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Research Article

Use of imaged capillary isoelectric focusing technique in development of diphtheria toxin mutant CRM197

Polysaccharide based-vaccines have been successful in providing protection in adults from bacterial infections, however they are not as effective in infants or young children. To enhance the immune response in these high risk groups, the polysaccharide is conjugated with a carrier protein such as cross-reacting material 197 (CRM197). The CRM197 protein has been well-characterized biochemically and biophysically using various analytical techniques however, none of these have been CE-based methods. Of the various CE techniques, imaged capillary isoelectric focusing (icIEF) is a method that has been used extensively in the field of protein-based drug development as a tool for product identification, stability monitoring, and characterization. Applications of icIEF technique using Convergent Bioscience icIEF instrumentation with whole-field imaging technology are presented and discussed in this paper. These applications include rapid method development to establish a CRM197 identity test for product release, a concentration assay for upstream and downstream in-process product development, and CRM197 stability with respect to its charge heterogeneity under accelerated temperature stress. The data presented demonstrates the utility of the icIEF method as a multifunctional assay because it can screen for better product candidates during early stage clonal selection as well as support in-process and final product characterization throughout CRM197 development.

Keywords:
Carrier protein / Cross-reacting material 197 / Diphtheria toxin / Imaged capillary isoelectric focusing / p*I*

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1 Introduction

Polysaccharide based-vaccines have been successful in providing protection in adults from bacterial infections, however they are not so effective in high risk population such as infants, young children, and elderly or immune-compromised people. Poor immune response has led to the advanced development of conjugated polysaccharide to protein carrier vaccines that induce T-cell dependent responses to boost immunogenicity in these high risk groups [1, 2]. Currently, there are at least five different carrier proteins used in the conjugate-based vaccine industries, diphtheria toxoid [3], tetanus toxoid [4], protein D from *Haemophilus influenza* [5], outer membrane protein complex from *Neisseria meningitides* [6, 7], and cross-reacting material 197 (CRM197), a nontoxic diphtheria toxin variant [8, 9].

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Abbreviations: CRM197, cross-reacting material 197; **icIEF**, imaged capillary isoelectric focusing

CRM197 has been used as a carrier protein in many vaccine conjugates such as pneumococcal (Prevnar™), meningococcal (Menveo®, Menjugate®, Meningitec®), as well as *H. Influenzae* type B (HibTITER®, Vaxm-Hib®) [10]. The nontoxic, CRM197, is a result of one amino acid substitution of glycine to glutamic acid at position 52 [11]. The protein contains 535 amino acid residues with a molecular weight of 58.4 kDa and theoretical p*I* of 5.8. CRM197 is generally cultured in *Corynebacterium diphtheriae* for manufacturing purposes and is secreted into the cell culture supernatant. The CRM197 protein is then recovered by filtration or centrifugation for further purification using various downstream chromatographic processing steps. More recently a large titer of CRM197 production from *Psudomonas fluorescens* using the Pfenex expression system has been described [12,13]. The biochemical characteristics and preclinical performance appears to be identical to CRM197 produced from *C. diphtheriae* [14].

The biochemical and biophysical characterization of CRM197 has been studied for many years using many different analytical techniques [15, 16]. However, none of them

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have included CE methods to the best of our knowledge. CE-based methods have increasingly been applied to the analysis of a variety of different types of proteins. Imaged capillary isoelectric focusing (icIEF), is one CE-based method that has been used extensively in the field of protein-based drug and vaccine development as a tool for product identification, stability monitoring, and characterization [17, 18]. It is now becoming a standard tool to evaluate protein charge heterogeneity [19]. It is routinely used in quality-control environments in addition to analytical product development laboratories to assess charge heterogeneity, process consistency, and product integrity. Furthermore, assay validation requirements can be easily satisfied in good manufacturing practice environments [20].

