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Electrochemical immunosensor for the detection of staphylococcal enterotoxin B using screen-printed electrodes

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Staphylococcal enterotoxin B (SEB) is responsible for large number of food poisoning cases throughout the world. SEB is an exotoxin and it is one of the several harmful substances produced by the bacterium *Staphylococcus aureus*. Therefore, it is required to develop methods for the sensitive, reliable, reproducible, cheaper, easy to use and rapid detection of SEB. An electrochemical immunosensor has been studied for the fast detection of SEB using disposable screen-printed electrodes. Ascorbic acid-2-phosphate (AA-2P) is used as a new substrate for the voltammetric detection of SEB. In the alkaline buffer solution the alkaline phosphate (ALP) enzymatic hydrolysis product of AA-2P is ascorbic acid (AA). Ascorbic acid is an electroactive substance and gives differential pulse voltammetric oxidative response at +380 mV (versus Ag/AgCl). The potential +0.39 V corresponds to the oxidation of AA. Indirect sandwiched enzyme linked immunosorbent assay (ELISA) has been used for the detection of SEB. In this method, anti-rabbit IgG for SEB (capturing antibody) is first immobilized on the surface of SPE followed by reacting with SEB (antigen) to form antigen-antibody complex. After that, anti-mice IgG for SEB (secondary antibody) are added, followed by ALP-conjugated anti-mice IgG (revealing antibody). The optimal conditions for ALP enzymatic reaction and the volumetric detection have been optimized. It is found that the response of voltammetric immunosensor is proportional to the SEB concentration in the range 0.1–100 ng/mL and the detection limit was found to be 0.1 ng/mL.

Keywords: Electrochemical immunosensors, Staphylococcal enterotoxin B, Enzyme linked immunosorbant assay (ELISA), Ascorbic acid, Differential pulse voltammetry

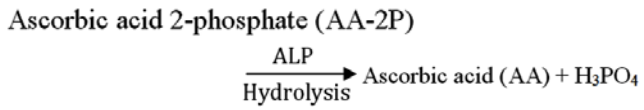
Nowadays, alkaline phosphatase (ALP, EC. 3.1.3.1) is an important enzyme that is widely used for the immunosensing of various biological warfare agents such as anthrax, H1N1 influenza, Staphylococcal Enterotoxin B (SEB), ricin, brucella, BoNT, α-1-fetoprotein, malaria etc[1-8]. In this paper, ALP is used to detect SEB which is classified as a category B type of biological warfare agent and also known as food borne pathogen. SEB causes so many diseases in human beings which depend on the route of exposure. When a person comes in contact of SEB via inhalation route the symptoms may appear like high fever, chills, muscle pain, headache, non-productive cough (which is up to four weeks), retrosternal pain, shortness of breath and at high exposure level there is shock and death[9]. But, when a person comes in contact of SEB via ingestion route the symptoms may appear like intense nausea, vomiting, cramping, abdominal pain, diarrhea etc[10]. SEB causes staphylococcal food poisoning (SFP) and it is easily soluble in water and can be easily aerosolized. The LD_{50} value for inhalation route is 20 μg/kg and the ED50 value for ingestion route is 0.3 μg/kg.

SEB is highly stable in the environment and it is a 24–29 KDa protein. It is highly resistant to temperature fluctuations such as it can withstand at boiling temperature for several minutes and in freeze dry condition can be remain active for one year. It is necessary to detect SEB below 100 ng/mL concentration because above this concentration SEB shows its effects on human beings[11]. The structure of SEB is shown in Fig. 1.

Some popular methods which are widely used for the detection of SEB are given in the Table 1. These methods mainly include multiplexed sandwich ELISA with QDs, Piezoelectric crystal immunosensor, electrical percolation-based biosensor, micro-fluidic electrophoretic chip based immunoassay, surface plasmon resonance, electrochemical detection (based on bio-magnetosomes), electrochemiluminescent-

immunosensor etc. The detection limit and the required assay time are given in the Table 1.

