



## Better Guidance in Antibody Therapeutics Process Development Using Differential Scanning Calorimetry

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Determining the impact of pH and ionic strength on the stability of proteins is crucial for the development of robust purification procedures and formulation strategies. Capillary Differential Scanning Calorimetry (Capillary DSC) is uniquely suited to provide such information and is of particular utility for the development of therapeutic proteins. Described here is a DSC investigation of the pH/ionic strength stability profiles of two antibody formats, IgG1 and IgE, used as drugs. DSC uniquely allows the investigation of the stability of *individual domains* from multi-domain proteins like antibodies. Here, it is shown by DSC that the constant domain fragment (Fc) of IgE is more sensitive to low pH/high salt conditions than the IgG1 Fc. These results indicate that purification and formulation strategies commonly used for IgG antibodies are not amenable to IgE. The study demonstrates how DSC can be used to guide process development activities for antibodies and other protein therapeutics.

### Summary of Application Note

The production and purification of immunoglobulins of the gamma isotype, particularly human IgG1, for diagnostic or therapeutic applications is now fairly routine. Alternately, immunoglobulin E (IgE) based therapeutics have only recently begun to build some momentum.<sup>1-3</sup> Standard methods for producing, handling, and formulating IgE or the constant region of IgE (Fc $\epsilon$ ) at an industrial scale, have not been established. While much is known concerning the biochemical function and regulation of IgE, it is not known whether Fc $\epsilon$  is similar to Fc $\gamma$  in terms of its thermostability or pH sensitivity<sup>2</sup> and whether standard methods for handling IgG will also apply to IgE.

IgE is important for host defense against parasites and for protective inflammation. Yet, IgE-mediated signaling through its receptors is also a focal point of inflammatory allergic disease.<sup>4</sup> The constant region of IgE, Fc $\epsilon$ , is a homodimer containing duplicate pairs of three unique Ig-fold domains (C $\epsilon$ 2, C $\epsilon$ 3, and C $\epsilon$ 4) and is responsible for binding its two receptors, Fc $\epsilon$ RI and CD23 (also known as Fc $\epsilon$ RII). (Figure 1.)

This application note focuses on the utility of Differential Scanning Calorimetry (DSC) to instruct multiple aspects

of the biotherapeutic development processes of IgG and IgE. In particular, it is shown that DSC provides insights for handling, purifying, and formulating IgG and IgE drug products. The ability of circular dichroism (CD) to contribute to these findings is contrasted to what can be discerned using DSC. DSC provides the capability of investigating protein stability at the level of individual domains within multi-domain proteins, an aspect that is less transparent in data obtained by CD.

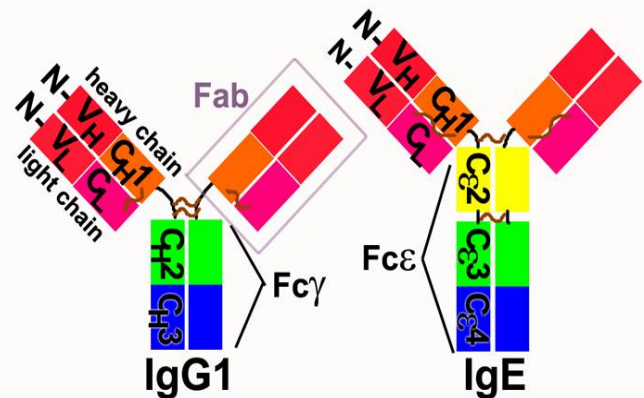


Fig. 1. Schematic diagrams of IgG and IgE

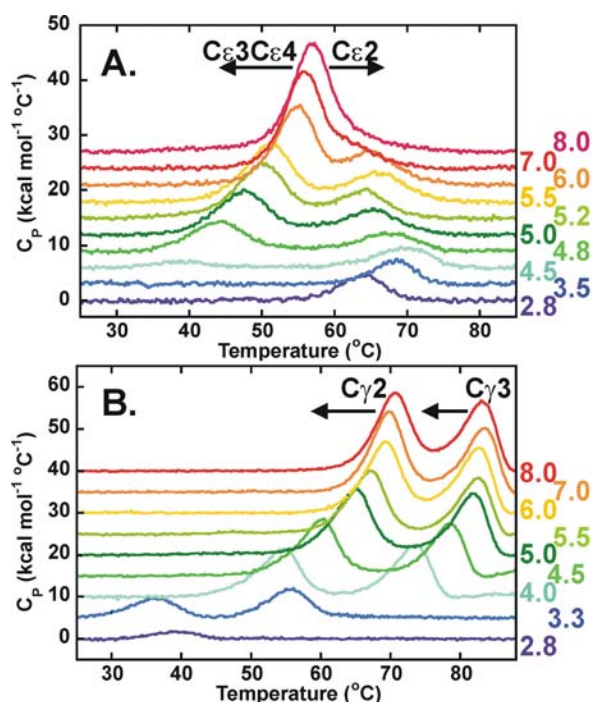
## Materials and Methods

Purified Fc $\epsilon$ , Fc $\gamma$  and Fc $\gamma$ -C $\epsilon$ 2 proteins were generated by Biogen Idec as described previously.<sup>5</sup> Details on CD (Jasco) experiments were described previously.<sup>5</sup> The entire set of Capillary DSC experiments – over 400 scans at different pH values, were performed over a four-month period with very little effort. It took approximately 3 hours labor to set up the experiments for Fc $\epsilon$  and Fc $\gamma$ , including the measurement of protein concentrations, dilutions, and setup of the plate; the remainder of the experiment was performed by the automation of the Capillary DSC itself. Full details described in Reference 5.

