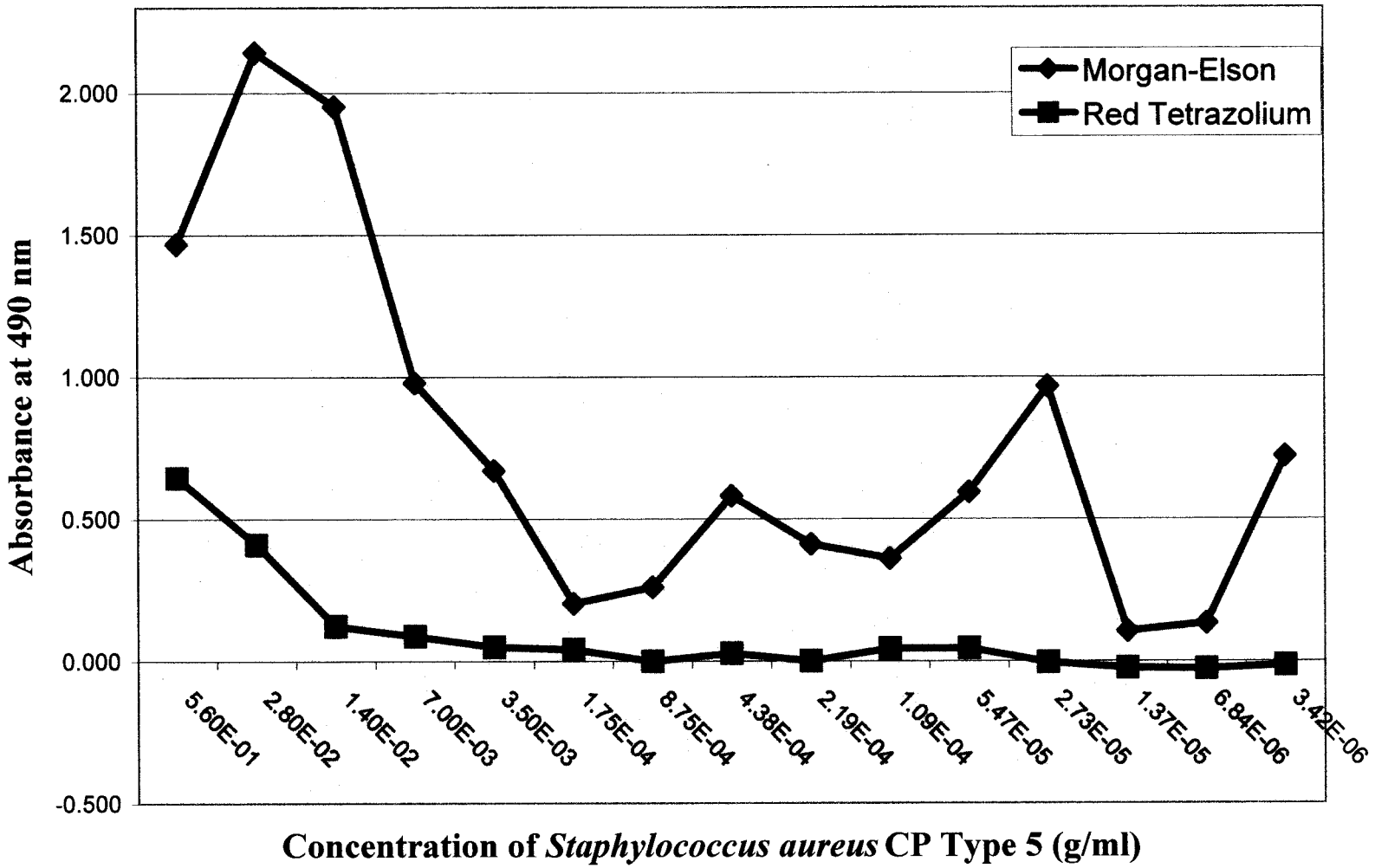

Morgan-Elson
Red Tetrazolium
2.000
1.500
1.000
0.500
0.000
-0.500
Absorbance at 490 nm
5.60E-01
2.80E-02
1.40E-02
7.00E-03
3.50E-03
1.75E-04
8.75E-04
4.38E-04
2.19E-04
1.09E-04
5.47E-05
2.73E-05
1.37E-05
6.84E-06
3.42E-06
Concentration of *Staphylococcus aureus* CP Type 5 (g/ml)

Figure 15: Absorbance of Fractions Tested with Tetrazolium Red from DEAE Sephacel Column Equilibrated with 0.05 M Sodium Acetate, 0.10 M NaCl, pH 6.0

Crude CP was adsorbed onto a DEAE Sephacel column and washed with 5 volumes of starting buffer. After approximately one column volume of starting buffer had been applied, the eluent was cloudy so fraction collection was begun and continued throughout the wash. The fractions obtained were tested using the Tetrazolium Red test and the absorbance was read at 490 nm. *N*-acetyl D-glucosamine was used as positive control. Equilibrating buffer was used as a negative control and its absorbance value was subtracted from each sample.

