Purification of *Staphylococcus aureus* Type 5 Capsular Polysaccharide by DEAE Sephacel and Sephacryl Chromatography

by

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Submitted in Partial Fullfillment of the Requirements

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Youngstown State University

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Type 5 Capsular Polysaccharide by DEAE
Sephacel and Sephacryl Chromatography

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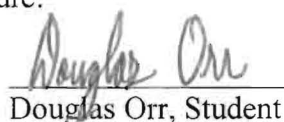

12/8/06

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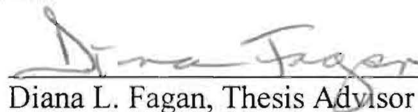

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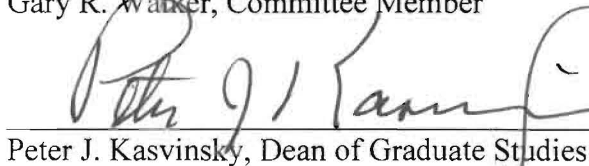

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ABSTRACT

Staphylococcus aureus (*S. aureus*) type 5 and 8 are an important cause of both nosocomial and community acquired infections. Out of the 40 million patients that undergo surgery, approximately 5% of these patients acquire *S. aureus* infections. Community acquired *S. aureus* bacteremia is considered to be even more serious than hospitalized infections comprising approximately forty percent of all *S. aureus* infections. Resistant strains toward recent antibiotics have been increasing in number and many antibiotics are ineffective. The development of new forms of treatment are needed for these resistant forms of bacteria. A resistant capsular polysaccharide (CP) layer located on the outer cell wall, aids the bacteria in pathogenesis. The purpose of these studies was to purify CP from *S. aureus* type 5. The bacteria was treated with DNAse, RNAse, protease, and lysostaphin to release the CP from the cell wall. Purification of lysates was accomplished by means of a DEAE charge separation and size exclusion chromatography. Collected fraction samples from chromatography purification were tested for the presence of organic molecules by an absorbance of 206 nm. The presence of reducing sugars by a red tetrazolium test, and contamination by teichoic acid was evaluated by a phosphate test. Anti-type 5 *S. aureus* monoclonal antibodies were used in an ELISA to test for specificity of purified CP. Monoclonal antibodies to type 8 *S. aureus* were developed to test preparations of purified type 8 CP. Purified CP will be used in future studies to determine the specificity of antibodies and phage display libraries used to develop immunotherapies for *S. aureus* infections.

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I would also like to thank my committee members, Dr. Gary Walker and Dr. David Asch, for the advice and guidance they have given to me throughout my course of study.

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July 22, 2004

Dr. Diana Fagan, Associate Professor
Department of Biological Sciences
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RE: IACUC Protocol Update Number: 05-03U05
Title: Production of scFv specific for S. *aureus* types 5 and 8

Dear Dr. Fagan:

The Institutional Animal Care and Use Committee of Youngstown State University has reviewed the aforementioned Protocol Update you submitted to continue the project for the above mentioned Protocol, and has issued ongoing approval for the period of June 27, 2004 through June 26, 2005.

You must adhere to procedures described in your approved request; any modification must first be authorized by the Animal Care and Use Committee.

Sincerely,

Peter J. Kasvinsky
Dean of Graduate Studies
Research Compliance Officer

PJK:cc

c: Dr. Walter Horne, Consulting Veterinarian
Dr. Robert Leipheimer, Chair, Department of Biological Sciences
Ms. Dawn Amolsch, Animal Care Technician

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LIST OF ABBREVIATIONS

BSA	Bovine Serum Albumin
CP	Capsular Polysaccharide
DEAE	Diethylaminoethyl
ELISA	Enzyme Linked Immuno Absorbant Assay
FCS	Fetal Calf Serum
HAT	Hypoxanthine-aminopterin-thymidine
Ig	Immunoglobulin
mAbs	Monoclonal Antibodies
MHC	Major Histocompatability Complex
MEM	Minimum Essential Medium
MRSA	Methicillin Resistant *Staphylococcus aureus*
OPI	Oxaloacetate Pyruvate Bovin Insulin
OD	Optical Density
PEG	Polyethylene glycol
PBS	Phosphate Buffer Saline
TMB	Tetramethylbenzidine
TBS	Tris (hydroxymethyl) Aminomethane
VRSA	Vancomycin Resistant Strain *Aureus*

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I. INTRODUCTION

Staphylococcus aureus (*S. aureus*), a gram positive bacteria, is an opportunistic bacterial pathogen that dwells on the skin and mucosal surfaces of healthy humans. Infection will occur when these barriers are breached and a foreign substance is introduced in the individual. Infection is also common in humans with weak immune systems (O'Riordan *et al.*, 2004). Individuals at risk for *S. aureus* infections include newborns, trauma victims, burn patients, drug abusers and people with low numbers of white blood cells. Healthy humans and animals have the ability to prevent normal colonized *S. aureus* from spreading throughout the body causing infection (Lee, 1996).

The significance of *S. aureus* can be seen in hospitalized individuals that have undergone surgery. Out of the 40 million patients that undergo surgery, 20% of these patients acquire at least one infection after the surgical procedure. *S. aureus* causes approximately 25% of these nosocomial infections. Patients commonly carry *S. aureus* in their nasal passages and transfer the bacteria to other individuals through the contact of hands. Patients that carry *S. aureus* are at a high risk of infection after surgical procedures (Perl *et al.*, 2002).

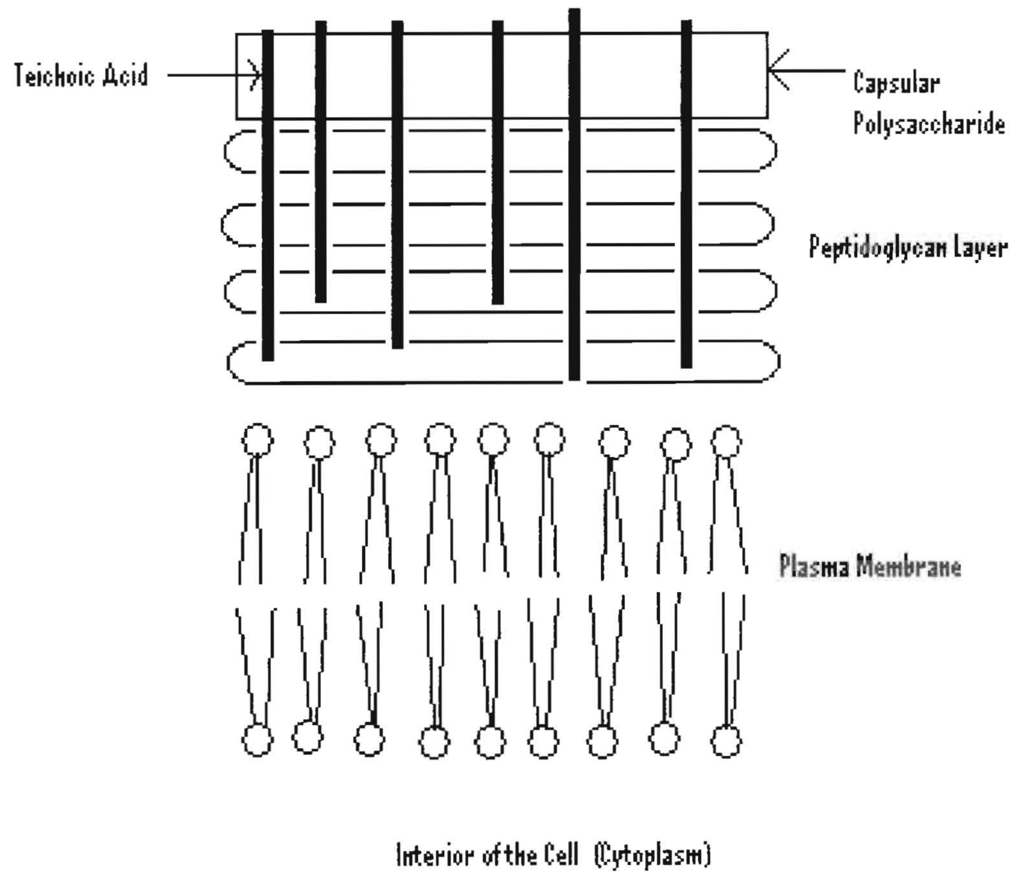
A study involving individuals who were considered to have community-acquired bacteremia from *S. aureus* resulted in a thirty-five percent mortality of the population. All of the individuals in the study were considered not to be intravenous drug users. Approximately ninety percent of the individuals in the study had at least one or more serious complications including acute renal failure, respiratory failure, endocarditis, and pneumonia (Willcox *et al.*, 1998).

The body's initial natural response to *S. aureus* is the migration of leukocytes to the area of infection due to the expression of adhesion molecules located on endothelial cells of blood vessels. This response is due to both the bacteria and tissue based immune cells called macrophages and neutrophils. When infection occurs, molecules called cytokines are produced by tissue macrophages that recruit inflammatory cells to the sites of infections to attempt to eradicate the bacteria (Lowy, 1998).

S. aureus resist destruction from the immune system by having a resistant structure called a capsule (Figure 1). This outer layer of the cell wall of *S. aureus* is composed of carbohydrates. The carbohydrate layer, or capsule, is exposed to the environment. The capsular polysaccharide (CP) assists the bacteria by adhering to host tissue and helping to avoid phagocytosis (ingestion and destruction of an antigen by white blood cells). There have been eleven *S. aureus* CP that have been discovered, and about 75% of the bacteria causing hospital acquired infections have CP that is type 5 or type 8. Serotype 5 and 8 are prevalent in both hospitalized patients and community infections. These types can also be found in infections in cows, rabbits, poultry, pigs, and horses. These capsular types can be identified by using specific antibodies to help stabilize and visualize the polysaccharides and performing electron microscopy (Cunnion *et al.*, 2002). Purified type 5 and 8 CP are almost identical in structure, consisting of trisaccharide repeating units comprised of N-acetyl mannosaminuronic acid, N-acetyl L-fucosamine, and N-acetyl D-fucosamine. The differences in the capsule between type 5 and type 8 are attributed to different linkages between the sugars and different sites of O acetylation (Watts *et al.*, 2005). Type 5 and type 8 are also referred to as micro capsules, which are used to distinguish these isolates from serotypes 1 and 2 mucoid strains and is

FIGURE 1. Capsular carbohydrate of *S. aureus*: Figure 1 shows a description of a typical cell wall of gram positive bacteria (such as *S. aureus*). The projections extending out from the peptidoglycan layer and into the capsular polysaccharide layer are teichoic acid. The capsular polysaccharide layer provides a protective structure for the bacteria and resists phagocytosis (process involving engulfing of a foreign structure such as a bacterium by white blood cells leading to the destruction of the foreign structure) by phagocytes.

Gram Positive Bacterial Cell Wall



Interior of the Cell (Cytoplasm)

based on the small amount of capsular material produced.

S. aureus infections can cause a wide variety of diseases. Symptoms of infection of the bacteria can range from pimples and furuncles to toxic shock syndrome (TSS) and sepsis. The type of disease caused by *S. aureus* is determined by specific virulence factors that include hymolysins, leukocidin, exfoliative toxins A and B, and pyrogenic toxins (Orwin *et al.*, 2001). Some other examples of infections that can be seen includes cutaneous infections, boils, wound infections, prosthetic-device related infections, and many of these can be severe life-threatening infections (Lee, 1996).

The bacteria produce various toxins that contribute to the symptoms of infection. Cytotoxins cause pore formation and induce proinflammatory changes in tissues, which might contribute to sepsis, the spreading of the disease throughout the system. Superantigens are molecules that have the capability of binding to MHC class II proteins, which will cause T cell proliferation and the release of cytokines. The superantigen toxins that are secreted by *S. aureus*, such as enterotoxin that causes both toxic shock syndrome and food poisoning, will bind to the MHC (major histocompatability complex) proteins of the host. After the superantigens bind to the host MHC, they will bind to the ß chain located on the variable region of the T-cell receptor resulting in expansion of T-cells which results in the release of numerous cytokines by T cells as well as macrophages. Tissue damage can result when large quantities of cytokines are released. Once the bacterial toxins bind to the MHC molecules, they will eventually lead to a variety of different symptoms within the host such skin separation from connective tissue leading to skin erythema (Lowy, 1998).

The exomolecules secreted by the bacteria that cause disease also contribute to the survival of the bacteria within the host. These exomolecules have been studied in vitro and have been shown to be produced only when the bacteria is in higher densities. When there are low densities of bacteria, the organism will express surface molecules to adhere to and colonize the cells of the host. Examples of these adherent molecules include fibronectin-binding proteins and fibrinogen-binding protein (Balaban *et al.*, 2001). *S. aureus* have MSCRAMM (microbial surface components recognizing adhesive matrix molecules) cell surface adhesins (Hall *et al.*, 2003). There are eight *S.* aureus structurally related MSCRAMMs that have been identified, four of these molecules are fibrinogen-binding MSCRAMMs, which have been characterized as FnbpA, FnbpB, ClfA, and ClfB (Perkins *et al.*, 2001). MSCRAMM can recognize and specifically bind to certain extracellular components of host tissues or artificial implanted materials that have been serum-conditioned (catheters, artificial joints, and vascular grafts). After *S. aureus* adheres to the host tissues, gene expression is altered in the bacteria, leading to an increased resistance to the individual's immune system (Hall *et al.*, 2003). The diverse expression of exomolecules as well as surface molecules associated with *S. aureus*, contributes to the difficulty of treatment.

