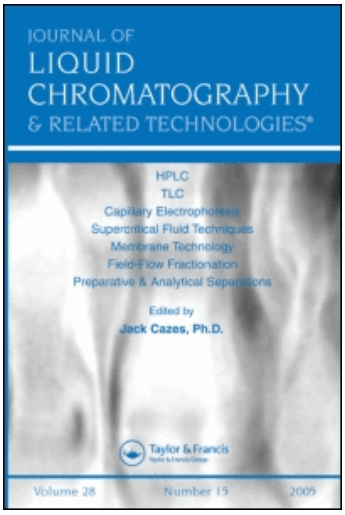

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ADVANTAGES AND LIMITATIONS OF DERIVATIZATION OF PEPTIDES FOR IMPROVED PERFORMANCE AND DETECTABILITY IN CAPILLARY ISOELECTRIC FOCUSING (CIEF)

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ABSTRACT

Several proteins of varying molecular weights (Mr) were shown to produce a single species, or multiple species which behaved as a single species, upon analysis with capillary isoelectric focusing (cIEF) after derivatization with a large molar excess of the derivatization reagent, 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC). Increased molar excesses of reagent were required as the molecular weight (Mr) of the sample increased. The derivative products exhibited acidic pI shifts, improved peak efficiencies, and lowered (improved) detection limits

when compared to the native species. In at least one case, a derivative product (not fully tagged) was shown to exhibit antibody (Ab) recognition when challenged with an Ab, raising the possibility of using these derivatives in affinity recognition studies (e.g., affinity CE, immuno-CE, and so forth). Problems were encountered with precipitation during derivatization and focusing. This problem was more pronounced with the more basic proteins. This would appear to limit the applicability of this reagent as a universal derivatization reagent for use with cIEF studies. The results presented herein represent a promising technique, and they offer advantages as well as certain limitations. Though not yet a perfect approach towards improved analysis and identification of peptides in cIEF, these results indicate tangible opportunities for further optimization.

INTRODUCTION

Proteins are linear arrays of amino acids bonded to one another in a head-to-tail fashion. Although the linking of the amino acids results in the loss of the ionizable amino and carboxylic acid groups of an individual amino acid, unmodified and linear proteins may contain at least one ionizable amino and carboxylic acid group. As a result, every unmodified peptide or protein has an isoelectric point (pI). The presence of amino acids containing ionizable side chains within the protein also contributes to the pI value for a protein. Also, the folding pattern of a protein, i.e. secondary and tertiary structures, can affect the ionization properties of these side chains by altering the immediate environment in which these residues reside. Therefore, isoelectric points can significantly vary from protein-to-protein. The determination of the isoelectric point of a protein is important in protein characterization studies and can often offer insight into structural differences between closely related species, e.g. various isoforms of a single protein.

Capillary isoelectric focusing (cIEF) is the miniaturization of the classical slab gel isoelectric focusing (IEF) method;[1-6] proteins and peptides can be separated under the influence of an electric field within a pH gradient established through the use of carrier ampholytes. By these techniques, cIEF affords several advantages over IEF performed in traditional slab gels, including being less manually intensive, amenable to automation, having faster analysis times, improved quantitative capabilities via on-line detection systems, and requiring less materials, such as ampholytes. cIEF has become an increasingly popular technique in recent years, and there have been several publications describing optimization of operational protocols, conditions, theory and instrumentation.[7-20] Also, cIEF has become more widely used in applications towards characterization of antibodies,[21-23] glycosylated proteins,[24-27] metalloproteins,[28-30] and other proteins that are difficult to analyze with slab gel IEF.[31] Similarly, in an effort

to truly automate the classic 2-D slab gel technology, various mass spectrometric detectors have been coupled to cIEF, allowing for accurate pI and Mr determinations.[32-41] Additionally, cIEF technology has recently been miniaturized onto a glass chip.[7] Clearly, these technologies will play a greater role in future protein characterization studies.

Currently, cIEF is performed through two distinct approaches, as determined by the order in which the focusing and detection steps occur. Single step cIEF involves the focusing of a sample within a capillary under the influence of residual electroendosmotic flow (EOF).[42-45] In this case, protein zones become focused first and are then swept past the detector. Two step cIEF involves first focusing a sample within a capillary under conditions in which there is minimal, if any, EOF present. The focused proteins then have to be mobilized past the detection window during a separate step, once focusing has been completed. This mobilization step has been accomplished through either chemical[1,47-51] or physical means.[46,52-56] These various cIEF protocols have been compared and contrasted.[7,57]

One of the shortcomings associated with cIEF analysis of protein species is the lack of useful and practical limits of detection. Since cIEF is a concentrative technique, one would anticipate that cIEF would exhibit improved detection limits compared to typical CZE protocols. However, the ampholytes needed to form the pH gradient in cIEF significantly absorb UV radiation below 254 nm. As a result, one is forced to monitor the progress of a cIEF analysis at wavelength less-than-ideal for protein determinations, i.e. 280 nm. The result is that protein concentrations need to be on the order of >1 mg/mL in order to be easily analyzed via cIEF with on-line UV detection. Up until this point, attempts at improving the detectability of proteins analyzed via cIEF through complete derivatization have not been reported. This report discusses the use of a derivatization reagent, 6-aminoquinolyl-N-hydroxysuccimidyl carbamate (AQC) in excess, to derivatize proteins and peptides to yield predominantly a single product, as determined by MALDI-TOFMS and cIEF analysis. The derivatized proteins and peptides exhibited improved detection properties relative to their native forms, and in some cases, retained biological activity when challenged with an appropriate Ab.

We hasten to add in concluding the Introduction section, that there are vast difficulties in deriving a single, homogeneously and fully tagged peptide or protein. This becomes more difficult the larger the protein becomes. Thus, to the very best of our own knowledge, there are no reports in the literature of CE or cIEF wherein the formation and analysis of a single, fully tagged peptide or protein by cIEF has been reported.[66] There are, of course, some reports utilizing incompletely and nonhomogeneously tagged peptides in CE (e.g., SDS-CGE), such as at the N-terminal alone, but all of the lysine groups were never demonstrated to be fully tagged.[61-66] This has always remained a synthetic prob-

lem in the CE of proteins, to derive a single, fully, and homogeneously tagged species that then behaves ideally in various CE modes. It should be added that the approaches being described herein are fully compatible with conventional, flat bed isoelectric focusing with any form of detection that follows.

EXPERIMENTAL

Chemicals and Reagents

All materials and reagents were obtained as the purest grade available and used as received, except where noted. 6-Aminoquinolyl-N-hydroxy-succinimidyl carbamate (AQC) standard was obtained from Waters Corporation (Milford, MA, USA) and synthesized according to literature reports.[58] Human insulin was from the United States Pharmacopoeia (Rockville, MD, USA). Acetonitrile (99.99%, HPLC grade), sodium borate, pH 2-11 ampholytes (product # A-8078), urea, cytochrome C (horse heart), myoglobin (horse heart), b-lactoglobulin B (bovine milk), trypsin inhibitor (soybean), methyl red (recrystallized from toluene, m.p. 178-179 °C), bovine serum albumin (BSA, product # A-7638), monoclonal anti-BSA (mAb) IgG (clone BSA-33), and monoclonal anti-human insulin IgG (clone K36aC10) were all from Sigma Chemical Company (St. Louis, MO, USA). Note: pH 2-11 ampholytes (product # A-8078) were no longer available from Sigma, as of January, 1999. Antibodies obtained in ascites fluid were affinity HPLC purified against a protein G column using conditions described elsewhere.[59] The monoclonal Ab (mAb) derivatized as part of this study has been described before.[23] Water used for all experiments was deionized and distilled from a Corning Glass Works (Corning, NY, USA) Megapure MG-1 water purification system. The FC-PN surfactant was obtained through the generosity of J&W Scientific (Folsom, CA, USA).

Equipment

Capillary isoelectric focusing experiments were performed under the influence of residual EOF, i.e. single-step mobilization, using an ISCO Model 3850 Capillary Electropherograph (Lincoln, NE, USA).[42-44] Electropherograms were collected via a Macintosh Plus 1 Mb computer using Dynamax software from Rainin (Woburn, MA, USA). The Reacti-Therm heating module used in derivatizations was from Pierce Chemical Company (Rockford, IL, USA).

Derivatizations of Proteins with AQC[58]

Typically, lyophilized protein samples were dissolved in 200 mM borate buffer (boric acid adjusted to pH 8.8 with 10.0 M NaOH) to a concentration of

Table 1

Derivatization Conditions for Several Proteins

Protein	Volume Sample	Volume Borate AQC	Volume Protein:AQC	Molar Ratio
Human insulin	5 µL	10 µL	34 µL	1:20
b-Lactoglobulin B[a]	5 µL	10 µL	30 µL	1:55
BSA[a]	5 µL	40 µL	60 µL	1:398
Porcine pAb[a]	5 µL	25 µL	10 µL	1:300
mAb[b]	3 µL	30 µL	40 µL	1:1000

[a] Lyophilized proteins were dissolved in 200 mM, pH 8.80 borate buffer to a concentration of 20 mg/mL. [b] Obtained in 20 mM, pH 7.0 phosphate buffer at a concentration of 5.7 mg/mL. Concentrated approximately four fold via microcentrifguation.

