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Competitive immunoassay for staphylococcal enterotoxin A using capillary electrophoresis with laser-induced fluorescence detection

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Abstract

Staphylococcal enterotoxins are a family of toxic proteins secreted by *S. aureus*. Using capillary electrophoresis (CE) linked with laser-induced fluorescence, a highly sensitive and selective assay using antibody–antigen recognition was developed for the determination of Staphylococcal enterotoxin A. Staphylococcal enterotoxin A (SEA) was chemically labeled with fluorescein and the product was used as a fluorescent tracer. A competitive assay was developed to detect SEA at concentrations between 0.3 n*M* and 6.5 n*M* with standard deviations of less than 5%. The detection limit was found to be 3 amol with the potential improvement by further optimization of the assay. No cross-reactivity between staphylococcal enterotoxin B and the SEA antibody was found at the concentrations used for the CE immunoassay. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Immunoassay; Laser-induced fluorescence; Enterotoxins; Toxins; Proteins

1. Introduction

The staphylococcal enterotoxins (SEs) are a family of serologically distinct toxins (designated A–E) secreted by various strains of *Staphylococcus aureus*. They are proteins with molecular masses of 27 000 to 30 000 and isoelectric points (p*I*) ranging from 7 to 8.6 [1]. These toxins are the primary cause of food poisoning in humans producing emesis and diarrhoea [1,2]. These biological effects make the detection of these toxins very important from the standpoint of public health concerns. Since very little toxin is needed to cause illness in humans (<1 μg/kg) [1], a sensitive and selective technique is needed. A number of modern techniques revolve around the use of immunoassays. Radioimmunoassays (RIAs) used in the early 1970s were selective and relatively sensitive (ng/ml range) but required the use of radioactive tracers of the toxins [3,4]. These techniques proved to be undesirable due to the use of radioactive materials along with being labor intensive and expensive. The enzyme-linked immunosorbent assay (ELISA) technique for SE detection has also been developed [5,6] leading to the production of a commercial assay for four of the SEs. ELISA gives many desirable features for the detection of SEs including high selectivity and sensitivity (0.1 ng/ml) and the use of non-radioactive reagents, but ELISA is still labor intensive requiring many incubation and washing steps. The focus of this work is to develop a technique that combines the selectivity of immunoassays, the separation capability of capillary electrophoresis, and the sensitivity of laser induced fluorescence for the detection of SEA. A competitive assay

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in which a tracer of fluorescently labeled SEA is used to monitor the SEA will be demonstrated in this paper. The CE separation of the labeled tracer from antibody complexes eliminates the washing steps common to ELISA, thus reducing the labor and time necessary for the assay.

2. Experimental

2.1. Instrumentation

The CE–laser-induced fluorescence (LIF) detection system used for this work has been described elsewhere [7]. Briefly, the electrophoresis was driven by a high voltage power supply (CZE1000R, Spellman, Plainview, NY, USA), and carried out in a fused-silica capillary of 40 cm×20 μm I.D.×150 μm O.D. (Polymicro Technologies, Phoenix, AZ, USA) with an electric field of 500 V/cm. The detection end of the capillary was inserted into a sheath flow cuvette that was grounded. A 4 mW laser beam from an argon ion laser (Model 2214-65, Uniphase, San Jose CA, USA) was focused with a 10×-microscope objective approximately 200 μm below the tip of the capillary. The sheath fluid, identical to the running buffer, was hydrodynamically introduced through the sheath flow cuvette at a few microlitres per hour. Fluorescence was collected using a high-numerical aperture microscope objective (60×, 0.7 NA, Universe Kogaku, Oster Bay, NY, USA), spectrally filtered with a bandpass filter (515DF20) and restricted using a 200 μm radius pinhole. The beam was then split using a polarizing beamsplitter (Melles Griot, Nepean, Canada) to two photomultiplier tubes (R1477 Hamamatsu Photonics, Japan) for measuring horizontally and vertically polarized light. The data collection from the two photomultipler tubes (PMTs) and the CE high voltage was controlled using a PCI data acquisition board and a LabView software (National Instruments, USA) in a Power Macintosh computer. Fluorescence signals from the two PMTs provided fluorescence polarization information, which was used to distinguish large molecules [e.g. antibody-bound staphylococcal enterotoxin A (SEA)] from smaller molecules (e.g. free fluorophores). Subsequently, data from only one PMT was used for quantitation.

