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Capillary electrophoresis laser-induced fluorescence for screening combinatorial peptide libraries in assays of botulinum neurotoxin A

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Abstract

Botulinum neurotoxin serotype A (BoNT/A) is a proteolytic enzyme that induces muscle paralysis. It is a cause of food poisoning, a potential bioterrorist threat and, in low doses an emerging pharmaceutical product. No effective treatment is currently available for BoNT intoxication. Previously we developed a BoNT/A light chain enzyme assay using a peptide substrate based on the SNAP-25 protein target, with HPLC separation and UV detection of assay products, and applied the method to screen combinatorial peptide libraries for inhibitory activity to BoNT/A. We now report on development of a capillary electrophoresis laser-induced fluorescence (CE-LIF) method for measuring BoNT/A activity. The enzyme assay products were labeled with CBQCA dye followed by CE separation on a bare fused silica column in a HEPES-based buffer and LIF detection. All assay products were separated in CE within 8 min compared to incomplete separation of assay products within 1 h by HPLC. The labeled products showed linear dependence of intensity *versus* concentration, and quantitative mole-fraction assignments. We used the CE-LIF method to screen combinatorial peptide libraries for potential modulating effects on BoNT/A peptidase activity. With some of the libraries, peptides co-migrated with assay products and interfered with quantitation. In such cases, interference was reduced by substituting sodium dodecyl sulfate (SDS) for Tween-20 in the running buffer. Separation in the capillaries then occurred by micellar electrokinetic chromatography (MEKC). The CE-LIF method is quick and lends itself to high-throughput or microfluidic formats.

Keywords: Capillary electrophoresis; Fluorescence; Botulinum neurotoxin; Micellar electrokinetic chromatography; Combinatorial peptide libraries

1. Introduction

Botulinum neurotoxin serotype A (BoNT/A) is a protein toxin derived from *Clostridium botulinum* that induces muscle paralysis by blocking release of neurotransmitter acetylcholine. BoNT/A comprises a 50 kDa light chain (amino acid residues 1-448) and a 100 kDa heavy chain (aa 449-1296), held together by a single disulfide bond (C430–C454). The heavy chain has a transmembrane region (aa 659-681) that inserts into the neuronal membrane permitting the light chain to enter the interior of the neuron. Enzymatic reduction of the disulfide bridge within the neuron initiates the proteolytic activity of the light chain contained in the HELIH zinc-binding domain (aa 223-227). The activated light chain cleaves SNAP-25 (25 kDa SyNaptosomal-Associated Protein), thereby inhibiting acetylcholine release from the neuron [1]. SNAP-25 is a component of the SNARE complex (Soluble *N*-ethylmaleimide-sensitive factor Attachment protein REceptor) that is responsible for docking and fusion of synaptic vesicles prior to neurotransmitter release. The SNARE complex is a four-helix bundle composed of two small proteins, vesicle-associated membrane protein (VAMP) and syntaxin, plus the larger protein, SNAP-25, which turns back on itself and provides two of the four helices of the bundle [2,3]. BoNT/A cleaves the Gln197−Arg198 bond near the C-terminus of SNAP-25.

Assays of BoNT/A that use various peptide substrates have been reported [1,4,5]. Hayden et al. [6] used a 17-amino acid

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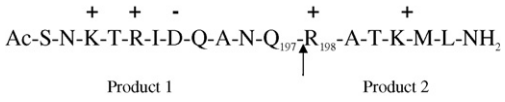

Fig. 1. Peptide sequence of substrate and product for BoNT/A Assay. Nominal positive and negative charges on the amino acids are indicated by (+) or (−). The arrow indicates the cleavage site by activated BoNT/A light chain. Ac and NH_2 on the ends of the peptide represent acetyl and amide capping of the N and C termini, respectively. The 17-mer substrate peptide represents residues 187-203 in SNAP-25.

substrate, derived from SNAP-25, and consisting of residues 187-203 [4,5] which contains the glutamine–arginine (Q–R) scissile bond (Fig. 1). The activated light chain cleaves the substrate into two products of 11 and 6 amino acids. The substrate has been used *in vitro* to detect peptidase activity with the activated light chain of BoNT/A and used as a tool to design and discover novel inhibitors in combinatorial peptide libraries [6–8]. Previously HPLC was the method of analysis used to measure the concentrations of substrate and products in reaction mixtures [6,8]. Several limitations of HPLC such as the length of time for analysis (approximately 1 h per sample) and the inability to completely resolve and easily quantitate substrate and products in reaction mixtures, especially in the presence of combinatorial peptide libraries, impeded the search for inhibitor compounds.