20 mg/mL. Samples were then diluted further with additional borate buffer. AQC (10 mM in ACN) was then added and the resulting mixture was vortexed for 5-10 seconds. The sample was allowed to stand at room temperature for at least one minute followed by heating at 55°C for 10 minutes. The optimal amount of AQC solution needed for each derivatization was determined when only one predominant derivative peak was observed in the resultant cIEF electropherograms. Specific derivatization conditions for each of the proteins used in this study can be found in Table 1.

cIEF Analysis

Samples were prepared by diluting with concentrated cIEF buffer (8-16% (v/v) pH 2-11 ampholytes and 0.08- 0.11% (v/v) FC-PN surfactant. This mixture was then diluted with an equal volume of 8.0-10.0 M urea. Samples were briefly vortexed and centrifuged prior to manual injection into the instrument.

The run buffer conditions consisted of 3.0 - 6.0% (v/v) pH 2-11 ampholytes, 0.04 - 0.06% (v/v) FC-PN surfactant and 4.0-5.0 M urea. Reversed polarity was used with an anolyte of 200 mM phosphoric acid (H3PO4) and a catholyte of 200 mM sodium hydroxide (NaOH). A Microsil FC or DB-1 coated capillary (J&W Scientific, Folsom, CA, USA) of 50 µm i.d. and 60 cm length (20 cm to detector with reversed polarity) was used for the separations. A running voltage of -20 kV was used with 280 nm UV detection. The capillary was rinsed with 200 mM H3PO4 anolyte and acetonitrile when the perfor-

mance of the method deteriorated over prolonged use, as dictated by the observance of excessive migration times or precipitation within the capillary.

Matrix Assisted, Laser Desorption Ionization, Time of Flight Mass Spectrometry (MALDI-TOFMS) Analysis

Samples were prepared for MALDI-TOFMS analysis, following derivatization, through either microcentrifugation using Mr cut-off filters (derivatized BSA), or with analytical scale HPLC, using conditions previously described.[60] Samples were observed and collected as single peaks at 280 nm UV detection.

Matrix assisted, laser desorption ionization, time of flight mass spectrometry (MALDI-TOFMS), was performed on a PE Biosystems (PE Biosystems Division of Perkin-Elmer Corporation, Framingham, MA, USA) Voyager RP Biospectrometry Workstation. The matrix used was 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid, Sigma Chemical Company, St. Louis, MO, USA). A Nitrogen laser operating at 337 nm was used to ionize the samples.

RESULTS AND DISCUSSION

Protein Derivatizations

The derivatization of proteins, in general, has been problematic due to the fact that a mixture of products is usually realized.[61-66] Perhaps no capillary based separation method for proteins could benefit more from a successful derivatization protocol than cIEF. Unfortunately, for the cIEF practitioner, the ampholytes used to establish the pH gradient within a capillary absorb significantly below 250 nm. Thus, one is forced to monitor proteins at a wavelength in which the aromatic side chains on the amino acid residues absorb, e.g. 280 nm, instead of performing detection for proteins at a wavelength in which the peptide bond absorbs, e.g. 200 - 210 nm, as can be done in CZE analysis. Where one would expect a lower detection limit regarding direct UV detection for cIEF versus CZE based upon the concentrative nature of cIEF, the detection limits between the two techniques are actually comparable.[6] Until the introduction of UV transparent ampholytes into the marketplace, derivatization appears to be a reasonable approach towards realizing improved protein detectability in a cIEF format. This has been attempted before; however, due to the fact that conditions for the formation of a single, fully tagged product were not realized, the final improvements in cIEF performance and detectability have not yet been described.[61-66] The UV and FL properties of AQC and its derivatives have been reported.[58,74]

Lysines, with pKa of approximately 10.5, are sufficiently nucleophilic under conditions of moderate pH that many derivatization protocols have been tailored toward these groups.[66] Along with arginine and histidine, lysine

residues account for most of the basic character for proteins or peptides. The pI of a protein can be estimated through the use of mathematical models and based upon knowledge of the primary sequence of the protein of interest and the pK values of those ionizable side chains.[67-68] Using these tools, one is able to estimate the pI shift a protein would experience upon the complete tagging of all lysine residues. Using these models, one can calculate for several examples, a shift of several pI units to more acidic values, between the native species and a fully tagged derivative in which all of the lysine residues have been removed. Any cIEF conditions used would need to be able to detect derivatized proteins possessing more acidic pI values.

cIEF Conditions

Previously, we have performed cIEF under the influence of residual EOF.[23,42-43,69] This approach has been termed single step cIEF, since protein focusing and resultant mobilization past a point of detection occur simultaneously. The primary advantages of single step cIEF are speed of analysis and simplicity of the instrumentation required. Additionally, single step cIEF can be performed using uncoated, fused silica capillaries, which are more rugged than coated capillaries. Unfortunately, single step cIEF using uncoated fused silica capillaries suffered from a relative inability to accurately monitor proteins of acidic pIs.[23] It was thought that as the zeta potential at the capillary wall varied along the length of the capillary, according to the ampholyte established pH gradient, then the EOF varied as well. The use of coated capillaries moderated this disparity in EOF and allowed for the separation of more acidic proteins down to pIs of 4.7 (ovalbumin); however, linearity between migration time and protein pI was not achieved down to these acidic pI values (pI < 5.1), under the optimized conditions (Experimental).

Initial attempts in developing a method for the analysis of acidic species were based upon those previously reported.[21,23,69] The type of ampholytes used in any cIEF separation determines the useful pH range of the resultant cIEF process. If one has prior knowledge of the pI of an analyte of interest, then one can use ampholytes having a narrow pH range, e.g. pH 6-8. If one is analyzing a mixture of analytes having a broad pI distribution, then it is advantageous to use ampholytes possessing a wider pH range. For this study, it was determined that pH 2-11 ampholytes resulted in reduced analysis times for all species encompassing a wide pH range. The disadvantage of using the pH 2-11 ampholytes was that they contained a greater amount of species that absorbed at 280 nm, which resulted in a noisier baseline. This would not, of course, be a problem in cIEF-ESI-MS applications, as for example in applying said technique to proteomics.

Early on in this study, it was apparent that solubility was problematic for some species during the cIEF process, which is not an uncommon problem in

cIEF.[3-9] Several solutions to the problem of sample precipitation have been proposed. These have mainly involved the addition of solubilizing agents to the run buffer, such as urea and nonionic surfactants. The inclusion of urea into the cIEF run buffer has been one of the more widely employed strategies to solve the problem of analyte precipitation[21,24,27,70-71] and was used here as well. There are, perhaps, some problems in using urea in the run buffer to improve solubilities of tagged proteins, wherein these have become very hydrophobic after AQC tagging. Alternatively, some proteins, when fully focused, become so concentrated that they are then insoluble in the run buffer. It is always possible that the native (untagged) or fully tagged proteins become denatured during the cIEF runs because of the presence of a high concentration of urea. That does not, of course, affect the change in peak shape (narrowing), migration times, pI shifts, and limits of detection, though it may well affect the recognition of a protein by its Ab or vise-versa. If fully tagged proteins are to be used in immuno-CE assays, then they need to remain Ab active, even after complete tagging and the conditions needed to run them in cIEF. Evidence presented below suggests that this denaturation does not occur, at least not in the case of BSA and its Ab. It may be that 4-5 M urea, as used here, is not sufficient to cause complete denaturation of BSA and/or its Ab during the time of cIEF analysis.

Identification of Suitable pI Markers

The use of pI markers was necessary in order to evaluate the ability of any developed method to resolve species of differing pIs. The standard markers used were selected because they produced one predominant peak upon cIEF analysis, comprised a wide range of pI values, remained in solution at their pI under the conditions used, and remained stable as a mixture. The primary protein standards used were cyctochrome C (horse heart, pI 9.3), myoglobin (horse heart, pI 7.4), b-lactoglobulin B (bovine milk, pI 5.3), and trypsin inhibitor (soybean, pI 4.6). The use of synthetic markers has been proposed as an alternative to protein markers,[72-73] since they have the potential to be obtained cheaply and in bulk. One such indicator was methyl red (pI 3.8), which was determined to be suitable as an acidic marker.

The cIEF method was evaluated with a series of pI standard markers. The standards were sufficiently resolved from one another and possessed reasonable peak shapes. The migration times of the standards were plotted against their pI in order to evaluate the ability of the cIEF method to produce a linear calibration curve over the pH range of interest. As was found in earlier work and is typical of single step cIEF protocols,[6,57,69] non-linearity was observed over the pI range of the markers used, particularly for the more acidic species. Although a method which possessed full linearity regarding migration time versus pI over the entire pI range examined would have been ideal,[52] it appeared that the use of standard pI markers to bracket species of interest would suffice for pI determinations. In addition, manual loading of the sample into the capillary led to

variations in sample loading, which adversely affected migration time, peak height and peak area, reproducibility of the standards, and was further incentive to use pI standards to bracket unknowns.

