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PhD by Published Work

Statement of contribution by David Paul Gervais

Paper to be considered as part of the PhD by Published Work:

Gervais D, King D. Capillary Isoelectric Focussing of a Difficult-to-Denature Tetrameric Enzyme Using Alkylurea-Urea Mixtures. *Analytical Biochemistry*, 2014. *In press*; DOI 10.1016/j.ab.2014.08.004.

Background: Measurement of the amount of deamidation (asparaginyl and glutaminyl) in *Erwinia chrysanthemi* L-asparaginase is an important facet of understanding the degradation profile of the enzyme. Current HPLC techniques measure the enzyme in the native, tetrameric state, which may confound quantitation due to buried residues (the most labile asparagine is internal to the tetramer) or conformational differences leading to charge variants. Therefore, a more robust technique was required.

Contribution of candidate: DP Gervais had the lead role in this research. He determined the instrumentation necessary to make the deamidation measurements, procured the instrument, ran the instrument and generated most of the data. He directed the research in this area including creating and running the forced-deamidation procedure and made the observation that the enzyme was not fully unfolded in the 8M urea denaturant. He analysed all the data, wrote the manuscript, and served as corresponding author with the journal reviewers. D King provided support to running the iCE instrument.

I agree that David Paul Gervais made the aforementioned contribution to this paper.

Name	**Signature**	**Date**
Darryl King		14 AUG 2014

Full title: Capillary Isoelectric Focussing of a Difficult-to-Denature Tetrameric Enzyme Using Alkylurea-Urea Mixtures

Short title: Protein Capillary IEF with Alkylurea-Urea Mixtures

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Abstract

Capillary isoelectric focussing (cIEF) is normally run under denaturing conditions using urea, to expose any buried protein residues that may contribute to the overall charge. However, urea does not completely denature some proteins, such as the tetrameric enzyme *Erwinia chrysanthemi* L-asparaginase (ErA), in which case electrophoresis-compatible alternative denaturants are required. Here, we show that alkylureas such as N-ethylurea provide increased denaturation during cIEF. The cIEF analysis of ErA in 8M urea alone resulted in a cluster of ill-resolved peaks with isoelectric points (pI) in the range 7.4 – 8.5. A combination of 2.0 – 2.2M N-ethylurea and 8M urea provided sufficient denaturation of ErA resulting in a main peak with a pI of 7.35 and an acidic species minor peak at 7.0, both comparing well with predicted pI values based on the sum of protein residue pK_a values. Recombinant deamidated ErA mutants were also demonstrated to migrate to pI values consistent with predictions (pI 7.0 for one deamidation). The quantitation of ErA acidic species in samples from full-scale manufacturing (1.0 – 3.5% of total peak area) was found to be reproducible and linear. Use of alkylureas as denaturing agents in CE and cIEF should be considered during biopharmaceutical assay development.

Keywords: deamidation; L-asparaginase; alkylurea; capillary isoelectric focussing; capillary electrophoresis

1. Introduction

Capillary isoelectric focussing (cIEF) is a useful technique for the determination of protein isoelectric point (pI). First described by Hjertén and Zhu [1], the technique has evolved into one of the mainstays of protein analysis, particularly in the biopharmaceutical industry, where there are numerous examples of its use [2, 3]. The technique is useful for separation of glycoforms, characterisation of protein microheterogeneity and quantitation of protein species which have charge modifications resulting in pI shifts in either the acidic or basic direction [4].

The technique involves injection of a protein analyte into a capillary in the presence of ampholytes and a polymeric matrix, usually methylcellulose-based. As in slab-gel IEF, the ampholytes set up a pH gradient through the capillary when an electric current is applied, using an acidic anolyte (usually dilute acid) and a basic catholyte (usually dilute base). The separation occurs as proteins migrate to their point of zero net charge within the capillary. The capillary is often neutral, hydrophilic and coated (with methylcellulose) to reduce distortion during the separation [5]. Internal standards are usually employed to enable calculation of analyte pI [6]. In most cIEF instruments, the separation is conducted and the entire contents of the capillary are mobilised by pressure, chemical or other means past a detection window [4]. This mobilisation step can lead to distortion and band spreading as the column of fluid moves through the capillary. In recent years, manufacturers have introduced whole-capillary imaged cIEF instruments [7, 8] which avoid this distortion by capturing images of the entire capillary separation zone at 280nm wavelength. This technique has the added advantage of being able to view the separation in real-time such that the end of the focus time can be easily ascertained, and aids in method development.

Isoelectric focussing, and cIEF in particular, are particularly attractive techniques with regards to characterisation of protein deamidation. Deamidation is a frequently-occurring degradation pathway in proteins and peptides [9], most frequently affecting asparagine (Asn or N) residues and, to a lesser extent, glutamine (Gln or Q). In the deamidation of an Asn residue, the C-terminal peptide bond nitrogen attacks the carboxylate group of the side chain, creating a succinimide ring intermediate. This intermediate is then hydrolysed to result in a mixture of aspartic acid (Asp or D) and iso-Asp [10]. The result of this reaction is a lowering of the overall protein pI, due to the change of residue charge from neutral (Asn) to negative (Asp). In some proteins, deamidation may reduce activity or have other deleterious effects, and therefore is important to understand, monitor, and control.

Recently, efforts have focussed on understanding the deamidation of the enzyme L-asparaginase (E.C. 3.5.1.1) from *Erwinia chrysanthemi* (ErA) [11, 12]. ErA is a 140,000Da homotetramer with an isoelectric point of pH 8.6 in the native form [13]. Each subunit of the tetramer consists of 327 amino acids, and the enzyme is only active in the tetrameric form. ErA is a biopharmaceutical product marketed as Erwinase™ or Erwinaze™ and is used in the clinical treatment of acute lymphoblastic leukaemia (ALL) [14, 15]. Native capillary zone electrophoresis (CZE) and weak cation-exchange high-performance liquid chromatography (WCX-HPLC) of ErA both give rise to a number of acidic species which, in the main, cannot be definitively determined to be deamidated.