But, all of these methods are very costly, cumbersome and required the well-trained personnel. Enzymes are excellent biocatalyst and in order to get reproducible results and high sensitivity with accuracy, the enzymatic immunoassay is greatly preferred. ALP exists in different tissues which is one of the most important enzymes. ALP uses a wide range of substrates these are mainly phosphorylated compounds such as phenyl phosphate, p-nitrophenyl phosphate (PNPP), 1-napthol phosphate, p-aminophenyl phosphate etc.,[12-13] that can be easily hydrolyzed by ALP. In this study, ascorbic acid-2-phosphate (AA-2P) is proposed as a new model substrate for ALP activity. The enzymatic hydrolysis reaction of AA-2P is shown in the reaction as given below:



The hydrolysis product i.e., ascorbic acid (AA) is also electroactive and gives the differential pulse voltammetry (DPV) peak response at +380 mV. In this paper, this new electrochemical substrate (AA-2P) was applied for the detection of SEB at various concentrations using ALP enzymatic activity. More the concentration of SEB, more will be the ALP immobilized on the SPE and that will hydrolyze more amount of AA-2P that will give more AA and this can be detected by DPV. Under optimized conditions 0.1 ng/mL detection limit was achieved. The obtained electrochemical signals showed that the developed method having the practical utility which gives the reliable results. This strategy may find widespread and promising applications in other immunosensing systems that involve ALP. This method can also be applied to the detection of various toxins and antigens in clinical samples.

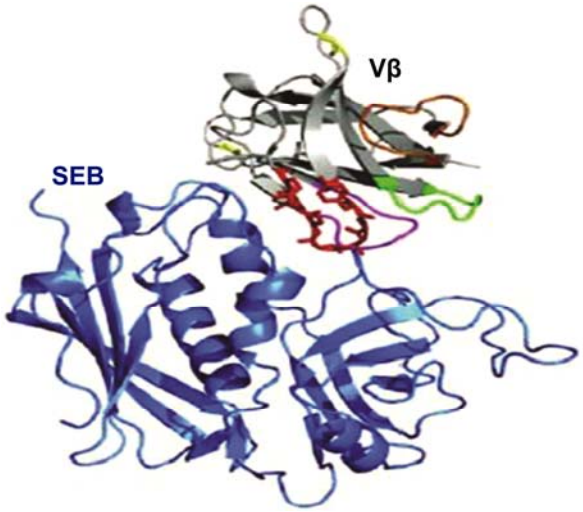


Fig. 1 — Structure of SEB.

Materials and method

Reagents and apparatus

Polyclonal rabbit anti-SEB antibodies were used as capturing antibodies, SEB was used as antigen, and monoclonal mice anti-SEB antibodies were used as secondary antibodies. These antigens and antibodies were purified and estimated in the biotechnology division of DRDE, laboratory. IgG was purified from the serum using the "protein A antibody" purification kit (Sigma Aldrich, USA). Details of experimentation are provided in the electronic supplementary data. In our laboratory, solutions of capturing antibodies, SEB and revealing antibodies were prepared in 0.1 M Tris–HCl buffer (pH 7.2). Alkaline phosphatase conjugated anti-mice IgG (Sigma code no. A-2418), ascorbic acid 2-phosphatase (AA-2P) and bovine serum albumin (BSA) was obtained from Sigma (St. Louis, MO, USA). Tris–HCl (0.1 M, pH 7.2) buffer was used for immunoreactions and washing. All chemicals those are not mentioned above were of analytical reagent (AR) grade and used without further purification. Ultrapure triple-distilled Millipore water was used to prepare all aqueous solutions throughout the experiments. AA-2P

Table 1 — Methods reported in the literature for SEB detection

S.No.	SEB methodologies	Sensitivity	Assay time	Reference
1	Multiplexed sandwich ELISA with QDs	30 ng/ mL	~1000 min	17
2	Piezoelectric crystal immunosensor	2.5 µg/ mL	~5 h	18
3	Electrical percolation-based biosensor	5 ng/ mL	~1–2 h	19
4	Micro-fluidic electrophoretic chip based Immunoassay	8.4 ng/ mL	~20 min	20
5	Surface plasmon resonance	10 ng/ mL	~10 min	21
6	Electrochemical detection (based on bio-magnetosomes)	0.017 ng/ mL	-	22
7	Electrochemiluminescent- immunosensor	0.01 ng/ mL	~3–5 h	23

solution was freshly prepared just before use to minimize the non-enzymatic hydrolysis.