Peptide and Protein Derivatizations

AQC is a derivatization reagent that was initially developed to aid in amino acid analysis.[58] The reagent is reactive to all forms of primary and secondary amines, Figure 1. Recently, AQC has been shown to rapidly and completely tag all available sites for several peptides and smaller proteins for improved performance and detection in HPCE.[74] The conditions used for these derivatizations involved the use of denaturing conditions with SDS to unfold the analyte and allow the reagent easier access to derivatization sites. The use of SDS is largely avoided in isoelectric focusing since SDS associated with a protein imparts substantial negative charges to the SDS-protein complex, resulting in undesirable focusing properties and inaccurate estimations of the pI. Therefore, derivatizations for this study were performed on non-denatured proteins and peptides possessing a wide range of Mr.

The primary structure of human insulin is well known[75] and has a Mr of 5807.6 Da. Human insulin contains one lysine residue in addition to the two amino termini present on each peptide chain and these should be accessible for reaction. As a result, there are seven (2n -1, where n = 3 for human insulin) possible products that can be formed upon derivatization with AQC. Ideally,

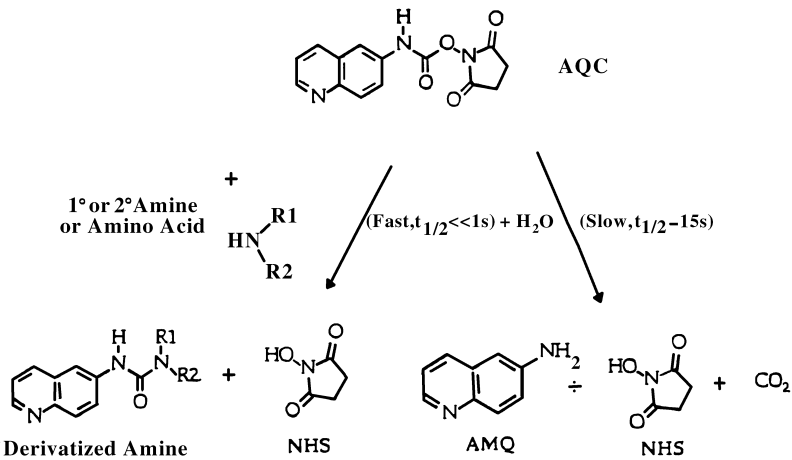

Figure 1. Structure of AQC and reaction diagram for a typical derivatization of an amine under aqueous conditions.

optimization of the derivatization conditions would result in only the fully tagged product.

Native human insulin was shown to give only one peak upon cIEF analysis, Figure 2 (A). The insulin was then bracketed with cytochrome C (pI 9.3) and b-lactoglobulin B (pI 5.3) and analyzed in the same manner, Figure 2 (B).

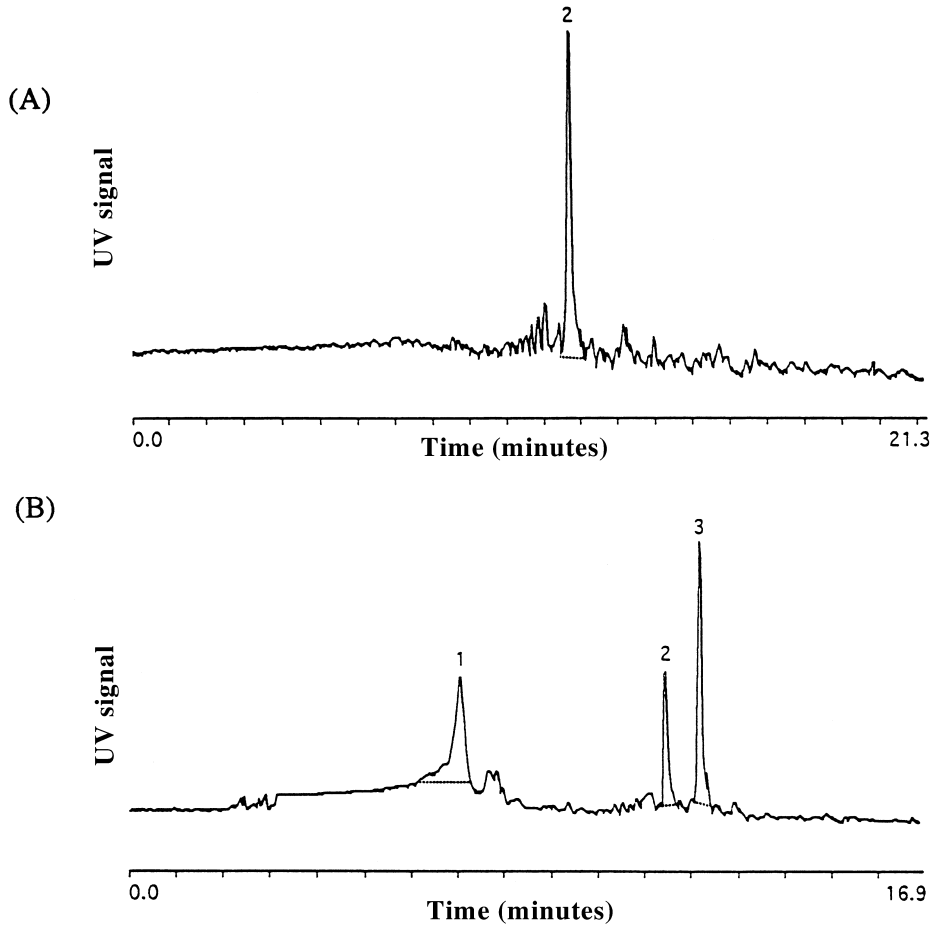

Figure 2. cIEF analysis of native human insulin, (A), and bracketed with pI markers, (B). Peak assignments: cytochrome C (1, pI 9.3), native human insulin (2), and b-lactoglobulin B (3, pI 5.3). Conditions: DB-1 coated capillary, 60 cm/20 cm effective length, 50 mm i.d., -20 kV potential, 20 mM NaOH catholyte, 20 mM H3PO4 anolyte, 280 nm detection. Buffer components were 3.0% pH 2-11 ampholytes, FC-PN 0.01% surfactant, and 4.0 M urea.

The pI of native human insulin was found to be 5.94 +/- 0.07 (n=7), which was more basic than the pI reported in other studies, pI 5.30-5.35, but not as basic as the pI determined for a slab gel IEF study in which 6.0 M urea was added to the buffer.[76] An average efficiency of 1.40 x 10^5 +/- 33.2% (n=3) plates was found for native insulin. The less-than-ideal precision of these results, and that for the tagged insulin (vide infra), most likely resulted from variations in manually loading the sample into the capillary, which may be improved with automated injections.

Native insulin was derivatized with a 20-fold molar excess of AQC. The derivatization reaction mixture and a reaction blank (no insulin) were analyzed with cIEF, Figure 3. As was generally observed for every sample, the derivatization blank (no insulin), Figure 3 (B), produced two peaks derived from the AQC reagent, while the derivatized native insulin appeared to produce only one species. The first peak observed in the blank, Figure 3 (B), was readily observed in all derivatization reactions and was determined to be 6-aminoquinoline (AMQ), which is produced upon hydrolysis of excess AQC.[58] The second peak often became readily visible only after standards were added to the sample, or was observed as one broad peak that would occasionally merge into the baseline. This second peak has not been definitively identified, but it is suspected that it is related to an autoderivatization product, i.e., the product formed from the reaction between AMQ and AQC, rather than N-hydroxysuccinimide, another known product of AQC hydrolysis. Additionally, the rise in the baseline observed at the end of the cIEF run buffer blank was attributed to a refractive index change, which coincided with the end of the pH gradient and the beginning of the presence of anolyte within the capillary.

Figure 4 shows a case where native insulin was spiked into a derivatized insulin sample and was shown to migrate with a pI different from the derivative. Considering this result, it was apparent the derivatization of insulin with AQC resulted in a derivative species having a lower pI value than the native form. This was the expected result, since the tagging of the basic lysine residues and amino termini as a result of derivatization would be expected to yield a species with a more acidic isoelectric point.

