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DESIGN AND EVALUATION OF IMMUNOGENIC *Escherichia coli* HEAT- STABLE ENTEROTOXIN (STa) AND CHARACTERIZATION OF THE IMMUNE RESPONSE IN LABORATORY ANIMALS

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ABSTRACT

DESIGN AND EVALUATION OF IMMUNOGENIC *Escherichia coli* HEAT-STABLE ENTEROTOXIN (STa) AND CHARACTERIZATION OF THE IMMUNE RESPONSE IN LABORATORY ANIMALS

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Strains of Enterotoxigenic *Escherichia coli* (ETEC) that produce heat-stable enterotoxin (STa) are an important cause of diarrheal disease in humans and animals. They are responsible for a significant proportion of diarrheal cases among infants, neonatal animals, and travelers going from non-endemic to endemic areas. The development of effective strategies to reduce the incidence and severity of ETEC-caused diarrhea has been hampered by the lack of an effective vaccine or immunotherapeutic agents against this enteric pathogen. The lack of an effective vaccine against the ETEC is largely due to the diverse antigenic structure of the ETEC strains and the poor immunogenicity of the STa enterotoxin, which is the predominant enterotoxin in 75% of ETEC strains and is the immediate mediator of ETEC-caused diarrhea.

Attempts to induce an appropriate immune response against the STa involve the design of immunogenic STa-carrier conjugates. However, based on the protocols reported in previous studies, only low titers of STa-antisera have been obtained. In this study, we used highly purified *E. coli* STa to produce STa-carrier conjugates through covalently cross-linking the STa to a modified bovine serum albumin (BSA) carrier protein. Four different conjugation protocols were evaluated. The most

effective STa-conjugate was selected based on its retention of STa biological activity, conjugation efficiency, and stability.

The selected STa-BSA conjugate was used to immunize rabbits for antibody production. This STa-conjugate induced a high immune response against the native STa. The harvested rabbit sera showed a neutralization capacity of 3×10^4 STa mouse units/ml serum using a suckling mouse assay, and a specific binding titer of 10^{-6} using indirect ELISA. These levels of STa neutralization and binding capacity are higher than what has been reported in previous studies. This study demonstrated the feasibility of designing an effective immunogenic ETEC-STa conjugate that can be used for the development of immunotherapeutic reagents and /or a potential vaccine against the ETEC-STa.

DEDICATION

To my almighty and beloved **ALLAH** who has been my shield, my strength, and my very present help in time of need. Thank you my **LORD** for helping me throughout this dissertation and for giving me a hope beyond hopes.

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LIST OF ABBREVIATIONS

ANPR = Atrial natriuretic peptide receptors

BSA = Bovine serum albumin

cAMP = Cyclic adenosine monophosphate

2CIATP = 2 choloradenosine trihosphate

CFA = Colonization fimbriae of human ETEC strains

CFTR = Cystic fibrosis transmembrane conductance regulator

cGMP = Cyclic guanosine monophosphate

Cl^- = Cholride ion

CS = Coli surface antigen

CT = Cholera toxin

EAEC = Enteroaggressive *E. coli*

EAST1 = Enteroaggregative heat-stable enterotoxin

ECD = Extracellular domain

EDAC =1-ethyl-3[3-dimethylaminopropyl] carbodiimide hydrochloride

ETEC = Enterotoxigenic *E. coli*

GC-C = Guanylyl cyclase type C

GTP = Guanosine triphosphate

HCO_3^- = Bicarbonate ion

HPLC = High performance liquid chromatography

ICD = Intracellular domain

K^+ =Potassium ion

LT = Heat-labile enterotoxin

LT = Heat-labile toxin

MU = Mouse unit

MWCO = Molecular weight cutoff

Na^+ = Sodium ion

NMR = Nuclear magnetic resonance

PBS = Phosphate buffer solution

PDE3 = phosphodiesterase

PKC = Protein Kinase

SMA = Suckling mouse assay

STs = Heat-stable enterotoxins

STb = Heat-stable enterotoxin B

STa = Heat-stable enterotoxin isolated primarily from bovine

STp = Heat-stable enterotoxin isolated primarily from porcine

STh = Heat-stable enterotoxin isolated primarily from human

SuBSA = Succinylated bovine serum albumin

TD = Transmembrane domain

TFA = Triflouroacetic acid

Amino Acid Abbreviations

A ALA Alanine

C CYS Cysteine

D ASP Aspartic Acid

E GLU Glutamic Acid

F PHE Phynylalanine

G GLY Glycine

H HIS Histidine

I ILE Isoleucine

K LYS Lysine

L LEU Leucine

M MET Methionine

N ASN Asparagine

P PRO Proline

Q GLN Glutamine

R ARG Arginine

S SER Serine

T THR Threonine

V VAL Valine

W TRP Tryptophan

Y TYR Tyrosine

CHAPTER I

INTRODUCTION

Statement of the Issue and Rationale

Enterotoxigenic *Escherichia coli* (ETEC) is a major enteropathogen that causes potentially fatal diarrhea in both human and animal neonates (Moon and Bunn 1993, Tacket et al. 1994). It is also responsible for a large proportion of diarrheal disease among adult travelers (Tacket et al. 1994). Therefore, strategies to reduce the incidence and severity of ETEC diarrhea have been considered an important public health priority (Tacket et al. 1994). A large proportion of ETEC diarrhea is caused by heat-stable enterotoxin (STa), a small peptide (2kD), which is an important virulence determinant in enterotoxin-mediated diseases (Sears & Kaper 1996 and Giannella & Elizabeth 2003). Upon infection, the STa producing- ETEC adheres to the epithelium of the small intestine via one or more colonization factor antigens or pili surface proteins. Once established, ETEC elaborates heat-stable enterotoxin (STa), which acts on a specific intestinal membrane bound receptor, guanylyl cyclase C, initiating a cascade of altered metabolic pathways. This may result in secretory diarrhea among affected adults but can cause fatal dehydration in neonates.

Methods for the treatment and control of ETEC diarrhea are still a matter of debate among veterinarians, livestock producers and in the animal industry in general. The use of sub-therapeutic doses of antibiotics may help protect animals from some, but not all, of these bacterial strains. Moreover, the use of antimicrobials at sub-therapeutic levels has been linked to the problem of emerging antibiotic resistance among several bacterial species, including ETEC strains.

While there are several reagents that are in use against ETEC diarrheal disease in animals, most of these reagents are based on surface structures of the ETE strains. However, the development of a broad-spectrum vaccine against ETEC remains elusive (Walker et al. 2007). Two major technical problems contribute to this deficiency. The first involves the production of immunogenic preparations of antigens with the ability to confer broad-spectrum protection against ETEC infections. The second is the challenge of achieving effective mucosal immunization (Walker et al. 2007) due to the multiplicity, antigenic diversity, and high prevalence of unidentifiable forms of specific colonization antigens responsible for mucosal adherence (Deneke et al. 1981, Levine et al. 1980, Thomas and Rowe 1982). Against this background, there is an urgent need to define a new common antigenic determinant that could provide broad protection against ETEC-STa-induced diarrhea.

Saeed et al. (1985) demonstrated that calf scour could be experimentally induced by a highly purified STa preparation, supporting the notion that ETEC STa is the immediate mediator of diarrhea in claves. Additionally, several studies have demonstrated a significant correlation between STa-producing ETEC strains and diarrhea, and that 75% of ETEC strains produce STa either alone or in combination with heat-labile enterotoxin (LT) (Wolf 1997). Thus, the inclusion of STa in colonization factor-based ETEC vaccines or the production of neutralizing STa-antibodies would potentially offer immune protection against ETEC-caused diarrhea. However, this approach has been a challenge, partly because of the haptenic nature of STa (molecular weight <2 kDa), which fails to elicit an antibody response (De Weck 1974 and Pereira et al. 2001 and Boedeker 2005). Additionally, the correlation

between STa toxicity and antigenicity (Takeda et al. 1993) hampers the ability to produce a safe STa/CFAs vaccine. However, it was hypothesized that the poor immunogencity associated with the STa molecule could be improved by conjugation of the STa to a suitable macromolecule (carrier protein) (Elanger 1980 and Pauillac et al. 1998).

Therefore, antibody-based therapy (passive immunization) targeting the STa antigen could be used to reduce the impact of ETEC-STa induced diarrhea and avoid the safety issue associated with active immunization with CFA/toxin based–vaccine. Attempts to conjugate the STa to a carrier protein have been reported (Clements, 1990; Houghten et al. 1984, 1985; Klipstein et al. 1982, 1983; Sanchez et al. 1986, 1988), however no sufficient details were presented on the efficiency and /or the characteristics of these conjugates.

In this study, STa was purified to homogeneity and its properties were characterized. The purified STa was then covalently cross-linked to modified BSA using 4 different protocols of peptide-carrier conjugation. We have characterized the STa-conjugates for biological activity, conjugation efficiency, and stability of the conjugates. The best STa-conjugate was then used for immunological studies.

General Objectives

- Purify and characterize *E. coli* STa.
- Design and characterize an immunogenic *E. coli* STa.
- Produce and characterize specific antibodies against *E. coli* STa in laboratory animal models.

Specific Aims

I. Production of high performance liquid chromatography - purified *E. coli* STa

- Verify bacterial strain as an ETEC STa-positive strain by identification of the STa encoding gene.
- Adoption of bioreactor *E. coli* culture method for the production of STa and study the growth kinetics at different pH levels.
- Biological and molecular confirmation of STa specificity and identity.

II. Design and characterization of an immunogenic *E. coli* STa

- Evaluate four different protocols of peptide conjugation to carrier protein.
- Molecular characterization of the most effective STa- conjugates.

III. Production of specific antibodies with high neutralizing capacity against *E. coli* STa

- Study the neutralization capacity of harvested polyclonal antibodies against *E.coli* STa.
- Study the kinetics of the *E. coli* STa antigen-antibody interaction:
  - ELISA STa-IgG binding capacity assay.
  - Determination affinity and avidity index of STa antibodies.

Potential Benefits of the Study

Enterotoxigenic *E. coli* (ETEC) that produces STa is a major cause of severe diarrhea in neonatal animals, children, and adult travelers. The production of specific antibodies with a high neutralization capacity against STa can help to reduce the incidence and economic losses associated with ETEC STa-induced diarrhea. An

effective immunogenic STa may have potential as an ETEC vaccine that may confer broad protection against ETEC-STa-induced diarrhea.

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CHAPTER II

REVIEW OF LITERATURES

Magnitude and impact of diarrheal diseases

Diarrheal diseases are one of the major causes of human death on a global scale. They are the leading cause of childhood death, resulting in 18% of all deaths in children under the age of five (Bryce, 2005). In some populous developing areas, they are responsible for more years of potential life lost than all other causes combined (WHO, 1990). According to the *Global Burden of Disease*, in 1990, diarrheal diseases were a leading cause of disability-adjusted life year (DALY), second only to lower respiratory infections (est. 99.2 million DALYs lost) (Murray, 1997).

Additionally, the National Animal Health Monitoring System (NAHMS, 1994 &1996) survey and the USDA (1997) identified diarrheal diseases as the most common infectious cause of neonatal calf mortalities (est.75% of mortalities). Decades of research have been dedicated to obtaining a better understanding of the neonatal calf diarrhea complex. However, despite improvements in the identification of the infectious agents, management practices, and treatment and prevention strategies, the complex remains the most common and costly (est. $120 million annually) disease affecting newborn calves in the United States (NAHMS, 1996).

The list of enteropathogens that can potentially cause diarrhea is quite large and diverse. There are six major pathogens that cause diarrhea in newborn animals: enterotoxigenic *Escherichia coli*, *Rotavirus, Coronavirus, Cryptosporidium parvum, Salmonella spp.*, and *Clostridium perfringens type C* (Snodgrass et al. 1986).

Diarrhea caused by Enterotoxigenic *Escherichia coli* (ETEC) is problematic in both animal and human populations. In animal neonates it causes severe profuse watery diarrhea, potentially fatal dehydration and metabolic acidosis (Argenzio, 1985). Additionally, ETEC is a major cause of traveler's diarrhea (Gorbach et al. 1975) and a leading enterpathogen responsible for gastroenteritis outbreaks on cruise ships (Addiss et al. 1989 and Koo et al. 1996).

General Overview of *Escherichia coli*

Escherichia coli is a common member of the normal flora of the large intestine. Most *E. coli* strains are avirulent and remain benign commensals because they lack specific genetic elements encoding for virulence factors. However, strains become virulent upon acquiring bacteriophage plasmid DNA encoding enterotoxins or invasion factors. The mechanisms by which virulent *E. coli* strains acquire such genetic elements seem to be horizontal genetic transfer including phage transudation, transposition, conjugation of plasmids and simple recombination (Seifert and DiRita, 2006). Virulent *E. coli* strains cause a variety of disease conditions including plain, watery diarrhea, inflammatory dysentery, hemolytic uremic syndrome, septicemia, pneumonia and meningitis (Salyers and Witt 1994).

Virulence Attributes of ETEC

Pathogenicity of *E. coli* is a complex multifactorial mechanism involving a large number of virulence factors that vary according to the pathotype. They include attachment functions, host cell surface modifying factors, invasins, and many different toxins as well as secretion systems which export toxins and other virulence factors and pilot them to the target host cells. These virulence attributes are often

organized into large genetic blocks on the chromosome, known as pathogenicity islands, on a large plasmid, or on phage DNA and can be transmitted horizontally between strains (Seifert and DiRita, 2006). In this regard, *E. coli* becomes virulent upon the acquisition of plasmids that encode genes for two virulence determinants, namely colonization factors (adhesins), and enterotoxins. Both enterotoxins and CFs work in concert to cause diarrhea in both humans and animals.

Two classification schemes are currently used to classify the different strains of *E. coli*, serotyping and virotyping (Lior 1996 and Levine 1987). Serotyping of *E. coli* occupies a central place in the history of these pathogens and is mainly based on antigenic differences in the highly variable bacterial surface molecules such as lipopolysaccharides (O antigens) and flagella (H antigen) (Lior 1996). Specific combinations of O and H antigens define the serotype of an *E. coli* strain and are widely used for tracing outbreaks of enteric diseases. More than 170 different "O"-specific somatic antigens, 56 different types of flagellar "H" antigens and 103 different capsular "K" antigens in *E. coli* have been characterized (Nataro and Kaper, 1998). Some *E. coli*, particularly the ETEC strains, produce mannose resistant (MR) fimbriae that may be used for serological identification. Some of these MR fimbriae (e.g. K88 and K99) were once identified as K antigens before their chemical composition was known. They are presently defined as F (fimbrial) antigens, which also include the mannose sensitive type 1 fimbriae group (Levine et al. 1983).

Virotyping is a phenotypic classification scheme based on virulence characteristics of *E. coli* that includes patterns of bacterial attachments on host cells, the effect of attachments on host cells, production of toxins and invasiveness.

Currently there are six virotypes: Enterotoxigenic *E. coli* (ETEC), Enteropathogenic *E. coli* or Attaching and Effacing *E. coli* (EPEH, AEEC), Enterohemorrhagic *E. coli,* Shiga toxin producing *E. coli or* Virotoxigenic *E. coli* (EHEC, STEC, VTEC), Enteroaggregative *E. coli* (EAEC), Enteroinvasive *E. coli* (EIEC) and diffusely adherent *E. coli* (DAEC) (Nataro and Kaper, 1998). In this literature review, we will largely cover the different characteristics of ETEC strains.

Structure, Classification, and Antigenic Types of ETEC

ETEC organisms are Gram-negative, short rods, not visibly different from the *E. coli* found in the normal flora of the large intestine (Bettelheim 1994). Virulence-associated attributes are fimbriae and enterotoxins. Genes for both attributes are carried on a plasmid and play a crucial role in the pathogenesis of calf scours (Levine et al. 1983 and Nataro and Kaper 1998). All ETEC strains contain plasmids, but this is not a distinguishing feature unless gene probe techniques are used to detect specific virulence-associated genes on these plasmids (Nataro and Kaper, 1998).

Pilus or Fimbrial Antigen (F antigen)

A common trait of ETEC strains is the expression of several surface-associated proteins, antigenically unrelated to O and H antigens, called Fimbriae (Pili) antigens. Fimbriae, which can be seen in electron micrographs as fuzzy coat, are filamentous structures that protrude as hair-like structures from the bacterial surface which are much thinner (3-4 nm) and usually more rigid than flagella (Follett and Gordon, 1980). They are composed of proteins (pilin) that are tightly packed into an array that is shaped like a helical cylinder and their assembly occurs on the outer cell membrane of the bacteria through a specialized pathway of the general secretion

system, the alternate chaperone/ usher pathway (Pitek et al. 2004 & Planet et al. 2006).

Commensal *E. coli* strains usually produce so-called common pili, while pathogenic ETEC possess specialized host-specific pili (Levine et al. 1983). Virulence-associated fimbriae are antigenically unrelated to common pili. They are too small to be seen by light microscopy and act as ligands to bind specific complex carbohydrate receptors on the epithelial cell surfaces of the small intestine. Since this interaction results in colonization of the intestine by ETEC, with subsequent multiplication on the gut surface, these pili are termed adhesins or colonization factors antigens (CFAs).

A variety of CFs has been described in ETEC strains of animal origin. F4 (K88), F5 (K99), F6 (987P), F41, F42, F165, F17 and F18 are produced by *E. coli* that cause acute diarrhea in domestic animals. ETEC strains expressing K-99 are pathogenic for calves, lambs and pig, whereas K-88 expressing organisms are able to cause disease only in pigs (Cassels & Wolf 1995). Human ETEC strains also possess an array of colonization fimbriae (CFAI, CFAII and CFAIV) (de Craaf & Gaastra 1994).

Nearly all ETEC from calves produce F5 (CFA-K99) and STa (Gyles 1996). In addition to K99, several other fimbriae designated as F41, F92b, Att25, and F210 have been identified on calf ETEC but are much less common (Morris et al. 1983). F41 has been characterized and it mediates attachment to calf enteroytes in vitro and to the small intestine of newborn lambs (Morris et al. 1983). There may be a relatively small group of other fimbriae that also mediate adhesion to calf enterocytes,

although K99 is the most common attachment factor on bovine ETEC. This is not surprising, since a similar situation occurs in ETEC isolated from pigs, where K88 and 987P, and at least two other fimbriae known as K99 and F41, also mediate adhesion to pig enterocytes (Moon et al. 1977). Age-dependent resistance to diarrhea caused by F5 positive ETEC develops rapidly in calves (Runnels et al. 1980).

Genes coding for the production of CFAs reside on the ETEC virulence transmissible plasmids, usually on the same plasmids that carry the genes for one or both of the two types of *E. coli* enterotoxin, heat-labile enterotoxin (LT) and heat-stable enterotoxin (ST). Most F4 (K88) strains produce LT while most F5 (K99) and F6 (987P) ETEC strains produce ST. Two types of STs are produced, the majority of ETEC strains produce STa thought a few produce STb. Therefore, most cases of ETEC diarrhea in newborn calves are caused by *E. coli* possessing a F5 (K99) and an ST gene (Gyles, 1996).

Somatic (O) Antigen

Somatic antigens are specific heat-stable and composed of polysaccharide chains linked to the core lipopolysaccharide (LPS) complex common to all Gram-negative bacteria. "O" antigen specificity is determined by sugar or amino-sugar composition and by the sequence of these outer polysaccharide chains (Morris et al. 1983).

In normal smooth strains, the core LPS is buried beneath the O antigen while O-minus mutants, called rough strains, the core LPS is exposed. "O" antigens are not single antigens, but they are composed of several antigenic components and, therefore, are called O group antigens. Different O groups may share some of these

antigenic components; hence, there is considerable cross-reactivity among *E. coli* O antigens; also, many O groups of *E. coli* are cross-reactive or identical with specific O groups of *Shigella, Salmonella, or Klebsiella* (Gyles, 1996).

O antigens form the basis of serotyping classification of *E. coli.* More than 170 different O-specific antigens have been defined but only groups 8, 9, 20, 26, 101 and 141 are common on calf ETEC (Nataro and Kaper, 1998).

The role of "O" antigen in the pathogenesis of diarrhea is not clear, however, it is assumed that strains that possess these antigens offer unknown advantageous conditions for the plasmids that carry the genetic elements encoding for enterotoxin and fimbrial production (Evans et al. 1977).

Capsular (K) antigens

Capsular antigens are acidic polysaccharides that encapsulate the somatic antigen on some but not all ETEC (Acres, 1985). They appear as a dense mat of fibrous projections under electron microscope (Hadad and Gyles, 1982a). The process of *E. coli* serotyping has encountered difficulty because K antigens prevent the agglutination of the "O" antigens by the homologous sera. They are further subdivided into three groups based on their heat stability; (i) The L-type K antigens are completely destroyed by heat at 100 °C for 1 hour, thus rendering the O antigen agglutinable in O antisera. They retain no antigenicity and lose their ability to combine with homologous K antisera. (ii) The B-type K antigens are also destroyed by heat at 100 °C for 1 hour and lose their antigenicity. However, they retain their ability to bind or combine with their homologous antisera. (iii) A-type K antigens are not inactivated by heat at 100 °C, but require heat at 121 °C for 2.5 hr before the O

antigen becomes agglutinable. There are 103 different K antigens recognized in *E. coli*, however only 11 have been identified commonly on calf ETEC strains (Orskov et al. 1977). The role of K antigens in infection by ETEC is not known, but they appear to be important in extra-intestinal infections.

