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Homogeneous fluorescent derivatization of large proteins

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Abstract

A method of homogeneously derivatizing large proteins for highly sensitive analysis is described. Homogeneity of the derivative was realized by tagging all the free amino groups of proteins. With this method, α-chymotrypsinogen A, ovalbumin and bovine serum albumin were derivatized with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC). Prior to the derivatization, all the proteins were reduced and alkylated. After reacting the resulting unfolded proteins with excessive amounts of AQC, the samples were analyzed with matrix assisted laser desorption ionization–time of flight-mass spectrometry (MALDI–TOF-MS) to determine the derivatization degree. The results indicated that all three proteins had been, or had almost been, fully derivatized. HPLC and CE were used for characterizing these protein derivatives. Under the optimized fluorescence detection conditions, the detectability of the tagged proteins was 2400–6200 times better than that detected at UV 280 nm, 170–300 times better than detected at UV 214 nm, and 150–420 times better than measured with their native fluorescence. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Fluorescence has become one of the most sensitive methods for detecting proteins separated by HPLC and CE [1,2]. There are currently two main fluorescent methods available for protein analysis. One is to use the native fluorescence emitted by the aromatic amino acid residues, i.e. tryptophan, phenylalanine and tyrosine [3–5], and the other is to tag the proteins to form a derivative that can emit fluorescence. Although the fluorescence emitted from the native protein can be intensive, not all proteins generate a useful native fluorescent signal. By selecting better reagents, the derivatized proteins can emit more intense fluorescence than the aromatic groups of proteins.

There have been many publications reporting the use of derivatization methods for improving a protein's detectability [1,6–10]. While the method has been demonstrated efficient in enhancing the sensitivity of protein analysis, the products of almost all the tagging reactions were found heterogeneous, i.e. multiple derivatives were produced from the reactions [11–16]. The heterogeneity in the tagged products was demonstrated by observing multiple peaks when the products were analyzed by HPLC and/or CE. This has limited the application of such methods for protein analysis.

The formation of multiple protein derivatives can be caused by several factors. One is related to the

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fact that there are usually many tagging sites in each protein molecule available for the reaction. Among all the groups in proteins selected for derivatization, the amino group is most commonly used. However, virtually all proteins contain multiple amino groups. For example, bovine serum albumin (BSA) has 60 amino groups in each molecule [17]. Another factor is the three-dimensional structure of proteins, which makes it difficult for some amino groups to be fully accessible to the reagent. As a result, a mixture of derivatives is usually formed which contains products tagged with a different number of tags or at different sites.

Several approaches have been taken to solve this problem. Post-column derivatization of proteins has been reported [18–20]. Since the proteins were tagged after separation, multiple peak formation caused no problems with subsequent detection. In another approach, Bank proposed adjusting the derivatization buffer to a lower-than-normal value, which would discriminate derivatization of ε-amino groups from α-amino groups of small peptides [21]. However, this method has only been reported for small peptides. Dovichi's group reported attaching the label to small peptides by first taking the peptide through one cycle of the Edman degradation reaction [16]. Wu and Brand converted the N-terminal, α-amino group into a reactive carbonyl group using a transamination reaction, which then reacted with a chromophore [22]. Amir et al. used *tert.*-butylazidoformate to protect ε-amino groups from reacting with the fluorescent probe they used [23]. However, these methods have only been used for small peptides. The current progress in tagging proteins and peptides has recently been reviewed [24].

Our group has recently reported fully derivatizing peptides and small proteins, such as insulin and lysozyme, by using 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC), a very active derivatization reagent [25–27]. The derivatization was simply done by mixing proteins with AQC with or without SDS present. AQC was originally used as a fluorescent reagent for amino acid analysis [28–30]. We selected it as the fluorescent reagent for peptides and proteins because of its reactivity in tagging amino groups, its simple derivatization procedure, the strong fluorescent intensity of the derivatives, and its commercial availability. However, the established derivatization procedure for small proteins was shown inefficient when applied to larger proteins, such as α-chymotrypsinogen A (CTA) and bovine serum albumin (BSA). We report here our latest work on fully derivatizing these larger proteins by reducing and alkylating them before the derivatization step was performed. To the best of our knowledge, this is the first time that large proteins have ever been homogeneously derivatized.

2. Experimental

2.1. Chemicals

Three proteins, CTA, ovalbumin and BSA, were used as models for the current work, all from Sigma (St. Louis, MO), and used without further purification. Dithiothreitol (DTT) and sodium iodoacetate were also purchased from Sigma. The AccQ-Tag reagent kit, including AQC and a borate derivatization buffer (0.2 *M*, pH 8.8), was from Waters (Milford, MA). Other chemicals were the products of either Sigma or Fisher Scientific (Pittsburgh, PA).