Studies involving animal experimentation were conducted in compliance with the regulation of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) and have the approval of the Institutional Animal Ethics Committee (IAEC). The care and maintenance of all the animals were as per the approved guidelines of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals, India).

Electrochemical measurements were carried out with a conventional three-electrode cell. It comprises a platinum rod as an auxiliary electrode, an Ag/AgCl/saturated-KCl as reference electrode and SPE as a working electrode. An Autolab PGSTAT potentiostat/galvanostat (Eco Chemie, The Netherlands) was used for DPV and cyclic voltammetric (CV) experiments. SPEs (3 mm in diameter) prepared in house were used for the immobilization of biomolecules. A magnetic stirrer controller (model no. TH100) and a pH meter (Eutech Instruments, Singapore) were utilized in this study. All electrochemical experiments were performed at room temperature.

Preparation and affinity purification of SEB antigen

In the biotechnology division of our establishment, SEB was produced by *S. aureus* strain ATCC14458. The toxin gene was amplified for cloning and expression under the optimized conditions. A strain of *Escherichia coli (E. coli)* SG13009 (pREP4) was selected as the host (kanamycin resistant) to achieve high yield of recombinant SEB. The expressed recombinant SEB (r-SEB) was tagged with 6 x histidine for affinity purification as per manufacturer's instructions (Qiagen, Germany) and cloned in the pQE30UA vector (ampicillin resistant). Affinity chromatography method was used for the purification of r-SEB. The cell pellet was resuspended in Ni-NTA buffer (1:10 w/v, pH 8) for this purpose[9]. The recombinant protein was eluted with Ni-NTA buffer (pH 4.5). The purity of the obtained protein was checked by the SDS-PAGE method. The yield was calculated by bicinchoninic acid (BCA) protein estimation method[11]. Details of experimentation are provided in the electronic supplementary data.

Immunization of animals and preparation of antibodies against r-SEB

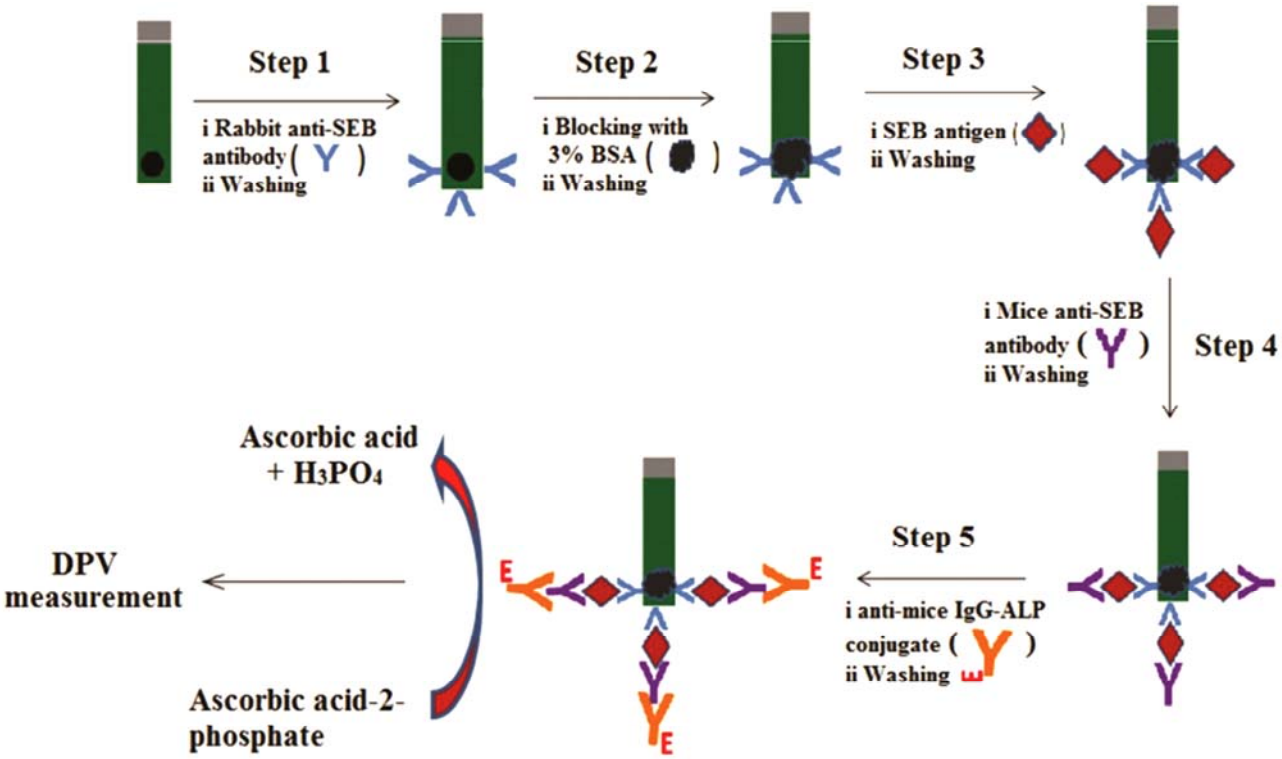
At an interval of one week (7 days) the female BALB/c mice (20–25 g) were intraperitoneally immunized. At an interval of two weeks (14 days), New Zealand White rabbits were subcutaneously immunized[9]. For the immunization, doses were given to animals and after two weeks and a final booster, animals were bled. Rabbit polyclonal antibodies and mice monoclonal antibodies were produced. The obtained antibodies were purified by affinity chromatography. Their concentrations were estimated by BCA protein assay kit. Details of experimentation are provided in the electronic supplementary data. This study had the approval of the Institutional Animal Ethics Committee (IAEC)[11].

