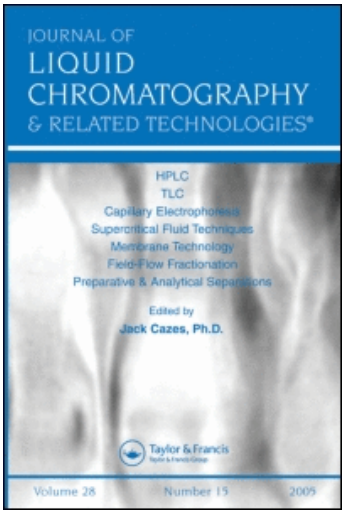

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INVESTIGATION OF CHEMICAL MODIFICATION OF PEPTIDE AMINE GROUPS FOR COMPATIBILITY WITH FLUORESCENT LABELING AND CAPILLARY ELECTROPHORESIS

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ABSTRACT

Two means for chemical modification of amine groups are investigated as to their compatibility with capillary electrophoresis (CE) and fluorescent derivatization of polypeptides. The chemical modification reagents, which are currently under examination for protein modification and subsequent peptide mapping, are selected such that there is minimal impact (permanent modification of amine groups with little or no effect on pK_a, relative size, solubility, etc.) or no impact (clean removal of the reagent after fluorescent derivatization) on the subsequent electrophoretic behavior of the fluorescently labeled peptide. Capillary electrophoresis conditions that permit the separation of the three possible amine modification products of the model peptide studied, performic acid oxidized insulin B-chain (two primary amine groups), are demonstrated. The distribution of formed products can thus be probed by CE.

To assess the characteristics of the modification strategies the conjugation reaction between fluorescein isothiocyanate (FITC) and the peptide was examined. The model peptide was then chemically modified by reductive dimethylation or reductive dihydroxypropylation (DHP) prior to fluorescent labeling. Permanent modification by dimethylation results in derivatives that feature CE properties identical to the unmodified peptide; while conditions suitable for removing the DHP blocking group, leaving the attached fluorescent label intact, are shown. These reagents hold promise for directing and controlling the fluorescent labeling of peptides resulting from chemical or enzymatic digestions.

INTRODUCTION

Capillary electrophoresis is an important bioanalytical technique that is rapidly emerging as a powerful tool for a variety of analytical situations.[1-3] Capillary electrophoresis has been successfully used for the separation of small ions,[4] peptides and proteins,[5,6] and nucleic acids.[7,8] The well-known attractive features of CE include sub-microliter sampling volumes, short analysis time, high separation efficiency, and correspondingly low mass detection limits. Such characteristics have been utilized for analysis in extreme, sample-limited micro-environments such as individual cells.[9-11]

A limitation to current CE technology is adequate detection of the migrating solutes. The most commonly employed detection scheme, UV-Vis absorbance suffers from the small injection volumes and limited optical pathlength, which lead to relatively high detection limits (10^{-6} - 10^{-7} M).

As an alternative, laser-induced fluorescence (LIF) detection provides for a substantial improvement in terms of both concentration and mass detection limits over UV detection, with mass detection limits at the zeptomole level.[12,13] While native fluorescence detection of proteins and peptides has been demonstrated,[14,15] the technique is limited to those solutes with an appropriate fluorescent moiety and requires a certain degree of experimental sophistication to isolate the emission signal. Thus, derivatization of the target analytes with fluorogenic or fluorescent reagents is commonly utilized to introduce fluorescence that can be probed with an inexpensive LIF detection design.[12,16-18] The most common derivatization reagents for proteins and peptides involve reaction of primary amine groups by a variety of chemistries.[19]

Pre-column derivatization stills appears to be the approach of choice for attaching a fluorescent moiety to the analyte.[18,20] Post-column fluorescence detection schemes for CE must conform to the strict volumetric requirements associated with CE and are limited in the range of available fluorogenic probes, although successful experimental arrangements have been described.[20] One of the major identified problems with pre-column fluorescent derivatization arises when the analyte contains more than one reactive site. In such cases a complex mixture of fluorescent products may be generated from a single analyte due to indiscriminate attachment of varying numbers of labels at several different sites.[20-23] For conjugation of amine-reactive probes to proteins or peptides, a number of possible reaction products may result from conjugation via the *n*-terminal amine and/or the ε-amino group of lysine side-chains. All the possible different derivatives[21] are probably not realized (this will depend on reaction conditions such as dye:solute concentration ratio, pH etc.) and each distinct formed product may or may not separate from other individual products. Regardless, the resulting electropherogram can be substantially complicated by the presence of multiple derivatives and sensitivity will suffer when the signal is diluted over the entire suite of formed products. Furthermore, such effects can severely limit peptide mapping applications, where reproducibility is of utmost importance.

Banks and Paquette utilized CE with UV detection to investigate solution control of the reaction of fluorescein isothiocyanate (FITC) with myoglobin,[22] and in continued studies evaluated the relative effectiveness of attaching fluorescein to a target amine via different reactive moieties: isothiocyanate, dichlorotriazine and succinimidyl ester.[23] A careful study of the kinetics of FITC conjugation with myoglobin, in which CE was used to separate the FITC derivatives, indicated pseudo-first order kinetics for myoglobin, however, the kinetic order with respect to FITC was 1.3,[22] indicating that FITC is not incorporated in the stepwise manner that would permit selective labeling but with more of a concerted reaction mechanism. While their data does not bode well for solution methods of controlled labeling, they suggested that micro-preparative CE might be beneficial for isolating the small quantities of a particular product (i.e. single label) for further studies.