This icIEF uses whole capillary imaging technology to detect the focused protein at 280 nm. Two important advantages of using icIEF are rapid method development times and high reproducibility compared to performing cIEF in traditional CE instruments. In conventional CE systems, once the isoelectric focusing separation occurs, the proteins still need to be mobilized to pass the single point UV detector which can cause extra line broadening and increases analysis time (20 min in icIEF vs. 60 min in traditional cIEF). Another advantage of icIEF is the ability to monitor the proteins focusing progress after sample injection so that separation dynamics such as adsorption and precipitation can be visualized [21, 22].

In this paper, we report a wide range of icIEF technique applications in the development of CRM197 carrier protein for a conjugate-based polysaccharide vaccine. Furthermore, it demonstrates how using this one method can increase the efficiency of analytical testing and guide process and/or formulation development. This technique has been used to identify CRM197 for product release, evaluate CRM197 product stability based on charge heterogeneity profile, and determine CRM197 concentration during cell culture and downstream purification development.

2 Materials and methods

2.1 Reagents

All methylcellulose containing solutions and the p*I* markers 3.59, 4.65, 9.6, 9.77 were obtained from ProteinSimple (Santa Clara, USA). Pharmalyte ampholytes (pH 3–10 and pH 4–6.5) were purchased from GE Healthcare (Uppsala, Sweden). CE-water grade was purchased from Microsolv (Eatontown, NJ, USA). Urea, glycerol and p*I* marker 7.6 were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2 Sample preparations

CRM197 protein was produced from *P. fluorescens*. The CRM197 final product was purified using various chromatography steps to ≥99% by the Vaccine Process Research and Development group at Merck Research Laboratories (Merck & Co., West Point, PA, USA). Their final product sample concentrations were measured using UV/Vis spectrophotometry with known extinction coefficients.

2.3 IcIEF procedure

Detailed description of this method including a schematic diagram of the instrumentation has been published previously [21, 23]. All icIEF runs were conducted on Convergent Bioscience iCE280 Analyzer (ProteinSimple) with a Prince autosampler (ProteinSimple). The IEF separation was conducted using a 50 mm long, 100 μm id × 200 μm od silica capillary coated with fluorocarbon (ProteinSimple) to eliminate EOF that is required to perform icIEF in general. The catholyte consists of 0.1 M NaOH in 0.1% methylcellulose and the anolyte is 0.08 M phosphoric acid in 0.1% methylcellulose. The absorbance of the focused proteins is detected at 280 nm. The sample mix (preparation described below) was vortexed for 30 ± 15 s followed by centrifugation at 10 000 rpm for 5 min to remove bubbles. A 150 μL aliquot of sample was transferred to a glass autosampler vial, placed in the vial holder, capped and placed in the autosampler carousel at 8°C for analysis. The sample injection time ranged from 170 to 240 s (dependent on the capillary installation) and the injection pressure was 1000 mbar for all samples. The capillary was conditioned prior to each sample injection with 0.5% methylcellulose for a variable period of 210–315 s depending on capillary installation. The sample containing the ampholyte mixture is injected into the capillary using pressure and a prefocus voltage of 1500 V is applied for 1 min to remove salt and the focus voltage of 3000 V is applied for 8 min to create the pH gradient. The spectral image was collected approximately every 34 s. At the end of separation, the capillary was rinsed with 0.5% methylcellulose for 180 s. The capillary is cleaned by pressure washing with deionized water for 10 min and dried by applying air pressure for 5 min. The dry capillary is stored at ambient temperature.

2.3.1 Concentration assay

A working ampholyte solution was prepared by mixing 400 μL of narrow range ampholytes pH 4–6.5 with 200 μL of broad range ampholytes pH 3–10. The sample mix was prepared by mixing 70 μL of 1% methylcellulose, 9 μL of working ampholyte solution, 40 μL of glycerol, 1 μL of p*I* marker 4.65, and 1 μL of p*I* marker 7.60 with various volume of CRM197 to make standard curves and various amount of water to obtain a final volume of 200 μL for each sample. The standard curve ranges from 62.5 to 500 μg/mL. The samples were prefocused for 1 min using a voltage of 1500 V followed by 8 min at 3000 V. The accuracy of the assay was evaluated by adding 125 μg of CRM197 to a CRM197 sample of known concentration and calculating the percentage recovery. The percentage recovery was determined for both crude and purified CRM197 material.

