Characterization of EcoCRM™, an *E. coli* Expressed CRM_{197} Conjugate Vaccine Carrier Protein

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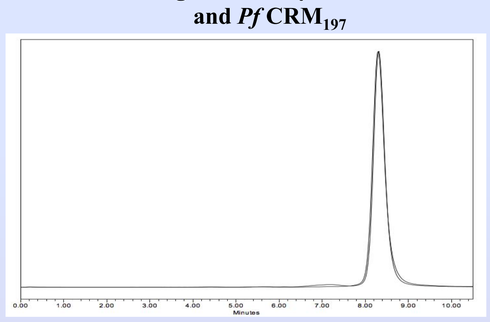
INTRODUCTION

CRM_{197}, a genetically detoxified diphtheria toxin, is widely used as a carrier protein in conjugate vaccines. Effective conjugate vaccines against *Streptococcus pneumoniae*, *Haemophilus influenzae* b and *Neisseria meningiditis* have been made using the strain specific capsule polysaccharide linked to this carrier protein. The cost of CRM_{197} production represents a significant contribution to the cost of goods for these vaccines however. Fina BioSolutions has improved the production process by development of a highly efficient *Escherichia coli* expression system for CRM_{197}, along with a simple purification scheme, whereby we have achieved expression yields of grams per liter.

Here, we show that EcoCRM™ maintained equivalency to "native" (expressed in *Corynebacterium diphtheriae)* or *Pf* CRM_{197} (expressed in *Pseudomonas fluorescens)* for composition and sequence (amino acid analysis and peptide mapping), stability (differential scanning calorimetry), molecular weight (multi-angle light scattering, and mass spectrometry), structure (circular dichroism, intrinsic fluorescence). EcoCRM™ also maintained solubility at high concentrations and over a broad pH range. We also confirmed the carrier function of EcoCRM™ using conjugation to *Salmonella* LPS derived O-polysaccharide as a model system.

Size Exclusion Chromatography

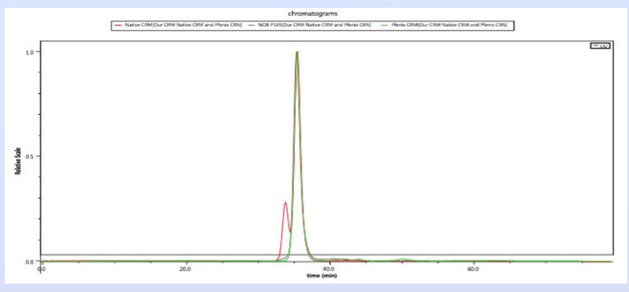
Chromatogram overlay of EcoCRM™ and *Pf* CRM_{197}



By SEC HPLC, both Pf CRM and EcoCRM™ show comparable separation patterns and <1% dimer.

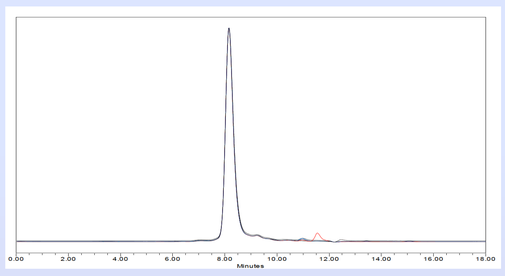
Size Exclusion Chromatography- Multi Angle Light Scattering (SEC MALS)

Chromatogram overlay of EcoCRM™, "native" CRM_{197} and *Pf* CRM_{197}



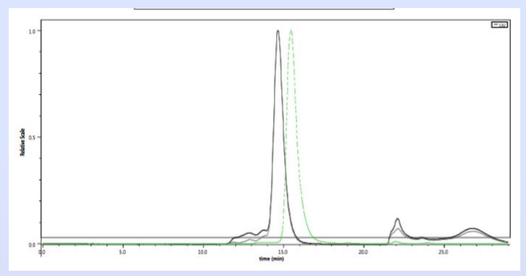
Chromatograms of EcoCRM™, native CRM and Pf CRM all overlay. While native CRM exhibited dimerization due to sample age, Pf CRM and EcoCRM™ had virtually no aggregation. The MW calculated by MALS was within experimental range of theoretical MW of 58,408. MW of native CRM was not calculated due to dimerization.