In cIEF method development for deamidation for proteins such as ErA, one difficulty is the lack of available denaturants compatible with electrophoresis. Analysis under denaturing conditions ensures that protein residues are exposed and therefore a true measure of the protein charge is realised. The aim of the present work was to use cIEF under denaturing conditions to understand the extent of any deamidation in ErA, as well as

develop a robust method of quantification of deamidation in this medically-important enzyme product.

2.0 Materials and Methods

All reagents were from Sigma (Dorset, UK) unless otherwise indicated.

Production of Enzyme:

Purified samples of ErA were manufactured and provided by Development & Production, Public Health England, Porton Down, UK. Recombinant wild-type (WT) ErA and ErA mutants were produced using previously published procedures [12].

Capillary Isoelectric Focussing (cIEF) Analyses:

Analyses of ErA samples were performed using an whole-capillary-imaged cIEF system (model iCE3 with PrinCE autosampler) from ProteinSimple (Toronto, Canada). The pI markers used throughout this work were proprietary, small molecular weight, UV absorbent markers (pI values 5.85, 6.1, 6.6, 9.5 and 9.77) obtained from ProteinSimple. Samples were prepared immediately before analysis using the following procedure. A master mix (MM) was prepared fresh daily with the composition 8M urea, 0.35% methylcellulose, and 4% Pharmalytes 3-10. N-ethylurea was added to this MM as desired. The analyte samples were prepared using 200µL MM with 1µL high-pI marker (9.5 or 9.77) and 1µL low-pI marker (5.85, 6.1 or 6.6), and protein sample was added to a target concentration in the range 0.1 – 0.2mg/mL. These samples were vortexed briefly to ensure complete mixing and centrifuged at 10,000rpm for 3min to remove air bubbles before analysis. Samples were focussed for 1min at 1.5kV followed by 12 – 13min at 3kV, and A280 images of the capillary were taken using the ProteinSimple software. The

resulting electropherograms were first analysed using the iCE3 CFR software (ProteinSimple) and pI values were assigned (linear relationship between the pI markers). The data were downloaded into Empower 2 (Waters, Elstree, UK) processing software for electropherogram integration. Empower data analyses were conducted using Savitsky-Golay smoothing of the electropherogram traces.

Circular Dichroism Analyses:

Circular dichroism (CD) analyses were performed using a Jasco J-715 spectropolarimeter at Alta Bioscience (Birmingham, UK). Samples were denatured in mixtures of urea and ethylurea, held for at least 10min, and analysed by CD. The analyses were carried out between 190 and 280nm, and appropriate mixtures of urea and ethylurea without protein were used for blank analyses.

Computational Predictions of Theoretical pI Values:

Theoretical pI values were calculated using the ErA sequence [12] and the internet-based tools at the European Molecular Biology Open Software Suite (EMBOSS, http://www.ebi.ac.uk/Tools/seqstats/emboss_pepstats/) [16, 17, 18] or the tools available at the Swiss Institute for Bioinformatics (SIB ExPASy, http://web.expasy.org/compute_pi/) [19 – 22].

Forced Deamidation of ErA:

Samples of high-concentration (35 – 40mg/mL protein by A280) ErA were combined with 2M NaOH at a ratio of 10:1 enzyme to base, and mixed gently. The samples were then heated at 70°C for 1min using a dry heat block (Grant Instruments, Cambridge, UK), removed and placed on ice. The samples were then immediately combined with master

mix to proceed directly to cIEF analysis, with samples run as described above in duplicate or triplicate.

3.0 Results and Discussion

In development of any denaturing cIEF method, the usual starting point is to attempt a separation in urea, usually in the concentration range 3 – 8M. The main goal is to ensure that the analyte protein is as unfolded as possible without inducing precipitation, so that the analyst can compare the theoretical pI with the measured cIEF pI. ErA is known [23] to be difficult to fully denature in 8M urea through previous work with analytical ultracentrifugation. However, it was unclear whether the extent of denaturation in 8M urea would be sufficient to expose enough surface charge to fully characterise charge variants in cIEF. Therefore, samples of ErA were analysed using 8M urea as the denaturant in 0.35% methylcellulose and 4% Pharmalytes 3-10. The profiles obtained (Figure 1) were reproducible and demonstrated a number of peaks in the pI region of 8.4, and a low-pI peak at 7.4. The cluster of peaks at pI 8.4 matched the previously-determined native (not denatured) ErA isoelectric point of 8.6 [13]. Therefore it was initially thought that the low-pI peak at 7.4 might represent deamidated or other degraded, acidic species.

In order to further understand the electropherograms obtained for ErA in 8M urea, mutant versions of ErA [12] were analysed in 8M urea. These mutant forms of the enzyme included genetically-induced single deamidation sites (either N41D or N281D) as well as a double-deamidated mutant (both N41D and N281D). Earlier work on these mutants had demonstrated that the N281D deamidation resulted in a loss of stability at urea concentrations greater than 2M, while variants with an intact Asn_{281} were stable after

exposure to 7.2M urea [12]. The cIEF analyses of these mutants (Figure 2) in 8M urea appeared to agree with these earlier observations, and suggested that the cIEF profiles of mutants lacking the N281D deamidation did not represent fully-denatured enzyme. Those mutants carrying the N281D mutation, and therefore fully denatured in 8M urea, were found to have pI values in cIEF, as shown in Figure 2, that compared well with the range of theoretical pI values calculated using protein sequence and amino acid side chain pK_a values (Table 1). However, those without the N281D mutation had apparent cIEF pI values that were outside of these theoretical ranges. It is important to note that the theoretical pI values calculated in Table 1 are presented for two different sets of pK_a values and algorithms; there is disagreement in the literature about the 'correct' values to use. Therefore, for the purposes of this study we consider the two pI values in Table 1 for each protein as upper and lower bounds of a possible range of theoretical values.

Furthermore, there are differences in the electropherogram profiles of these enzymes in 8M urea. The profiles in Figure 2 for the two enzymes without the N281D mutation are very similar to that of the ErA presented in Figure 1, while those with the N281D mutation appear somewhat different, as one predominant main band. This suggested that the low-pI (7.4) peak identified in Figure 1 may not be due to an acidic modification to one or more residue(s), but instead representative of complete protein denaturation, with the higher (pI 8.4) group of peaks representing partially-denatured species. Further supporting this idea was the observation that the pI of the low-pI peak (7.4) in Figure 1 compares well with the range of values expected for the wild-type enzyme shown in Table 1.