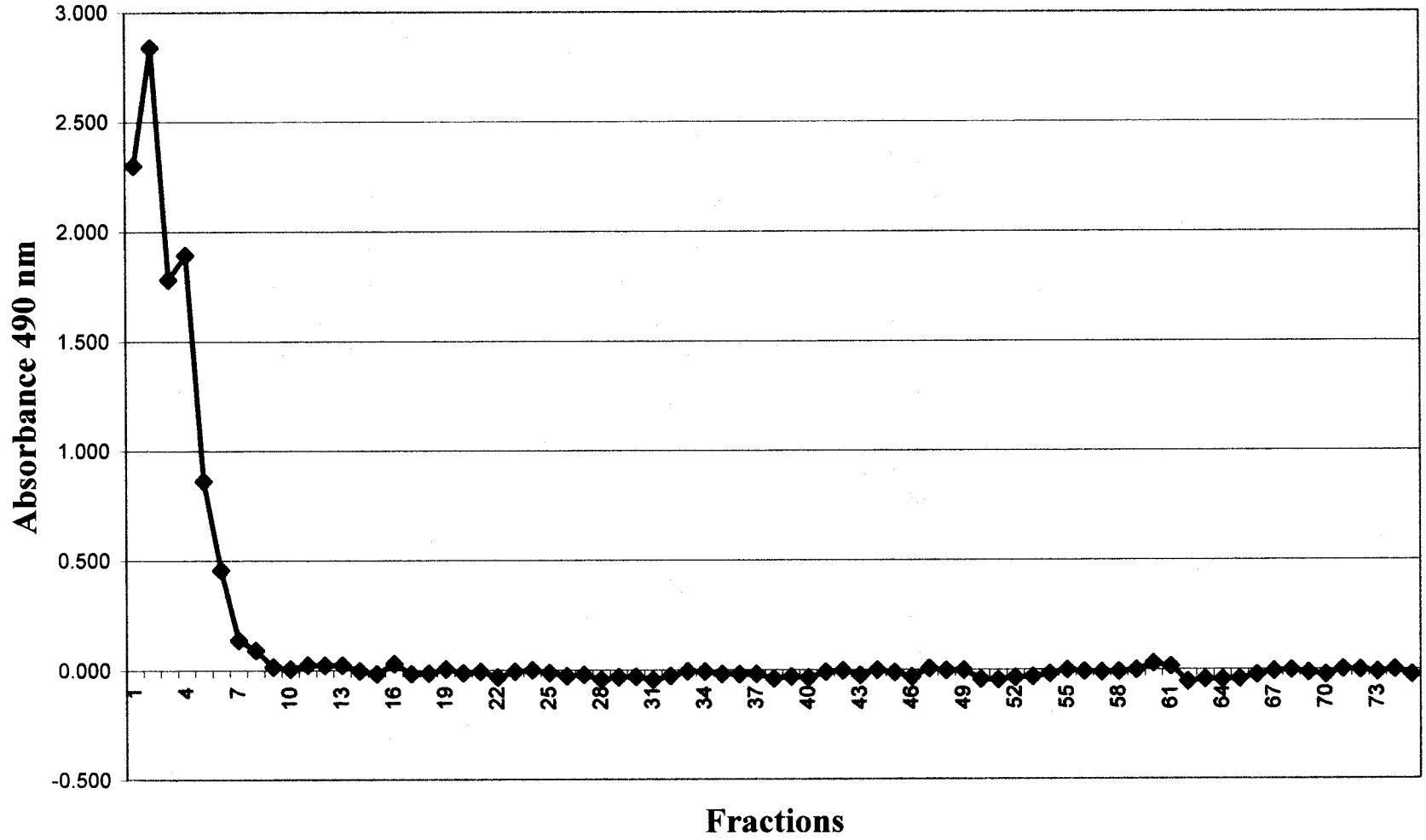
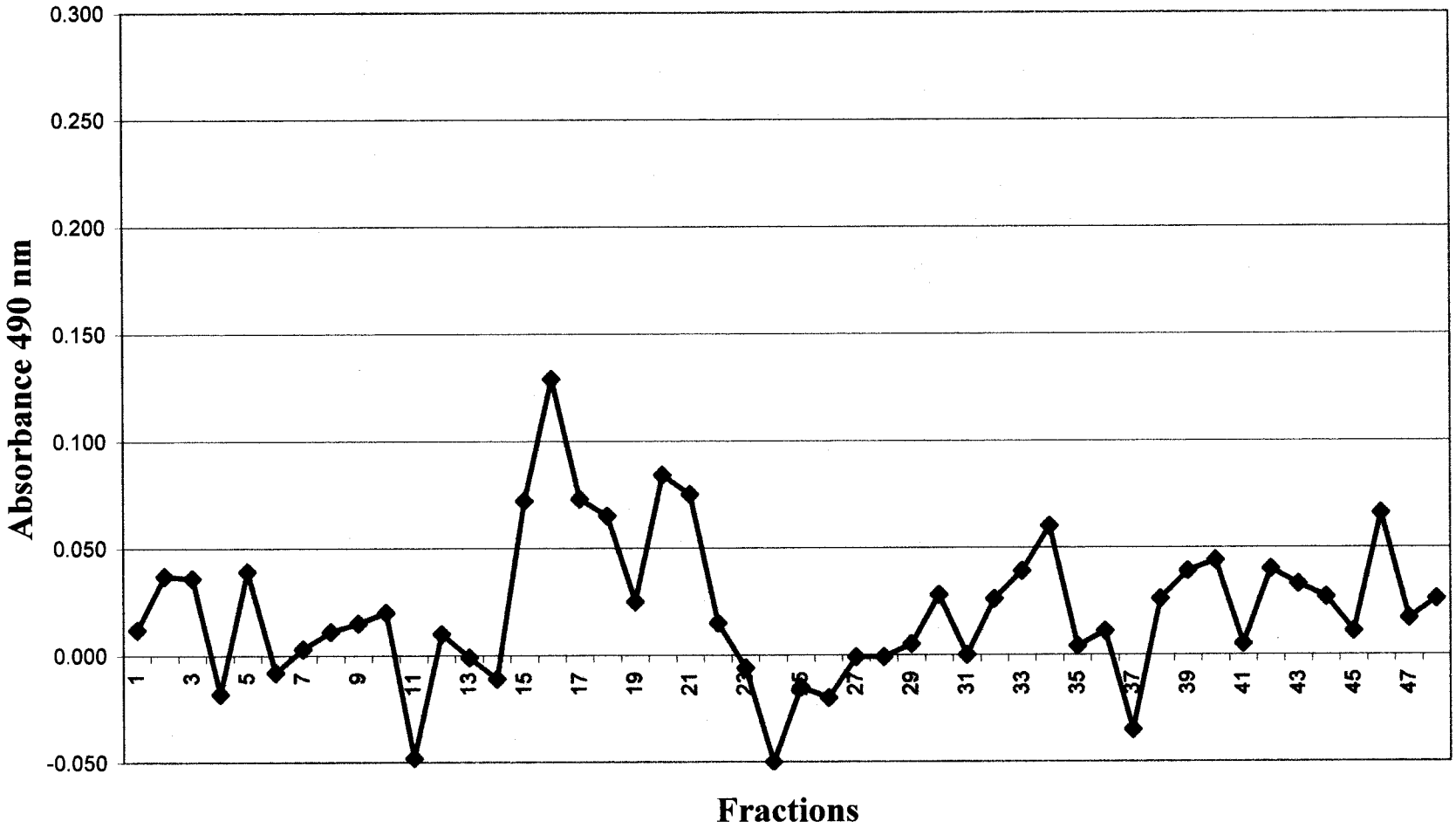