Enterotoxins of Enterotoxigenic *E. coli*

Two enterotoxins have been identified among the extracellular products of *E. coli* isolated from human and other mammals with diarrheal diseases- one is a high molecular- weight type (86.500 kD), namely heat-labile enterotoxin (LT) and the other is a low-molecular weight type called heat-stable enterotoxin (STs). The LT is closely related to cholera toxin (CT), sharing approximately 80% protein sequence identity in the A and B subunits and like CT toxin, stimulates adenylate cyclase and is inactivated by heating. On the other hand, STs completely differ from CT, they act more rapidly, and resist inactivation by heating (Levine et al. 1983; Saeed et al 1984; Sears & Kaper 1996 and Nataro & Kaper, 1998).

Heat- Stable Enterotoxins (STs)

ETEC strains produce a diverse family of closely related potent peptides toxins that induce secretory diarrhea in humans and animals. STs were initially defined by their resistance to inactivation by boiling for 30 min (Stine and Nataro, 2006). They are classified into two structurally, functionally and immunologically unrelated subtypes, STa (STI) and STb (STII). Table 1 shows the basic differences between STa and STb (Sears and Kaper, 1996 & Nataro and Kaper, 1998).

Table 1. Difference between *E. coli* Heat-Stable Enterotoxin A (STa) and Heat-Stable Enterotoxin B (STb)

Properties	STa = STI	STb = STII
Size	<2kDa	5.1 kDa
Number of amino acid residues	18-19 amino acids	48 amino acids
Mechanism of synthesis and secretion	Pre-pro form 72 amino-acid precursor followed by two consecutive peptidase cleavages before extracellular diffusion of 18-19 amino acid mature toxin	71 amino-acid precursor processed into 48 amino acid mature toxin secreted extracellularly without further processing
Number of cysteine residues	Six cysteine residues Three S-S bonds	Four cysteine residues Two S-S bonds
Toxic domain	Hydrophobic: 11-14 amino acid especially Ala 13	Charged amino acids: especially lys-22, lys 23, arg 29 and asp-30
Solubility	Methanol soluble	Methanol Insoluble
Activity	Active in mice, infant, piglet and calves	Inactive in suckling mice but active in rats and ligated piglet intestinal segment. Have no effect on human small intestine.
Trypsin effect	Resistant	Sensitive
Mechanism of action	Act on guanylyl cyclase C	Does not act on cyclic nucleotides. Ca, PGE2 and serotonin may be its mode of action
Effect on enterocytes:	No effect	Loss of villus epithelial cells and partial villus atrophy (Nagy and Fekete 1999)
Prototypes	STaP (procine isolates) and STaH (human isolate)	None
Producing strains	ETEC and other bacteria	Only ETEC

Genes encoding for both classes are found predominantly on plasmids, and some STa-encoding genes have been found on transposons. So and colleagues (1979, 1980 & 1981) demonstrated that the STa gene is embedded within a functional transposon, and the presence of STa on widely variable plasmids supports this as the dominant mechanism for ongoing transfer.

Enterotoxigenic *E. coli* STa (STI)

There are two slightly different toxin prototypes under this designation: STIa (STp) and STIb (STh). Both variants are structurally, functionally and antigenically related. They share a core 13 amino acid-sequence that is necessary and sufficient for enterotoxic activity but differ in their N-terminal sequence. While STp is produced by porcine, bovine and human ETEC, STh is only produced by human isolates (Nataro and Kaper, 1998).

A large family of STa-related toxins has been also identified in various organisms. All share similar size (ranging from 15-30 amino acids) and a highly conserved 13-amino-acid C- terminal region, which is essential for their toxicity (Table 2) (Stine and Nataro, 2006). The biochemical, physiological and immunological properties of the various STa enterotoxins are remarkably similar. They have four conserved amino acids (N-P-A-C) (Stine and Nataro, 2006) that mediate binding to the membrane guanylyl cyclase-C (Greenbreg et al. 1997). Most of them have six cysteine residues located in the same relative positions and linked intramolecularly by three disulfide bonds, which suggests that these enterotoxins have similar tertiary structure (Shimonishi et al. 1987). The secretory potency and heat

stability of the STa enterotoxins are determined by conserved core sequence (Yoshimura et al. 1985 and Shimonishi et al. 1987).

Table 2. The STa Family of Toxins

Toxin and host	No. Amino acids	Sequence	Reference
STaH ETEC	19	N-S-S-N-Y-C-C-E-L-C-C-**N-P-A-C**-T-G-C-Y	Aimoto et al 1982
STaP ETEC	18	N-T-F-Y-C-C-E-L-C-C-**N-P-A-C**-A-G-C-Y	Takao et al 1983
STa ETEC (bovine)	18	N-T-F-Y-C-C-E-L-C-C-**N-P-A-C**-A-G-C-Y	Saeed et al 1984
Citrobacter Freundii	18	N-T-F-Y-C-C-E-L-C-C-**N-P-A-C**-A-G-C-Y	Guarino et al 1989
Yersinia enterocolitica	30	S-S-D-Y-D-C-C-D-Y-C-C-**N-P-A-C**-A-G-C	Takao et al 1985
V. cholera Non-O1	17	I-D-C-C-E-I-C-C-**N-P-A- C**-F-G-C-L-N	Yoshimura et al 1986
V.cholera non-O1 Hataka	18	L-I-D-C-C-E-I-C-C-**N-P-A-C**-F-G-C-L-N	Arita et al 1991
V. mimicus	17	I-D-C-C-E-I-C-C-**N-P-A-C**-F-G-C-L-N	Arita et al 1991
E. coli EAST-1	38	...A-S-S-Y-A-S-C-I-W-C-T---T-**A-C**-A-S-C-H-G	Savarino et al 1993
Conus geographus 13	13	E-C-C-**N-P-A-C**-G-R-H-Y-S-C	Gray et al 1981
Guanylin (human)	15	P—G-T-C-E-I-C-C-AY-A-**A-C**-T-G-C	Greenberg et al 1997

Adopted from Nataro & Stine (2006)

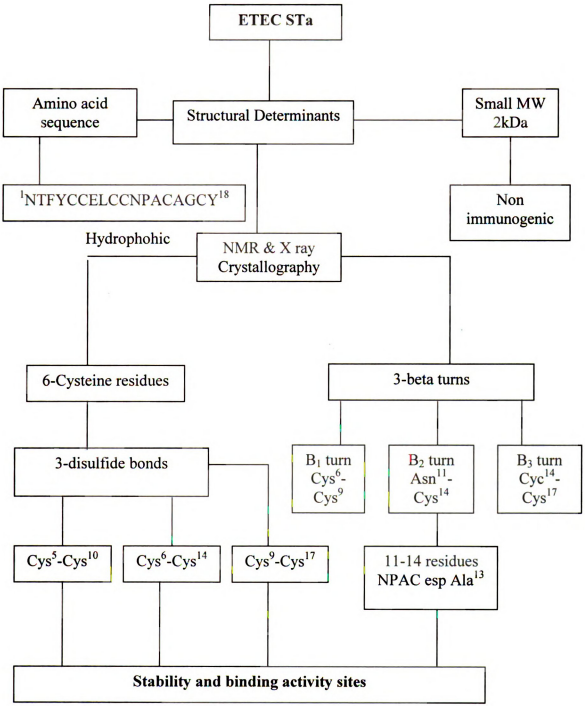
Structural determinants and biochemical properties of STa (Figure 1)

STa is a small peptide (molecular weight of less than 2 kDa) that has 18- or 19- amino acids (Sato & Shimonishi, 2004). STa is produced as a 72 amino-acid precursor that is cleaved by signal peptidase 1 to a 53-amino- acid-peptide (pro-STa) (Rasheed et al. 1990). Prior to secretion by the bacteria, three intramolecular disulfide bonds crucial to toxin activity are formed in the periplasm by DsbA, chromosomally encoded protein (Yamanaka et al. 1994). A second undefined proteolytic event occurs extracellularly to process the pro-STa to produce biologically active STa of 18 or 19 amino acids which is released by diffusion across the outer membrane (Rasheed et al. 1990).

The structural characteristics of biologically active STa were extensively studied (Gariepy et al. 1986; and Osaki et al. 1991) on weakly toxic and nontoxic STa analogs (Sato et al. 1994). Using X-ray diffraction analysis, Sato and Shimonishi, (2004) studied the molecular structure of the toxic domain of fully active STa molecule, which consists of the native sequence from Cys^{5} to Cys^{17} and revealed that the peptide molecule forms a ring-shaped peptide hexamer. The outer surface of this peptide is occupied exclusively by the central portion of the side chains of Leu^{8}, Asn^{11}, Pro^{12}, and Ala^{13}, proposed to be a binding site to the receptor protein, guanylyl cyclase C, GC-C while the inner surface of the peptide hexamer consists of a single invariant Gly residue (Osaki et al. 1991; Sato and Shimonishi, 2004).

Overall the STa molecule has a hydrophobic nature with certain structural elements that are functionally critical. One of which is the occurrence of six cysteine residues participating in three disulfide bridges (Garpiey et al. 1987; Okamoto 1987;

Figure 1. Structural Determinants of *Escherichia coli* STa



Osaki et al. 1991; Sato et al. 1994 and Sato & Shimonishi, 2004) suggesting that this toxin has tertiary structure (Shimonishi et al. 1987). Listed in descending order of importance in terms of their respective contribution to enterotoxic activity, the disulfide linkages between Cys^{6} and Cys^{14}, Cys^{5} and Cys^{10}, and Cys^{9} and Cys^{17} are all necessary for stabilizing the STa molecule and expressing full STa toxic activity (Garpiey et al. 1987). In addition, the second β-turn at residues 11 to 14 (especially Ala^{13}), is proposed to be crucial to the STa toxicity and its interaction with GC-C (Osaki et al. 1991 and Sato et al. 1994).

Amino acid sequence of STa purified from several strains of bovine ETEC was reported by Saeed and colleagues (1983, 1985a & b). STa from bovine ETEC was found to consist of 18 amino acids and was similar to STa purified from ETEC of human and porcine origin.

ETEC (STa) is methanol and acetone soluble. It resists proteolytic (pronase and trypsin) enzymes (Jacks and Wu, 1974), heat and acids but not alkalines. The toxin loses biological activity on treatment with reducing agents, such as *p*-mercapto-ethanol, dithiothreitol or after performic acid oxidation suggesting the presence of disulfide bridges (Eldeib et al. 1986).

Antigenicity and antigenic determinants of *E. coli* STa

E. coli STa is poorly immunogenic because it has low molecular weight (Gyles 1971; Evans et al. 1973; and Alderete & Robertson, 1978). The antigenic determinants of STa have been analyzed (Takeda et al. 1993). Three distinct antigenic sites of STa have been recognized, one near the *N*-terminus, another in the core functional region, and the third in the *C*-terminal, Asn-Tyr^{4}-Leu^{8} -Asn^{11}-Pro^{12}-Ala^{13}-

Cys^{14}-Tyr^{18}. Characterization of various STa epitopes revealed that the *N*-terminus residues, which are not essential for the biological activity of STa in suckling mice (Aimoto et al. 1982; Shimonishi et al. 1987 and Yoshimura et al. 1985 & 1987), but possess an important antigenic determinant (Takeda et al. 1993).

Structural-Function Relationship of *E. coli* STa

Four characteristic structural features of the STa molecule greatly contribute to its function: hydrophobicity, single invariant glycine residue, conserved 6-cysteine residues, and geometrical shape of this molecule (Sato and Shimonishi, 2004).

Hydrophobicity of the STa creates a favorable situation for an interaction with GC-C on biological membranes. The side chains of the amino acids of the binding region of ST (Asn^{11}, Pro^{12} and Ala^{13}), which are constant for numerous bacteria, form a prominent, isolated cluster that projects from the surface to the outside of the molecule and allows binding with GC-C because of their hydrophobic nature. Site directed mutagenesis studied the contribution of these residues to the toxic activity of the STa molecule and found that replacement of Ala at position 13 by Gly or Leu residue led to loss of enterotoxigenic activity of the toxin (Sato and Shimonishi, 2004) suggesting that Ala^{13} has a key role in the interaction of STa with its receptor domain. Other amino acid residues were tested for their role in STa-receptor binding (Takeda et al. 1991 & 1993 and Yoshino et al. 1994).

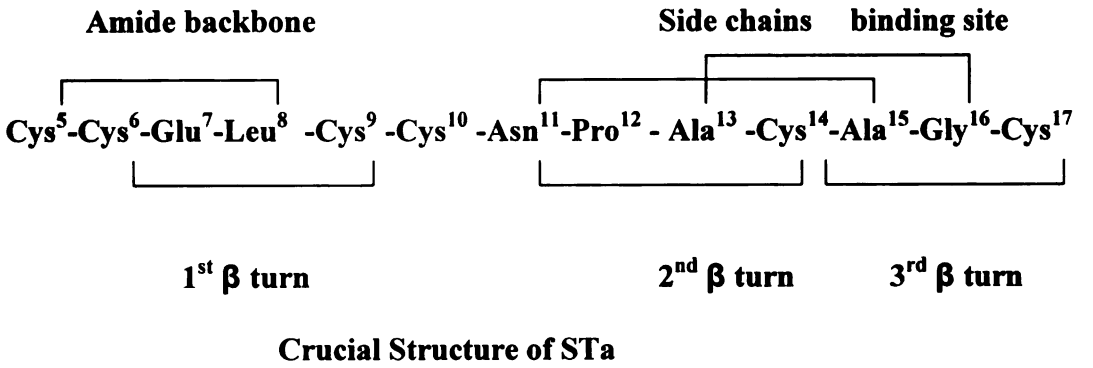
The inner surface of the peptide hexamer is occupied by a single invariant Gly. The backbone conformation in this region is unique for the Gly residue and other amino acid substitution may disrupt the formation of hexamer structure by steric hindrance (Sato and Shimonishi, 2004).

STa has 6-cystiene residues connected with each other though three intramolecular disulfide bonds (Figure 2) which are crucial for the spatial structure of STa and for the expression of toxicity. Yamasaki et al. (1990) studied the contribution of each disulfide bond to the toxic property of STa. The authors found that peptides with only one disulfide bond are not biologically active, but peptides with a disulfide bond in the second β-turn and one other disulfide bond had distinct activity. A peptide that lacks a disulfide bond in the second β turn is not toxic. Clearly, disulfide bonds are essential to stabilize the spatial structure of STa are necessary for STa toxicity and stability.

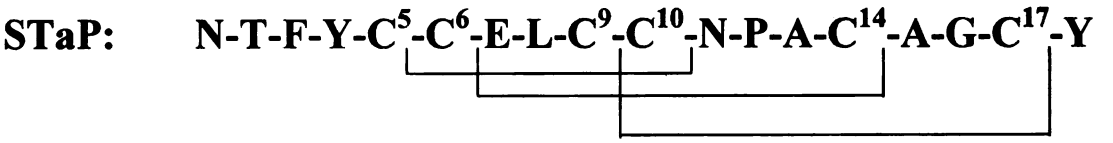
The finding that native ST forms a self-associated hexamer is consistent with other structure-activity relationships, which are difficult to rationalize using the monomeric structure of STa analogous (Sato and Shimonishi, 2004).

Receptor of ETEC-STa: Guanylyl Cyclase C (GC-C)

The major receptor for STa is a membrane-bound (particulate) guanylyl cyclase C (GC-C), (Field et al. 1978; Hughes et al. 1978; Schulz et al. 1990 and Vaandrager, 2002, Giannella and Elizabeth 2003) which belongs to a group of receptor-linked enzymes that catalyzes the conversion of guanosine 5'-triphosphate (GTP) to second messenger cyclic guanosine 5'-monophosphate (cGMP) in response to diverse signals, such as peptide hormones. In addition to GC-C, this family includes also the atrial natriuretic peptide receptors (GC-A & GC-B) (Lowe et al. 1990 a&b) and the olfactory (GC-D) (Yang et al. 1996) and retinal cyclases, (GC-E, & GC-F) (Oliveira et al. 1994 and Yang et al. 1996).



Crucial Structure of STa



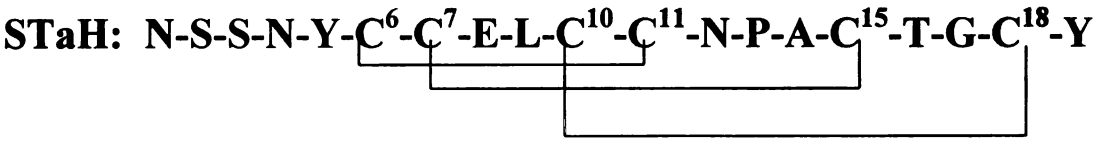


Figure 2. Intramolecular cysteine-disuflide linkage for STaP and STaH

(Sato et al. 1994)

All these GCs are receptor-linked enzymes with one membrane-spanning region and share a conserved intracellular catalytic domain (ICD) but they differ in their extracellular ligand-binding domains (ECD) because they are activated by different signals (Lucas et al. 2000; Schulz et al. 1990 and Vaandrager, 2002) (Figure 3).

The size and nature of the STa receptors have been an area of intensive investigation resulting in the identification of at least one definitive STa receptors located in the apical membrane of the intestinal epithelial cells (Schulz et al. 1990; Thompson & Giannella, 1990; and Cohen et al. 1993). Binding of ligands (STa, guanylin and uroguanylin) to the ECD stimulates the intracellular enzymatic activity. Molecular cloning from various mammalian species revealed that the primary structure of GC-C (120-kDa protein unglycosylated and a 140 to 160-kDa protein after *N*-linked glycosylation) is composed of an extracellular *N*-terminal receptor domain (ECD), a single transmembrane domain (TMD) and a cytosolic domain (ICD). ICD comprises a kinase homology domain (KHD) which is linked by an α-helical putative dimerization domain to the *C*-terminal catalytic domain (Schulz et al. 1990; de Sauvage et al. 1991; Garbers, 1999; Lucas et al. 2000).

GC-C contains a unique ECD comprising approximately 40% of the total protein, which contains 8-10 *N*-linked glycosylation sites and functions as the binding site for GCC-ligands (Schulz et al. 1990 and Lucas et al. 2000). The ligand recognition sites and the importance of carbohydrate moieties on GC-C were studied by site directed mutational analysis of the ECD and revealed that the *N*-linked carbohydrate moiety at N379 plays an essential role in stabilizing the functional structure of ECD.

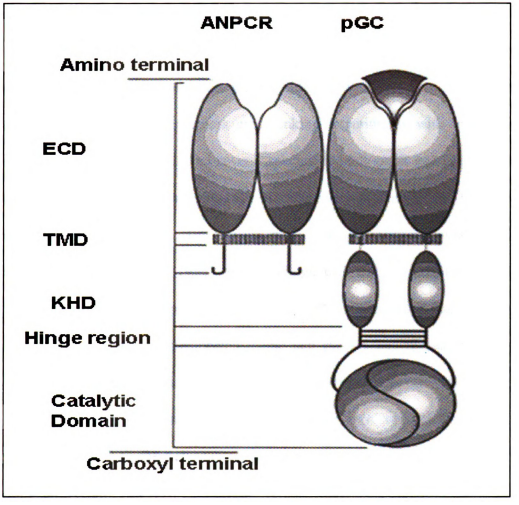


Figure. 3. Domain structure of guanylyl cyclases (Lucas et al. 2000)

It also revealed that the ligand specifically binds to the region residue 387-393. Both the *N*-linked carbohydrate moiety at N379 and residue 387 to residue 393 regions are located close to the transmembrane portion of GC-C on the external cellular surface which explain their pivotal role in ligand binding and signaling processes (Hasegawa et al. 1999 and Hasegawa & Shimonishi, 2005).

Solubilization and STa receptors cross-linking experiments with intestinal tissues have been repeatedly identified several species of both small and large STa-binding proteins, 45 to 80kD and 120-160kD respectively (Cohen et al 1993; de Sauvage et al. 1992, Hakki et al. 1993; Ivens et al. 1990; Thompson & Giannella, 1990 and Vaandrager & de Jonge 1994). Study of biogenesis, cellular localization and functional activation of STa receptors (Runder et al. 1996) demonstrated that only the full length of STa receptor is translocated to the cell surface and is thus the only species available to interact directly with the extracellular environment. The smaller protein forms are likely to represent premature termination products of STa receptor that are not transported to the cell surface. The generation of antibodies to GC-C furthermore suggested that low molecular weight STa binding proteins were proteolytic fragments of GC-C (de Sauvage et al. 1991 and Vaandrager et al. 1993). The study of STa receptor biogenesis also showed that ligand stimulation of GC-C does not lead to recruitment of additional receptor to the cell surface. This proves that STa receptor is rapidly internalized and recycled back to the cell surface after binding STa (Runder et al. 1996 and Urbanski et al. 1995).