2.2. Reduction and alkylation of proteins

The reduction and alkylation of proteins was performed based on a method reported by Takeda et al. [31]. CTA (10 mg) and ovalbumin (10 mg) were each first dissolved in 3.5–4.0 ml of a buffer containing 0.2 *M* phosphate, 8 *M* urea and 1 m*M* EDTA, pH 8.0. BSA (7.5 mg) was first dissolved in 0.2 ml water, as it was not completely soluble in the buffer. DTT was then added to the protein solutions for reduction (CTA/ovalbumin/BSA: 5.2/1.8/4.0 mg). After flushing with nitrogen, the samples were incubated at room temperature for 2 h. Finally, sodium iodoacetate was added to the samples for alkylation (CTA/ovalbumin/BSA: 14/5.0/11.2 mg). The reaction was performed in the dark for 40 min. To stop the reaction, an excess amount of DTT (approximately two times the molar amount of iodoacetate) was added to the samples, and, immediately after that, the reduced and alkylated (RA) samples were transferred to Slide-A-Lyzer 10K dialysis cassettes from Pierce (Rockford, IL) for

overnight dialysis. The dialysis was performed either against water containing 0.05% triethylamine (TEA) or 0.2 *M* borate (pH 8.8) containing 12.5 m*M* sodium dodecyl sulfate (SDS).

2.3. Derivatization of the RA samples

For the derivatization of RA CTA, 320 μl of a dialyzed RA CTA solution in the borate–SDS buffer (concentration: 0.71 mg/ml) was mixed with 240 μl of 10 m*M* AQC. A 100-μl amount of a dialyzed RA ovalbumin in the TEA buffer (1.1 mg/ml) was mixed with 300 μl of 0.2 *M* borate, pH 8.8, 100 μl of 10 m*M* AQC, then 400 μl more AQC solution. A 600-μl amount of the borate buffer was added to 200 μl of a RA BSA solution (TEA buffer, 0.75 mg/ml), followed by adding 200 μl of the AQC solution. After the derivatization, all the samples were heated at 55°C for 10 min. A part of each sample (~0.5 ml) was then transferred to a 10K Slide-A-Lyser cassette, and dialyzed overnight against 1500 ml 0.05% TEA in water. The reduced, alkylated and derivatized (RAD) CTA and RAD ovalbumin cassettes, in which a precipitate was observed, were then moved to a solution of 0.05% TEA/acetonitrile at 8/5 (v/v) for 30–40 min or until the precipitate disappeared.

2.4. MALDI–TOF-MS conditions

The MALDI–TOF-MS was performed with a Voyager-DE STR system from PE Biosystems (Framingham, MA). A delayed extraction mode was used for MW measurement of the proteins. The matrix used was a saturated sinapinic acid solution in one part of formic acid, three parts of water and two parts of iso-propanol. Before being analyzed by MS, the matrix was mixed with an equal volume of each sample. Mode of operation of MS system: linear. Extraction mode: delayed (300 ns). Polarity: positive. Accelerating voltage: 25 000 V. Grid voltage: 90%. Laser intensity: 2200–2550. Number of laser shots: 100/spectrum. Calibration type: external. Low mass gate: 2000–4500.

2.5. Determination of protein concentration

Concentrations of the RAD proteins were measured by hydrolyzing the proteins and analyzing the amino acids in the resulting hydrolysates, and using an AQC precolumn derivatization method similar to a previously reported one designed for separating amino acids in cell culture fluids [30]. A 2–5-μg amount of protein was transferred for hydrolysis and derivatization, which were carried out as previously reported [28].

2.6. Flow injection analysis (FIA) of native and derivatized proteins

FIA was carried out on a Waters HPLC system, which included a Model 2690 Separation Module connected to a knitted open tubular reactor (KOT) [32,33]. The volume of the KOT was ~0.5 ml. Depending on the type of samples injected, different detectors were connected to the KOT for detection. If the sample injected was a native protein, a Model 486 UV detector was employed, with its wavelength set at 280 nm. If the sample was derivatized, however, the UV detector was replaced with a Model 474 scanning fluorescence detector. The fluorescence detector was equipped with a 5- or 16-μl flow cell, with its excitation and emission wavelengths adjusted to 250 and 395 nm, respectively. When a native protein solution was injected, a 50 m*M* phosphate buffer, pH 6.85, was prepared as the mobile phase. When a derivatized protein sample was analyzed, the mobile phase was replaced with a combination of the phosphate buffer (10–80%), 6 *M* urea (20%) and acetonitrile (0–70%). The flow rates of the mobile phases were both set at 1 ml/min. Before FIA was carried out, all the RAD stock solutions were dialyzed to remove any interfering components from the samples.

2.7. HPLC

Two HPLC systems, both from Waters, were set up for running the tagged and native proteins. While the first system was the same as used for FIA, as described above, except that the KOT was replaced with a column, the second system was composed of a Model 616 pump, a Model 715 autosampler and a Model 470 fluorescence detector. Depending on the chromatographic mode used, the column connected to both systems was either a Symmetry 300 C_{18} reversed-phase column (3.9×150 mm) or a Shodex

KW-804 size exclusion chromatography (SEC) column (Waters). For reversed-phase separation of the tagged CTA, the mobile phase contained 40–50% of 50 m*M* Tris, pH 7.0, 30% of acetonitrile and 20–30% of 6 *M* urea. When the same system was used for running native CTA, however, the mobile phase was changed to a combination of 58% of 0.1% trifluoroacetic acid (TFA) in water and 42% of 0.085% TFA in acetonitrile. For SEC of the tagged proteins, the mobile phase was composed of three eluents: 50 m*M* phosphate with a pH of 6.85 (30%), acetonitrile (50%) and 6 *M* urea (20%). For SEC of the native proteins, however, the mobile phase was replaced with a combination of 50 m*M* phosphate (85%) and 1 *M* sodium chloride (15%). The flow rates for all the modes of chromatography were set at 1 ml/min. The settings of the fluorescence detector were the same as used for FIA.