3.000
2.500
2.000
1.500
1.000
0.500
0.000
-0.500
Absorbance 490 nm
1
4
7
10
13
16
19
22
25
28
31
34
37
40
43
46
49
52
55
58
61
64
67
70
73
Fractions

Figure 16: Absorbance of Fractions Tested with Tetrazolium Red Eluted from DEAE Sephacel Column with 0.05 M Sodium Acetate, 0.15 M NaCl, pH 6.0

Elution buffer was then applied to the DEAE Sephacel column and eluent was collected in 6 ml fractions. The fractions obtained during the elution wash were tested using the Red Tetrazolium test and absorbance was read at 490 nm. *N*-acetyl D-glucosamine was used as a positive control. Elution buffer was tested and used as a negative control and its absorbance value was subtracted from each sample.


0.300
0.250
0.200
0.150
0.100
0.050
0.000
-0.050
Absorbance 490 nm
1
3
5
7
9
11
13
15
17
19
21
23
25
27
29
31
33
35
37
39
41
43
45
47
Fractions

polysaccharide bound to the DEAE Sephacel column. The fractions obtained were tested with the Red Tetrazolium test and a peak was observed at fractions 4-8 (Figure 17). These fractions were pooled and saved for further analysis.

The CP positive fractions were next examined for the presence of cell wall contaminant teichoic acid. The teichoic acids present in *S. aureus* are poly-ribitol phosphates. The fractions positive for CP, as determined by the Red Tetrazolium test, from each elution were then tested for the presence of phosphate to determine if teichoic acid was present (Figure 18). PBS was used as a positive control. The CP-positive fractions that eluted with the equilibrating buffer contained an amount of phosphate similar to that of crude polysaccharide. The first CP-positive fractions to elute from the column and the CP-positive fractions from the elution buffer contained trace amounts of phosphate. The CP-positive fractions from the 0.05 M sodium acetate, 0.20 M NaCl elution contained no phosphate and hence no teichoic acid. The fractions that tested positive for the presence of CP and contained no traces of teichoic acid were lyophilized and sent to CS Bio Company for solvolysis to produce trisaccharide fractions from the CP. Future studies will examine the NMR spectra of the solvolyzed carbohydrate to determine the integrity of its structure and purity.

Figure 17: Absorbance of Fractions Tested with Tetrazolium Red from the DEAE Sephacel Column eluted with 0.05 M Sodium Acetate, 0.20 M NaCl, pH 6.0

A higher salt buffer 0.05 M sodium acetate, 0.20 M NaCl, pH 6.0 was applied to the DEAE Sephacel column to check for CP that had not yet eluted. Fractions obtained from this wash were tested with the Tetrazolium Red test and absorbance was determined at 490 nm. *N*-acetyl D-glucosamine was used as a positive control. The higher salt buffer, used as a negative control, was tested and its absorbance value was subtracted from each sample.