2.3.2 Identity test

A working ampholyte solution was prepared by mixing 400 μL of narrow range ampholytes pH 4–6.5 with 200 μL of broad range ampholytes pH 3–10. The sample mix was prepared by mixing 70 μL of 1% methylcellulose, 9 μL of working ampholyte solution, 40 μL of glycerol, 10 μL of CRM197 (5 mg/ml), 71 μL of water, 1 μL of p*I* marker 4.65, and 1 μL of p*I* marker 7.60. The samples were prefocused for 1 min using a voltage of 1500 V followed by 8 min at 3000 V.

2.3.3 Anti-CRM titration binding study

A CRM197 sample, an unrelated mAb, and an anti-CRM197 mAb were diluted to 1 mg/mL in water. In four separate vials, anti-CRM197 mAb was added to CRM197 in molar ratios of 1:0.3, 1:0.7, 1:1, and 1:1.6 of CRM197:anti-CRM197 mAb. A fifth vial was prepared by adding an unrelated mAb to CRM197 at a molar ratio of 1.6 unrelated mAb:1 CRM197. The samples were incubated at room temperature for 5 min.

The sample mix was prepared by mixing 70 μL of 1% methylcellulose, 9 μL of broad range ampholytes covering the pH range of 3–10, 40 μL of glycerol, 120 μL of the CRM197:mAb solutions, 1 μL of p*I* marker 3.59, and 1 μL of p*I* marker 9.77. CRM197, the unrelated mAb, and the anti-CRM197 mAb were prepared independently using similar sample preparation conditions. The samples were prefocused for 1 min using a voltage of 1500 V followed by 8 min at 3000 V.

2.3.4 CRM197 stability

A stability study was performed using formulated CRM197 at 40 mg/mL. Samples were placed at −70°C, −20°C, 4°C, and 22°C for 3 months. At each time point a small aliquot of CRM197 sample was taken for charge heterogeneity testing using icIEF.

3 Results and discussion

3.1 CRM197 icIEF method development

Method development of CRM197 icIEF was initiated using a commonly used broad range ampholyte that covers a pH range of 3–10. The method was optimized further by mixing the broad range ampholytes with narrow range ampholytes spanning a pH range of 4–6.5 at a 1:2 ratio, respectively. This optimization was performed to increase the resolution since the theoretical p*I* of CRM197 is about 5.8. After establishing the ampholyte mixture and ratio of addition the focusing time was evaluated in the range of 6 to 16 min at a constant voltage to determine the optimum focusing time. During assay optimization the main peak, based on the peak of maximum total area, was observed at a p*I* of approximately 5.77. However, multiple irreproducible peaks were focusing near a the p*I* range of 6.6–6.9 (see Fig. 1A).

It is known that when the protein is at its p*I*, it has a tendency to precipitate due to electroneutrality. It is thought that the peaks around p*I* 6.6–6.9 are conformational variants of CRM197 that are partly denatured in electrophoretic environments and lack stability when a voltage is applied across the capillary. We have found that adding 20% glycerol as a protein stabilizer to the sample mixture provides reproducible results by eliminating the peaks at p*I* 6.6–6.9 and thus only one peak remains at the theoretical p*I* of 5.91 (Fig. 1B). This observation that all of the sample material focuses at a p*I* of 5.91 when 20% glycerol was added is supported by the finding that the peak area of middle trace (20% glycerol) is identical to the sum of total peak areas in upper trace (no glycerol) shown in Fig. 1C. The hypothesis that the irreproducible peaks focusing between a p*I* of 6.6 and 6.9 are denatured CRM197 was tested by treating CRM197 with urea. Interestingly, when 8 M urea is added to the sample preparation all CRM197 material is focused at a p*I* of 6.8 (lower trace in Fig. 1C) supporting the hypothesis that the peaks observed at p*I* 6.6–6.9 are related to various partly denatured CRM197 conformations. This observation suggests that the addition of 8M urea uniformly denatures all CRM197 populations while adding 20% glycerol presumably helps reversing the partly disordered CRM197 population structures to their native state.