STa-GCC receptor binding kinetics has been studied (Giannella et al. 1983 Frantz et al. 1984; Carpick & Gariepy 1991 & 1993; and Al-Majali et al. 1999 a&b).

Giannella (1995) showed that the binding constant of STa is two log orders less than that of CT for its receptor and accounts for the rapid reversibility of ST_a-induced secretion and the relative "permanency" of CT-induced secretion (Fasano, 1999). Previous studies of affinity chromatography fail to recover the functional STa receptors from affinity matrices which contain STa covalently cross-linked to a solid support (Thompson, 1987 and Hugues & Waldman 1992). This suggests that the STa-receptor interaction is an irreversible process. However, Hugues et al. (1992) showed that bile salts completely dissociated ST-receptor interaction despite the low recovery of total ST binding activity, thus supporting the theory of reversibility of STa-receptor interaction.

Several groups of workers have studied the age-dependence, density and distribution of STa receptors in different models including human, pig, rat, rabbit and calf intestines (Cohen et al. 1986; Forte et al. 1989; Jaso-Friedman et al. 1992; Katwa and White 1992; Krause et al. 1994 and Al-Majali et al. 1999 a, b&c). In all of these models, a consistent increase in STa receptor density along the brush border membrane was observed in immature host. This coincides with the period of high susceptibility to STa-induced diarrhea that occurs in the early life of human and animals. Study of distribution and characterization of STa-specific receptors on enterocytes and brush border membrane vesicles (BBMVs) from different intestinal segments of newborn calves found that higher density and affinity of STa receptors on enterocytes and BBMVs derived from the ileum than other intestinal segments (Al-Majali et al. 1999 a&b). Moreover, ileal villous epithelial cells have approximately twice as many receptors as crypt cells for the enterotoxin (Giannella et

al. 1983 and Frantz et al. 1984). This strongly explains why STa act more rapidly than LT.

The endogenous agonist for GC-C is 15- amino- acid hormone called guanylin, which contains four cysteines and is less potent than STa in activating GC-C and stimulating chloride secretion (Carpick & Gariepy 1993; Currie et al. 1992 and Forte & Currie 1995). Guanylin presumably plays a role in basal gut homeostasis, and STa opportunistically utilize GC-C to alter ion transport in the gut. The STa enterotoxin brings its secretory effect through the GC/cGMP pathway.

Intestinal fluid and electrolyte homeostasis

Most of enteropathogens, including ETEC, target normal processes of intestinal water and electrolyte transport. Consequently, an understanding of the physiology of intestinal fluid and ion transport is essential.

Fluid is both secreted, to provide an environment that promotes the digestion and absorption of ingested nutrients, and absorbed, to avoid excessive fluid loss and resulting dehydration (Berne & Levy 1996 and Montrose et al. 1999).

Intestinal absorptive mechanisms

Sodium ions play a central role in net nutrient and water intestinal absorption (Chang & Bookstein 2000). Glucose, amino acids and other nutrients are absorbed, through an ion selective channel, or by a process coupled transport of Na^+ via a Na^+/H^+ cation exchanger and Cl^-/HCO_3 anion exchanger. All of these pathways involve Na/K-ATPase pumps on the basolateral membrane of the enterocyts, (Chang & Bookstein 2000).

Intestinal secretory mechanisms

The primary driving force for secretory fluid fluxes in the intestine is the movement of Cl^- from the blood circulation to the intestinal lumen. Chloride accumulate in the cytosol above its electrochemical equilibrium in response to the sodium concentration gradient established by the basolateral Na, K- ATPase and exit across the apical membrane when Cl^- channels at that site open in response to changes in second messengers. The most important of these Cl^- channels is the cystic fibrosis transmembrane conductance regulator (CFTR) (Barrett and Bertelsen, 2003).

Role of cyclic nucleotides in intestinal homeostasis

The important role of both cAMP and cGMP in the control of intestinal epithelial ion transport has been documented (Rao et al. 1981 and Donowitz and Welsh 1986). Cyclic AMP (cAMP) is generated from ATP in response to the activation of adenylate cyclase while cGMP is generated from GTP through the action of guanylyl cyclase. Both cyclases are membrane bound enzyme and are activated in the presence of appropriate agonists.

The cAMP-dependent Cl^- secretion is induced through protein kinase A (PKA) (Berger et al. 1993, Shlatz et al. 1979) while cGMP-induced Cl^- secretion has been documented to occur via a protein kinase GII (PKGII) pathway (Selveraj et al. 2000). Both PKA and PKGII appear to be capable of phosphorylating and thereby activating of CFTR in the presence of appropriate levels of intracellular signaling.

Absorptive mechanisms may also be affected by changes in cyclic nucleotide, although in this case it can be inhibitory. Na^+/H^+ exchangers (NHE3) are also regulated by phosphorylation events via cAMP-induced PKA action; however this

activation has an inhibitory effect on NHE3 with the subsequent inhibition of Na absorption. Therefore, activation of PKA leads to inhibition of NHE3 and activation of CFTR stimulating Cl^- secretion and inhibiting neutral Na^+/Cl^- absorption (Yun et al. 1995).

Pathogenesis of ETEC-STa Diarrhea

ETEC diarrhea occurs in the first few days of life, particularly in the most colostrum-deprived calves. Three criteria must be met for ETEC to produce illness: the ETEC must express adhesive factors that allow it to adhere to the small intestine; the ETEC must possess the plasmid that encodes for the enterotoxin(s), and sufficient numbers of the ETEC must be ingested. The pathogenesis of ETEC diarrhea involves three steps: establishment of infection, intestinal colonization, and finally elaboration of diarrheagenic enterotoxin(s) leading to an acute profuse watery diarrhea and potentially fatal dehydration, metabolic acidosis and electrolyte imbalances (Argenzio 1985).

Infection with ETEC

Calves contract infection with *E. coli* from the environment during or shortly after birth, often by fecal-oral route of transmission (Smith, 1965 and Butler & Clarke, 1994). Under normal circumstances, nonpathogenic types of *E. coli* are among the first bacterial species to gain access the intestinal tract, and they are prevalent throughout the intestine by the end of the first day of life with marked increase in number from the proximal to the distal portion of the small intestine (Smith, 1965 and Acres, 1985). In healthy calves, the majority of these nonpathogenic *E. coli* are suspended in intestinal contents and are constantly

propelled caudally by peristaltic and flow of ingesta and their number rarely exceed 10^7/g intestinal content. However, when ETEC are ingested they multiply and colonize the small intestine in larger numbers. Rapid establishment of *E. coli* infection is favored by several characteristics of newborn calves including a relatively high abomasal pH, sluggish intestinal motility and the absence of competing microflora (Acres, 1985).

Intestinal colonization

The ability to produce enterotoxin alone is not sufficient for ETEC to cause diarrhea. The bacteria must also able to colonize the mucosal surface of epithelial cells of the small intestine. This depends on adhesive factors that are composed of nonflagellar, flimentous fimbriae that are protein in nature. These fimbriae bind to specific receptors in the membrane of cells of the small intestine and allow ETEC to overcome the peristaltic cleansing mechanisms of the intestine. In normal calves, the majority of *E. coli* are in the lumenal content and only 10-20 % attached to the mucosa. In contrast, in claves with enteric colibacillosis the situation is reversed and 80 % to 90% of ETEC are attached the mucosa (Hadad and Gyles, 1982a&b). The dynamics of this process have been studied in colostrums-deprived calves where it appears that colonization begins at the ileal-cecal junction within 3 hours of infection and progress anteriorly to involve up to 60% of the small intestine by 16 hours post infection.

The precise molecular mechanism of attachment is not well understood, however bacterial fimbrial are definitely involved in this process (Smith & Huggins, 1978 and Hadad & Gyles, 1982a&b). Both the surface of ETEC bacterial cells and

intestinal epithelial cell possess a negative net charge which tends to repel each other. To overcome this physicochemical property, adhesive organelles, fimbriae K99, protrude from the bacteria cell surface and initiate attachment by extending across the zone of repulsion to reach specific complex receptors on the epithelial cell surfaces of the small intestine and act as a ligand to bind the bacterial cell to the intestinal surface (Acres, 1985 and Donnenberg, 2000). Receptors are probably sugar residues located in the cell membrane of the epithelial cells and those which bind to K99 or F41 fimbriae appears to contain sialic acid (Faris et al. 1980). Following primary attachment, ETEC multiply and form microcolonies that cover the intestinal villi (Chan et al. 1982 and Bellamy & Acres, 1979). Colonization of the posterior half of the intestine is the central event in the ileum and there may be as many as 10^9-10^{10} ETEC/g of intestinal contents (Smith and Huggins, 1978).

The degree of intestinal colonization varies among ETEC strains and is probably affected by serotype as well as physiological and nutritional factors in the small intestine (Chan et al. 1982, Isaacson, 1980 and Bellamy & Acres, 1979). Maximum colonization seems to occur with strains that are fimbriated and encapsulated (Smith & Huggins, 1978 and Hadad & Gyles, 1982b). In addition, some strains posses only one type of fimbriae whereas others posses more than one type which may intensify colonization and increase virulence (Orskov et al. 1977).

Elaboration of enterotoxin: Pathophysiology of STa action

When large numbers of ETEC colonize the small intestine, sufficient STa is produced to cause diarrhea. Presumably, enterotoxin is released by the bacteria and diffuses toward the brush border where it binds to receptor sites on the membrane of

the intestinal epithelial cells. Enterotoxin alters normal movement of ions and water across the intestinal mucosa by exerting a hormone-like effect on the enterocytes (Moon, 1974 & 1978). Fluid and electrolytes constantly move across the intestinal mucosa in two directions: from the intestinal lumen to the blood (absorption), and from blood to intestinal lumen (secretion). Secretory and absorptive fluxes occur simultaneously, and the net movement of fluid is the difference between the two processes (Finco et al. 1973 and Moon 1994).

The villus-crypt unit is the functional apparatus through which these fluxes occur (Finco et al. 1973). Immature enterocytes in the crypt are the main secretory cells, whereas more mature villous enterocytes are responsible for digestion and absorption. In normal calves, absorptive fluxes exceed secretory fluxes creating a state of net absorption (Finco et al. 1973 and Acres, 1985). STa appears to act by stimulating secretion as well as reducing absorption, and this dual effect reverses the normal pattern causing a net secretion of fluid into the small intestine and resulting in hypersecretory diarrhea (Rao et al. 1980).The total volume of each unidirectional flux in normal calves 4-6 week of age is between 80-144 liters/day (Bywater, 1973). Calves infected with ETEC may lose between 1.0-2.7 liters of diarrheal fluid in 24 hours, which represents only 1-2% of the normal unidirectional flux volume (Fisher and Martinez, 1975). This illustrates that only a small percentage change of the normal capacity of the small intestine is required to cause severe diarrhea.

STa induces secretory diarrhea by activating guanylyl cyclase-C and increasing cGMP (Figure 4). Disruption of the gene encoding GC-C in mice resulted in resistance to ST-induced diarrhea, demonstrating that GC-C is absolutely required

for ST-induced intestinal secretion (Mann et al. 1997 and Schulz et al. 1997). Cystic fibrosis transmembrane conductance regulator (CFTR), chloride channel, is a key component mediating the enterotoxigenic effect of STa (Golin-Bisello et al. 2005). In the absence of functional CFTR, ST and cGMP analogs fail to induce diarrhea (Quinton, 1990). Cyclic GMP activates CFTR and promotes chloride efflux, which presumably drives water transport into the lumen of the intestine. The intermediate steps involved in the CFTR phosphorylation are controversial, though the roles for both cGMP-dependent kinases and cAMP-dependent kinases have been reported (Sears and Kaper, 1996).

GMP-dependent kinases (PKG) appear to be the principal molecular target of cGMP in the signal sequence leading to CFTR activation. Both PKG-Iα and PKG-II phosphorylate CFTR, in vitro, with similar kinetics, suggesting the absence of a specific PKG-mediated function in this process (French et al. 1995). However, PKG-II., but not PKG-Iα colocalizes with GC-C in brush borders of enterocytes and activates CFTR in excised membrane patches of various cell lines transfected with CFTR (Lohmann et al. 1997) suggesting that PKG-II is a major physiological mediator of CFTR activation in small intestine (Pfeifer et al. 1996; Vaandrager et al. 1997 & 2000). The role cAMP-dependent kinases (PKA) are also reported. They may be activated either directly by cGMP or indirectly by local accumulation of cAMP in response to inhibition of PDE3 by cGMP and leads to CFTR activation. Vaandrager et al (2000) reported that STa induce electrogenic chloride secretion in the colon and jejunum of PKG II-deficient mice.

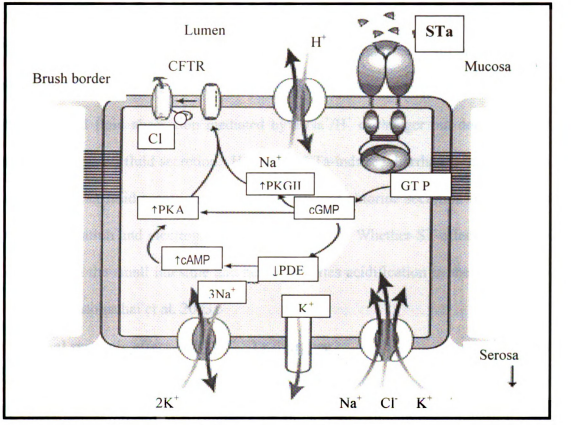


Figure 4. Pathophysiology of *E. coli* STa diarrhea. "Enterotoxigenic *E. coli*, containing plasmids encoding a member of the homologous peptide family of STs colonize the intestine after the consumption of contaminated food and/or water. Bacterial colonization leads to production of ST in the gut lumen, which specifically recognizes and binds to the extracellular domain of GC-C, expressed in the brush border membranes of intestinal mucosa cells from the duodenum to the rectum. Interaction of ST to the extracellular domain of GC-C is translated across the plasma membrane into activation of the cytoplasmic catalytic domain resulting in the production and accumulation of [cGMP]. This cyclic nucleotide binds to and activates PKG II, also localized in the intestinal cell brush border membrane. Also, cGMP may activate PKA, either directly or by inhibiting a cAMP-specific PDE and inducing the accumulation of cAMP. The CFTR that is colocalized with GC-C and PKG II in brush border membranes is a substrate for that protein kinase and PKA. CFTR is a chloride channel, and its phosphorylation by PKA or PKG results in a persistent open state, permitting chloride to flow down its concentration gradient from the intracellular to the extracellular compartment. Other ion channels and transporters in the cell maintain the electroneutrality of ST-induced chloride efflux. Vectoral water flux from the basolateral to the apical surface is driven by these ionic conductances, resulting in the accumulation of fluid and electrolytes in the intestinal lumen and secretory diarrhea" (Lucas et al. 2000)

In addition to CFTR activation, recent evidence suggests a role for inhibition of brush border membrane electroneutral sodium absorption, possibly mediated by a Na^+/H^+ exchanger, in mechanisms underlying STa-induced fluid and electrolyte secretion (Vaandrager et al. 2000). Lucas and colleagues (2001 & 2005) reported that STa reduced fluid absorption mediated by a Na^+/H^+ exchanger but does not act by causing increased fluid secretion. Hence, the STa-induced diarrhea is the result of an increase in net fluid secretion due to stimulation of chloride secretion in response to CFTR activation and electrogenic chloride transport. Whether ST affects the Na^+/H^+ exchange in the small intestine and hence modifies acidification in the lumen is still unknown (Laohachai et al. 2003).

Potential systemic effect of ETEC-STa diarrhea

The systemic effects of ETEC-STa diarrhea, which eventually contribute to deaths, are precipitated by a single event, the loss of extracellular fluid (ECF). Four major potentially fatal can be identified due to the loss of ECF: dehydration, electrolyte imbalances, metabolic acidosis and negative energy balance (Fisher, 1965, Barber et al. 1975; Moon et al. 1978; Argenzio, 1985; Booth and Naylor, 1987; Groutides and Michell 1990 and Grove-White, 1996).

Metabolic acidosis is an important consequence of ETEC diarrhea and a number of factors contribute to its development. A major factor is the loss of bicarbonate ions in feces and additionally, an increase in the production of lactic acid in poorly perfused tissue and reduction in renal hydrogen ions excretion secondary to dehydration (Booth and Naylor, 1987).

Hyperkalemia is a complex sequence to ETEC diarrhea and occurs despite a significant loss of potassium ions (K^+) in the feces. This paradoxical situation is the result of acidosis and compromised renal function. A shift of potassium ions from intracellular to ECF compartment in exchange with the excess extracellular hydrogen ions occurs to maintain cell electroneutrality (Lewis and Phillips, 1972). This redistribution of K^+ ions in response to acidosis causes a reduction in the resting membrane potential leading to serious and eventually lethal effects on cardiac muscle function (Fisher 1965, Fisher and McEwan, 1967 and Phillips and Knox, 1969).

Hyponatremia and hypochloremia result from significant fecal loss of sodium and chloride ions. Hypoglycemia frequently occurs in the last stage severe ETEC diarrhea near death. Anorexia, decreased absorption of nutrients, minimal glycogen reserves, inhibited gluconeogenesis, and increased anaerobic glycolysis in poorly perfused tissues may all contribute to the negative energy balance (Hall et al. 1992).

Current Approaches for Controlling ETEC-STa- induced Diarrhea

Several approaches for the treatment and control of ETEC diarrhea are briefly described below:

Antibiotic-based therapy: The use of sub-therapeutic doses of antibiotics may help protect animals from some but not all of the bacterial strains. However, the use of antimicrobial agents at sub-therapeutic levels has been linked to the problem of emerging antibiotic resistance among several bacterial species, including ETEC strains.

Mechanism-based therapy: Therapeutic trails were also explored in an attempt to approach ETEC-STa diarrhea through its putative receptors (Mechanism-based

therapy). Mann et al. 1997 and Schulz et al. 1997 showed that disruption of the gene encoding GC-C in mice resulted in resistance to ST-induced diarrhea. However disruption of genes involved in the regulation of intestinal secretion may have adverse effect on other host's cell function. One study showed the GCC knockout- mice were resistant to STa-induced diarrhea but they suffered of over 33 percent stunted growth compared to normal mice (Pfeifer et al. 1996).

Another study targeted the path of the signaling cascade mediated by STa using 2-chloroadenosine triphosphate (2-chloroATP) to interrupt STa stimulation of chloride current and water secretion in Caco-2 cells (Zhang et al. 1999). However, models of STa-induced secretion developed *in vivo* are particularly unsuited to examine the therapeutic efficacy of prodrug (2ClAdo) that requires time-dependent transport and metabolic conversion to the active moiety of 2-chloroATP. Moreover, the pharmacokinetic barriers to maintain this active moiety in enterocytes *in vivo* limit examination of the utility of this approach to prevent STa-induced diarrhea.

Immunity-based therapy

Active immunization

Active immunization through vaccination is an effective way to control infectious agents. However, the current strategy for development of a broad-spectrum vaccine against ETEC targeting the pili antigen has been challenged by the numerous intestinal colonization factors expressed by ETEC and continuous antigenic drift of pili antigens (Boedeker, 2005 and Walker et al. 2007).

Passive immunization

Antibody-based therapy, which includes pathogen-specific and non pathogen-specific modalities, plays an important role in modern therapy against several diseases, including ETEC. However, due to the lack of common ETEC antigen candidates, there are no immunotherapeutic reagents that can confer broad protection against the wide array of ETEC strains. The use of STa toxin as a common antigen to confer a broad protection against 75% of ETEC is rationalized. However, antibodies raised against STa toxin have been limited by several factors including the poor immunogenicity of the STa, and the fact that the design of an effective immunogenic STa is dependant on a successful conjugation reaction. Antibody-based therapy against STa is also challenged by the large amount of STa antibodies required to neutralize the local effect of the STa (Svennerholm and Steele, 2004).

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CHAPTER III

PURIFICATION AND CHARACTERIZATION OF HEAT-STABLE ENTEROTOXIN (STa) OF ENTEROTOIXIGENIC *Escherichia coli*

Introduction

Enterotoxigenic strains of *Escherichia coli* (ETEC) have been implicated as causative agents of diarrhea in human infants (Guerrant et al. 975 and Sack et al. 1975), diarrhea in neonatal domestic animals (Smith and Halls, 1967 and Whipp et al. 1975) and in "travelers" diarrhea (Jiang et al. 2000). ETEC strains are capable of producing at least two distinct forms of extracellular enterotoxins. One form is called heat-labile enterotoxin (LT), which is structurally and functionally similar to cholera toxin (CT) (Gyles 1971 and Evans et al. 1973), and the other form is heat-stable enterotoxin (STa), which is a low-molecular-weight (< 2kD) peptide that is non-immunogenic and is not neutralized by antibodies specific to CT (Gyles 1971 and Evans et al. 1973 and Naline et al. 1974). Seventy five percent of tested ETEC strains have been found to produce STa alone or in combination of LT (Wolf, 1997). Therefore, STa is considered to be an important virulence determinant in enterotoxin-mediated diarrheal diseases caused by *E. coli* (Sears & Kaper, 1996 and Giannella & Elizabeth, 2003).