One solution to multiple label incorporation is to manipulate separation conditions such that all formed products from a particular analyte elute or co-migrate as a single peak, resolved from other sample components.[24] For example, Li and co-workers[25] heated peptide derivatives of 6-aminoquinoline (6-AQ) prior to HPLC separation to realize a single peak; although it was unclear if the reaction mixture was comprised of derivatives labeled at different sites[25] or if heating the reaction mix facilitated the hydrolysis of by-products from incomplete labeling of tyrosine. Similarly, we recently reported[26] the size-

based separation of FITC-labeled proteins by capillary gel electrophoresis under denaturing conditions. Each protein migrated as a single zone, despite indiscriminate labeling with FITC. In this case the contribution to molecular weight due to incorporation of multiple labels at different sites along the protein backbone was negligible. This approach is adequate for denaturing separation conditions, however, the limited versatility of this strategy somewhat restricts applications of other types of CE that do not utilize such conditions.

Pinto, et al.[27] utilized oxidized insulin chain B as a test peptide to demonstrate the use of an Immobilon CD membrane to preconcentrate the peptide from dilute solution. Fluorescent labeling directly on the membrane was performed to attempt to limit the number of incorporated labels. While sub-attomole detection limits were reported, the product was not demonstrated to be homogeneously labeled exclusively at a single amine site. In another approach from the same group[21] a single fluorescent label was unequivocally fixed to the η-terminus of a target peptide by taking the peptide through one cycle of the Edman degradation prior to fluorescent labaling. Interfering amines groups were blocked and a fresh η-terminal was produced on the truncated peptide to which the single fluorescent was attached. However, after an Edman cycle an amino acid residue is truncated from the peptide and the ε - amine groups are permanently modified with a relatively bulky moiety. Thus, mobility and perhaps other physical features of the peptide are altered.

Chemical modification of proteins and peptides by various means has been utilized for a wide variety of different studies.[28-35] Recently, Whitesides' group has developed the concept of a protein charge ladder,[29-31] in which protein amine groups are acetylated to varying degrees with acetic anhydride.[30] The charge ladders have been used to determine effective charge in solution,[29] estimate η-terminal pK_a[30] and probe the effectiveness of different wall-coatings for protein separations.[30] Means and Feeney have reviewed many different methods for chemical modification of protein amino groups,[28,32-33] in particular reductive alkylation[33] and dimethylation[32] as a means to study active sites or introduce isotopic labels. Oates and Jorgenson further utilized reductive alkylation to improve the recovery and limit fluorescent derivatization of lysine residues for high-sensitivity amino acid composition analysis.[35]

In this paper we seek to examine the reaction of a small peptide, oxidized insulin chain B (InsB), and FITC to investigate the fundamentals of solution control of this reaction. Subsequently, chemical modification of the peptide amine groups is examined for compatibility with fluorescent labeling and CE/LIF analysis. Conditions for separation of all formed FITC products by free-solution CE are demonstrated. In this manner, the distribution of products and reaction kinetics can be easily probed. The chemical modification

approaches described here are currently being investigated[36] as a means to control the number and position of fluorescent labels attached to peptides resulting from specific chemical or enzymatic digestion of chemically modified proteins. Here we show results from initial investigations as to the characteristics of the formed derivatives and the compatibility of the strategy with peptide and protein samples.

MATERIALS AND METHODS

Materials

Insulin B-chain (performic acid oxidized), phosphoric acid, boric acid, phthalate, and citrate for buffer solutions were purchased from Sigma Chemical Co. (St. Louis, MO). Carboxyfluorescein succinimidyl ester (CFSE) was from Molecular Probes, Inc. (Eugene, OR). Fluorescein isothiocyanate, sodium cyanoborohydride, glyceraldehyde, and formaldehyde were purchased from Aldrich Chemical Co. (Milwaukee, WI) and used without further purification.

Capillary Electrophoresis

The CE system used for this work was built in-house and consisted of a Bertan (Hicksville, NY) Series 230 high voltage power supply and a variable wavelength Linear Instruments (SpectraPhysics, Piscataway, NJ) Model UV200 UV-Vis absorbance detector, operating at 200 nm. Uncoated fused silica capillaries (Polymicro Technologies, Inc., Phoenix, AZ), 50 μm internal diameter, served as the separation capillary. The total length of the capillary was 45 cm, 30 cm to the detector. The capillary was rinsed with 0.1 M KOH for 3 min between runs, followed by a 3 min buffer rinse prior to hydrodynamic injection (15 cm for 10 secs). The current was dependent on the buffer composition, but was kept below 50 μA.

FITC Conjugation

For the kinetic measurements FITC was conjugated to InsB under two sets of reaction conditions. In the first, an aliquot of a polypeptide stock solution (5 mg/mL in water) was diluted into various buffers of different pH values ranging from 6.0 to 11.0 to a final concentration of 0.125 mM. A 10-fold excess of FITC (0.01M in acetone) was added to each of the solutions, in the dark.

During the course of the reaction, aliquots (10 μL) were taken every 15 minutes to 30 minutes, and diluted with 100 mL electrophoresis running buffer for analysis by CE.

In the second set of reactions to determine the kinetic participation of FITC, 50 mM pH 10.0 borate buffer served as the reaction medium. The final concentration of polypeptide was made 0.125 mM and 5, 10, 15, 20, 25 equivalents of FITC were added to each of the polypeptide solutions. Again, 10 μL aliquots were taken every 10 or 15 minutes, diluted 1:10 in the run buffer and analyzed by CE.

Conjugation with Carboxyfluorescein Succinimidyl Ester (CFSE)

For CFSE conjugation to InsB, CFSE (0.01 M, in DMF) was made 2.5 mM and mixed with 0.25 mM InsB or DHP-InsB at pH 10 for 60 min at room temperature.

Reductive Dimethylation

Amine groups of InsB were dimethylated by reaction of formaldehyde in the presence of sodium cyanoborohydride to selectively reduce the formed Schiff's bases. Complete dimethylation (both the η-terminal and ε-amino groups) of 0.25 mM InsB was accomplished at a reaction pH of 7.4 with 2.0 mM formaldehyde and 2.2 mM sodium cyanoborohydride at 40°C for 30-40 min. Complete dimethylation of the η-terminus of InsB was carried out under the same conditions as above except that the reaction medium was buffered at pH 5.5 and the reaction was stopped after 15-20 min. For subsequent reaction steps (i.e. FITC conjugation), the modified peptide was isolated from reactants by passage through a Bio-Gel, P-2 column cast in a Pasteur pipette.

