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Site-specific fluorescent derivatization and liquid chromatographic–mass spectrometric characterization of Long R^3 IGF-I for bioanalytical applications

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Abstract

Recombinant Long R^3 IGF-I was derivatized with fluorescein isothiocyanate (FITC) at a single location by careful selection of reaction conditions (i.e. pH, and FITC/protein amino group ratio). High-performance liquid chromatography (LC) and electrospray mass spectrometry (MS) were used to confirm the extent of fluorescein conjugation. The protein conjugate was isolated and subjected to cyanogen bromide (CNBr) cleavage, followed by LC–MS to determine the site of modification. The isolated species of Long R^3 IGF-I-FITC was labeled at the N-terminal Met residue. Recognition of this fluorescent analog by monoclonal anti-IGF-I was preserved, indicating its potential for immunodiagnostic applications.

Keywords: Derivatization, LC; Long R^3 IGF-I

1. Introduction

Human insulin-like growth factor I (IGF-I) is a basic polypeptide consisting of 70 amino acid residues and three disulfide bonds. IGF-I is a potent growth factor both in vivo and in vitro, with activity modulation by at least six specific binding proteins and a host of proteases [1,2]. The potent anti-apoptotic function of IGF-I is achieved via stimulation of a Type I IGF receptor which contains a tyrosine kinase domain and is linked to the ras-raf-MAPK signalling cascade [3]. Disruption of this complex regulatory system has been associated with various cancers, particularly cancers of the breast and prostate [4,5]. Development of a fluorescent IGF-I conjugate may thus be used in various bioanalytical applications such as early disease detection, and the elucidation of disease mechanisms.

The use of fluorescent probes offers a number of advantages over radioisotopes: safety, longer shelf life, inexpensive disposal. In addition, fluorescence-based high-throughput screening instruments are commercially available. Microtitre plate-based fluorescence polarization and capillary electrophoresis instrumentation with laser induced fluorescence detection are well suited to the clinical laboratory.

A variety of fluorescent derivatization approaches have been used in CE-based protein analysis. The key difficulty encountered in derivatization is the production of a single desired product [6]. The presence of numerous reactive moieties in the protein target often results in a host of derivatization prod-

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ucts, complex electropherograms, poor peak quantitation and resolution [7–9]. For example, targeting free amino functional groups may result in a complex mixture of reaction products. It has been demonstrated that for a molecule with n primary amino groups, 2^n-1 reaction products can be formed [9].

Two opposite strategies have been used in an effort to address this issue. The first involves a ''homogeneous labeling'' approach whereby one attempts to produce a fully tagged product by using a large molar excess of derivatizing reagent relative to protein in conjunction with relatively harsh conditions (i.e. elevated temperature, high pH, longer reaction times, in the presence of denaturing agents) [10]. This has been successfully applied in the derivatization of large proteins such as bovine serum albumin, ovalbumin, α-chymotrypsinogen A, and insulin using 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate [11,12]. Potential problems associated with this approach include the reduced solubility of the multiply labeled target, quenching, and a reduction or loss of immunorecognition.

A second approach involves the use of limited, stoichiometric amounts of labeling reagent, pH values which exploit pK_a differences between various functional groups, and milder reaction conditions [13]. This method is more conducive to the production of biologically active protein conjugates. Selectivity towards the N-terminus of an analyte can be achieved by careful selection of reaction pH and the ratio of probe to protein. Human insulin has been selectively labeled at its N-terminus at pH 7.0, and FITC/insulin ratio of 3:1 [13]. Since insulin is structurally similar to IGF-I, and contains a comparable number of derivatizable sites, it was deemed possible to achieve similar labeling selectivity with IGF-I and FITC.

Fluorescein has found widespread use in bioanalytical applications as a fluorescent probe due in part to its relatively high molar absorptivity and quantum yield, water solubility, and efficient excitation by relatively inexpensive argon ion lasers [13]. Use of the amine reactive dye, FITC, to produce fluorescein thiocarbamylated conjugates is a common labeling strategy and was thus selected for this study.

In developing a fluorescent IGF-I conjugate, it was desirable to elucidate the most cost-effective process. Recently, an efficient expression system which produces a potent, biologically active IGF-I fusion peptide analogue in *Escherichia coli* has been described [14]. The 9110 Da analogue is referred to as Long R^3 IGF-I and is composed of the entire IGF-I sequence (Glu-3 replaced by Arg), and an N-terminal 13 amino acid residue linker. This paper describes for the first time: (1) conjugation of Long R^3 IGF-I with FITC; (2) LC purification of the fluorescein-labeled analogue; (3) structural characterization of the labeled analogue by chemical cleavage and LC–MS; (4) demonstration of the binding of monoclonal anti-IGF-I to this fluorescein conjugate for use in immunodiagnostic applications.

2. Experimental

2.1. Chemicals

Nanopure water was obtained from a Sybron/Barnstead, 18 MΩ-cm water purification system (Sybron/Barnstead, Boston, MA, USA). HPLC-grade acetonitrile was purchased from Fisher (Fair Lawn, NJ, USA), trifluoroacetic acid (TFA) and anhydrous dimethylformamide were obtained from Aldrich (Milwaukee, WI, USA). Boric acid, sodium tetraborate, monobasic and dibasic sodium phosphate, cyanogen bromide, ethylenediaminetetraacetic acid (EDTA), FITC (Isomer I), and Long R^3 IGF-I were obtained from Sigma (St Louis, MO, USA). Monoclonal anti-IGF-I (Clone 82-9A) was purchased from Oncogene Research Products (Cambridge, MA, USA).