The purification and characterization of STa have been complicated by the complexity of various growth media used for toxin production and by laborious purification procedures such as salt precipitation, ultrafiltration, ion exchange and molecular sieve chromatography. These procedures are time consuming and have a

relatively low recovery yield of homogenous STa (Staples et al. 1980, Saeed et al. 1983, and Saeed & Greenberg 1985).

In this study, we adopted the growth medium and purification methods reported by Staples et al. (1980) and modified by Saeed et al. (1983) and Saeed and Greenberg (1985) under controlled growth conditions using a 36 L Bellco bioreactor.

Material and methods

Animals

Swiss Webster Mice: A group of 20 Swiss-Webster (fifteen females and five males) was used to establish a colony as a source of suckling mice for STa bioassay. Exhausted females and males were continuously replaced with younger animals to ensure production efficiency of infant mice litters by the colony.

Reagents

All reagents were obtained from commercial sources and were of analytical grade. Mobile phases used for purification of STa include HPLC-grade methanol, triflouroacetic acid, as well as the other chemical ingredients listed under this section.

Verifying the ETEC K99$^+$ Strain

Basic PCR protocol described by Olsvick and Strockbine (1993) and Salvadori et al. (2003) was used to detect the STa gene and verify the strain as STa-producing *E. coli*.

Bacterial Strains: An ETEC strain was isolated from a clinically diarrheic neonatal calf and was provided by A. M. Saeed (Molecular Epidemiology Laboratory, National Food Safety Toxicology Center (NFST), Michigan State University (MSU),

East Lansing MI. A control strain (K-12 *E. coli*) was kindly obtained from the Bacterial Evolution Laboratory, NFST, MSU, East Lansing- MI.

DNA Extraction (Template) by boiling lysis: ETEC and K-12 strains were grown on Tripticase Soy Agar slants overnight at 37°C. A uniform bacterial colony from both strains was taken and suspended in 1 ml sterile Milli Q water and boiled for 10 min, then left in ice for 5 min, followed by centrifugation at 13,000 rpm for 4 min. The supernatant was taken and kept at -20 °C until use (Holmes and Quigley,1981).

Primer selection and preparation: Two different sizes of STa primer, 244bp and 127bp (Table 3), were obtained from Integrated DNA Technology Inc. (Coralville, IA). Both primers were prepared according to the manufacturer's instructions.

Table 3. PCR primers used to detect STa gene

Primer	Sequence (5-3)	Base pair	Reference
STa	5'- TCC GTG AAA CAA CAT GAC GG-3' 5'- ATA ACA TCC AGC ACA GGC AG-3'	244	Salvadori et al. 2003
STI	5'-TTA ATA GCA CCC GGT ACA AGC AGG-3' 5'-CTT GAC TCT TCA AAA GAG AAA ATT AC-3'	127	Olsvick, and Strockbine 1993

***PCR program*:** PCR running conditions for detection of the STa gene are presented in Table 4 using PCT-100 Programmable Thermal Controller (MJ Research Inc).

Table 4. PCR running conditions for detection of STa gene

Step	Temperature (°C)	Time (min)
Pre-denature	95	5
Denature	95	1
Annealing	60	1
Extension	72	1
Final extension	72	10
Storage	4	24 hours

Number of PCR cycles 29 before storage

PCR reaction: The PCR reaction was performed as described in table 5 using Fisher *ex*ACTGene Complete PCR kit.

Table 5. PCR reaction for detection of STa gene

Component	Amount/sample (µl)	# of Samples	Amount/two rxns Volume (µl)
10x buffer A	2	2.5	5
dNTP (10mM)	0.4	2.5	1
Sta-F (20µM)	0.4	2.5	1
Sta-R (20 µM)	0.4	2.5	1
25 mM $MgCl_2$	0	0	0
Fisher Taq polymerase	0.1	2.5	1
Ultrapure water	14.7	2.5	36.75
Total volume	20		45
Template	2		

Agarose Gel Electrophoresis Analysis of PCR Products: The analysis of the PCR products was performed in 2% agarose gel electrophoresis using the Horizontal Gel Electrophoresis System, Life Technology (Cat # 11068-012). Briefly, two percent of agarose was prepared (1.5 gm/75 ml 1xTAE electrophoretic sequence grade) and ethidium bromide was added at concentration 3 µl/ 50 ml. The reagent was poured into the electrophoretic chamber and filled with 1xTAE. Five volumes of PCR product were mixed with 1 volume of gel loading buffer and loaded into the wells along with a 1.5 kb ladder. The agarose gel was left to run at the appropriate voltage (100-160 volts) for 30-45 min and then examined under UV light.

Purification and Characterization of *E. coli* STa

STa was purified according to the protocol reported by Staples et al. (1980) and modified by Saeed et al. (1983) and Saeed and Greenberg (1985).

Seed culture and frozen stock of ETEC preparation

Casamino acid-yeast extract-salts (CAYE) seed culture was used for optimal growth of the ETEC strain (Giannella, 1976). The ETEC strain was grown on 500 ml of CAYE, incubated at 39 °C for 24 hours on a rotary shaker at 120 rpm, then mixed with glycerol at a final concentration of 15%, then aliquoted into 10 ml samples and frozen at -80 °C (frozen stock).

Batch medium (Asparagine salt medium) and growth conditions (Staples et al. 1980):

Table 6. Composition of optimal minimal medium for STa production (g/L)

NaCl	2.52	Na_2SO_4	0.14
Na acetate	10.00	$MgSO_4$	0.05
$K_2HPO_4.3H_2O$	8.12	$MnCl_2$ 1%	0.5 ml
Asparagine	5.00	$FeCl_3$ 1%	0.5 ml

We used the Asparagine-salt medium (ASM) reported by Staples et al. (1980) that was compared with other growth media by Saeed et al. (1983) and found to offer several advantages, including high level of STa production along with minimal contaminating proteins that facilitated the STa-purification process. Each batch consisted of 30 liters of culture-innoculated ASM grown in a 36L omni vessel under different pH conditions (7.4, 8 and 8.6) using a Bellco bioreactor (Bellco Glass Inc. Vineland NJ). Preculture was prepared by inoculating 10 ml of frozen stock of ETEC into 1L of CAYE broth and was incubated at 39 °C for 24 hours on a rotary shaker at 120 rpm. The preculture medium was then transferred into 30 liters of batch medium and kept at 39°C under continuous agitation at 120 rpm, aeration and oxygenation

were at a rate of 5 L/min and 600 ml/min, respectively, through a sintered metal dispersion ring. Foam, speed of agitation, temperature and PO_2 were controlled using respective Bellco control modules. Samples were taken every two hours to determine the growth kinetics under various pH levels.

Preparation of cell free filtrate

After 24 hours of incubation, the growth medium was immediately filtered by tangential flow filtration through a 0.2-μ cassette in Millipore Pellicon System (Millipore Crop, Bedford, MA). Cell free filtrate was kept on ice throughout the time of filtration to minimize bacterial growth and enzymatic activity. Samples from the cell-free filtrate were collected for determination of total protein and STa content using suckling mouse assay.

Amberlite XAD-2 Batch Adsorption Chromatograph

Cell free filtrate was desalted and the hydrophobic STa was concentrated using Amberlite XAD-2 batch adsorption chromatography. Amberlite XAD-2 resin was first washed extensively with purified water to remove any preservative and powdery contaminants. Then 500g were suspended into 15 L of cell free filtrate in a 20- L carboy and kept overnight at 4 °C under gentle stirring. Resin was poured from the carboy into a 40 cm long glass column and washed with 5 L of Milli Q water. The contaminants loosely bound to the resin were eluted with 1L of 1% acetic acid in 20% methanol/water (v/v). A stepwise elution system was applied to elute the STa starting with 1L of 1% acetic acid in 80% methanol/water (v/v) followed by 1L of 1% acetic acid in 99% methanol/water (v/v) and finally 1 L of 50% acetone/water (v/v). The last three fractions were pooled and concentrated by flash evaporation & freeze-drying.

The resin was degassed for 5 minutes after each solvent was added to drain completely before further addition of solvent. Samples were collected for determination of the total protein and testing for STa biological activity in suckling mouse.

Acetone Fractionation

Lyophilized crude STa was dissolved in 20 ml of 25% of acetic acid. Acetone was added to bring the final volume to 100-150 ml. After standing 1 hour at 4 °C, the sample was centrifuged at 10,000g for 30 minutes at 4 °C. The supernatant fraction was evaporated to remove the acetone and was then freeze-dried. Samples were collected for protein determination and STa biological activity.

Reversed-Phase Batch Adsorption Chromatography (MCI-gel)

An intermediate purification step was applied to the acetone STa-rich fraction to achieve a further level of STa purity. The lyophilized crude STa was solubilized in 100 ml of 0.1% of 20% HPLC-grade methanol. To this solution, 100g of Reverse-Phase Methacrylate Adsorbent Polymer Resin (340 °A 30 μm Mitsubishi Chemical Corporation, Cat # CHP2MGY-01L) was slowly added under gentle mixing and the slurry was kept at 4 °C for 2 hours under gentle shaking. The slurry was poured into a 10-mm-i.d.x 25-cm-long glass column. The column was washed with 300ml of 0.1%TFA/H_2O (v/v). Stepwise elution of the proteins was performed with 100 ml of 0.1%TFA of 20, 40, 60, 80 &100% MeOH (v/v). Fractions were collected separately from each elution step. The methanol and TFA were evaporated and the residues were tested for protein and STa biological activity.

Preparative Reverse-Phase High Performance Liquid Chromatography (RP-HPLC)

RP-HPLC was performed on Waters Associate Liquid Chromatography System equipped with multi-solvent delivery pumps, an automated gradient programmer 600S controller, Model 486 tunable absorbance detector using 7 μm, 300 Å, 25cm x 10 mm i.d Vydac C8 preparative columns (Sorbent Technologies, Inc, Atlanta, GA). Samples from RP-methacrylate adsorbent polymer resin were applied on RP-C8 column and STa was eluted by gradient system with 0.1% TFA in water as solvent A & 0.1%TFA in 80% methanol as solvent B (0-30% 5min and 30-80% in 80 min). The UV absorbing peaks were detected at 214 nm. Peaks were collected separately and the methanol was evaporated and then freeze-dried. The freeze-dried substance was reconstituted into physiological saline and tested for protein content and STa biological activity.

STa assessment for biological activity

Detecting and quantifying STa biological activity was done using a reference standard in vivo model test, suckling mouse assay, according to Dean et al. (1972) and Giannella, (1976). Newborn Swiss Albino suckling mice (2-3 days old) were randomly divided into groups (three each). Samples from RP-HPLC were serially diluted 1/100; 1/10,000 & 100,000 and 10 μl of 0.2% Evans blue (w/v) was added per ml. Each suckling mouse was inoculated orally by 100-μl sample using a 1 ml syringe and a 20-μ-diameter polyethylene tube. Each sample dilution was tested in triplicate. After 2-hour incubation at room temperature, the mice were euthanized by carbon

dioxide in a CO_2 chamber and the intestine (not including the stomach) from each newborn mouse was removed and weighed.

The ratios of intestinal weight to remaining body weight of the three mice were determined. Animals with no dye in the intestine or with dye within the peritoneal cavity at autopsy were discarded. One unit of ST activity (one mouse unit) is defined as the minimal amount of toxin that produces an intestinal weight/carcass ratio of ≥ 0.083.

Criteria for homogeneity of purified STa

Homogeneity of the purified STa from preparative runs was validated by analytic aquapore RP-300Å Perkin Elmer C8 column. Additionally, the exact molecular weight of STa was determined by matrix-assisted laser desorption ionization-time of flight mass spectroscopy (MALDI-TOF/MS). The purified STa was then submitted for amino acid sequencing.

Results and Discussion

Detection of STa gene

PCR amplification verified that the tested strain carried the gene encoding for STa after analyzing the product on gel electrophoresis. Two amplicon bands of 127 bp and 244 bp were detected under UV light for the tested strain, which were not apparent for the control strain (*E. coli* K-12) (Figure 5).

Culture analysis

Growth kinetics experiments were conducted on 30L batch cultures under various pH values (7.4, 8 and 8.5). Samples were taken every two hours and the growth pattern was determined by counting the total cell count (CFU/ml) using a

robotic spiral plate and computer linked camera (Q counter). As Figure 6 indicates, tested ETEC growth was maximal in medium in which the initial pH was adjusted to 7.4. This level of growth was associated with higher level of crude STa as verified by the suckling mouse assay.

Purification and Characterization of *E. coli* STa

Table 7 shows the summary of the purification scheme of STa for the ETEC *E. coli* in 30 L batch culture.

Amberlite XAD-2 Batch Adsorption Chromatography

This step yields a high specific activity of the crude STa (8.70×10^3 MU/mg protein) compared with the STa specific activity in the cell free filtrate (1.22×10^3 MU/mg protein).

Acetone fractionation

Acetone fractionation resulted in further purification of the STa by removing additional amount of non-STa protein that was precipitated in acetone. Samples were taken for protein determination and STa biological activity. Specific activity of STa increased to 88.7×10^3 MU/mg protein.

Reversed Phase-Batch Adsorption Chromatography (MCI-gel)

Specific activity of the STa at this step of purification increased from 88.7×10^3 to 112×10^3 MU/mg protein. This step allowed for a larger sample load on preparative RP-HPLC. Up to 15 mg of the crude STa cleaned by this procedure could be used as a single load in RP-HPLC without overloading the column or losing the resolution. This has led to a considerable reduction in the number of HPLC runs needed to purify STa.

Preparative Reverse-Phase HPLC Chromatography (RP-HPLC):

Sixty percent methanol MCI-gel STa-rich fractions were loaded on a preparative C8 column for further purification. Figures 7 and 8 describe the elution profiles of STa. Elution with an increasing methanol gradient resulted in number of absorbance peaks at 214 nm (Figure 7), the last of which was found to contain enterotoxin activity. The enterotoxin peak began to elute at approximately 55-60% methanol after 35 minutes retention time. This peak was collected and after methanol evaporation, was freeze-dried. It was then reconstituted into physiological saline and tested for STa biological activity and protein concentration. Further improvement in the STa specific activity (885 x 10^4 MU/mg) was achieved in this step. The biological activity was demonstrated to be 0.113 ng per one mouse unit of STa minimal effective dose (MED) in 2-3 day-old inoculated Swiss Webster suckling mice.

Criteria for homogeneity of purified STa

- Analytic C8 column: Pooled peaks from several preparative RP-HPLC runs were tested on an analytic aquapore RP-300Å Perkin Elmer C8 column to demonstrate a single symmetrical peak (Figure 8).
- ***Mr-value determination using Matrix assisted laser desorption ionization-time of flight/mass spectroscopy:*** A lyophilized HPLC-purified sample was analyzed by MALDI-TOF/MS to determine the molecular weight and the result is shown in Figure 9. The observed signal at 100% MS intensity with m/c = 1972.1 indicates that the M*r* of the purified product is 1972.1 Da, which is compatible with the M*r* (1969-1972) value calculated from amino acid composition of the STa, confirming the purity and identity of the purified

product as the STa molecule. This was in agreement with the findings of Takao et al. (1983).

- ***Amino acid sequence:*** Further confirmation of the homogeneity and identity of the purified product was performed by determination of amino acid sequence. A lyophilized HPLC-purified sample was submitted for amino acid sequence analysis and the results showed the 18 amino acid residues of the STa molecule were matching the reported sequence.

Conclusion

This protocol includes concentrating the cell free filtrate using Amberlite XAD-2 batch adsorption chromatography (BAC), acetone fractionation, and Methacrylate polymer resin BAC and finally through RP-HPLC. Chemical analysis of the purified preparations matched the reported structure for this type of enterotoxin. The biological activity was demonstrated to be less than 0.2 ng per one Mouse Unit of the STa in 2-3 day-old inoculated Swiss Webster suckling mice. In summary, purification of STa to homogeneity was accomplished and the purity of the produced STa was documented through amino acid sequencing, and mass spectroscopy.

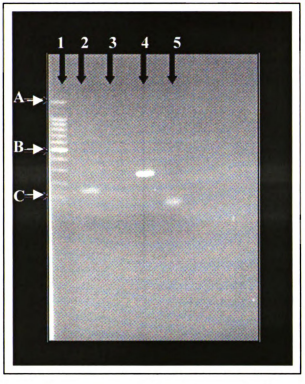

Figure 5. Agarose gel electrophoresis of PCR product

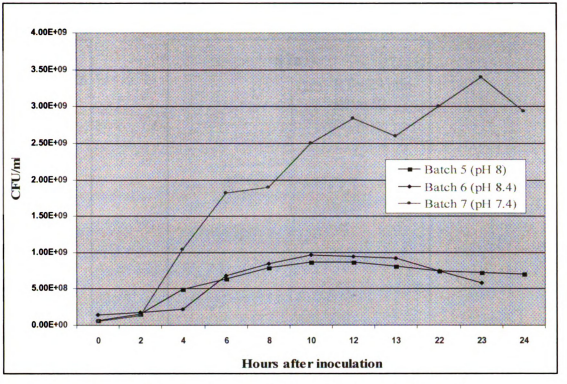

Figure 6. Growth kinetic of ETEC on 36L batch ASM under different pH using Bellco bioreactor

1. 1500 bp ladder (DNA marker) 2. Clinical *E. coli* isolate tested with 127bp primer 3. Control *E. coli* strain (K12) tested with 127bp primer, 4. Clinical *E. coli* isolate tested with 244 bp primer, 5. Control *E. coli* strain (K12) tested with 244bp. A. 1500 bp ladder band B. 500 bp ladder band C. 100 bp ladder band

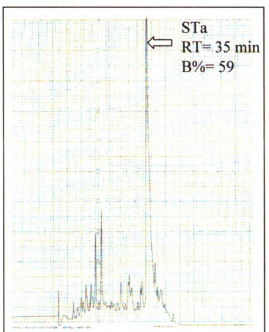

Figure 7. Revere-phase-high performance liquid chromatography elution profile of 60% HPLC-grade methanol- MCI gel - STa-rich fraction on preparative C8 Vaydac column

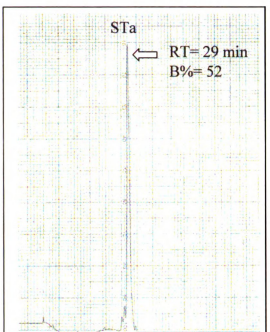

Figure 8. Elution profile of biologically active *E. coli* heat-stable enterotoxin peaks on analytic aquapore reverse-phase C 8 Perkin Elmer column

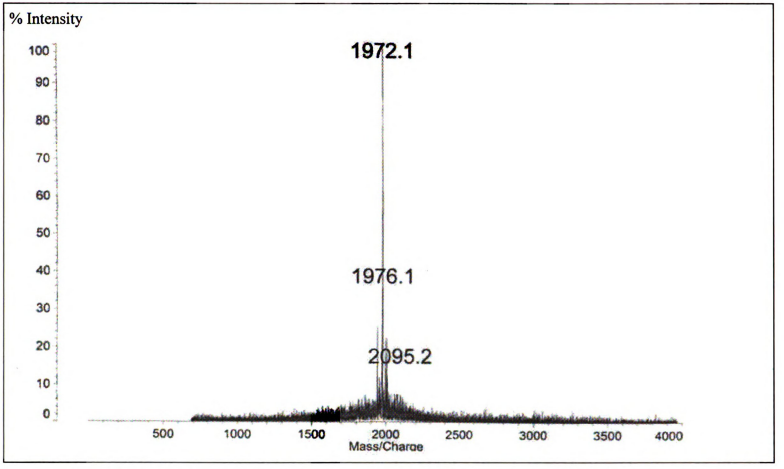

Figure 9. Matrix assisted laser desorption ionization-time of flight mass spectroscopy profile of *E. coli* heat-stable enterotoxin

Table 7. Summary of the purification of *E. coli* STa per batch

Step	Volume ml	Titer	Total MU/ 10^7	Protein Conc /mg	Sp Ac MU x 10^3/mg	MED ng	Purification fold
Cell Free Filtrate	30 x 10^3	10^{-2}	3	24,660	1.22	822	1
Amberlite XAD-2 BAC	120	10^{-4}	1.2	1,378	8.70	114.9	7.13
Acetone Fractionation	60	10^{-5}	6	676.8	88.7	11.28	72.67
60%MCI-gel F	30	10^{-5}	3	267.6	112.10	8.92	91.88
RP-HPLC	80	10^{-6}	80	90.4	8849.56	0.113	7253.28

MU = Mouse Unit = minimal amount of toxin producing intestinal weight to remaining body weight ratio $\geq$ 0.085

Sp Ac = Specific activity = total mouse unit/protein concentration

MED = Minimal effective dose = protein concentration per mg/total mouse unit per million

BAC = Batch absorption chromatography

Purification fold = specific activity of STa from each step/ specific activity of STa in the cell free filtrate

Protein assay was done by Lowery method (Lowery 1951) using Perkin Elmer Spectrophotometer

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CHAPTER IV

DESIGN AND CHARACTERIZATION OF AN IMMUNOGENIC *Escherichia coli* HEAT-STABLE ENTEROTOXIN (STa)[1]

Introduction

Heat-stable enterotoxin (STa) is an important virulence determinant in enterotoxin-mediated diseases caused by *E. coli* (Sears & Kaper, 1996; Nataro & Kaper, 1998 and Giannella & Elizabeth, 2003). The immune system has difficulty in eliciting antibody against STa because of its small molecular weight (<2 kDa) (De Weck, 1974 and Pereira et al. 2001). However, proteins of molecular size 1,000-3,000 Da can be made immunogenic by conjugation to a suitable macromolecule (carrier protein) (Elanger, 1980 and Pauillac et al. 1998). A wide range of carrier proteins and several procedures for conjugation have been reported for the preparation of peptide-carrier conjugates using various cross-linkers. The selection depends on the chemistry of conjugation and the location of antigenic determinants (epitopes) within the native peptide.