Gel electrophoresis and transfer was performed using a mini-gel unit with a mini transfer cassette (Bio-Rad, Missisauga, Canada).

2.2. Reagents

All solutions were prepared using 18.2 MΩ distilled deionized water from a Maxima Ultra-pure Water System (Elga, Topsfield, MA, USA). Tetraborate (TB) and Tris–borate–EDTA (TBE) buffers were made from reagent grade materials. SEA, staphylococcal enterotoxin B (SEB), fluorescein isothiocyanate (FITC), rabbit SEA antibody (SEAAb), anti-rabbit IgG conjugated to horse-radish peroxidase (ARb-HRP) and 3,3′-diaminobenzidine tablets (DAB) were obtained from Sigma (Missisauga, Canada).

2.3. Procedures

2.3.1. Labeling of SEA

A 50 μl aliquot of the SEA protein (0.5 mg/ml in PBS) was introduced into a microtube. Fifty microlitres of a 10^{-4} *M* FITC solution made in carbonate buffer (pH 9.0) was added to the SEA. After mixing, the solution was allowed to incubate at room temperature for 30 min. The resulting mixture was then loaded onto a Pharmacia PD-10 column filled with G-25 gel-filtration media equilibrated with PBS. The labeled protein was excluded from the gel-filtration media and eluted using 4 ml of PBS. Purity was analyzed by gel electrophoresis with western blotting.

2.3.2. Formation of the SEA–SEAAb complex

A fixed amount of the fluorescein labeled SEA (FSEA) was added to varying amounts of SEAAb in a sodium phosphate buffer (pH 7.4) containing 0.02% sodium azide and protein stabilizer. These mixtures were allowed to incubate at room temperature for at least 30 min and analyzed by CE. Incubation for more than 30 min did not yield more of the SEA–SEAAb complex (data not shown). When an appropriate antibody:FSEA ratio was achieved, a series of samples containing FSEA+ SEAAb and unlabeled SEA was made for the competitive assay.

2.4. Gel electrophoresis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was done on the SEA, FSEA, and SEB samples. The procedure used is fully described elsewhere [8]. Briefly, 20 μl of the undiluted samples and a sample with protein standards (Low range standards, Bio-Rad) were boiled for 5 min with 20 μl of gel-loading buffer. The gel-loading buffer contained 100 m*M* Tris (pH 6.8), 200 m*M* dithiothreitol (>99%, Sigma) 4% SDS (electrophoresis grade, Sigma), 0.2% bromophenol blue (electrophoresis grade, Sigma), and 20% glycerol (ACS grade, BDH, Toronto, Canada). The samples were then loaded onto two 12% polyacrylamide gels and run at 100 V for 2 h. The first gel was stained with Commassie blue. The proteins in the second gel were electrophoretically transferred onto a poly vinylidene difluoride (PVDF) membrane (Bio-Rad) for western blotting. After the transfer, the PVDF membrane was incubated with blocking buffer containing 5% skim milk powder, 25 m*M* tris-buffered saline and 0.02% Tween-20 (enzyme grade, Sigma) to block non-specific binding. The membrane was then incubated with 1/1000 dilution of the SEAAb in blocking buffer for 2 h and then with 1/1000 dilution of the ARb-HRP (also in blocking buffer) for 45 min. The blot was then visualized after a 2 min incubation with DAB.

3. Results and discussion

3.1. Purity and reactivity of the FSEA and SEA protein

The purity and reactivity of the SEA and FSEA proteins were analyzed by SDS–PAGE. Fig. 1A shows the Commassie blue stained gel displaying the components of SEA and FSEA. The SEA lane shows the expected bands at around MW 27 000. The heterogeneity of SEA is not unexpected and has been previously shown in SEA and other SEs [1]. The SEA lane also shows the presence of another band around the MW 50 000 range. This band is likely due to the presence of Protein A which has a