2.2. Instrumental procedures

All HPLC separations were carried out with Vydac C_{18} (5-μm d_p) columns: 250×4.6 mm and 250×1.0 mm for conjugate purification and CNBr digest analysis, respectively. A HP 1090 Series II/M HPLC system with binary DR5 solvent delivery and HP 1050 series multiple wavelength diode-array UV–visible absorbance detector (Hewlett-Packard, Mis-

sissauga, ON, Canada) was used for all LC separations. The following gradient was used in all HPLC determinations: 0 −15 min (100% to 15% A), where A was 0.05% TFA in nanopure water and B was 100% acetonitrile/0.05% TFA. Flow rates of 1.00 and 0.040 ml/min were used for conjugate purification and CNBr digest analysis, respectively.

Samples were injected manually using a Rheodyne 7125 manual valve injector (Hewlett-Packard) and a 100-μl glass, gastight syringe (Hamilton, Reno, NV, USA). For Long R^3 IGF-I conjugate analysis and purification, a 100-μl stainless steel injection loop was used, and for CNBr digest analysis, a 20-μl injection loop was used. Peaks were monitored by UV absorbance at 215 nm.

Electrospray mass spectra were obtained using a Finnigan SSQ 7000 single-quadrupole mass spectrometer (Thermoquest, Mississauga, ON, Canada). Long R^3 IGF-I conjugate analysis was carried out on-line by splitting the LC eluent into a 50 μm I.D. bare-fused-silica capillary, reducing flow rates to approximately 40 μl/min. Analyses of CNBr digests were carried out on-line without flow splitting. Positive ion detection was employed in all cases, scanning over an m/z range of 300–2500 at a scan rate of 2 s. The following conditions were used in all analyses: 4.5 kV spray voltage, 210 °C capillary temperature, 275 kPa sheath and 103 kPa auxiliary gas pressures (prepurified N_2), electron multiplier set at 1200 V, centroid mode.

Monoclonal anti-IGF-I recognition was analyzed using an ATI-Unicam (Mississauga, ON, Canada) Model Crystal 310 capillary electrophoresis system with a 48-position peltier-cooled autosampler. Laser-induced fluorescence of the protein conjugate was achieved using a detection system described previously [15]. Briefly, the output beam of a Uniphase (San Jose, CA, USA) air-cooled, light-controlled 4 mW Ar^+ laser (Model 2012-4SLL) was focused onto the window of a 50-μm I.D./186-μm O.D. 60 cm long (45 cm to detector) bare-fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA). Fluorescence was collected at 90° to the excitation beam, spatially filtered using a 800-μm pinhole, and spectrally filtered with two optical filters (515EFLP and 535DF35) (Omega Optical, Brattleboro, VT, USA). Fluorescence was detected using a Hamamatsu (Bridgewater, NJ, USA) Model R1477 photomultiplier tube set at −800 V.

2.3. Preparation of Long R^3 IGF-I–fluorescein conjugate

A 100 m*M* FITC stock solution was prepared by dissolving 1 mg FITC in 25.6 μl of anhydrous DMF. A 10 m*M* substock was made by performing a 10× dilution of this stock, and was used for all derivatizations. Conjugations were initiated by adding 1.76 μl of 10 m*M* FITC to a solution containing 204.8 μl of 100 m*M* phosphate (pH 7.00)/400 μ*M* EDTA and 40 μl of 1 g/l Long R^3 IGF-I. Upon addition of FITC, tubes were protected from light, gently vortexed for 3 h, then allowed to react overnight at room temperature. Single-labeled Long R^3 IGF-I was purified by HPLC, lyophilized, and subjected to CNBr digestion.

2.4. CNBr cleavage

CNBr cleavage reactions were carried out on unlabeled and labeled Long R^3 IGF-I as previously described [16]. Briefly, a small crystal of CNBr was added to a solution containing 10 μl of 1 g/l unlabeled protein and 190 μl of 0.12 N HCl. In the case of labeled protein, the lyophilized product (ca. 10 μg) was resuspended in 200 μl of 0.12 N HCl, with the subsequent addition of a small crystal of CNBr. All cleavage reactions were carried out in an oven at 38 °C, protected from light. Reactions were stopped after 3 h by quenching with 500 μl of nanopure water. Samples were lyophilized and resuspended in 50 μl of nanopure water prior to LC–MS analysis.

2.5. Immunorecognition of labeled Long R^3 IGF-I

Purified Long R^3 IGF-I conjugate (e.g. 10 μg) was resuspended in 100 μl of nanopure water and 20 μl transferred to a glass CE sample vial. To this vial was added either 1 μl of 100 μg/ml monoclonal anti-IGF-I (prepared in sterile phosphate-buffered saline, pH 7.4) or sterile phosphate-buffered saline (control). Vial contents were mixed and analyzed immediately on the Crystal CE system. Injections

were carried out at 50 mbar for 0.1 min, followed by a 20 kV separation using a 100 m*M* borate (pH 8.50) run buffer.