3.2 Concentration assay

During CRM197 development, its titer yield needs to be analyzed early in the process upstream samples such as lysates as well as in the downstream process purification steps. This poses an analytical challenge since crude lysate samples contain many other native cellular proteins as well as lipids, carbohydrates, and cell culture media. ELISA methods could potentially be used, however, this technique is usually time consuming and is dependent on two or three reagents such as specific antibodies and reference standard. Initially, RP HPLC was attempted and more than ten different types of RP columns were screened to evaluate CRM197 concentration. However, due to the strong binding affinity of CRM197 to the columns, protein carry-over was an issue in determining the protein concentration. The strong binding affinity of CRM197 to RP column in acidic environments is somewhat expected since lowering the pH of CRM197 causes the protein to undergo large conformational changes by exposing its hydrophobic regions. Therefore, a low pH RP HPLC column will bind CRM197 very tightly [24, 25].

The established icIEF method above has been evaluated for fitness of use as a titer assay during this CRM197 process development. The standard analytical parameters such as linearity, LOD, LOQ, reproducibility, and accuracy were evaluated. Figure 2A illustrates the electropherograms for the five CRM197 concentration standards and its calibration curve demonstrates linearity over the selected range (63–500 μg/mL) with correlation coefficients ($r^2 > 0.990$). The