Capillary electrophoresis (CE) is a powerful separation method for bioanalysis, having high resolution, low sample requirement and short run times and has proven to be an effective technique for analysis of proteins and peptides [9,10]. The principle of CE separation is the differential electrophoretic mobilities of the analytes in an electric field [11]. The mobilities are dependent on the charge-to-mass ratios of the molecules. The physico-chemical properties of the peptide substrate and products (Table 1) were calculated based on the Offord model for peptide electrophoretic mobility [12,13] using the expert protein analysis system (ExPASy) [14]. Detection methods in CE are predominantly UV absorption or fluorescence emission. High sensitivity of fluorescence-labeled biomolecules is achievable in the sub-picomolar range for CE using laser-induced fluorescence (LIF) [15,16]. For CE-LIF analysis of peptide substrates and products, two avenues were available: first, an end-labeled peptide whereby the only species detectable in LIF would be the intact substrate and the peptide fragment that carried the fluorescent label. This approach has been used to develop an assay for cleavable nucleic acid probes using CE-LIF [17]. However, preliminary studies for this work suggested that the peptidase activity of the BoNT/A light chain was sensitive to end-located substituents such as fluorescein dye. We also considered the possible effect that the label could have on competition between substrate and peptide libraries for the enzymatic site of the activated light chain. In this work we preferred substrate and products to be free of fluorescence label during the BoNT/A enzyme reaction. Hence we pursued a second approach that employed post-reaction labeling of substrate and products with the primary amine-reactive dye 3-(4-carboxy-benzoyl)-2-quinolinecarboxaldehyde (CBQCA). CBQCA is non-fluorescent until it forms an adduct with a primary amine in the presence of cyanide ion. CE-LIF analysis of amino acids and peptides labeled with CBQCA has been reported [15,18,19]. The same labeling reagent has been used in CE-LIF for characterization of VAMP-thioredoxin fusion protein, developed as a potential substrate for BoNT/B [20]. A detailed CE-based study of BoNT/B light chain (LC) activity using UV detection has been reported, although no results were given for quantitation in the presence of potential modulator compounds [21].

Table 1
Physico-chemical characteristics of peptide substrate and cleavage products

Compound	Charge[a] (at neutral pH)	MW[b] (Da)	p*I*[b]
Substrate	+3	1989	11.00
Product 1	0[c]	1288	8.46
Product 2	+3[c]	719	11.00

Note: CBQCA labeling changes the *m*/*z* ratio peptides by decreasing the net positive charge on lysine residues and adding mass (*ca*. 300 Da per adduct).

[a] Sum of nominal charges.

[b] Molecular weight (MW) and isoelectric point (p*I*) were calculated by ExPASy ([12]).

[c] Cleavage of the peptide bond introduces a carboxyl group on P1 and a free amino group on P2, which modifies the charge.

The strategy behind the use of combinatorial peptide libraries was: (i) to block the proteolytic site of BoNT/A, e.g., substrate inhibitors or selective chelation of zinc; (ii) to block the interaction of BoNT/A with the SNARE-binding recognition motif; or (iii) to interact with possible allosteric sites on the enzyme. The libraries had the structure acetyl–X_1–X_2–linker–X_3–X_4–NH_2, where X_1–X_4 were mixtures of selected amino acids and the linker was a flexible "hinge" 4-aminobutyric acid. The purpose of the hinge was to disrupt secondary structure in the library peptides thus allowing a greater range of conformational orientations to the individual molecules and hence a greater range of interactions [6–8]. We used the CE method to screen for modulating effects of combinatorial peptide libraries developed against the SNARE recognition motif of BoNT/A. Here we describe a technique for measurement of peptidase activity by CE-LIF that provides substantial improvements regarding time, resolution and quantitation compared to HPLC. When the technique was applied to measurement of peptidase activity in the presence of the peptide libraries, modulating properties of the libraries could be observed.