3. Results and discussion

3.1. FITC labeling of Long R^3 IGF-I

Derivatization of Long R^3 IGF-I with FITC can produce 15 different conjugate species. This is attributed to the presence of one N-terminal α-amino and three Lys ε-amino groups. In the development of a bioanalytical fluorescent conjugate, it is highly desirable to preserve immunorecognition and/or biological activity. Functional epitope mapping of IGF-I by anti-IGF-I monoclonal antibodies, and the production of novel recombinant fusion protein analogues of IGF-I have indicated that certain residues or regions of residues are important for binding of the IGF-I receptor [14,17]. These studies have shown that the N-terminus is not involved in receptor binding. The selective introduction of fluorescein on the α-amino group of Long R^3 IGF-I is thus expected to have minimal impact on bioactivity.

In order to target the N-terminus of Long R^3 IGF-I, a variety of FITC to protein amino group ratios, and reaction pH values were employed. A 10-fold molar excess of dye at pH 7.0 resulted in the production of multiple labeled species which were poorly resolved by HPLC (data not shown). Performing the derivatization at pH values of 8.5 and above also proved undesirable, with the dual disadvantage of multiple conjugates and increased FITC hydrolysis (data not shown). Production of single-labeled Long R^3 IGF-I appeared to be achieved by simultaneously lowering the FITC to protein amino group ratio and decreasing the reaction pH to 7.0. Performing the reaction at pH 7.0 seemed to best exploit pK_a differences between the α- and ε-amino groups, whereas lowering the FITC to amino group ratio served to limit the amount of available dye. Repeat derivatizations carried out under these conditions produced similar reaction product profiles and relative peak heights. Assuming similar absorbance characteristics at 215 nm between unlabeled, singly-labeled, and multiply-labeled analyte, yield of the

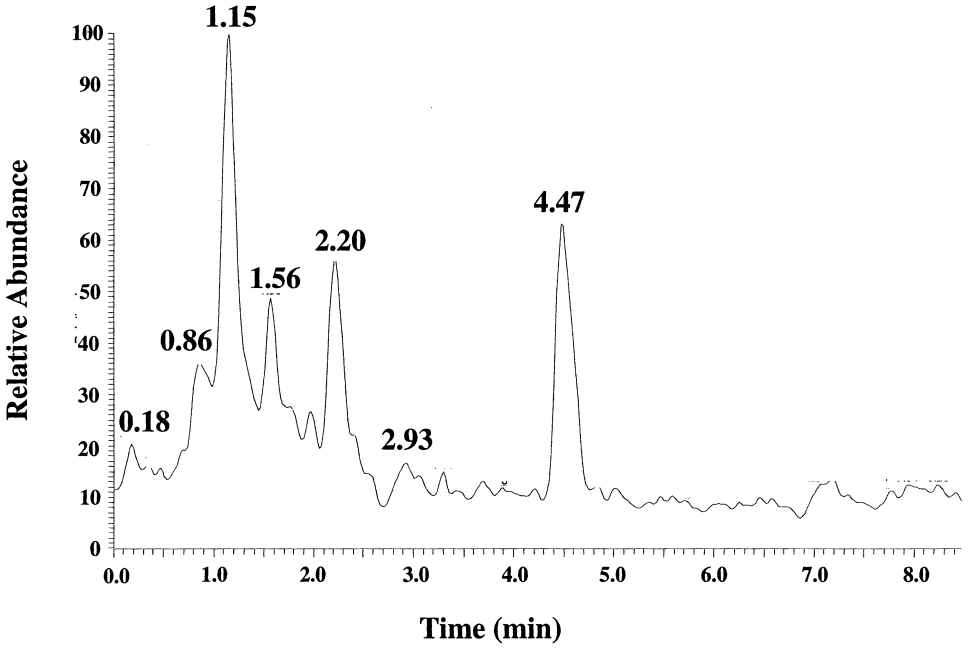

Fig. 1. TIC measurement of LC separated Long R^3 IGF-I/FITC reaction products.