In order to arrive at suitable conditions for the cIEF analyses of deamidated variants of ErA, a relatively complete unfolding of the enzyme structure was desired. It was thought that accurate quantitation of the ErA charge variants could not be achieved if some of the charged residues, such as the labile Asn_{281}, were still partially buried in the protein

structure during cIEF. Therefore the goal of this work was to define a set of conditions under which all (or nearly all) ErA protein residues could participate in the charge-based cIEF separation, such that each charge variant species (due to changes in charge at the sequence level, and independent of charge differences due to structural effects) would result in a separated cIEF peak. It is important to note that this strategy cannot necessarily distinguish deamidation from other post-translational modifications (PTM) and that one charge-variant peak, while being uniform in overall charge, may represent more than one PTM.

Based on this strategy and these observations, it was concluded that the denaturing power of 8M urea was not sufficient for cIEF analyses of ErA. Although the analyses of ErA as described above were reproducible, due to the incomplete denaturation it was not clear what the various cIEF peaks in the electropherogram represented with respect to deamidation and PTMs. Therefore, it was decided that a more powerful denaturant was required. Guanindine hydrochloride is an effective and powerful denaturant for ErA, but it cannot be used for cIEF separations due to the ionic character of the molecule, particularly at the high concentrations required for protein denaturation.

Previous work on two-dimensional gel electrophoresis [24] demonstrated the increased chaotropic power of urea and thiourea mixtures for electrophoretic analysis of sparingly-soluble membrane and nuclear proteins. A mixture of 8M urea with added 2M thiourea improved the analysis and resolution of difficult proteins. The use of thiourea-urea mixtures was therefore attempted in the context of ErA cIEF analysis. The data obtained (data not shown) were encouraging, as lower-pI peaks (pI 7.4) were obtained for the WT ErA samples with a lack of higher-pI species, but the high UV background caused by the thiourea prevented this system from being taken into further development.

Alkylureas, such as N-methylurea and N-ethylurea, had been previously as well as more recently investigated as protein denaturants by several investigators [25, 26]. These compounds lack the UV-absorbing thione group found in thiourea, and have substituted alkyl groups on one of the urea amines. The strength of the substituted ureas as denaturants was found to increase with increasing alkyl chain length. However, the solubility of these substituted ureas in water is appreciably lower than unsubstituted urea. Therefore, similar to thiourea, these alkylureas are most effective as denaturants when used in urea-alkylurea mixtures.

A cIEF screen of mixtures of 8M urea with N-methylurea, N-ethylurea and N-butylurea was conducted. It was determined that 8M urea with added 2M N-ethylurea (Figure 3) completely denatured ErA such that the electropherogram profile changed from that shown in Figure 1 to a profile with one predominant peak at pI 7.4. As the concentration of N-ethylurea was increased from zero up to 2M, the partially-denatured species grouped at pI 8.4 were observed to disappear and the fully-denatured species at pI 7.4 appeared. A similar effect was observed with N-butylurea (data not shown), but the butylurea electropherogram baseline was not as stable as that for N-ethylurea, and the butylurea proved more difficult to dissolve during sample workup. Addition of up to 2M N-methylurea to 8M urea was not sufficient to cause complete denaturation as observed for ethylurea.

In order to more fully understand the structural effects of ErA denaturation in mixtures of urea and N-ethylurea, circular dichroism (CD) experiments were carried out in concentration ranges similar to those described above for Figure 3. Due to the high concentrations of urea and ethylurea present in these samples, reliable CD spectra were not realised below between 210-220nm (Figure 4). However, the data obtained for the

higher wavelengths illustrate the increasing loss of protein structure as the ethylurea concentration is increased, adding confirmation to the results observed by cIEF.

As a test of the urea-ethylurea system in ErA cIEF analyses, the recombinant ErA WT and deamidated mutants were analysed using a mixture of 8M urea and 2.2M ethylurea as the denaturant. A slightly increased concentration of N-ethylurea was utilised to ensure that complete denaturation was observed and to provide a degree of robustness in the assay for routine analyses. The data (Figure 5) confirmed that the more powerful denaturant had fully exposed the surface charges in ErA and the mutants. Unlike in 8M urea alone, the two single-deamidation mutants N41D and N281D both migrate to the same point (pI 7.0) in the electropherogram, independent of the location of the mutation in the primary sequence and consistent with the idea that the proteins are fully denatured. The double-deamidation mutant migrates to a pI further into the acidic region (6.8) than the single-site mutants, and the WT electropherogram constitutes one main peak at a pI of 7.35. All of these cIEF determined pI values sit within the ranges calculated based on linear amino acid sequence in Table 1, further suggesting that complete denaturation of all four proteins has been achieved. The electrical current profile obtained during electrophoresis in urea-alkylurea mixtures was not significantly different to the profile for urea alone (data not shown), further adding to the potential of these compounds in cIEF.

In order to further explore the idea that ErA deamidation could be quantitated and measured in the urea-ethylurea cIEF system, a forced degradation study was conducted. In earlier work [12], it was shown that WT ErA could be irreversibly thermally denatured at temperatures of 65 - 70°C, with accompanying loss of quaternary, and probably tertiary, structure. It was known from these earlier experiments that even after brief exposure (1 – 2min) to these high-temperature conditions, the denatured enzyme remained soluble. Therefore, a forced-deamidation experiment was designed by combining these thermal

conditions with high pH. High pH and the presence of hydroxyl ion is known to accelerate the deamidation reaction, and ErA has one Asn residue in the sequence GN_{281}GIVPPDEEL which is known to be labile to deamidation, with several other Asn residues that are also prone to a lesser extent [11].

The data from the forced deamidation experiment (Figure 6) demonstrated that the urea-ethylurea cIEF system is appropriate for understanding and quantification of ErA deamidation. The control electropherogram depicts a typical ErA analysis, with a main species at pI 7.35 and an acidic species at pI 7.0. Thermal denaturation and degradation with sodium hydroxide generated peaks with pI values consistent with either one affected residue (pI 7.0), two affected residues (pI 6.8), and three affected residues (pI 6.6). Samples post-degradation were tested in SDS-PAGE (data not shown) to confirm that truncation of the 35kDa subunit had not occurred and that the low-pI species represented full-length polypeptide. The pI values from these forced-deamidation experiments compare well with those of the deamidated mutants shown in Figure 5.

