Published in final edited form as:

J Immunol Methods. 2011 March 7; 366(1-2): 100–105. doi:10.1016/j.jim.2011.01.012.

Design and Characterization of Highly Immunogenic Heat-Stable Enterotoxin of Enterotoxigenic *Escherichia coli* K99⁺

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Abstract

In this study, STa peptide of enterotoxigenic *Escherichia coli* K99⁺ was purified and successfully covalently cross-linked to modified bovine serum albumin after thorough evaluation of three different hapten-carrier conjugation protocols. Dimethylformamide (DMF) based STa-conjugation protocol demonstrated higher biological activity (10×10^6 STa Total Mouse Units [MU]) and 100% conjugation efficiency. A range of conjugation ratio of 4–12 STa molecules per one molecule of BSA was achieved and confirmed by matrix-assisted laser desorption ionization-time of flight/mass spectroscopy (MALDI-TOF/MS). This conjugate was used for immunization of ten rabbits for STa antibody production. A high antibody binding titer (10^6) against STa was obtained with neutralization capacity of 3×10^4 STa MUs/ml serum. These levels of high STa binding and neutralizing antibodies titers propose the potential use of this conjugate for development of immunotherapeutic reagents and/or STa-based vaccine against ETEC K99⁺.

Keywords

ETEC; Heat-stable enterotoxin; STa antibody; STa conjugate; MALDI-TOF/MS; ELISA

1. Introduction

Enterotoxigenic *Escherichia coli* (ETEC) is a major enteropathogen that causes potentially fatal diarrhea in neonatal calves (Moon and Bunn, 1993; Gyles, 1996). It is also responsible for a large proportion of diarrheal disease among children and adult travelers (Tacket et al., 1994). A significant proportion of ETEC diarrhea is caused by a small hydrophobic peptide (<2 KDa), heat-stable enterotoxin (STa), which is an important virulence determinant in enterotoxin-mediated diarrheal diseases (Sears and Kaper, 1996). Upon infection, the STa producing- ETEC adheres to the epithelium of the small intestine via one or more colonization factor antigens (CFA) or pili surface proteins. Although K99⁺ is the most

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Conflict of interest

There is no conflict of interest

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common colonization factor on bovine ETEC, there is a relatively small group of other fimbriae that also mediate adhesion to calf enterocytes (Morris et al., 1983).

Once established, ETEC elaborates STa, which acts on a specific intestinal membrane bound receptor, guanylyl cyclase C, initiating a cascade of an altered metabolic pathway (Giannella and Elizabeth, 2003) resulting in secretory diarrhea and potentially fatal dehydration in neonatal calves.

Methods for 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. Therefore the use of immune- based therapy is considered promising approach for combating against ETEC.

While several reagents that are in use against ETEC, most of these reagents are based on CFA (pili) which did not confer broad protection against ETEC strains. This is because of the antigenic diversity and high prevalence of unidentifiable forms of specific CFAs of ETEC (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 ETEC- induced calf diarrhea could be experimentally induced by a purified STa preparation, supporting the notion that STa is the immediate mediator of diarrhea in calves. Additionally, several studies have demonstrated a significant correlation between STa-producing ETEC strains and diarrhea (Wolf, 1997). Thus, the inclusion of STa in CF-based ETEC vaccines or the production of STa- neutralizing antibodies would potentially offer immune protection against ETEC-caused diarrhea. However, this approach has been challenged because of the haptenic nature of STa (M.W. <2 KDa), which fails to elicit an antibody response (Pereira et al., 2001; Boedeker, 2005).

Several attempts to render STa immunogenic through hapten-carrier conjugation protocols however, only limited success was reported (Alderete and Robertson, 1978; Frantz and Robertson, 1981; Lockwood and Robertson, 1984; Sanchez et al., 1988; Clements 1990; Löwenadler et al., 1991 and Pereira et al., 2001). Moreover no sufficient details were presented on the efficiency and the characteristics of these conjugates. In this study, we defined an improved protocol for efficiently coupling native STa peptide to carrier proteins after evaluation of three different conjugation protocols and tested its efficiency for eliciting high STa antibody response.