Before antibiotics were used for treatment, mortality following *S. aureus* was approximately 90%. The most effective cure for *S. aureus* is antimicrobial agents. Unfortunately, resistance to antibiotics has been increasing, and today many antibiotics cannot be used (Lowy, 1998). Community acquired *S. aureus* bacteremia comprises approximately forty percent of all *S. aureus* infections and is considered to be more serious than hospital acquired infections (Willcox *et al.*, 1998). Methicillin-resistant *S.*

aureus (MRSA) infections have been associated with health care settings, but recently this strain has been found in individuals without risk factors. These community-acquired MRSA infections have been reported to be seen in populations of correctional facility inmates, homosexual males, and athletes. Molecular typing has shown the majority of community-acquired infections have been caused by MRSA associated with Panton-Valentine leukocidin virulence factor and MRSA containing the chromosome cassette mec type IV allele. These two strains are commonly seen in community-acquired infections but not hospital-acquired infections (King *et al.*, 2006). Strains that are resistant to methicillin (MRSA) have been shown to have increasingly less susceptibility to vancomycin (referred to as VRSA isolates). These isolates have been seen in several countries, but identification of the mechanism for resistance has been difficult due to the conflicting observations relating to the properties of the VRSA isolates (Sieradzki *et al.*, 2003).

S. aureus resistance is brought about due to poor use of antimicrobials. Some *S. aureus* infections require only localized treatment. Abscesses can be drained and foreign bodies removed instead of requiring treatment with antibiotics. Hospitalized infections are sometimes not promptly cared for. Rapid identification of infected patients and strict enforcement of infection control measures minimize the threat of nosocomial spread of infection. Some of these measures can include strict guidelines for the use of antibiotics with *S. aureus*, education, feedback from physicians, and information at prescription order sites. Improper seclusion measures have also lead to the direct transfer of resistant organisms between patients, indirect transfer, and healthcare worker carriage of resistant organisms. Unrestricted use of antibiotics will lead to the occurrence of more resistant

strains of *S. aureus*. Overuse or misuse of new antimicrobials with different forms of treatment can result in the development of resistance against these antimicrobials (Smith, *et al.*, 1999).

Resistance to antibiotics by *S. aureus* is often transferable and usually due to the acquisition of plasmids and/or transposons. Transfer of resistance between staphylococcal strains has been shown to occur in experiments *in vitro* by conjugation (cell to cell contact to exchange genetic material), transduction (incorporation of genetic material through a virus), and transformation (transfer of genetic material through DNA fragments). Conjugative transfer appears to be most significant *in vivo*, where resistance determinants are spread by the conjugative plasmids (Khan *et al.*, 2000). New treatment methods are needed for these new antibiotic resistant strains.

Different forms of vaccines can be used to produce resistance against *S. aureus*. One type of vaccine is the whole-cell vaccine that uses live or killed *S. aureus* cells. Antibodies (molecules that bind to foreign material within a host) would be produced by the host against the vaccine, enhancing phagocytosis by binding to the bacteria and allowing for recognition by white blood cells (Lee, 1996). A study by Greenberg (*et al.*) created a whole bacteria vaccine for *S. aureus* methicillin resistant strain to test for protection against endocarditis. The bacteria was killed by formalin and administered to rabbits intravenously. The rabbits were vaccinated once a day for three consecutive days then repeated one week later. The rabbits were given a booster a few days after the second doses were administered. Blood samples were obtained before immunization and one week after the booster. Antibody production was demonstrated in the rabbits, but the

antibodies had no effect on the course of bacteremia. Similar results were seen in unimmunized rabbits (Greenberg *et al.*, 1997).

Another vaccine that has been produced is the capsular vaccine which uses a purified capsular carbohydrate. Antibodies produced by the host will bind to the capsule of the bacteria, stimulating phagocytosis of the bacteria (Lee, 1996). Capsular polysaccharide (CP) antigens are classified as thymus-independent-2 (TI-2) antigens. The CP antigens can potentially cause an immune response in adults, but a much weaker immune response in infants and young children (Garcia-Ojeda *et al.*, 2000).

Fattom and coworkers (Fattom *et al.*, 1992) purified type 5 and type 8 CP by means of anion-exchange chromatography and gel filtration. Young female mice (6-8 weeks old) were immunized biweekly subcutaneously with the CP (used as a vaccine). Mice were bled before the second injection and one week after the last two injections. An ELISA was used to measure serum antibodies. The study showed that the type 5 vaccine did not elicit any antibodies in the mice. Type 8 CP elicited low levels of antibodies after the first injection and a slightly higher level after the following two injections (Fattom *et al.*, 1992). Supportive evidence from this article suggests that a capsular vaccine alone does not elicit an adequate immune response due to T-cell independency. Capsular polysaccharides alone are poor immunogens (Lee, 1996).

Finally, a vaccine can be produced by linking the polysaccharide to an immunogenic protein carrier, such as a tetanus toxoid. A response will occur involving the production of T cell-dependent antibody toward the polysaccharide antigens that are causing the infection.

Fattom and coworkers (Fattom *et al.*, 1992) developed a vaccine from type 5 and type 8 CPs covalently bound to *P. aeruginosa* and used the vaccine on young mice (6-8 weeks old). CPs were purified by anion-exchange chromatography and gel filtration. Mice were immunized biweekly subcutaneously with the CP (used as a vaccine) conjugated with an exoprotein from *Pseudomonas aeruginosa.* Mice were bled before the second injection and one week after the last two injections and sera was analyzed by means of an ELISA for the presence of antibodies against *S. aureus*. The conjugates did not elicit antibodies after the first injection, but after the second and third injections there was a significant increase in antibodies (Fattom *et al.*, 1992).

The conjugate vaccines result in the production of anti-capsular antibodies; however, there are still problems with these vaccines. The conjugate vaccines can result in toxic effects when used in the host (Fattom *et al.*, 1992). The process of conjugating the CP to the carrier is a tedious and time consuming procedure which can be avoided by using a whole cell vaccine to get similar results. The conjugate also requires the use of an adjuvant (a substance added to increase or elicit an immune response) for antibody production within a host. These vaccines are also not always effective due to the population that the vaccine targets. Children, elderly, and immunocompromised individuals lack a sufficient T cell response toward the vaccine due to the low number of T cells (Lee, 1996).

Monoclonal antibodies can be used to treat patients not helped by vaccines, including the immunocompromised, elderly, or young individuals. Carbohydrate vaccines do not elicit a strong immune response in these individuals. Our research involves the production of hybridomas that produce specific monoclonal antibodies that

will bind to *S. aureus* type 5 or 8. The antibodies produced by our hybridomas may be used for the treatment of the general population of individuals including young, elderly, and immunocompromised (Isaacs, 2001).

B cells produce immunoglobulins that bind to only one epitope (binding region located on the antigen that binds to the antibody). When a B cell encounters an antigen that has the epitope it is specific for, appropriate signals will stimulate the B cell to divide several times and produce multiple identical antibody-secreting plasma cells. In general, an organism will produce millions of different B cells, each having distinctive epitope-specific immunoglobulins. This characteristic gives the organism immunological diversity. Each immunoglobulin has a high degree of specificity for a certain epitope. Immunization of an organism results in the production of a large quantity of different immunoglobulins, each kind reacting with a particular epitope. This mixture of different antibody types are called polyclonal antibodies.

Antibody-producing B cells can be fused with immortal myeloma cells to produce a hybridoma. A chemical reagent (polyethylene glycol) is used for cell fusion, and the cells are placed in a media that selects for the HGPRT enzyme. The HGPRT is not found in myeloma (cancer) cells, therefore they will not grow. The enzyme is found in B cells; however, B cells do not grow well in culture. Hybridomas will survive in the selective media because the cells contain HGPRT from the B cell parent and information for unlimited growth from the myeloma cell parent. These cells will also acquire the genetic information for antibody production from the B cell parent. The cells can be cloned and expanded resulting in cells that will produce a monoclonal antibody population. The immunoglobulins produced will then bind to the same epitope. The cells can then be

cultured for a large scale production of mAbs. The result is a long lived cell that will continue to produce monoclonal antibodies against the targeted antigen (Köhler *et al*., 1975).

In 1975, Köhler and Milstein published their method of producing monoclonal antibodies (mAbs) through use of spleen cells and myeloma cells from a mouse. Their method permitted the isolation of high amounts of pure and specific antibodies adding to the methodology of antibodies in the detection, and treatment of disease. Monoclonal antibodies are now being used in the medical field for a variety of different treatments and tests including diagnosis of cancer *in vivo* (within the host) and immunotherapy of human disease (Waldman, 1991). Some examples of immunotherapy include using antibodies alone (for passive therapy) or as active immunogens in vaccines. Monoclonal antibodies can be used to carry toxic agents to their targets, aiding in the destruction of pathogens. They have also been used clinically for manipulating the immune response to produce immunosuppression before transplants (Goldenberg, 1994).
Monoclonal antibodies that distinguish surface molecules facilitating cell to cell interactions are effective as immunosuppressive agents (Waldman, 1991).

Monoclonal antibodies (mAb) have been actively used in the treatment of bacterial infections and the inhibition of accumulated neutrophils. By reducing neutrophil accumulation, tissue damage will be prevented (Waldmann *et al*., 2000); however, infection may spread. In a clinical trial involving 200 patients having bacteremia, mortality rates were reduced in mAb treated patients. Monoclonal antibodies specific for leukocyte adhesion molecules can also be used to halt the neutrophil accumulation that results in the damage of tissues in animal models with bacterial

meningitis, hemorrhagic shock, and myocardial reperfusion injury. MAbs are used to prevent and reverse allograft rejection, used for immunotherapy of autoimmune and graft-versus host diseases, and other various techniques (Goldenberg, 1994).

A commonly used mAb is OKT3, which is directed against the CD3, a molecule that transmits signals after antigen-binding to the T cell receptor complex that is located on almost all circulating T cells. This mAb drug is used for the treatment of renal allograft rejection. The drug showed a 93% reversal of minor rejection episodes compared to other non-mAb drugs that have shown a 75% reversal in rejection (Waldmann, 1991).

A drug called rituximab, used for the treatment of autoimmune disorders, binds to tumor cells and attracts killer cells to the infected cells. Rituximab, also known as rituxan, is an antibody with anti-tumor properties. Reports have demonstrated the high rates of efficacy with this drug when used for initial therapy toward the treatment of lymphoma. The evidence suggests that the drug's primary mechanism of anti-tumor action is through binding to the tumor cells and recruiting killer cells through process called the antibody-dependent cellular cytotoxicity mechanism. This method involves antibodies that are needed to bind to both the antigen and a killer cell for cell killing to occur (Waldmann *et al.*, 2000).

Nelles and coworkers (Nelles *et al.*, 1985) created monoclonal antibodies against *S. aureus* type 5 and type 8. Mice were immunized three times with formalin fixed *S. aureus* bacterial vaccines, and spleen cells were extracted and fused with mouse myeloma cells. The fused cells were selectively grown in a hypoxanthine-aminopterin-thymidine (HAT) media for hybridoma selection. Bacterial agglutination was used to

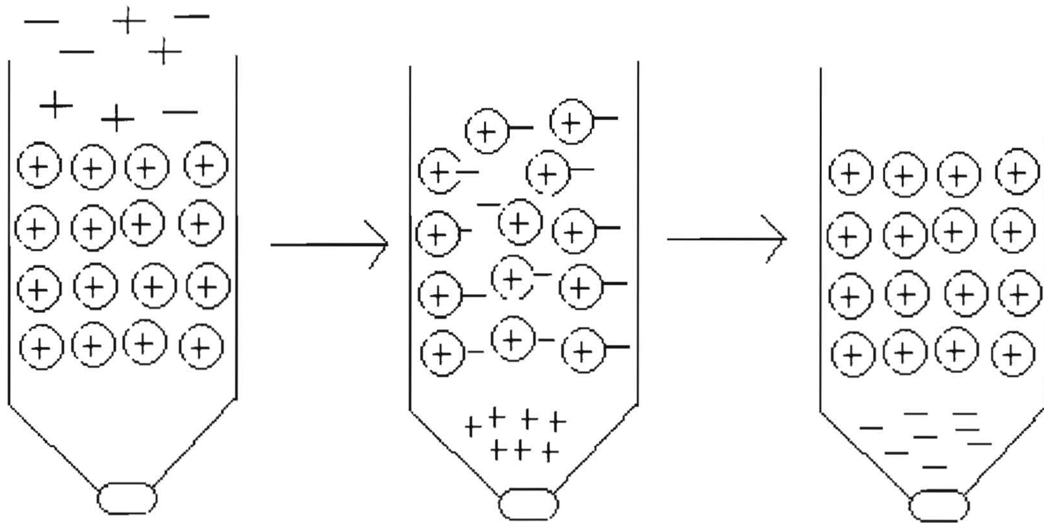
screen hybridoma cell culture supernatants for anti-*S. aureus* antibody activity. An ELISA (which is a method that uses an enzymatic reaction to detect the binding of an antibody to an antigen, and is interpreted by means of absorbance) was used to determine the subclasses of the immunoglobulins. Type 5 *S. aureus* capsular polysaccharides were purified and tested for binding to the monoclonal antibodies by an ELISA. The monoclonal antibodies in the study were shown to be reactive with the type-specific capsular polysaccharides. First, the antibodies bound to the surface antigens on the whole bacteria demonstrated by agglutination assays. Second, the serological specificity of the monoclonal antibodies in the agglutination assays and ELISA correlated with the capsular serotypes assigned by using polyclonal antisera to perform typing studies (Nelles *et al.*, 1985).

In previous studies in our lab, mice were inoculated with type 5 *S. aureus* twice and the spleen was removed. Spleen cells (lymphocytes) were fused with myeloma cells and grown in the selective HAT media. After cloning hybridomas, the cells were tested using an ELISA for binding to type 5 *S. aureus* whole bacteria. Comparing the hybridomas to the positive controls (serum from mouse immunized with *S. aureus*) and negative controls (no primary antibodies were added, so no binding to *S. aureus* type 5 occurred). Positive clones that produced antibodies specific for *S. aureus* type 5 and resulted in a high absorbance value in the ELISA, were used for identifying purified capsule.