Solubility at high concentrations



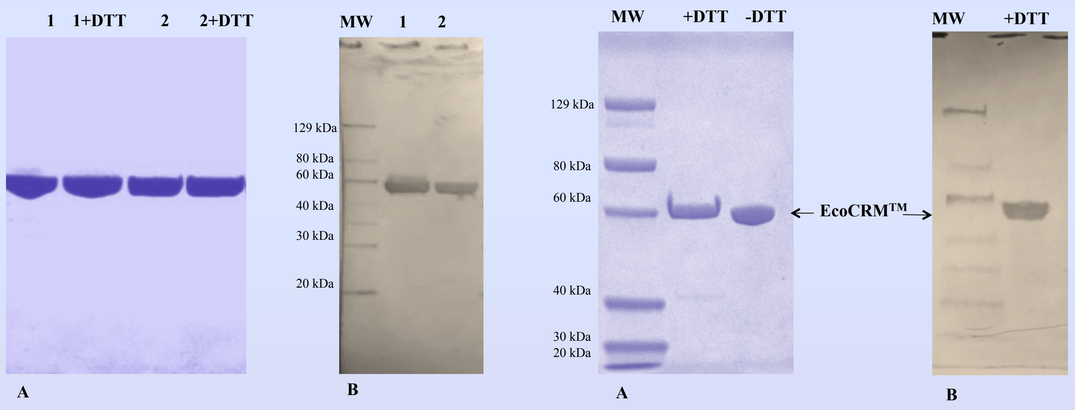
EcoCRM™ was prepared in buffers from pH 6 to pH 9, without additives, and concentrated to 28 mg/ml using an Amicon Ultra device. Recovery after concentration was >95% (BCA assay) and the monomeric state of the protein was confirmed by SEC HPLC.

High concentrations after modification



EcoCRM™ was labeled with a 30 fold molar excess of the NHS maleimide reagent (GMBS) and concentrated to 30 mg/ml at pH 6.8 using an Amicon Ultra spin device. Protein recovery was >95% (BCA assay). Monomeric state of the modified protein was confirmed by SEC HPLC.

EcoCRM™: SDS-PAGE and WB



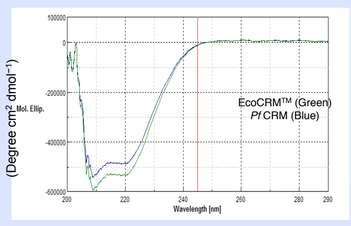
1- *Pf* CRM_{197} (*Pseudomonas*) 2- EcoCRM™ (*E. coli*)
A: SDS-PAGE, reduced vs non reduced, Coomassie Blue staining
B:Western Blot, detection with polyclonal rabbit@CRM_{197} (AIC Biotech)

EcoCRM™ : Stability at 4°C

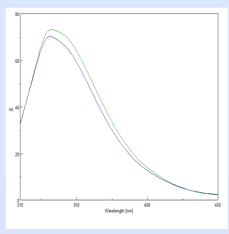
EcoCRM™ shows minimal "nicking" and degradation after 60 days at 4°C

EcoCRM™ : Secondary structure

Circular dichroism spectra



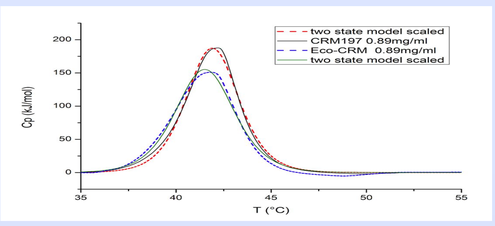
Intrinsic Fluorescence



The similarity of the far UV CD spectra indicates similar percentages of alpha-helix and beta-sheet of EcoCRM™ and *Pf* CRM.

Tryptophan fluorescence (excitation at 295 nm) is diagnostic of the conformational state of the protein as the emission peaks are influenced by the microenvironment. The overlay of the the spectra of EcoCRM™ and *Pf* CRM are indicative of similar conformations for each.

Protein Thermal Denaturation Differential Scanning Calorimetry



EcoCRM™ and "native" CRM_{197} have similar Tm and ΔH values within experimental error, Indicating that they have similar thermal stability. This confirms that EcoCRM™ has its full complement of disulfides.