2.8. Capillary zone electrophoresis (CZE)

A Quanta 4000 capillary electrophoresis system was used for the CZE separation of the native as well as the AQC tagged proteins. A 60-cm×50-μm uncoated capillary was installed on the system. The running buffer was 10 m*M* tetraborate, pH 9.2, the detection was carried out at UV 214 nm, and the run voltage was set at 20 kV. The injections were made with a hydrostatic mode, with an injection time set at 15–45 s.

3. Results and discussion

3.1. Sample preparation

Before derivatization, all the model proteins were fully unfolded by reducing the disulfide bonds and alkylating the resulting thiol groups. When selecting an alkylation reagent, sodium iodoacetate was preferred rather than iodoacetamide because it and therefore, its products, would be more hydrophilic. In contrast to its acid form, sodium iodoacetate was easier to use, as no pH adjustment of the reaction mixture was necessary.

After being reduced and alkylated, the proteins were dialyzed to remove excess reagents and chemicals from the RA samples. Preliminary experiments showed that RA samples were apt to precipitate if an inappropriate dialysis buffer was used. Since the samples would be subject to a derivatization reaction after being dialyzed, this compatibility was another factor to consider when selecting the buffer. Two buffers were found that could keep the RA proteins in solution, while also being compatible with the derivatization reaction. One was a 0.2-*M* borate buffer containing 12.5 m*M* SDS, while the other was 0.05% TEA. When the dialyzed RA samples were tagged with AQC, the first buffer had the advantage of making the reaction readily occur, as no additional borate buffer was required. The main disadvantage of the first buffer was the presence of SDS, which was difficult to remove from the samples. In the current studies, both buffers were used for the dialysis step.

During the derivatization procedure, no precipitation was found for the proteins, even though they were derivatized at a relatively high temperature. After the dialyzed samples were derivatized with a large excess AQC (at least eight times the total molar amount of the amino groups), the samples were further prepared for MALDI–TOF-MS analysis. Dialysis was again used as the method to remove SDS, sodium borate or other components that would interfere with the analysis. Initially, the 0.05% TEA was tried as the dialysis buffer. However, a precipitate was found in all the reduced, alkylated and derivatized (RAD) samples. Further experiments showed that the precipitate could be redissolved if dialyzed against a mixture of the TEA buffer with acetonitrile at a 8:5 ratio. This two-step-dialysis (precipitating and redissolving) procedure was then used as an optimized sample preparation method for all the RAD samples. The acetonitrile-containing buffer was not selected for use from the beginning of the dialysis, as it was suggested that, in the long run, the high concentration of acetonitrile in the buffer would dissolve the material supporting the membrane. Moreover, precipitation of the proteins was thought to be helpful for efficiently removing SDS. When the second dialysis step was performed against the acetonitrile-containing buffer, the dialysis was stopped immediately after the precipitate disappeared (~30 min).

3.2. MALDI–TOF-MS measurement of tagged amino groups of the model proteins

To measure the extent of modification to the reactive groups of the model proteins (disulfide bonds, amino groups) by the reduction and alkylation and by derivatization with AQC, the native proteins, as well as their RA and RAD samples, were analyzed by MALDI–TOF-MS. The measurement principle was based on the fact that the modification of iodoacetate would result in an increase of 59 Da in MW of the native protein, and that one AQC tag to an amino group of a protein would result in an increase of 170 Da in MW of the derivatized molecule. The numbers of the alkylated thiol groups and the tagged amino groups were then determined from the differences in MW before and after modification.

CTA has a molecular weight (MW) of 25 666. There are 15 amino groups (including the aliphatic epsilon-amino of lysine and the alpha-amino group of the N-terminal amino acid) and five disulfide bonds on each CTA molecule [34]. The most updated molecular weight of BSA is 66 430 [17]. Each BSA molecule has 60 amino groups and 35 cysteine residues. As previously reported [35], there are 17 disulfide bonds on each BSA molecule. Ovalbumin has an MW of 44 287. As can be seen from its reported sequence [36–38], it has 21 amino groups and three disulfide bonds. However, its total amino group number is actually 20, since its N-terminus is acylated [39].