The control electropherogram in Figure 6 depicts a cIEF analysis of a typical ErA sample. The low-pI peak at 7.0 represents an acidic variant of the main peak which, due to the denaturing conditions employed, is likely to be a consequence of a charge difference at the primary structure level. The levels of these acidic species, of which deamidation is the most likely cause, in routine ErA analyses like this control sample are shown in Table 2. The general acidic species content of these ErA samples, representing 9 individual ErA batches, is low (under 4%).

In order to assess the robustness and reproducibility of assays using ethylurea in denaturing cIEF, several further experiments were conducted. Samples were prepared according to the procedure described above and subjected to repeat injections from the

same sample container. The reproducibility data (Figure 7) demonstrated the robustness of the assay in 2.0M ethylurea but indicated that for the higher concentration of 2.2M ethylurea, the percent acidic species quantified was on the increase after several hours exposure to the assay reagents (ethylurea, urea and ampholytes). This apparent increase was confirmed by further injections of materials at 24h and 48h post-sample preparation, which showed a dramatic increase in the percent acidic species above those shown in Figure 7 (data not shown). This effect may be due to a reaction of the unfolded protein with the ampholytes, protein carbamylation during prolonged exposure to urea, or simply an accelerated pH-induced deamidation reaction due to the exposure of labile residues to solvent. As a consequence of these observations it was decided to prepare ErA samples for analysis immediately before running the electrophoresis.

The linearity of the assay (Figure 8) was also assessed using samples of ErA prepared by serial dilution and analysed in triplicate. The UV response of ErA main peak (pI 7.35) and the acidic peak (pI 7.0) was linear across a broad range and demonstrated that the assay is suitable for the quantitation of acidic or deamidated ErA variants.

4.1 Concluding Remarks

In designing an analytical procedure to quantify protein deamidation and acidic species content, it is important to ensure that the protein structure is such that the influence on charge from all residues is included. For most proteins, urea is sufficient for this purpose, but for some difficult-to-denature proteins, additional chaotropic power is needed. For isoelectric focussing applications, the additional denaturant must be compatible with the high voltages utilised, ruling out many chaotropes such as guanidine hydrochloride and potassium thiocyanate. For CE applications such as cIEF, the denaturant must also have a

low UV absorbance in the 220 – 280nm range so that the protein analytes can be detected. Alkylureas, such as N,N-methylurea, N-ethylurea and N-butylurea, are compatible with electrophoresis, having been used in two-dimensional gel electrophoresis as discussed previously. In this work we demonstrate the utility of these compounds as denaturants for cIEF using the difficult-to-denature enzyme ErA.

Using this technique, the resulting ErA pI values fell within the range of theoretical pIs (calculated by two techniques) for the wild-type and deamidated forms, allowing easier interpretation of the electropherograms with respect to protein modifications including deamidation. Although results for ErA acidic species cannot be definitively classed as deamidation using this technique alone, the method has been proven to be capable of detecting deamidated species, if present. The alkylureas should form a much-needed additional tool in the development of cIEF assays for protein acidic species quantitation.

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FIGURES

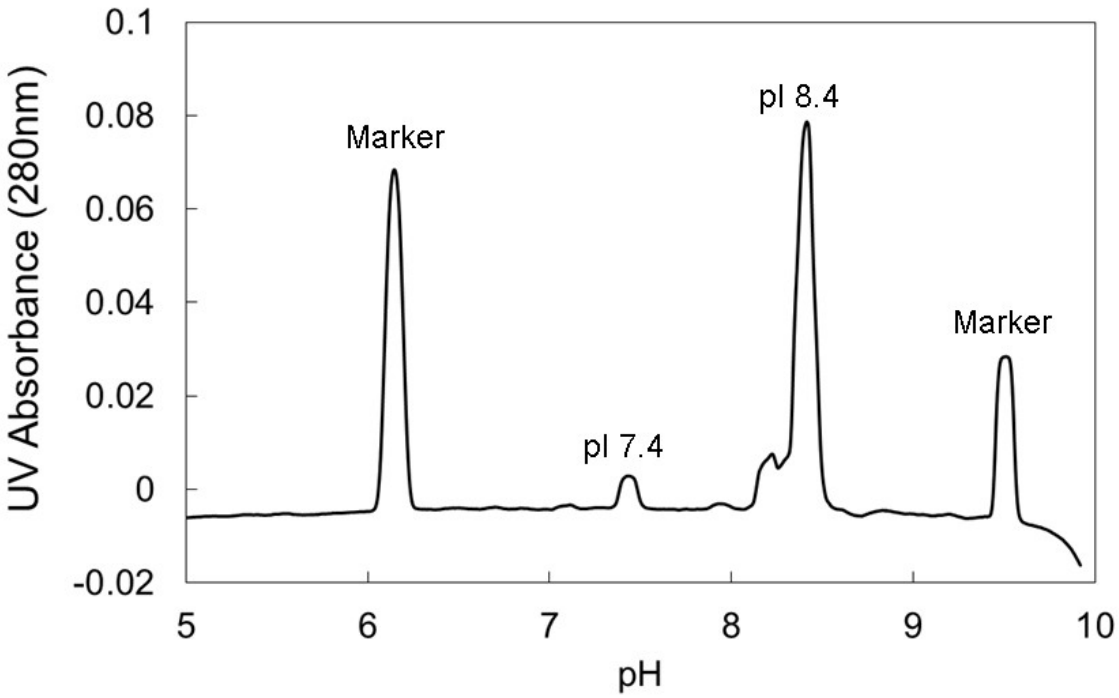

Figure 1. Typical ErA cIEF Profile in 8M Urea. The absorbance on the y-axis is presented in AU. The positions of the major species at pI 7.4 and 8.4 are indicated, along with the two internal standard pI marker peaks (pI 6.1 and 9.5).