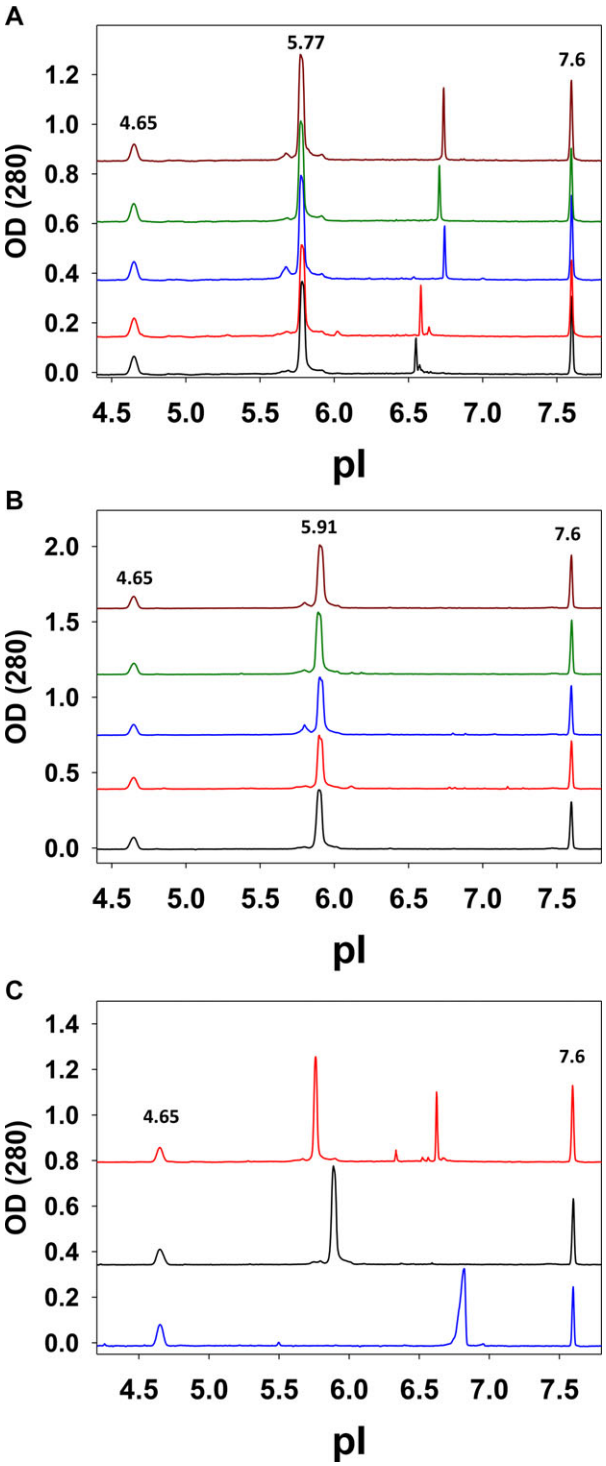

Figure 1. (A) Five different lots of CRM197 without 20% glycerol are illustrated to show the inconsistency of peaks around p*I* 6.6–6.9. The CRM197 main peak at an approximate p*I* of 5.8 is very reproducible. (B) After adding 20% glycerol to the ampholyte mix all five lots of CRM197 focus consistently and reproducibly at p*I* 5.9. (C) Upon denaturation by adding 8 M urea to CRM197 the p*I* shifts to 6.8 (lower trace) indicating that the inconsistent peaks observed around p*I* 6.6–6.9 (upper trace) is likely due to partly denatured CRM197 conformations. Upon adding 20% glycerol all material focuses at p*I* 5.9 (middle trace).

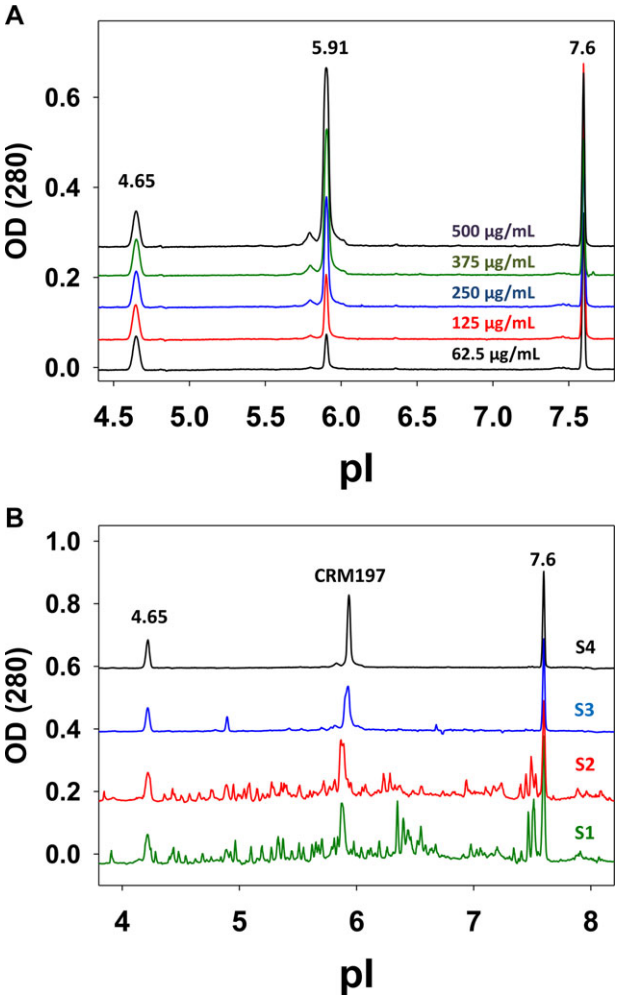

Figure 2. (A) Electropherograms of CRM197 standards in the concentration range of 62.5–500 µg/mL demonstrating assay linearity with an $r^2 > 0.990$. The two p*I* markers are 4.65 and 7.6. (B) IcIEF electropherograms of two upstream lysate samples (S1 and S2) and two downstream process purification samples after chromatographic elution (S3 and S4).

RSD of peak area for three injections of each concentration standard was <10% throughout all experiments. The estimated method LOD and LOQ are 0.8 µg/mL and 16 µg/mL, respectively.