Methyl red and trypsin inhibitor were added to the AQC/insulin reaction mixture and analyzed with cIEF, Figure 5. The pI of the insulin derivative was found to be 4.41 +/- 0.03 (n=10). In addition, the number of theoretical plates associated with the derivatized insulin peak was 1.02 x 10^6 +/- 31.3% (n=3). The improved efficiency with which derivatized human insulin was analyzed was most likely due to the inability of basic sites (lysine residues and amino termini) to interact, post-derivatization, with residual free silanol groups on the surface of the capillary. As a result, secondary interactions between the capillary and the protein played less of a role in the separation. This is not to suggest that the lone arginine and two histidine residues were not prone to such

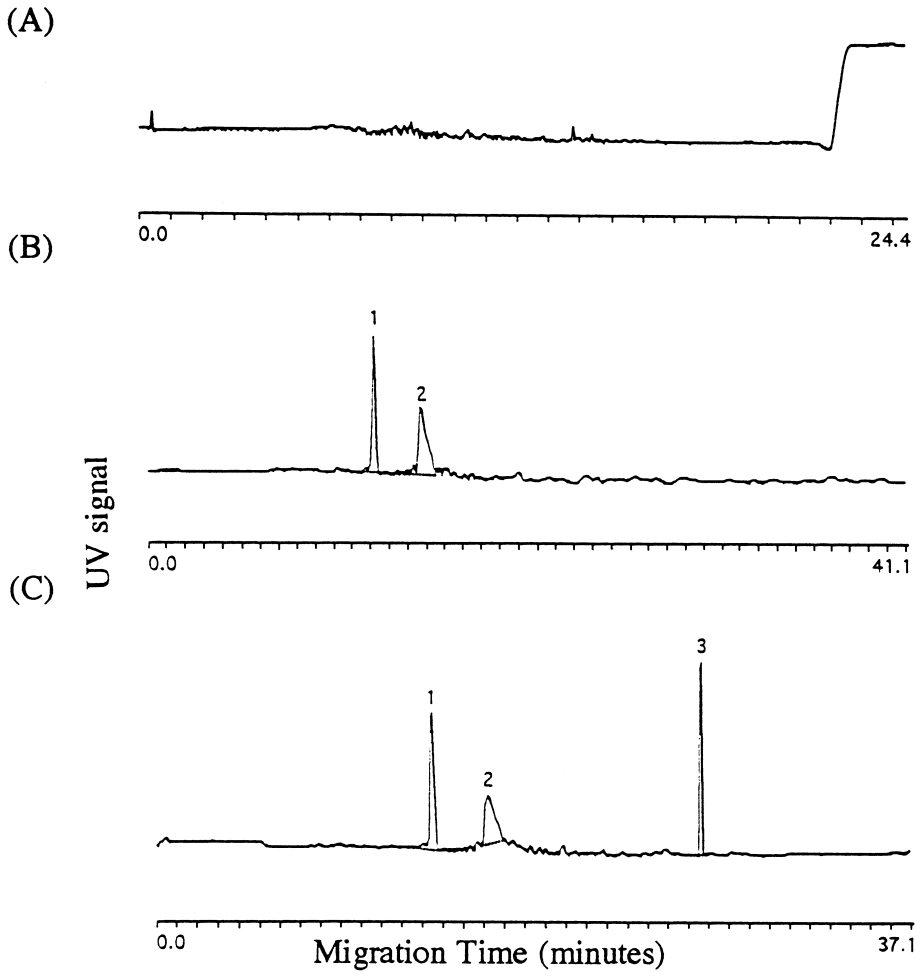

Figure 3. cIEF analysis of run buffer blank, (A), derivatization blank (no human insulin), (B), and human insulin derivatization, (C). Peak assignments: AQC derived (1 and 2), and derivatized human insulin (3). Conditions were the same as those reported in Figure 2.

secondary interactions, nor, does this discount any possible hydrophobic interactions between the derivative and the coated capillary; however, it appeared that secondary interactions between the derivative product and capillary were reduced upon derivatization, as indicated by the increased peak efficiency. Although it may seem to be unorthodox to consider separation efficiencies when dealing with a concentrative technique, such as cIEF, we feel an interpre-

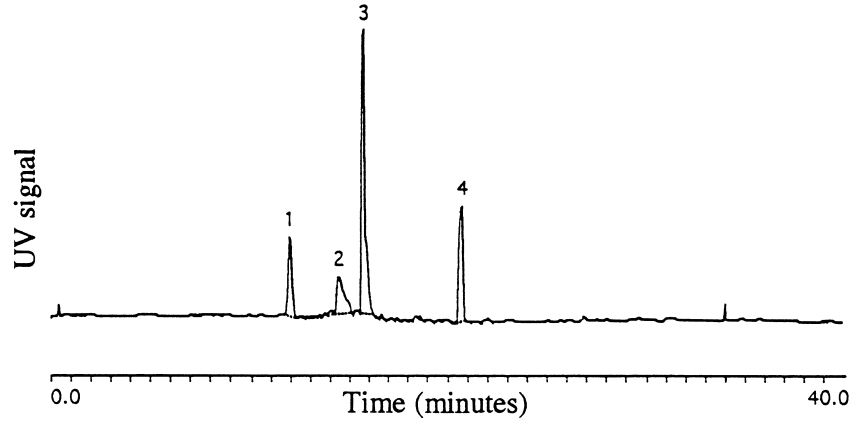

Figure 4. cIEF analysis of a human insulin derivatization reaction mixture spiked with native human insulin. Peak assignments: AQC derived (1 and 2), native human insuiln (3), and derivatized human insulin (4). Conditions were the same as those reported in Figure 2

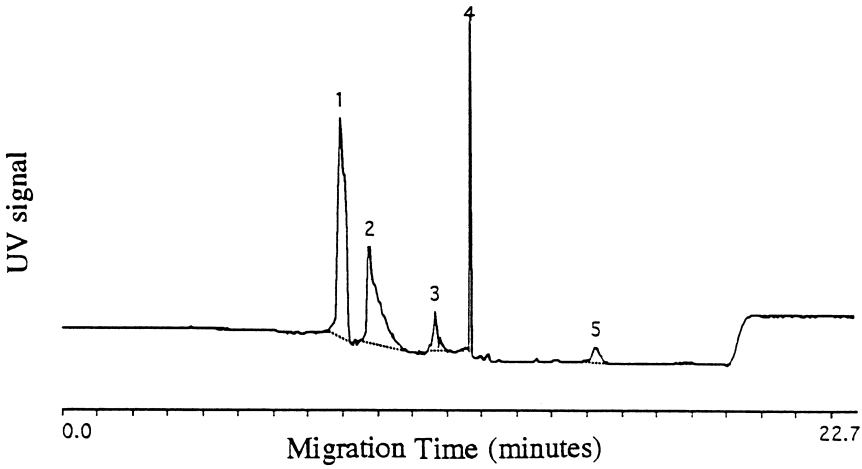

Figure 5. cIEF analysis of derivatized human insulin bracketed with pI markers. Peak assignments: AQC derived (1 and 2), trypsin inhibitor (3), derivatized insulin (4), and methyl red (5). Conditions were the same as those reported in Figure 2.

tation of such data is appropriate when one takes into account the fact that single-step cIEF is performed under residual EOF present within the capillary. One would expect that the focused protein zones are subject to the same secondary interactions between the protein and capillary wall normally observed with free zone capillary electrophoresis. Results illustrating the reduction in pI (more acidic values) and improvement in separation efficiency (higher plate counts) upon derivatization for several proteins are summarized in Table 2.

The limit of detection (LOD) for the derivatized human insulin was estimated to be 24.7 μg/mL with direct UV detection. By comparison, the limit of detection of the native human insulin was measured to be approximately 0.05 mg/mL. In addition, the limit of derivatization, defined here as the concentration of native protein below which no derivative product was observed after derivatization, was determined to be 0.71 mg/mL for the native insulin. Results demonstrating improved detection limits, as well as the limit of derivatization,

Table 2

Summary of pI and Plate Efficiency Results

Sample	pI	Efficiency (N, Plates)
Native h-insulin	5.94 +.- 0.07 (n=7)	1.40×10^5 +/- 33.2% (n=3)
Deriv. h-insulin	4.41 +/- 0.03 (n=10)	1.02×10^6 +/- 31.3% (n=3)
Native b-lact. B	5.3 (literature value)	6.18×10^4 +/- 56.4% (n=3)
Deriv. b-lact.Bb	3.88 +/- 0.08 (n=3)	1.04×10^6 +/- 56.4% (n=3)
Native BSA	6.02 +/- 0.06 (n=3)	1.60×10^4 +/- 27.5% (n=3)
Deriv. BSA	4.29 +/- 0.02 (n=3)	2.22×10^5 +/- 4.8% (n=3)
Native pAb	7.79-7.17 +/- 0.09-0.12 (n=3)	ND
Deriv. pAb	4.33 +/- 0.07 (n=3)	9.16×10^5 +/- 64.2% (n=3)
Native mAb	7.24-6.98 +/- 0.02-0.02 (n=3)	ND
Deriv. mAb	3.95 +/- 0.01 (n=3)	8.50×10^5 +/- 66.8% (n=3)

ND = not determined. For the above data, n=3 represents a single sample injected in triplicate, for n=7 or 10, these were samples determined over several days. Native insulin was determined over the course of three different days, and derivatized insulin was determined over at least three different days using three different derivatization reactions.

for several proteins are summarized in Table 2. Limit of derivatization refers to the smallest amount (mass) of analyte that can be found to react with an excess of AQC reagent under typical reaction conditions within a reasonable timeframe. This forms a part of limit of detection of the original analyte, but it is different from derivatizing a large concentration or mass of analyte and then diluting its solution to realize the actual limit of detection. Limit of derivatization refers to the overall rate of a bimolecular reaction (in the case of AQC), where the concentration of each reagent appears in the overall rate equation to the first order. As the concentration of the analyte reactant becomes less and less, the overall rate of the reaction also becomes less, until no reaction is observed in a reasonable timeframe. At that concentration of analyte, one has reached the limit of derivatization. It does not have to do with loss of the analyte on the walls of the reaction vessel, though this could play a role at low concentrations. It really refers to the concentration at which the rate of reaction is so slow to effectively be unusable and no derivative can be observed to form. It is not that one cannot detect the derivative, it is no longer formed in sufficient amounts to be detected. There is a very real difference between not being formed and not being detected, and analysts should really discuss both limits of detection and derivatization, which is rarely the case in the literature. Limit of detection is often lower than limit of derivatization, because when measuring limit of detection, one derivatizes the analyte at a high concentration and then just dilutes until a derivative species is no longer detectable. That is very different than derivatizing at lower and lower levels (concentrations) of analyte and noting when one can no longer see that derivative formed.