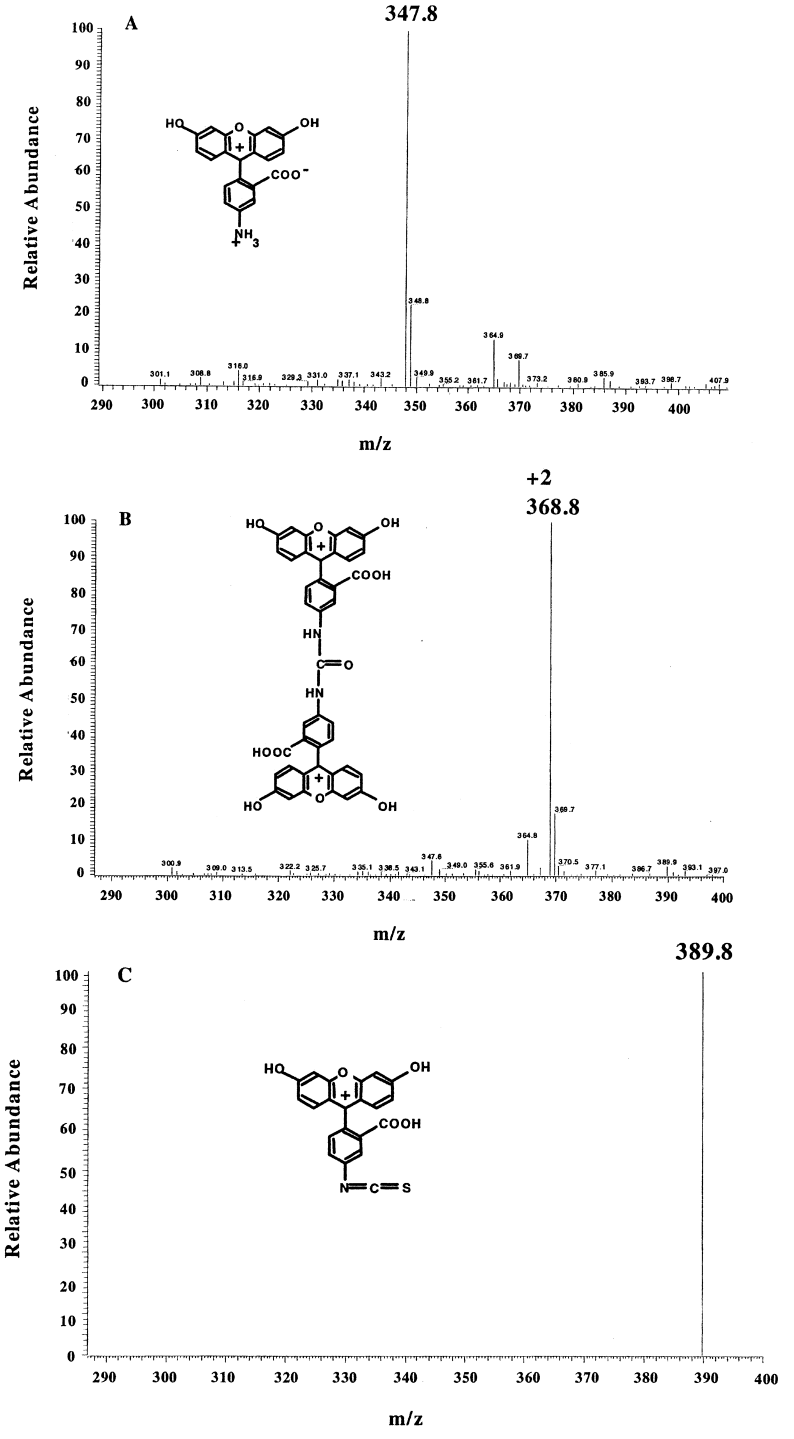

Fig. 2. Extracted ion mass spectra for FITC and its hydrolysis products: (A) 0.20–0.33 min of TIC measurement corresponding to fluorescein amine; (B) 2.82–3.03 min of TIC measurement corresponding to difluorescein thiourea; (C) 4.37–4.64 min of TIC measurement corresponding to unreacted FITC.