2. Materials and methods

2.1. Chemicals and reagents

Unless otherwise noted all chemicals and reagents were purchased from Sigma–Aldrich Canada Ltd. (Oakville, Ont., Canada).

2.2. BoNT/A substrate, products and combinatorial peptide libraries

The botulinum neurotoxin A substrate peptide (S, see Fig. 1) and product peptides (P1; Ac-SNKTRIDQANQ) and (P2; RATKML-NH_2) were obtained from Dr. Dennis McMaster at

the University of Calgary Peptide Synthesis Laboratory (Calgary Alta.). Ac and NH_2 on the ends of the peptide represent acetyl and amide capping of the N and C termini, respectively. The S and P2 peptides were readily dissolved in sterile distilled water at a stock concentration of 10 mg/mL. P1 peptide required the addition of 0.005% Tween-20 to solubilize. Stock peptides were stored at 4 °C for up to 5 days or longer at −20 °C.

Materials for synthesis of the combinatorial peptide libraries were obtained from Nova Biochemicals Inc. (Mississauga, Ont.). The peptide libraries were synthesized by previously described solid phase methods using standard Fmoc chemistry and MBHA resin [6,7]. Peptides were simultaneously cleaved from the resin and deprotected using trifluoroacetic acid (TFA). Products were precipitated with ether, washed with ether, filtered, dried *in vacuo* over KOH, and stored at −20 °C. The combinatorial peptide libraries shown in the electropherograms herein had chemical compositions given by Ac–X_1–E_2–linker–W_3–R_4–NH_2 (designated as library 33-5) and Ac–X_1–K_2–linker–W_3–R_4–NH_2 (designated as library 33-8) where X represents a mixture of 12 amino acids present in the libraries in equal amounts [6,8]; E, W, R, K are the single letter conventions for the amino acids glutamic acid, tryptophan, arginine, lysine; the subscript represents the position in the peptide chain starting from the N-terminus. Ac and NH_2 on the ends of the peptide represent acetyl and amide capping of the N and C termini, respectively. For modulation studies of BoNT/A LC assays, stock solutions of the libraries were freshly reconstituted (0.5 mg in 0.2 mL 5% aqueous DMSO) to approximately 5 mM (total for the library) and the appropriate volume of stock solution was added to LC assays to give a final concentration of 0.5 mM (total). No significant effects were observed in the LC assays by the presence of 0.5% DMSO (not shown).

2.3. Botulinum neurotoxin a assays

Stock solution of intact BoNT/A (List Biological Laboratories Inc., distributed by Cedarlane Ltd., Hornby, Ont., Canada) was prepared in deionized water (NanoPure filtration Barnstead) at a concentration of 50 μg/mL (*ca*. 330 nM) and stored at 4 °C for up to 2 days. Reduction/activation of BoNT/A was accomplished by adding 30 μL of intact toxin stock solution (typically 1–2 μg) to 30 μL of 50 mM HEPES pH 8 containing 20 mM fresh dithio-DL-threitol (DTT) and incubating at 37 °C for 30 min. After the first reduction/activation step, 390 μL of 50 mM HEPES buffer pH 6.6 containing 1 μM zinc sulfate was added to the reduced toxin solution, followed by 750 μL of 1 μM zinc sulfate. The mixture (final volume 1200 μL) was incubated at 37 °C for 5 h. The 17-mer peptide, Ac-SNKTRIDQANQ$_{197}$-R$_{198}$ATKML-NH_2, which represents SNAP-25 (residues 187-203), was used as the substrate for the enzyme (light chain). Each assay tube (typically 15 tubes total) contained 10 μL of 0.5 mM substrate, 80 μL of activated enzyme solution and 10 μL of deionized water or 10 μL of the combinatorial peptide library solution (5 mM total peptide). The concentration of activated LC enzyme in the assay tubes was nominally 5 nM but some adjustment in concentration was made to yield *ca*. 30–70% cleavage of substrate in the positive controls. Inhibitory assays using combinatorial peptide libraries were conducted by adding the library compounds to the enzyme assay immediately prior to substrate. After 30 min incubation at 37 °C the reaction was terminated with 100 μL of 1% TFA.