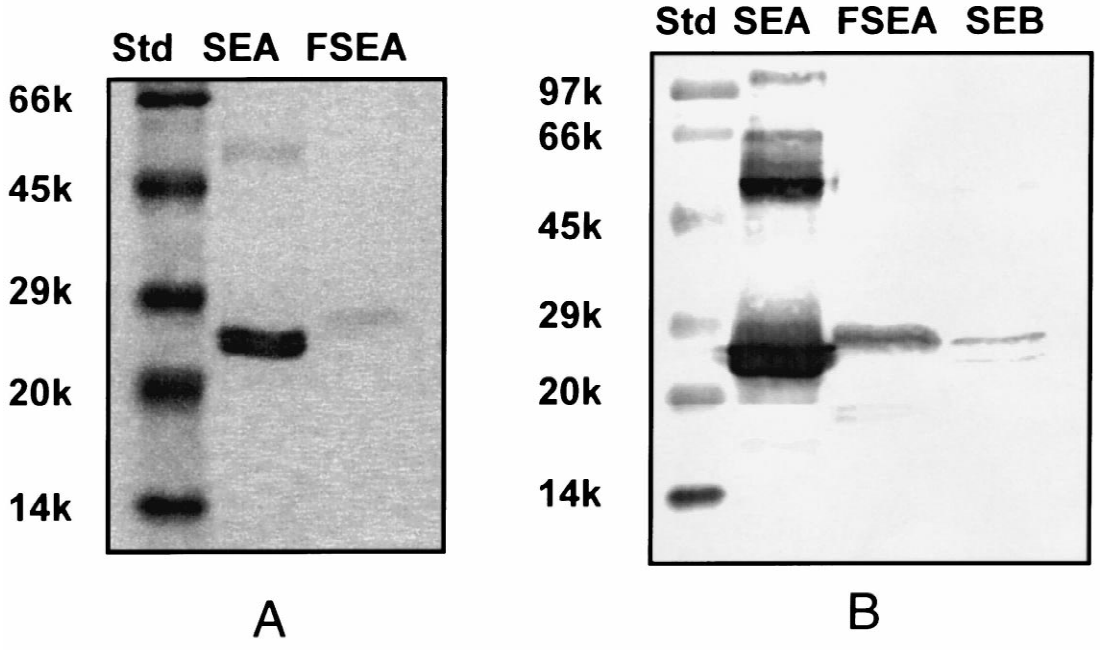

Fig. 1. SDS–PAGE of SEA and fluorescently labeled SEA (FSEA). (A) Commassie blue stained gel. (B) Western blot analysis probed with rabbit anti-SEA antibody (SEAAb) and visualized with anti-rabbit horse-radish peroxidase and 3,3′-diaminobenzidine. Each lane was loaded with 10 μl of undiluted sample. k=kilodaltons.

molecular mass around 48 000 and is also secreted by *Staphylococcus aureus* [9]. The FSEA lane shows a faint band at the expected molecular mass range (~28 000). The weaker signal was due to the dilution of the protein from the column purification process.

To investigate the activity of the SEA antibody (SEAAb) to the fluorescently labeled SEA (FSEA), western blot analyses were performed on FSEA, SEA, and SEB. Visualized components in the western blot are expected to be complexed by the SEAAb. Fig. 1B shows a series of bands for the SEA lane. A strong band was found between the MW 20 000–29 000 range representing SEA. There is also a component seen at the MW>45 000 range. This result confirms that this component is most likely Protein A as it is expected to bind to the Fc site on rabbit IgG [9]. The FSEA lane shows a major band at the predicted position (~28 000), indicating the activity of FSEA with the antibody and a second unknown band with a smaller molecular weight. The SEA antibody was also reacted with SEB and is shown in the fourth lane. The SEA antibody seems to have some cross reactivity with SEB as shown by two faint signals at the >20 000 region. These weak