Cross-reactivity study

To check the cross reactivity of the prepared purified antibodies, western blotting method was used. Cross-reactivity was not observed with other staphylococcal enterotoxins such as (SEA, SEC1, SEC2, SEC3, SED), *E. coli* SG13009, non-enterotoxigenic *S. aureus* strain ATCC6538P and other microorganisms, namely *Bacillus subtilis, Bacillus cereus, Enterococcus faecalis* and *Vibrio cholarae*. Hence, the produced antibodies had strong affinity and specificity for SEB detection only as compared to other commercial antiserums as available from M/S Toxin Technology, USA[9,11].

Fabrication of electrochemical immunosensors

Indirect sandwich ELISA protocol was used in this study for the electrochemical immunosensing of SEB, as shown in Scheme 1. The analytical procedure was as follows, first of all a known quantity (5 µL) of rabbit anti-SEB polyclonal antibodies (i.e., primary antibodies) were physically adsorbed on screen-printed electrode (SPE) surface at the optimized concentration of 125 µg/mL. Then, the electrodes were kept at 37 °C for 1 h in the incubator. Physical adsorption is possible due to the roughness of SPE electroactive surface where the antibodies were immobilized. We report here that there is no significant effect of physical adsorption on antibody binding activity, analytical sensitivity, limit of detection, stability and performance of the immunosensor. Physical adsorption is successfully done on directly putting the primary antibodies on SPE surface. After the incubation electrodes were washed with (Tris-HCL buffer, pH 7.2, 0.1 M) for three times to remove the unbound antibodies. 3% BSA in (0.1 M Tris-HCl, pH 7.2) was used to reduce the nonspecific binding effect. The third step is to incubate with 5 µL of diluted concentrations of SEB antigen in buffer solution for 15 min at 37 °C. This results in the formation of stable immunocomplex i.e., selective



Analytical principle for the electrochemical immunosensing of SEB using indirect sandwich ELISA protocol in buffer medium. (Step 1) Physical adsorption of primary antibodies i.e., Rabbit anti-SEB antibodies, (Step 2) Blocking with 3% BSA, (Step 3) Target antigen i.e. SEB and formation of immunocomplex, (Step 4) Addition of secondary antibodies i.e., Mice anti-SEB antibodies and formation of sandwich immunocomplex, (Step 5) Incubation with revealing antibodies i.e., ALP conjugated anti-mice IgG and finally (Step 6) i.e., DPV based detection.