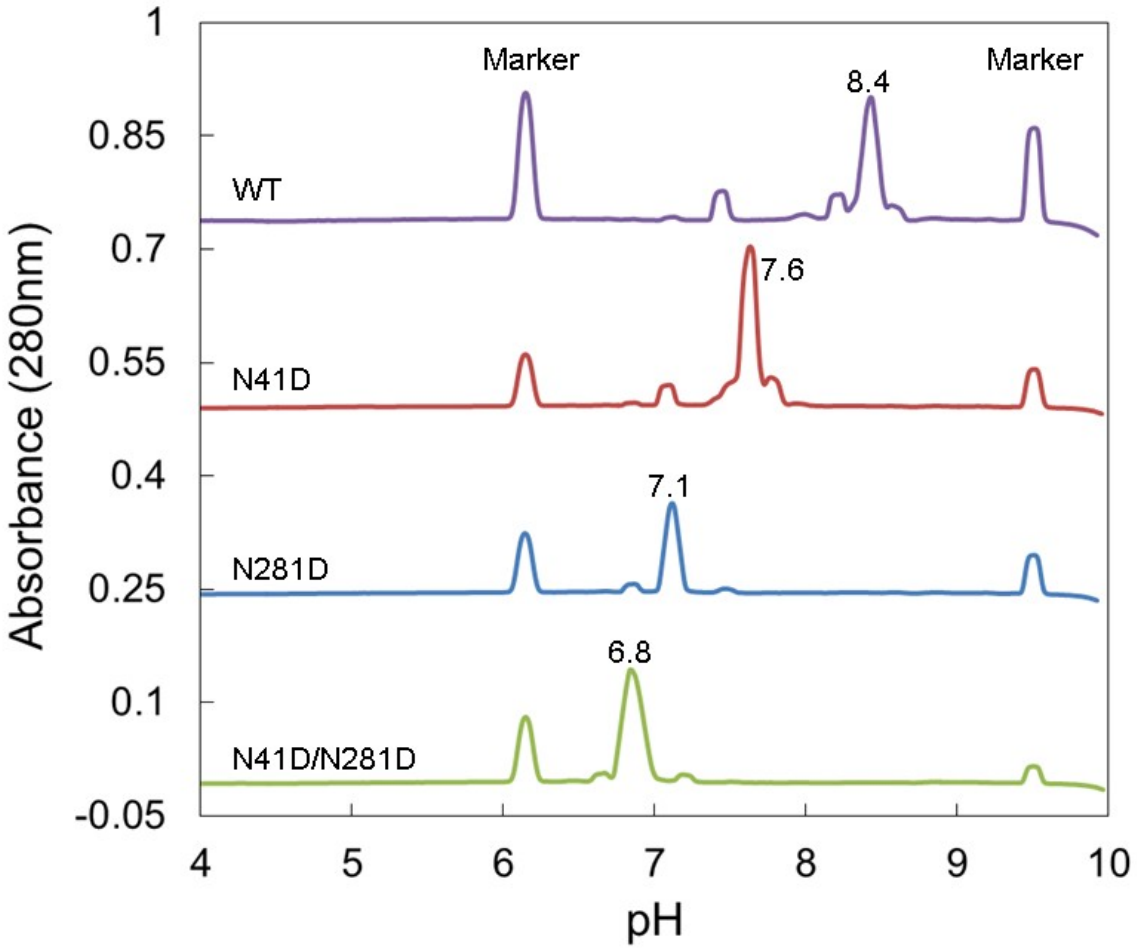

Figure 2. cIEF Analyses of ErA Recombinant Deamidated Mutants in 8M Urea. The absorbance on the y-axis is presented in AU. The isoelectric point of the main species in each trace is indicated, along with the position of the two marker peaks (pI 6.1 and 9.5).