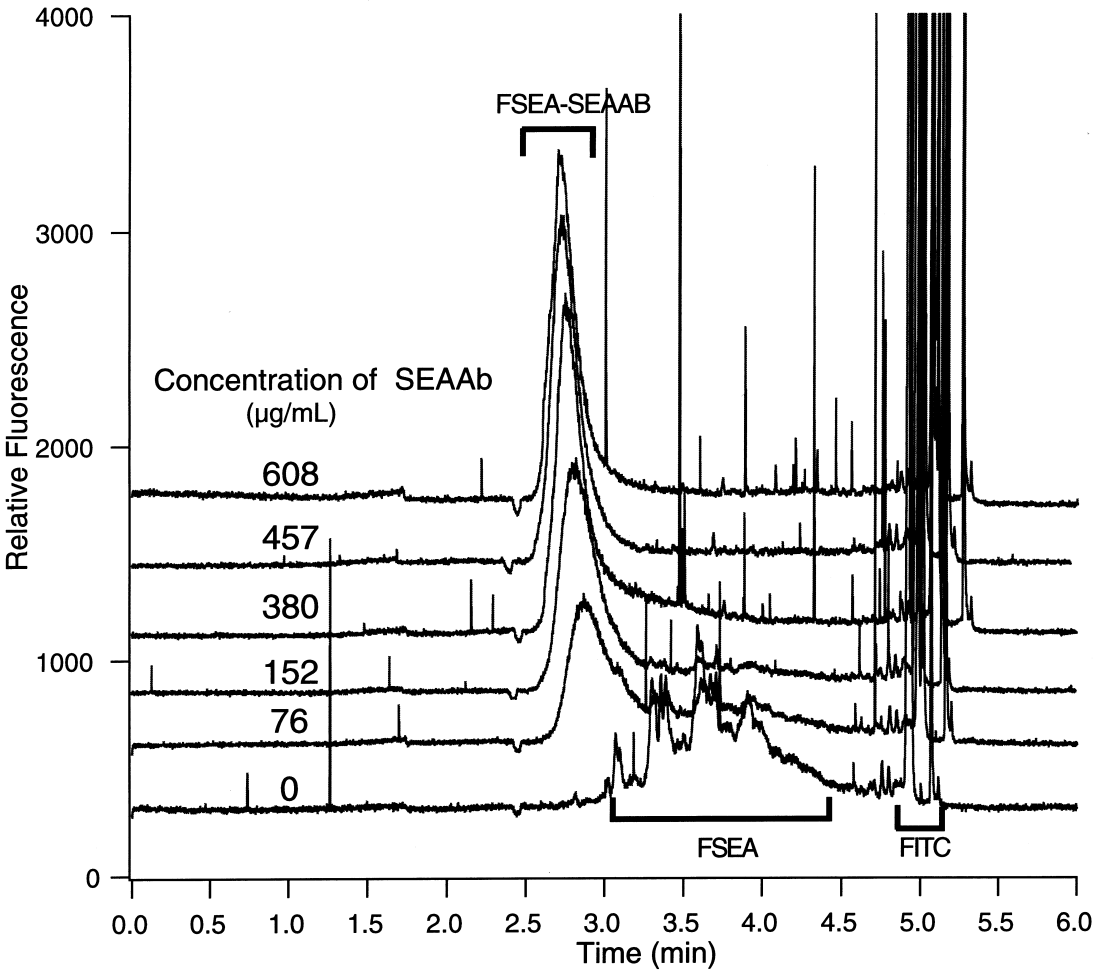

Fig. 2. Capillary zone electrophoresis analyses of fluorescently labeled SEA (FSEA) and its mixture containing varying amounts of anti-SEA antibody (SEAAb). A 40 cm capillary (20 μm I.D.×150 μm o.d.) was used for separation with an electric field of 500 V/cm. Separation buffer contained 2× tris–borate–EDTA at pH 8.5. Samples were electrokinetically injected with 10 kV for 5 s. The SEA sample was from a 250-fold dilution of the original sample.

signals however show that the cross reactivity is minimal when compared to the SEA lane.

Fig. 2 shows electropherograms from capillary zone electrophoresis analyses of fluorescently labeled SEA (FSEA) and its complex with anti-SEA antibody (SEAAb). When no SEAAb is present, the electropherogram shows a series of peaks between 3.0 and 4.5 min. This set of peaks represent FSEA as they are shown to react and disappear with the addition of SEAAb. The multiple peaks for the FSEA indicate FITC labeling at a number of different reactive sites on the SEA molecule. FITC coupling to proteins is through primary amine groups which are numerous on most proteins. Thus, the multiple labeling was expected. The sharp peaks at 5.0 min had similar migration times to those of a standard FITC solution (data not shown) and represent the free fluorophore.