Reductive Dihydroxypropylation

Dihydroxypropylation was accomplished by selectively reducing the Schiff's bases resulting from the condensation of glyceraldehyde with the InsB amine groups. Complete dihydroxypropylation of the η-terminus was achieved by reaction of 0.5 mM InsB, pH 5.5, with 2.5 mM glyceraldehyde in the presence of 5.0 mM sodium cyanoborohydride at 40°C for 1 hour. Prior to separation, the InsB DHP-derivatives were passed through a Bio-Gel column, as above. Complete coverage of both amine groups was accomplished by reductive dihydroxypropylation at pH 7.4.

Periodate Oxidation

The DHP-modified InsB (with or without a fluorescein label) was brought to pH 7.2 and a concentration of 0.1 mM. Sodium metaperiodate was added to a concentration of 1.0 mM and the reaction mixture was incubated in the dark for 20 min at room temperature. Passage through the Bio-Gel column stopped the reaction and served to remove reagents and by-products. The efficacy of the DHP removal was confirmed with electrospray mass spectrometry, free-solution CE and reaction of re-generated amines with FITC.

RESULTS AND DISCUSSION

Fluorescent Derivatization of InsB and Identification of the Reaction Products

Since the two amine groups of InsB are expected to have significantly different pK_a's,[29] it should be possible to separate and evaluate the distribution of formed products with CE using an operating buffer whose pH is between the pK_a values of the two amines. As we are interested in the monitoring the unreacted InsB as well as FITC-conjugates, UV-Vis detection at 200 nm was utilized in these studies. The difference in amine pK_a's should allow for a degree of selectivity in the formation of products as well. Resolution of the two possible single FITC-derivatives (η-terminal FITC and ε-FITC) from the double-FITC conjugate and unreacted InsB was achieved in less than four minutes in borate buffer pH 9.5. In Figure 1 are shown three electropherograms representing the free-solution CE profiles of the FITC-InsB conjugation reaction carried out at pH 7.25, 9.5, and 10.5. The first peak at a migration time of about 1.8 min is acetone which serves as the neutral marker. The time scales for this and subsequent electropherograms have been adjusted such that the acetone peaks are aligned. When the FITC reaction is performed at low pH only one product peak appears in the electropherogram which is assigned as the η-terminal FITC conjugate (peak 2). As the pH increased to 9.5 at least two new products begin to appear. These are assigned as the ε-FITC (peak 3) and the double-FITC conjugates (peak 4). After 6-8 hrs. reaction time, at high pH (pH 10.5), complete conversion to a single peak corresponding to the double-FITC conjugate is achieved. The new peak that appears between these two is thus attributed to the ε-amine conjugate, which is consistent with the higher basicity of the ε-amino group.[29] The peaks labeled with an asterisks are impurity peaks that are present in the stock InsB samples when purchased and FITC-derivatives of the impurity which appears to contain reactive amine groups.