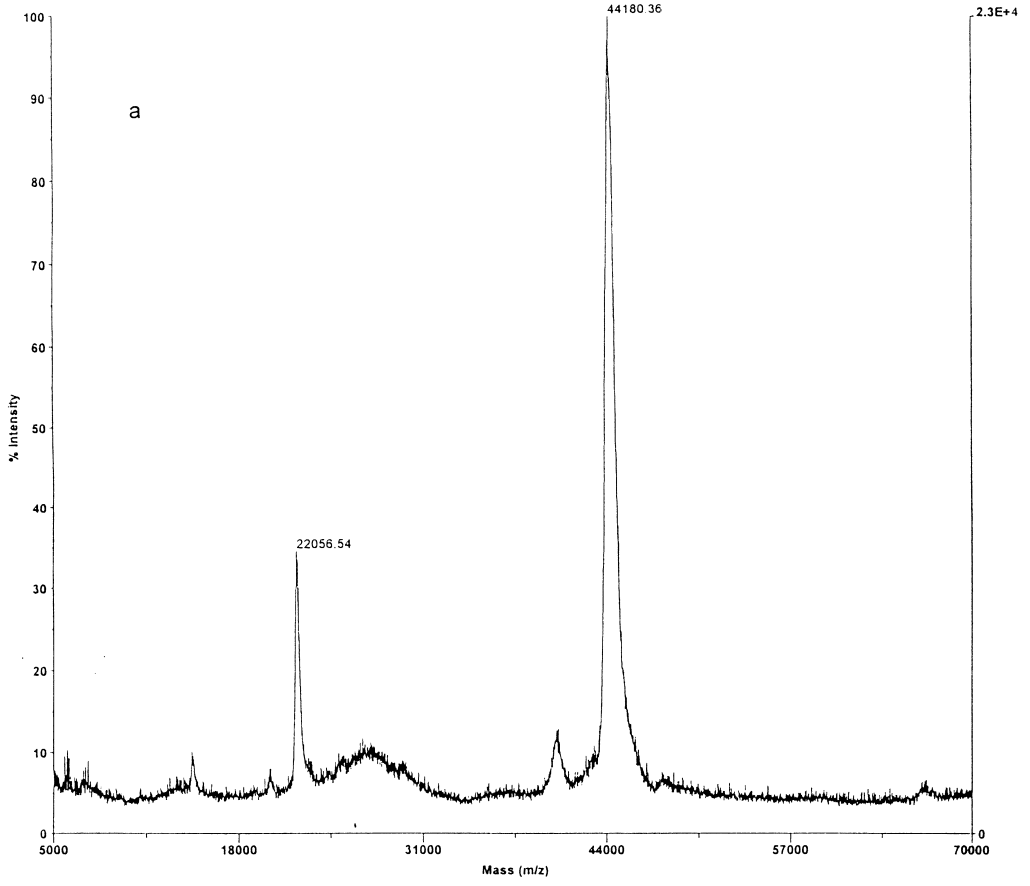

Fig. 1. MALDI–TOF-MS spectra of ovalbumin. (a) Native ovalbumin (0.36 mg/ml); (b) RA ovalbumin (1.0 mg/ml); (c) RAD ovalbumin (0.068 mg/ml). Matrix: saturated sinapinic acid.

Shown in Fig. 1 are the spectra of native (a), RA (b), and RAD (c) ovalbumin. In (a), The peak with a MW of 44 180 corresponds to the native ovalbumin. There is another peak in (a) which has a MW of 22 056. It is believed to be the peak of the doubly-protonated ovalbumin. As can be seen from (b) and (c), the measured MWs of the RA and RAD ovalbumins were 44 400 and 47 832, respectively. Except for the main RAD ovalbumin peak, there were no other peaks observed in the mass range of 44 000–48 000 in (c). The spectra of CTA and BSA were similar to those of ovalbumin (spectra not shown). Table 1 summarizes the measured MW of all the native, RA and RAD proteins, as well as the calculated number of thiol alkylations and amine modifications.

As seen from Table 1, the disulfide bonds of CTA have all been reduced and alkylated, and all the free amino groups have been derivatizated. There might be one intact disulfide bond left on the ovalbumin molecules after alkylation, but the extent seemed to be enough for fully tagging all the free amino groups on the molecules. With most disulfide bonds of BSA broken, the number of amino groups that were tagged with AQC was measured as 58.0 ± 0.5 ($n=2$), very close to 60, the number of total amino groups on BSA.

From these MALDI–TOF measurements, one can see that, by reduction and alkylation, all the amino groups of CTA and ovalbumin have been completely derivatized. For BSA, the tagged number of amino groups is still 2.0 less than that of the theoretical