The objectives of this study are to purify the capsular carbohydrates from type 5 *S. aureus* and test the CP after DEAE purification (Figure 2) and after size exclusion purification (Figure 3) to demonstrate binding to antibodies specific for *S. aureus* type 5.

The purified CP will be used for future screenings for hybridomas that bind to *S. aureus* type 5. Previously produced monoclonal antibodies against type 5, will be used to develop an ELISA for testing column fractions for the presence of CP. In addition, we have begun to produce monoclonal antibodies against type 8 *S. aureus* which we have tested for binding to formalin treated bacteria. Our studies in lab will hopefully lead to products useful for the treatment of *Staphylococcus aureus* type 5 and type 8 infections in immunocompromised individuals.

FIGURE 2. DEAE Sephacel column: The sample is loaded into the column with positively charged DEAE cellulose beads. Positively charged molecules will not bind to the beads and will be eluted from the column as well as weak negatively charged molecules. Highly Negatively charged molecules, such as capsular polysaccharides, will bind tightly to the DEAE beads. By increasing the salt concentration of the buffers (used to wash the column) from 0.05 M NaCl to 0.15 M NaCl, strong ionic bonds from the capsular polysaccharides will be disrupted and elute from the column.

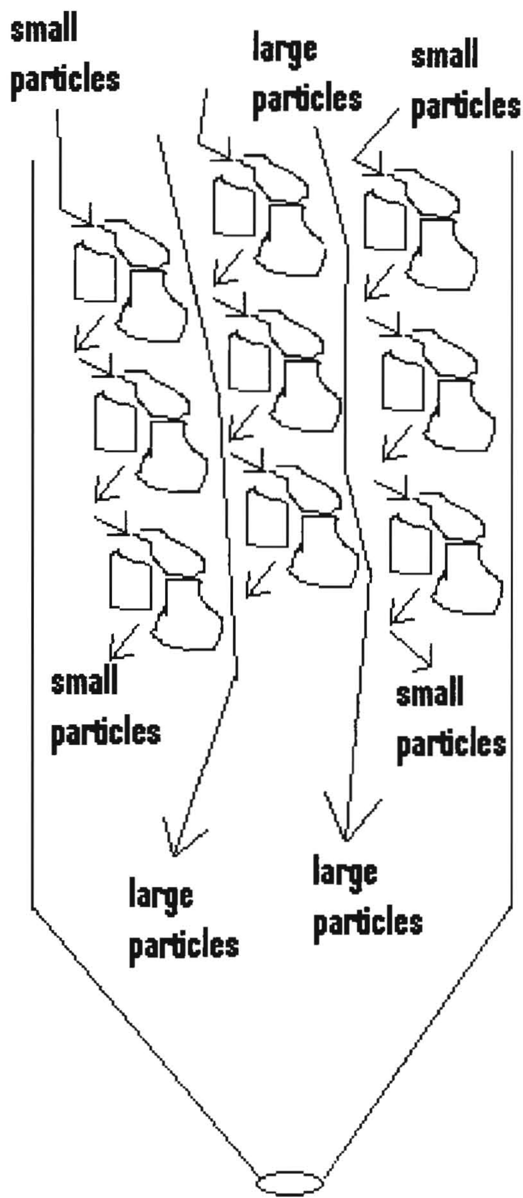


Charged sample loaded into positively charged cellulose beads.

Negatively charged particles bind to positively charged beads.

Increase in NaCl concentration disrupts ionic bonds, and negatively charged particles are eluted off.

FIGURE 3. S-300 Sephacryl Size Exclusion column: Capsular polysaccharides in the fraction samples pass through the column due to the large size of the carbohydrates. Smaller molecules are trapped in the porous beads and elute off of the column more slowly.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II. MATERIALS

Goat anti-mouse Ig was purchased from Becton, Dickinson and Company; (Sparks, MD). Nutrient agar, columbia broth, sodium chloride (NaCl), sodium phosphate monobasic (NaH_2PO_4-H_2O), sodium phosphate dibasic anhydrous (Na_2HPO_4), glycerol (glycerin), RNase (ribonuclease A; ribonuclease 1; from bovine pancreas; type I-A), sodium acetate ($C_2H_3O_2Na_3H_2O$, trihydrate), DEAE sephacel solution (Diethylaminoethyl Sephacel, anion exchanger), red tetrazolium (2,3,5-triphenyltetrazolium chloride; $C_{19}H_{15}N_4Cl$), glucose (D-(+)-Glucose; $C_6H_{12}O_6$, anhydrous), OPI (OPI media supplement; formulated with 0.15g oxaloacetate, 0.05g pyruvate, and 0.0082g bovine insulin), bovine insulin, Tween-20 (polyethylene-sorbitan monolaurate), phorbol myristate acetate (phorbol 12-myristate 13-acetate; $C_3H_{56}O_8$), DNase (Deoxyribonuclease 1; from bovine pancreas), 3,3`,5,5`- tetramethylbenzidine (TMB) liquid substrate, bovine serum albumin (BSA), anti-mouse polyvalent immunoglobulins (peroxidase conjugated antibody produced in goat), sodium hypoxanthine, aminopterin and thymidine (HAT media) were purchased from Sigma Chemical Co. (St. Louis, MO). Sodium hydroxide was purchased from MCB Manufacturing Chemists, Inc, associate of E. Merk (Cincinatti, OH). Hydrochloric acid (2M HCl), and sulfuric acid (H_2SO_4) were purchased from VWR Scientific (West Chester, PA). Serotype 5 *Staphylococcus aureus* (ATCC# 49521) subsp. *aureus* Rosenbach (Designations: Lowenstein), U937, MRC-5, P3X myeloma cells, and serotype 8 *Staphylococcus aureus* were purchased from American Type Culture Collection (Manassas, VA). Ethanol (Alcohol; ACS/USP grade) was purchased from Pharmco Products Inc. (Brookfield, CT). $CaCl_2$ (Calcium Chloride, analytical reagent) was purchased from Mallinckrod, Inc. (Paris, Kentucky). Ammonium molybdate

(heptamolybdate, tetrahydrate; 4-hydrate, crystal; $(NH_4)6Mo_7O_{24}.4H_2O$), ascorbic acid (L-(+)-Ascorbic acid; $C_6H_8O_6$), sodium carbonate (Na_2CO_3; anhydrous) was purchased from JT Baker (Phillipsburg, NJ). Sodium pyruvate (MEM sodium pyruvate solution), trypsin-EDTA (0.5% trypsin; 5.3mM EDTA and RPMI Medium 1640 powder with L-glutamine, without sodium bicarbonate), MEM (minimum essential medium eagle, with Earle's salts, with L-glutamine), and L-glutamine (200mM) were purchased from Gibco (Grand Island, NY). Tris (hydroxymethyl) aminomethane (TBS) was purchased from Fisher Scientific (Suweene, GA). Formalin was purchased from Fisher Health Care, Biochemical Sciences Inc, (Swedesbord, NJ). Hydrogen peroxide (H_2O_2) was purchased from Mallinckrodt, Inc. (Paris, Kentucky).

III. METHODS

Preparation of Columbia Salt Broth (2% NaCl)

Columbia broth (35g) and NaCl (20g) were dissolved in 350 ml of deionized water (Fattom *et al.*, 1990). The solution was heated slightly until components were completely dissolved. The mixture was transferred to a graduated cylinder, and deionized water was added until the total volume of the solution was 1000 ml. The mixture was prepared for autoclaving by separating the solution into 300 ml portions inside 1.0L Erlenmeyer flasks. The flasks were plugged with cotton, and autoclaved at 121°C for 15 min. The broth was cooled before use (Maniatis *et al.*, 1982).

Stock Preparation of *Staphylococcus aureus* (50 ml of bacterial paste)

Columbia salt broth was inoculated using a frozen stock of *S.* aureus. *S. aureus* was grown overnight at 37°C with shaking to produce a stock culture (good for two weeks at 4°C). A bacterial suspension (100 µL) of *S. aureus* type 5 was transferred from the stock to a Columbia salt plate and spread evenly creating a bacterial lawn. The process was repeated for 20 plates, and the plates were incubated at 37°C overnight. PBS (5 ml) was added to the first plate and a plastic pipette was used to gently scratch the surface of the entire plate, releasing the bacteria from the plate. The bacterial suspension was transferred to a 50 ml centrifuge tube. PBS (5 ml) was used to wash the first plate, rinsing any remaining bacteria from the plate, and transferred to the next plate. The process was repeated until all plates were rinsed and transferred to a 50 ml centrifuge tube. Tubes with the bacterial suspension were centrifuged at 13,000 X *g* for 30 minutes and the supernatant was discarded. The bacterial paste was transferred to a 50 ml conical tube and stored at -20°C freezer. The process of bacterial plating and scraping was repeated until 50 ml of bacterial paste was obtained for carbohydrate purification (Maniatis *et al.*, 1982).

Purifying Carbohydrate from Type 5 Capsule

Cells were suspended at a wet weight of 0.5 g/ml in buffer containing 0.05 M tris-2 mM $MgSO_4$. Buffer was added to the conical tubes of bacterial paste (*S. aureus* type 5) and vortexed to create a suspension. The bacterial suspension was poured into a 500 ml flask with tris buffer (total volume of 100 ml of buffer + 50 ml of bacterial paste) and autoclaved at 121°C for 1hr. Lysostaphin (25 µg/ml) was added and incubated at 37°C

overnight with constant shaking. To confirm cell death, a control sample of the solution was streaked on a plate for 2 days at 37°C. DNAse and RNAse were added, each to a final concentration of 40 μg/ml and the incubation continued for 3hrs. Protease (0.5 mg/ml) was added to the solution, which was then placed on a shaker and incubated overnight at 37°C. The mixture was pelleted by centrifuging at 13,500 X *g* for 30 min at 4°C. The supernatant was transferred to dialysis tubing, and mixture was repeatedly dialyzed against Milli-Q water. The reaction mixture was dialyzed with stirring at 4°C for 2 days and then filtered through a 0.45-μm-pore-size membrane. The sample was precipitated with 25% cold ethanol in the presence of 5mM $CaCl_2$ by placing on ice for 20 min. The mixture was then centrifuged at 25,000 X *g* at 4°C for 30 min. The supernatant was collected and was precipitated with 75% cold ethanol (in presence of 5mM $CaCl_2$) and placed on ice for 20 min. The mixture was centrifuged for 30 min at 25,000 X *g* at 4°C. The supernatant was discarded, and the precipitate was dissolved in mill-Q water. The 75% ethanol precipitate was dialyzed several times against water at 4°C and freeze-dried. The powder (CP) was dissolved in 8 ml of 0.05M sodium acetate with 0.05M NaCl (pH= 6.0) and stored at 4°C (Fattom *et al.*, 1990).

A DEAE Sephacel solution was prepared by removal of fines and degassing. Degassing was accomplished by pouring solution into Erlenmeyer flask that was connected to a vacuum pump. The 2.5-50cm tube (250 ml) was assembled for chromatography. The column was packed by adding DEAE to the top. After the DEAE settled, the top surface was gently swirled and more DEAE was added. The "packing" process was repeated until the line of the concentrated DEAE reached the top. Once the column was packed a cap was placed on the top of the column (Fattom *et al.*, 1990).

The solution containing capsular polysaccharide (CP) was applied to the DEAE-Sephacel column (3 X 19 cm) equilibrated in the same buffer (0.05 M sodium acetate + 0.05 M NaCl with a pH of 6.0). The column was washed with approximately 700 ml (or 5 column volumes) of starting buffer. CP was eluted with 0.05 M sodium acetate and 0.15 M NaCl (6.0 pH) and 7 ml fractions were collected. Absorbance of the fractions was determined at 206 nm wavelength using a blank of deionized water (Fattom *et al.*, 1990).

Carbohydrate Test Using Red Tetrazolium

Red tetrazolium (0.5% aqueous solution) was made by adding 0.5g of red tetrazolium to 100 ml of Milli Q water and was stored in refrigerator (in amber or foil–covered bottle). The carbohydrate solution (200 µl) to be tested was placed in a test tube along with 1 ml of 0.5% red tetrazolium (2,3,5-triphenyltetrazolium chloride) and 200µl of 10% sodium hydroxide solution. The test tube was placed in a beaker of boiling hot water for 10 min. An aliquot (100µl) was transferred to a 96 well flat bottom plate and the absorbance was read at 490 nm. The negative control in the experiment was water and the positive controls were 0.1 mg/ml glucose and crude extract before chromatography (Fieser & Williamson, 1987).

Phosphate Test

Reagent C was made by mixing 1 volume of 6N sulfuric acid with 2 volumes of distilled water, 1 volume of 2.5% ammonium molybdate, and 1 volume of 10% ascorbic acid. Water was used as a negative control and the positive controls were the original CP

sample (containing teichoic acid), and PBS. The sample (200 µl) was dissolved in 0.5 ml 2M HCL and placed in a boiling water bath for 30 min. This solution was adjusted to 4 ml using distilled water (the sample in acid was added to the water). Reagent C (4 ml) was pipetted into each tube. Parafilm was used to cap tubes. The tubes were mixed, placed in a rack and incubated at a 37°C for 1.5-2 hrs. The tubes were removed and observed for a change in color. This was measured using a spectrophotometer at an absorbance of 820 nm (Chen *et al.*, 1956).