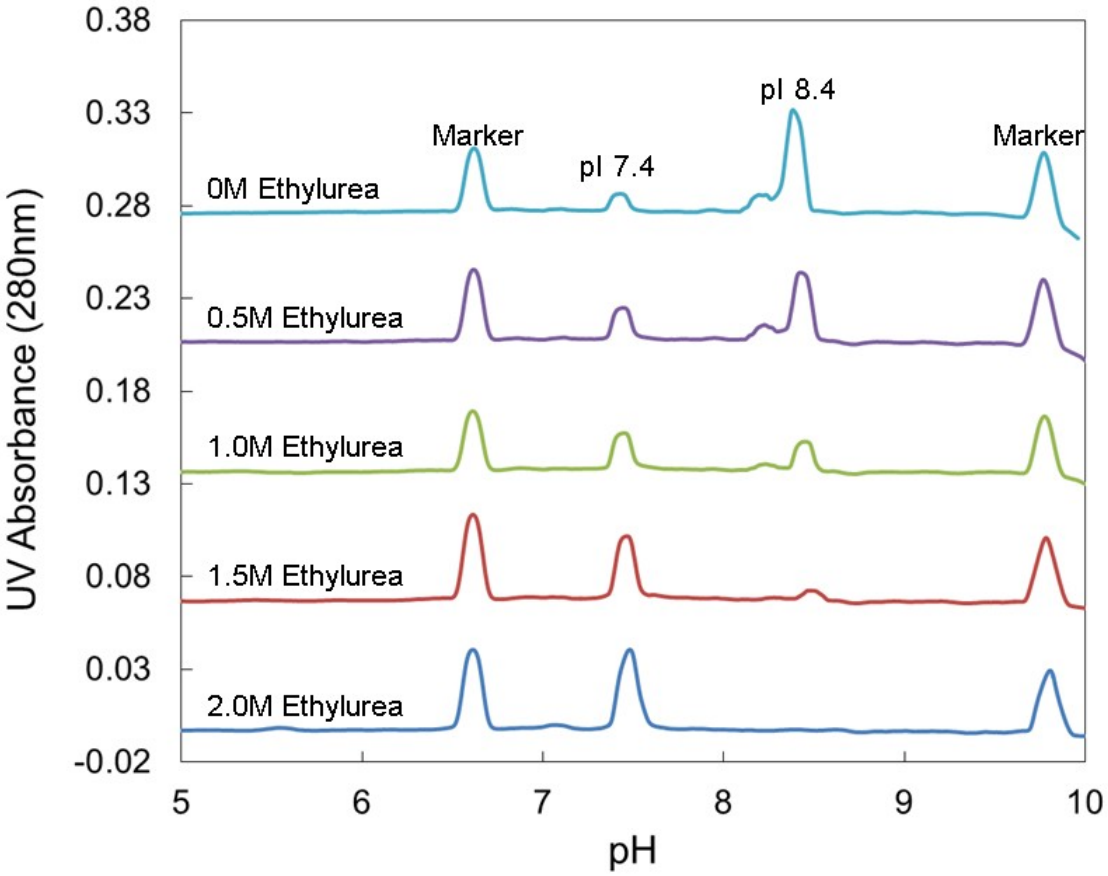

Figure 3. Effect of N-ethylurea on cIEF Profiles of ErA. The analyses were carried out using the same sample of ErA diluted in different denaturants. All analyses contained 8M urea as the denaturant, with additional added N-ethylurea as indicated in the figure. The absorbance on the y-axis is presented in AU. The position of the two pI marker peaks (pI 6.6 and 9.77) is also indicated in the figure.

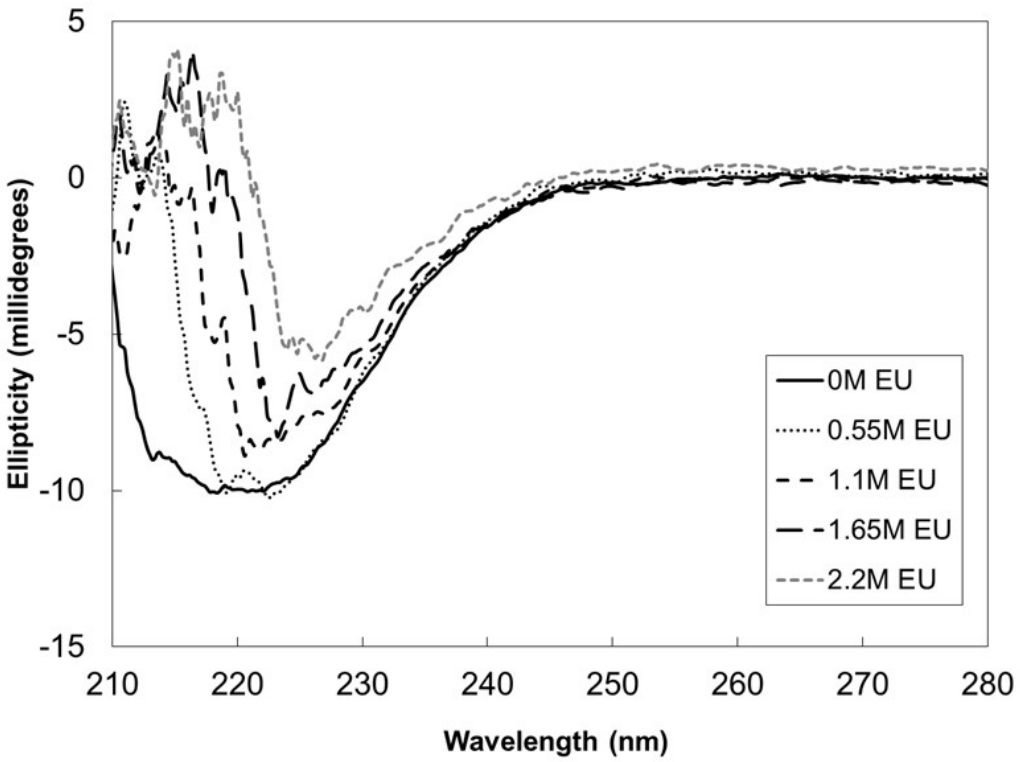

Figure 4. Circular Dichroism Analyses of ErA in 8M urea containing various amounts of N-ethylurea. The signals in the low-wavelength region had a high signal-to-noise ratio (confirmed by analysis of a blank, data not shown) due to the presence of the two ureas.

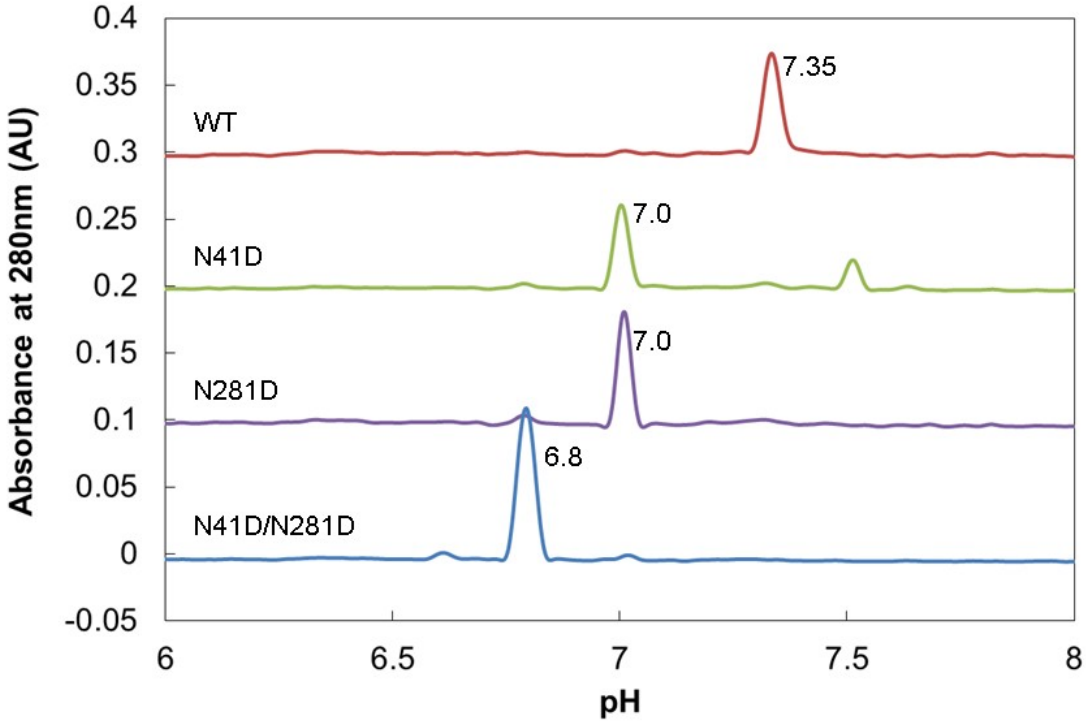

Figure 5. cIEF Analyses of ErA Deamidated Mutants in Urea-Ethylurea denaturant. The concentrations of urea and N-ethylurea were 8.0M and 2.2M respectively. The pI values for the major peaks are indicated. The pI markers used in this experiment were 5.85 and 9.50.