2. Materials and Methods

2.1. Chemicals and Reagents

All chemicals and reagents were of analytical grades and obtained from Sigma Chemical Company St. Louis Mo, USA.

2.2. Purification of STa

A modified STa purification protocol (Saeed et al., 1983) was adopted. In brief ETEC-K99⁺ were grown in 30L of asparagines salt medium under controlled growth conditions using 36L Bellco bioreactor. STa was purified in 4 different steps: 1) Extracting the cell free filtrate by tangential flow filtration 2) STa capture and concentration by Amberlite XAD-2 batch adsorption chromatography (BAC) and acetone fractionation 3) Intermediate STa

purification step on MCI-gel reverse phase BAC 4) Final STa purification step via reverse phase high performance liquid chromatography on Grace Vydac C8 preparative column. The biological activity of STa was determined by suckling mouse assay (SMA) according to Dean et al., 1972 and Giannella, 1976. Sample that gives gut weight to remaining body weight ratio (SMA) 0.083 is considered positive for STa.

2.3. Chemical Modification of BSA

New carboxyl moieties were introduced to BSA to ensure binding of STa through its amino terminal using two different protocols: succinylation and hyper-succinylation (HS) reactions according to Habeeb (1967a) and Fuentes et al. (2005) respectively.

2.4. Size Exclusion-High Performance Liquid Chromatography (SE-HPLC)

Twenty five μ l (1mg/ml) of both native and succinylated BSA (suBSA) were loaded to SE-HPLC using equilibrated Bio-Sil® SEC-125 HPLC column (BioRad Hercules, CA) and hooked to the Waters Associate Liquid Chromatography System. Isocratic elution system was applied at flow rate 1 ml/min. The peak absorbance was monitored at UV 280 nm.

2.5. Coupling of STa to Succinylated BSA

DMF-based protocol—Briefly 140 mg of suBSA suspended in 10 ml of anhydrous DMF and stirred magnetically in a tight-capped foil-wrapped bottle for 4 hours. A solution of *p*-nitrophenol (PNP) (65 mg/0.5ml) in DMF was added and magnetic stirring continued for 15 minutes. A solution of organic solvent carbodiimide (DCC) (50 mg/0.5ml) was added to suBSA at a molar ratio of 120:1 and the reaction was allowed to continue 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 M.W. cutoff 12-14 KDa and then freeze-dried.

Imidazole-based protocol—Briefly STa peptide and the carrier protein (suBSA) were dissolved in 0.5 M *N*-methyl-imidazole pH 6.0 at a molar ratio 100:1. After the addition of water soluble carbodiimide (EDAC) at a molar ratio 50:1 STa, the mixture was stirred for 30 minutes at room temperature followed by dialysis against distilled water at 4 °C using a dialysis membrane M.W. cutoff 12-14 KDa and then freeze-dried.

HS BSA-based protocol—In this coupling protocol, 3 mg of HS 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 composite was dialyzed against PBS using dialysis membrane M.W. cutoff 12-14 KDa and then freeze-dried

2.6. Gel Filtration Chromatography (GFC)

A freeze-dried STa conjugate sample from each protocol was reconstituted into PBS and passed through equilibrated Sephadex G-25M packed columns of a nominal MW exclusion limit of 5000 for protein (G.E. Healthcare, Buckinghamshire, UK). The STa conjugate was then eluted and the effluent was collected and tested for biological activity. Samples that demonstrated high biological activity was subjected for further characterization using amino acid compositional analysis and MALDI-TOF/MS.

2.7. MALDI-TOF/MS and Amino Acid Compositional Analysis

A hundred μg of freeze-dried samples of suBSA and DMF-based STa conjugate were subjected to MALDI-TOF/MS to determine precisely M.W. of suBSA before and after conjugation and consequently conjugation ratio. Another sample of DMF-based STa conjugate was analyzed for amino acid composition.