Carbohydrate ELISA

Plates (96 well, flat bottomed, tissue culture treated) were coated with 100 µl/well of the antigen solution containing 1 µg/ml of purified CP in PBS. The plates were incubated for 2 hrs at room temperature. The solution was removed and the plates were blotted with paper towels. Unreacted protein-binding sites in the wells were blocked with 200µl of 1% bovine serum albumin (BSA) in PBS and incubated for 30 min at 22°C. The plates were washed with PBS-tween (PBS containing 0.05% Tween-20) three times. The first well was a blank containing the 100µl of the sample buffer (PBS with 2% BSA and 0.05% Tween-20). Monoclonal antibody supernatants were added and left for 2 hrs at 37°C to allow for antibody binding. The wells were washed with PBS-tween three times, and 100µl of anti-mouse Ig-PO (horseradish peroxidase-conjugated rabbit anti-mouse IgG) diluted 1:1,000 in sample buffer was added to all wells. The wells were left for 30 min at 37°C and then washed three times with PBS-tween. Tetramethylbenzidine (100µl) substrate was added to the wells and incubated for 30 min at 22°C. H_2SO_4 (50µl

of 2N) was added to all wells, and the absorbance was read at 450 nm (Thakker *et al.*, 1998).

Whole Bacteria ELISA (Trypsin method)

S. aureus, type 8, was grown in Columbia broth with the addition of 2% NaCl. The bacteria were incubated overnight at 37°C with shaking. Cells were centrifuged at 13,000 X *g* at 4°C for 30 min. Cells were washed 3 times with PBS and treated with 3% formalin rotating end over end overnight at 4°C. Cells were washed 3 times in PBS and treated with trypsin (1 mg/ml) at 37°C overnight (Sinha *et al.*, 1999) with shaking. Cells were washed 3 times with PBS and the optical density of the bacterial suspension was brought up to 1.0 at 550 nm. The cell suspension (100 µl per well) was added to three rows (36 wells) of a 96 well flat bottomed tissue culture treated polyvinyl microtiter plate and incubated at 37°C overnight. Plates were centrifuged at 2,000 X *g* for 15 min. Wells were washed 3 times with PBS and incubated with 1% BSA in PBS (200µl) at room temperature for 1hr. Wells were aspirated, then coated with a 1:1000 dilution of mouse sera, or 1:100 dilution of hybridoma supernatant, and incubated at 37°C for 2.5 hrs. Plates were washed with PBS containing 0.5% Tween-20 three times. Wells were then incubated with peroxidase-conjugated anti-mouse immunoglobulin (1:1,000 in PBS containing 0.05% Tween-20 and 1% BSA) for 2 hrs at 37°C, and then washed with PBS. Tetramethylbenzidine (100µl) substrate was added to each well. The reaction was stopped after 10 min (or when color developed) with addition of 50 µl of 2N sulfuric acid. Absorbance was read on a spectrophotometer at 450 nm (Nelles *et al.*, 1985).

Cell Fusion (Hybridomas)

A mouse was immunized three times with formalinized *S. aureus* type 8. The spleen from the inoculated BalbC mouse was placed in a petri dish with sterile RPMI media. The spleen was transferred to a 24 well plate and the cells were released by using a syringe plunger. The cells were transferred to a 15 ml conical tube (2 ml of cells) and allowed to sediment for 5 min. The cells remaining in the solution were centrifuged at 400 X *g* for 8 min and the supernatant was aspirated. Tris NH_4Cl (1 ml) was added to the pellet to lyse the red blood cells. After 2 min, the cells were underlaid with fetal calf serum (FCS) and centrifuged at 400 X *g* for 8 min. The FCS and cell fragments were aspirated and the viable cells were washed 3 times with PBS. Spleen cells were added to P3X myeloma cells in a 5:1 ratio and were washed 3 times in RPMI media. Cells were pelleted and a 50% polyethylene glycol (PEG) solution (containing 0.5 g PEG + 0.5 ml RPMI media + 50 μl DMSO) was slowly added over a one minute period. Warm RPMI (8 ml) was added slowly over a five minute period. The mixture was centrifuged at 200 X *g* for 8 minutes and resuspended in 24 ml of P3X media (1 ml of 200 mM L-glutamine + 1 ml of 100 mM oxaloacetate pyruvate insulin + 20 ml FCS + 80 ml RPMI). The mixture was transferred (0.5 ml per well) to a 48 well tissue culture treated plate and placed in a 37°C CO_2 incubator overnight. Cells were incubated with 24 ml HAT (for 50 X HAT, 100 μM hypoxanthine + 0.4 μM aminopterin + 16 μM thymidine) + 0.25 ml OPI (containing 1 mM oxaloacetate + 0.45 mM pyruvate + 0.2 U/ml insulin) media (0.5 ml 2X HAT per well). Two weeks after the first cell feeding, cells were fed with a solution containing 50 ml of 1 X HAT + 0.1 ml OPI (1 ml of solution per well). Cells were observed every two days (if media was acidic, yellow, cells were fed with 1 ml of 1 X

HAT) for one week. When cells reached 2/3 confluency, supernatant was saved for ELISA testing and the cells were expanded until 10^6 cells were available for freezing (Harlow & Lane).

IV. RESULTS

Purification of the capsular polysaccharide (CP) was accomplished through a multistep method. The purification method involves the use of enzymes, such as protease and lysostaphin, to separate the capsule from the intact bacteria. Once the CP was separated from the bacterium, the sample was fractionated by DEAE column chromatography. DEAE is made up of positively charged beads that bind to negatively charged carbohydrates from the capsule. The negatively charged molecules are then eluted by increasing the salt concentration in the elution buffer. After DEAE purification, the collected product was analyzed by determining the fractions at 206 nm. This step detected the presence of organic substances in the sample. In figure 4, peaks show the presence of organic substances in the sample, suggesting that carbohydrate from the capsule was collected.

Previous studies (Fieser &Williamson, 1987) used a substance called tetrazolium red to determine the presence of carbohydrate in the sample. We found the previously used method produced precipitate in the presence of the salt solution. The tetrazolium red test for reducing sugars (Fieser & Williamson, 1987) was tested as an alternative method. In Figure 5, serial two-fold dilutions of glucose and capsular extract were tested with tetrazolium red to determine the sensitivity of the test. Glucose could be detected until a dilution to a concentration of 0.025 mg/ml. No precipitate was seen in samples containing capsule extract and tetrazolium red was determined to be an acceptable test for reducing sugars in the column fractions.

The red tetrazolium test was then used to determine the presence of reducing carbohydrates in the column fractions. Figure 4 shows the overlaid OD and red

FIGURE 4. Optical Density and Red Tetrazolium Test: Type 5 *S. aureus* capsular polysaccharide was cultured using NaCl Columbia salt agar plates. Bacterial paste (50 ml) was used for purification. Digestions, including lysostaphin, DNAse, RNAse, and protease were used to release the capsule. The lysed *S.* aureus mixture was dialyzed against tris buffer containing 0.05M Tris (hydroxymethyl) Aminomethane + 0.002M $MgSO_4$. Mixture was removed from dialysis, frozen, then placed in the lyophilizer for freeze drying. Lyophilized powder was dissolved in loading buffer containing 0.05M sodium acetate + 0.1M NaCl. A DEAE column was washed with loading buffer, and then the sample was applied to the column for charge purification. Elution buffer containing 0.05M sodium acetate + 0.15M NaCl was used to elute carbohydrates from the capsule off of the column. A fraction collector was used to collect the samples. The collected fractions, approximately 7 ml each (total of 63 fractions), were tested for organic molecules measured by absorbance at 206 nm (—▲—) and for the presence of reducing sugars using the red tetrazolium test measured at absorbance of 490 nm (—●—). The red tetrazolium test will determine if there are carbohydrates present in the sample.

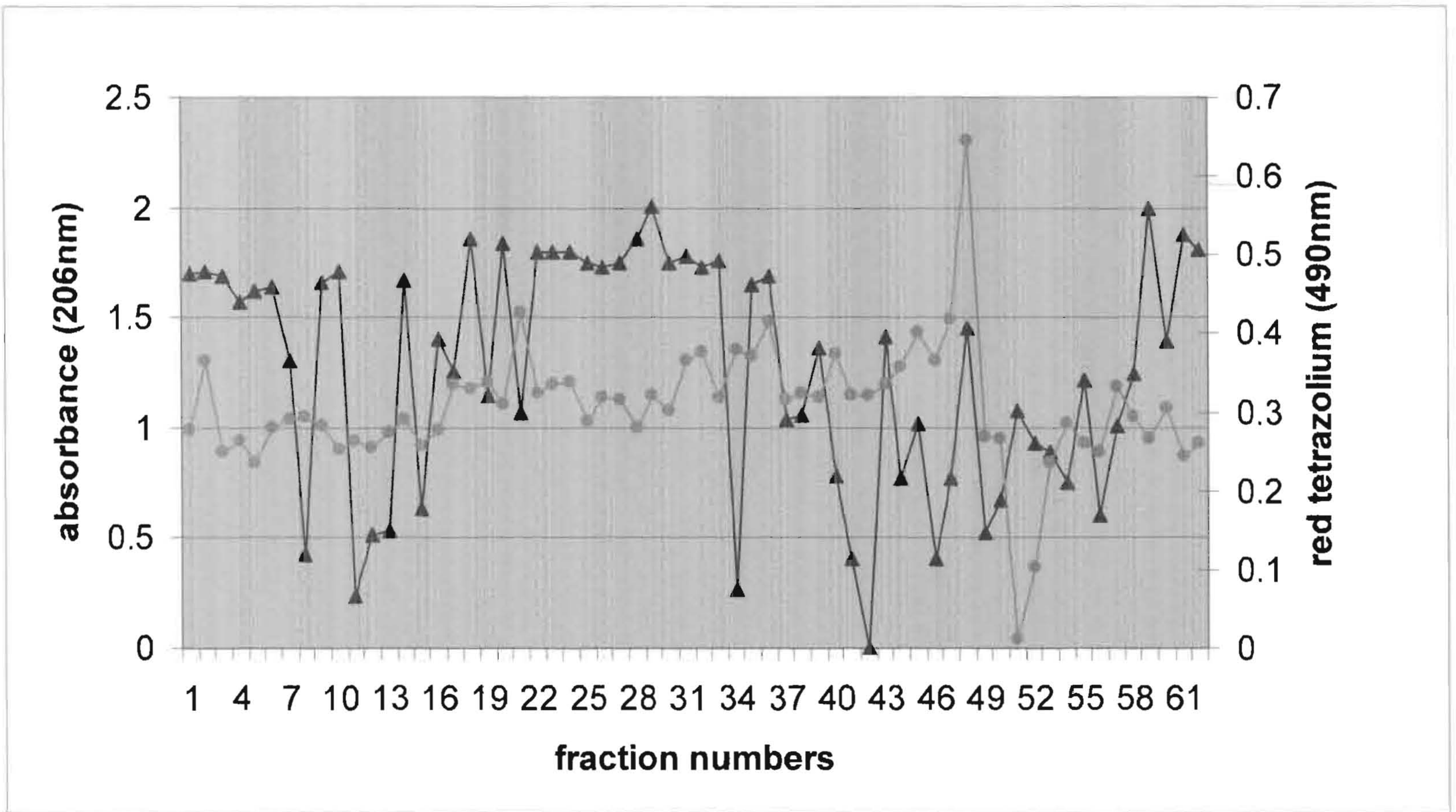
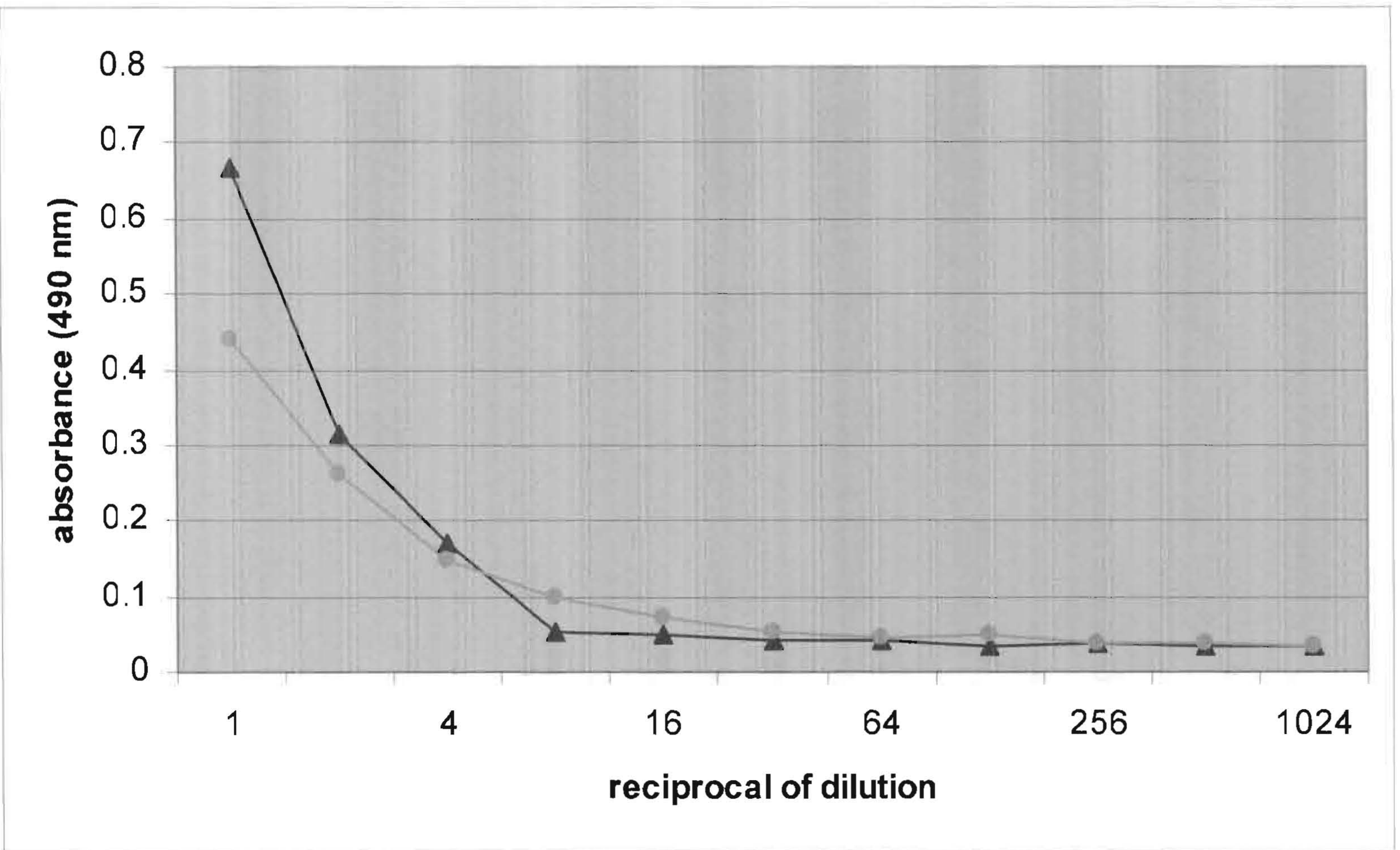

absorbance (206nm)
2.5
2
1.5
1
0.5
0
red tetrazolium (490nm)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
1 4 7 10 13 16 19 22 25 28 31 34 37 40 43 46 49 52 55 58 61
fraction numbers