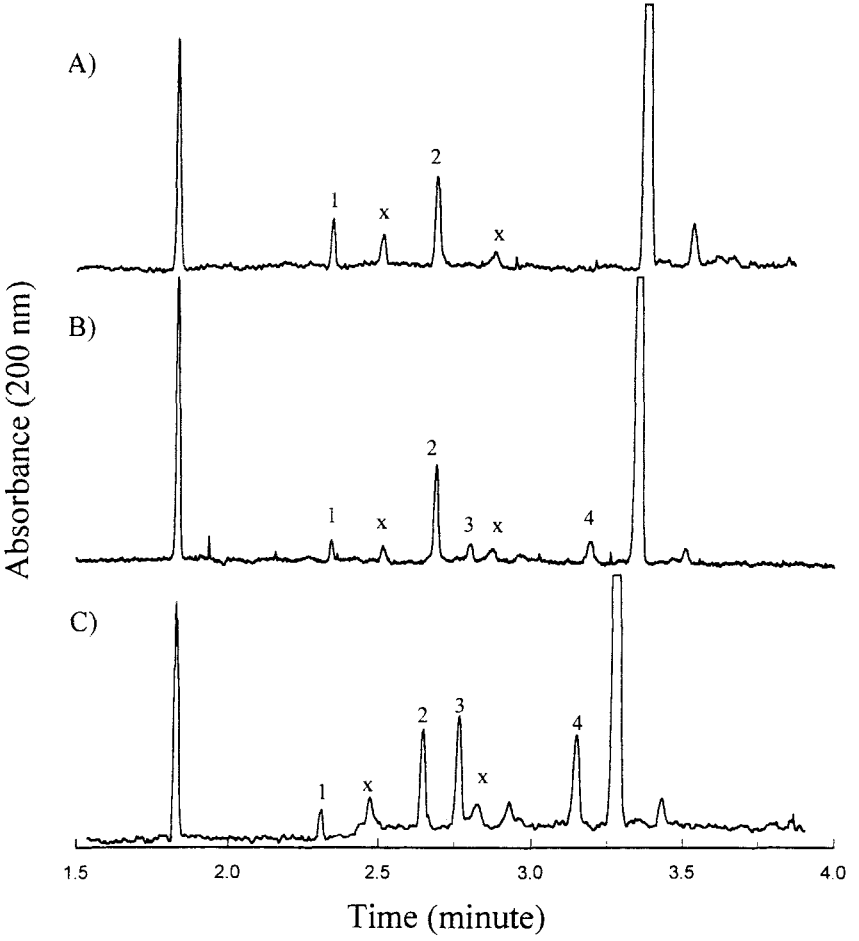


Figure 1. Free-solution electropherogram (borate buffer pH 9.5) of performic acid oxidized insulin B-chain conjugated with FITC (10-fold excess, over the peptide) in buffers of different pH. A) Reaction buffer pH 7.25; B) reaction buffer pH 9.5; C) reaction buffer pH 10.6. Peak identifications: 1) unmodified InsB; 2) *n*-terminal FITC single conjugate; 3) ε-amine FITC single conjugate; 4) double FITC conjugate. The peaks labeled with an "X" are impurity peaks (and FITC derivatives thereof) found in the commercial InsB sample. The peak at 1.8 min is acetone, while the off-scale peak at ca. 3.3 min (and those with longer migration times) are due to excess FITC and degradation products of FITC. Capillary: 50 μm inner diameter, 45 cm long (30 cm to the detector); injection by hydrodynamic pressure (15 cm, 20 sec). Detection is UV-Vis absorbance at 200 nm.

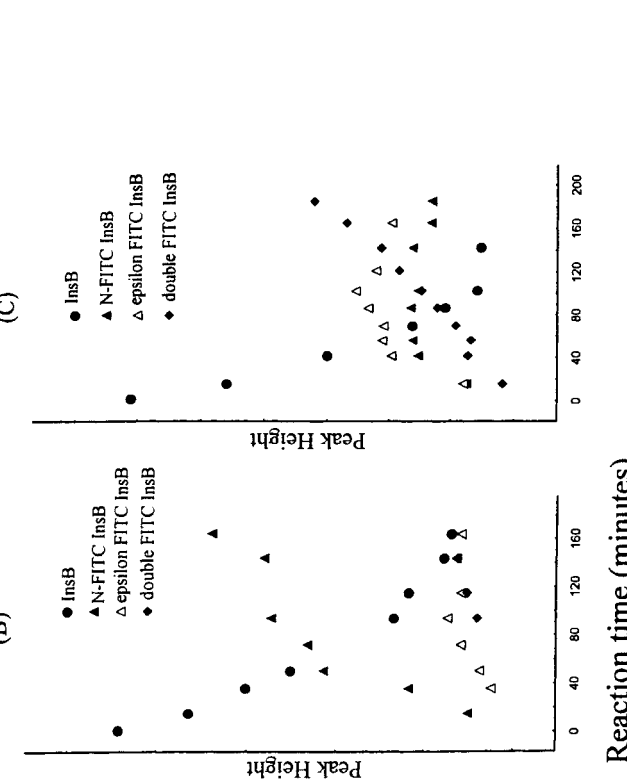


Figure 2. Observed time profiles of the consumption of InsB and production of the three possible FITC-InsB products (measured as peak height in the free-solution electropherogram) as a function of FITC reaction buffer pH, A) reaction pH 7.5, B) reaction pH 9.5, C) reaction pH 10.5.

Determination of the Reaction Rate Constants and Pk_a's of Both Amino Groups

Time profiles for the consumption of InsB during conjugation with FITC at different reaction pH values are shown in Figure 2. There is no appreciable concentration of either the ε- or double-FITC conjugate at low FITC reaction pH, whereas upon increasing the reaction pH to 9.5 new peaks begin to appear for the ε-conjugate and, after an induction period, for the double FITC derivative. Further increasing the reaction pH to 10.6, reveals a reversal in the relative reactivities of the two amine groups as the ε-amine becomes more fully activated at higher pH (ε-FITC conjugate peak height is greater than that of the η-terminal, see Figure 1C). These data can be modeled at each reaction pH by the pseudo-first order kinetic expression shown in equation 1, below.

$$\text{Rate} = -d[\text{InsB}]/dt = k_{obs}[\text{InsB}] \quad (1)$$

Where k_{obs} is the observed first order rate constant for the consumption of InsB which can be experimentally determined from the slope of a plot of ln[InsB] vs. time and is detailed by equation 2, below.

$$k_{obs} = [k^a K_a^a / (K_a^a + [H^+])] + [k^e K_a^e / (K_a^e + [H^+])] \quad (2)$$

Where k^a and k^e are the pseudo-first order rate constants (FITC in excess) for the η-terminal and ε-amine groups, respectively and K_a^a and K_a^e are the acid dissociation constants for the η-terminal and ε-amine groups, respectively.

As predicted by Equation 2, increasing the FITC reaction pH increases the observed rate constant - paralleling the activation of the ε-amino group. Thus, a plot of k_{obs} vs. pH yields a titration curve (data not shown) which can be fit to equation 2 and from which the individual pseudo-first order rate constants and pK_a values can be determined; these are $k^a = 0.0084\ min^{-1}$, $K_a^a = 1.01 \times 10^{-7}$ ($pK_a = 7.0$), $k^e = 0.011\ min^{-1}$ and $K_a^e = 1.14 \times 10^{-10}$ (pKa = 9.9). The experimentally determined K_a values agree quite well with published values for insulin,[29] although the calculated ε-amine K_a for InsB is about 1.2 pH units lower than the reported value for the η-amino group of insulin. This is possibly due to the different environment of this residue in the oxidized B-chain relative to the intact insulin species. When the pH is increased above 11.5 FITC rapidly hydrolyzes which makes complete reaction of available amine groups difficult and sets a functional upper limit to the pH of the FITC reaction. The lower limit to the reaction pH is determined by the limited FITC solubility below pH 6.0.