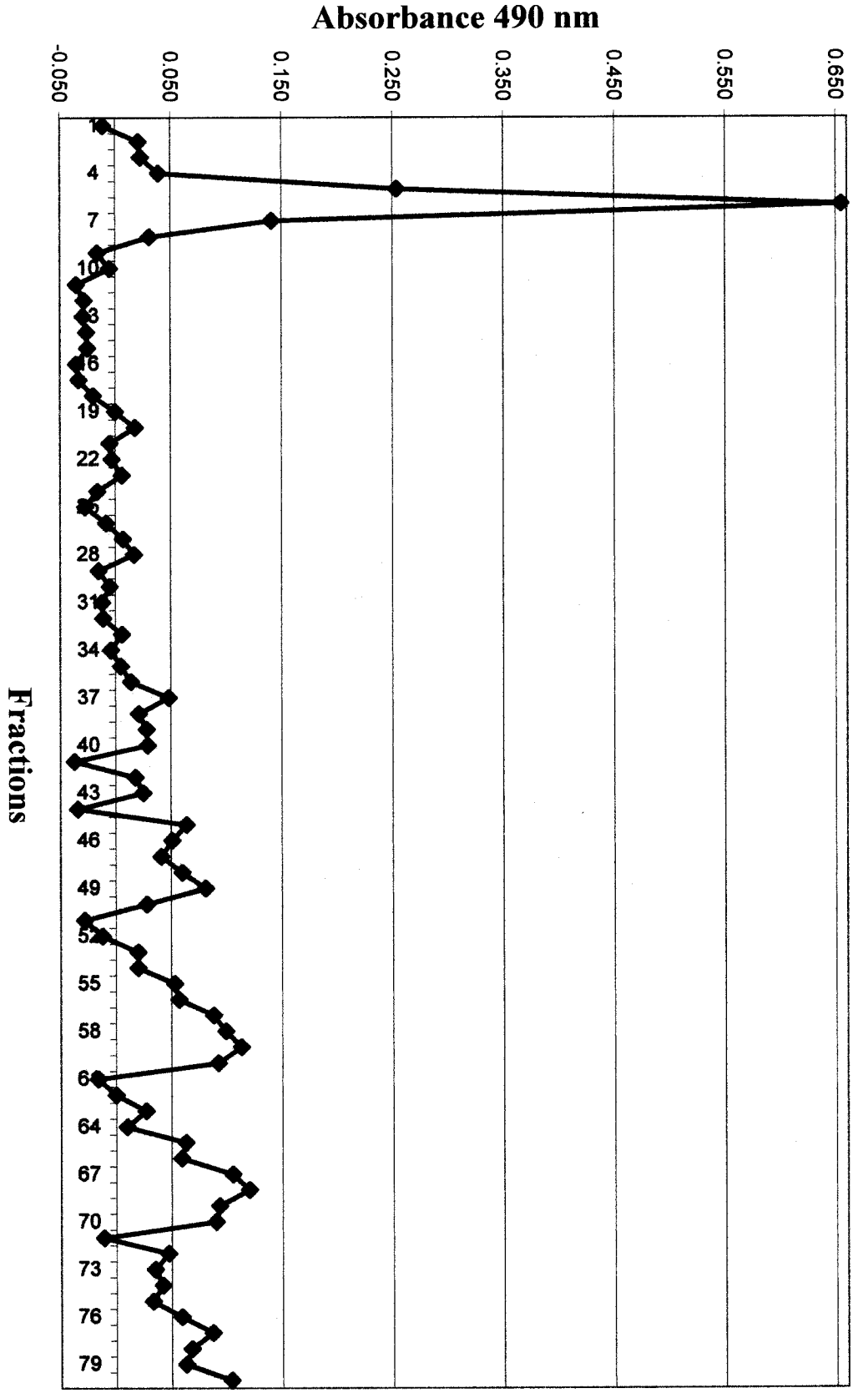
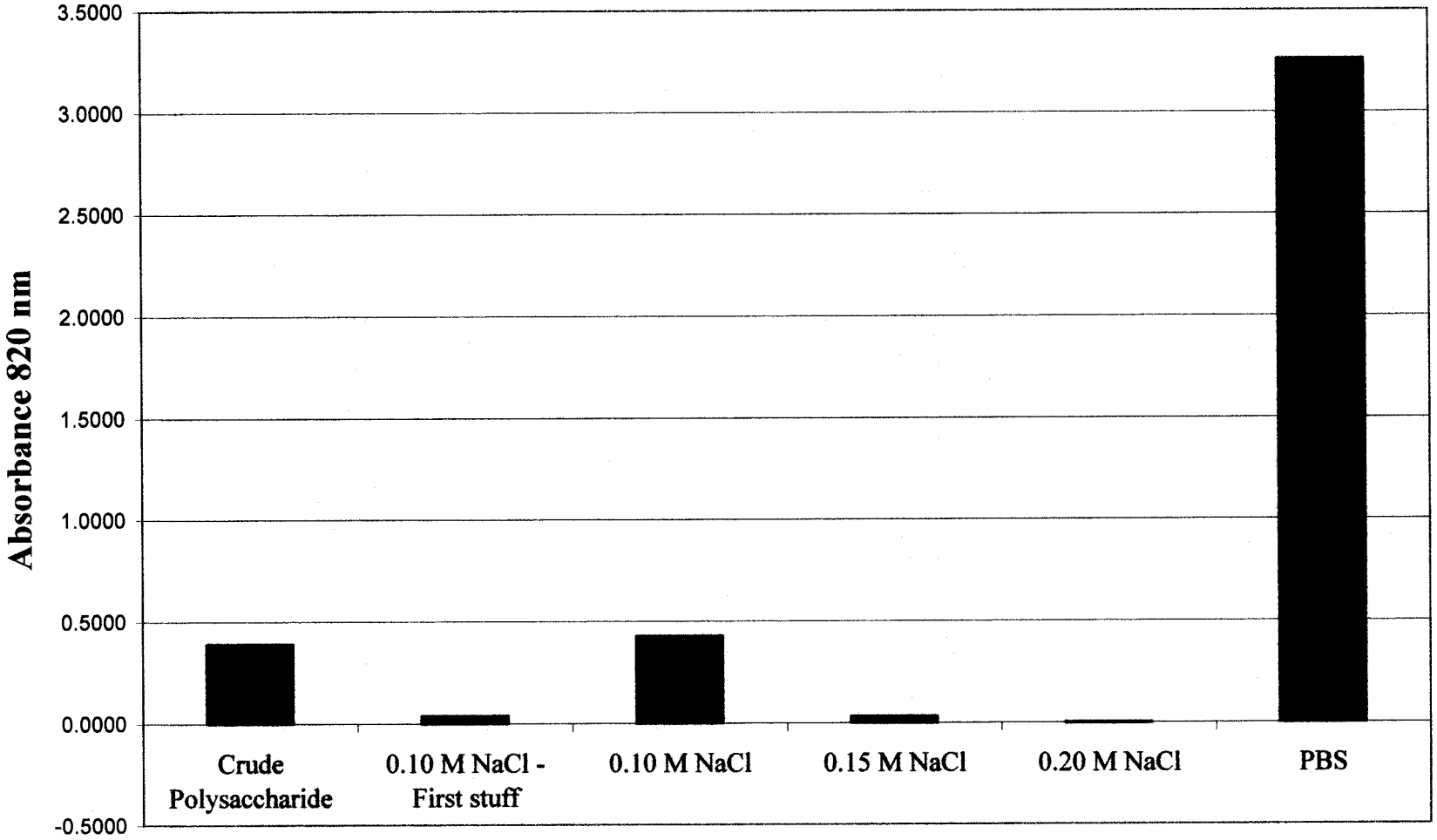

Absorbance 490 nm
-0.050
0.050
0.150
0.250
0.350
0.450
0.550
0.650
Fractions
1
4
7
10
13
16
19
22
25
28
31
34
37
40
43
46
49
52
55
58
61
64
67
70
73
76
79

Figure 18: Test for Teichoic Acid

The fractions positive for CP, as determined by the Red Tetrazolium test, from each elution were pooled and tested for the presence of phosphate, which would indicated the presence of cell wall contaminate teichoic acid. Absorbance was determined at 820 nm. PBS was used as a positive control.