2.8. Antibody Production against STa

Ten rabbits were inoculated with a mixture of DMF-based STa conjugate (1 mg/ml PBS) and Freund's complete adjuvant (1:1) at multiple intradermal sites. Each rabbit then received a booster dose at three-week intervals with a mixture of STa conjugate (0.5 mg/ml PBS) and Freund's incomplete adjuvant (1:1). Rabbits received boosters until STa-neutralizing antibodies titers were detected. Standard operating procedures for immunization of rabbits in compliance with the guidelines and recommendations for the Institutional Animal Care & Use Committee of Michigan State University were followed.

2.9. STa- Serum Neutralization Assay

The STa-serum neutralization capacity was determined using SMA test. Briefly, three aliquots of 50 μl of rabbit serum were incubated with 25, 50 and 75 μl of STa (20 MU/ 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 assay. In addition, two controls were included; one had the 25 μl STa mixed with similar volume of PBS and the other control had 25 μl STa mixed with similar volume of serum from non immunized rabbit. Three mice were used for each test and control samples. All samples were treated similarly. Positive sample for STa antibodies gives SMA value < 0.080. Neutralization end titer of tested serum is defined as the highest serum dilution that neutralized one MU of STa. Neutralization capacity is defined as the total MUs of STa that were neutralized per one ml serum. Neutralization specific activity is defined as the total MUs of STa that were neutralized per one mg serum protein.

2.10. STa Antibodies Confirmation Using ELISA

The ELISA plates were coated with 2.5 μg of STa/100 μl 0.05 M 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 $\mu\text{l}/\text{ml}$) 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 ($\bar{x} + 2 \text{ SD}$) O.D value higher than the OD of the baseline serum sample, was reported as antibody end titer for each tested serum sample.

2.11. Statistical Analysis

Generated ELISA OD data were subjected to statistical analysis for the calculation of the mean and standard deviation using Microsoft office Excel 2003.

3. Results

3.1. Mr Value of STa

A lyophilized HPLC-purified sample that showed SMA > 0.080 was analyzed by MALDI-TOF/MS to determine the M. W. and the result is shown in Figure 1. The observed signal at 100% MS intensity with m/c = 1972.1 indicates that the Mr value of the purified product is 1971.1 Da, which is compatible with the Mr value (1969–1972) calculated from amino acid composition of the STa, confirming the purity and identity of the purified product as STa molecule.

3.2. SE-HPLC and Mr Value of suBSA

Figure 2 showed the elution profile of both modified BSA (suBSA) (retention time = 2.99 min) and native one (retention time = 4.20 min) on SE-HPLC. The faster elution of suBSA indicates that the M. W. of suBSA has undergone a significant increase. This result was confirmed by MALDI-TOF/MS which determined precisely the Mr value of suBSA as 72.40 KDa (Figure 3).

3.3. Characteristics of STa Conjugates

3.3.1. STa conjugation efficiency—Conjugation efficiency was extrapolated from the total mouse units retained in the conjugate after extensive dialysis and GFC considering that 2 ng of STa is the minimal amount of STa that could produce one MU (SMA = 0.080). The conjugation protocol based on the DMF method demonstrated higher level of retained STa biological activity (10×10^6 Total MUs) compared to other protocols (Table 1).

3.3.2. Conjugation ratio—The conjugation ratio is defined as the number of STa molecules covalently cross-linked to one molecule of suBSA. The amino acid composition of the STa conjugate was empirically determined by measuring the concentration (μ moles) of each residue detected in a known volume of sample. Well-recovered residues, arginine and methionine, were used to quantify the concentration of each residue in the conjugate sample. 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. This result was confirmed by MALDI-TOF/MS.

MALDI-TOF/MS accurately determined the Mr Value of suBSA before and after STa conjugation (Figure 3). STa conjugate sample showed median Mr Value over 80 KDa. The molecular weight difference (Δ M.W.) of suBSA before and after STa conjugation was calculated as 8342.5 Da. 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. = $8342.5/1972 = 4-5$ (Figure 3).

3.3. Characterization of the Immune Response against STa

Three weeks after the primary immunization with DMF-based STa conjugate, slight binding titers were detected based on ELISA assay. By 12th week post immunization, both high antibody binding (10^6) and neutralizing titers of STa antibody were detected. Up to 3×10^4 MUs of STa could be completely neutralized by one ml of serum.