3.2. Optimization of the assay

In order for the competitive assay to be quantitative, it is required that the antibody be in limiting quantity. It has also been shown that the best detection limit of competitive assays is achieved with a minimal amount of fluorescent tracer that competes for the limiting amounts of antibody [10]. To balance these two criteria, titrations of the antibody and the tracer were performed to assess the optimal concentrations of these two reagents.

The titration of the SEAAb was done using a fixed concentration of 250-fold dilution of the original FSEA. This dilution was chosen as it was the lowest concentration to give a signal that could be easily distinguished from the background. Fig. 2 shows the formation of the FSEA-SEAAb complex by the appearance of a new peak with a migration time just before the FSEA peaks. The quantitative results plotted in Fig. 3 show a characteristic titration curve with a plateau region at high SEAAb concentrations. Assuming the plateau region reflects 100% binding of the FSEA, antibody concentrations of <200 μg/ml would be reasonable starting concentrations for the assay.

Starting with an antibody concentration of 200 μg/ml, a titration of the FSEA was carried out. Fig. 4 shows the series of electropherograms. The FSEA-SEAAb complexes can be clearly distinguished from the baseline with as low as 5000-fold diluted FSEA. Fig. 5 shows that the titration curve has two distinct linear portions separated by a breakpoint located at around 1000-fold dilution. Although the best limit of detection would be located at the higher dilution factors, a better working range would lie where the dilution of the FSEA is less than 1000-fold. The sensitivity of the assay appears to be better in this

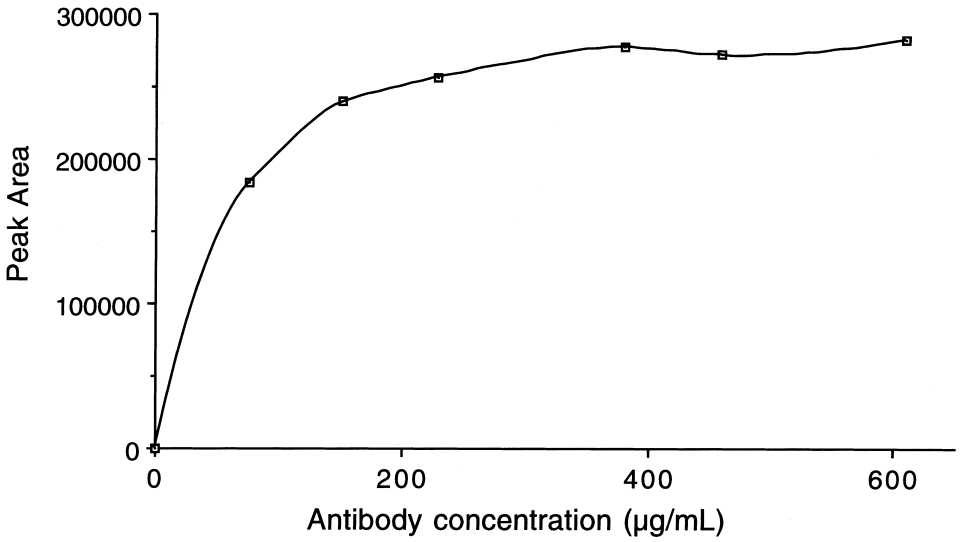

Fig. 3. Titration curve for SEAAb. All conditions were the same as shown in Fig. 2.