Absorbance 820 nm
3.5000
3.0000
2.5000
2.0000
1.5000
1.0000
0.5000
0.0000
-0.5000
Crude Polysaccharide
0.10 M NaCl - First stuff
0.10 M NaCl
0.15 M NaCl
0.20 M NaCl
PBS

Discussion

It has been found in some animal models that CPs enhance the virulence of the bacterium, while in others the capsule deficient strains were more virulent. The culture conditions under which the bacterium was grown have also been shown to affect the virulence of the bacteria. CP5-deficient strains of *S. aureus* Reynolds were found to be equally as virulent as its CP5 producing parental strain in a mouse renal abscess model (Albus *et al.*, 1991). However, it was later shown that the parental strain was grown in a manner that allowed little CP production. When CP-deficient and parental *S. aureus* strain Reynolds were grown on solid media, enhanced expression of the CP was observed compared to broth grown cells. When the cells grown on solid media were used to intraperitonealy or intravenously inject mice, the parental strain sustained a higher level of bacteremia than did the CP-deficient strain (Thakker *et al.*, 1998). In another mouse model, a CP5-deficient Reynolds strain had reduced ability to cause arthritis and septicemia (Nilsson *et al.*, 1997). However in a rat model of endocarditis the CP5-deficient strains were found to be more virulent than the wild-type CP5 producing strains. This is thought to occur because the CP hinders the bacteria's cell surface adhesion proteins from anchoring the bacteria to host proteins on the damaged heart valves (Baddour *et al.*, 1992). In a mouse colonization model, a CP-deficient strain Reynolds was less effective in colonizing the nares of mice compared to the wild-type strain (Kiser *et al.*, 1999). Mice injected with purified *S. aureus* CPs 5 or 8 alone did not produce serum antibodies (Fattom *et al.*, 1990). In a study by Nemeth and Lee, mice immunized with whole killed *S. aureus* CP5 cells produced high levels of anti-CP antibodies but were not protected from *S. aureus* infection (Nemeth and Lee, 1995).

well on immunocompromised individuals, the population at the highest risk of *S. aureus* disease. Children under two years of age do not respond well to active immunizations due to their immature immune systems. The elderly, patients undergoing renal dialysis, burn patients, and patients with HIV, cancer, or other immune debilitating diseases do not respond well to active immunizations because of their weakened immune response. Passive transfer of hyperimmune Ig would be the best choice for these patients because Ig would be available to offer immediate protection. In this study, the capsule of *S. aureus* was purified and will be used in later studies to develop monoclonal antibodies against *S. aureus* for passive transfer of immunity.

S. aureus CPs belong to a family of surface polysaccharides found on Gram-positive bacteria called teichuronic acids (Ellwood and Tempest, 1972). These CPs have a uronic acid component and are covalently attached to the cell wall PG. The amount of CP synthesized has been shown to be dependent on culture conditions. Synthesis of the teichuronic acid class of CPs is enhanced by growth in medium low in phosphate, such as Columbia broth (Stringfellow *et al.*, 1991). Other investigators have used blood cultures such as brain heart infusion or tryptic soy broths which contain 2.5 mg phosphate per ml and do not favor expression of these CPs (Fattom *et al.*, 1990; Ueda *et al.*, 1998; Ichiman *et al.*, 1991; Yoshida *et al.*, 1987; Dassy *et al.*, 1991).

In this study, the bacteria were grown to late exponential phase of growth because it was previously shown that the CP is produced at the end of the exponential phase of the bacterial growth curve and is enhanced by O_2 availability (Dassy and Fournier, 1996; Herbert *et al.*, 1997). The CP was removed from the bacterial cell wall by autoclaving the bacterial cell pellet followed by lysostaphin treatment according to a method first

described for *S. aureus* CP type 5 by Fournier (Fournier, Hannon *et al.*, 1987). Lysostaphin works by enzymatically degrading the bacterial cell wall, which causes release of the CP (King *et al.*, 1980; Huber & Huber, 1989). Other investigators used ultrafiltration to concentrate the bacterial suspension after autoclaving followed by precipitation of the CP with 80% ethanol (Havaei and Hancock, 1994; Reynaud-Rondier *et al.*, 1991). The CP has also been extracted by sonic oscillation or mechanical agitation with glass beads followed by lysostaphin treatment to remove the CP from the bacterial cell wall (Morse, 1962; Yoshida *et al.*, 1987; Ichiman *et al.*, 1991; Ueda *et al.*, 1998; Lee *et al.*, 1987; Fournier *et al.*, 1984). The bacterium has also been killed by the addition of liquid phenol for 24 hours or treatment with hot dilute acid, followed by ultracentrifugation and lysostaphin treatment (Fournier *et al.*, 1984; Fattom *et al.*, 1993). We chose to autoclave the cells to remove the CP because it is a proven method used by many investigators. We also chose this for ease of method and because it did not require the use of an ultrafiltration unit or an ultracentrifuge to pellet the CP after phenol extraction or acid hydrolysis.