2.4. HPLC analysis

Samples were run on a BioRad (Hercules, CA, USA) Hi-Pore C_{18} reverse phase 304-column (25 cm × 4.6 mm) with a flow rate of 1 mL/min and a column temperature of 30 °C. Eluent A was 0.1% TFA in deionized water; eluent B was 0.1% TFA/70% acetonitrile. The gradient profile was generated by running 100% eluent A for 2.5 min, followed by a linear gradient to 45% eluent B for 28.5 min, then increasing to 100% B for 6 min.

2.5. Fluorescent dye labeling

Admixtures of substrate and synthesized products (i.e., S, P1 and P2) in 1% TFA were neutralized by titration with a minimal volume of 2 M NaOH. Aliquots of these admixture samples (10 μL) were subsequently labeled with CBQCA Protein Quantitation kit (Molecular Probes Inc., Eugene OR). Stock solution was prepared by dissolving CBQCA in sufficient dry DMSO to yield 40 mM. For CBQCA labeling, each 10-μL sample aliquot was reacted with 5 μL of 5 mM KCN, 1.5 μL of 100 mM *N*-ethylmaleimide and 10 μL of 5 mM CBQCA in HNTE buffer (50 mM HEPES pH 8.4, 20 mM NaCl, 0.005% Tween-20, 1 mM EDTA). Samples were agitated for 30 min at room temperature in the dark on a Nutator shaker, then transferred to microvials for CE-LIF analysis. The products of BoNT/A LC assays were labeled in a similar fashion. The labeling conditions provided a theoretic minimum dye-to-amine ratio of eight in the presence of peptide libraries. All of the labeling reactions were conducted pre-column. With on-column CBQCA-labeling we did not obtain satisfactory fluorescent intensities (data not shown).

2.6. CE-LIF analysis

CE-LIF analyses were performed using a Beckman 5010 CE instrument (Beckman Coulter Inc. Fullerton CA) equipped with an argon ion laser for excitation at 488 nm and detection at 520 nm. Samples were run on a 37 cm × 50 μm i.d. bare fused silica column (PolyMicro Technologies, Phoenix AZ) with a 30-cm separation length and a column temperature of 25 °C. The column was conditioned with 0.1 M NaOH for 10 min and HNTE running buffer for 30 min before use. During sample analysis, the column was rinsed for 1 min with 0.1 M NaOH and 2 min with running buffer before sample injection. The sample was injected at 0.5 psi for 9 s for a sample injection volume of 19.8 nL, determined by the Beckman instrument software. A plug (11 nL calculated volume) of running buffer was injected at 0.5 psi for 5 s after the sample. The sample was then separated at 18 kV (486 V/cm). Data analysis was performed using P/ACE Version 1.2 software (Beckman Coulter Inc.) on duplicate or triplicate samples. Peak heights and peak areas were calculated and plotted as required.

3. Results

3.1. Comparison of CE and HPLC for separation of BoNT/A reaction peptides

CE separations of substrate and synthesized products were carried out initially using UV (214 nm) detection. With UV detection the absorbance of product, P2, was routinely masked by the background absorbance of the HNTE running buffer although peaks for substrate, S, and product, P1, were visible (data not shown). Similar masking effects were reported for UV detection in CE separations of reaction products of BoNT/B LC in HEPES buffer [21]. Hence in order to facilitate detection and quantitation of products, detection methods based on laser-induced fluorescence were developed.