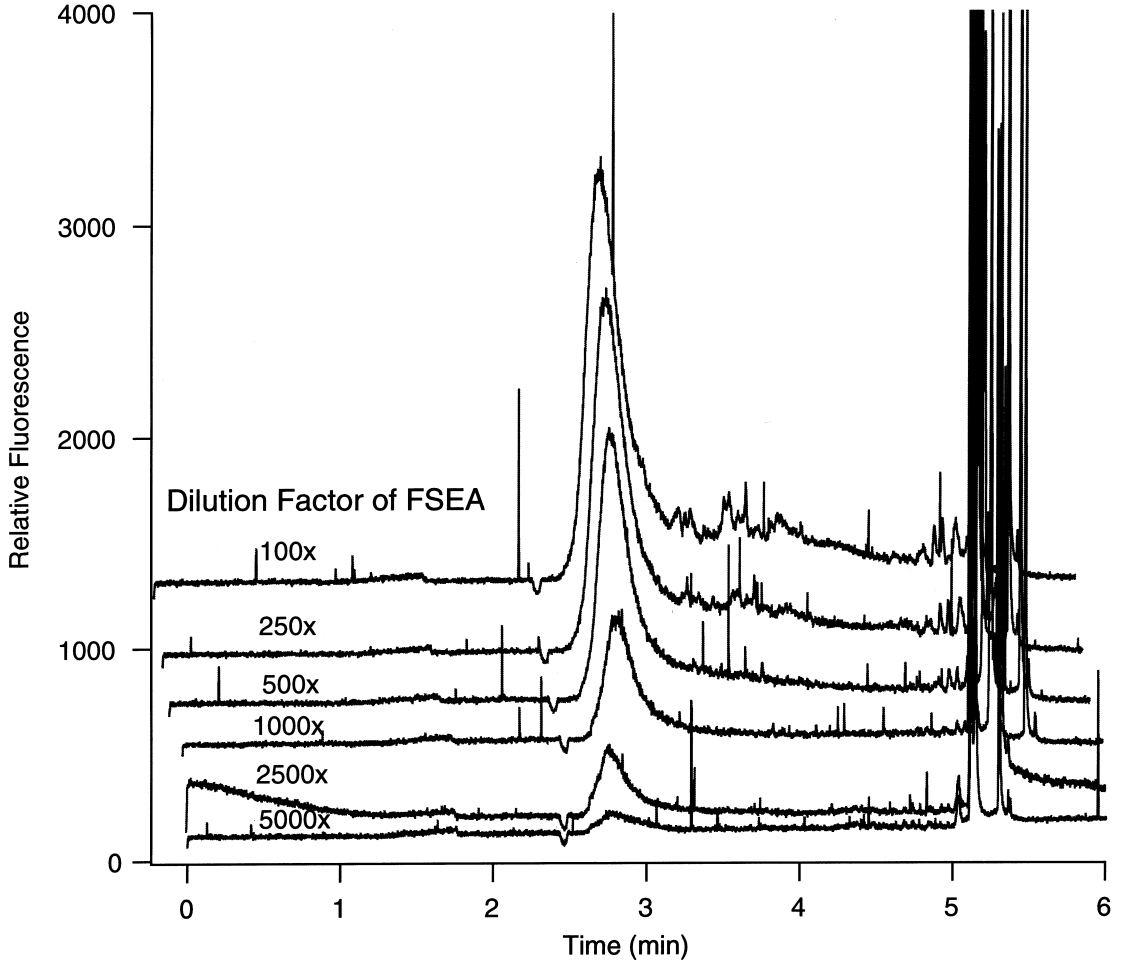

Fig. 4. Titration of FSEA. The concentration of SEAAb was 200 μg/ml for all traces. All other conditions were the same as shown in Fig. 2.

region (shown from the larger slope, giving a better analytical response). However, if low detection limits are needed, good analytical signals can be produced at the higher FSEA dilution factors (Fig. 5).

3.3. Competitive Assay for SEA

Using the results obtained in the previous sections, a competitive assay for SEA was performed. A 1/250 dilution of FSEA and 200 μg/ml of SEAAb were used as the starting reagents for the assay. The results of the assay are shown in Fig. 6. Each point on the graph represents a minimum of 3 replicates with the standard deviations shown as error bars. The graph displayed linearity between 0.3 to 6.5 n*M* of SEA added. The limit of detection for this analysis was then estimated to be around 0.3 n*M* SEA. Since the injection volume for the sample is approximately 10 nl, the mass sensitivity was calculated to be 3 amol. The limit of detection can be lowered with the use of less FSEA and antibody, however the analytical signal will be more difficult to measure thus giving poorer reproducibility. The current parameters gave a standard deviation of less than 5% as shown in Table 1 and thus may be more applicable for quantitative work. To show the selectivity of the assay, Fig. 7 compares the addition of 40 n*M* of SEB to FSEA+SEAAb with a sample containing just