Limits of detection for fully tagged peptides in CZE-LIF (laser induced fluorescence) have been described by us in a related publication, which utilized various ratios of aqueous buffer to acetonitrile in order to improve the FL response of AQC tagged products.[74] Those results clearly demonstrated a lowering of LODs for all fully tagged peptides, in comparison with UV for both the tagged and native species. It is well known that such derivatives exhibit FL quenching in purely aqueous buffers/solutions, and that the addition of an organic solvent can improve such results.[74] If satisfactory FL responses could be realized with the inclusion of an organic modifier without overly distorting the pH gradient, then one could perform LIF analysis at the optimum wavelength conditions for AQC derivatives, without any concern about ampholyte quenching interferences.

Limits of derivatization, as opposed to limits of detection, relate to what lowest level of analyte can actually be found in solution, in a reasonable timeframe, and then be efficiently converted to the desired derivative. Though LODs relate to forming a derivative at a high concentration and then determining the lowest level that can be detected, it is often lower than the limit of derivatization. For all practical purposes, unless one can derivatize a species at the level desired, it does not really matter what is its LOD, since it is still impossible to find the analyte to first derivatize. There may be ways, as suggested

below, to lower the limits of derivatization, with any given LOD, but unless that can be reduced below the LOD, then the LODs are really meaningless analytical reference points. Perhaps not enough has been discussed in the literature relating LOD to limits of derivatization, as we are now attempting to make clearer. Most analysts have tried to lower LOD without giving the same concern to lowering limits of derivatization, a real mistake.

The derivatization protocol was extended to larger proteins, such as beta-lactoglobulin B and bovine serum albumin (BSA) with similar results, Table 2. Antibodies, which presumably have a Mr of approximately 150 kDa, were also included in this study. A native polyclonal Ab was analyzed via cIEF and at least seven separate species were shown to be present, Figure 6 (A). These separate species were not baseline resolved, and the possibility existed that an even greater number of species were present in the sample. The pI of the native species was determined by bracketing the sample with cytochrome C (pI 9.3) and trypsin inhibitor (pI 4.6) and performing cIEF analysis, Figure 6 (B). The pI range for the native pAb was estimated to be from 7.79 +/- 0.09 to 7.17 +/- 0.12 (n=3).

The pAb was derivatized with increasing amounts of AQC, Table 1, until a single species was observed with cIEF analysis, peak 2 in Figure 7(A). In Table 1, the column on the far right (molar ratio of protein:AQC) indicates the ratio of moles of reagent to one mole of protein (μM to μM). Although the results indicated a single derivative species, there was a likely chance that the derivative peak observed was comprised of multiple species that were not resolved under the conditions employed. Still, the apparent reduction in the degree of sample heterogeneity after derivatization was a bit startling. It was unclear if the heterogeneity observed in the native sample was mostly due to differences in amino acid composition or differences in the carbohydrate composition between the various components of the pAb sample; however, the fact that the derivatized pAb behaved as though it were a single species suggested that the initial heterogeneity may have been due to variations in total lysine content between species within the sample, as opposed to variations in the numbers of sialic acid moieties. The derivatization procedure may have neutralized the effect that lysine variability had on the number of species present and reduced the number of species observed, post-derivatization.

Several standards, b-lactoglobulin B (pI 5.3), trypsin inhibitor (pI 4.6), and methyl red (pI 3.8), were added to the crude derivatization reaction mixture and the resultant mixture was analyzed, Figure 8. The migration positions of trypsin inhibitor, peak 4 in Figure 8, and methyl red, peak 6 in Figure 8, were only considered in determining the pI of the derivatized pAb, peak 5 in Figure 8. The pI of the derivatized pAb was determined to be 4.33 +/- 0.07 (n=3). The efficiency of the derivative pAb peak was likewise determined to be 9.16×10^5 +/- 64.2% (n=3) plates. Despite the large variation in efficiency, the smallest number of plates calculated for the derivative peak within a single run for this

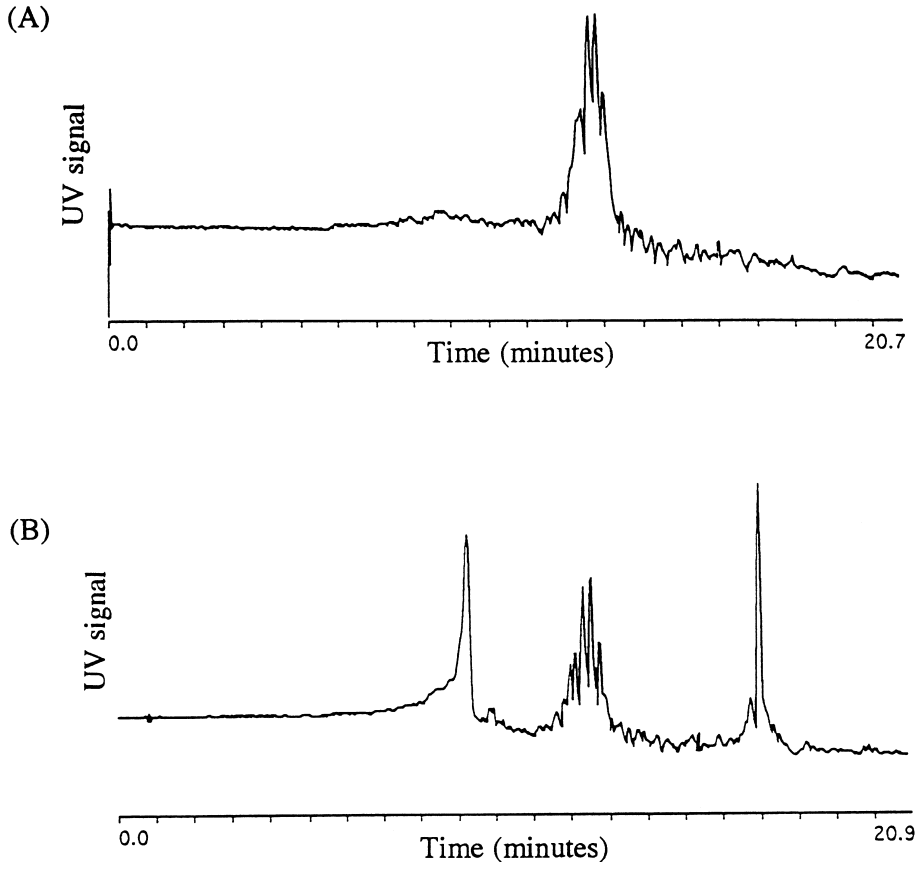

Figure 6. cIEF analysis of native porcine pAb, (A), and bracketed with cytochrome C (pI 9.3) and trypsin inhibitor (pI 4.6), (B). Conditions: microsil FC coated capillary, 60 cm/20 cm effective length, 50 mm i.d., -20 kV potential, 200 mM NaOH catholyte, 200 mM H3PO4 anolyte, 280 nm detection. Buffer components were 6.0% pH 2-11 ampholytes, 0.04% FC-PN surfactant, and 5.0 M urea.

analysis was 5.08 x 105 which was several times greater than the number of plates calculated for the beta-lactoglobulin B and trypsin inhibitor standards for the same run.