Fig. 2a shows CE separation of an admixture of the substrate and synthesized products using pre-column CBQCA labeling for LIF detection. The substrate contained the greatest number of nominal positive charges (Fig. 1) and hence displayed the shortest migration time at 2.6 min. The labeled P2 peak was observed at 2.8 min; labeled P1 at 3.2 min. Near baseline separation of all the main labeled peptide product peaks was achieved in CE-LIF within 3.5 min separation time. The total time for one CE-LIF run, including column rinses and injections, was approximately 8 min. Although labeling shifted the migration times slightly longer (by *ca*. 0.1–0.2 min) with respect to unlabeled peptides, the order stayed the same (data not shown). The other peaks in Fig. 2a were attributable to fluorescence arising from CBQCA-labeled components of the HEPES-based buffer used in the analysis (see Fig. 2b). The peaks for the species of interest, i.e., the substrate and product peptides, were sufficiently separated from the buffer peaks that no problems were encountered in making assignments. The presence of the wide buffer peak at 5 min was useful as an internal reference marker for the labeling reaction, electrophoretic mobility and electroosmotic flow. The fluorescence emission of the CBQCA-labeled products was stable at 4 °C up to 14 days (data not shown). Fig. 2c (inset) shows the partial separation of a BoNT/A LC assay mixture by HPLC with UV detection at 210 nm. Products (22.8 min) and substrate (24.2 min) were separated, although P1 and P2 peaks were difficult to resolve. The total time in HPLC for separation and column rinse was about 60 min.

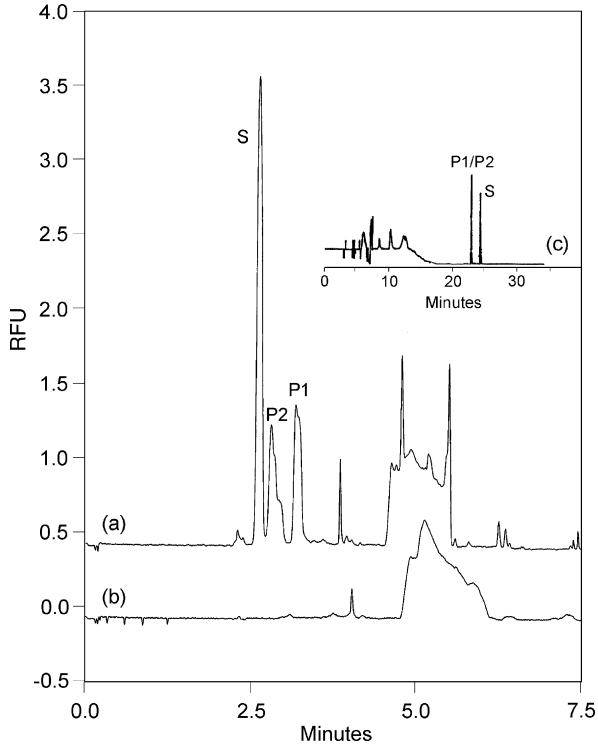

Fig. 2. (a) CE separation and LIF detection of an admixture of CBCQA-labeled substrate and synthesized products. S, substrate; P1, product 1; P2, product 2. (b) HEPES-containing running buffer (described in Materials and Methods section as HNTE buffer) reacted with CBQCA reagent. (c) HPLC of BoNT/A LC assay products. Samples were prepared and analyzed as described in the text.

3.2. Quantitation of BoNT/A LC products in CE-LIF

A critical issue for the work was whether the labeling process and CE analysis of the BoNT/A assay products could be performed quantitatively. To this end, admixtures of substrate and product fragments were prepared from stock solutions, labeled with CBQCA, and analyzed by CE-LIF. The concentrations were adjusted to simulate the mole fractions of BoNT/A LC cleavage of substrate; i.e., the admixtures were prepared at constant total concentration (S + P1) of 100 μM and then diluted 1/1 with running buffer in CE. Thus the concentrations of S, P1 and P2, were given by:

$$[S] + [P1] = 100\,\text{mM}; \quad [P1] = [P2]. \tag{1}$$

where [ ] denotes concentration of the species in square brackets. The plots of fluorescence intensities obtained from the electropherograms *versus* concentration over the range 0–100 μM were shown to be linear for the CBQCA-labeled assay products, i.e., substrate, S, and cleaved peptide fragments P1, P2 (Fig. 3). For the series of P1 mole fractions from 0 to 1.0, the ratio, R_{P1}, of integrated intensity of P1, A_{P1}, normalized to the sum of the intensity of S and P1 at each mole faction, $A_S + A_{P1}$, is given in Eq. (2) as:

$$R_{P1} = \frac{A_{P1}}{A_S + A_{P1}} \tag{2}$$

and shown in Fig. 3 (inset) as a linear plot ($R^2 = 0.9935$). These results demonstrate that CBQCA labeling and CE-LIF analysis provides a quantitative method of measuring the products of BoNT/A LC reactions.