Conjugation of STa to several types of carrier protein have been reported, however, no sufficient details were presented on the efficiency and the characteristics of these conjugates (Houghten et al. 1984, 1985; Klipstein et al. 1982, 1983; Sanchez et al. 1986, 1988; Clements, 1990 and Thompson and Giannella, 1990). In this study, purified STa peptide was covalently cross-linked to the modified bovine serum albumin (BSA) through the amino terminal of the STa to preserve the biologically active moiety that is perceived to be associated with carboxyl terminus of the peptide.

[1] Manuscript of this chapter is under preparation

This study aims to design and characterize an efficient immunogenic STa conjugates based on the evaluation of four different conjugation protocols.

Background and principles of peptide -carrier conjugation

Conjugation of proteins involves the linkage of two or more molecules to form a novel complex which has the combined properties or features of its individual components (Hermanson, 1996). Ideally, there are two critical criteria that should be met in any conjugation procedure. The first is that the coupling process should provide a high yield of well-defined and reproducible composition without inactivation of the peptide molecule. Secondly, it should yield a stable linkage between the peptide and carrier (Pauillac et al. 1998 and Fuentes et al. 2005).

The strategies of the coupling process rely on the presence of functional moieties, which can be used to facilitate the conjugation of the peptide to a carrier protein. These moieties include carboxylic acids, amines, thiols, anhydrides, *N*-maleimides, imino esters and others. Depending on the functional moiety used, the reactive group may be the ε-amino group of a lysine residue, α-amino of an *N*-terminal (NH_3,), sufhydryl [SH] group of cysteine, or the carboxylic acid [COO^-] groups (Asp, Glu, or alpha-carboxyl). These moieties are capable of forming covalent bonds between two protein species (Dick & Beurret 1989 and Wong, 1991).

Cross- linking reagents

Cross-linkers contain reactive ends to specific functional groups (primary amines, sulfhydryls, etc.) on protein molecules (Wong, 1991 and Hermanson, 1996). Several cross-linkers are available and can be used to covalently link one molecule to

another. Most commonly used are carbodiimide derivatives, glutaraldehyde (GA), and *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS).

Carbodiimide derivatives, both the water soluble derivative, 1-ethyl-3-(3-dimethylaminopropyl carbodiimide hydrochloride (EDAC) and the organic soluble derivative, *N,N*-dicyclohexylcarbodiimide (DCC) are commonly used. Carbodiimides are heterobifunctional reagents that are mainly used for coupling –COOH and $-NH_2$ groups (Goodfriend et al. 1964 and Kurzer et al. 1967). They can activate the side chain carboxylic groups of aspartic and glutamic acid, as well as the carboxyl terminal group, to make reactive sites for coupling with primary amines (Yamada et al. 1981).

Glutaraldehyde is a bifunctional coupling reagent that links two compounds through their amino groups (Habeeb & Hiramoto, 1968; Richard & Knowles, 1968 and Russel and Hopwood, 1976). Although glutaraldehyde provides a highly flexible spacer between the peptide and carrier protein for favorable presentation to the immune system, it is a very reactive compound and will react with Cys, Tyr and His to a limited extent and the result is a poorly defined conjugate (Molin et al. 1978).

The *m*-Maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) is a hetero-bifunctional reagent that can be used to link peptides to carrier proteins via cysteines (Carlson et al. 1978; King et al. 1978 and Yoshitaki et al. 1979). The coupling takes place with the thiol group (SH) of cysteine residues.

Carrier Proteins

Carrier proteins are macromolecules capable of stimulating the immune cells to elicit an antibody response (Harlow and Lane, 1988). Peptides lack the T-cell

epitopes, which are essential for priming $CD4^+$ T helper cells. The carrier molecule provides the T-cell antigenic determinants for T-cell signaling, proliferation and release of mediators which activate specific B cells to stimulate antibody production in response to the immunogenic peptide-carrier conjugate (De Silva et al. 1999). Structurally, peptide-carrier conjugates have native antigenic determinants of the carrier as well as the peptide epiotpes. Introducing this conjugate into a suitable host generates peptide-specific antibodies (Brinkley, 1992) as well as carrier antibodies.

Peptides have been coupled to several carrier proteins. The most commonly selected carriers are bovine serum albumin (BSA) and keyhole limpet hemacyanin (KLH). BSA is one of the most widely used carriers for the design of peptide-carrier conjugates. It is highly antigenic (M.W. ~67 kD) and can be easily modified to introduce a new moiety for specific coupling procedures (Habeeb 1967a&b). However, BSA is used as a blocking agent in many experimental immunoassays (ELISA), so antibodies raised against peptide-BSA conjugates will limit the application of the standard ELISA procedure and different blocking agents are needed.

Although KLH is large (1.3×10^7 Da) and immunogenic, it may precipitate during cross-linking making it difficult to use in some cases. Moreover, the serum antibody response to this carrier often obscures that of the antigen of interest (De Silva et al. 1999) resulting in a reduced antigen-specific antibody concentration in the IgG fraction. Ovalbumin (OVA), rabbit serum albumin (RSA) thyroglobulin (TG) or synthetic carriers such as multiple antigenic peptides (MAPS) can also be used as carrier proteins (Wong, 1991 and Hermanson, 1996).

It is important to recognize that the immune system reacts to the peptide-protein carrier as a whole and that there will be a portion of response directed against the conjugated peptide as well as the carrier protein. Thus, coupling should be done to keep the antigen of interest in as native a condition as possible (Harlow and Lane, 1988). De Silva et al. (1999) showed that during preparation of peptide-carrier conjugates, one must strive to preserve the integrity of the antigen structure since many applications involving the antibodies require discrimination with very minimal conformational differences.

In this study, BSA was modified in order to incorporate extra functional carboxyl groups and used as a carrier to cross-link the STa molecules through their amino terminals (Figure 10). Four different conjugation protocols were evaluated to design a successful conjugate that retained high biological activity for further use in the study of its immunogenicity.

Materials and methods

Reagents

All reagents were obtained from commercial sources and were of analytical grade. Bovine serum albumin (BSA), succinic anhydride (SA), dioxane, *N,N*-dicyclohexylcarbodiimide (DCC), 1-ethyl-3-(3-dimethylaminopropyl carbodiimide hydrochloride (EDAC), *N*-methy-imidazole, dimethyformamide (DMF), 2-(*N*-morpholino) ethanesulfonic acid buffer (MES), triethylamine (ET_3N), *p*-nitrophenol, sodium azide (NaN_3) and phosphate buffer saline (PBS) tablets were obtained from Sigma Chemical Co. (St. Louis, MO). STa was purified and a detailed methodology was described in the previous chapter.

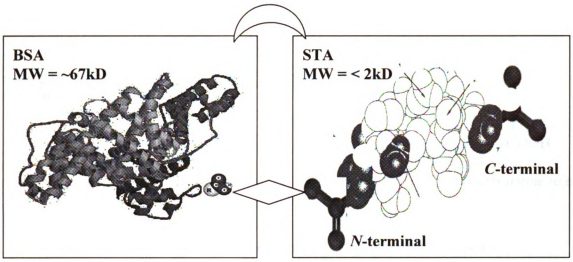

Figure 10. Illustration showing the conjugate of bovine serum albumin carrier protein- *E. coli* STa peptide

Procedure for covalently cross-linking STa with modified BSA

Chemical modification of bovine serum albumin

Bovine serum albumin was chemically modified to introduce new carboxyl moieties using two different protocols:

- Succinylation
- Hyper-succinylation

Succinylation of Bovine Serum Albumin

Basis of reaction: Succinic anhydride (SA) reacts rapidly with the ε-amino groups of lysines and the α-amino groups of the *N* -termini of proteins at pH 7-9, forming an amide bond by replacing the amino group with a carboxyl (Riordan & Vallee 1964, Gounaris & Perlmann 1976 and Merck & Co., Inc 1996) (Figure 11). Thus, introducing a succinic anhydride moiety on BSA will afford a protein derivative with more carboxyl groups and hence increase the possibility to link STa

from its amino terminal and preserve its antigenic determinants associated with the carboxyl terminal of the molecule.

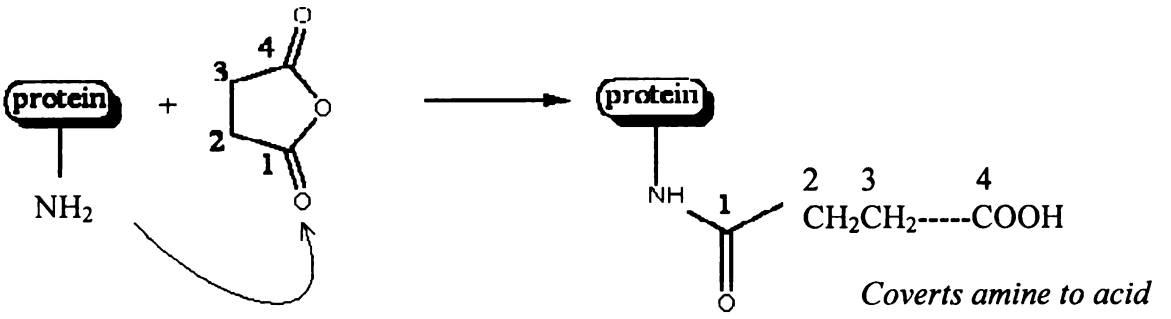

Figure 11. The action of succinic anhydride upon the amino groups of a protein

Procedure: The methods followed were those described by Habeeb (1967a, b). Briefly, one gram of BSA was dissolved in 200 ml of 0.2 M borate buffer, pH 9.3. A 20 ml solution of dioxane with 5.4 g succinic anhydride was added in small aliquots over a period 30 minutes, the reaction mixture was stirred magnetically while maintaining the pH at 9.3 through the addition of 3 M NaOH. Following the last addition of succinic anhydride, the acylation reaction was allowed to continue for 45 minutes. The solution was then dialyzed at 4°C against several changes of 0.01 M triethylamine using dialysis tubing with a M.W. cutoff of 12-14 KD. The dialyzed preparation was first freeze-dried and then further dried in a dessecator over phosphorous pentoxide (P_2O_5). Samples were taken and reconstituted in PBS buffer (pH 6.8) for size exclusion chromatography and mass spectroscopy.

***Hyper-succinylated Bovine Serum Albumin** (HS-BSA)*

Basis of reaction: An extensive modification of the BSA by introducing a large number of succinyl moieties [COO^-] on the carrier protein could be achieved in

the hyper-succinylation reaction. The hyper-succinylation reaction was carried out in two steps. The first step involves the production of hyperaminated BSA by conversion of all free carboxyl groups on the BSA (aspartic and glutamic acids) into amino groups. The second step was the production of hypersuccinylated BSA by addition of succinic anhydride to the hyperaminated BSA to convert all amino groups (newly introduced, free, and *N*-terminal) into carboxyl groups.

Procedure: Native BSA was treated with 1mM of ethylenediamine at pH 4.75 in the presence of 10 mM EDAC. This hyperaminated protein molecule was then treated with 100 mmoles of succinic anhydride at pH 8.0 for two hours to produce a hyper-succinylated BSA molecule (Fuentes et al. 2005).

Coupling of *E. coli* STa to modified BSA

Four different conjugation protocols were used to covalently cross-linking STa to modified BSA. They were evaluated on the basis of stability of covalent bond, retained STa biological activity and the conjugation efficiency.

Protocol 1: Using Dimethyformamide (DMF) as a solubilizer for the peptide and carrier protein

Atassi (1981) described the use of DMF to solubilize several synthetic peptides before cross-linking them to carrier proteins. This protocol enhances the coupling of the amino terminal ends of synthetic peptides to the carrier protein. In this study, the carrier protein (suBSA) was solubilized in DMF and then treated with *p*-nitrophenol to activate the carboxyl groups on the carrier.

Procedure: Applying this procedure, we have used the purified native STa peptide and have solubilized it in DMF prior to addition to the modified suBSA. The mixture was kept stirring overnight at room temperature. This design was meant to encourage

the STa coupling through its amino terminals based on nucleophilic attack at the reactive ester groups of the modified suBSA forming amide linkages. In a typical reaction, we used 140 mg of suBSA suspended in 10 ml of anhydrous DMF and stirred it magnetically in a tight-capped foil-wrapped bottle for 3-4 hours. A solution of *p*-nitrophenol (65mg/0.5ml) in DMF was added and magnetic stirring continued for 15 minutes. A solution of DCC (50 mg/0.5ml) was added to suBSA at a molar ratio of 120:1 and the reaction was allowed to continue stirring at room temperature for 3 hours. One hundred mg of STa in 1 ml DMF was added to the activated suBSA at a molar ratio of 24:1. Shortly after, 1 ml of triethylamine was added. The reaction mixture was stirred overnight, at room temperature, while protected from direct light. The next day, 30 ml of Milli Q water was added and the mixture was dialyzed extensively against distilled water at 4°C using a dialysis membrane with a 12-14 kD M.W. cutoff and then freeze-dried. Samples were taken for measurement of the STa biological activity of the conjugate using suckling mice assay (SMA), protein determination, biochemical and molecular characterization.

Protocol 2: Imidazole-based protocol

One coupling procedure (Dean et al. 1990) described the use of imidazole to stabilize the carrier protein and minimize the formation of polymers due to the acylation process when the cross linker and the peptides intended for cross-linking are added.

Procedure: We used 0.5 M *N*-methyl-imidazole pH (6.0) to dissolve the STa peptide and the carrier protein suBSA at a molar ratio (100:1). After the addition of EDAC (molar ratio: 50 mol EDAC/mol STa), the mixture was stirred for 30 minutes

at room temperature followed by dialysis (M.W. cutoff 12-14 kD) against distilled water at 4°C.

Protocol 3: Hyper-succinylated BSA-based protocol

Fuentes et al. (2005) reported that an increase in the numbers of succinyl groups [COO^-] on the carrier protein can enhance the cross linking of the peptides from their amino terminal. The procedure for hyper-succinylation of BSA was described above.

Procedure: In this coupling protocol, 3 mg hypersuccinylated-BSA was dissolved in 2.5 ml of 5 mM sodium phosphate buffer pH 7 and mixed with 2.5 ml of the STa peptide (0.5 mg/ml) in dioxane. EDAC was then added gradually to reach a concentration of 100 mM. After that, the conjugated composite was dialyzed using a tube with a M.W. cutoff 12-14 kD against distilled water at 4°C.

Protocol 4: Conventional peptide-carrier coupling protocol

In this coupling procedure, suBSA, EDAC and MES buffer were used (Uptima interchim, 2007).

Procedure: SuBSA carrier protein was dissolved in 0.1M MES buffer pH 5 to a final concentration of 10mg/ml. Two milligrams of STa peptide were added to 2 mg of suBSA carrier protein. Then, EDAC (10 mg /ml in cold distilled water) was added at a ratio of 0.5 mg of EDAC per mg of total protein. The reaction was kept stirring for 2-3 hours at room temperature before dialysis at 4 °C against PBS using a tube of 12-14 kD M.W. cutoff.

Dialysis

The products of BSA modification and STa-modified BSA conjugation reactions were subjected to extensive dialysis to remove the small molecular weight reactants (<14 kD). Dialysis tubing with nominal M.W. cut-off 12-14 kD was purchased from Fisher Scientific (Pittsburg, PA). STa-SuBSA conjugate was subjected to extensive dialysis against Milli Q purified water using a dialysis membrane of 12-14kD M.W. cutoff. Molecular species of 14kD or higher were retained inside the dialysis tube and all other reactants below 14 kD including uncoupled (free) toxin were dialyzed out.

Gel Filtration Chromatography (GFC)

PD-10 columns, Sephadex G-25M packed columns, of a nominal molecular mass exclusion limit of 5000 for protein were purchased from G.E. Healthcare (Buckinghamshire, UK). These columns are designed to separate proteins based on their molecular weight. The columns were equilibrated and developed by following the manufacturer's instructions. The dialyzed STa conjugate samples were passed through PD-10 Sephadex G-25 GFC column to purify the STa-suBSA conjugates from free STa, then assessed for biological activity and protein concentration.

A freeze-dried STa-suBSA conjugate sample was reconstituted into 2.5 ml of PBS and passed through a PD-10 column to separate the unconjugated STa peptide from the portion that was successfully cross linked to the carrier. The STa-carrier conjugate was then eluted with 3.5 ml PBS and the effluent was collected and tested for biological activity in a suckling mouse assay.

Size Exclusion-High Performance Liquid Chromatography

SE-HPLC was performed to compare the molecular size of both native and modified BSA. Bio-Sil® SEC-125 HPLC 300 x 7.8mm filtration column (Cat # 125-0060) was purchased from BioRad (Hercules, CA) and hooked to the Waters Associate Liquid Chromatography System equipped with multi-solvent delivery pumps, an automated gradient programmer 600S controller and a tunable absorbance detector (Model 486). The column was equilibrated with 0.05 M sodium phosphate (diabasic), 0.05M sodium phosphate (monobasic), 0.15M NaCl and 0.01 M NaN_3 at pH 6.8. Isocratic elution system was applied at flow rate 1 ml/min. The peak absorbance was monitored at UV 280 nm.

Amino acid compositional analysis

To determine the conjugation ratio of STa to the modified BSA, a freeze-dried STa-suBSA conjugate sample was subjected to amino acid compositional analysis at the Research Technology Support Facility (RTSF) at Michigan State University.

Matrix-assisted laser desorption ionization/time of flight mass spectroscopy (MALDI-TOF/MS)

Two hundreds micrograms of freeze-dried STa-suBSA conjugate sample were subjected to MALDI-TOF/MS analysis at the MSU-RTSF laboratory to determine precisely the molecular weight of the conjugate and use this figure to calculate the number of STa molecules that covalently cross-linked to one BSA molecule (conjugation ratio).

Protein Assay

Protein assays were performed according to the Lowery method (Lowry et al. 1951), using a Perkin Elmer Lambda 3A UV/US Spectrophotometer.

STa-suBSA conjugates activity bioassay

The biological activity of the STa-suBSA conjugates was determined using the suckling mouse assay as described by Dean et al. (1972), Giannella, (1976) and modified by Saeed et al. (1983).

Results

Table 8 shows the summary of conjugation experiments and their evaluation. More details on the DMF conjugation protocol are presented below.

Characteristics of the modified BSA

The results from SE-HPLC showed that modified BSA with succinic anhydride was eluted faster (RT= 2.99 min) than native BSA (RT= 4.20 min), suggesting that the molecular weight of suBSA had undergone a significance change (Figure 12). Change in the molecular weight of the succinylated BSA was also confirmed by MALDI-TOF/ MS. It was found that suBSA has a M.W. of 72.40 kD in comparison to native BSA which has a M.W. of ~67.00 kD (Figure 13).

Characteristics of *E. coli* STa-suBSA conjugates

Dialysis and Gel Filtration Chromatography

A summary of the results is presented in Table 8. The conjugation protocol based on the DMF method showed a higher rate of conjugation efficiency and higher level of retained STa biological activity compared to other conjugates (Table 8).

Further characterization of the DMF-based conjugation protocol is described below.***Amino acid compositional analysis***

A dialyzed conjugate sample based on the DMF protocol was subjected to amino acid compositional analysis. Table 9 shows the *p*mole concentration and retention time of each amino acid residue.

Calculation of STa Peptide to suBSA Ratio

The conjugation ratio is defined as the number of STa molecules covalently cross-linked to one molecule of succinylated BSA. The amino acid composition of the conjugate was empirically determined by measuring the *p*moles of each amino acid detected in a known volume of sample. Well-recovered residues, arginine and methionine, were used to quantify the concentration of each residue (*p*mole) in the conjugate sample. The number of STa molecules in the conjugate sample was calculated using the Arg & Meth residues not present within the sequence of the STa peptide. Table 10 gives the approximate number of coupled STa molecules to one molecule of modified BSA. Based on the data of amino acid compositional analysis, it was found that approximately 4-5 STa molecules were coupled to each molecule of suBSA.