## Results

### pH Dependent Unfolding of Fc $\gamma$ and Fc $\epsilon$

Broad pH/salt stability is a prerequisite for many affinity protein purification processes performed in the industrial setting. Poor tolerance to unusual pH or salt conditions can result in aggregated or non-functional protein. The tolerance of Fc $\epsilon$  to various pH/salt conditions is important information for determining an appropriate and scalable purification scheme for IgE/Fc $\epsilon$ -containing proteins. To study the affect of pH on the secondary structure of Fc $\epsilon$ , CD spectra were taken of the protein under buffer conditions ranging from pH 4.5 to 7.4. Between pH 5.2 and 7.4, the spectra of Fc $\epsilon$  were identical and contained a single minimum between 216 and 217 nm indicative of significant  $\beta$ -sheet and typical of Ig-domains. At pH 5, the Fc $\epsilon$  spectrum shifted in a random coil direction (the minimum shifted towards 200 nm), and at pH 4.5, the spectrum suggests the protein is predominantly random coil.<sup>5</sup> Based on the pH-dependent unfolding, we investigated whether Fc $\epsilon$  may have an attenuated stability between pH 7.0 and 4.5.

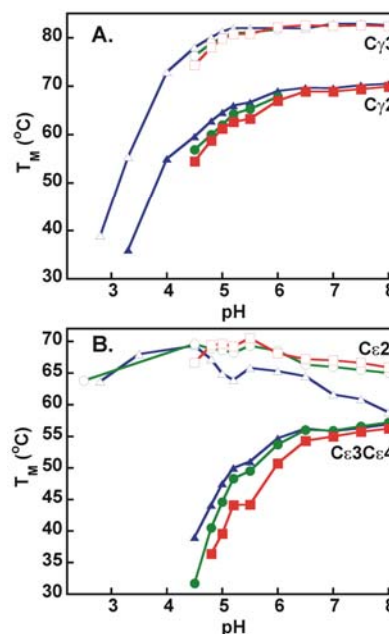


**Fig. 2.** A, B. pH-dependent DSC traces of Fc $\epsilon$  (A.) and Fc $\gamma$  (B.) in the presence of 15 mM NaCl. Reprinted from Reference 5 with permission of American Society of Biochemistry and Molecular Biology.

Thermal denaturation of Fc $\epsilon$  at various pHs was monitored by far-UV CD. At pH 7.0, there was one transition for the unfolding of all three domains (C $\epsilon$ 2-4). A similar transition was observed at pH 6.0, though the apparent  $T_M$  decreased by 1 °C. Thermal unfolding of Fc $\epsilon$  at pH 5.2 resulted in a much broader transition that began 6 °C lower than at neutral pH. Only at pH 4.8 were two transitions clearly evident.<sup>5</sup>

Based on the initial CD results, detailed pH-dependent stability studies were initiated for both Fc $\epsilon$  and Fc $\gamma$  using DSC. The unfolding transitions of both Fc $\epsilon$  and Fc $\gamma$  were found to be irreversible and scan rate dependent (data not shown) suggesting that irreversible aggregation affects the apparent  $T_M$ s of both proteins.<sup>6,7</sup> Unlike what could be determined using CD, Fc $\epsilon$  was shown to contain two independent unfolding transitions at all pHs below 8.0 (Figure 2A). One of these transitions was destabilized at low pH and high NaCl while the other was not.

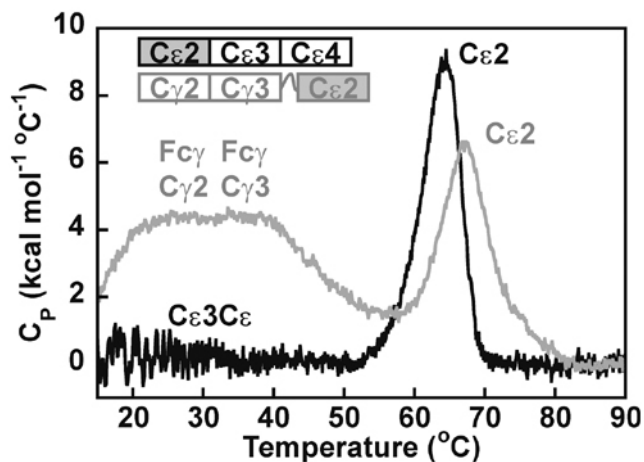
The domains involved in the pH sensitive transition were completely unfolded at pH 4.5 as expected based on the structural data obtained using CD – demonstrating how DSC can be important not only for understanding the stability of folded domains, but their folding status as well. Fc $\gamma$  (from IgG1) was shown to unfold via two separate transitions; the low temperature transition belonging to the C $\gamma$ 2 domain and the high temperature transition belonging to the C $\gamma$ 3 domain (Figure 2B). The C $\gamma$ 2 transition was identified by the effect that deglycosylation had on its thermostability (unpublished results) and the C $\gamma$ 3 transition by its unusually high thermostability.<sup>8</sup> Both Fc $\gamma$  domains were shown to be pH and NaCl sensitive. Unlike Fc $\epsilon$ , the Fc $\gamma$  domains did not become intrinsically unfolded until the pH was reduced below 3.0 – suggesting (1) why antibodies elute from protein A resins at pH values below 3.5, and (2) why cation exchange chromatography is a suitable for IgG (Figure 3).