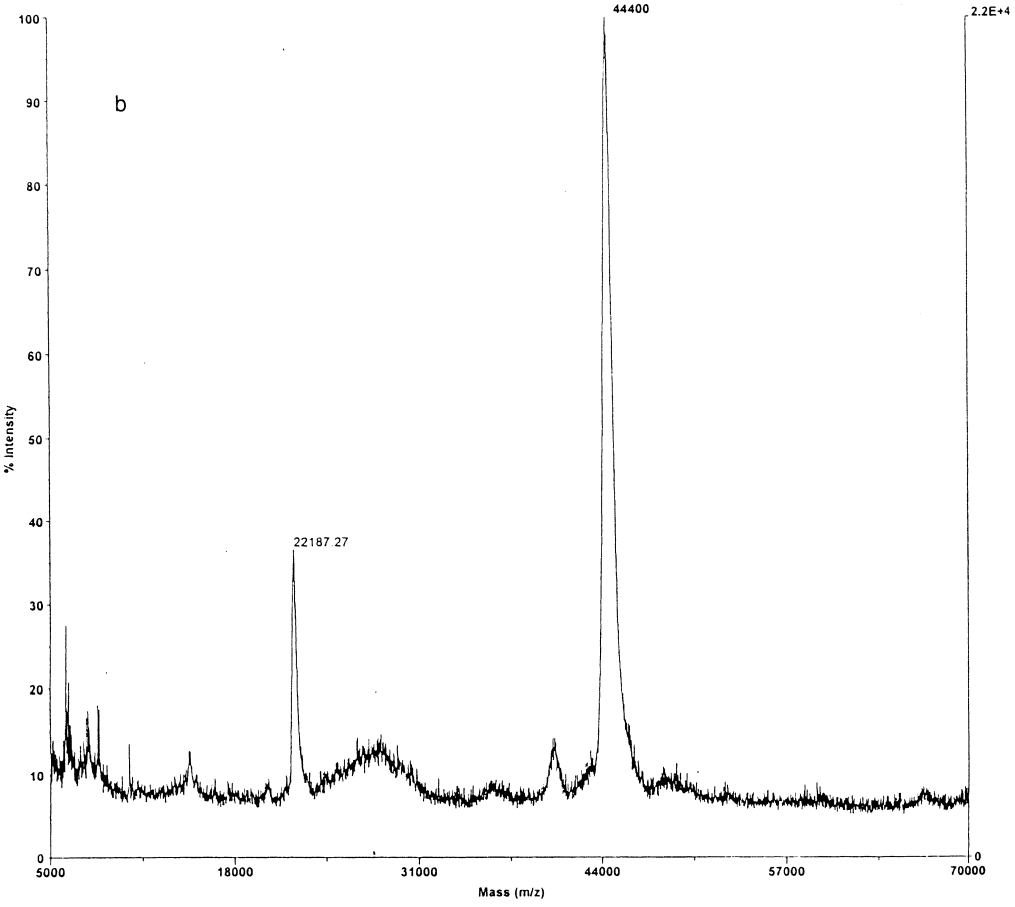

Fig. 1. (*continued*)

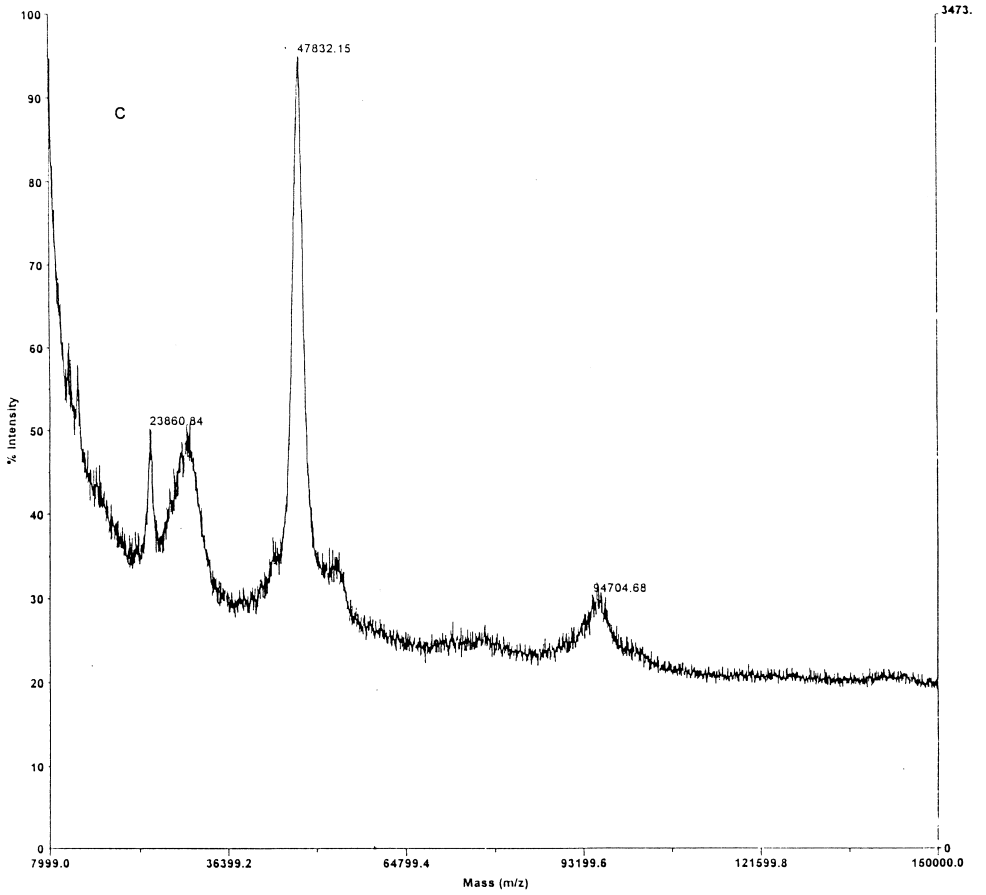

Fig. 1. (*continued*)

Table 1
MALDI–TOF-MS data of the model proteins and calculation results

Protein	CTA			Ovalbumin			BSA		
	Native	RA	RAD	Native	RA	RAD	Native	RA	RAD
Measured MW	25647	26 262	28 854	44 180	44 400	47 832	66 432	68 226	78 078
Calculated number of alkylations		10.4			3.7			30.4	
Number of cysteine residues per molecule		10			6			35	
Calculated number of AQC derivatizations		15.2			20.2			58	
Number of free amino groups per molecule		15			20			60	

number. This might result from incomplete reaction, measurement error, and/or the unavailability of those groups to tagging.

Besides the method described above, a simpler derivatization method was also tried for BSA's homogeneous derivatization. In this method, native BSA was reacted with AQC directly without being reduced and alkylated first. However, a MALDI–TOF-MS spectrum of the resulting tagged BSA indicated that the derivatization was less complete than that with reduction and alkylation involved (data not shown). Using the difference between the MW of the native BSA and that of tagged BSA indicated from the apex of the BSA peak, the number of amino groups that were derivatized was only 44, far fewer than the number tagged in RAD BSA (58.0).