Isolating FITC to determine its participation on the rate equation was accomplished by holding pH constant (10.0) and varying the molar excess of FITC. Since the observed rate constant increased linearly with FITC concentration ($r^2 = 0.998$), first order participation of FITC can be inferred for this particular reaction.[37]

The observed stepwise reaction indicates the possibility of controlling the conjugation reaction such that complete conversion of analyte to a specific, single-label, product (η-terminal) can be achieved.[36] Indeed these findings should be applicable to fluorescent labeling of tryptic peptides, since cleavage by trypsin on the carboxyl side of lysine residues will yield a peptide with only an η-terminal and a single ε-amine group near the c-terminal end of the peptide, a species structurally very similar to InsB (arginine terminated tryptic peptides will contain only the η-terminal amine group). Unfortunately, with an increase in the number of amine side chains, i.e. as found with intact proteins or other large polypeptides, such control will not be possible since the probability of simultaneously attaching multiple labels (due to the wide range of pK_a values occupied by amine side chains in proteins and polypeptides) increases. In fact, specific control of the acetylation of bovine insulin (which contains only three amine groups) to a homogeneous product modified at a single amine site was not achieved using solution parameters.[29] Thus for selective conjugation using solution control of the reaction parameters the difference in pK_a's of competing amine moieties should be greater than ca. 1.5.

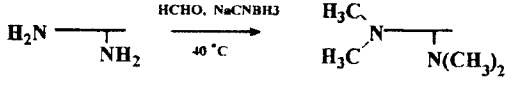
Chemical Modification of Amine Groups

For the formation of fluorescently labeled proteins and peptides the preferred product is a single-label derivative in which the label position is well-controlled.[20] Formation of the exhaustively derivatized analyte is probably not the ideal situation for subsequent separation and LIF detection.[35] This is particularly true for capillary electrophoresis where the hydrophobic nature of many fluorescent derivatives may lead to solubility and/or recovery problems.[35] Also the fluorescence signal from multiple labels attached to a single solute does not necessarily increase with the number of tags. Furthermore, exhaustive modification of amine groups may lead to a significant alteration of the physical properties (pI, activity, solubility, etc.) of the protein or peptide.

An approach we are actively pursuing to control the fluorescent label attachment to peptides for LIF-based peptide mapping involves chemical modification of interfering reactive group(s) along the protein backbone prior to digestion. The modification reactions under examination were chosen to have little, or no, effect on the peptide electrophoretic mobility (reductive

dimethylation)[36] or such that the protective group can be easily removed after fluorescent labeling regenerating the intact amine (reductive dihydroxypropylation). While Oates and Jorgenson[35] utilized dimethylation of amine groups to improve recovery in amino acid composition analysis, protective groups have not been investigated in any detail as a tool to aid in controlled derivatization reactions for subsequent analytical separation. Some reasons for this may include the extra sample preparation and cleanup steps required, and also the effect of the protective agent on the protein or peptide itself. There is no sense in generating a product with bulky protective groups so one does not have to deal with bulky fluorescent groups.

A common, straightforward, approach to protein and peptide modification is reductive alkylation with formaldehyde.[28,32-33] Reductive methylation[28,32-33,35] of amines with formaldehyde as the alkylating agent and cyanoborohydride to selectively reduce formed Schiff's bases (Equation 3), rapidly and cleanly dimethylates primary amine groups. Modification with formaldehyde does not significantly alter the pK_a of the modified amine nor add appreciable mass to even small polypeptides. That is solubility, pI and electrophoretic mobility of the native and dimethylated species should be almost identical. The dimethylated amine will, however, be inert to reaction with primary amine specific chemical reagents, in particular amine reactive fluorescent probes.[35]



(3)

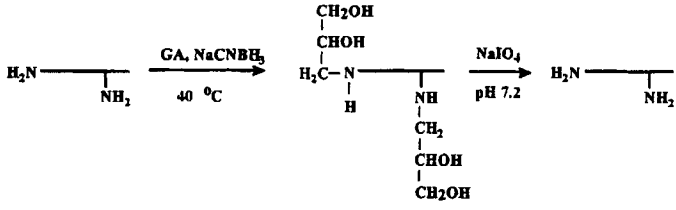
Complete dimethylation of both InsB amine groups can be achieved in 30 min at pH 7.4 and 40°C. Dimethylation of the InsB η-terminal exclusively was achieved within 15 min at pH 5.5 and 40°C. Confirmation of the extent of the reaction was provided by electrospray mass spectrometry and the reactivity of modified InsB towards amine probes in buffers of different pH. The most notable feature of formaldehyde modification is that the pK_a of the modified amine group is not altered nor is the overall molecular size of the peptide substantially increased by the modification, therefore the electrophoretic mobility of the peptide is unaffected by dimethylation.

This was confirmed by free-solution CE analysis of the formed products when the reaction was carried out at pH 5.5 and 7.4 (dimethylation of the *n*-terminal only and of both the η-terminal and ε-amine, respectively) in operating buffers whose pH values were above and below the pK_a values of the amine groups (pH 6.0, 7.5, 9.5, 10.5).

Spiking of unmodified InsB into the sample mixture did not reveal any additional peaks in any of the operating buffers above, thus indicating identical migration behavior of unmodified and dimethylated InsB. Therefore, dimethylation can be utilized to modify peptide amines without affecting electrophoretic mobility and other properties of the peptide, such as solubility, pI, etc.

Reductive dimethylation of the η-terminal will then permit rapid conjugation with FITC solely at the ε-amino group at a high pH (11.0), before hydrolysis of FITC becomes problematic. The electrophoretic properties of the dimethylated derivatives can then be evaluated relative to the unmodified peptide. In Figure 3 is shown a series of electropherograms, with the time scales adjusted to the neutral marker, corresponding to ε-FITC conjugation of the η-terminal dimethylated peptide. Experiments in which this single FITC derivative was spiked with the mixture of products formed by FITC labeling of unmodified InsB, in CE operating buffers of different pH, confirmed the co-migration of the dimethylated peptide with the ε-amino conjugate of the unmodified InsB. Thus, reductive dimethylation can be carried out rapidly and cleanly, is compatible with subsequent fluorescent derivatization, and does not alter the electrophoretic mobility of the modified peptide. Direct kinetic analysis of the ε-FITC conjugation indicated a pseudo-first order rate constant (k^e) for FITC conjugation of 0.012 min^{-1}, closely matching that indirectly determined for the InsB.