**Fig. 3.** pH-dependence of the Fc $\epsilon$  and Fc $\gamma$  domain thermostabilities as represented by their  $T_M$  values. A. Fc $\gamma$  domain  $T_M$ s: C $\gamma$ 2 at 15 (▲), 150 (●), and 750 (■) mM NaCl; C $\gamma$ 3 at 15 (▲), 150 (○), and 750 (□) mM NaCl. B. Fc $\epsilon$  domain  $T_M$ s: C $\epsilon$ 3C $\epsilon$ 4 at 15 (▲), 150 (○), and 750 (■) mM NaCl; C $\epsilon$ 2 at 15 (▲), 150 (○), and 750 (□) mM NaCl. Reprinted from Reference 5 with permission of American Society of Biochemistry and Molecular Biology.

In the presence of high salt, the C $\gamma$ 2 and C $\gamma$ 3 domains of Fc $\gamma$  and the C $\epsilon$ 3 and C $\epsilon$ 4 domains of Fc $\epsilon$  were destabilized. This was seen as a small shift in their T<sub>M</sub> values at 150 and 750 mM NaCl relative to 15 mM NaCl in the intermediate pH range between 5.0 and 7.0 (Fig. 3A). These small stability differences are unlikely to have a major effect on the *in vitro* half-life of Fc $\gamma$  within this pH range because the T<sub>M</sub> of both the C $\gamma$ 2 and C $\gamma$ 3 domains remained above 60 °C.

The pH sensitive domains of Fc $\epsilon$  were identified as the receptor binding domains (C $\epsilon$ 3 and C $\epsilon$ 4) by performing DSC experiments with an Fc $\gamma$ -C $\epsilon$ 2 fusion protein. One domain of both Fc $\gamma$ -C $\epsilon$ 2 and Fc $\epsilon$  remains stably folded at pH 2.5 (Figure 4). Based on the experiments described above, it is known that the Fc $\gamma$  domains are intrinsically unfolded below pH 3.0. By default, this identifies the C $\epsilon$ 2 domain of Fc $\epsilon$  as the pH insensitive domain. These results were confirmed by limited proteolysis of Fc $\epsilon$  at pH 4.5.<sup>5</sup>



**Fig. 4.** DSC traces of Fc $\epsilon$  (black line) and Fc $\gamma$ -C $\epsilon$ 2 (grey line) performed using samples dialyzed against the same pH 2.5 phosphate buffer. Schematic diagrams of the Fc $\epsilon$  and Fc $\gamma$ -C $\epsilon$ 2 proteins are shown above the DSC curves Reprinted from Reference 5 with permission of American Society of Biochemistry and Molecular Biology.

In high salt, the C $\epsilon$ 2 domain of Fc $\epsilon$  was slightly more thermostable. C $\epsilon$ 2 was especially stabilized at neutral pH and 750 mM NaCl with a T<sub>M</sub> more than 7 °C higher than the T<sub>M</sub> measured in 15 mM NaCl (Fig. 3B). In contrast, NaCl significantly destabilized the C $\epsilon$ 3C $\epsilon$ 4 domains between pH 5 and 6 (Fig. 3B). C $\epsilon$ 3C $\epsilon$ 4 began to unfold at pH 5.0 in low salt. In high salt, the unfolding transition was shifted 0.5 pH units (to pH 5.5), precluding the use of cation exchange chromatography as a viable purification step for IgE or Fc $\epsilon$ -containing proteins.

## Conclusions

In this study, we showed that Fc $\epsilon$  demonstrated an unusual pH-sensitivity that resulted in the unfolding of its receptor binding domains at 2 pH units higher (i.e. pH 5.0) than what was observed for Fc $\gamma$ . The pH/salt sensitivity of Fc $\epsilon$  determined by DSC provides valuable information for choosing purification strategies, handling procedures, and formulations for IgE-based proteins and suggests that standard IgG protocols will not be amenable. The pH stability data for Fc $\gamma$  also suggests a likely mechanism for IgG time-dependent aggregation during standard affinity purifications (e.g. most commonly on protein A resins) that include low pH elution and hold steps.

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