4. Discussion

Our data showed that DMF-based STa conjugation protocol was more efficient for designing highly immunogenic STa conjugate based on total mouse units and conjugation ratio than other conjugation protocols. Additionally, DMF-based STa conjugate was capable of eliciting higher immune response against STa than previously reported studies (Alderete and Robertson, 1978; Frantz and Robertson, 1981; Lockwood and Robertson, 1984; Sanchez et al., 1988; Clements 1990; Löwenadler et al., 1991 and Pereira et al., 2001).

First step in this protocol was BSA modification by introducing carboxyl moieties to ensure binding of STa molecules from its amino terminal. Data of MALI-TOF/MS on suBSA showed 5000 Da increasing in the molecular size compared with native BSA (~67KDa), indicating 8% increases in the M.W. of modified BSA. This suggests that an extensive modification of BSA was achieved by conversion of free amino groups into carboxyl ones (Habeeb, 1967b). The subsequent step in the design of STa conjugate was the cross-linking of STa to suBSA. This reaction was initiated by incubation of suBSA with PNP and DCC for three hours to provide reactive ester groups that could easily form amide linkages with STa molecules. Covalent attachment (amide linkages) of STa molecules to suBSA was documented by amino acid composition analysis and MALDI-TOF/MS.

We believe strongly that DMF-based STa conjugate was efficient because of: 1) The use of DMF has facilitated the solubility of the hydrophobic STa molecules, solving a problem encountered with the other conjugation protocols and this is in agreement with the finding of Lateef et al, (2007). 2) Incubation of suBSA with PNP and DCC provide long space arm of reactive ester groups that could easily form amide linkages with STa molecules. 3) Finally, STa molecules may have been efficiently oriented on the BSA via linkage through their amino terminals. Such orientations have preserved the biologically active moiety of the STa and explain the high immune response against STa.

In short, we have succeed to design a well-defined stable, active and immunogenic STa conjugate based on a thorough understanding of the molecular structure of the STa peptide and after careful evaluation of several peptide-carrier conjugation protocols. Results of 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. Further studies are needed to study the effect of this conjugate on intestinal mucosal immunity.

Acknowledgments

This project was supported by the National Institute of Health-USA. I gratefully acknowledge, Ronald Erskine Ph.D. & Daniel Grooms Ph.D. Department of Large Animal Clinical Sciences, Robert Roth Ph.D. & Patricia Ganey Ph.D. Department of Pharmacology and Toxicology, and Joseph Leykam Ph.D. and Eric Lund at Department of Biochemistry, Michigan State University, East Lansing MI.

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Figure 1. Matrix assisted laser desorption ionization-time of flight mass spectroscopy profile of *E. coli* heat-stable enterotoxin

Figure 2.
Size exclusion chromatography profile of native and suBSA molecule
Rt = retention time
SuBSA = succinylated bovine serum albumin

Figure 3.
Matrix- assisted laser desorption ionization-time of flight mass spectroscopy profile of
suBSA and STa conjugation ($M_r = 72404.3$ and 80746.8 Da respectively)
 Δ M.W. = 8342.5 Da

Table 1

Methods and Results of Different STa Peptide-Carrier Conjugation Protocols

	DMF Method	HS-BSA Method	Imidazole Method
Carrier	suBSA	HS-BSA	suBSA
Cross linker	DCC	EDAC	EDAC
Medium	<i>N,N</i> , DMF	5 mM PB (Na ₂ HPO ₄ /NaH ₂ PO ₄)	Imidazole
pH	7–9	7	7.2
Reactants	10 mg STa + 14 mg suBSA + 6.5 mg <i>p</i> -NP	5 mg STa + 12 mg HS-BSA	10 mg STa + 3.3 mg suBSA
Total STa MU	10×10^6	48×10^4	4×10^6
Protein Assay	1.96 mg/ml	0.277 mg/ml	1.8 mg/ml
Conjugation ratio	4–5:1	4:1	-
Conjugation efficiency based on:			
1. Protein assay	~52–64%	~ 26%	~ 40.6%
2. Retained biological activity	100%	~20%	40%
Reference	Atassi et al. 1981	Fuentes et al. 2005	Dean et al. 1990