The driver for the icIEF concentration assay was to develop a method suitable for measuring CRM197 titer in both upstream and downstream process intermediate samples. The electropherograms are shown in Fig. 2B for two upstream cell lysate samples (samples 1 and 2) and two downstream samples after chromatographic processing steps (samples 3 and 4). The two upstream lysate samples are clearly impure and contain many other proteins, however, the CRM197 peak is observed at p*I* ~5.8 (pure CRM197 was spiked to further confirm the peak in crude lysates). The method was further evaluated with respect to its reproducibility for 3 days and yielded a <15% RSD of concentration measurements for upstream samples 1 and 2 and a RSD of <5% for samples 3 and

4. The spike recovery was 80% for sample 1 and ranges from 90 to 110% for the other three samples. The icIEF method clearly provides high resolution separation as it is shown that many other protein peaks are observed in lysate samples. This again demonstrates the high efficiency separation power of CE technology in general. Furthermore, this icIEF method does not suffer from protein carry over as observed in RP HPLC. Hence, the method was also utilized to quickly screen the highest cell clone producer and the optimal fermentation condition to provide maximum CRM197 titer.

3.3 Product identity test

Biological product development regulations state that product identity is required for a site that produces multiple different products. The identity test should be highly specific and be based on unique aspects of its particular protein molecular structure. The identity test is normally qualitative in nature. Traditionally, ELISA is used as an identity test for biological products but this technique is usually time and labor intensive and requires specific reagent(s). Another method commonly used for protein-based product identity test is peptide mapping using RP HPLC after trypsin digestion. Although this method is better and slightly faster than ELISA, it still requires 2 days due to the trypsin digest treatment. This icIEF method for product identification is easy to perform in a quality control environment, is a relatively fast experiment that requires no sample pretreatment, and is a much more robust technique compared to labor intensive methods such as ELISA and trypsin digest RP HPLC.

It has been demonstrated that the icIEF method can be used as an identity test in therapeutic proteins such mAb to replace the ELISA and the RP HPLC trypsin digestion peptide analysis methods [18, 19]. The product release identity test criteria using icIEF is usually determined by a combination of both p*I* value and electropherogram profile matching with a reference standard profile. Furthermore, the specificity of this CRM197 identity test can be elucidated further by binding the mAb that targets CRM197 as demonstrated in Fig. 3. Upon titration of CRM197 with its specific anti-CRM197 mAb, the electropherogram profiles change and stop changing at about 1:1 mole ratio (Fig. 3A). In addition, the acidic peaks of this CRM197 have also shifted together with its main peak indicating that these acidic forms are also active in terms of binding to its anti-CRM197 mAb. In contrast, a nonspecific mAb does not bind to CRM197 as shown in Fig. 3B. Note that the observed CRM197 p*I* value is lower than 5.9 in Fig. 3B because these experiments were performed using only ampholytes in the pH range of 3–10. In addition, p*I* markers differing from the markers used in the final method were used in these experiments. It is known that the p*I* value of proteins may shift slightly depending on the ampholyte range and p*I* markers used.

As demonstrated above, CRM197 in general contains charge heterogeneity and it is well-known that some acidic forms of certain proteins reduce the binding ability to their antigens and hence lower potency [26, 27]. The assessment of acidic forms with respect to potency is normally evaluated using a combination of two or three analytical methods and is a very tedious process. Here we illustrate the use of an icIEF method to characterize the direct binding of CRM197 with its specific antibody. Hence, this identity test using icIEF method provides two advantages. First, it confirms correct p*I* with its charge heterogeneity profile compared to reference standard and second, it demonstrates its binding activity similar to ELISA methods. This assay helps tremendously in providing a quick answer regarding the binding activity of the CRM197 acidic or basic forms.