Scheme 1

antigen-antibody interaction. In the next step, 5 µL of secondary antibodies i.e., mice anti-SEB monoclonal antibodies were added at the optimized concentration of 100 µg/mL. This was followed by incubating the electrodes for 15 min with alkaline phosphatase conjugated anti-mice IgG (1:10 dilution) at 37 °C. Electrodes were washed with 0.1 M Tris-HCl buffer of pH 7.2 after each and every step to remove the unbound biomolecules. In the next step, the substrate AA-2P is added for the ALP catalyzed hydrolysis reaction which gives the product ascorbic acid (AA) which can be determined by the DPV measurement.

Electrochemical detection

SEB antigen is quantified by indirect sandwich ELISA method and the amount of ascorbic acid formed during the enzymatic reaction is directly proportional to the amount of SEB captured in the sandwiched immunoassay. All the electrochemical measurements were performed at 25 °C using autolab PGSTAT potentiostat/galvanostat workstation. A conventional three electrode system, comprising a platinum wire as counter electrode, an Ag/AgCl (sat. KCl) as reference electrode and SPE as working electrode. These electrodes were used throughout the experiments. All the potentials mentioned are referred to the Ag/AgCl electrode used in this paper. DPV measurements were carried out in Tris-HCl electrolyte buffer solution (pH 7.2) over a scan range from 0.0 to 1.1 V with a pulse width 0.06 s, pulse period 0.2 s, potential step of 5 mV, amplitude of 25 mV and at a frequency of 15 Hz. The obtained current signal was used for the quantitative analytical measurement for the immunosensing of SEB.

Results and discussion

Optimization of substrate (AA-2P) concentration

DPV experiments were performed to optimize the substrate (AA-2P) concentration at which it shows maximum peak currents for the ALP activity. For this purpose, standard solution of AA and AA-2P was prepared at various concentrations such as 5 mM,

10 mM and 15 mM in Tris-HCL buffer. This buffer medium without AA-2P was used as blank. The prepared solutions and buffer itself were scanned in the potential range from 0 to 1.1 V (vs. Ag/AgCl) and the results are shown in Fig. 2. The oxidative peak was observed at +0.39 V and +0.86 V for AA & AA-2P, respectively. It is found that the maximum peak current response was obtained at 10 mM AA and 10 mM AA-2P concentration and this is optimized for the further electrochemical immunosensing studies.

Cyclic voltammetry and Differential pulse voltammetry analysis

The electrochemical behavior of AA and AA-2P was determined by cyclic voltammetry and differential pulse voltammetry analysis on the screen-printed electrodes (SPE) in the Tris-HCl buffer medium and the result are shown in Fig. 3. It is clearly shown in both the Fig. 3a and 3b that there is wide range of potential difference between the substrate AA-2P and the product AA. Since the product AA could be easily detected by the electrochemical methods without the interference from the substrate i.e., AA-2P. The oxidative peak current was decreased with the repetitive scans at the multiple sweep CVs of AA and AA-2P solution. It occurs due to the formation of oxidative product of AA which is adsorbed on the SPE surface and caused the fouling of the electrode surface. For AA-2P electrochemical analysis, the active area of electrode surface was decreased due to the electro-polymerization of phenolic radicals[12].

In order to get the reproducible results the electrode should be used only one time for the analysis. SPEs are cheaper and because of this they can be used for the detection of any antigen in the field. Since, the detection of SEB is highly selective and sensitive and this is one of the most suitable strategies for the determination of SEB concentration with good reproducibility and the fast DPV technique gives the current response within 5 s.