3.3. RAD protein's fluorescence responses as a function of % acetonitrile (ACN) in the mobile phase

One major factor that significantly affected the fluorescence responses of the RAD proteins was the concentration of acetonitrile in the mobile phase. This can be seen from Fig. 2, in which the FIA peak responses of all three proteins were plotted as a function of the acetonitrile concentration in the mobile phase. To measure the FIA peak heights under fluorescence detection, RAD samples with various concentrations (BSA: 4.4 μg/ml, ovalbumin: 4.1 μg/ml, CTA: 5.5 μg/ml) were injected into the FIA system. Urea was employed in the mobile phase to keep the RAD proteins in solution. In Fig. 2, acetonitrile affected the fluorescence intensity of each protein in a very similar way. The fluorescence signals were very small when the acetonitrile concentration was low in the mobile phase (<20%). However, they were significantly increased when higher concentrations of acetonitrile were present in the mobile phase. The signals reached the highest level at an acetonitrile concentration around 60%, and then decreased when the concentration was higher, probably because of the insolubility of the proteins in a solvent containing mostly acetonitrile. The fluorescence intensities of these proteins were 60–170 times higher in 60% acetonitrile than those in a purely aqueous solution.

The above significant effect of acetonitrile concentration on the fluorescence intensity of the RAD proteins could be caused by one of two factors, the aqueous quenching effect and the emission spectral shifts as a function of solvent changes [40]. We demonstrated that the spectrum of RAD ovalbumin had similar profiles under different acetonitrile concentrations (data not shown), which made us believe that the effect was probably caused by the quenching effect.

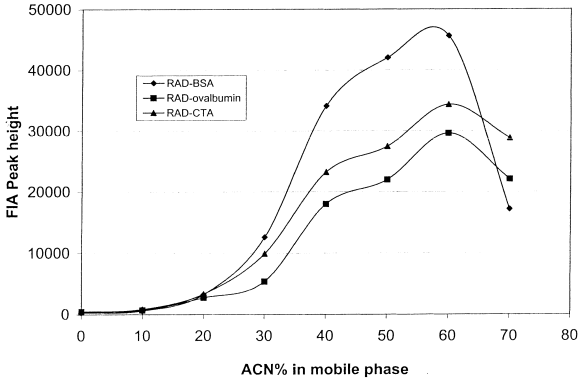

Fig. 2. RAD protein fluorescent response as a function of acetonitrile concentration. RAD BSA: 4.4 μg/ml; RAD ovalbumin: 4.1 μg/ml; RAD CTA: 5.5 μg/ml. Injection volume: 10 μl. Mobile phase contained 20% of 6 *M* urea and 10–80% of 50 m*M* phosphate. Flow cell of the fluorescence detector: 5 μl. See Experimental section for other conditions.

3.4. Chromatography of the tagged proteins

The purpose of chromatography, and CE as described below, of the tagged proteins was to check their homogeneity. If there were multiple peaks found in a chromatogram (electropherogram) of a tagged protein, the protein was then probably heterogeneously derivatized. However, if there was only one peak in the chromatogram, the protein was then probably derivatized homogeneously. Fig. 3a is a reversed-phase chromatogram of a RAD CTA sample, in which only one main peak was found. This peak has a plate count of 3650±130. To keep the protein in solution, the mobile phase was at a basic pH, and urea was added to the mobile phase. Shown in Fig. 3b is a UV chromatogram of a native, untagged CTA sample separated with the same

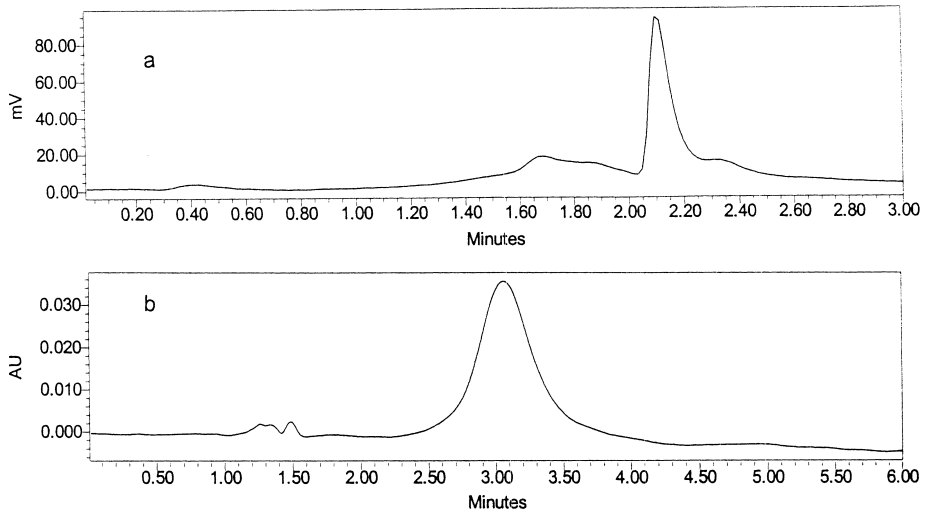

Fig. 3. Reversed phase chromatogram of CTA. (a) RAD CTA (110 μg/ml); (b) native CTA (2 mg/ml). In (a), mobile phase: 30% of 50 m*M* phosphate, pH 6.85+50% acetonitrile+20% of 6 *M* urea; injection volume: 1 μl; detection: fluorescence (16-μl flow cell). In (b), mobile phase: 85% of 50 m*M* phosphate, pH 6.85+15% of 1 M; injection volume: 10 μl; detection: UV 280 nm. See Experimental section for more detailed conditions.