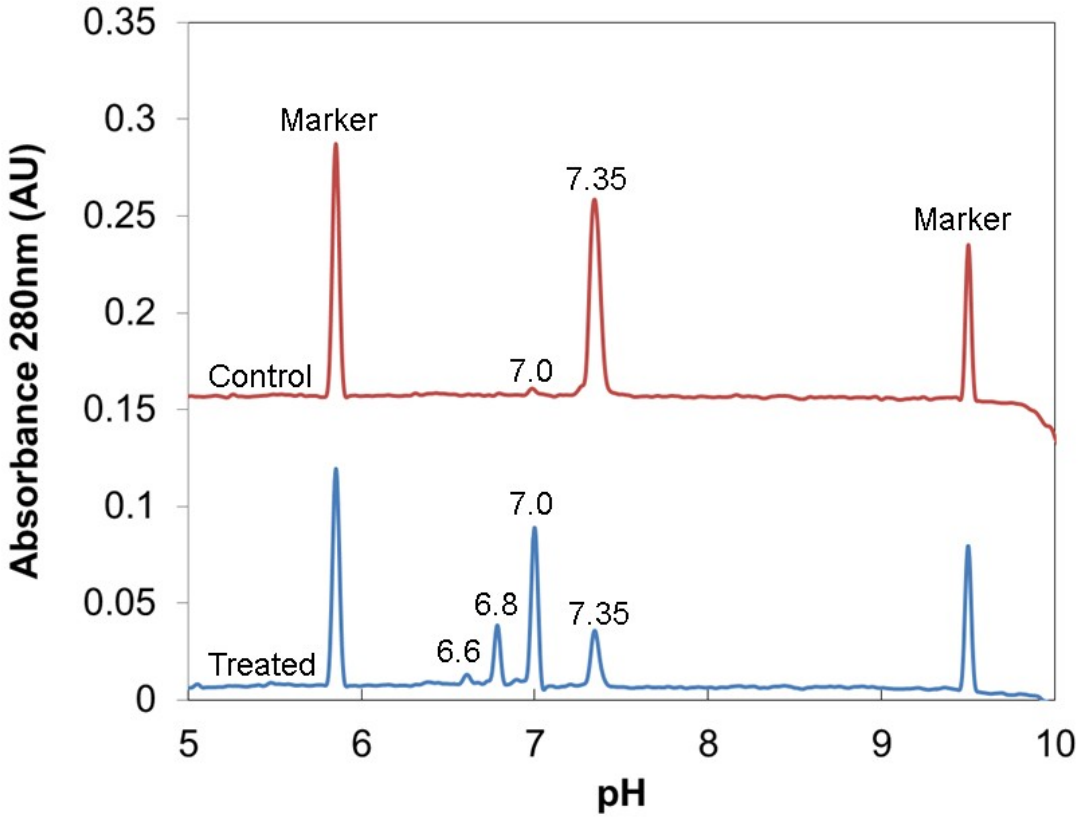

Figure 6. cIEF Analyses of ErA Forced Deamidation. The treated sample was created by mixing a 10µL aliquot of 38mg/mL ErA with 1µL 2M NaOH, mixing and heating for exactly 1min at 70°C immediately prior to mixing with master mix and analysis. The control sample was not heated or adjusted with NaOH. The pI values determined during integration are depicted in the figure, along with the position of the two pI markers (5.85 and 9.5).