Differential Pulse Voltammetric (DPV) detection of SEB

DPV experiments were performed for the detection of SEB at various concentrations which is captured in the sandwich immunosensing layer. It is observed that

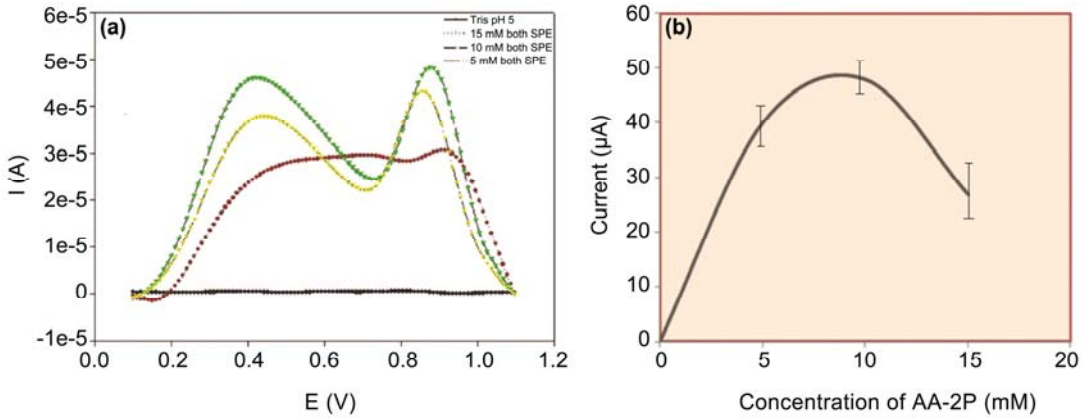


Fig. 2 — (a) Optimization of the solution having equal concentration of AA and AA-2P and (b) The calibration curve.

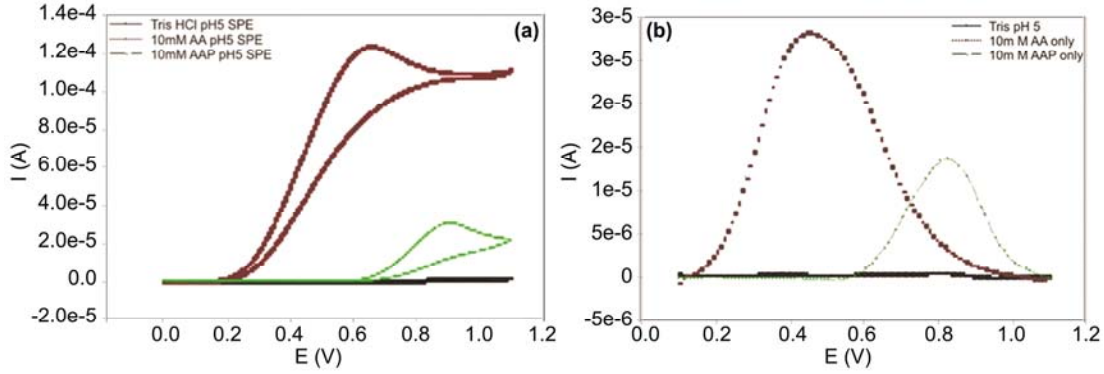


Fig. 3 — (a) Cyclic voltammograms (CVs) for 10 mM AA, 10 mM AA-2P and blank with the SPE in 0.1 M Tris-HCl buffer solution of pH 5 and (b) is the Differential Pulse Voltammograms for 10 mM AA, 10 mM AA-2P and blank with the SPE in 0.1 M Tris-HCl buffer solution of pH 5.