column, but with a TFA mobile phase, a more commonly used mobile phase for reversed-phase HPLC of proteins. Under conditions similar to those used for RAD CTA and native CTA samples, the ovalbumin and BSA samples were also injected into the system. The RAD ovalbumin peak was very similar to that of RAD CTA (figure not shown). However, the RAD BSA did not elute from the column, probably due to its high hydrophobicity after derivatization. A main protein peak was observed when the RAD BSA was run with SEC (figure not shown). These results suggested that the RAD proteins were most likely homogeneously derivatized.

3.5. CZE of the tagged proteins

Figs. 4 and 5 show the electropherograms of the RAD ovalbumin and BSA, respectively, as well as their native proteins. The RAD ovalbumin peak in Fig. 4a and RAD BSA peak in Fig. 5a were the only protein-related peaks observed. This was demonstrated by the fact that all the other peaks disappeared after the samples were dialyzed and reinjected (data not shown). One common characteristic of both RAD protein peaks was that they both migrated more slowly than their native forms. The likely explanation is that after the amino groups were tagged, a protein's mobility became smaller under the given CZE conditions since they were less positively charged. The plate count of the RAD BSA peak (12 130±2040) was greater than native BSA (5140±280). However, a comparison of the RAD ovalbumin peak (plate count 2160±350) with its native form was not made, since there were several peaks in Fig. 4b, the electropherogram of the native ovalbumin, probably due to several isoforms existing in the sample.

A RAD CTA sample was also run with the same CZE system as used for RAD BSA and ovalbumin, and a peak corresponding to the CTA was observed, with a peak shape similar to other RAD proteins (figure not shown).

In contrast to what we observed for CZE peaks of derivatized peptides and small proteins, which were extremely sharp [25], the CZE peak shapes of these larger proteins were broad. The reason for this phenomenon was unknown. However, the peak shapes of these derivatized, large proteins were indeed improved, as compared with those of the

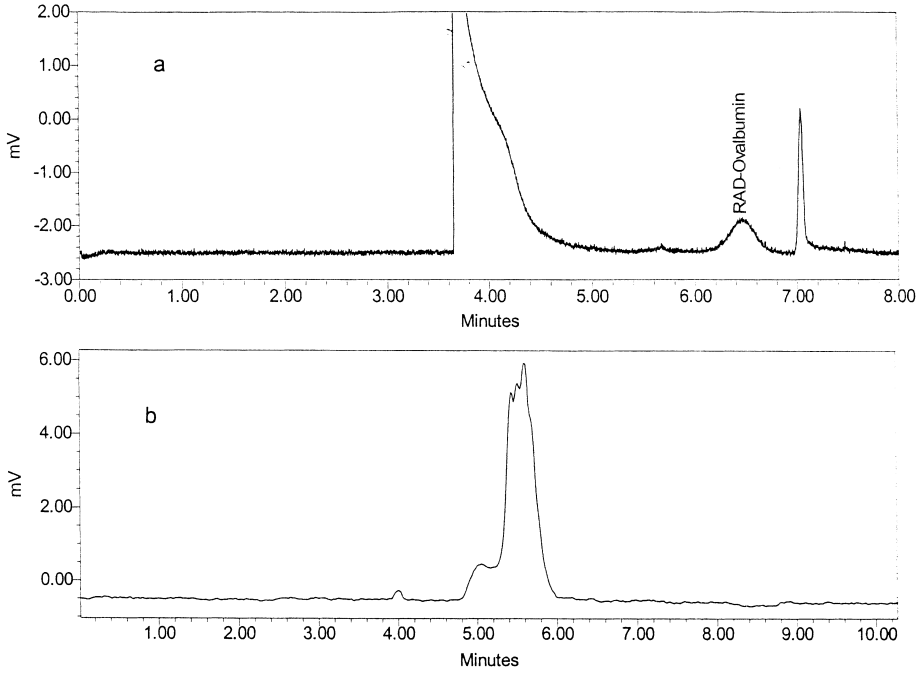

Fig. 4. CZE of ovalbumin. (a) RAD ovalbumin (0.22 mg/ml), 15-s injection; (b) native ovalbumin (2 mg/ml), 45-s injection. Capillary: 60 cm×50 μm (uncoated). Buffer: 10 m*M* tetraborate, pH 9.2. Voltage: 20 kV. Detection: 214 nm. Injection: 15 s.