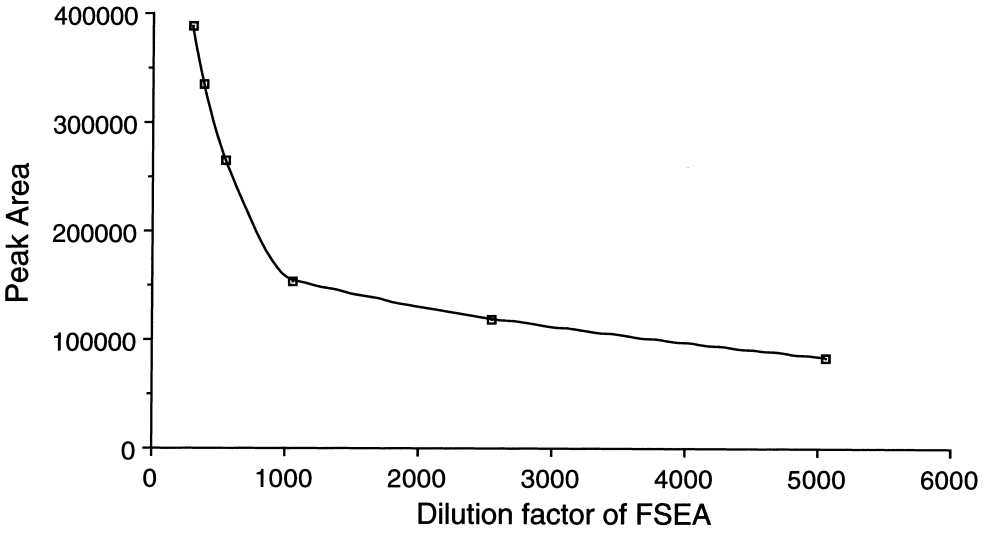

Fig. 5. Titration curve of FSEA. All conditions were the same as shown in Fig. 4.

FSEA+SEAAb. The two electropherograms in Fig. 7 are virtually identical showing no cross-reactivity of SEB with the SEAAb. This was unexpected considering the results of the western blot study, however, the concentrations used in western blot are at least 1000 times more concentrated that the CE immunoassay.

4. Conclusions

The competitive immunoassay using CE–LIF was found to be a sensitive and selective method for the determination of SEA. The assay requires very little sample preparation and minimal incubation time. The limit of detection is in the amol range with a

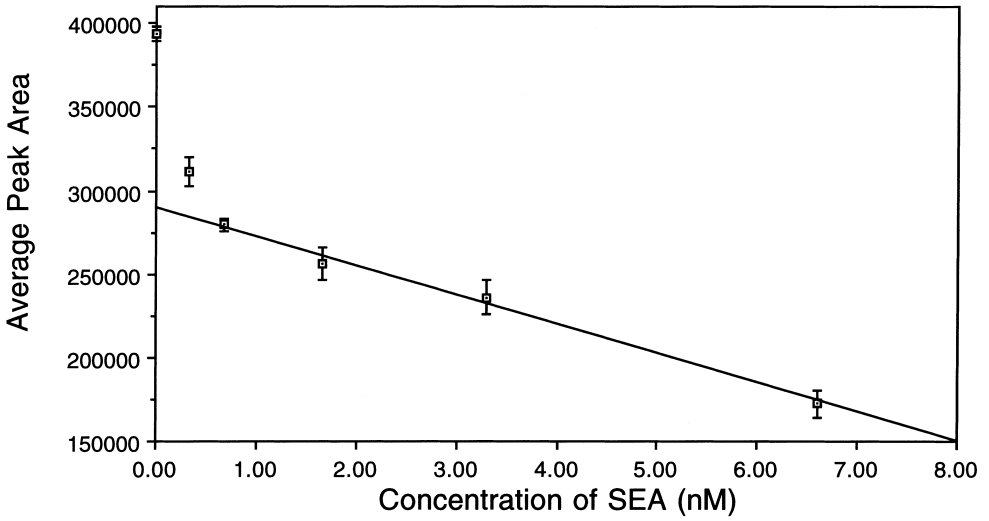

Fig. 6. Competitive calibration curve for SEA. All points represent average of at least three replicates with the standard deviation shown as error bars. Each sample contained 250-fold dilution of FSEA and 200 μg/ml of SEAAb. Linear regression line drawn through last five points. All other conditions were the same as shown in Fig. 2.