Figure 5. Validation of Method for Testing Carbohydrates in Sample: Serial dilutions of a glucose positive control (0.025 mg/ml) and a type 5 capsular polysaccharide crude extract were tested by a red tetrazolium test at an absorbance of 490 nm. The glucose positive control (—▲—) and the CHO sample before DEAE purification (—◆—) were subjected to serial two-fold dilution prior to testing. The type 5 CP was from a sample lysate that was dialyzed but not charge purified by means of a DEAE column chromatography. The test was performed to show validity of the red tetrazolium test by comparing the two curves of serial dilution samples for similarity. Samples (one tube containing 200 μl of the glucose control and the other tube containing 200 μl of the CHO sample before DEAE chromatography) with red tetrazolium and NaOH were boiled. Solutions (200 μl) from each test tube were placed in a 96 well flat bottom plate. Serial dilutions from each tube were brought across each row containing the following concentrations: 1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, 1:512, and 1:1024. The samples were tested simultaneously after the dilutions were distributed to each well. The samples were read at an absorbance of 490 nm.


0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
absorbance (490 nm)
1
4
16
64
256
1024
reciprocal of dilution

tetrazolium data that was used to group the collected fractions into peaks. Fractions that contained high OD and low red tetrazolium (such as fractions 49-51) were determined to have the presence of some other substance than carbohydrate and were discarded. Fractions 1-48 and 52-63 were determined to have relatively high absorbances following the red tetrazolium test suggesting that there are carbohydrates present in these fractions. Fractions were then pooled in peaks, and the peaks were tested for phosphate (Chen *et al.*, 1956) to determine if they were contaminated with teichoic acid, a phosphate containing carbohydrate found in the cell wall. Fractions 1-3, 4-16, 17-25, 26-43, 44-48, and 52-63 were categorized as peaks 1-6 respectively.

Following acid hydrolysis to release phosphate from teichoic acid, the phosphate assay developed by Chen and coworkers (Chen *et al.*, 1956) was used to measure inorganic phosphate. We attempted to modify the assay to a microplate method to allow measurement of large numbers of samples quickly. This test is usually read at 820 nm, which is not available on our microplate reader. In order to compare results at 820 nm with the wavelength available on our microplate reader (630 nm), we measured phosphate levels of serial 2-fold dilutions of PBS (Figure 6) and unpurified capsular extract (Figured 7). A comparison of the values obtained in Figures 6 and 7 at the two wavelengths showed similarities when the phosphate was measured in PBS. However, the assay was much less sensitive when analyzing phosphate in the capsular extract at 630 nm when compared to the values seen at 820 nm. Our results suggested that we could not convert the method to a microtiter test at this time.

The results of the phosphate test of column fractions are shown in Figure 8.

Figure 6. Comparison of Phosphate Test at 820 nm and 630 nm: Serial dilutions (expressed as reciprocals on the x-axis) of a positive control for phosphate (phosphate buffered saline) was read at 820 nm (—▲—) and 630 nm (—◆—). The first sample used concentrated PBS (phosphate concentration in the sample was 0.1 M) and the next sample began the dilution at 1:2. A total of 10 dilutions were used, and the same dilution samples were read at both wavelengths for comparison. HCl (2M) was placed in each glass tube along with PBS in one tube and crude CHO extract in the second tube. The tubes were vortexed and placed in boiling water. After the tubes were cooled, each tube volume was brought up to 4.0 ml with addition of Milli-Q water. Reagent C containing 6N sulfuric acid, 2.5% ammonium molybdate, and 10% ascorbic acid was added to each tube (4.0 ml per tube). Absorbance was read at 820 nm and 630 nm for each tube in the serial 2-fold dilutions.

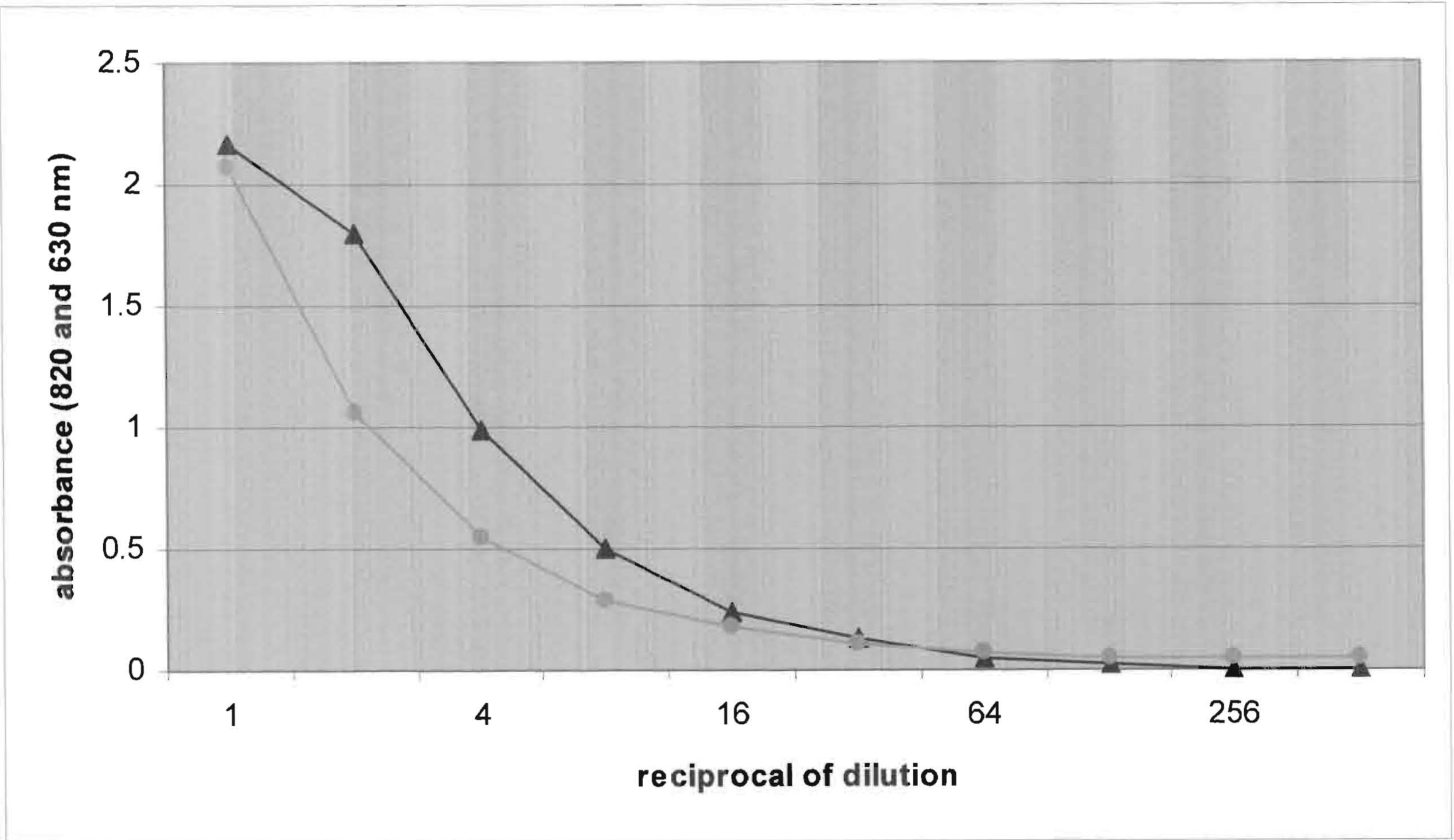

absorbance (820 and 630 nm)
2.5
2
1.5
1
0.5
0
1
4
16
64
256
reciprocal of dilution

Figure 7. Serial 2-Fold Dilution of Polysaccharide Before Purification by a DEAE Sephacel Column Tested for Phosphate at 820 nm and 630 nm: A serial dilution of type 5 *S. aureus* sample lysate that was dialyzed but not purified by means of a DEAE column chromatography. The serial dilution sample (reciprocal dilution on the x-axis) was tested for phosphate (Figure 6) and read at an absorbance of 820 nm (—▲—) and 630 nm (—◆—). The first sample for both wavelengths was concentrated, and the second sample began the dilution (at 1:2). A total of eleven dilution samples (the first being concentrated) were read at two different wavelengths.

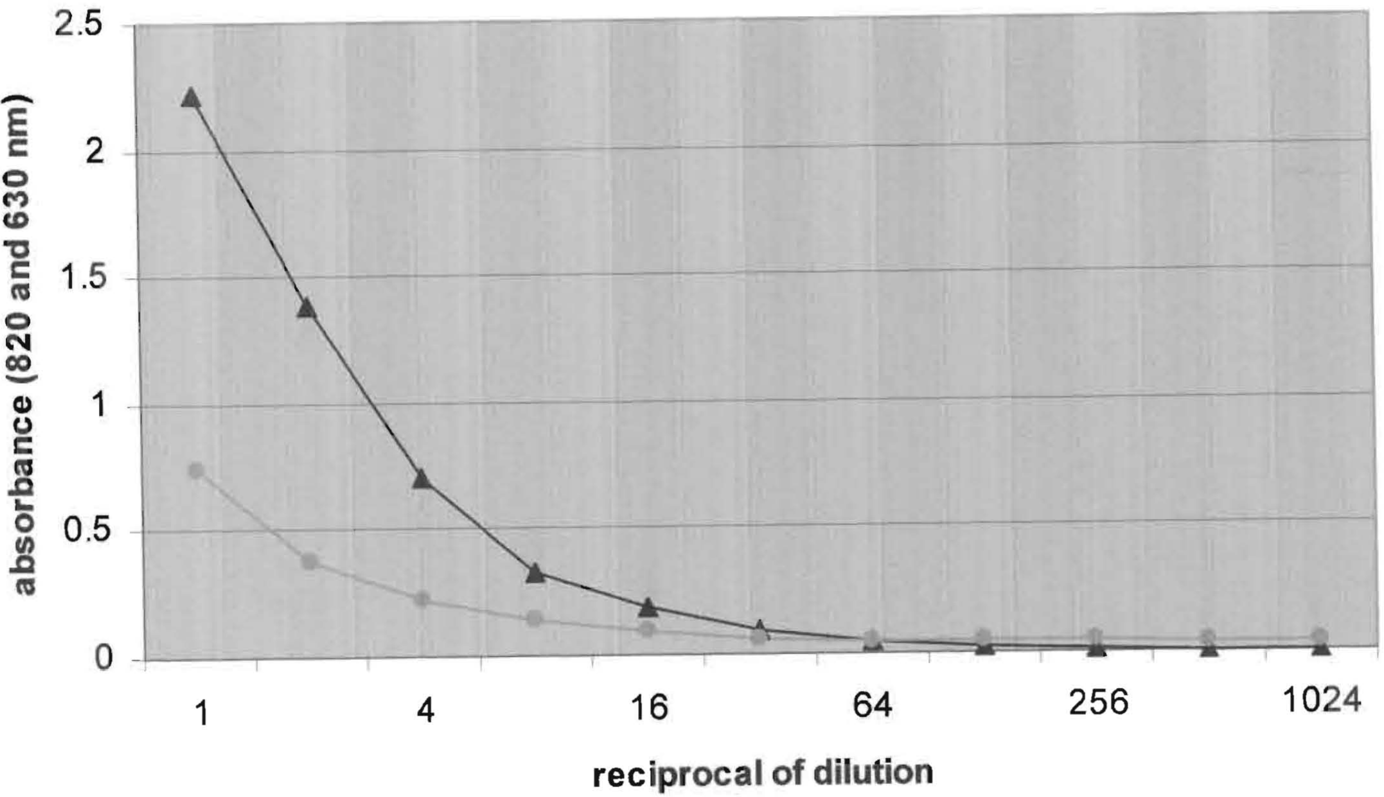
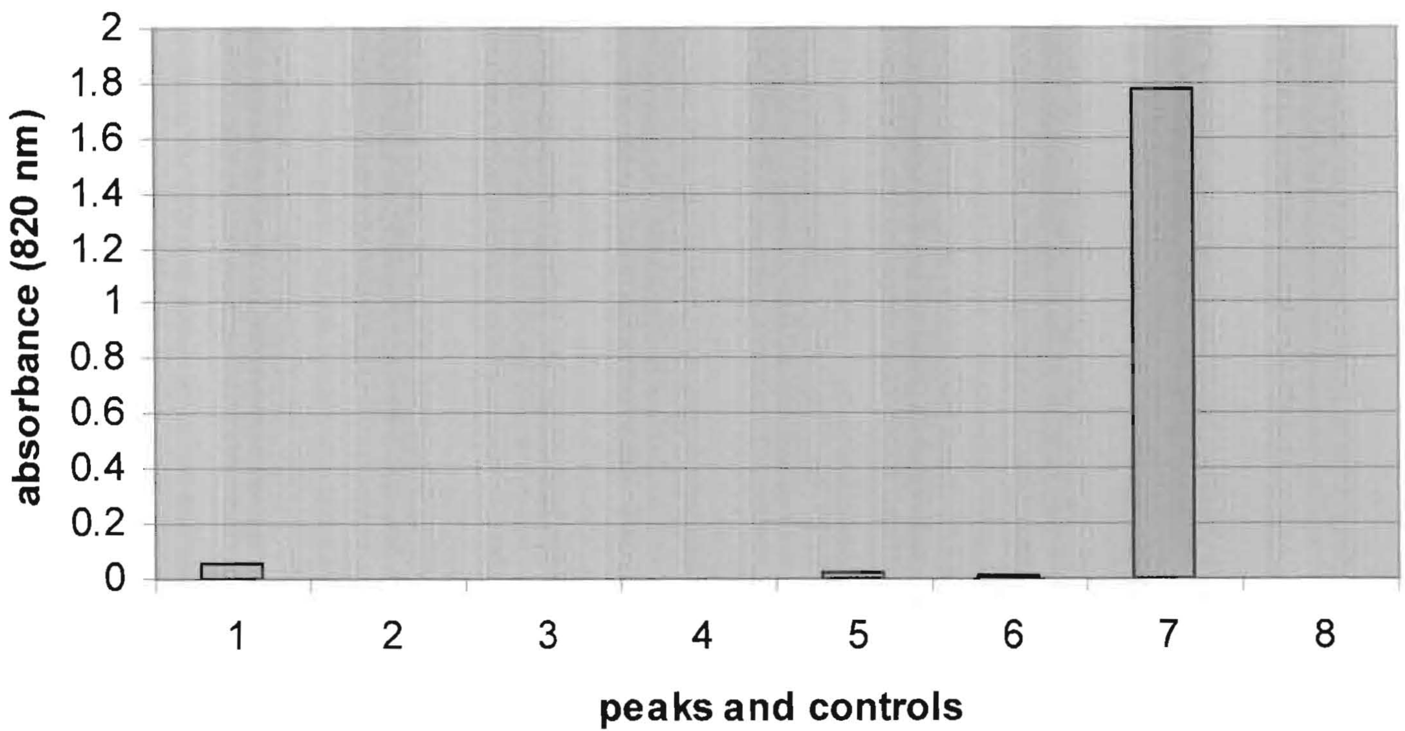