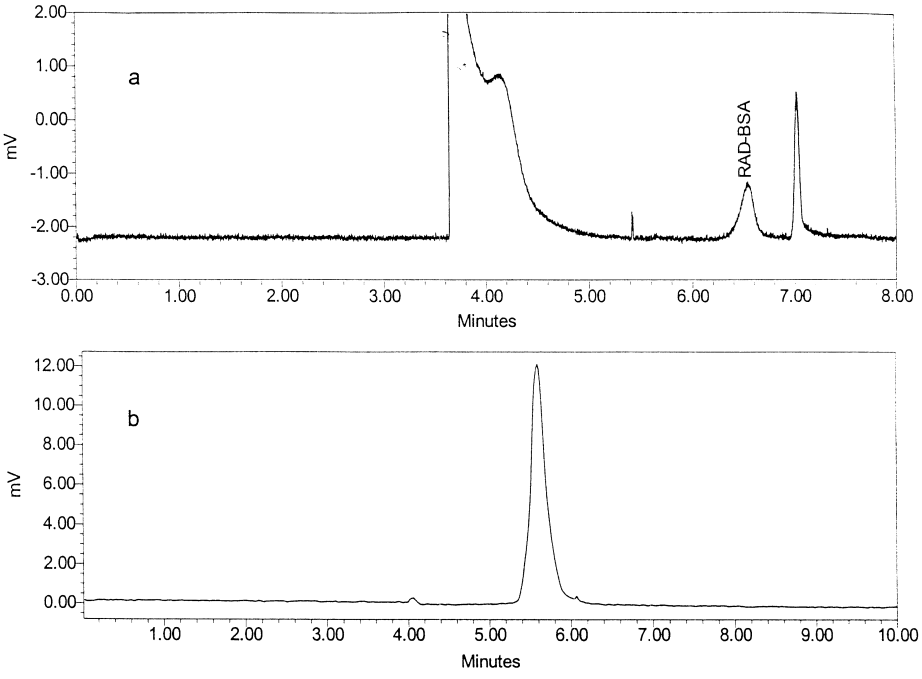

Fig. 5. CZE of BSA. (a) RAD BSA (0.25 mg/ml), 15-s injection; (b) native BSA (2 mg/ml), 30-s injection. See Fig. 4 for the running conditions.

Table 2
FIA-derived detection limits of native and RAD proteins

Protein (detection)	Detection limit[a]	
	ng	n*M*
Native BSA (280 nm)	50	80
Native BSA (214 nm)	1.6	2.5
Native BSA (fluorescence)	2.2	3.3
Tagged BSA (fluorescence)	0.0081	0.012
Native ovalbumin (280 nm)	46	100
Native ovalbumin (214 nm)	1.6	3.6
Native ovalbumin (fluorescence)	1.4	3.3
Tagged ovalbumin (fluorescence)	0.0092	0.021
Native CTA (280 nm)	26	100
Native CTA (214 nm)	3.3	13
Native CTA (fluorescence)	4.6	18
Tagged CTA (fluorescence)	0.011	0.042

[a] Detection limits were calculated based upon three times the noise level.

same proteins in their native form, perhaps due to the removal of the amino groups in these proteins.

3.6. Comparison of detectability of the tagged and native proteins

A comparison of the detectability of the tagged and native proteins was made by FIA. The native proteins were detected by both UV (214 and 280 nm) and fluorescence (excitation wavelength: 280 nm, emission wavelength: 337 nm), all with 50 m*M* phosphate, pH 6.85, as the mobile phase. The tagged proteins were monitored only with fluorescence. Table 2 lists the calculated detection limits (signal:noise 3:1) for each protein. Considering the interference of the FIA peak found in each blank sample, the protein samples were prepared with a concentration of 4.1 μg/ml so that the concentrations were high enough to make sure that most of the response was due to the proteins. Although the final sample concentrations were much higher than the measured detection limits, the detection limit data were still meaningful since they were only used for sensitivity comparisons. One can see that, by tagging with AQC, a protein's detectability could be improved by as much as 2400–6200 times than detected at UV 280 nm, 170–300 times than at UV 214 and 150–420 times than detected with native fluorescence.

As can be seen from Table 2, the RAD BSA, which had the largest number of tags of the model proteins, had the lowest molar detection limit. On the other hand, the RAD CTA, which had the fewest tags, had the highest molar detection limit. This indicated, under the detection conditions, a higher tagging level would improve detectability of the tagged molecules.

4. Conclusion

Via reduction and alkylation, all the model proteins studied have been fully, or nearly fully derivatized. Although the whole procedure is somewhat time-consuming, and tagging all the amino groups of protein may not be the best way of performing derivatization, it seems that, currently, this is the only way that possibly prepares a homogeneous derivative of large proteins. It is expected that the same method can be applied to other large proteins for high sensitivity analysis. Since proteins can now be homogeneously derivatized, they can be readily analyzed using HPLC, CE and other separation techniques with better sensitivity.

The potential problems in applying this technique may be the solubility and hydrophobicity of the RAD proteins. Another problem is the quenching effect of the tagged molecules. Although these problems can be solved by using appropriate solvents, this may limit the application of the technique in cases where these solvents cannot be used.

Limited by the availability of instruments, LIF

detection was not used in the current report. It expected that, when a LIF detector is employed for the detection of the RAD proteins, the detectability of these proteins will be significantly improved, especially when a correct excitation wavelength is selected.

5. Nomenclature

ACN	acetonitrile
AQC	6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate
BSA	bovine serum albumin
CTA	α-chymotrypsinogen A
CZE	capillary zone electrophoresis
DTT	dithiothreitol
FIA	flow injection assay
KOT	knitted open tubular reactor
MALDI–TOF	matrix assisted laser desorption ionization–time of flight
MS	mass spectrometry
MW	molecular weight
RA	reduced and alkylated
RAD	reduced, alkylated and derivatized
SDS	sodium dodecyl sulfate
SEC	size exclusion chromatography
TEA	triethylamine
TFA	trifluoroacetic acid

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