EcoCRM™ : LC MS Peptide Mapping

Eco-CRM: 99% sequence coverage

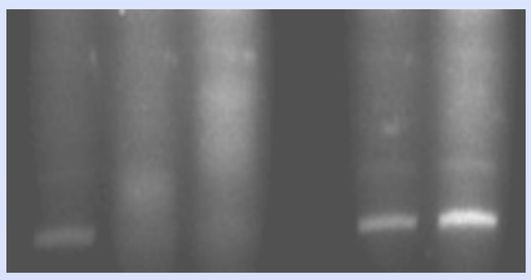
M/GADDVVDSS KSFVMENFSS YHGTKPGYVD SIQKGIQKPK SGTQGNYDDD WKEFYSTDNK YDAAGYSVDN ENPLSGKAGG VVKVTYPGLT KVLALKVDNA ETIKKELGLS LTEPLMEQVG TEEFIKRFGD GASRVVLSLP FAEGSSSVEY INNWEQAKAL SVELEINFET RGKRGQDAMY EYMAQACAGN RVRRSVGSSL SCINLDWDVI RDKTKTKIES LKEHGPIKNK MSESPNKTVS EEKAKQYLEE FHQTALEHPE LSELKTVTGT NPVFAGANYA AWAVNVAQVI DSETADNLEK TTAALSILPG IGSVMGIADG AVHHNTEEIV QSIALSSLMV AQAIPLVGEL VDIGFAAYNF VESIINLFQV VHNSYNRPAY SPGHKTQPFL HDGYAVSWNT VEDSIIRTGF QGESGHDIKI TAENTPLPIA GVLLPTIPGK LDVNKSKTHI SVNGRKIRMR CRAIDGDVTF CRPKSPVYVG NGVHANLHVA FHRSSSEKIH SNEISSDSIG VLGYQKTVDH TKVNSKLSLF FEIKS

CRM: 95% sequence coverage

GADDVVDSSK SFVMENFSSY HGTKPGYVDS IQKGIQKPKS GTQGNYDDDW KEFYSTDNKY DAAGYSVDNE NPLSGKAGGV VKVTYPGLTK VLALKVDNAE TIKKELGLSL TEPLMEQVGT EEFIKRFGDG ASRVVLSLPF AEGSSSVEYI NNWEQAKALS VELEINFETR GKRGQDAMYE YMAQACAGNR VRRSVGSSLS CINLDWDVIR DKTKTKIESL KEHGPIKNKM SESPNKTVSE EKAKQYLEEF HQTALEHPEL SELKTVTGTN PVFAGANYAA WAVNVAQVID SETADNLEKT TAALSILPGI GSVMGIADGA VHHNTEEIVA QSIALSSLMV AQAIPLVGEL VDIGFAAYNF VESIINLFQV VHNSYNRPAY SPGHKTQPFL HDGYAVSWNT VEDSIIRTGF QGESGHDIKI TAENTPLPIA GVLLPTIPGK LDVNKSKTHI SVNGRKIRMR CRAIDGDVTF CRPKSPVYVG NGVHANLHVA FHRSSSEKIH SNEISSDSIG VLGYQKTVDH TKVNSKLSLF FEIKS

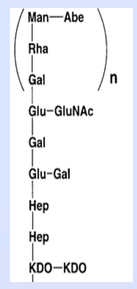
Peptide maps of EcoCRM™ and "native" CRM were compared. Following trypsin, chymotrypsin or Glu-C digestion, the peptides were analyzed by nano LC/MS/MS. Over 95% coverage was achieved. The peptide maps were identical except for the N-terminal fragment.
EcoCRM™ was found to have approximately 50% of N formyl methionine as the starting amino acid.

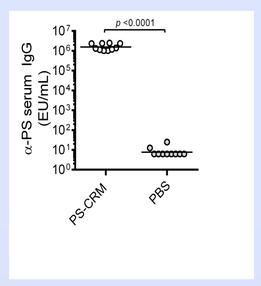
EcoCRM™ : Nuclease Activity



The nuclease activity of EcoCRM ™ was assayed by 30 min incubation with linearized DNA and electrophoresis on 1% agarose. Tetanus toxin heavy chain fragment (TTHC) expressed and purified from *E.coli* was used as a negative control. EcoCRM™ but not TTHC caused degradation of the DNA fragment.

Conjugates Immunogenicity





S. typhimurium core outer polysaccharide (COPS)) was covalently linked to EcoCRM™ via its KDO group (A).

Groups of 10 female outbred CD-1 mice were immunized 3 times with 2.5 µg of conjugated COPS at 28 day intervals. Serum was taken 21 days after the final dose and assessed for anti- *S. typhimurium* COPS IgG titers by ELISA (B).

SUMMARY

EcoCRM™

- ✓Purified from *E. coli* as single chain monomeric protein
- ✓Remains monomeric at pH 6-9 even at high concentrations
- ✓Excellent immunogenicity and carrier function

Equivalence with CRM_{197} from other sources:

- ✓*Amino acid sequences match except for the presence of f-Met*
- ✓*Possesses intrinsic nuclease activity*
- ✓*Biophysical similarity: DSC, CD, intrinsic fluorescence*

→ *An economical alternative* ←

Acknowledgement
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