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Study on Electrochemical Immunoassay methodology for Protein A: A modified approach towards detection and quantification of *Staphylococcus aureus* in food samples

Majumdar Tania, Agarwal Shubhra, Chakraborty Runu and Raychaudhuri Utpal*
Department of Food Technology and Biochemical Engineering Jadavpur University, Kolkata-700032, INDIA

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Abstract

In the present work, an amperometric electrochemical detection and quantification of Staphylococcus aureus (S.aureus) present in food samples have been done. The method was based on sandwich enzyme immunoassay (EIA) technique where the enzyme label was used to catalyze the dephosphorylation of substrate NADP$^+$ to NAD$^+$. NAD$^+$ so formed then catalytically activates an NAD$^+$-specific redox cycle by incorporating an enzyme amplification step with ethanol, diaphorase and alcohol dehydrogenase. The NADH so formed further reduces the mediator, ferricyanide which induces a redox cycle at Platinum (Pt) electrode. The response obtained had a linear relationship to the increasing concentration of the protein antigen of S.aureus NCIM 2602 in pure culture as well as in artificially contaminated food samples. The study was also extended for naturally contaminated milk samples and S.aureus contamination for this case gave similar result as of the known strain. The detection limit was found to be 10 CFU/ml of S.aureus.

Keywords: Protein A, *S.aureus* NCIM 2602, sandwich electrochemical immunoassay, amperometric detection, food samples.

Introduction

Research on microbial safety implied in the areas like food industry, water and environment quality control and clinical diagnosis[1]. Among these, the food industry is the area where most attention has been focused[2]. Contamination of food by pathogenic microorganisms results in various foodborne diseases[3]. There are some microorganisms that can survive even in pesticide contaminated site[4]. The microbiological safety of food products are considered very important for both the human and animal food chains[5]. *Staphylococcus aureus* (*S.aureus*) is one of the major food borne pathogens and is a leading cause of gastroenteritis resulting from the consumption of contaminated food. *S.aureus* is also responsible for suppurative infections such as boils, abscesses and wound infections[6]. The ability to determine the occurrence of food contamination due to foodborne pathogens at every stage of food production, processing, and distribution is crucial to improving the safety of our food constituents[7]. The test for the presence of microorganisms in drinking water was performed by conventional microbiological method like most probable number (MPN) determinations[8]. Other conventional microbiological methods based on plate count, biochemical tests, isolation and enrichment procedures are time consuming, labour intensive and inefficient. For food samples like milk, meat, cheese etc rapid and sensitive methods of analysis are required[9]. A successful method for detection of such pathogens is by applying the specific antibody against the antigen produced by the pathogen and further by adding an enzyme conjugated specific secondary antibody to detect the target antigen. This method is called 'sandwich' enzyme linked immunoassay[10]. However, in this method an upstream cell enhancement procedure has to be added to detect low numbers of bacteria in food[11,12]. Therefore to increase the sensitivity of the immunoassay procedure and make developments facilitating their ready automation, immunoassay was combined with electrical methods. This combination forms the basis of immunosensors[13], which have been used for the detection of various antigens with high sensitivity and specificity[14-20].

An amperometric electrical immunoassay procedure specific for protein A with the strains *Staphylococcus aureus* NCDO 949, NCDO 1022 and NCDO 2044 was developed[9]. Protein A (SpA), cell wall protein of *S.aureus* has got high binding affinity for Fc region of human Immunoglobulin G (IgG)[21] and interferes with the immune response. SpA is present in around 95% of all pathogenic strains of *S.aureus*[22]. The molecular size ranges from 50-60 kDa[23-26].

In the present work, a study on electrochemical detection system with good stability for quantification of a specific pathogenic strain of *S.aureus* NCIM 2602 has been made. Signal response variation against the variation in pH, temperature and incubation time was obtained at Platinum (Pt) electrode by using an amperometric biosensor detector. The study was made for pure culture of *S.aureus* as well as artificially contaminated food samples. A parallel observation was obtained for naturally contaminated milk sample.

Material and Methods

Reagents and Chemicals: Human Immunoglobulin G (IgG) (Sigma), rabbit anti-protein A antibody (Sigma), anti-rabbit immunoglobulin G alkaline phosphatase (AP) conjugate (Santa

cruz Biotechnology), nicotinamide adenine dinucleotide phosphate (NADP) SRL, diaphorase (Sigma), alcohol dehydrogenase (SRL), diethanolamine, Tween 20, lysostaphin (Sigma), Dnase I (Fermentas), phosphate buffer saline (PBS, pH 7.5), Bovine serum albumin (BSA) (sd fine-chem), sodium citrate buffer (pH 7.5), Potassium ferricyanide ($K_3[Fe(CN)_6]$) (Rankem), mannitol salt agar media (Himedia).