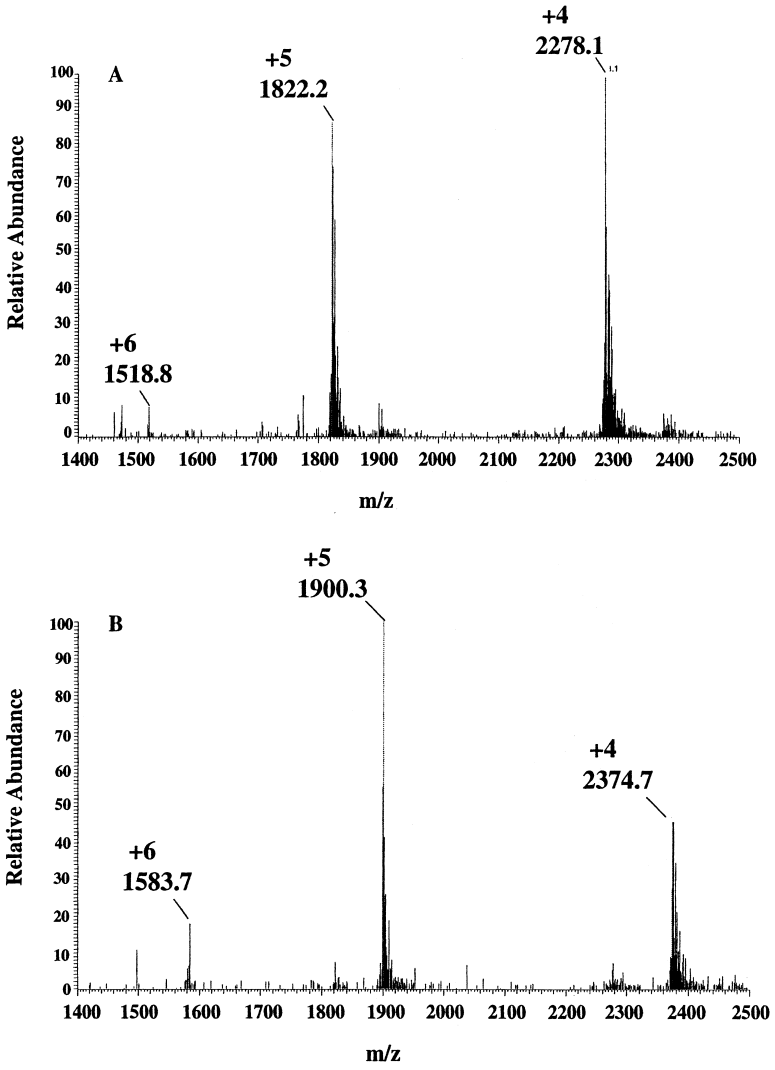

Fig. 3. Convolved mass spectra of: (A) Peak at 0.99–1.38 min of LC separated Long R^3 IGF-I/FITC reaction TIC measurement. Calculated mass of 9107.7±0.4 mass units (mu). Corresponds to unreacted Long R^3 IGF-I. (B) Peak at 2.06–2.31 min of TIC measurement for the LC separated Long R^3 IGF-I/FITC reaction. Calculated mass of 9496.4±1.0 mu. Corresponds to single-labeled Long R^3 IGF-I.

mono-labeled product was approximately 30%. The yield of multiply-labeled protein was approximately 16%. It was estimated that 54% of Long R^3 IGF-I remained unmodified.

3.2. LC–MS characterization of single-labeled Long R^3 IGF-I

The products of the 1:1 FITC/Long R^3 IGF-I conjugation reaction (pH 7.0) were analyzed by LC–MS. The reconstituted chromatogram from the total ion current (TIC) measurement indicated the presence of multiple peaks (Fig. 1). Unreacted FITC and its hydrolysis products, fluorescein amine and difluorescein thiourea, were identified from extracted ion mass spectra (Fig. 2). It is necessary to purify labeled Long R^3 IGF-I from these species since they are known bioassay interferents, binding non-specifically to various biofluid proteins. In producing a single-labeled Long R^3 IGF-I competitive immuno-

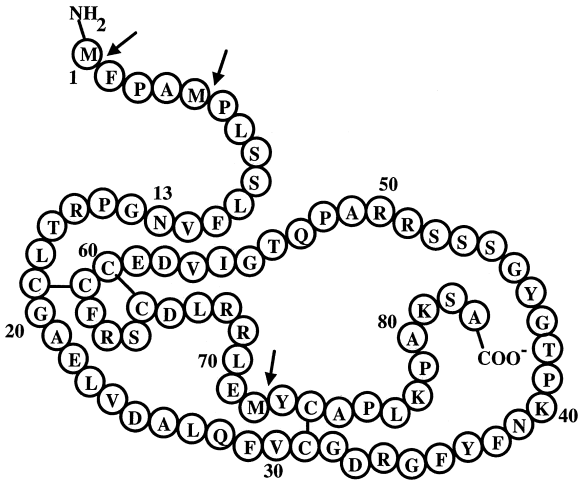

Fig. 4. Amino acid sequence of Long R^3 IGF-I. Disulfide bonds for correctly folded Long R^3 IGF-I are shown (—). Arrows indicate CNBr cleavage sites.

assay reagent, it is equally important to adequately remove any remaining unlabeled protein from its labeled counterpart. Failure to do so would result in an over-estimation of the amount of IGF-I present in a particular biofluid. The peaks corresponding to unlabeled and single-labeled Long R^3 IGF-I were identified by LC–MS analysis (Fig. 3). The LC conditions established in this study thus provide for the rapid separation of labeled Long R^3 IGF-I from unreacted FITC, various FITC hydrolysis products, as well as unlabeled protein. Additional peaks were observed at 0.86 and 1.56 min of the TIC measurement (Fig. 1) with masses of 9125 and 9132 Da, respectively. Whereas the 9125 Da species is believed to correspond to singly oxidized Long R^3 IGF-I, the 9132 Da peak could not be identified.

After initial LC–MS characterization of the Long R^3 IGF-I/FITC derivatization reaction, it was necessary to determine the modification site in LC purified single-labeled protein.

3.3. Structural characterization of single-labeled Long R^3 IGF-I by chemical cleavage and LC–MS

Cyanogen bromide selectively reacts with the sulfur of the thioether side chain of methionine, resulting in cleavage of the methionyl peptide bond. The fragments produced by the action of CNBr