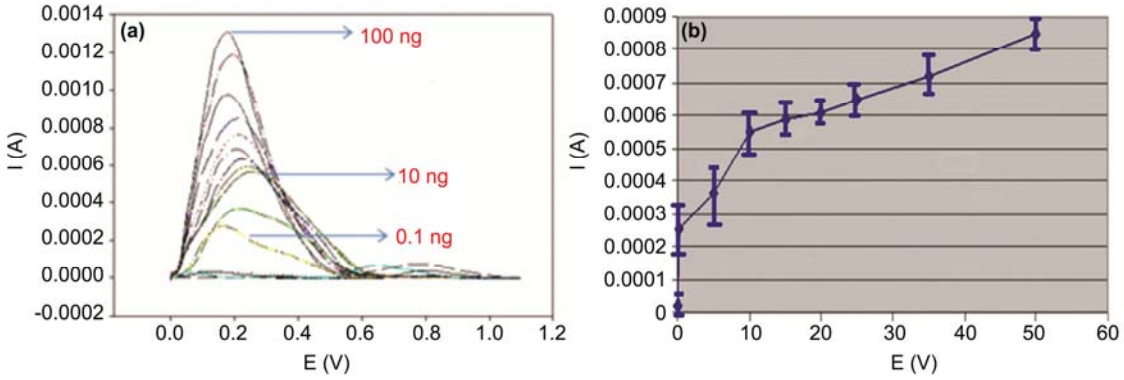


Fig. 4 — (a) Oxidation current measured by DPV with various concentrations of SEB in 0.1 M Tris-HCl buffer solution of pH 7.2 and (b) Calibration graph of current measured and SEB concentration.

the voltammetric peak current is increased with increasing the concentration of SEB and under optimized conditions 0.1ng/mL detection limit was achieved (Fig. 4). A small oxidative peak current was observed at +0.86 V (shown in Fig. 4a) which is due to the remaining AA-2P that is not hydrolyzed in the enzymatic reaction. At higher concentrations of SEB, this peak was not observed which is due to the complete hydrolysis of AA-2P by the ALP catalytic reaction. It is important to note that the oxidative peak of AA-2P do not affect the detection of SEB because it is measured only by the product i.e., AA. Since, the ALP shows maximum catalytic activity in the alkaline medium therefore, the pH of Tris-HCL buffer medium in these immunosensing studies is 8.8. The total immunosensing analysis required only 2 hr. In order to get the reproducible result, substrate AA-2P is freshly prepared in the immunosensing experiments. SEB antigen was detected in the range of 0.1–100 ng/mL under optimized conditions.

Reproducibility and stability

Many washing steps are required in the ELISA protocol and the SPE is needed to be handle very carefully otherwise it may produce imperfections in the immunosensing layer that may also vary the results. Various concentrations of SEB i.e., 0.1 ng/mL, 1 ng/mL, 5 ng/mL, 10 ng/mL, 15 ng/mL, 20 ng/mL, 25 ng/mL, 35 ng/mL, 40 ng/mL, 50 ng/mL, 75 ng/mL, 80 ng/mL and 100 ng/mL were used for the immunosensing. To find out the reproducibility, simultaneously three experiments were performed separately for the detection of 10 ng/mL SEB concentration on different SPE. The relative standard deviation (RSD) was calculated and it is found to be less than 5%, which signifies that the developed method shows acceptable and satisfactory reproducibility and can be applied for the real sample analysis of any antigen.

The cut-off value is calculated by the formula given by Snyder *et al.*,[16]:

Cutoff value = (3×standard deviation) + mean absorbance of blank i.e., (3×0.00005 + 0.00003 = 0.00018).

The readily achievable detection limit was found to be 0.1 ng/mL of SEB with this method. Electrode-to-electrode reproducibility was established because of very low signal variation and use of same batch of designed SPE. The dilution used for the ALP conjugated anti-mice IgG is also stable when they are not in use and stored them at 4 °C. No significant signal variation was observed after three weeks when using the same batch of biomolecules. This is very much promising approach for the determination of SEB for practical applications and security purpose from biological warfare agents[14,15].

Conclusions

This paper has established the practicability for the detection of SEB using ALP enzymatic activity by differential pulse voltammetric method with ascorbic acid 2-phosphate (AA-2P) used as a new electroactive, commercially available, inexpensive and environmental non-toxic substrate. The results show that AA-2P can be used as the suitable substrate for the routine ALP immunoassay with satisfactory results. The detection limit obtained by this method for SEB is also acceptable and quite useful. This paper provides a potential for ALP based electrochemical immunoassay for the analysis of SEB with disposable SPE as a working electrode and can

also be used for developing an electrochemical immunosensor for on line measurement of any antigen or analyte of interest.

Supplementary Data

Supplementary data associated with this article are available in the electronic form at: http://nopr.niscair.res.in/jinfo/ijca/IJCA_59A(02)174-180_SupplData.pdf.

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