Table 1
Results from competitive assay. For experimental conditions, see Fig. 6

[SEA] (n*M*)	Mean peak area (arbitrary units)	Replicates	Standard deviation (arbitrary units)	Deviation (%)
0.00	393 576	8	4263	1.1
0.33	311 173	4	8698	2.8
0.67	279 596	4	3942	1.4
1.65	256 249	3	10025	3.9
3.30	235 794	4	10483	4.4
6.60	172 232	4	8569	5.0

standard deviation of less than 5%. This assay can be easily modified for the detection of other SEs for which there is an antibody and other toxins such as cholera enterotoxin, and botulinum toxins.

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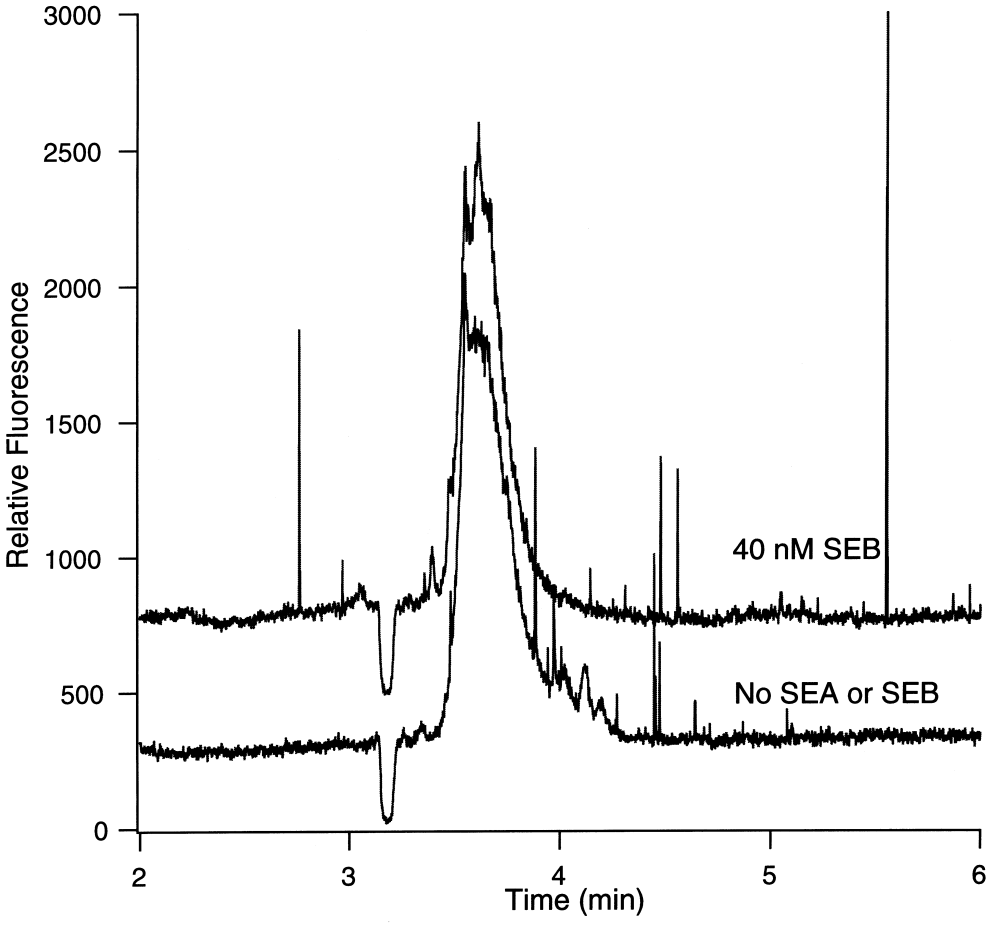

Fig. 7. Cross-reactivity of SEAAb to SEB. Each sample contained 250-fold dilution of FSEA and 200 μg/ml of SEAAb. All other conditions were the same as shown in Fig. 2.

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