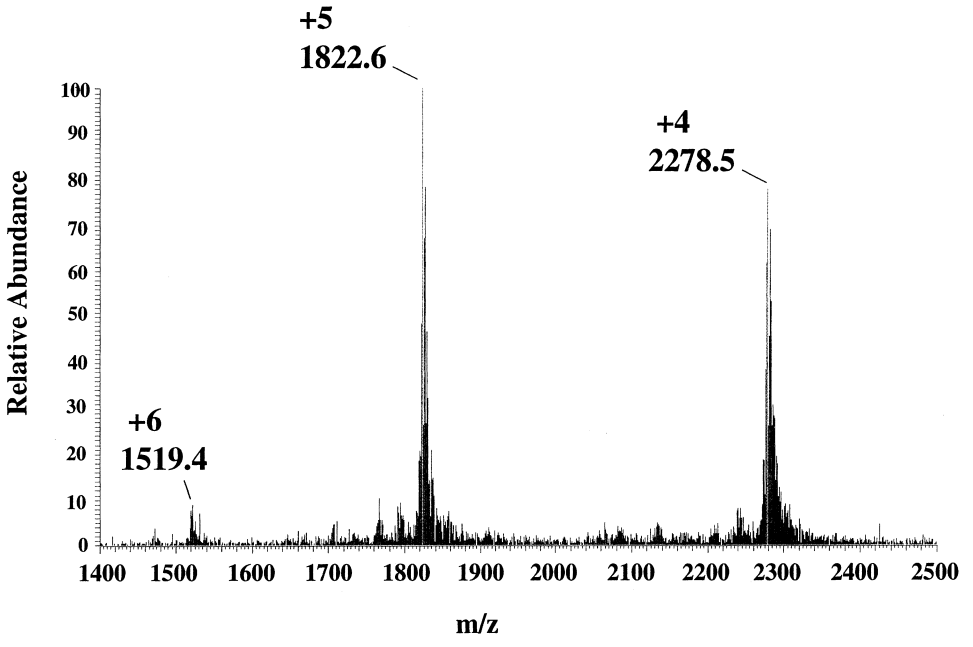

Fig. 5. Unlabeled Long R^3 IGF-I (No CNBr). Convolved mass spectrum of peak at 5.00–6.09 min of TIC measurement. Calculated mass of 9110±0.9 mu.

contain C-terminal homoserine or its lactone except for the C-terminal peptide of the protein [18]. This chemical cleavage reagent is well-suited to LC–MS analysis of single-labeled Long R^3 IGF-I. Complete CNBr cleavage of Long R^3 IGF-I should produce only three fragments (Fig. 4) [19]. Furthermore, since the first residue at the N-terminus is Met, conjugation of the α-amino group is expected to produce a fragment consisting of fluoresceinated homoserine lactone. Determining the site of modification could thus be achieved by LC–MS using a single-quadrupole instrument.

In order to characterize the CNBr cleavage reaction, unlabeled protein was incubated in the presence and absence of CNBr and subsequently analyzed by LC–MS. As expected, Long R^3 IGF-I remained intact in the absence of CNBr (Fig. 5). In the presence of cleavage reagent, protein was digested into three fragments (Met^1, residues 2–5, and residues 6–83). Both of the larger fragments were detected with masses of 417 and 8533 Da, respectively (Fig. 6). It was thus determined that a 3-h cleavage reaction at 38 °C was sufficient to completely cleave the protein.

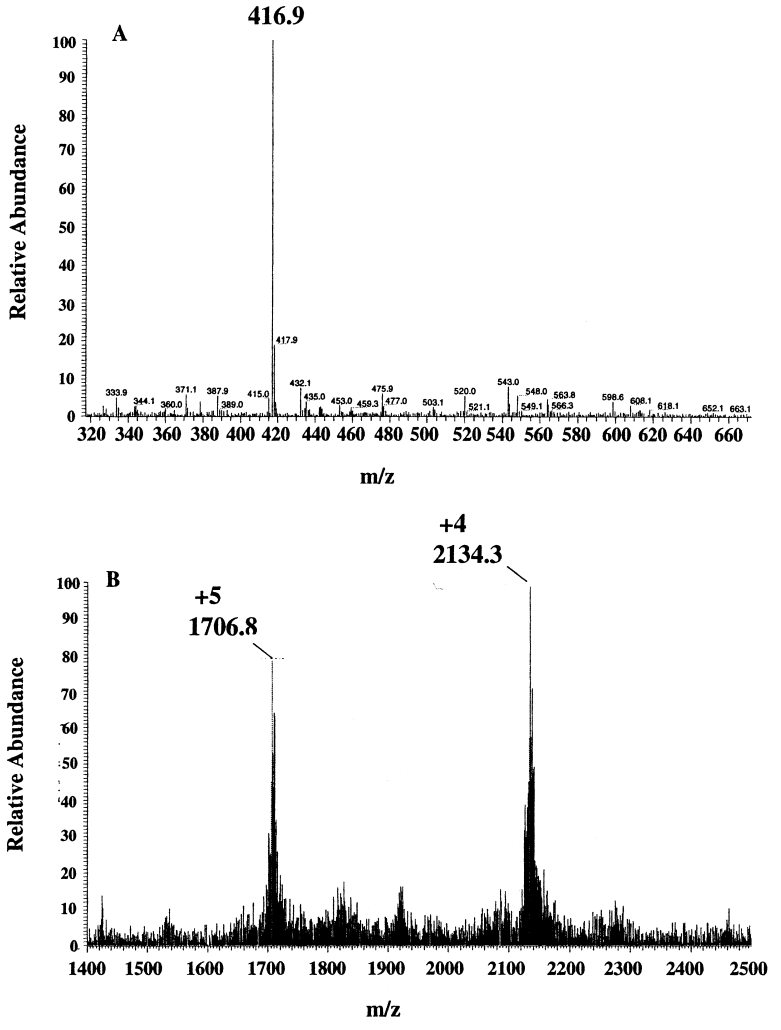

Fig. 6. Unlabeled Long R^3 IGF-I CNBr digestion. (A) Peak at 2.81–3.73 min of TIC measurement. Fragment corresponding to residues 2–5. (B) Convolved mass spectrum of peak at 4.16–5.78 min of TIC measurement. Calculated mass of 8533±2.0 mu, corresponding to residues 6–83.