3.3. Analysis of BoNT/A LC products in the presence of combinatorial peptide libraries

CE-LIF was carried out on samples derived from actual BoNT/A LC reactions containing substrate, cleaved products and enzyme in the presence of combinatorial peptide libraries. Prior to CE analysis the entire LC product mixture was reacted with CBQCA. Thus intact S, P1, P2 plus BoNT/A and peptide libraries (if present) were rendered fluorescent. Fig. 4a shows

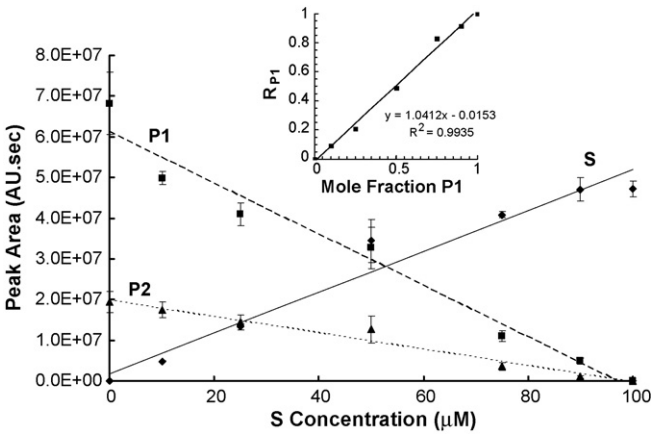

Fig. 3. Standard curves of CBQCA-labeled substrate and products separated and detected in CE-LIF. The standard curves were obtained from the peak areas (arbitrary units s) of a series of electropherograms in which S + P1 = 100 μM, P1 = P2 (see Eq. (1)). The curve ascending from left to right is the linear regression of substrate intensities ($r^2 = 0.9635$). As concentration of substrate increases from 0 to 100 μM, the concentrations of P1 and P2 decrease from 100 to 0 μM. The top curve descending from left to right is P1 ($r^2 = 0.9705$); bottom descending is P2 ($r^2 = 0.9724$). Inset. Quantitation of admixtures of intact substrate, S, and product, P1 by CE-LIF. The *x*-axis represents the mole fraction of P1, where S + P1 was constant at 100 μM, i.e., when P1 = 0.25, S = 0.75. The *y*-axis is the normalized intensity, R_{P1}, calculated according to Eq. (2). The plot was derived from data displayed in Fig. 3.

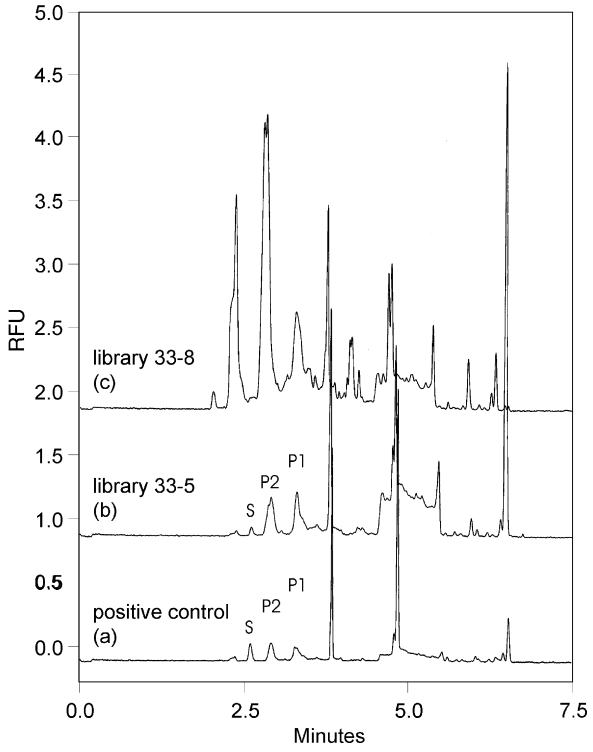

Fig. 4. CE-LIF separation and detection of products of BoNT/A LC assay in the presence of peptide libraries. Samples were labeled with CBQCA and run as described in the text. (a) Positive control sample (no library): S, P1, P2 peaks were readily assigned. (b) With library 33-5: the products were separated and resolved from the library peptides. (c) With library 33-8: products could not be separated and resolved from the library peptides.