absorbance (820 and 630 nm)
2.5
2
1.5
1
0.5
0
1
4
16
64
256
1024
reciprocal of dilution

FIGURE 8. Phosphate (Teichoic Acid) Test on Pooled Fractions: Fractions from Figure 5 were pooled into peaks (1-6) following the red tetrazolium test. Each peak, or sample, was grouped based on their similar absorbance value in the red tetrazolium test. Fractions 49-51 were discarded due to their low presence of carbohydrates. The peaks were then tested for phosphate (Figure 6).

sample 1	fractions 1-3
sample 2	fractions 4-16
sample 3	fractions 17-25
sample 4	fractions 26-43
sample 5	fractions 44-48
sample 6	fractions 52-63
sample 7	pos contr= PBS
sample 8	neg contr= water


Teichoic Acid Test
absorbance (820 nm)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1
2
3
4
5
6
7
8
peaks and controls

Peaks 1-6 were tested for the presence of phosphate and all samples were found to either contain no phosphate or insignificant levels of phosphate when compared to the controls. Peak 7, the positive control, contained only PBS. Peak 8, the negative control, contained only water.

The ELISA currently used to test monoclonal antibody specificity was modified to test for the presence of CP in column peaks. An ELISA (Figure11) was used to demonstrate the ability of a type 5 specific monoclonal antibody to recognize and bind to the purified CP sample after DEAE chromatography purification. Binding of monoclonal antibodies to the sample was demonstrated in all column peaks except peak 6. Column peaks 1-5 and column peak 7 contained the CP sample following DEAE purification. The values shown for column peaks 1-7 are an average from duplicate experiments. The positive control, type 5 *S. aureus* whole bacteria, had an absorbance value greater than 1.00 and is not depicted on the chart.

The sample peaks that were positive for carbohydrates and negative for phosphate were combined and dialyzed to remove any unwanted salts. The samples were resuspended in a 0.05M sodium acetate (pH 0f 6.0) buffer and applied to a size exclusion (S-300-HR) column containing sephacryl beads (Moreau *et al.*, 1990). Larger molecules, such as the capsular polysaccharides, elute off of the column first while the smaller particles are trapped in the porous beads, taking longer to elute. The fractions that were collected were again tested for absorbance at 206 nm and for carbohydrate using the red tetrazolium test. The data from the OD and tetrazolium red test were overlaid and examined in Figure 12. Fractions 75-79 were discarded due to their relatively high OD and low red tetrazolium absorbance. The fractions were then grouped into peaks (1-7).

Peak 1 contained fractions 1-12, peak 2 were fractions 13-25, peak 3 were fractions 26-36, peak 4 were fractions 37-48, peak 5 were fractions 49-56, peak 6 were fractions 57-63, and peak 7 were fractions 64-75. Fractions 76-79 were discarded due to a high OD absorbance and low red tetrazolium absorbance.

The peaks were tested for the presence of phosphate using the teichoic acid test. The two different absorbance values were used to compare the original method to our microtiter method. Absorbance was read at 820 nm (Figure 9) and 630 nm (Figure 10). The negative control (peak 8) was water, and the positive control (peak 9) was PBS. Both tests resulted in similar readings and showed that peaks 1-6 (sample peaks) were similar to the negative control. The absorbencies read at both absorbance values (630 nm and 820 nm) indicate that there is either no phosphate or an insignificant amount of phosphate contamination in the samples.

FIGURE 9. Phosphate (Teichoic Acid) Test on Pooled Fractions (read at 820 nm) After Size Exclusion Purification: The fractions collected from the red tetrazolium and OD test were pooled into peaks (1-7) based on their similarity of absorbance in the red tetrazolium test. The seven peaks were evaluated for their presence of phosphate in the teichoic acid test (Figure 6) at an absorbance of 820 nm and compared to negative (water) and positive (PBS) controls.

sample 1= fractions 1-12
sample 2= fractions 13-25
sample3= fractions 26-36
sample 4= fractions 37-48
sample 5= fractions 49-56
sample 6= fractions 57-63
sample 7= fractions 64-75
sample 8= neg contr= water
sample 9= pos contr= PBS

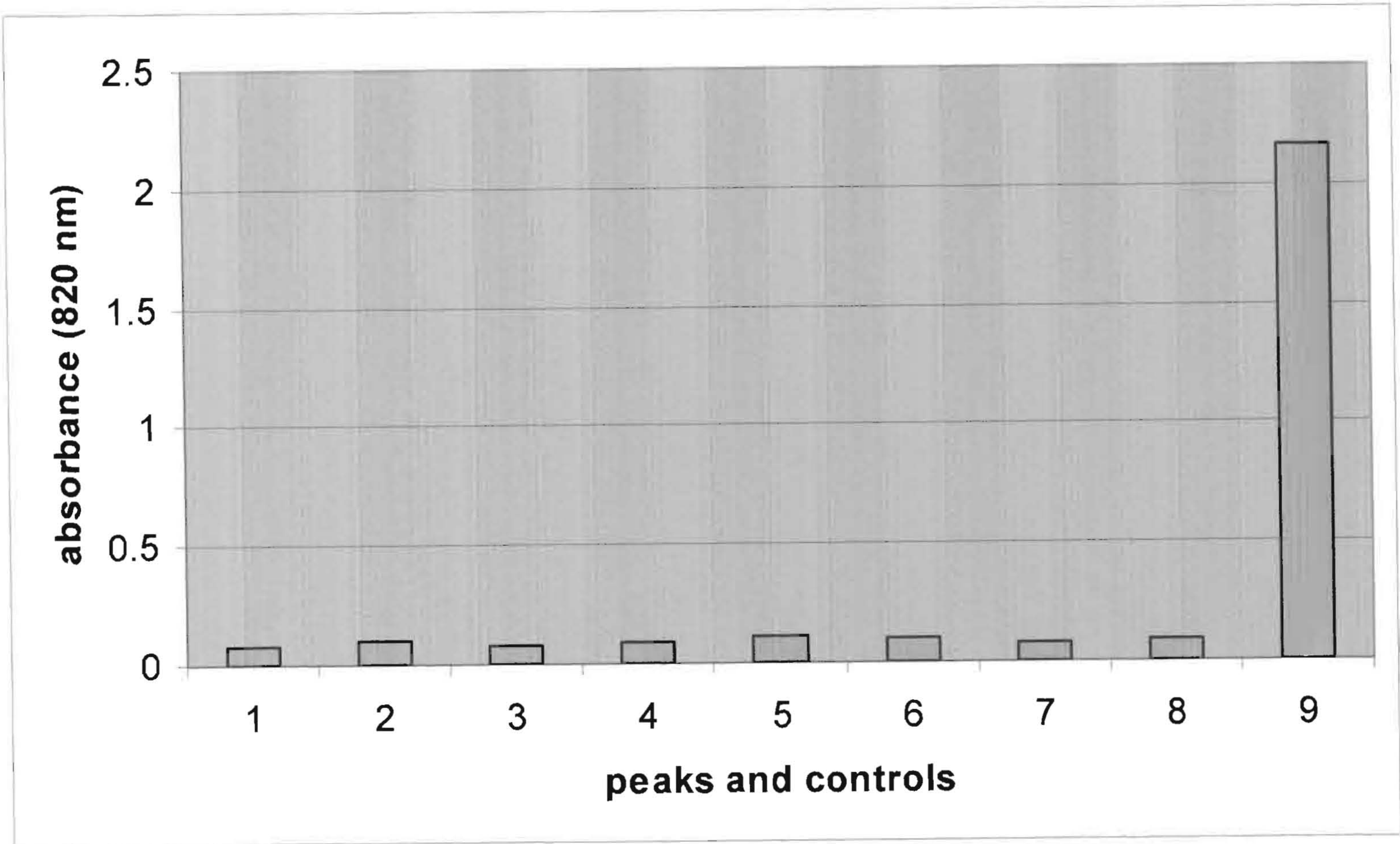

absorbance (820 nm)
2.5
2
1.5
1
0.5
0
1
2
3
4
5
6
7
8
9
peaks and controls

FIGURE 10. Phosphate (Teichoic Acid) Test on Pooled Fractions (read at 630 nm) After Size Exclusion Purification: Teichoic acid test (see Figure 6) was read at 630 nm for comparison of two different wavelengths. Samples 1-7 were evaluated for the presence of teichoic acid and compared to the controls.

sample 1= fractions 1-12
sample 2= fractions 13-25
sample3= fractions 26-36
sample 4= fractions 37-48
sample 5= fractions 49-56
sample 6= fractions 57-63
sample 7= fractions 64-75
sample 8= neg contr= water
sample 9= pos contr= PBS