Purified single-labeled Long R^3 IGF-I was incubated in the presence and absence of CNBr to determine the location of the fluorescein label. Contrary to the unlabeled protein control described above, in the absence of cleavage reagent, the single-labeled protein was cleaved. A fragment of 8977 Da, corresponding to residues 2–83 of Long R^3 IGF-I was observed (Fig. 7). It is postulated that this was attained via an Edman reaction. In this instance, FITC rather than phenylisothiocyanate was previously coupled to the N-terminal Met. Subsequent cleavage (acidic conditions, 38 °C) to release the original N-terminal amino acid as fluoresceinated Met (loss of 520 Da) would produce an 8977 Da fragment. The fragment corresponding to fluoresceinated Met was however not detected. This may have been due to loss on the C_{18} column and/or poor ionization.

CNBr cleavage of single-labeled Long R^3 IGF-I resulted in the formation of three fragments. Two fragments corresponding to residues 2–5 and 6–83, were identified by LC–MS (Fig. 8). These fragments match those previously observed for cleavage of unlabeled protein, indicating the absence of a fluorescein label on residues 2–83. Further evidence of N-terminus labeling (i.e. residue 1) resided in the third fragment, with m/z of 474.8. This fragment can be attributed to the 2+ charged state of fluoresceinated MFPAM (residues 1–5) whereby Met^1 has been oxidized to methionine *S*-oxide and is labeled with fluorescein. It should be noted that CNBr will not cleave at oxidized Met residues. Due to the absence of side-chain primary amines in residues 1–5, the only possible location of fluorescein in this fragment is the N-terminus. The above evidence supports selective modification of the N-terminus of Long R^3 IGF-I whereby an expected mass of 9496 Da was obtained (Fig. 3).

3.4. Monoclonal anti-IGF-I binding to single-labeled Long R^3 IGF-I

Following LC–MS characterization of the site of modification, it was necessary to determine if this bioanalytical reagent could be recognized by an anti-IGF-I antibody. Purified single-labeled Long R^3 IGF-I was incubated with monoclonal anti-IGF-I and binding analyzed by capillary electrophoresis with laser-induced fluorescence (CE–LIF) (Fig. 9). Addition of antibody resulted in a substantial decrease in the fluorescence intensity of free, labeled Long R^3

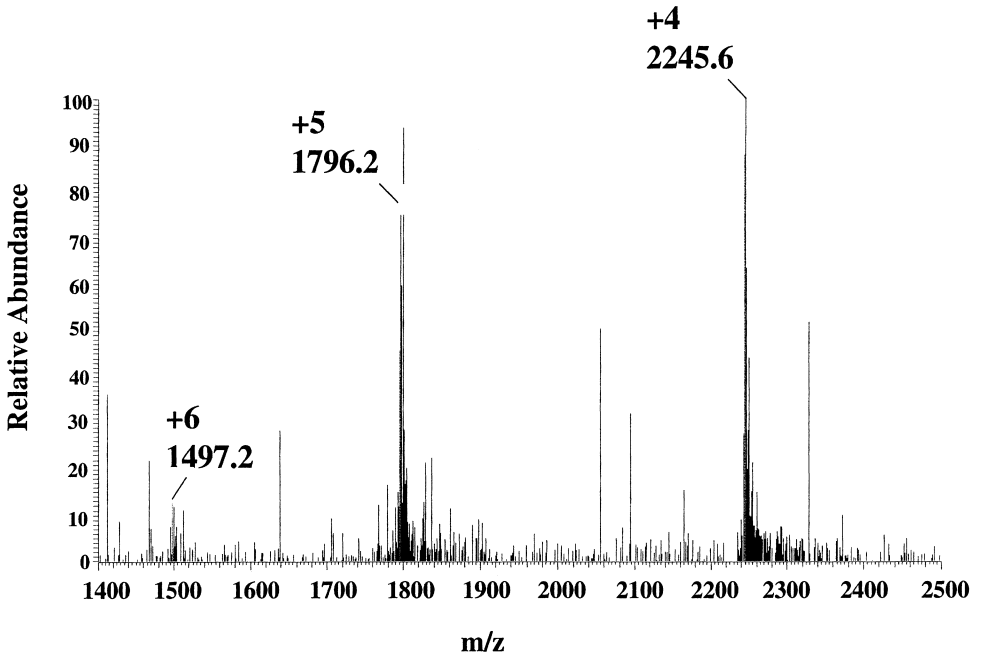

Fig. 7. Labeled Long R^3 IGF-I (No CNBr). Convolved mass spectrum of peak at 5.79–6.72 min of TIC measurement. Calculated mass of 8977±0.7 mu, corresponding to residues 2–83.