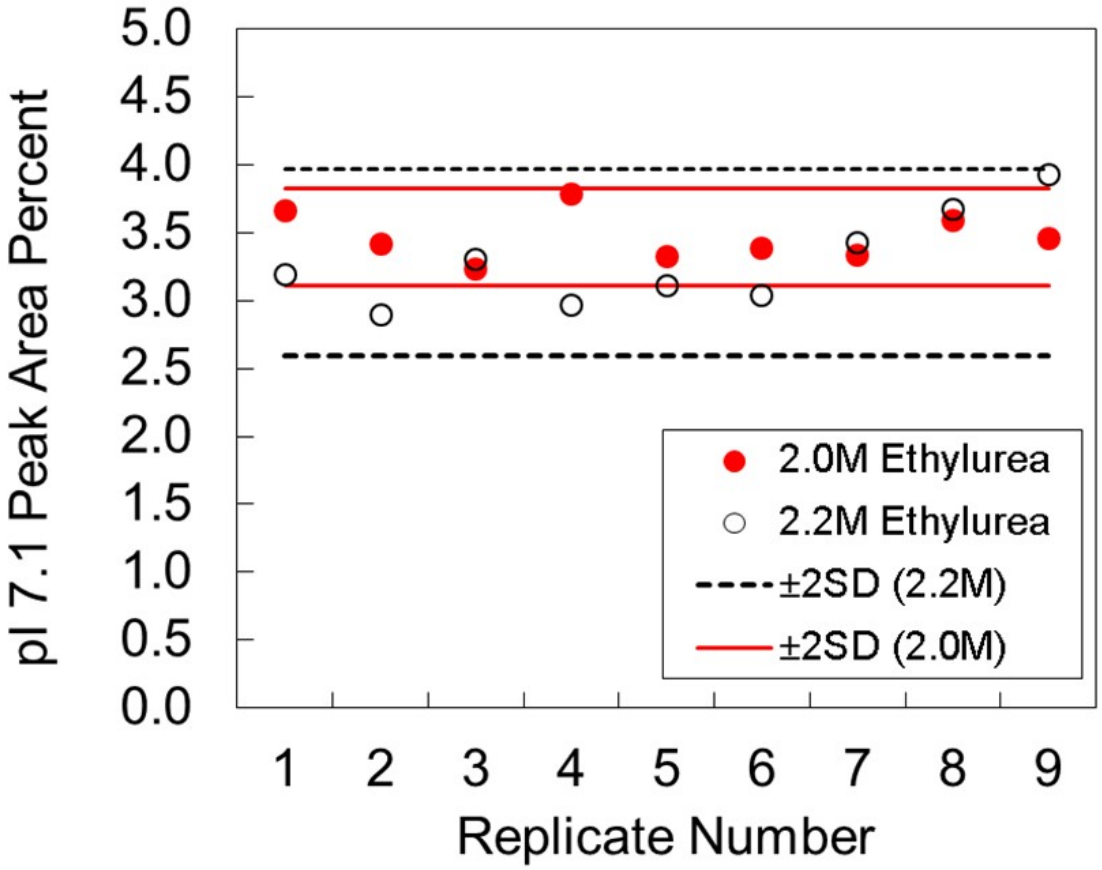

Figure 7. Reproducibility of cIEF Analyses of ErA. The analyses were carried out using 8M urea plus additional N-ethylurea (2.0 or 2.2M) as the denaturant. The chromatograms were integrated and the acidic peak at pI 7.0 was quantified for all runs. Limits representing plus and minus two standard deviations (SD) around the mean are shown for both data series.

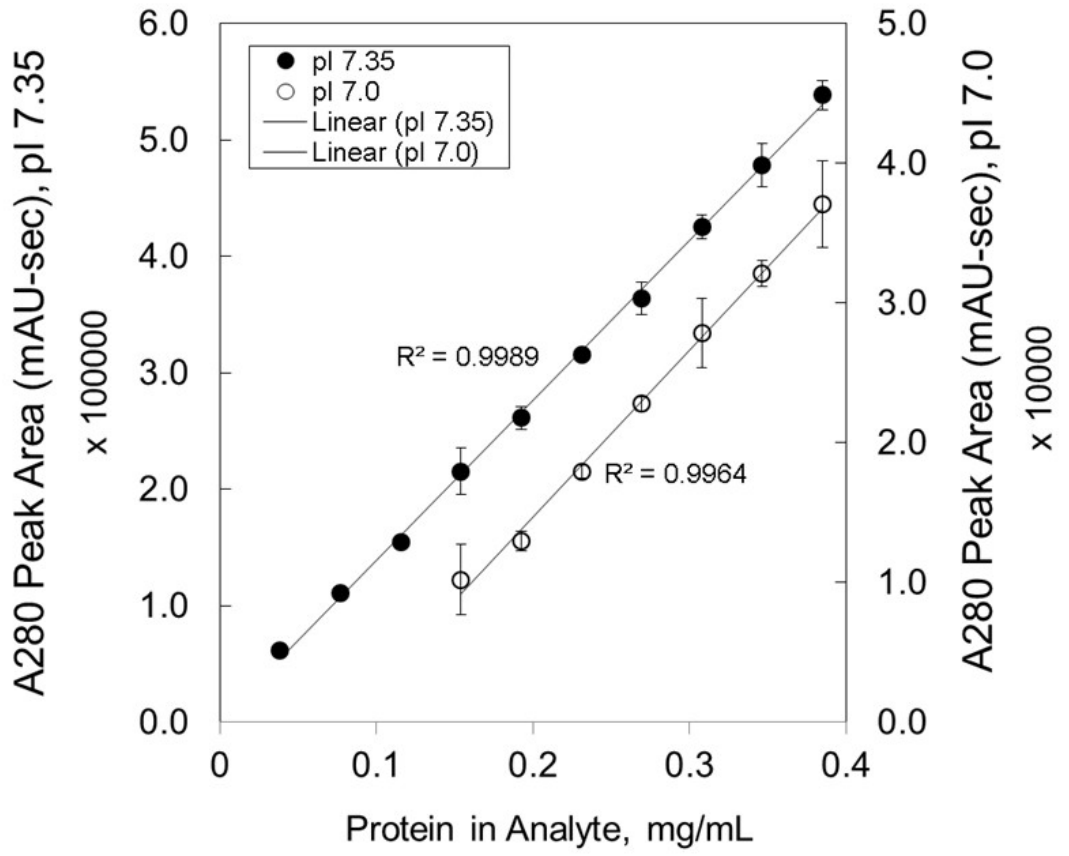

Figure 8. Linearity of cIEF Analyses of ErA. The analyses were carried out using 8M urea plus additional N-ethylurea (2.2M) as the denaturant in all runs. The chromatograms were integrated and the peaks (acidic peak at pI 7.0 and main peak at 7.35) were quantified for all runs. Each data point in the graph represents the mean of three replicate runs. Limits representing plus and minus one standard deviation (SD) around the mean are shown for each data point. The pI 7.35 data corresponds to the left-hand axis and the pI 7.0 data to the right-hand axis.

Table 1. Predicted pI Values for ErA and Recombinant Deamidated Mutants. Values were calculated using the ErA sequence and the web servers at the Swiss Institute of Bioinformatics or the European Molecular Biology Open Software Suite.

Protein	Predicted pI (EMBOSS)	Predicted pI (ExPASy)
ErA WT	7.87	7.23
ErA N41D	7.18	6.68
ErA N281D	7.18	6.68
ErA N41D N281D	6.81	6.36

Table 2. Acidic Species Content of ErA Batches from Full-Scale Manufacture Measured Under Denaturing Conditions. The data were gathered from cIEF analyses of ErA samples using 8M urea and 2.2M ethylurea as the denaturant system. The peak areas for the main (pI 7.35) and acidic (pI 7.0) species were integrated and data are presented as area percent of the pI 7.0 peak. Each batch was analysed in triplicate, and the data are presented as the mean and one standard deviation of the analyses of each batch.

Batch	pI 7.0 Area Percent (Mean) (n=3)	Standard Deviation for Three Replicates
A	3.4%	0.2%
B	2.4%	0.1%
C	1.5%	0.6%
D	1.2%	0.2%
E	0.9%	0.3%
F	1.6%	0.1%
G	2.7%	0.3%
H	3.5%	0.0%
I	2.2%	0.2%