Since the primary sequence of the pAb and the number of lysine residues was unknown, it was not possible to determine the degree of tagging of the pAb upon derivatization. For this reason, MALDI-TOFMS analysis was not attempted on either this pAb or the mAb described below. The evidence pre-

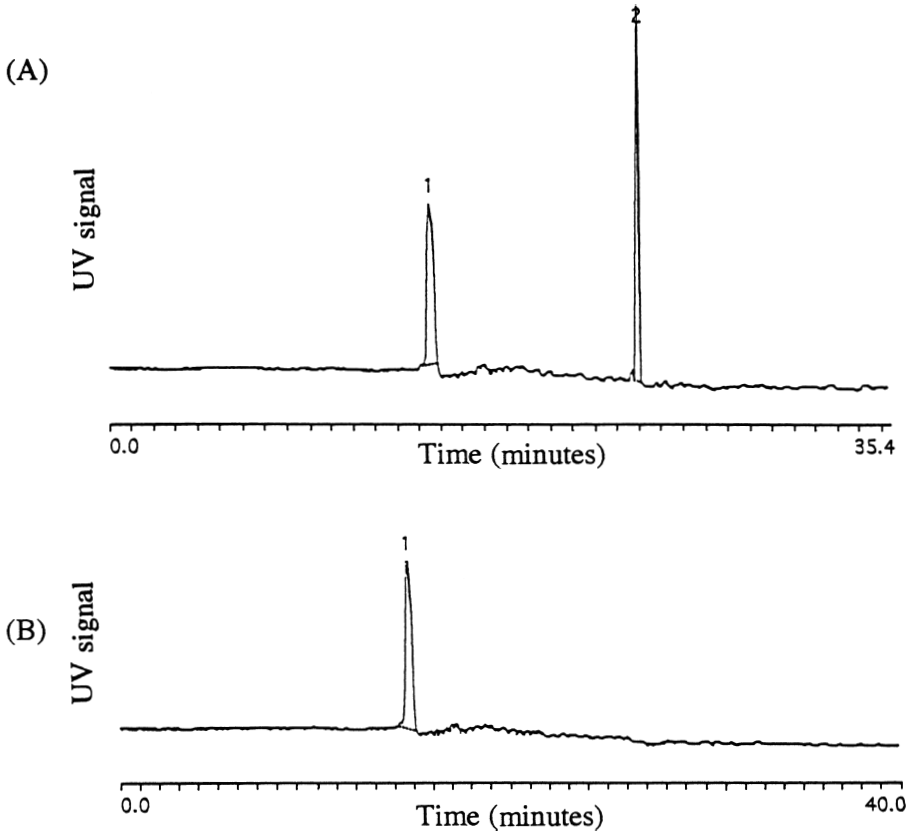

Figure 7. cIEF analysis of AQC derivatized pAb, (A), and a derivatization blank, (B). Peak assignments: AQC derived (1) and derivatized pAb (2). Conditions were the same as those reported in Figure 6.

sented for the derivatization of BSA strongly suggested incomplete tagging of these antibodies, or any protein possessing significant tertiary structure, in the absence of harsh denaturing conditions, e.g., SDS and thiol reduction and alkylation. The results for all of the proteins successfully derivatized are summarized in Tables 2 and 3.

Affinity Recognition of Derivatized Species

The mAbs against BSA and human insulin, obtained in ascites fluid, were HPLC affinity purified against a protein G column. These antibodies were then

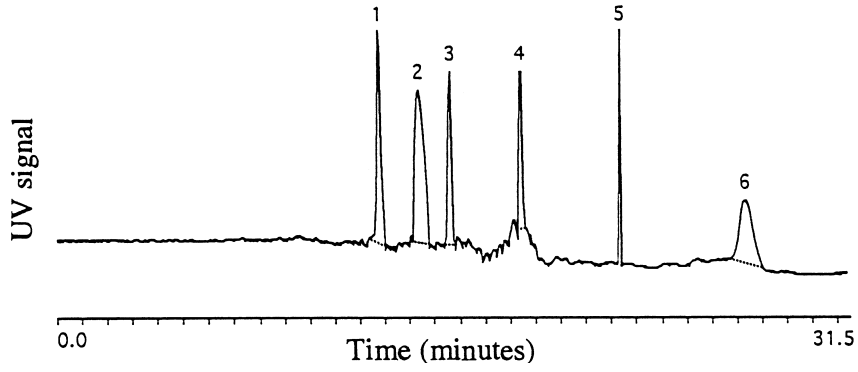

Figure 8. cIEF analysis of derivatized pAb bracketed with pI markers. Peak assignments: AQC derived (1 and 2), b-lactoglobulin B (3, pI 5.3), trypsin inhibitor (4, pI 4.6), derivatized pAb (5), and methyl red (6, pI 3.8). Conditions were the same as those reported in Figure 6.

challenged with derivatized BSA and human insulin. This was done in order to determine whether or not a derivatized species retained any Ab recognition (bioactivity) post-derivatization. Provided that such an interaction could exist post-derivatization, then the derivatization protocol developed could be an

Table 3

Summary of Detection Limits and Derivatization Limits

Sample	Limit of Detection[a]	Limit of Derivatization[b]
Native h-insulin	0.05 mg/mL	0.71 mg/mL
Deriv. h-insulin	27.7 µg/mL	N/A
Native b-lact. B	0.10 mg/mL	0.67 mg/mL
Deriv. b-lact. B	2.7 µg/mL	N/A
Native BSA	0.10 mg/mL	0.55 mg/mL
Deriv. BSA	10.3 µg/mL	N/A

[a] Determined to be that concentration of serially diluted derivative product that produced a discernible signal (S/N = 3). [b] Determined as the lowest concentration of native protein prior to derivatization that could be observed upon cIEF analysis after derivatization.

approach towards easily synthesizing labeled proteins for use in any process that relies upon an affinity recognition step, e.g. competitive immunoassays.[77-79] What has always been missing in immuno-HPLC or immuno-CE methods is the ability to generate a fully tagged protein or Ab which still retains recognition for its partner (antigen, Ag).[61-66]

A native mAb raised against BSA was affinity purified and found to be comprised of several isoforms upon cIEF analysis. This Ab was used to challenge native BSA under the cIEF conditions employed, and it was found to recognize the native Ag (data not shown). BSA was derivatized to form a product which behaved as (if it were) a single species under the cIEF conditions employed, and this was then challenged with the purified anti-BSA mAb. It is not clear if the cIEF conditions utilized here could discriminate between derivatized BSA species that differed in one or more tags. This is likely the reason that these BSA derivative species appeared as a single cIEF peak, when in reality, they were not. Later results (vide infra) by MALDI-TOFMS clearly showed that this was not really a single species, but rather consisted of multiple, differently tagged products. And, none of these appeared to be fully tagged, having less than 60 maximum possible sites reacted. Nevertheless, mAb addition resulted in a shift of the single BSA derivative towards more basic pIs, Figure 9. The determined pI of the mAb was more basic than the pI of the derivatized BSA, and thus any complexation between these two species would generate a species having a more basic pI than the derivative itself, as observed. Indeed, the first addition of mAb resulted in a splitting of the derivative peak, Figure 9(A), and a basic shift of part of the derivative as anticipated. In Figure 9(A), peak 4 is, we believe, uncomplexed, tagged BSA and peak 3 is likely that of the Ab complexed and tagged BSA. As more Ab was added to the solution in Figure 9 (A), there was again a shift towards more basic pI of the tagged BSA, as above, and the formation of more Ab complexed species, peaks 3-5, Figure 9 (B). However, without MS data, it is not clear what peaks 3-5 truly represented, other than Ab complexes of variously derivatized BSA (none fully tagged, as yet).

Eventually, enough mAb was added that no further migration of the BSA derivative was observed, and this coincided with the appearance of an unknown species (peak 6) in the electropherogram, Figure 9 (C), which may have been uncomplexed, excess mAb. We believe that peak 4 in Figure 9 (c) is now due to all of the variously tagged BSA species being fully complexed with mAb and coeluting, even though they have different degrees of tags on BSA. The smaller peaks on the basic side (left) of the major complex peak 4 in Figure 9 (C) appeared to be related to the BSA derivative. It was unclear if these peaks arose from multiple BSA derivative species (uncomplexed), or were due to the microheterogeneity observed for the mAb and then exhibited again by the complex.

Whatever the true origin of these smaller peaks in Figure 9 (C), the thrust of Figures 9 (A-C) is that a shift in pI values was observed for the original BSA

