Analytical Comparability Assessments of Commercially Available Recombinant CRM_{197} Proteins from Different Manufacturers and Expression Systems

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Introduction

CRM_{197}, a single amino acid mutant of diphtheria toxin, is a commonly used carrier protein in licensed conjugate vaccines. Until recently, this protein has been commercially available only as expressed in *Corynebacterium diphtheriae ("C7")* and *Pseudomonas fluorescens* (Pfenex Reagent Proteins), as secreted and periplasmic proteins, respectively. A new CRM_{197}, EcoCRM™ (Fina Biosolutions), is expressed in *E. coli* as a soluble, properly-folded intracellular protein and purified at high yields. To determine comparability of EcoCRM™ with the other two commercial sources of CRM_{197}, an assessment using a wide variety of physicochemical assays was performed. A comprehensive analysis demonstrates that recombinant CRM_{197}'s expressed in two heterologous systems (*E. coli* and *Pseudomonas fluorescens*) are overall highly similar in terms of primary sequence/post translational modifications, higher-order structural integrity, apparent solubility, and physical stability profile (vs. pH and temperature) with CRM_{197} from *Corynebacterium*.

The physicochemical assays established in this work to monitor the key structural attributes of CRM_{197} should also prove useful as complementary characterization methods (to routine quality control assays) to support future process and formulation development of lower-cost CRM_{197} carrier proteins for use in conjugate vaccines.

These results demonstrate that EcoCRM™ has the potential to be a low-cost source for CRM_{197} carrier protein for conjugate vaccines.

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Objective

CRM_{197}, a genetically detoxified diphtheria toxin, is a widely used conjugate vaccine carrier protein. Unlike toxoided protein, CRM_{197} has its full complement of lysines available for conjugation. Manufacturing conjugate vaccines is a complex process and multiple factors can affect the product e.g., polysaccharide composition, linker length, conjugation chemistry and the carrier protein structure. Ensuring the carrier protein is properly manufactured and well-characterized is critical to the consistent manufacture of conjugate vaccines.

CRM_{197} is translated as a 58 kDa polypeptide in that is commonly expressed in *C. diphtheria,* usually at low yield. As highly multivalent vaccines, such as ones for *S. pneumoniae*, use large amounts of carrier protein, the cost of CRM_{197} can make up a significant fraction of the overall cost of goods. This is an important consideration in efforts to make these vaccines affordable for low income countries.

In this study, we evaluated commercially available recombinant CRM_{197} molecules from three expression systems, the traditional *C. diptheriae ("C7 CRM") from List Labs)*, and two heterologous systems, *E. coli (EcoCRM™ from Fina Biosolutions)* and *P. fluorescens (from Pfenex CRM from Reagent Proteins)*. They were analytically characterized and compared in terms of structural integrity, solubility and conformational stability profiles using a wide variety of biochemical, biophysical assays.

Primary Structure Analysis

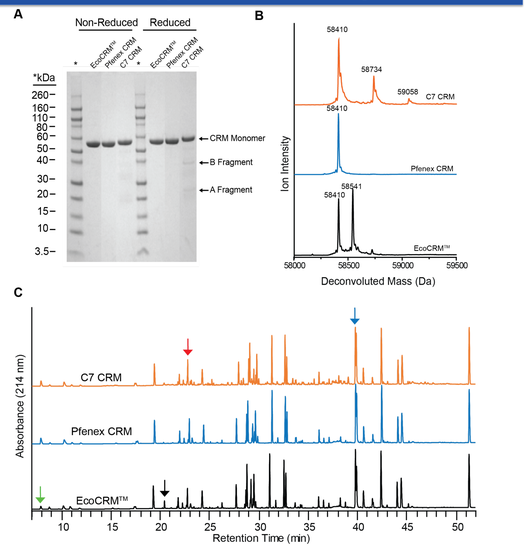

Figure 1. (A) SDS-PAGE analysis of the three CRM_{197} molecules under non-reducing and reducing conditions. (B) Representative intact mass spectrometry analysis of the non-reduced CRM_{197} molecules. (C) Representative LC peptide mapping chromatograms of non-reduced trypsin-digested CRM_{197} samples. The red and blue arrows indicate the two peaks containing Cys^{461}-Cys^{273} and Cys^{186}-Cys^{200} bonded peptides, respectively. The black and green arrows indicates an N-terminal peptide with or without a Met residue, respectively.