While it is clear that dimethylation can be carried out efficiently and rapidly with no apparent effect on mobility or other properties of the peptide, the protected amine is nonetheless permanently modified. Our goal is to understand and utilize reagents and develop strategies to direct fluorescent probes without permanently interfering with amine groups or other reactive moieties. To this end, we are investigating chemical modification using the alkylating agent glyceraldehyde (GA) to reversibly block amine groups. The formed dihydroxypropylated (DHP) amine (Equation 4) is susceptible to mild periodate oxidation, which frees the primary amine.[34] Thus it should be possible to attach a fluorescent reporter moiety and develop appropriate conditions to remove protective groups leaving the fluorescent label intact.



(4)

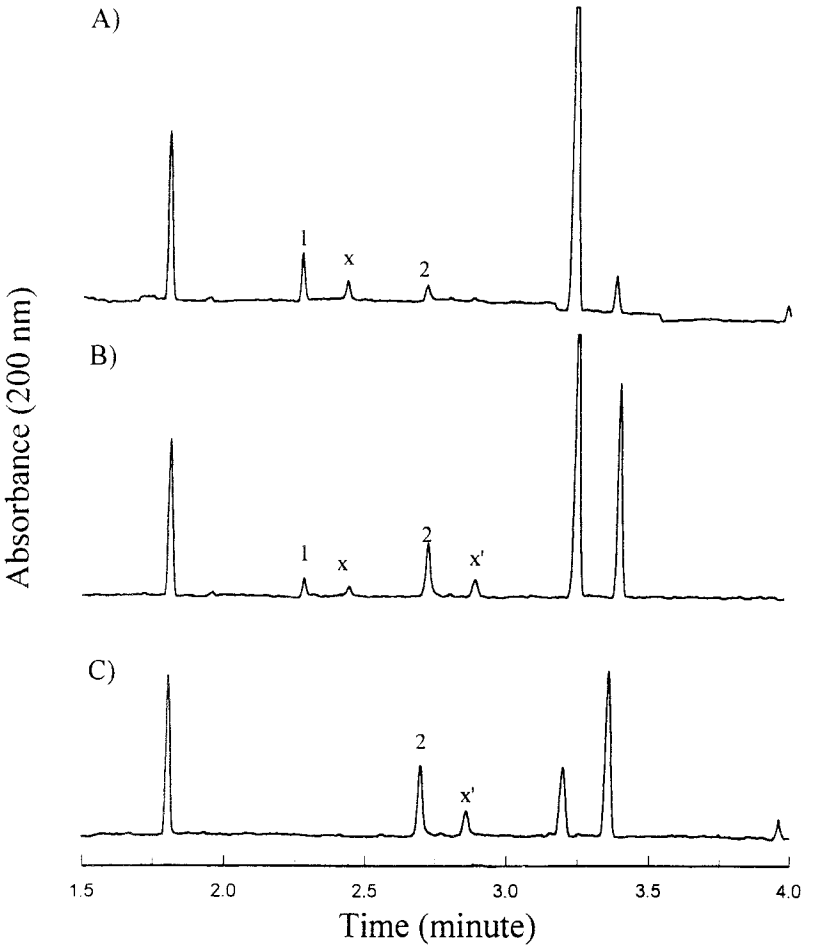


Figure 3. Electropherograms sampled during the course of the reaction of FITC (pH 11.0) with *n*-terminal N,N-dimethylated InsB. The electrophoretic conditions and peak identifications are the same as Figure 1, with the exception that the *n*-terminus of InsB was dimethylated. A) 20 min FITC reaction time; B) 140 min FITC reaction time; C) 240 min FITC reaction time. The large peaks appearing after 3 min are FITC and its degradation products.

Reaction with GA for 30-50 min, at pH 7.4 and 40°C, was sufficient for complete modification of InsB amine groups. Glyceraldehyde reaction at pH 5.5 at 40°C for 60 min resulted in modification of the η-terminus. Electrospray MS of the products confirmed attachment of a single DHP moiety to the peptide. Periodate oxidation (1 mM periodate for 20 min at pH 7.2 in the dark) of the DHP derivative regenerated the intact peptide amines. The nature of the regenerated peptide was again confirmed by electrospray MS (mass spectrum identical to unmodified peptide), by performing CE in different buffers with spiking of unmodified InsB (co-migration of oxidized product with unmodified), and reaction of the oxidized product with FITC to probe the regenerated reactivity of both amine groups. Further evidence that the oxidation conditions are sufficiently mild so as to not cause significant functional damage was detailed by Acharya et al.[34] They modified ribonuclease A (RNase A), a protein whose enzymatic activity is dependent upon several lysine groups through the active cleft, with GA to completely quench the RNase A enzymatic action. Upon periodate oxidation of DHP groups more than 90% of the enzymatic activity was regained.[34] Based on these data we believe that periodate oxidation, under the mild conditions necessary to remove the DHP group, will have minimal effect on peptide functional groups.