STa Conjugate Analysis by MALDI-TOF/MS

Lyophilized conjugate samples based on DMF and HS-BSA protocols were subjected to MALDI-TOF/ MS to accurately determine the molecular weight (M*r* Value). Both samples showed median M*r* Values over 80 kDa (Figure 14 & 15). Figure 16 shows the molecular weight differences for the modified BSA before and after STa peptide conjugation (ΔM.W. = 8342.5 Da). This difference was attributed to

the contribution of the STa molecules. Based on this data, the median number of STa molecules successfully crossed linked to one molecule of suBSA was calculated from the following equation: Δ M.W. /STa M.W. = 8342.5/1959 = 4 -5. Four to five STa molecules were successfully crossed linked to one molecule of modified BSA based on DMF and HS-BSA protocols.

STa conjugate activity bioassay & Conjugation efficiency

We concluded that the STa biological activity and the conjugation efficiency of the conjugate were highest in the DMF protocol. A summary of conjugation efficiency based on the tested protocols expressed largely by the conjugation ratios and the retained STa biological activities of the conjugates is presented in Table 8.

Discussion

Numerous attempts have been made to render STa immunogenic, including chemical coupling and genetic fusions to appropriate carrier proteins (Houghten et al. 1984, 1985, Sanchez et al. 1986, 1988, Clements, 1990). However, results of these studies showed limited success since the uncontrolled cross-linking process led to the loss of the biological activity of STa as a part of the conjugation process (Pereira et al. 2001). Additionally, these studies showed no sufficient details on the efficiency and the characteristics of the produced STa conjugates.

The objective of this study was to design and characterize a well-defined, stable and active STa conjugate for further study of its immunogenicity in laboratory animal models. We have evaluated several conjugation protocols to achieve a stable biologically active STa conjugate through carefully planned cross-linking of the STa

peptide to a modified carrier protein using BSA, carbodiimide derivatives and different solvents.

Given the perceived molecular structure of the STa peptide and the desire to crosslink it through its amino terminus, we have selected carbodiimide coupling reagents. Other coupling reagents, glutaraldehyde and *m*-Maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS), may affect the 3-demientional structure of the STa peptide. Glutaraldehyde binds non-specific amino groups and this leads to polymerization of peptide and/ or carrier protein, which results in a poorly defined conjugate (Molin et al. 1978). Cysteine residues on the STa peptide play a crucial role in the biological activity and the stability of STa peptide. Thus, using MBS as a hetero-bifunctional reagent targeting thiol group on cysteine residue (Carlson et al. 1978; King et al. 1978 and Yoshitaki et al. 1979) may disrupt the disulfide bonds and affect the biological moieties on the STa peptide. BSA is widely used as a carrier protein in conjugation reactions because it is highly antigenic and can be easily modified to introduce a new moiety for specific coupling procedures (Habeeb 1967a&b). Therefore, the use of carbodiimide and BSA, in our conjugation reaction was justified based on a thorough understanding of the molecular structure of the STa peptide.

In this study, BSA was modified by introducing succinic moieties, and its modification was confirmed using size exclusion chromatography and MALDI-TOF/MS. The data showed a 5000 Da difference in the molecular size between the modified and native BSA molecules, indicating an 8% increases in the M.W. of the modified BSA. This suggests that an extensive modification of the free amino groups

was achieved with the addition of succinic anhydride (Habeeb, 1967b). The subsequent step in the design of STa-BSA conjugate was the cross-linking of STa to the modified BSA. This reaction was initiated by incubation of the modified BSA with *p*-nitrophenol and DCC for three hours to provide reactive ester groups that could easily attach the STa from its amino terminal, forming amide linkages. The use of DMF was shown to enhance the solubility of reactants including peptides and carrier proteins ((Lateef, 2007). We believe that the use of DMF as a solvent reagent may have facilitated the solubility of the hydrophobic STa molecules, solving a problem encountered with the other solvents and coupling media.

The STa-conjugate was tested for its protein content and biological activity. Based on the protein estimation, there was a conjugation efficiency of 52-64%, which is higher than previously reported (Frantz & Robertson 19981; Frantz et al. 1987; and Thompson and Giannella, 1990). Moreover, this conjugate showed a higher biological activity than any activity reported in the previous STa-conjugates (Table 8). Based on these results, it is clear that most of the biological activity of the STa introduced into this reaction was retained in the conjugate even after extensive dialysis, GFC and SEC.

Covalent attachment of STa molecules to modified BSA was documented by amino acid composition analysis and MALDI-TOF/ MS. A median value for the conjugation ratio of 4-5:1 STa:suBSA has been determined based on amino acid analysis and MALDI-TOF/MS (Figure 16). Based on the results of the biological activity of this conjugate, we believe strongly that STa molecules may have been more efficiently oriented on the BSA carrier molecule via linkage through their amino

terminals. Such orientation, achieved through the DMF protocol, has preserved the biologically active moiety of the STa and may offer an explanation for the relatively low yield of STa conjugate produced by other protocols in this study. The ineffective preservation and presentation of the STa biologically active moiety on previously studied STa conjugates may also explain the sub-optimal immune response against STa in laboratory animals (Alderete and Robertson 1978; Lockwood and Robertson 1984; Houghten et al. 1984, 1985; Sanchez et al. 1986, 1988; Clements, 1990; Löwenadler et al. 1991 and Pereira et al. 2001).

In summary, we have designed a well-defined STa-conjugate based on a thorough understanding of the molecular structure of the STa peptide. After careful evaluation of several peptide-carrier conjugation protocols, we have defined the conditions for a conjugate that expressed a high STa biological activity in suckling mice. Its stability and biochemical attributes were characterized using GFC, amino acid analysis and MALDI-TOF/mass spectroscopy.

Table 8. Summary of conjugation experiments: Evaluation of four different conjugation protocols

	SuBSA/DMF Method	HSBSA/PB Method	SuBSA/Imidazole Method	Conventional Method
Carrier	suBSA	HS-BSA	suBSA	SuBSA
Cross linker	Organic soluble carbodiimide (DCC)	Water soluble carbodiimide (EDAC)	Water soluble carbodiimide (EDAC)	Water soluble carbodiimide (EDAC)
Medium	*N,N*, DMF	5 mM PB (Na2HPO4/NaH2PO4)	Imidazole	0.1 M MES
pH	7-9	7	7.2	5
Stating reactants	10 mg STa + 14 mg suBSA + 6.5 mg *p*-NP	5 mg STa + 12 mg HS-BSA	10 mg STa + 3.3 mg suBSA	2 mg STa + 2 mg suBSA
Total STa MU	10×10^6	$48x10^4$	$4x10^6$	$3x10^5$
Protein Assay	1.96mg/ml	0.277mg/ml	1.8mg/ml	1.2mg/ml
Conjugation ratio	4-5:1	4:1	-	-
Conjugation efficiency (Lowery protein)	~52-64%	~ 26%	~ 40.6%	~30%
Conjugation efficiency (retained biological activity)	100%	~20%	40%	30%
Reference	Atassi et al 1981	Fuentes et al 2005	Dean et al. 1990	Uptima, 2007

DCC= N,N, dicyclohexyl carbodiimide. EDAC=1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride
DMF= Dimethylformamide. PB= Phosphate buffer. MES=2-(*N*-morpholino)ethanesulfonic acid buffer
suBSA= succinylated BSA. HS= Hypersuccinylated BSA
P-NP= *P*-nitrophenol

Table 9. Amino acid compositional analysis of *E. coli* STa-su BSA carrier conjugate

Serial #	Name	RT	Amount pmole	Serial #	Name	RT	Amount pmole
1	Gln	1.78	5.983	**10**	Tyr	37.09	499.248
2	Glu	20.63	549.781	**11**	Cys	37.53	509.796
3	Ser	25.61	147.557	**12**	Val	37.85	297.137
4	Gly	27.42	329.482	**13**	Meth	39.214	38.214
5	His	29.29	174.214	**14**	Ile	43.65	118.619
6	Thr	30.47	311.339	**15**	Leu	44.80	576.721
7	Arg	31.57	219.833	**16**	Lys	46.87	333.965
8	Ala	31.886	507.974	**17**	Phe	38.22	386.2
9	Pro	33.18	377.778	**18**			

Table 10. Approximate contribution of STa molecules to one molecule of modified BSA: calculation of the conjugation ratio

Residue	Total # of residue in STa-suBSA conjugate sample	# of residue /one BSA molecule	# of residue /one STa molecule	Conjugation Ratio
Threonine	311.339/8.00 = 38.9	34 residues	1	38.9-34 = 4-5
Cysteine	509.796/8.00 = 63.6	35 residues	6	63.6-35 = 28.6/6 =4-5
Leucine	576.721/8.00 = 72	65 residues	1	72-65 = 7
Alanine	507.974/8.00 = 63.5	48 residues	2	63.5-48= 15.5/2 = 7-8

*Brown, 1975; Patterson and Geller, 1977; McGillivray et al. 1979; Reed et al. 1975 and Hirayama et al. 1990.

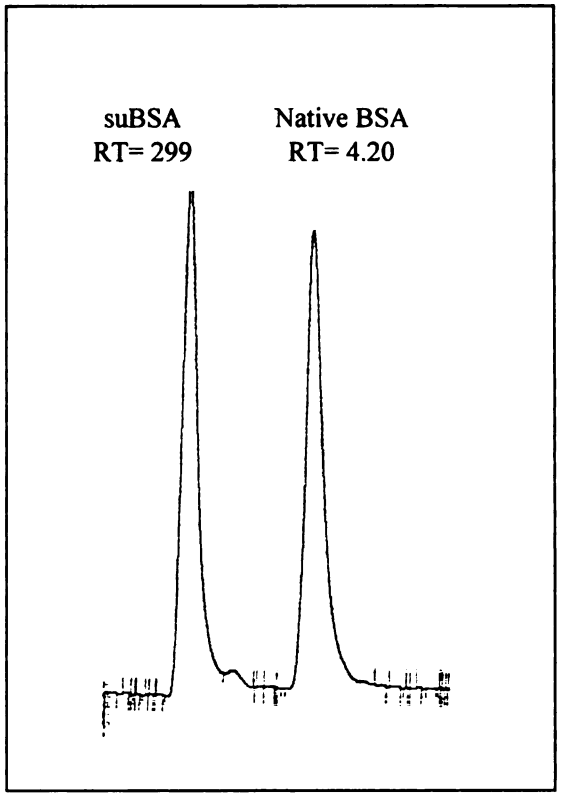

Figure 12. Size exclusion chromatography profile of native and succinylated BSA molecule

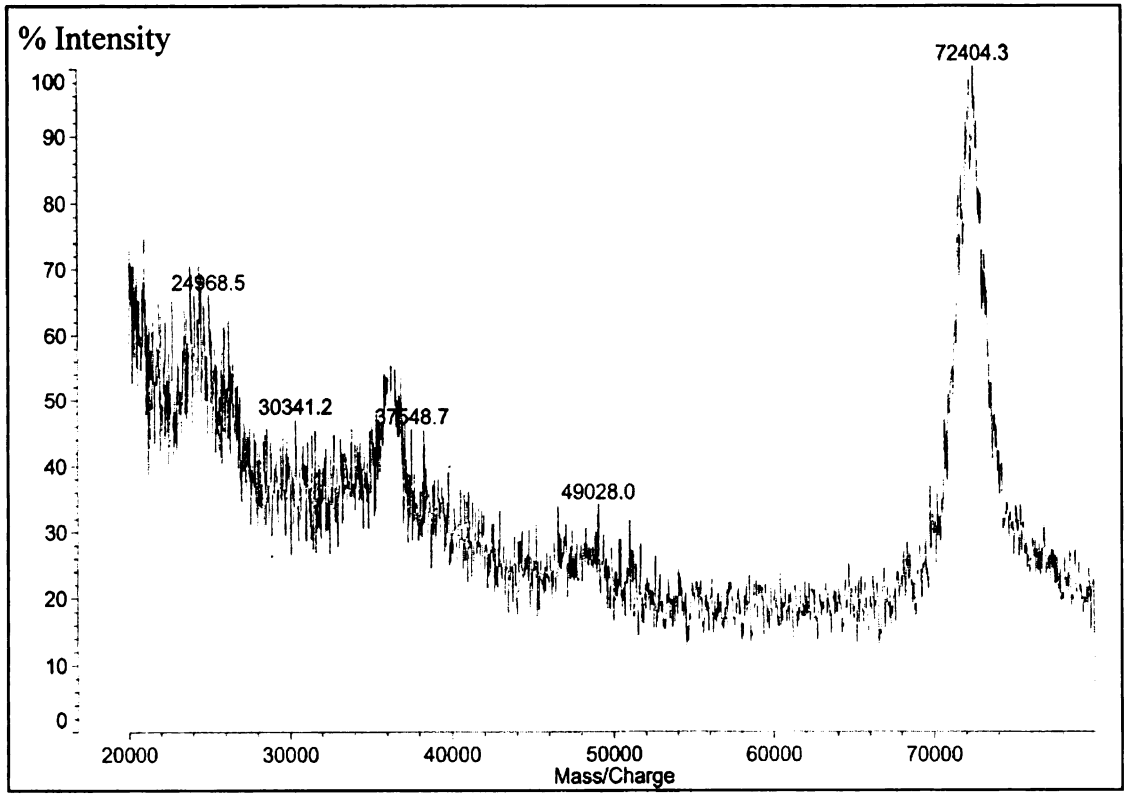

Figure 13. Matrix assisted laser desorption ionization-time of flight mass spectroscopy profile of succinylated BSA molecule

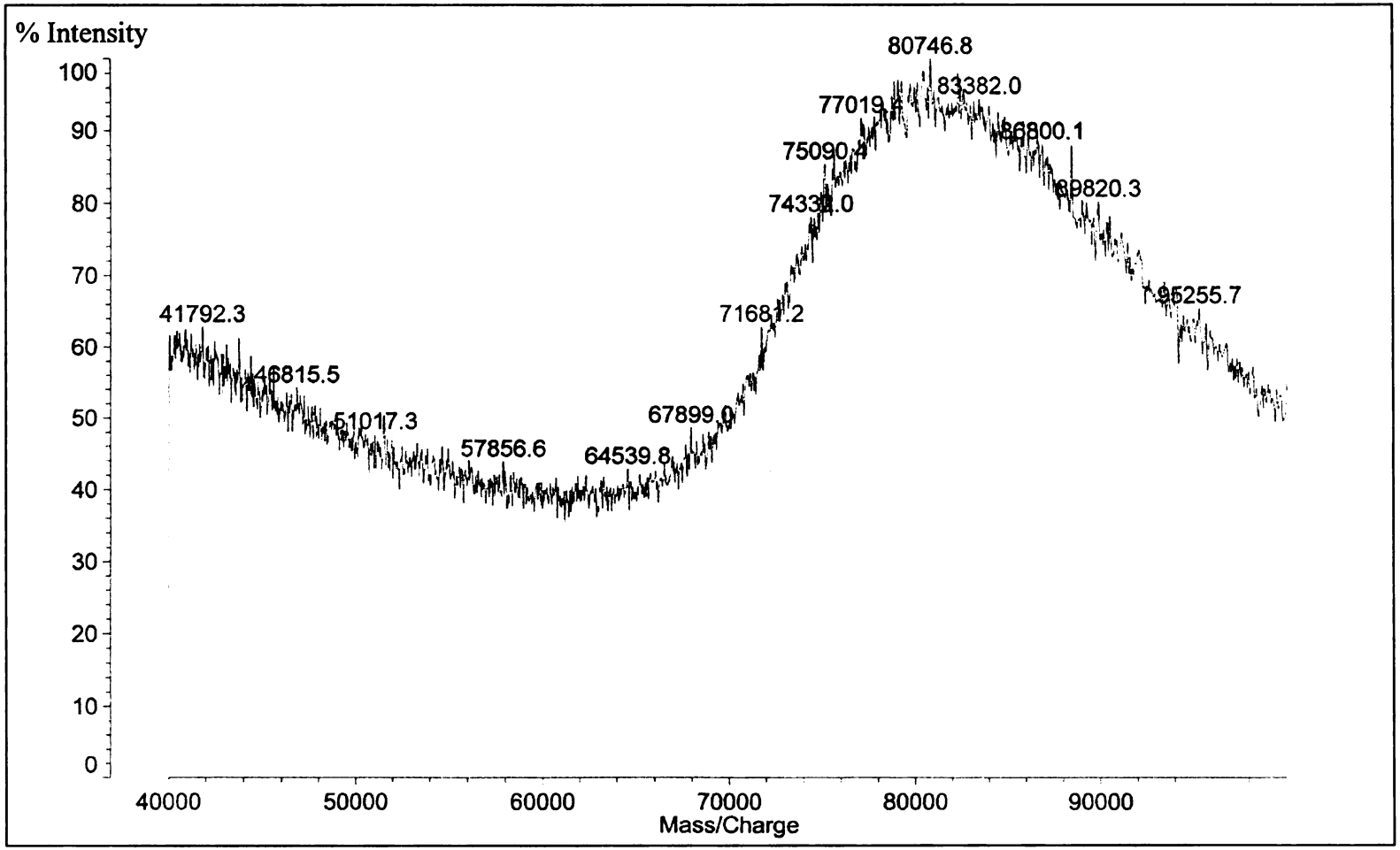

Figure 14. Matrix assisted laser desorption ionization-time of flight mass spectroscopy profile of *E. coli* heat-stable enterotoxin peptide-succinylated BSA carrier conjugate

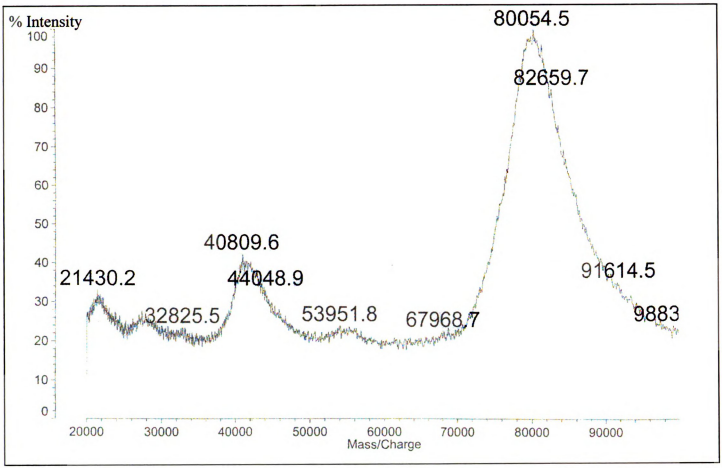

Figure 15. Matrix assisted laser desorption ionization-time of flight mass spectroscopy profile of *E. coli* heat-stable enterotoxin peptide-hypersuccinylated BSA carrier conjugate

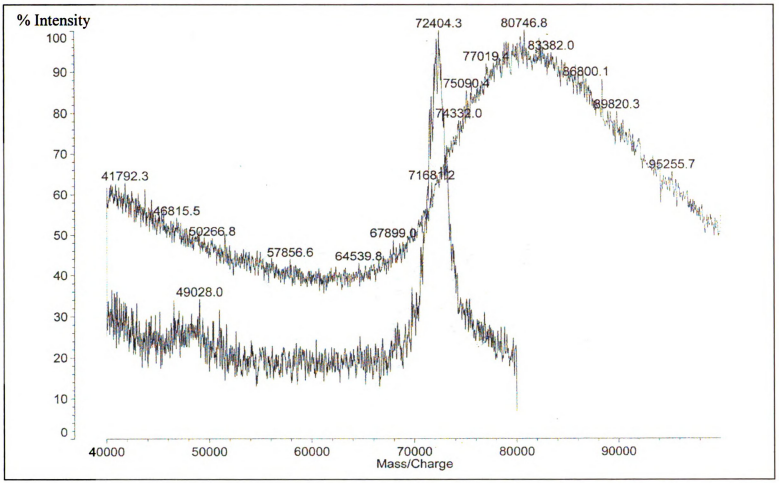

Figure 16. Calculation of Δ molecular weight and conjugation ratio of the *E. coli* heat-stable enterotoxin peptide-suBSA carrier conjugate

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CHAPTER V

PRODUCTION OF SPECIFIC ANTIBODIES AND CHARACTERIZATION OF THE IMMUNE RESPONSE AGAINST *Escherichia coli* HEAT-STABLE ENTEROTOXIN[1]

Introduction

Strains of Enterotoxigenic *Escherichia coli* (ETEC) that produce STa are among the most important causes of diarrheal disease in children, travelers and neonatal animals (Gyles, 1996, Kosek et al. 2003, Svennerholm and Steele, 2004). The changing epidemiology of ETEC strains including the emergence of new pathotypes (toxin/CFs combinations) presents serious challenges for vaccine development. The antigenic diversity of ETEC strains and the nonimmunogenic property of STa account for a high prevalence of ETEC-STa-diarrheal cases in endemic areas. ETEC strains are incriminated as the cause of 280-400 million diarrheal episodes in children below 5 years and additional 100 million episodes in children aged 4-14 years (Svennerholm and Steele, 2004) each year.