Charge Heterogeneity Analysis

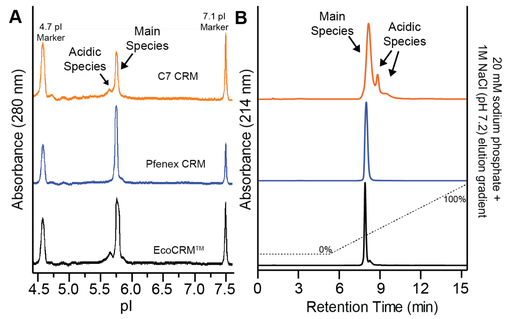

Figure 2. The overall charge heterogeneity of the three CRM_{197} samples were compared through both (A) capillary isoelectric focusing, and (B) anion-exchange chromatography. The migration of two pI markers (4.7 and 7.1) are indicated in the cIEF electropherogram for C7 CRM.

Size Analysis

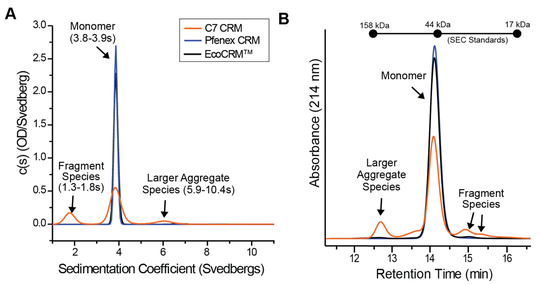

Figure 3. The size and distribution of monomer, aggregate and fragment species in the three CRM_{197} samples were compared through (A) sedimentation velocity analytical ultracentrifugation (SV-AUC), and (B) size exclusion chromatography (SEC). The elution times and molecular weight values of gel filtration standard proteins are shown above the SE chromatograms.

Higher Order Structure Analysis

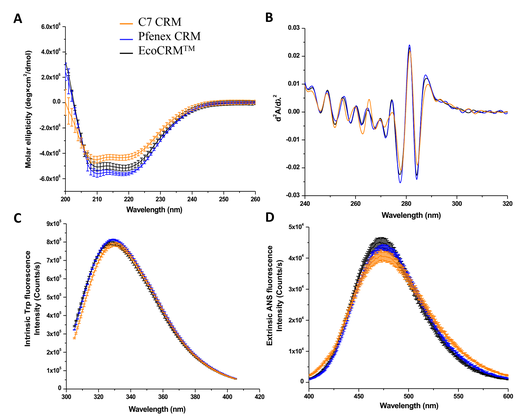

Figure 4. (A) The overall secondary structures were evaluated by Far-UV circular dichroism spectra, (B) the overall tertiary structures were monitored by both (B) Second derivative UV absorbance spectra, and (C) Intrinsic tryptophan fluorescence emission spectra. (D) The overall surface hydrophobicity of the samples were determined by Extrinsic ANS fluorescence emission spectra. The three CRM_{197} samples were compared at 10°C in PBS pH 7.2.

Physical Stability Analysis as a Function of pH and Temperature

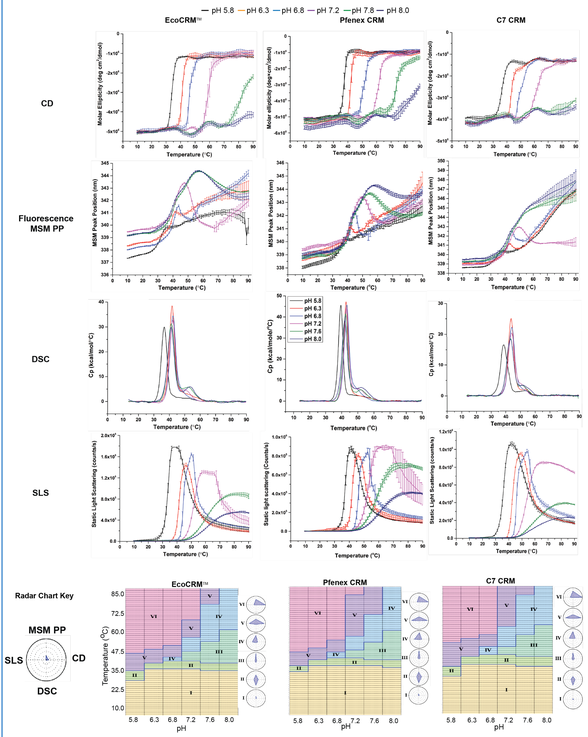

Figure 5. The molar ellipticity at 222 nm (CD), intrinsic Trp fluorescence MSM peak position, differential scanning calorimetry (DSC), and static light scattering at 295 nm (SLS) for EcoCRM™, Pfenex CRM, and C7 CRM in PBS buffer at six different pH s (5.8, 6.3, 6.8, 7.2, 7.6, and 8.0) were measured as a function of temperature (10-90°C). A radar chart[2] for each CRM_{197} protein was generated from the resulting data. Six distinct biophysical states of each protein (regions I-VI) were observed as a function of pH and temperature The contribution of each biophysical technique towards the different structural states of each CRM_{197} are indicated on each vertex in the key.