the electropherogram of the products of a positive control assay (only substrate and activated BoNT/A LC, no combinatorial peptide library). The peaks for S, P1, P2 were readily observable. Fig. 4b and c show electropherograms of the products of BoNT/A LC assays in the presence of two representative 12-component combinatorial peptide libraries out of a large number of screenings. Most of the libraries screened did not have significant migration overlap with the assay products; the reaction products were readily separated and identified as shown by library 33-5 (Fig. 4b). We noted that the BoNT/A LC activity actually increased in the presence of library 33-5 [8]. In a few libraries we observed significant interference of co-migrating library components with the labeled assay products. The greatest amount of interference occurred with the library designated 33-8 in which the labeled constituent peptides co-migrated with S and P2 (Fig. 4c). Calculations on ExPASy [14] determined that peptides in this library carried similar mass/charge ratios as the substrate and P2 peptides (calculations not shown).

The overlap problem was resolved by reformulating the HNTE separation buffer to 50 mM sodium dodecyl sulfate (SDS) and eliminating the Tween-20 detergent. The latter buffer formulation was referred to as HNSE. The addition of SDS detergent in the buffer changed the characteristics of the separation from capillary zone electrophoresis to micellar electrokinetic chromatography (MEKC) [22,23,24]. At 50 mM concentration SDS forms micelles; the negatively charged polar sulfate head groups are on the outside of the micelle structure. SDS, being negatively charged and contained throughout the column in the separation buffer, migrates counter to the electroosmotic flow. In the presence of SDS micelles, all the peptides in the sample, i.e., substrate, product fragments, and combinatorial peptide library, were partitioned between the micelles and free solution. The most positively charged peptide had the strongest interaction with the negatively charged heads on the SDS micelles. In MEKC the longer the time a molecule is held by the micelles, the greater is the time required to reach the detector. Neutral P1 (see Table 1) migrated faster in MEKC than positively charged S or P2 as shown in Fig. 5. The run times were longer than CZE and the migration order of substrate and products to the detector was reversed. For the admixture (Fig. 5b) labeled P1 migration time was 13 min, compared to about 3 min in CZE (HNTE buffer). The P2 migrated at 18 min in MEKC; S migrated at 23 min. The migration time of labeled library 33-8 was between 25–30 min (Fig. 5c). The MEKC separation of the BoNT/A LC assay products containing library 33-8 is shown in Fig. 5a wherein S, P1, P2 were readily resolved from one another and from the components of library 33-8. Overall the resolution and speed of MEKC separation in SDS buffer was still superior to that afforded by HPLC.

4. Discussion and conclusions

The rapid analysis of the reaction products of BoNT/A LC by CE-LIF presented several advantages over HPLC. All of the reaction products were readily separated by CE-LIF within 8 min on-column time. In contrast, separation of the reaction products by HPLC and subsequent column washing required