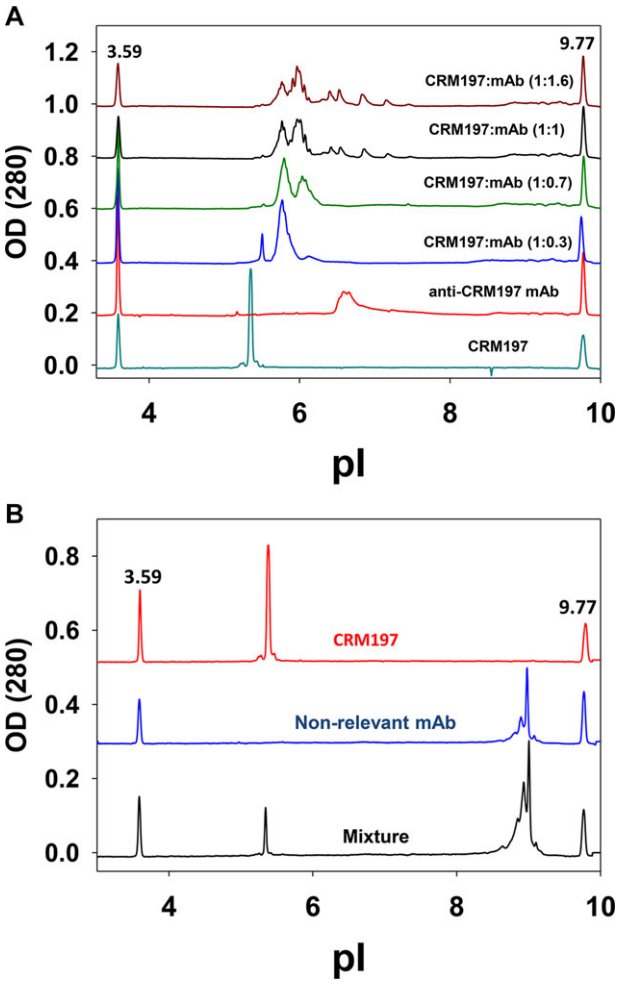

Figure 3. (A) Titration of anti-CRM197 mAb with CRM197. The two p*I* markers are at 3.59 and 9.77. The peak shape stops changing at a molar ratio of 1 to 1 as expected of an antigen–antibody binding complex. These results show a direct binding between CRM197 and its anti-CRM197 mAb. (B) IcIEF electropherograms of CRM197 (top trace), nonrelevant mAb (middle trace), and a mixture of both (bottom trace). There is no change in peak shape of both samples in the mixture indicating that these two species do not interact. Note that the CRM197 concentration is two times lower and the nonrelevant mAb concentration is two times higher in this mixture than samples in top and middle traces, respectively. These two experiments support the identity test assay for CRM197.

3.4 CRM197 stability

Stability-indicating assays are an essential part of vaccine development because they provide recommendations on storage conditions and expiry dating as well as guide formulation buffer optimization. The icIEF method is one stability-indicating assay that can be used as a tool in protein-based vaccines to monitor charge heterogeneity as part of product stability assessment. The CRM197 stability was performed at four different temperatures (−70°C, −20°C, 4°C, and 22°C) for 3 months. CRM197 precipitates immediately at 37°C because its thermo-denaturation temperature starts at 35°C and maximizes at about 45°C [16]. Representative electropherograms for 3 months of stability monitoring at 22°C is illustrated in Fig. 4A. It demonstrates that the acidic peaks (p*I* 5.77 and 5.82) have increased while the main peak (p*I* 5.9) has decreased during the 3 month storage period as shown in Fig. 4B. Similarly, the acidic variants increase when CRM197 is stored in liquid (4°C or 22°C) compared to when stored frozen (−20°C or −70°C). As a result of this short stability study, CRM197 bulk product is now stored at −70°C. It is interesting to note that other stability-indicating assays such as SEC-HPLC for aggregation, SDS-PAGE for clipping, and ELISA do not indicate any change after 3 months (data not shown).