Summary of Key Structural Attributes

Structural Attribute	Analytical Method	Measurement	EcoCRM™	Pfenex CRM	C7 CRM
Primary Structure	Mass Spectrometry	Intact Protein Mass (Da)	58410 ± 1 58541 ± 1*	58410 ± 1	58410 ± 1 58734 ± 1* 59058 ± 1*
		*Post-Translational Modification	N-terminal Met (+131 Da)	-	Glycation (+324, +648 Da)
Protein Charge Heterogeneity	Capillary Isoelectric Focusing	Main Peak (pI 5.6-5.7) (%) Acidic species (pI 5.5-5.6) (%) Basic species (pI 5.7-5.8) (%)	92 ± 0 7 ± 0 1 ± 0	99 ± 0 0 ± 0 1 ± 0	79 ± 1 21 ± 1 0 ± 0
	Anion Exchange Chromatography	Main Peak (%) Acidic Species (%)	92 ± 0 8 ± 0	100 ± 0 0 ± 0	75 ± 1 024 ± 1
Higher Order Structure	Circular Dichroism	Spectral minima at 10°C (nm) Tm (°C)	208 & 222 58.2 ± 1.0	208 & 222 57.9 ± 0.3	208 & 222 NA*
	Intrinsic Trp Fluorescence	Peak emission maximum at 10°C (nm) Tm (°C)	328 ± 1 41.2 ± 0.3	329 ± 1 42.3 ± 0.3	331 ± 1 43.2 ± 0.2
	Static Light Scattering	Tonset (°C)	43.0 ± 0.6	43.6 ± 0.3	45.2 ± 0.7
	Extrinsic ANS Fluorescence	Peak emission intensity (10^3counts/s) Tm (°C)	441 ± 7 40.8 ± 0.5	437 ± 8 41.4 ± 0.5	411 ± 16 42.5 ± 0.2
	Differential Scanning Calorimetry	Tonset (°C) Tm1 (°C) Tm2 (°C)	32.7 ± 0.3 42.0 ± 0.0 51.3 ± 0.2	34.8 ± 0.2 42.8 ± 0.0 51.1 ± 0.2	35.2 ± 0.6 44.0 ± 0.1 51.7 ± 0.1
Aggregate/ Particle Analysis	Size Exclusion Chromatography	Monomer (%) Aggregates (%) Fragment (%)	98 ± 0 1 ± 0 2 ± 0	100 ± 0 0 ± 0 0 ± 0	73 ± 1 13 ± 1 14 ± 0
	Sedimentation Velocity Analytical Ultracentrifugation	Monomer (%) Aggregates (%) Fragment (%)	99 ± 0 1 ± 0 0 ± 0	100 ± 0 0 ± 0 0 ± 0	68 ± 1 11 ± 1 21 ± 0
	Resonant Mass Measurement (0.2-2 μm)	Total particles after dilution (number/mL x 10^5)	1.7 ± 0.4	1.6 ± 1.2	5.1 ± 0.4
	Micro-Flow Imaging (2-100 μm)	Total particles after dilution (number/mL)	85 ± 56	84 ± 9	453 ± 163

Table 1. Summary of the key structural attributes (physicochemical and *in vitro* antigenicity) of EcoCRM™, Pfenex CRM, and C7 CRM. * Broad and slightly biphasic nature of the transition in C7 CRM prevented accurate calculation. The experimental data is presented in full in Hickey et al[1].

Conclusions

This study provides baseline data sets of the key structural attributes of CRM197 to enable future comparisons of the physicochemical properties of this carrier protein.

The data provides a basis for the acceptance of CRM_{197} produced in low cost expression systems, such as Fina Biosolution's EcoCRM™.

References

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Acknowledgement

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For more information on EcoCRM™ from Fina Biosolutions, please contact info@FinaBio.com or visit www.Finabio.com