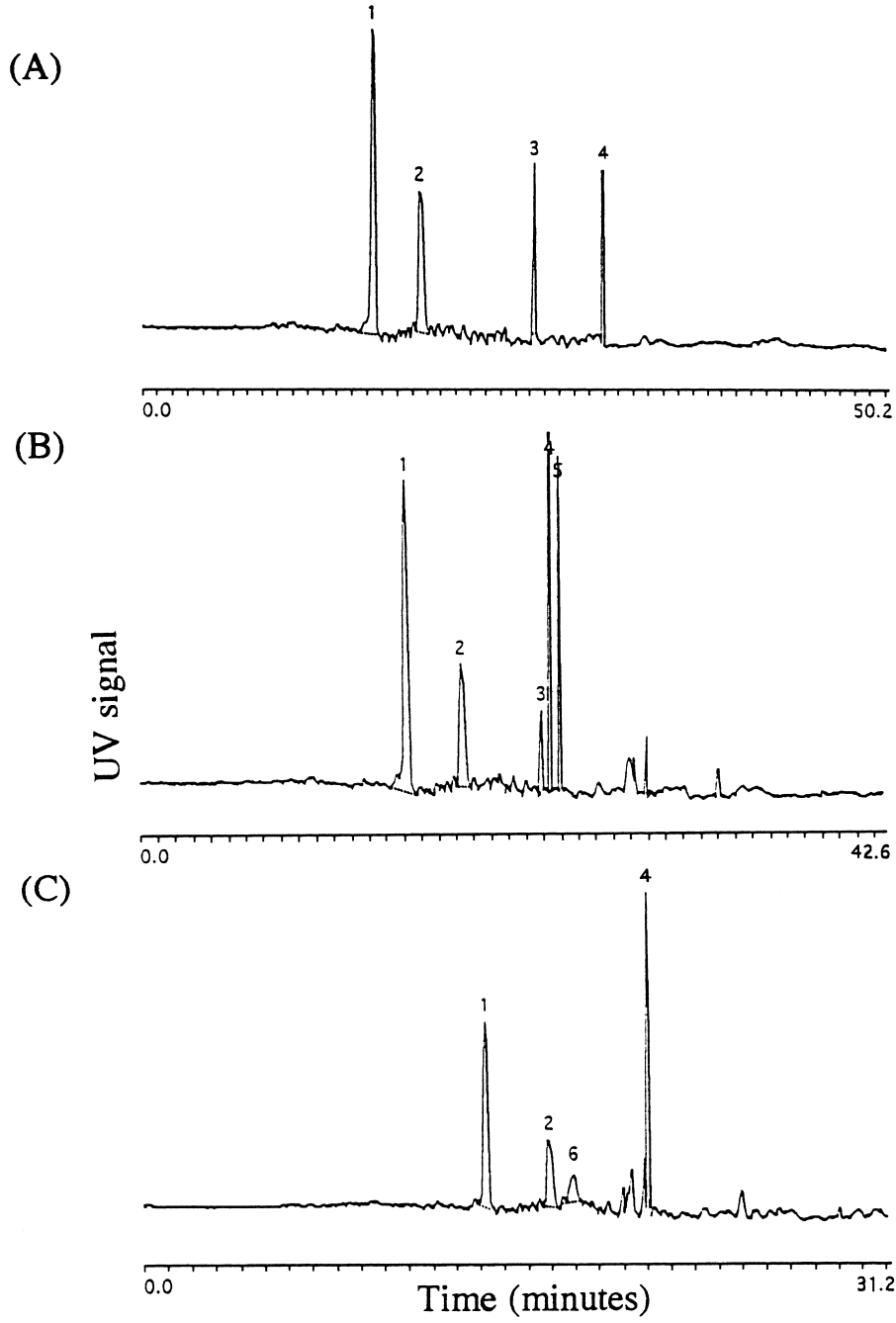

Figure 9. cIEF analysis of derivatized BSA after the inclusion of increasing amounts of anti-BSA mAb, (A-C). Peak Assignments: AQC derived (1 and 2), derivatized BSA free or complexed with mAb (3-5), and unknown (6). Conditions were the same reported in Figure 6.

derivatives as their complexes, when challenged with larger and larger amounts of mAb (most likely several isoforms of mAb present). The complex(es) possessed pI values more basic than the derivative itself and more acidic than the mAb (peak 6, Figure 9 (c)). This movement of the original BSA derivative peaks, as occurred with the native BSA (vide supra), is again taken as indicative of complexation via Ag-Ab recognition, suggesting that the tagged BSA species were indeed still Ab active. At least, some of these derivatives were Ab active, if not most of them. BSA is perhaps a difficult protein to fully tag, and we have shown that it usually formed multiple species, having different degrees of tagging (MALDI-TOFMS data, vide infra). It is, of course, not the correct species to use for affinity CE or immuno-CE, but these studies have clearly shown that the incompletely tagged BSA can be recognized by its mAb. It is not yet clear if the fully tagged BSA will also be recognized by this or other of its antibodies. Efforts are now underway to develop protocols for complete tagging of larger and larger proteins, such as BSA, which has not yet been shown 100% tagged in the current studies.[74,85]

In an effort to measure the pI of the derivatized-BSA/mAb complex, trypsin inhibitor (pI 4.6) was spiked into the sample, Figure 10. It was observed that the derivatized-BSA/mAb complex migrated on the basic side of trypsin inhibitor. Therefore, the pI of the derivatized-BSA/mAb complex (pI > 4.6) was significantly more basic than uncomplexed derivatized BSA (pI 4.29).

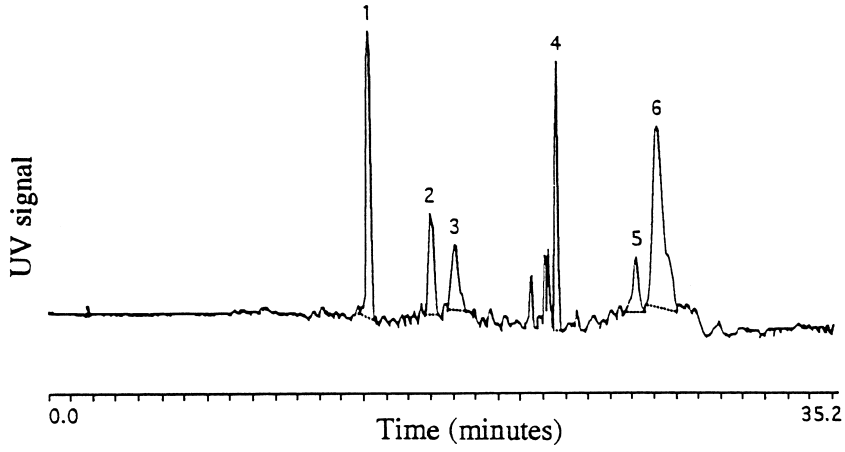

Figure 10. cIEF analysis of derivatized BSA complexed with anti-BSA mAb and spiked with trypsin inhibitor. Peak Assignments: AQC derived (1 and 2), unknown (3), major peak of derivatized BSA complexed with mAb (4), trypsin inhibitor impurity (5), and trypsin inhibitor (6). Conditions were the same as those reported in Figure 6.

In a similar fashion, a mAb against human insulin was affinity purified and used to challenge both the native and derivatized human insulins. Whereas, the native insulin was recognized by the purified Ab, the derivatized insulin was not recognized (data not shown). We believe that this was due to the presence of all three tags. Tagged insulin has been used in several studies reported in the literature as a suitable labeled, competitor for various separation based assays.[77-79] Although the authors in these studies claim to have used fully tagged insulin following HPLC purification, there seems to be some ambiguity as to whether the label was doubly or triply tagged. In any event, these authors did not provide definitive (i.e., MS) evidence as to the exact nature of the species they were using. The authors did make the point that many of the mAbs examined as potential agents in their assay failed to recognize labeled insulin after demonstrating affinity for the native species. Indeed, one published study indicated that a substantial portion of mAbs from a pool of antibodies raised against native insulin recognized the basic residues of native insulin.[80] Such an Ab, of course, would have a decreased chance of recognizing an epitope that had been derivatized at these sites, which quite likely may have been the case with the Ab used in this study. Alternative antibodies to insulin might recognize this fully tagged (3 tags) derivative species.

Ideally, one would like to be able to routinely form a homogeneously tagged, but not necessarily fully tagged, derivative that is still recognized by an Ab. Such a derivative species would then be ideally suited toward use in a competitive immunoassay format. A potential solution to the problem of derivatizing the epitope of the Ag would be to perform the derivatization reaction on an Ag-Ab complex. Derivatization of such a complex should, in theory, protect the Ag and yield a derivative product possessing an intact and unmodified epitope.[81] Of course, it is not yet clear if the reaction conditions needed for complete tagging would permit survival of the Ab-Ag complex intact.

MALDI-TOFMS Characterization of Derivative Species

The primary structures and Mr of human insulin, bovine beta-lactoglobulin B and BSA are well known.[75,82-84] As a result, the number of tags present on a derivative was determined through measurement of the Mr differences between the native and derivatized proteins and compared with the number of lysine residues and the amino terminus. This information was readily available using current MALDI-TOFMS methods and data handling routines.

Native insulin was found to have a Mr of 5,810.2 Da, which deviated from the theoretical value by approximately 2 Da, Figure 11. The species found 206 Da heavier than the insulin, Figure 11 (A), was most likely an adduct formed between insulin and sinapinic acid (Mr 208.2 Da), and was observed for several of the samples. The insulin derivative was analyzed under similar conditions and found to possess a Mr of 6,340.9 Da, Figure 11 (B). Considering that a suc-