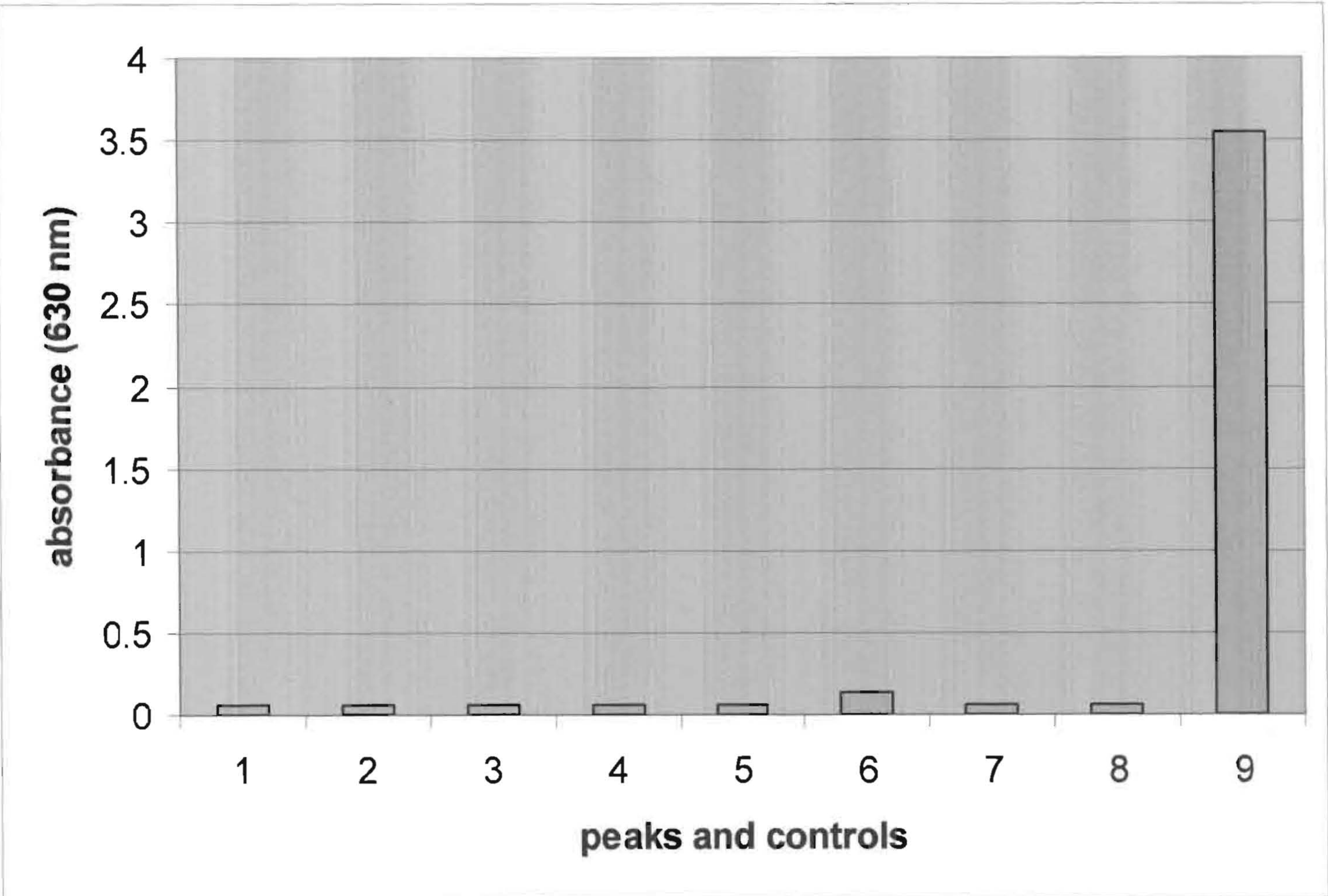
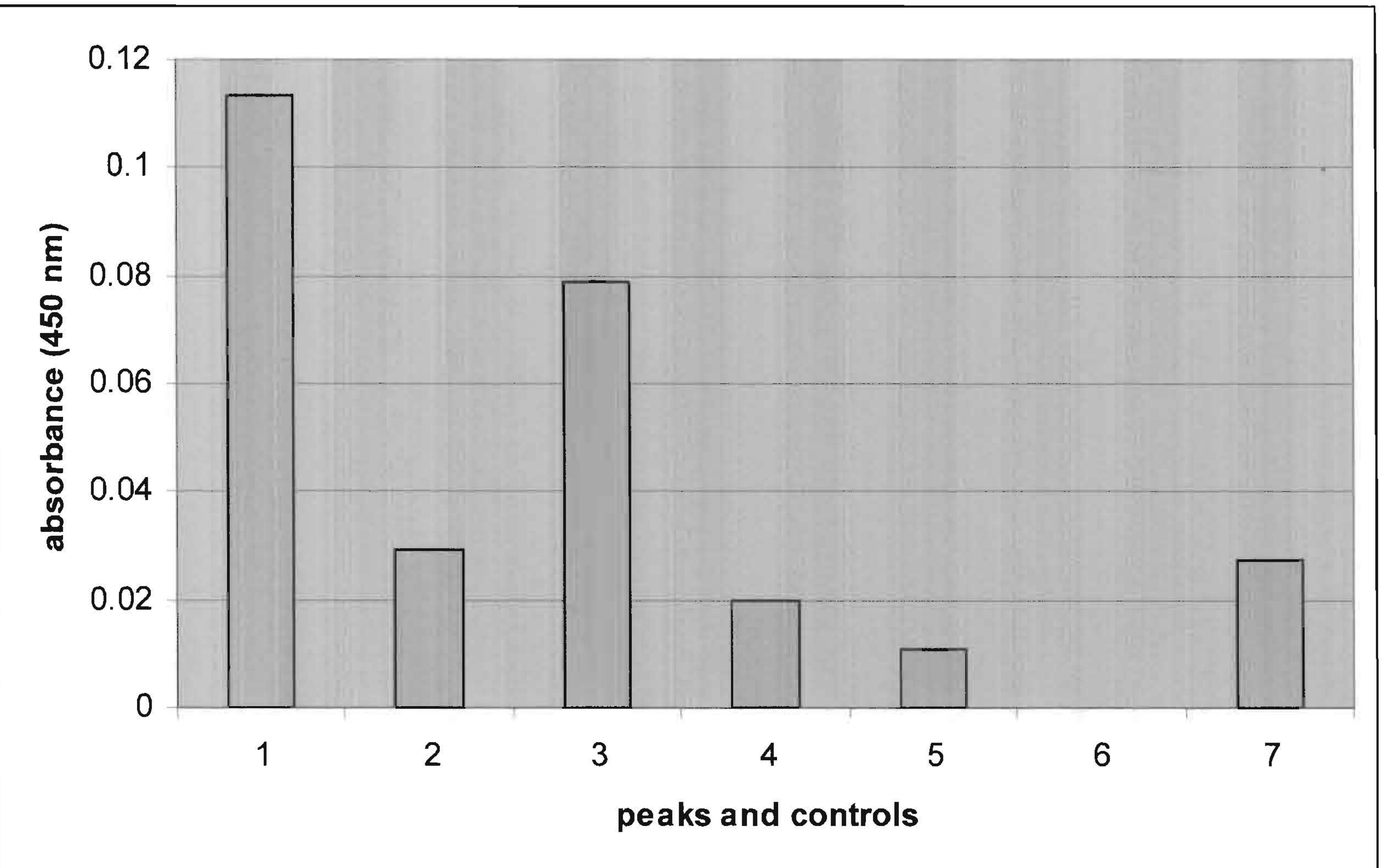

absorbance (630 nm)
4
3.5
3
2.5
2
1.5
1
0.5
0
1
2
3
4
5
6
7
8
9
peaks and controls

Figure 11. ELISA after DEAE Column Chromatography: Monoclonal antibodies were used to test collected peaks after DEAE column chromatography purification. Plates were coated with 100 µl of DEAE purified *S. aureus* type 5 samples, incubated, and blocked with blocking buffer. Monoclonal antibodies were added to the wells (100 µl) except the negative control. Anti-mouse Ig-PO was added (100 µl) to each well. After the addition of 100 µl of TMB color development was stopped with 50 µl of 2N H_2SO_4. Absorbance was read at 450 nm.



0.12
0.1
0.08
0.06
0.04
0.02
0
absorbance (450 nm)
1
2
3
4
5
6
7
peaks and controls

The absorbance values of each sample were closely similar, suggesting that there was a high quantity of carbohydrate in the type 5 sample. The results for this test also showed that there was a fairly steady decreasing curve for the positive control demonstrating the validity of the red tetrazolium test (the more concentrated sample had the highest absorbance and the least concentrated dilution had the lowest absorbance).

In Figure 13, an ELISA showed the binding of monoclonal antibodies that were from mice immunized with type 5 *S. aureus*. The monoclonal antibodies were tested in an ELISA for binding to the purified carbohydrate sample of type 5 *S. aureus* that was from sample peaks collected after size exclusion chromatography. Columns 1-7 show binding of antibodies against type 5 *S. aureus* that were tested for binding to purified CP. All sample peaks show binding to the antibodies suggesting that the sample is a purified CP.

Figure 14 shows an ELISA for hybridomas that were from mice immunized with type 8 *S. aureus* whole bacteria. Hybridomas that were cultured from immunized mice spleen cells and P3X myeloma cells were tested in an ELISA for binding to the whole bacteria of type 8 *S. aureus*. In column 3 a hybridoma pool, referred to as DV.T8.C4, showed the highest absorbance value and will be used in further research for testing for binding against purified type 8 CP.

FIGURE 12. Optical Density and Red Tetrazolium Test After Size Exclusion Purification: Size exclusion chromatography was accomplished using S-300 HR sephacryl beads with a 0.05M sodium acetate buffer. After dialysis, the collected fractions (containing 6.0 ml per fraction) were tested again for organic molecules at an absorbance of 206 nm (—▲—). Reducing sugars were measured by the red tetrazolium test (Figure 5) at an absorbance of 490 nm (—●—).

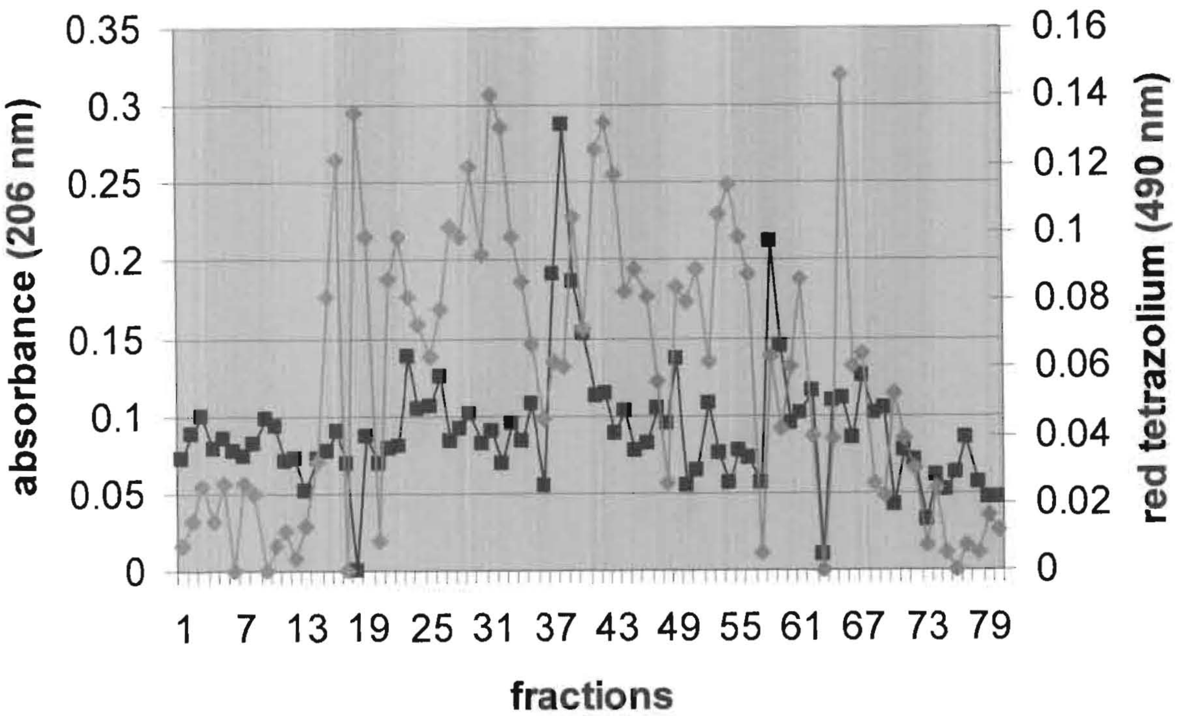

absorbance (206 nm)
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
1 7 13 19 25 31 37 43 49 55 61 67 73 79
fractions
red tetrazolium (490 nm)
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0

Figure 13. ELISA on Fraction Peaks After Size Exclusion Column: Peaks from fractions that were pooled after the size exclusion purification were tested for their ability to bind to monoclonal antibodies against type 5 *S. aureus* (Figure 11). Using an absorbance value of 450 nm, samples were compared to positive (taken from a sample of type 5 *S. aureus* before column chromatography purification) and negative (a sample well containing no carbohydrates) controls. The negative control (column 9) absorbance value (0.133) was subtracted from all of the samples, including the negative control. The positive control (column 8, indicated with "*") was a value that was greater than 1.00 absorbance.