Currently, there are no effective vaccines or immunotherapeutic reagents that confer a broad protection against ETEC-induced diarrhea. Intestinal colonization by ETEC is a prerequisite for the initiation of diarrhea. However, the colonization factors that facilitate the ETEC adhesion may undergo an antigenic evolution causing failure of the currently used CFA-based vaccines. Therefore targeting STa, as the immediate mediator of ETEC-induced diarrhea, may be the logical approach for the development of a new immunotherapeutic reagent or an effective vaccine against ETEC diarrhea (Boedeker, 2005 and Walker et al. 2007). Numerous attempts were made to render STa immunogenic, including chemical coupling and genetic fusions to appropriate

[1] Manuscript of this chapter is under preparation

carrier proteins (Houghten et al. 1984, 1985, Sanchez et al. 1986, 1988 and Clements, 1990) but the described chemical coupling reduced the STa-associated toxicity as a result of the STa conjugation to a carrier protein. The use of reported STa-conjugates did not result in the production of quality STa-neutralizing antibody from the immunized animals (Cárdenas & Clements, 1993 and Pereira et al. 2001). In this study, we have developed a biochemically-defined STa-suBSA conjugate and used it for immunization of New Zealand rabbits. The immune response during the immunization process was monitored by the specific binding and neutralization capacities of the produced antisera against purified native ETEC-STa.

Materials and methods

Reagents and instruments

STa- suBSA conjugates were designed and characterized as described in the previous chapter. All buffers ingredients, Freund's complete and incomplete adjuvant, alkaline phosphatase labeled goat-anti-rabbit IgG antibodies, *p*-nitrophenyl phosphate, fish gelatin, Tween-20 and ammonium thiocyanate (NH_4SCN) were obtained from Sigma Chemical Co. (St. Louis, MO). Costar 3590- 96-welll microtiter plates were obtained from Fisher Scientific (Fairlawn, NJ). Molecular Devices ThemoMax Microplate reader equipped with SOFT max Pro 2.6.1 was used to read the ELISA plate. A bleeding set (coagulant vacutainer tubes, adaptors and 20 gauge vacutainer needles) was obtained from B-D. (Franklin Lakes, NJ) and used for rabbit bleeding.

Animals

Ten female New-Zealand albino rabbits (2-4 kg) were obtained from Charles River Laboratories (Wilmington, MA) and were housed in approved-size-single cages at the Containment Facility of Michigan State University. Temperature was kept at 20°C ± 4°C, with 55% humidity. Rabbits were checked on a daily basis for their health status by qualified staff and veterinarians.

Immunization procedure

Standard operating procedures for handling and immunization of rabbits in compliance with the guidelines and recommendations for the Institutional Animal Care & Use Committee (IACUC) of Michigan State University were used.

A water-in-oil emulsion of STa-conjugate in Freund's adjuvant was prepared as follows: 20 mg of freeze-dried STa-suBSA conjugate was reconstituted in 10ml 0.01M PBS-pH 7.0 and added to 10 ml of Freund's complete adjuvant (primary immunization). The mixture was homogenized with a polytron at 15,000 rpm for up to 10 minutes or until a stable water-in-oil emulsion was obtained. The ten rabbits were inoculated at multiple intradermal sites as described by Vaitukaitis (1981) with 0.5 ml of the described emulsion. Each rabbit was similarly inoculated with a booster dose at three-week intervals with 0.5 ml of STa conjugates mixed with Freund's incomplete adjuvant. Rabbits received boosters until STa-neutralizing antibodies titers were detected.

Animal bleeding

Pre-immunization blood samples were collected after one weak of adaptation from the central ear artery according to Gordon (1981) using serum separator BD-

vacutainer tubes to obtain a reference baseline for serum titers. Blood samples were then collected three weeks after the primary immunization, approximately 4-5 days after each booster immunization. After collection, blood was allowed to clot for 60 min. at 37°C. The clot was then separated from the sides of the collection vessel and allowed to contract at 4°C overnight. The separated sera were collected by centrifugation at 2000 rpm for 30 min, aliquoted and kept at -20°C. The sera were tested for neutralization and binding capacity against STa using suckling mouse assay (SMA) and indirect ELISA binding assay respectively.

STa- Serum Neutralization Assay

The STa-serum neutralization capacity was determined using the SMA as described by Frantz et al. (1987). Briefly, three 50 µl aliquots of serum sample with were incubated with 25, 50 and 75 µl of STa (20 Mouse Units per µl) at 37°C for 2 hours. The contents of each tube were brought to a final volume of 0.5 ml with PBS, before bioassay. Three mice were used for each sample. In addition, two controls were included; one has the 25µl STa mixed with similar volumes of PBS instead of the tested sera and the 2nd control had 25 µl STa mixed with similar volume of base line serum of the corresponding rabbit. All samples were treated similarly.

Neutralization end titer of tested serum is defined as the highest serum dilution that neutralized one mouse unit of STa. Neutralization capacity is defined as the total mouse units of STa that were neutralized per one ml serum. Neutralization specific activity is defined as the total mouse units of STa that were neutralized per one mg serum protein.

Kinetics of the Rabbit Immune Response to ETEC STa

Antibody-capture ELISA for screening sera

An indirect antibody-capture ELISA in which STa antigen was bound to a solid phase and reaction with antibody-containing samples was allowed (Figure 20) in order to monitor the presence of STa antibodies in rabbit sera as described under ELISA protocol (Lefkovits, 1997).

The ELISA plates were coated with 2.5 μg STa a 100 μl 0.05M carbonate buffer, pH 9.6 (plate coating buffer) and incubated overnight at 4°C. Plates were washed four times with 0.01M PBS-0.05% Tween-20 and blotted dry. One hundred μl of 0.5% cold fish gelatin- 0.01M PBS-0.1%Tween-20 (blocking buffer) was added to each well to block nonspecific binding sites on the plastic surface and incubated at 37°C for 30 min. Plates were then washed with 0.01M PBS-0.1%Tween-20 (washing buffer) and blotted. Serum samples collected from all rabbits over the period of immunization were screened at ten-fold dilution with PBS-0.1% Tween-20. One hundred μl of each serum dilution (10^{-3}, 10^{-4}, 10^{5}, & 10^{-6}) was added to each well, in triplicate, and incubated at 37°C for 45 minutes. After four washings and blotting, 100 μl of 1000-fold diluted alkaline phosphatase-conjugated goat anti-rabbit IgG antibodies (1 μl/ml) was added to each well, incubated at 37°C for 45 min. Plates were washed as previously described and 100 μl of freshly prepared substrate solution (one tablet set of *p*-nitrophenyl phosphate (pNPP/PBS) / 5ml in 0.1M diethanolamine buffer, pH 9.8) was added to each well. The reaction was allowed to develop for 30 minutes at 37°C. The developed color was read at 405 nm with an ELISA plate reader (Molecular Devices "ThemoMax" Microplate Reader with SOFT

max Pro 2.6.1). Reciprocal value of the maximal dilution of serum that had a mean plus 2 Standard Deviations ($\bar{x}$ + 2 SD) O.D value or higher than the OD of the baseline serum sample, was reported as antibody end titer for each tested serum sample.

Avidity ELISA

Avidity (Functional Affinity) is the measure of the overall strength of interaction of an antigen with many antigenic determinants with multivalent antibodies (Goldblatt, 2001). In this study, the STa-antibody avidity was measured by comparing the amount of antibodies that could bind the STa antigen in the presence or absence of increasing concentrations of a chaotropic agent. In this test, a mild chaotropic agents, ammonium thiocyanate, was added in increasing molar concentrations to the antibody-antigen mixture. Antibodies of low avidity are more likely to dissociate from antigen-antibody complexes than those of higher avidity as described by Ferreira & Katzin (1995).

The procedure was performed in similar steps to the ELISA protocol described above. However, after the final incubation of the tested sera, four washings and blotting, 100 µl of three different molar concentrations of ammonium thiocyanate in PBS (5M, 2.5M, and 1.25M) were added to each well, in duplicates, incubated at 37°C for 15 minutes. Plates were processed then as described under the ELISA protocol. Avidity index was calculated as a percentage of the OD of the sample with different concentrations of ammonium thiocyanate and the OD of the sample without treatment. Serum samples obtained from rabbits before the initial immunization were used as baseline controls.

Statistical analysis

Generated ELISA OD data were adjusted and subjected to statistical analysis for the calculation of the mean and standard deviation. Plotting of the ELISA OD data were performed using Microsoft Excel (2003).

Results

Characterization of serum antibody response in STa-conjugate-immunized rabbits

Sera from immunized rabbits were tested for STa-serum neutralization capacity as described under the methods section. Tested sera from three rabbits showed a positive neutralization capacity in suckling mouse assay (gut weight to body weight ratio = 0.06 ± 0.001) (Figure 18) and up to 3 $x10^4$ mouse units of STa could be completely neutralized by one ml of serum (Figure 19). Neutralizing antibodies were first detected among these rabbits after 12 weeks post immunization (4^{th} immunization). These 3 rabbits were grouped retrospectively as "group one" based on the onset of the detectable neutralizing ST-antibody titers. At week 17-post immunization, another three rabbits had a detectable neutralization titer. These rabbits were grouped as "group 2". At week twenty-four, two more rabbits had mild neutralization capacity against *E. coli* STa. These rabbits were grouped as "group 3". Figure 20 shows *E. coli* STa-specific serum antibody neutralization bioassay from all rabbits.

Binding activity

Indirect ELISA was used to determine the binding capacity of anti-STa IgG. Figures 21-29 shows the results of binding data for sera from seven bleedings of all

rabbits that were giving antibodies against STa at various serum dilutions (10^{-3}-10^{-6}). The 10^{-4} dilution was recognized as the optimal dilution for screening the tested sera for STa-binding capacity Figure 21 (equivalence point of antigen antibody interaction).

Three weeks after the primary immunization, detectable neutralizing IgG-antibodies against STa were not observed. However, based on ELISA assay, slight binding titers were detected. Higher binding antibody titers were only detected 12 weeks post immunization (Figure 22). Other three rabbits showed late immune response at week 17 post immunization (Figure 23), however higher antibody binding titers (10^6) and STa-neutralizing antibodies (3 x 10^4 MU/ml serum) were then detected. By 24 weeks post immunization, other two rabbits mounted mild neutralization and binding titer against *E. coli* STa (Figure 24). Figures 25-29 shows the end titer of *E. coli* STa-specific serum antibody. Mean OD ± SD values of STa-specific serum antibody end titer of group 1, 2 and 3 rabbits after 24-week post immunization at various serum dilutions are shown in Table 11. Summary of STa-ELISA binding and neutralization end titers of rabbit sera immunized with STa-suBSA conjugate after the primary immunization and during the boosting intervals are presented in Table 12.

STa- binding avidity of the rabbit immune sera

The dissociation effect of ammonium thiocyanate on the STa-binding to its specific antibodies was demonstrated. The dissociation effect of the chaotropic agent on the STa-antibody binding using sera from a rabbit with the highest neutralizing antibody titer, is shown in Figure 30. Sera from other rabbits demonstrated similar

avidity patterns. The figure shows the time course evaluation of STa-IgG avidity using ammonium thiocyanate dose response. The increasing molar concentration of ammonium thiocyanate (1.25 M-5 M) needed to dissociate STa-IgG complex is depicted suggesting that the strength of the STa-IgG avidity developed gradually after multiple boosters with the STa-conjugate. A 5 M concentration of the chaotropic agent was determined to be the appropriate cutoff point in order to demonstrate the strength of the avidity of STa-specific serum antibodies. Mean values of the ODs of serum ELISA for the 3 groups of rabbits assayed using 5 M of the ammonium thiocyanate are depicted in Figure 31 and summarized in Table 13. It is noted that there is some variation in the patterns of dissociation of the STa- antibodies from sera of the 3 groups of rabbits, which are largely corresponding to the STa-neutralization and binding titers established for these sera.

The avidity index which is calculated by dividing the OD of the sera in the binding ELISA with 5 M of chaotrpic agent by the OD of the ELISA binding result for the same sera without treatment with the chaotrpic agent (Figure 32 & Table 13). By week 24-post immunization, sera from all 3 groups of rabbits demonstrated variable avidity indices. It was noted that sera from the first group of rabbits had the highest avidity index, which is associated with the high STa-neutralization and binding titers demonstrated for these sera.

Discussion

Construction of immunogenic ETEC STa has been reported by several investigators who used different chemical coupling protocols to link the STa to carrier proteins as well as the genetic expression of the STa with flagellin as a fusion protein

(Houghten et al. 1984, 1985; Sanchez et al. 1986, 1988; Clements, 1990; Klipstein et al. 1982, 1983 and Pereira et al. 2001). However, limited success in producing high titers STa antisera was reported in those studies. This could be attributed to the uncontrolled cross-linking process of the STa to the carrier proteins which led to the reduction or loss of the STa biological activity as a part of the conjugate (Pereira et al. 2001). An important objective of this study was to design and characterize an effective immunogenic STa conjugate using the major different peptide-carrier conjugation protocols. Based on the evaluation of four different conjugation procedures, a well-defined STa conjugate with high STa biological activity was produced.

Immunization of ten rabbits with this STa-conjugate led to the production of STa-specific neutralizing antibodies by eight animals. The STa-neutralization and specific binding titers of these sera were higher than previously reported by other investigators (Alderete and Robertson 1978; Frantz and Robertson 1981; Lockwood and Robertson 1984; Löwenadler et al. 1991 and Pereira et al 2001). However some variations in the onset and quality of the immune responses were noticed. Two rabbits demonstrated weaker STa neutralizing titer 20 weeks post immunization. It is not fully understood why some rabbits differed in their immune response to the STa immunogen. Such individual variations may worth additional investigation in future studies. Comparing data on the STa binding and STa neutralization titers of the sera produced in this study with data from previous reports is presented in Table 14.

Measurement of the avidity of the STa-antibodies using a chaotropic agent, ammonium thiocyanate at several increasing molar concentrations suggested that the

avidity of the STa neutralizing antibodies improved throughout the series of boosters administered to the rabbits. Comparison of the avidity of the serum antibodies demonstrated that the strength in the STa antibody avidity developed in time corresponding to the development of the STa-neutralizing and ELISA binding titers of the tested sera. This is consistent with the common knowledge about maturation of antibodies after immunization and continuous boosting protocols (Goldblatt 2001).

In summary, we have described the design of a highly defined STa-conjugate and its use in the induction of high STa neutralization and ELISA binding serum titers in immunized rabbits. The carefully designed STa-conjugate and the produced high STa-neutralizing serum antibodies can be evaluated for the design of effective vaccine and / or immunotherapeutic reagents against the STa-producing *E. coli* strains that are associated with a significant proportion of diarrheal disease worldwide.

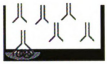
The ELISA plate was coated with 100 µl of STa, 20 µg/ ml 0.05M carbonate buffer, pH 9.6 (plate coating buffer) and kept overnight at 4 °C

wash four times with 0.01M PBS-0.05% Tween-20 and plotted to dry

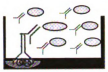
100 µl of 0.5% cold fish gelatin-0.01M PBS-0.1%Tween-20 (blocking buffer) was added

wash four times with 0.01M PBS-0.05% Tween-20 and plotted to dry

100 µl of ten-fold diluted serum (10^{-3} - 10^{-6}) was added to each well (triplicate) and incubated at 37 °C for 45 minutes



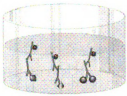
wash four times with 0.01M PBS-0.05% Tween-20 and plotted to dry



100 µl of 1000-fold diluted alkaline phosphatase-conjugated goat anti-rabbit IgG was added to each well, incubated at 37 °C for 45 min

wash four times with 0.01M PBS-0.05% Tween-20 and plotted to dry

100 µl of freshly prepared substrate solution (PNPP) was added to each well. And incubated at 37 °C 30 min.



Read the developed color at 405 nm with an ELISA reade (Molecular Devices ThemoMax Microplate Reader with SOFT max Pro 2.6.1)

Figure 17. Antibody Capture ELISA

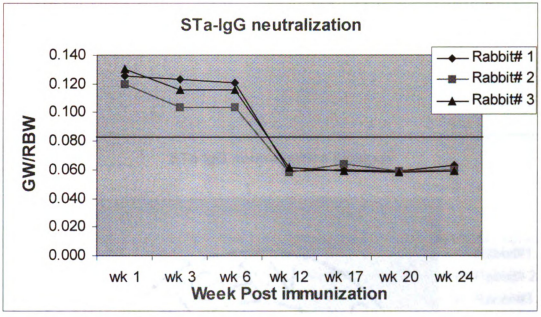


Figure 18. *E. coli* STa-specific serum antibody neutralization bioassay: Group 1 (rabbits. 1, 2, and 3). Straight horizontal line at 0.083 Gut weight: remaining body weight ratio, signifies the cut off value for STa- positive SAM.

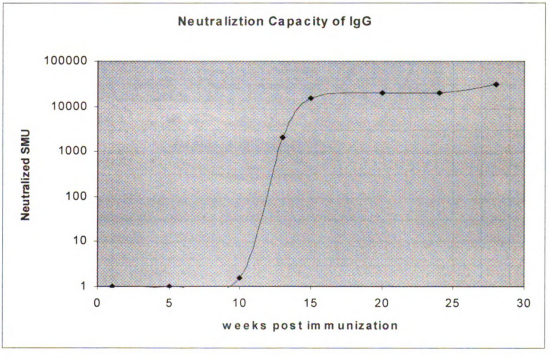


Figure 19. Neutralization capacity of *E. coli* STa-specific serum antibody. Points represent the mean of neutralization titers from sera of 3 rabbits

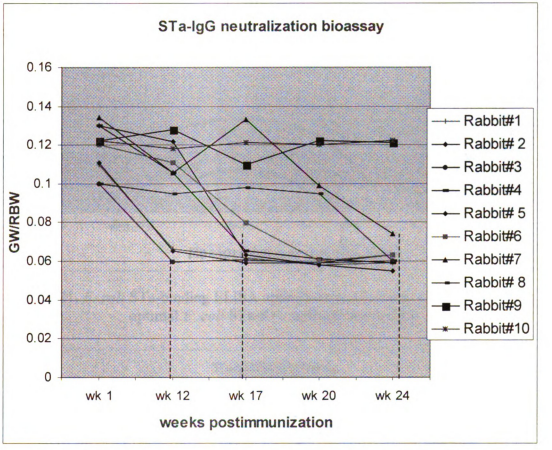


Figure 20. *E. coli* STa-specific serum antibody neutralization bioassay: All rabbits **Note**: Three rabbits showed STa neutralizing antibodies by 12 weeks, another 3 rabbits showed STa neutralizing antibodies by 17 weeks and finally 2 rabbits started to show STa neutralizing antibodies by 20 weeks post immunization
GW= Gut weight, RBW= Remaining body weight

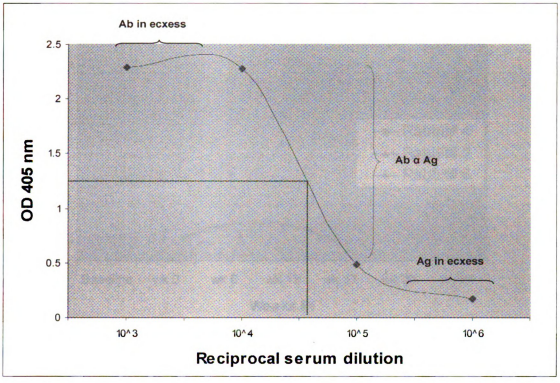


Figure 21. *E. coli* STa-binding ELISA optimization: screening serum dilution for optimal *E .coli* STa-STa antibody interaction

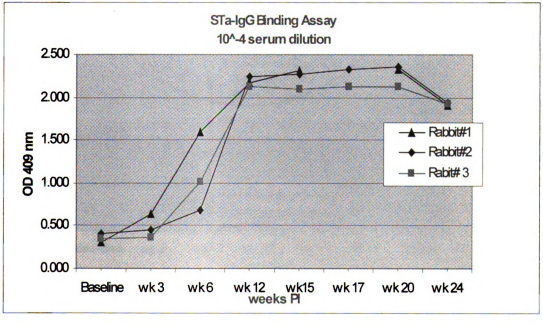


Figure 22. *E. coli* STa-specific serum antibody: 10^{-4} serum dilution of group 1 rabbits

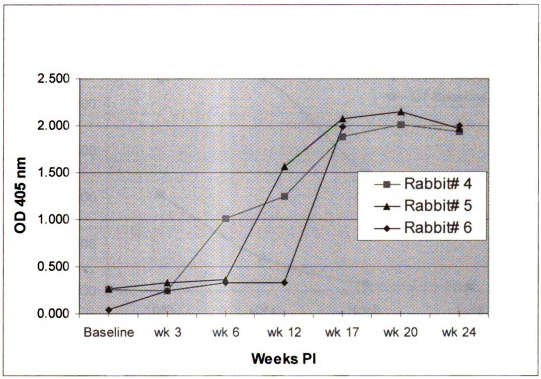


Figure 23. *E. coli* STa-specific serum antibody: 10^{-4} serum dilution of group 2 rabbits