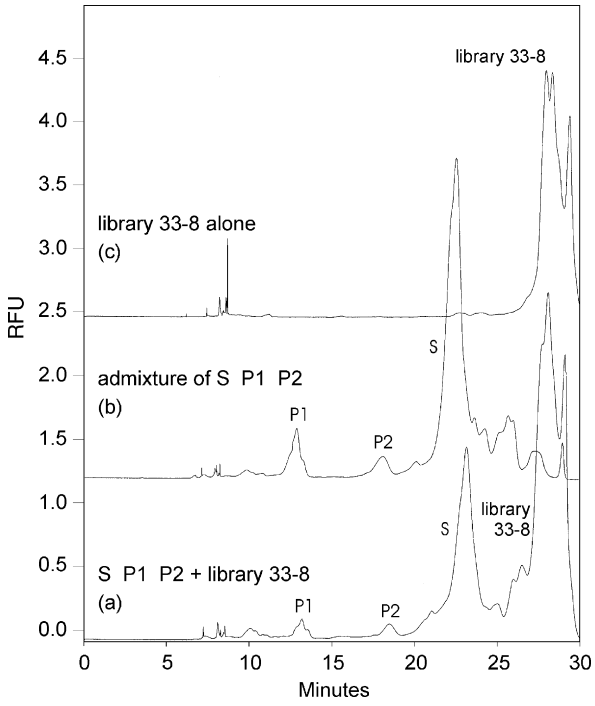

Fig. 5. MEKC separation and LIF detection of products of BoNT/A LC assay. All species were labeled with CBQCA. (a) BoNT/A LC assay products containing library 33-8 (same sample of assay products as used in Fig. 5c). With MEKC separation S, P1, P2 are separated and resolved from the peptide library. (b) Admixture of S, P1, P2, no library. (c) Library 33-8 alone, no S, P1, P2.

approximately 60 min column time, resulted in incomplete resolution products and provide less reliable quantitation of products in the presence of peptide libraries. CBQCA labeling was found to be an effective method for detection of the BoNT/A LC reaction products. An advantage of the method is that the dye itself is non-fluorescent; only upon reaction with primary amines in the presence of cyanide ion is a fluorescent adduct formed. This limits background interference from unreacted dye and eliminates the need to separate fluorescent adducts from excess labeling reagents. We observed in the electropherograms several large peaks arising from reaction of the dye with components of the buffer (HEPES, Tween-20, EDTA). We found that other CE buffers (e.g., borate) contained fewer fluorescent adducts when reacted with CBQCA (data not shown). In earlier work significant effort had been made to optimize an assay for BoNT/A LC [6,8]. In that work the reduction and activation reactions of the intact toxin were carried out in HEPES buffer. As this work was follow-on, it was not feasible to switch to a cleaner buffer system, one with non-amine-containing compounds and less reactive with CBQCA. Despite the presence of the non-identified peaks, the assignments and quantitation of the critical peaks were readily accomplished. The fact that the extraneous buffer peaks did not cause serious problems actually adds to the utility of the techniques. The fluorescent 7-aza-1-cyano-5,6-benzisoindole covalent adduct (maximum excitation and emission 450 and 550 nm, respectively) is stable for two weeks and is readily detectable with argon ion laser excitation and 520 nm detection. A caveat with CBQCA labeling is that all species containing primary amines are labeled. Multiple peaks or shoulders may be generated if an analyte contains more than one available primary amine site and labeling of sites is incomplete. For S, P1 and P2, one major peak was identified for each. In CE the bands for P1 and P2 were nonsymmetric; the shoulders suggested that heterogeneous labeling might have occurred.

In capillary zone electrophoresis mode, limited migration-overlap of the combinatorial peptide libraries with S, P1, P2 was observed, but it was not usually a significant problem. Since P1 contained a single labeling site, and it was well separated from both substrate and P2, it was chosen as a convenient reference point for assignments quantitations. We observed a few cases where overlap of the CBQCA-labeled libraries defeated resolution and quantitation by CZE of reaction products. This problem was resolved by MEKC separation. Although it required longer time, MEKC served as a useful back-up method for the difficult libraries. For this work, once the BoNT/A LC assay samples were quenched, labeled and cooled, they were stable for at least two weeks. Thus we routinely ran all samples in normal capillary electrophoresis; the difficult-to-resolve samples were subsequently re-run by MEKC.

CE-LIF presents the potential for convenient adaptation to high-throughput, multi-channel and microfluidic formats, which would further improve the ability to screen combinatorial peptide libraries for modulators of BoNT peptidase activity. To date there have been few reports regarding CE analysis of BoNT activity in the literature. The work has demonstrated the utility of CE-LIF for quantitation of BoNT/A LC activity. We are applying the results of the present work to screening and searching peptide and chemical libraries for potential inhibitors of BoNT/A. The methods are also useful for a range of proteolytic toxins, such as the other serotypes of BoNT, anthrax and tetanus.

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