Once optimal conditions for removing the DHP group from the peptide were established, the stability of FITC-peptide conjugates to periodate oxidation was investigated. The peptide was η-terminal modified with glyceraldehyde followed by ε-FITC conjugation at pH 11.0. The DHP group proved to be sufficiently stable at this pH to carry out rapid fluorescent labeling with FITC. Unfortunately when the η-DHP, ε-FITC-peptide was treated with periodate to remove the η-terminal DHP group, the FITC-amine linkage was found to be extremely labile. Since the FITC-Insb conjugate was not suitable for further applications involving periodate treatment, attachment of fluorescein to the peptide via the succinimidyl ester of carboxy-fluorescein (CFSE) was investigated. Banks and Paquette recently reported on the stability and reactivity of different reactive moieties carrying the fluorescein fluorophore[23] and found that the derivatives formed with CFSE were more stable in solution and at elevated temperatures, than corresponding FITC derivatives. Indeed, CFSE is recommended by Molecular Probes, Inc. as a superior reagent for bioconjugation of FITC to proteins and peptides.[19]

The CFSE linkage to InsB was found to be completely inert to periodate under the conditions necessary to completely remove the DHP blocking group as demonstrated in Figure 4. Figure 4A depicts the electropherogram of the CFSE conjugate with unmodified InsB. As noted by Banks for the reaction of CFSE with lysine,[23] CFSE appears to show some preference for the ε-amino groups. Thus the predominate species in this electropherogram is the ε-CFSE

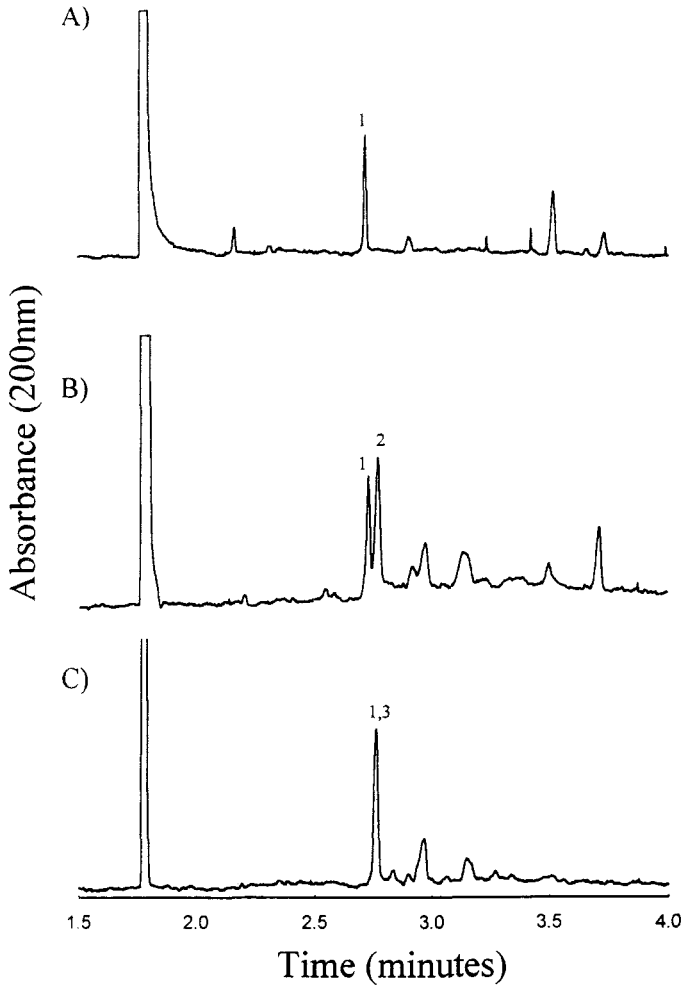


Figure 4. Free-solution electropherograms of the CSFE-InsB conjugates prior to and subsequent to modification by reductive dihydroxypropylation with glyceraldehyde and periodate oxidation. A) unmodified InsB reaction with CFSE for 60 min, pH 10; B) *n*-terminal DHP, ε-CSFE InsB derivative co-injected with mixture from A. C) Periodate oxidation to remove the *n*-DHP, co-injected with mixture from A. Peak identifications: 1)ε-CFSE InsB; 2) *n*-DHP, ε-CFSE InsB; 3) periodate oxidized *n*-DHP, εCFSE InsB. The electrophoresis conditions are the same as Figure 1.

derivative. Therefore, peak 1 in Figure 4 corresponds to the ε-CFSE derivative. The η-terminus was modified with DHP followed by conjugation with CFSE at the ε-amine to yield the η-DHP, ε-CFSE derivative. This derivative was co-injected with the ε-CFSE InsB derivative to demonstrate the small shift in migration time associated with DHP modification, Figure 4B. Treatment of the η-DHP, ε-CFSE InsB species with periodate allows removal of the DHP protective group while the fluorescent moiety remains intact. This is shown in Figure 4C, the co-injection of ε-CFSE InsB (peak 1) with periodate treated η-DHP, ε-CFSE InsB (labeled Peak 3). Both species co-migrate while the η-terminal amine of the periodate treated η-DHP, ε-CFSE InsB remains reactive to amine probes. Thus, attachment of fluorescein via the succinimidyl ester yields derivatives stable to periodate oxidation and suitable for use with the glyceraldehyde protection scheme.

CONCLUSIONS

The objective of these studies was the investigation of various aspects of chemical modification for subsequent use with fluorescent labeling and analysis by CE. Free-solution CE conditions were established to allow separation of the three possible conjugation products of FITC and the model peptide, performic acid oxidized insulin B-chain. With UV-Vis detection the consumption of InsB by FITC at different reaction pH values was monitored to allow for the determination of the pseudo-first order rate constants and pK_a values for each amine group. The fact that solution parameters (such as pH) can offer selectivity toward the η-terminal amine group of InsB, should be directly applicable to fluorescent derivatization of tryptic peptides for high sensitivity peptide mapping. When multiple amine groups whose pK_a values differ by less than approximately 1.5 are present, complete conversion of the starting material to a single-labeled product is probably not feasible. This is supported by earlier work[22,23] with a larger polypeptide, myoglobin (which contains 19 reactive amino groups), where the overall order of the FITC-conjugation reaction was determined to be greater than 2,[23] and controlled formation of a single-label derivative was not possible.[22]

In light of the above, we believe that chemical modification can play an important role in limiting the extent of fluorescent derivatization. The two chemical modification reactions described here were examined for compatibility with fluorescent derivatization and their effect on electrophoretic mobility (and presumably other physical properties) of the modified peptide. Both modification reagents described in this work are currently being utilized to direct the position of fluorescent probe attachment exclusively to the η-terminus in a peptide mapping strategy.[36] Here we show that reductive

alkylation with formaldehyde proceeds rapidly and cleanly to form a derivative whose electrophoretic mobility is unchanged as a result of amine modification. Reductive dihydroxypropylation with glyceraldehyde results in formation of blocked amine groups that can be regenerated upon mild periodate oxidation in the presence of the fluorescent label. We are currently modifying proteins with these reagents followed by chemical or enzymatic digestion to generate the peptide maps. Thus, the resulting peptides will have a single η-terminal site available for conjugation. Aspects of this approach to peptide mapping will be described in a separate report.[36] Furthermore, the succinimdyl ester of fluorescein (and possibly other fluorescent reporters) apparently reacts preferentially toward ε-amino groups. This feature has been noted for reaction with lysine,[23] we are further investigating this aspect of succinimidyl ester reactivity with other peptides and amino acids.