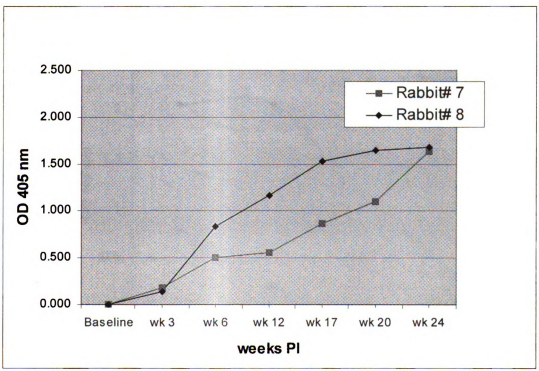


Figure 24. *E. coli* STa-specific serum antibody: 10^{-4} serum dilution of group 3 rabbits

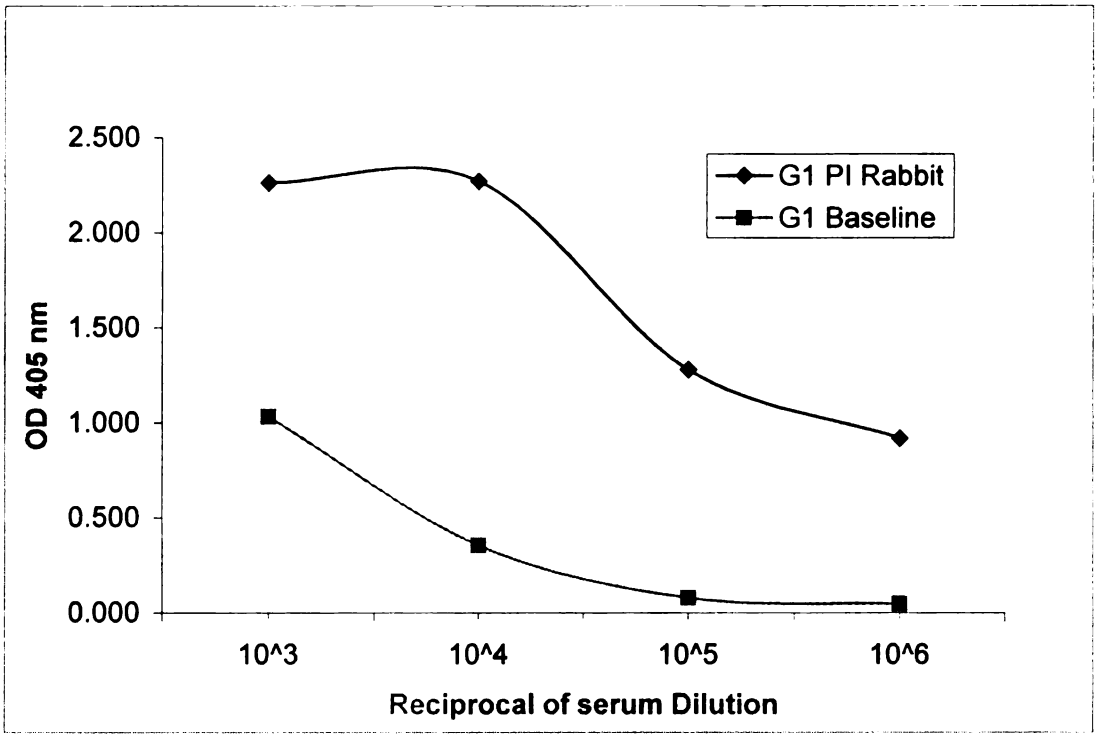


Figure 25. Mean OD value of group 1 rabbits after 20 weeks post immunization at various serum dilutions

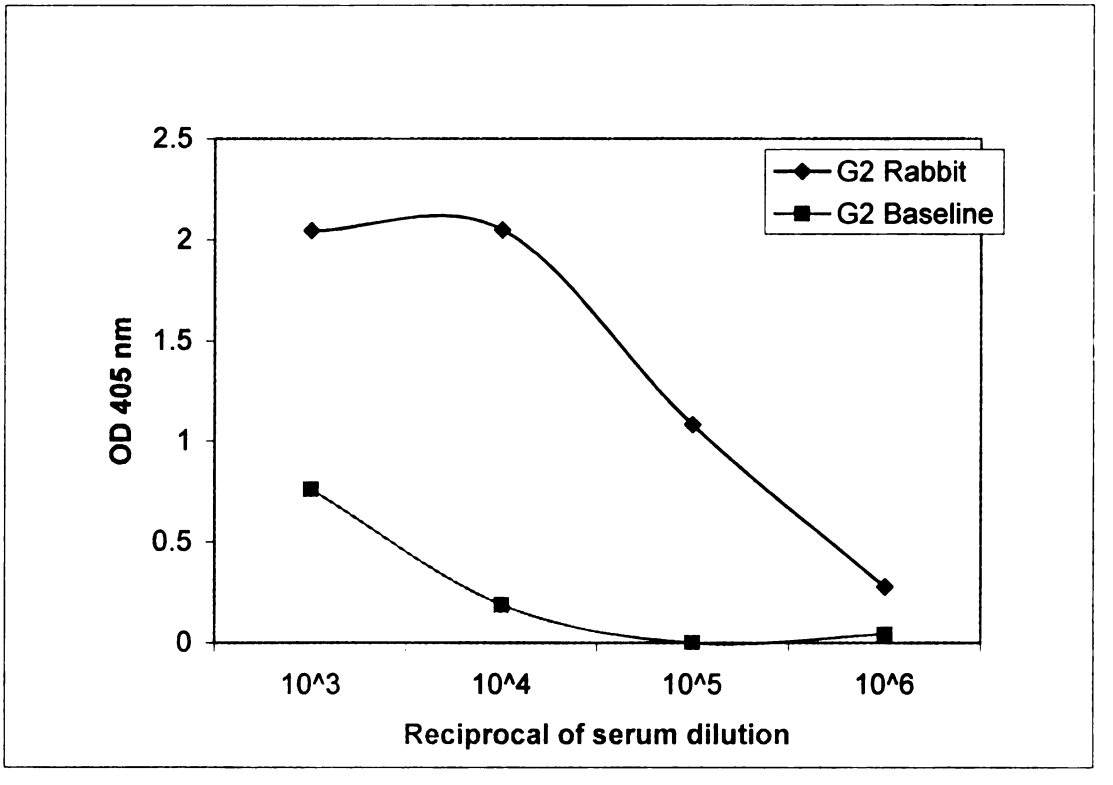


Figure 26. Mean OD value of group 2 rabbits after 20 weeks post immunization at various serum dilutions

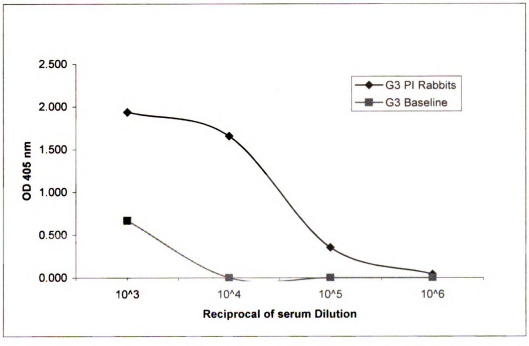


Figure 27. Mean OD value of group 3 rabbits after 24 weeks post immunization at various serum dilutions

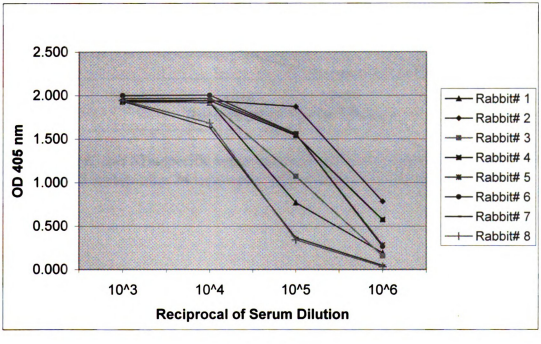


Figure 28. End titer of *E. coli* STa- specific serum antibody: 24 weeks post immunization from 8 rabbits

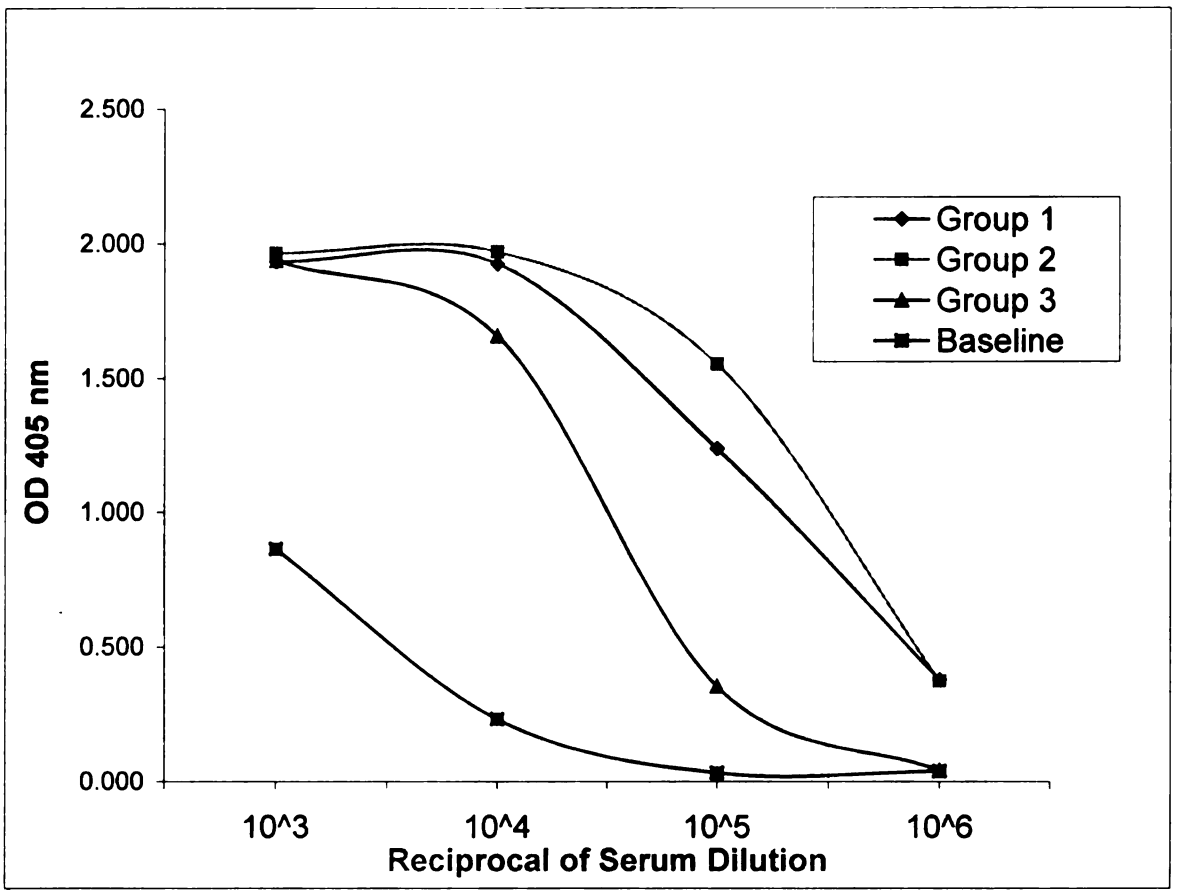


Figure 29. *E. coli* STa-specific serum antibody end titer: Mean OD value of group 1, 2 and 3 rabbits after 24 weeks post immunization at various serum dilutions

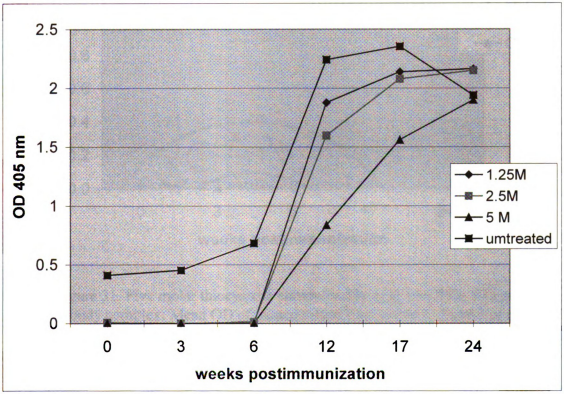


Figure 30. Time-course evaluation of the avidity of *E. coli* STa-specific serum antibody using ammonium thiocyanate dose response

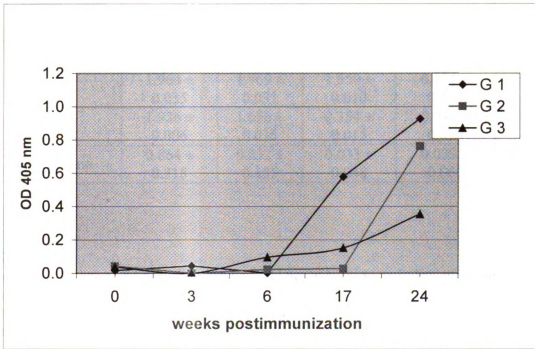


Figure 31. Five molar thiocyanate elution profile of *E. coli* STa- STa serum antibody complex: Mean OD of treated serum from group 1, 2 and 3 of rabbits

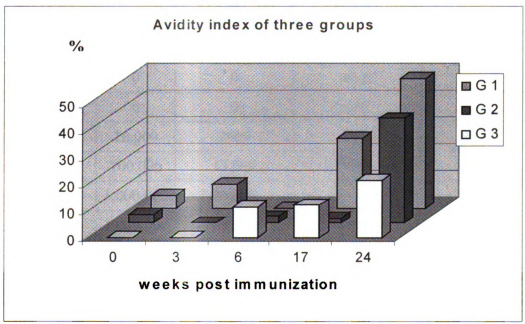


Figure 32. Avidity index of *E. coli* STa-specific serum antibody from group 1, 2 and 3 of rabbits using avidity ELISA procedure

Table 11: *E. coli* STa-specific serum antibody end titer: Mean OD ± SD value of group 1, 2 and 3 rabbits after 24- week post immunization at various serum dilutions

Group/serum dilution	10^{-3}	10^{-4}	10^{-5}	10^{-6}
G1	1.933 ± 0.013	1.923 ± 0.014	1.237 ± 0.568	0.379 ± 0.352
G2	1.964 ± 0.035	1.969 ± 0.031	1.550 ± 0.010	0.374 ± 0.173
G3	1.936 ± 0.006	1.656 ± 0.035	0.354 ± 0.015	0.040 ± 0.010
Baseline	0.864 ± 0.318	0.232 ± 0.155	0.033 ± 0.048	0.039 ± 0.060

Table 12. Summary of STa- ELISA binding and neutralization end titers of rabbit sera immunized with STa-suBSA conjugate after the primary immunization and during the boosting intervals. Data were generated by STa binding ELISA and STa neutralization using suckling mouse assay.

Bleeding	**Anti-STa Response**		**Protein Assay mg/ml**	**Neutralization Specific Activity**
	ELISA Titer	**Neutralized STa MU/ml serum (Titer)**		
Baseline	0	0	64.5	-
Week 3 PI	0	0	66.8	-
Week 6 PI	0	0	65.7	-
Week 12 PI	10,000	2000	83.5	23.95
Week 15 PI	100,000	15,000	69.6	215.52
Week 20 PI	1,000,000	20,000	65.00	307.69
Week 24 PI	1,000,000	20,000	65.00	307.69
Week 28 PI	1,000,000	30,000	65.00	461.54

Table 13. Summary of the development of STa antibody avidity after multiple boosters with the STa conjugate using 5 M ammonium thiocyanate ELISA dissociation assay.

	Baseline	Week 3	Week 6	Week 12/17	Week 24
	Avidity Index (AI) % after measurement OD at 405 nm				
Group 1	0.017 (4.70%)	0.043 (8.91%)	0.001 (0.03%)	0.578 (25.94%)	0.927 (48.21%)
Group 2	0.043 (3%)	0 (0%)	0.022 (2.24%)	0.027 (1.53%)	0.762 (38.71%)
Group 3	0.04 (0%)	0 (0%)	0.095 (11.36%)	0.151 (12.05%)	0.352 (21.30%)

Table 14. Neutralization capacity of sera from animals immunized with several STa-immuogenes and the end titers of the STa-neutralizing antibodies as reported in the literature.

References	Maximum neutralization titer	Amount of SMU	Neutralization capacity Total MU/ml serum
Löwenadler et al. 1991	1:55	30	1665
Lockwood and Robertson 1984	1:100	8	800
Frantz and Robertson 1981	1:5000 to 1:10,000	1	5000 to 10,000
Alderete and Robertson 1978	1:2,500	1	2,500
Pereira et al. 2001	1:50 for immunogene using native STa fusion protein	1	50
	1:4000 using mutated STa fusion protein	1	4,000
This study	1:30,000	1	30,000

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SUMMARY AND CONCLUSIONS

Enterotoxigenic strains of *E. coli* (ETEC) that produce heat-stable enterotoxin (STa) are a major cause of diarrheal disease in humans and animals. Economically, ETEC is among the important causes of diarrheal diseases in animals, including neonatal calves, pigs and lambs. Additionally, ETEC strains have been recognized to cause a significant proportion of "travelers" diarrhea. Currently, there are no effective vaccines or immunotherapeutic reagents that confer broad protection against STa-induced diarrheal disease. This is largely due to the antigenic diversity of surface antigens and the nonimmunogenic property of STa, which is the immediate mediator of ETEC diarrhea. To reduce the impact of STa-caused diarrhea, it was logical therefore to investigate the feasibility of designing an immunogenic STa conjugate to produce immunotherapeutic reagents and / or potential vaccine to combat the diarrheal disease caused by these enteropathogens.

This study was based on the hypothesis that the design of an effective STa immunogen is feasible, if based on the understanding of its molecular structure and the preservation of its bioactive configuration. This study has supported the stated hypothesis through the achievement of the objectives listed below.

1. Production of purified STa from Enterotoxigenic *Escherichia coli*

STa was purified to homogeneity and its purity was documented using amino acid sequence analysis, and MALDI-TOF mass spectroscopy. The minimal effective dose of the purified STa was less than 0.2 ng per one Mouse Unit using 2-3 day-old inoculated Swiss Webster suckling mice.

2. Design and evaluation of immunogenic Enterotoxigenic *Escherichia coli* STa

Conjugation of a STa -carrier has been reported. However, no sufficient details were presented on the efficiency and the characteristics of the produced STa conjugates. An important objective of this study was to design and characterize an effective immunogenic STa conjugate using the major different peptide-carrier conjugation protocols. Based on the evaluation of four different conjugation procedures, a well defined STa conjugate with high STa biological activity was produced. We confirmed the stability of the molecular structure of this conjugate using amino acid compositional analysis and MALDI-TOF mass spectroscopy.

3. Immunization of rabbits and the production of high titers of STa neutralizing and specific ELISA binding serum antibodies.

Numerous attempts have been made to render STa immunogenic, including chemical coupling to carrier proteins and genetically produced fusions proteins. However, only limited success was reported for the immunogenic potency of the STa-conjugates and the quality of the STa-specific antibody titers produced in immunized laboratory animals. We have used the produced STa-conjugate to immunize a group of New Zealand albino rabbits. The immunized rabbits produced higher STa-neutralizing serum titers than reported in previous studies within 15 weeks post immunization, indicating the effective immunogenicity of the designed STa-conjugate. Additionally, the kinetics of immune response of the rabbits to this conjugate, the STa-neutralization capacity and the quality of the antibodies of the produced sera, were characterized. Results of this study validated the value of the designed STa-suBSA conjugate as a potent immunogen.

Strength of the study

- Previously reported STa conjugation protocols did not consider the preservation of the STa biological activity in the conjugation process which, when used for immunization of laboratory animals, may have led to suboptimal quality of the produced STa antibodies. This study was based on thorough understanding of the molecular structure of the STa that was considered in the planning of the conjugation process of the STa to modified BSA. This approach ensured the effective crosslinking of the STa through its amino terminal end and thus had preserved its biologically active moiety.
- Additionally, the conjugation design may have enhanced the presentation of STa on the BSA carrier that was modified to have longer reactive groups for crosslinking with the STa peptide. This was demonstrated by the high retention of the STa biological activity in the STa conjugate using the suckling mouse assay, prior to the immunization process. Finally, the produced STa conjugate was proved to be highly immunogenic as it elicited higher STa neutralizing antibody titers in immunized rabbits than previously reported by other investigators.

Limitations

- The yield of STa from one batch of ETEC culture is not sufficient to enable extensive experimentation and evaluation of several methods for optimized protocols to produce the desired STa conjugates. Therefore, numerous batches of cultures were required.

- We have evaluated the crosslinking protocols that were reported for several synthetic peptides to carrier proteins. Those peptides were not structurally similar to the STa peptide. Therefore, we had to use large amounts of the precious STa to optimize the conjugation methods. Several initial attempts were not successful.
- The use of the produced STa conjugate was only evaluated in rabbits. Its use in immunizing other animal species could have further revealed its potential as a possible vaccine against ETEC-induced diarrhea.