Following autoclaving and lysostaphin treatment, the CP was dialyzed against Milli-Q water, lyophilized, resuspended in PBS and then enzymatically digested with DNase, RNase, and proteinase K (Fournier *et al.*, 1987; Albus *et al.*, 1988; Reynaud-Rondier *et al.*, 1991; Glibert *et al.*, 1994; Burgeot *et al.*, 2001) to get rid of contaminating bacterial cell components. The suspension was again dialyzed against Milli-Q water, and then lyophilized (Fattom *et al.*, 1990). The remaining crude polysaccharide was resuspended in 0.05 M sodium acetate, 0.1 M NaCl, pH 6.0 and applied to a DEAE Sephacel ion exchange column equilibrated in the same buffer. DEAE is a positively

charged anion exchanger that is chemically linked to a cellulose-based matrix. Other investigators have used DEAE linked to dextrose, agarose, or acrylamide based matrices, such as DEAE Spherodex, Sepharose 6MB, or DEAE Trisacryl M (Reynaud-Rondier *et al.*, 1991; Hancock and Cox, 1991; Fournier, Hannon *et al.*, 1987). In the past other investigators used a linear NaCl gradient ranging from 0.1 M to 0.5 M NaCl to elute the CP. Protein eluted first from the column followed by the CP, which for type 5 eluted at a NaCl concentration of 0.15 M (Fournier *et al.*, 1987). For this study a step gradient was utilized. The ion exchange column was washed with equilibrating buffer (0.05 M sodium acetate 0.1 M NaCl, pH 6.0) and the NaCl concentration was increased to 0.15 M to elute the CP (Fattom *et al.*, 1990).

To detect which fractions of the column eluate contained the CP, a variety of methods have been employed including capillary precipitation (Fattom *et al.*, 1990), competition ELISA (Gilbert *et al.*, 1994; Burgeot *et al.*, 2001), monitoring the absorbance at 206 nm (Reynaud-Rondier *et al.*, 1991), and immunodiffusion using an antibody raised in rabbits against whole killed mucoid *S. aureus* cells (Lee *et al.*, 1987). In this study, several methods were tested for detection of the CP in the column eluate fractions. No antibody to the CP was commercially available, which prohibited detection of the CP by capillary electrophoresis, immunodiffusion, or competitive ELISA as was done previously by other investigators (Fattom *et al.*, 1990; Lee *et al.*, 1987; Gilbert *et al.*, 1994; Burgeot *et al.*, 2001), as we cannot prepare sufficient quantities of necessary antibody because we do not have animal facilities for rabbits. Reynaud-Rondier *et al.* analyzed the column eluate fractions with a spectrophotometer at 206 nm. In this study,

no peak was observed when measuring the absorbance at 206. This method did not prove sensitive enough to detect the amount of CP in our eluate fractions.

The first method we examined to detect the CP was iodine vapor thin layer chromatography (TLC). Iodine has a high affinity for both unsaturated and aromatic compounds. Iodine crystals were placed in a Pasteur pipette and a stream of iodine rich air was blown at the surface of a plate that was spotted with a sample of the eluate fractions. Iodine vapor in the air oxidizes the substances spotted on the plate making them visible to the eye. A positive iodine test appears as a brown spot on a yellow background (Gordon, 1972). No brown or positive spots were observed (data not shown). Therefore, the iodine vapor TLC method was not used because it was not sensitive enough to detect the CP from the eluate fractions.

The remainder of the methods tested took advantage of the fact that the CP of *S. aureus* is a reducing sugar and can cause reductions by donating electrons to an acceptor, and thus were assays to identify reducing sugars. The first reducing sugar test we examined was the Benedict's test (Robyt and White, 1987). Benedict's solution is composed of copper sulfate, a blue soluble form of copper (Cu^{++}) that can undergo a reduction when heated in the presence of a reducing sugar, sodium carbonate, and sodium citrate. The free aldehyde groups of sugars are oxidized by the metallic ions present in the Benedict's solution, with the formation of colored cuprous oxide. When the blue copper ions are reduced, they change from the soluble blue color to reddish colored copper ions (Cu^{+}) that are insoluble. The color of the test solution changes from blue to green to orange to red-brown or rust color as more reduced copper ions are formed (Robyt and White, 1987). No color change was observed when testing the column eluate fractions.