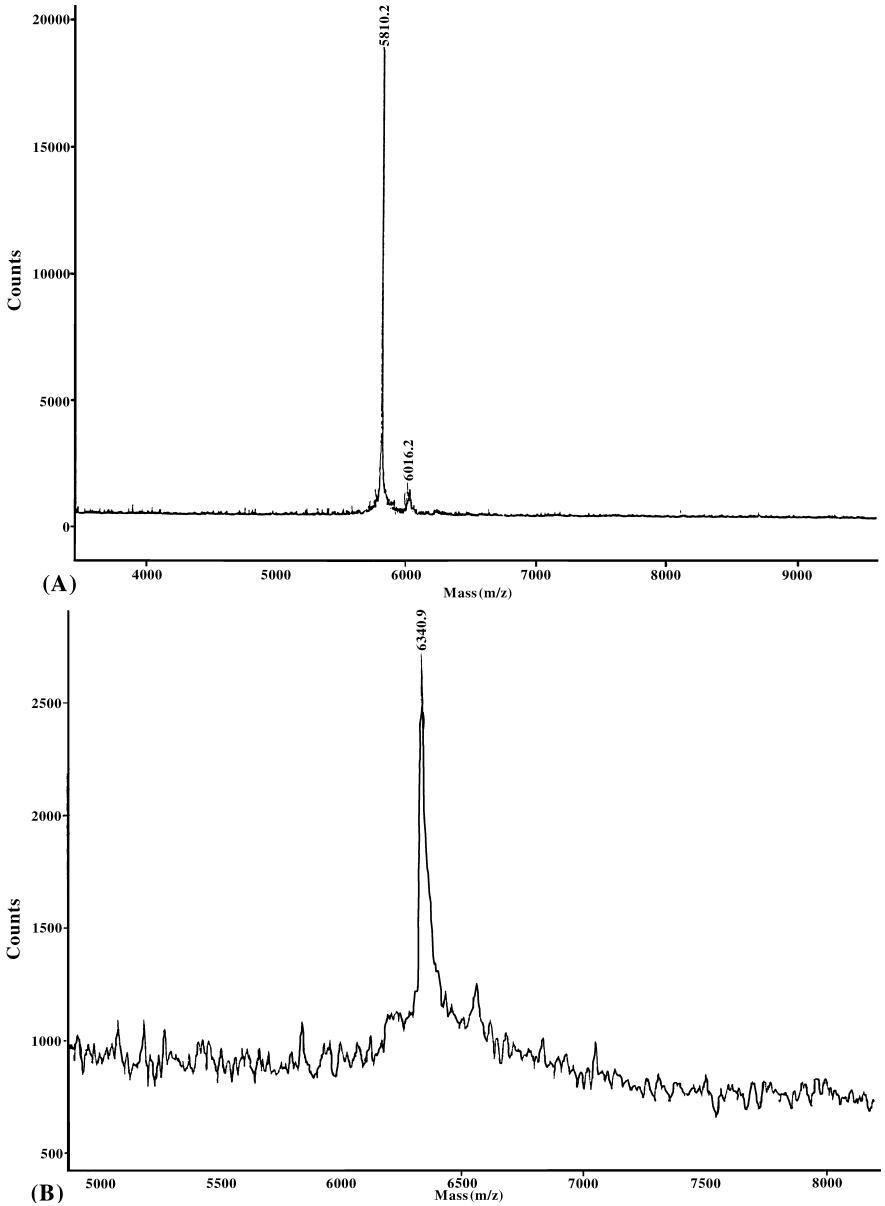

Figure 11. MALDI-TOFMS analysis of native and fully tagged human insulin. The spectra were generated on a Voyager RP Biospectrometry Workstation using an accelerating voltage of 2500 V, a grid voltage of 93.0%, a guide wire voltage of 0.175% and delayed extraction after 100 ns.

cessful incorporation of one AQC moiety into a protein or peptide coincides with an increase in the Mr of the sample by 170.16 Da, the difference in mass between the two species indicated that the insulin derivative had 3.1 tags on it, which was consistent with a completely tagged species.

Likewise, native beta-lactoglobulin B, which possesses 15 lysine residues and one amino terminus for a total of 16 potential reaction sites, was shown to have a Mr of 18,275.2 Da (18,277.29 calculated) with a matrix adduct as a shoulder centered at 18,484.7 Da. The derivative product was found to have been comprised of a minimum of four species having Mr of 20879.7, 21050.3, 21235.1, and 21404.5 Da, which corresponded with 15.3, 16.3, 17.4, and 18.4 tags, respectively. Although the theoretical number of tags for beta-lactoglobulin B was only 16, it was possible that the thermally labile AQC/tyrosine adducts (as encountered in amino acid derivatizations) may have been a bit more stable than normally expected within the protein framework and survived the heating step following derivatization. In any event, it appeared as though beta-lactoglobulin B did not possess such a complex tertiary structure as to hinder access to all derivatization sites by AQC. It was clear from the MS data that a single, fully tagged and homogeneous AQC product was not derived from beta-lactoglobulin, similar to the case for BSA.

As with the other samples of known primary amino acid sequence, BSA was also analyzed with MALDI-TOFMS. The native species, which possesses 59 lysine residues and an amino terminus, showed a predominant molecular ion at 66,341 Da. The BSA derivative produced a molecular ion peak centered at 73,917 Da, which was consistent with only 44 tags. Whereas the data for beta-lactoglobulin B suggested that the AQC reagent had access to many, if not all derivatization sites, it appeared as though the tertiary structure of BSA, which includes 19 disulfide bonds, prohibited access of AQC to significant portions of the molecule. In addition, the width of the BSA derivative peak at its base was twice that of the native species, which further suggested that the derivatized BSA, more than likely, was not comprised of a single species. Earlier work (data not shown) with a differently derivatized BSA had also clearly shown the presence of multiple products with the number of tags varying around 44.

Limitations of the Derivatization Protocol

There were several limitations encountered over the course of this study, which tempered the attractiveness of the protocol as a general method for protein derivatization and analysis via cIEF. One limitation was occasional precipitation of a derivatized sample, either during the derivatization reaction, or upon focusing. This was shown to be particularly problematic when trying to derivatize basic proteins, such as lysozyme (pI > 9.0), and was also sometimes encountered for normally well behaved proteins and derivatives. Additionally, the inclusion of urea into the run buffer was found to be necessary to prevent

precipitation during focusing for the majority of samples analyzed. Urea can complex or react with certain proteins, leading to a species with a different pI value and for its AQC derivatives.

Further difficulties arose when finding the optimal derivatization conditions for a particular protein. Each protein successfully analyzed in this study produced the best results using slightly differing conditions, whether that be determination of the molar equivalency of derivatization reagent needed, or establishing the proper volumes of borate buffer and derivatization solution. As a result, optimal derivatization conditions determined for one protein were not readily transferable to another protein. It is relevant to point out that the results presented in this study are from optimized derivatizations. Many times, a derivative analyzed under less than optimal derivatization conditions produced a multitude of peaks upon cIEF analysis. Derivatizations were not always 100% foolproof. The reaction-to-reaction reproducibility was better for the smaller proteins, as expected, and less so for the larger ones. We do not have exact statistics to demonstrate derivatization reproducibility, as yet.

Finally, the smallest protein studied was found to be fully tagged, while the same claim could not be made for larger ones. Complete tagging of all potential derivatization sites on a large protein would most likely become a possibility only after complete denaturation of such proteins, perhaps through detergent denaturation followed by disulfide reduction and alkylation, again using a high ratio of reagent to denatured/reduced protein. These studies are now in progress and shall be reported at a later date.[85]

CONCLUSIONS

Several proteins of varying Mr were shown to produce a single species, or multiple species which behaved as a single species, upon cIEF analysis, after derivatization with a large molar excess of AQC. Increased molar excesses of AQC were required as the Mr of the sample increased in order to achieve this result.

The derivative products exhibited acidic pI shifts, improved peak efficiencies, and lowered (improved) detection limits, when compared to the native species. In at least one case, a derivative product was shown to exhibit recognitive properties when challenged with an Ab, raising the possibility of using these derivatives in affinity recognition studies. At the present time, it is not easy to predict pI changes due to this type of derivatization, although a suitable equation and formula for such calculations could be eventually devised. Were it then possible to predict and observe similar pI shifts for known proteins, then the overall technique could be quite useful to confirm suspected structures for such analytes.

Problems were encountered with precipitation during derivatization and focusing. This problem was more pronounced with more basic proteins. This limitation would appear to limit the applicability of this reagent as a universal derivatization reagent for use with cIEF studies. Alternatively, perhaps less hydrophobic derivatives may overcome most or all of the current limitations described in this and other work.

Though LODs are only modestly improved in cIEF-UV, they should be substantially lowered in cIEF-LIF, a commercially viable approach now. However, the limits of derivatization would still impede truly lower LODs, overall, until ways are found to circumvent this lingering problem, not just with proteins, but with all analytes. These facts of life may relegate the described protocols to recognition studies, such as competitive immunoassays in HPLC or CE or CEC or just general immunoassays (ELISA), assuming that the fully tagged proteins are then recognized by some Ab.

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GLOSSARY

Ab	antibody
Ab-Ag	antibody-antigen complex
Abs	antibodies
Ag	antigen
AQC	6-aminoquinolyl-N-hydroxysuccinimidyl carbamate
AMQ	6-aminoquinoline
BSA	bovine serum albumin
cIEF	capillary isoelectric focusing
CZE	capillary zone electrophoresis
CE	capillary electrophoresis (HPCE)
Da	Dalton
EOF	electroendosmotic flow

FL	fluorescence
IEF	isoelectric focusing
MALDI	matrix assisted laser desorption ionization
mAb	monoclonal antibody
Mr	molecular weight (mass)
MS	mass spectrometry
pAb	polyclonal antibody
pI	isoelectric point
RPLC	reversed phase liquid chromatography
SDS	sodium dodecyl sulfate
TOFMS	time-of-flight mass spectrometry
UV	ultraviolet detection

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