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REFERENCES

1. W. G. Kuhr, C. A. Monnig, Anal. Chem., **64**, 389R (1992).

2. P. D. Grossman, J. C. Colburn, eds., **Capillary Electrophoresis: Theory and Practice**, Academic Press: San Diego, CA, 1992.

3. J. W. Jorgenson, K. D. Lukacs, Science (Washington, D.C.), **222**, 266 (1983).

4. P. Jandik, W. R. Jones, A. Weston, P. R. Brown, LC/GC Internat., **5**, 20 (1991).

5. A. T. Timperman, K. Khatib, J. V. Sweedler, Anal. Chem., **67**, 139 (1995).

6. K. C. Chan, G. M. Janini, G. M. Muschik, H. J. Issaq, J. Liq. Chrom., **16**, 1877 (1993).

7. N. Best, E. Arriaga, D. Y. Chen, N. J. Dovichi, Anal. Chem., **66**, 4063 (1994).

8. K. Ueno, E. S. Yeung, Anal. Chem., **66**, 1424 (1994).

9. L. Zhang, E. S. Yeung; J. Chromatogr. A, **734**, 331 (1996).

10. S. D. Gilman, A.G. Ewing, Anal. Methods Instrum, **2**, 133 (1995).

11. R. T. Kennedy; M. D. Oates; B. R. Cooper; B. Nickerson, J. W. Jorgenson, Science (Washington, D.C.), **246**, 57 (1989).

12. Y. F. Cheng, N. J. Dovichi, Science (Washington, D.C.), **242**, 562 (1988).

13. S. Wu, N. J. Dovichi, J. Chromatogr., **480**, 141 (1989).

14. T. T. Lee, E. S. Yeung, J. Chromatogr., **595**, 319 (1992).

15. A.T. Timperman, K. Oldenburg, J. V. Sweedler, Anal. Chem., **67**, 3421 (1995).

16. S. C. Beale, J. C. Savage, D. Wiesler; S. M. Wietstock, M. Novotny, Anal. Chem., **60**, 1765 (1988).

17. P. de Montigny, J. F. Stobaugh, R. S. Givens, R. G. Carlson, K. Srinivasachar, L.A. Sternson, T. Higuchi, Anal. Chem., **59**, 1096 (1987).

18. A. J. G. Mank, E. S. Yeung, J. Chrmoatogr. A., **708**, 309 (1995).

19. R. P. Haugland, **Handbook of Fluorescent Probes and Research Chemicals**, K. D. Larison, ed., Molecular Probes Inc., Eugene OR, (1992-1994).

20. I. S. Krull, R. Strong, Z. Sosic, B-Y Cho, S. C. Beale, C-C Wang, S. Cohen, J. Chromatogr. B: Biomed. Appl., (1997), submitted.

21. J. Y. Zhao, K. C. Waldron, J. Miller, J. Z. Zhang, H. Harke, N. J. Dovichi, J. Chromatogr., **608**, 239 (1992).

22. P. R. Banks, D. M. Paquette, J. Chromatogr. A, **693**, 145 (1995).

23. P. R. Banks, D. M. Paquette, Bioconjugate Chem., **6**, 447 (1995).

24. D. M. Pnito, E. A. Arriaga, D. Craig, J. Angelova, N. Sharma, H. Ahmadzadeh, N. J. Dovichi, C. A. Boulet, Anal. Chem., **69**, 3015 (1997).

25. G. Li, J. Yu, I. S. Krull, S. Cohen, J. Liq. Chromatogr. and Rel. Tech., **18**, 3889 (1995).

26. C-C. Wang, S. C. Beale, J. Chromatogr. A, **756**, 245 (1996).

27. D. M. Pinto, E. A. Arriaga, S. Sia, Z. Li, N. J. Dovichi, Electrophoresis, **16**, 534 (1995).

28. G. E. Means, R. E. Feeney, **Chemical Modification of Proteins**, Holden-Day, Inc., San Francisco, 1971.

29. J. Gao, M. Mrksich, F. A. Gomez, G. M. Whitesides, Anal. Chem., **67**, 3093 (1995).

30. E. Cordova, J. Gao, G. M. Whitesides, Anal. Chem., **69**, 1370 (1997).

31. J. Gao, F. A. Gomez, R. Harter, G. M. Whitesides, Proc. Natl. Acad. Sci. U.S.A., 91, 12027 (1994).

32. G. E. Means, R. E. Feeney, Biochemistry, **7**, 2192 (1968).

33. G. E. Means, R. E. Feeney, Anal. Biochem., **224**, 1 (1995).

34. A. S. Acharya, B. N. Manjula, Biochemistry, **26**, 3524 (1987).

35. M. D. Oates, J. W. Jorgenson, Anal. Chem., **62**, 2056 (1990).

36. J. W. Moore, R. G. Pearson, **Kinetics and Mechanism**, Wiley and Sons, Inc., New York, 1981.

37. S. J. Sudmeier; K. Sims, S. C. Beale, J. Chromatogr. A., submitted (1997).

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