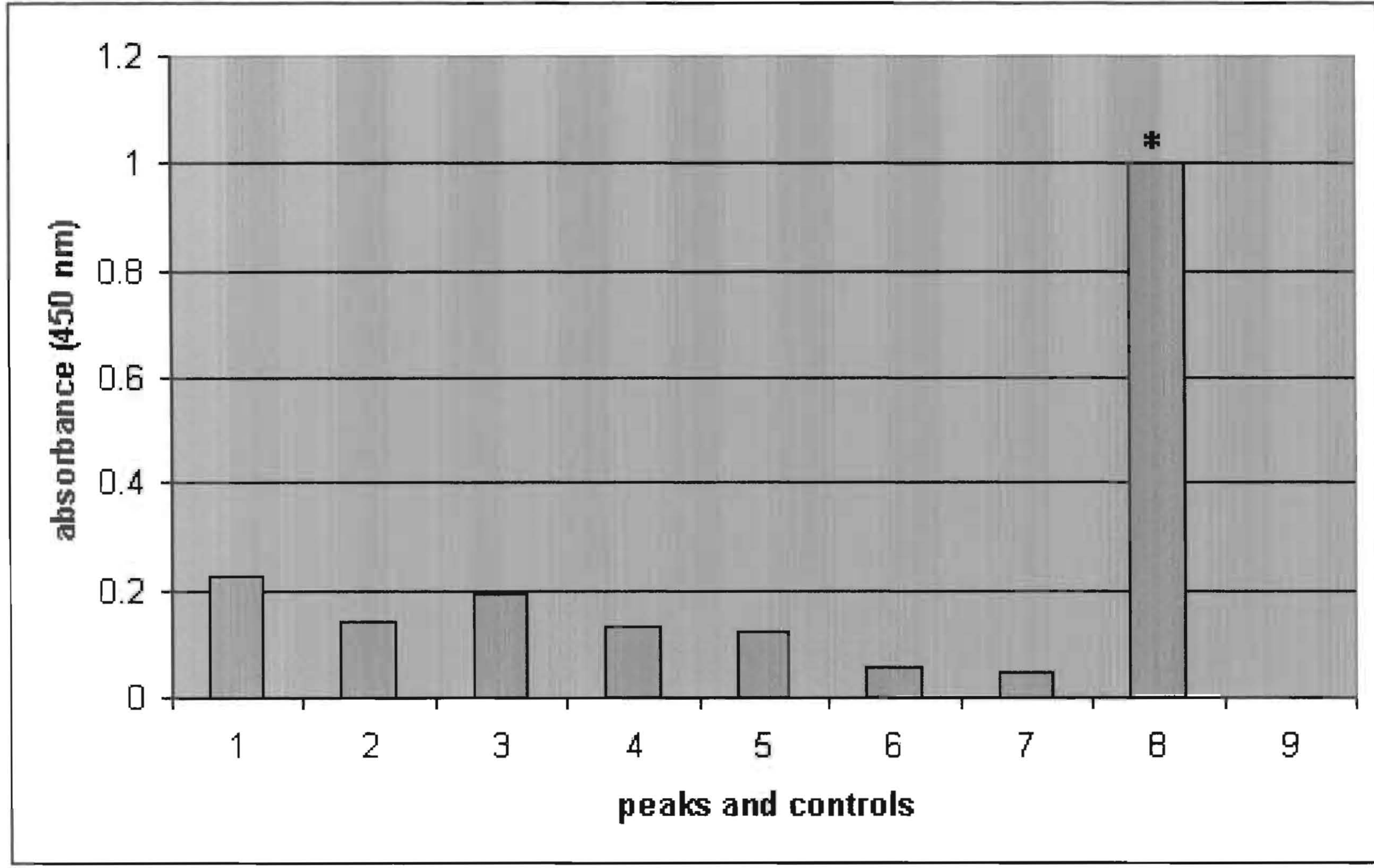
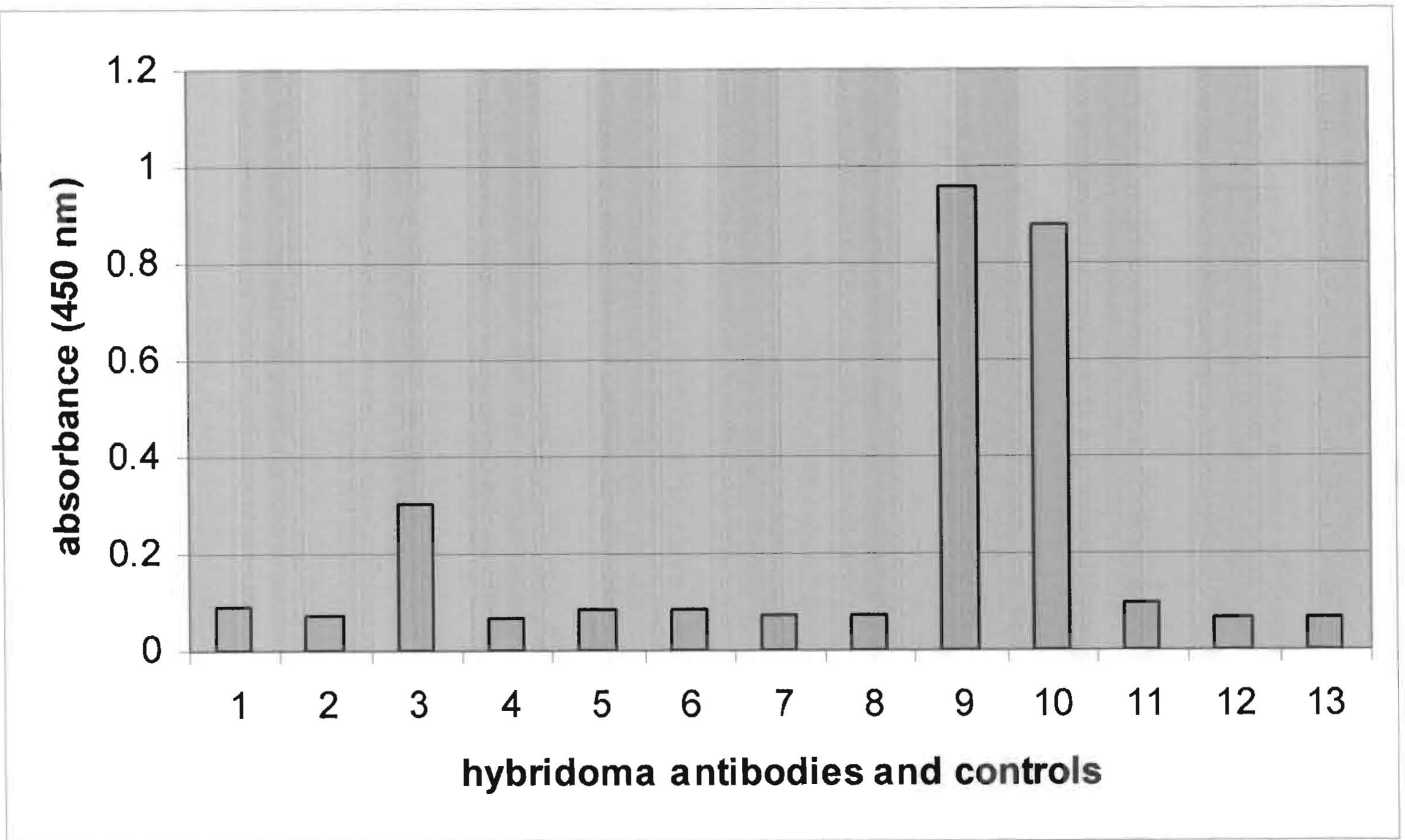

absorbance (450 nm)
1.2
1
0.8
0.6
0.4
0.2
0
*
1
2
3
4
5
6
7
8
9
peaks and controls

Figure 14. Testing Hybridoma Antibodies Against Whole Bacteria Type 8 *S. aureus* by means of an ELISA: Spleen cells from an immunized (with type 8 *S. aureus*) BalbC mouse were placed in media and transferred to a 24 well plate. The red blood cells were removed from the suspension. The spleen cells were added to P3X myeloma cells fused by addition of a solution containing PEG, RPMI, and DMSO. Cells were incubated with HAT + OPI media. Antibodies from hybridomas were tested for binding to type 8 *S. aureus* by an ELISA. Formalin fixed bacteria (*S. aureus* type 8) were treated with trypsin and added to a 96 well tissue culture treated polyvinyl microtiter plate. After 24hrs of incubation, the plate was centrifuged and washed with PBS (phosphate buffer saline). Cells were incubated with BSA (bovine serum albumin) in PBS to block nonspecific protein binding. Hybridoma supernatant was added to the wells for 2 hours at 25° C. Wells were washed and peroxidase conjugated with anti-mouse immunoglobulin was added and incubated for 2 hours at 25° C. The wells were washed and TMB (the substrate) was added to each well. Sulfuric acid was used to stop the reaction. The positive (serum from mouse immunized with type 8 *S. aureus*) and negative (no primary antibody used; only sample buffer) were used to compare to the results. The absorbance value of the antibodies and controls were read at 450 nm. Columns 9 and 10 are the positive controls and columns 11-13 are the negative controls. Columns 1-8 are DV.T8.C2, DV.T8.C3, DV.T8.C4, DV.T8.D3, DV.T8.D4, DV.T8.D5, DV.T8.D6, and DV.T8.E7 respectively.


absorbance (450 nm)
1.2
1
0.8
0.6
0.4
0.2
0
1
2
3
4
5
6
7
8
9
10
11
12
13
hybridoma antibodies and controls

V. DISCUSSION

The purpose of this study was to purify the capsular carbohydrates from type 5 *S. aureus* by means of DEAE and size exclusion chromatography. The sample was determined to be a purified CP by high absorbance of red tetrazolium and low absorbance of phosphate. Fournier and coworkers purified CP from type 8 *S. aureus* by similar methods using sephacel and size exclusion column chromatography (Fournier *et al.*, 1984). Unlike Fournier, our lab cultured bacteria on Columbia salt agar plates, whereas Fournier cultured bacteria in broth media. Studies have shown *S. aureus* grown on solid media have a higher capsule expression compared with *S. aureus* grown in liquid media (Ambrose *et al.*, 1988). The amount of CP remaining after size exclusion chromatography in these studies was 2.00 mg from 408 agar plates, compared with an amount between 0.5-2 mg/L obtained from bacteria grown in broth by Fournier and coworkers. Due to lack of materials, such as a 100 L fermentor, we were only able to obtain only 50 ml of bacterial paste from 408 plates, whereas Fournier used a large culture brought up in a 100 L fermentor. To detect CP, Fournier used a capillary precipitation method against rabbit anti-type 8 serum. Unlike Fournier, our lab used a carbohydrate ELISA method using antibodies from mice immunized with type *S. aureus* type 5 for identification of the CP. In addition, we have begun to produce monoclonal antibodies against type 8 *S. aureus* which we have tested for binding to formalin treated bacteria. Our studies in lab will hopefully lead to products useful for more specific ELISAs that can be used to detect *S. aureus* infections.

Unlike Fournier and coworkers who used serotype 8 Becker and Wright strain, our lab used serotype 5 Rosenbach strain. The procedures for capsular polysaccharide

extraction were similar. Using a series of reagents such as lysostaphin, protease, DNAse, and RNAse, capsule was released and dialyzed against Milli-Q water. The sample was purified by charge separation using a DEAE sephacel column with a NaCl step gradient to disrupt ionic bonds (Fournier *et al.*, 1984). The collected fractions after DEAE column chromatography were tested for optical density (Reynaud-Rondier *et al.*, 1991) at an absorbance of 206 nm to ensure that there were organic molecules present in the sample.

The red tetrazolium test (Fieser *et al.*, 1987) was used to show the amount of carbohydrates, or reducing sugars, that were present in the samples. Sample lysates before column chromatography were compared to a glucose control. The control and sample lysates were tested with red tetrazolium and read at a wavelength of 490 nm (see Figure 5). The gradual slopes of both serial two-fold dilutions demonstrated that the absorbance of 490 nm was an efficient wavelength for testing the presence of capsular polysaccharides (the absorbance showed both the presence of glucose sugar in the control and the CPs in the bacterial lysates). The results demonstrated that a concentration of 0.025 mg/ml was the lowest amount of CP that is detectable by the red tetrazolium test. By using the absorbance values of the serial two-fold dilution glucose control, an estimated concentration of the CP can be determined in the CP sample after column chromatography.

Since teichoic acid, which is a phosphate-containing molecule, is embedded within the CP of *S. aureus*, the sample had to be tested for this contaminant. A phosphate test (teichoic acid test) will detect carbohydrates that contain phosphates. The sample peaks were determined to have either low or no absorbance values for phosphate present in the sample.

The sample peaks were then passed through an S-300 Sephacryl Size Exclusion column (Fournier *et al.*, 1984), where smaller particles such as proteins and carbohydrates (contaminants) are trapped in the porous beads. Larger particles such as polymeric capsular carbohydrates are unable to be trapped in the small pores of the beads and will elute off of the column first (Cooper 1977). Fractions from the S-300 Sephacryl column (Size Exclusion column) were analyzed by the red tetrazolium and OD tests. The last fractions (76-79) were discarded due to a high 206 nm OD and low red tetrazolium absorbance values, which indicated the presence of contamination in these samples. The rest of the fractions were grouped into peaks according to data from the red tetrazolium (490 nm) test. Fournier and coworkers obtained their CP from the size exclusion column within fraction samples 65-85 and teichoic acid within fraction samples 90-110 (Fournier *et al.*, 1984). Our data shows high absorbance values for the tetrazolium red test for fraction samples 15-66 and low absorbance values for 75-79 (see Figure 12). Samples 76-79 were discarded due to their relatively high OD and low red tetrazolium absorbance. Absorbance values for teichoic acid for fraction samples 1-75 were low for teichoic acid (see Figures 9 and 10).

As part of these studies we investigated whether phosphate tests could be read at a lower wavelength. One spectrophotometer read microtiter plates, whereas the other spectrophotometer read individual samples only. A wavelength of 820 nm, the previously used wavelength, was possible on the spectrophotometer that only read individual samples. We wanted to adapt the method to a microtiter assay. However, the spectrophotometer that reads microtiter plates had only 630 nm filters. A comparison of both wavelengths was performed. A phosphate test was performed on serial 2-fold

dilutions of PBS (see Figure 6). The samples were read at 630 nm and 820 nm. The two different wavelengths resulted in similar absorbance values, suggesting that either wavelength (820 nm or 630 nm) could be used to read absorbance values of phosphate. Serial 2-fold dilutions were also completed comparing two different wavelengths (820 and 630 nm) for testing for the presence of phosphate in a teichoic acid test (see Figure 7) using a complex mixture of bacterial components. A sample lysate before column chromatography was read at both wavelengths (820 and 630 nm). In these tests, absorbance at 630 nm was significantly lower than that seen at 820 nm (see Figure 7). Testing a sample lysate for both wavelengths (820 and 630 nm) in a phosphate test, showed completely different results. Data from Figure 7 suggests that a wavelength of 630 nm cannot be used to test for teichoic acid in a phosphate test using a purified CP sample of *S. aureus*.

After the teichoic acid test the sample peaks were confirmed to bind to anti-type 5 *S. aureus* antibodies using an ELISA. The monoclonal antibodies were selected for antibody binding to type 5 *S aureus* CP and type 5 whole bacteria. All of the purified CP samples collected after DEAE column chromatography (Figure 11) had a greater absorbance value than the negative control, indicating that all samples have the presence of CP.

Purification of the capsular polysaccharide produced compounds necessary for further testing of monoclonal antibodies against type 5 capsular carbohydrate. Other laboratories have previously produced monoclonal antibodies to *S. aureus* capsule. Nelles and coworkers produced and characterized monoclonal antibodies against type 5 and 8 CP (Nelles *et al.,* 1984). Antibodies were produced from mice immunized with

formalin fixed bacteria. Spleen cells were removed and fused with myeloma cells. Unlike our lab, antibodies from hybridomas were selected by bacterial agglutination. Agglutination assays were accomplished on round-bottomed 96-well microtiter plates and read 24 hrs after incubation at 4°C.

We have also begun to produce hybridomas against type 8 *S. aureus* and we have demonstrated the presence of antibodies in hybridoma supernatants that bind to type 8 whole bacteria (Figure 13). We have successfully developed a hybridoma that has shown type 8 specificity in an ELISA. This hybridoma will eventually be cloned and tested for binding to the purified capsular polysaccharide of *S. aureus* type 8. Our goal is to culture hybridomas that will produce antibodies against the CP of type 5 and type 8 *S. aureus* that can be used to target the bacteria for destruction by phagocytosis. The antibodies could be used to treat patients who are immunocompromised or young individuals with *S aureus* infections.

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