Therefore, the Benedicts test was abandoned because it was not sensitive enough to detect the CP in the column eluate (data not shown).

The Morgan-Elson test for *N*-acetyl-2-amino sugars was next examined (Robyt and White, 1987). The basis of the test involves a brief heating in alkaline solution, which causes the sugar to undergo dehydration and double bond formation, followed by reaction with p-dimethylaminobenzaldehyde to give an intense cherry red color, which can be quantified spectrophotometrically. The Morgan-Elson test did prove to be sensitive enough to detect the CP in the eluate fractions, however there were a few drawbacks to this method. The red color that formed faded quickly so we automated this method in a 96-well plate to immediately read all fractions simultaneously. However, in some wells a white precipitate formed and settled to the bottom. This caused nonspecific increases in absorbance preventing us from reading all fractions with a multi-well spectrophotometer. In order to use this method to detect the CP each fraction sample would have to be reacted independently and then read in a cuvette in a spectrophotometer. This would prove to be very time consuming with over 100 fractions to be tested so we employed yet another method to detect the CP.

The next test examined involved Red Tetrazolium, a nearly colorless, water-soluble substance that oxidizes aldoses and ketoses and is thereby reduced. When Red Tetrazolium is reduced it forms an intensely red colored diformazan that is insoluble and precipitates out of solution (Fieser & Williamson, 1987). The Red Tetrazolium test was sensitive enough to detect the CP in the eluate fractions. The red color that formed did not fade as quickly as with the Morgan-Elson test and no precipitates were formed in the reaction. The sensitivity of the Morgan-Elson test was compared to the Red Tetrazolium

test using a standard curve of the crude CP or *N*-acetyl D-glucosamine, a positive control. Although the Morgan Elson test proved to be more sensitive, the Red Tetrazolium test was ultimately used to test for the CP due to the precipitates in the Morgan Elson test that skew the spectrophotometric results.

The next step in the CP preparation was to purify the CP from the cell wall contaminate, teichoic acid. Sodium periodate oxidation has been used by many investigators to destroy teichoic acid while leaving the *S. aureus* CP intact. Ethylene glycol is then added to get rid of the excess unreacted periodate. Some investigators have used sodium periodate treatment prior to running the crude CP over an ion exchange column (Gilbert *et al.*, 1994; Burgeot *et al.*, 2001; Havaei and Hancock, 1994) while others have first run the crude CP over the column, pooled the fractions positive for CP and then subjected the pools to sodium periodate treatment (Lee *et al.*, 1987). Other investigators have used a size exclusion chromatography column such as Sepharose CL-4B or Sephacryl S-300 to get rid of teichoic acid contamination (Fournier *et al.*,1987 ; Moreau *et al.*, 1990; Havaei and Hancock, 1994; Reynaud-Rondier *et al.*, 1991; Fattom *et al.*, 1990; Arizono *et al.*, 1991; Lee *et al.*, 1987). Fournier *et al.* purified crude CP over a DEAE Sephacel column as was done in this study. Fractions positive for the CP were pooled, dialyzed, lyophilized and again applied over a DEAE Sephacel column this time with a linear increase in NaCl concentration to get rid of teichoic acid (Fournier *et al.*, 1984). In this study, the crude CP was only run over one column and several peaks of CP elution were observed. The positive fractions in each peak were pooled, dialyzed, and lyophilized, keeping the peaks separate. Previous investigators have assayed for the presence of teichoic acid by capillary precipitation (Fournier *et al* 1984; Fattom *et al.*,

1990), or immunodiffusion (Lee *et al* 1987; Fournier *et al.*, 1987). In this study, each sample was tested for the presence of phosphate by the method of Chen to determine if contaminating cell wall component teichoic acid was present (Chen *et al.*, 1956). The CP sample that tested negative for phosphate and hence was free of teichoic acid was subjected to HF solvolysis to break the carbohydrate polymer into trisaccharides as was done by other investigators (Moreau *et al.*, 1990).

S. aureus remains a health problem despite the introduction of antibiotics and the need for a vaccine is warranted. In this study the CP was isolated from *S. aureus* cells by lysostaphin treatment and autoclaving. DNA, RNA, and protein were enzymatically digested and the crude polysaccharide was purified by anion exchange chromatography. Various biochemical methods were tested for detecting the CP in fractions of column eluate. Fractions that contained CP but were free of teichoic acid were treated with HF to break the CP in to trisaccharides. In the next steps of vaccine production the carbohydrate purified in this study will be coupled to a protein to improve the immune response. This carbohydrate-protein conjugate could then be tested for use as a vaccine, or could be used for the development of immune therapies for *S. aureus* infections.

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