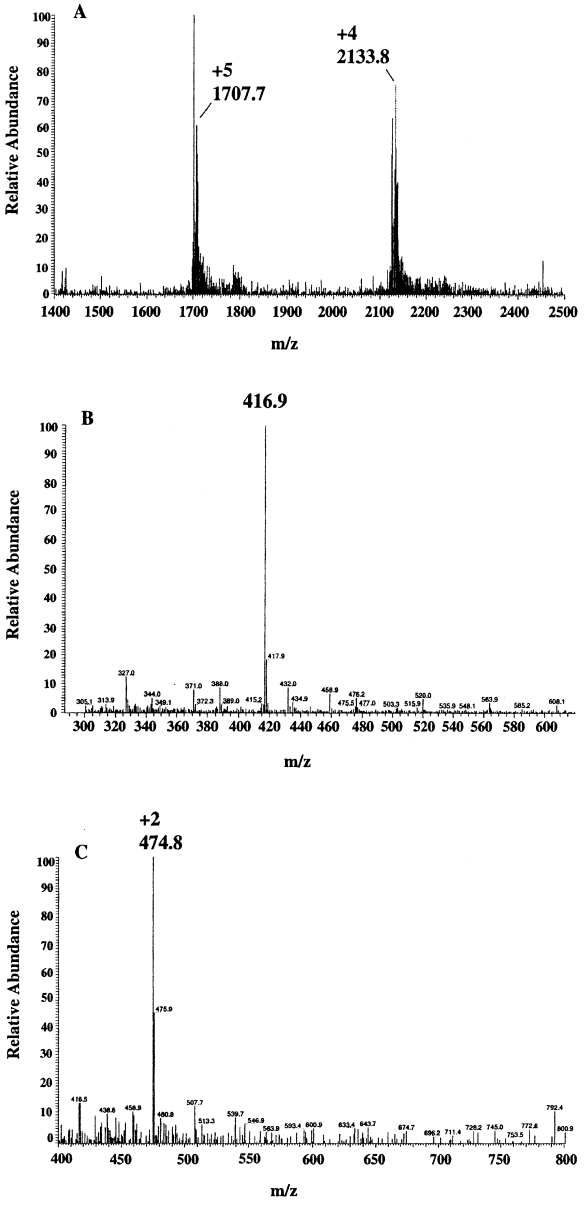

Fig. 8. Single-labeled Long R^3 IGF-I CNBr digestion. (A) Convolved mass spectrum of peak at 5.33–6.42 min of TIC measurement. Calculated mass of 8532.5±1.0 mu, corresponding to residues 6–83. (B) Peak at 3.6–4.7 min of TIC measurement, corresponding to residues 2–5. (C) Peak at 5.69–5.99 min of TIC measurement consisting of a 2+ charged state of fluoresceinated MFPAM (residues 1–5) whereby Met^1 has been oxidized to methionine *S*-oxide and is labeled with fluorescein.

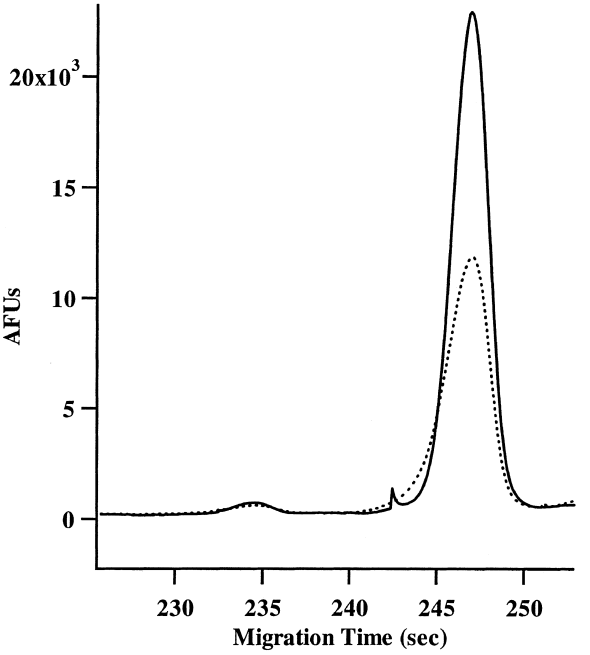

Fig. 9. CE–LIF electropherogram for the titration of purified single-labeled Long R^3 IGF-I with monoclonal anti-IGF-I. Solid line denotes labeled protein in the absence of antibody. Dashed line represents labeled protein after the addition of antibody. Arbitrary fluorescence units (AFUs) are on the *y*-axis.

IGF-I. Elimination of the labeled Long R^3 IGF-I could be achieved via the addition of approximately 3.5 μg of anti-IGF-I. This data indicated preserved immunorecognition of labeled analyte by monoclonal anti-IGF-I. The appearance of an antibody–protein complex could not be observed. This may have been due to fluorescence quenching during the binding reaction or co-migration with observed fluorescent impurities.

4. Conclusion

For the first time, a complete protocol for derivatization of Long R^3 IGF-I with FITC at a single site has been developed. This includes a specific derivatization procedure, LC purification conditions, and a LC–MS approach for the characterization of single-labeled protein. There is substantial LC–MS evidence that selective modification of the α-amino group of Met^1 can be achieved by performing the

conjugation reaction at pH 7.0, with a 1:1 FITC to protein amino group ratio. Future work includes improving the reaction yield, developing a CE–LIF IGF-I competitive immunoassay, and testing this bioanalytical reagent for its ability to bind the Type I IGF-I receptor.

Acknowledgements

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