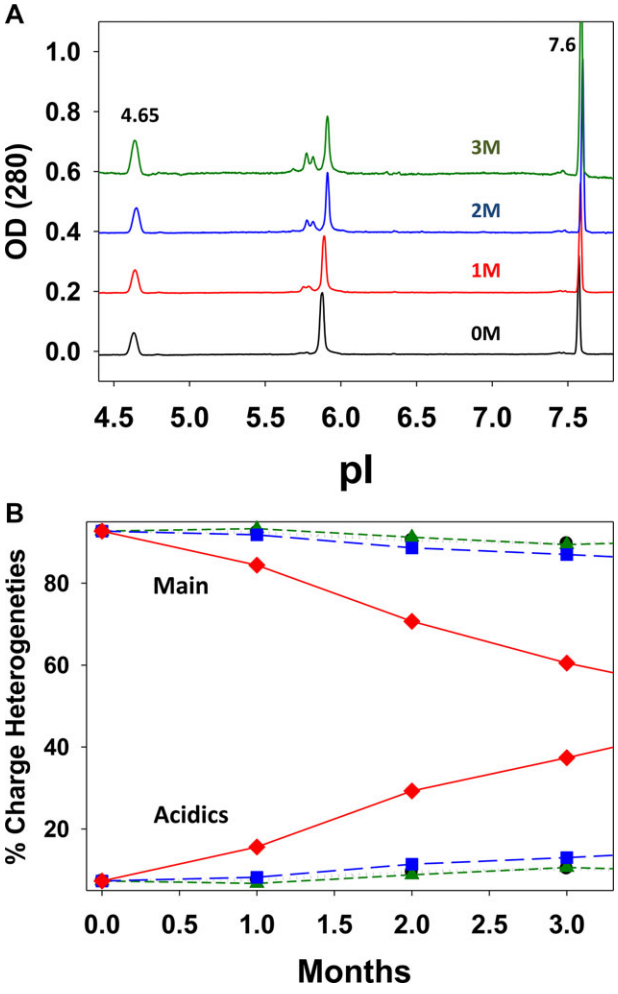

Figure 4. Stability of CRM197 upon heat-stress. (A) CRM197 electropherograms show an increase of acidic peaks around p*I* 5.77 and 5.82 upon storage at 22°C for 3M. (B) CRM197 stability curves at four different temperatures for 3 months. At 22°C (solid line, ◆), the percentage acidic peak area increase corresponds to the percentage main peak area decrease; at 4°C (long dash line, ■), the percentage acidic peak area increase is much slower indicating slight degradation over three months at this temperature; at −20°C (dash line, ▲) and −70°C (dot line, ●) the acidic peaks do not change indicating the this CRM197 is very stable at these two temperatures.

The increase of two acidic peaks is likely due to a chemical modification such as deamidation since no aggregation or clipping were observed. Deamidation occurs as a result of conversion of asparagine to aspartic acid or iso-aspartic and its rate of conversion depends on various conditions such as pH, temperature, amino acid sequence, and protein structure [28, 29]. The amino acid sequence of CRM197 contains two deamidation "hot spots NG" at the receptor domain. This type of stability information is useful in predicting the shelf life of a particular vaccine product with respect to charge isoform heterogeneity.

4 Concluding remarks

The icIEF technique has proven to be an indispensable tool for monitoring CRM197 protein products. It is becoming the platform method of choice to analyze protein charge heterogeneity and is quickly replacing the more traditional and labor intensive IEF slab gel method because of its higher resolution, quantitative, robust, faster analysis time, and automation. IcIEF analysis has been implemented in quality control environments for product identity release, stability testing, and formulation screening. It is also a very powerful characterization tool which has been demonstrated by the anti-CRM197 mAb binding evaluation performed here to evaluate activity. This single method can be utilized to analyze CRM197 protein concentration during upstream and downstream processing, identity testing, charge heterogeneity, activity with respect to anti-CRM197 mAb binding, and stability monitoring. As a result it has been demonstrated that icIEF provides both high efficiency and great analytical capacity (high information in a relatively short time), and finally provides product coverage for a wide spectrum of CRM197 protein product development areas (discovery, preclinical, clinical, and commercialization) in the vaccine industry.

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