Food samples: Milk was procured from Mother Dairy, Kolkata, cheese (Amul, Gujarat) and goat meat (local market, Kolkata).

Electrodes and Apparatus: All electrochemical measurements were performed in a three electrode arrangement including a platinum (Pt) working electrode, Ag/AgCl reference electrode and a Pt counter electrode. Electrodes were procured from bioanalytical systems (BASI, USA). Surface diameter of Pt working electrode was 1.6 mm. amperometric methods of detection were followed in all experimental measurements using amperometric biosensor detector (ABD), Model No. 3001 (Universal Sensors Inc, USA).

Isolation of protein: Pure culture of *S.aureus* NCIM 2602 was grown overnight in nutrient broth medium at 37^0C with shaking. Then bacterial culture was serially diluted and 10 µl of samples were plated on nutrient agar media and incubated for 24 hours at 37^0C for enumeration of colonies. Target antigen, protein A (SpA) was isolated from *S.aureus* NCIM 2602 using lysostaphin and Dnase I[27] and subsequently estimated by lowry method[28] and the molecular weight was determined by sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE)[29]. The protein was stored at – 70^0C for future use.

For use as test antigen, 10 g or 10 ml of food samples were inoculated with 0.1 ml of overnight grown *S.aureus* culture and incubated at 37^0C overnight. To 10 g or 10 ml of inoculated food sample, 90 ml of 0.01 M sodium citrate buffer (pH–7.5) with 0.05% (vol/vol) Tween 20 was added and homogenized for 2 min. Total protein was estimated by lowry method[28]. Further serial dilutions were prepared and boiled for 15 min to denature enzymes and liberate protein A in the solution[9]. For naturally contaminated milk sample, 10 µl of one day old milk sample (kept at 4 ^{0}C) was plated on mannitol salt agar media (MSA), selective media for *Staphylococcus* and incubated at 37^0C for overnight. Colonies were observed on MSA plate. Loopful of bacteria was added to nutrient broth and incubated at 37^0C with shaking for overnight. SpA was isolated, estimated, identified by the above mentioned procedure for *S.aureus* NCIM 2602 and diluted with 0.01 M sodium citrate buffer (pH–7.5) and 0.05% (vol/vol) Tween 20 to get different concentrations of the protein. The samples thus prepared were then subjected to amperometric measurements.

Amplified Electrochemical Immunoassay procedure: The immunoassay procedure was based on sandwich configuration involving four different stages[9], some modifications have been made to the protocol. 1ml of Human IgG (5 µg/ml) in PBS (pH 7.5) was coated on to the wells of sterile flat bottom 12-well, 3 ml capacity (Nunc) plates and allowed to adsorb antibody overnight at 4^0C. The plates were washed three times with PBS containing Tween 20 (PBST). 0.5% BSA in PBS (blocking agent) was added to each well then and incubated for another 1h. After the wells were washed with PBST thrice, 1ml of isolated SpA prepared in 0.01 M sodium citrate buffer (pH 7.5) containing Tween 20 with variable concentrations were added to the wells and given an incubation of 1h with gentle shaking at 30^0C. Target antigen was absent in control wells. After the wells had been washed thrice with PBST, 1ml of rabbit anti-protein A antibody (20 µg/ml) in PBST was added to each well and incubated for 1 h at 30 ^{0}C with gentle shaking. The wells were again washed with PBST, 1ml of anti-rabbit immunoglobulin G-AP conjugate (1.2 µg/ml) was added to the well and incubated again for 1 h. After the wells were washed with PBST, 200 µl of NADP (substrate) solution (240 µg/ml in 50 mM diethanolamine hydrochloride buffer [pH 9.8]), 600 µl of the amplifier solution (25 mM sodium phosphate buffer [pH 7.2] containing 40 µl of ethanol/ml, 150 µg of alcohol dehydrogenase/ml, 150 µg of diaphorase/ml) and 400 µl of $K_3Fe(CN)_6$ mediator (3 mM) in 0.1 M Tris hydrochloride buffer (pH 7.5) were added to the wells and incubated for 20 min at 30^0C. Figure- 1 describes the mechanism for amperometric response obtained against protein A bearing *S.aureus*[9]. Similar assay procedure was applied for pure culture of protein A bearing strain of *S.aureus*, food samples inoculated with *S.aureus* as well as food sample naturally contaminated with *S.aureus* and further examined for the presence of test bacteria by plate counts and the MPN method. The minimum protein concentration subjected to amperometric measurement was 5µg/ml which corresponded to approximately 10 CFU/ml.

Amperometric measurements: After addition of the reagents (substrate, amplifier and mediator) the assay was adapted for electrochemical detection with Pt as working electrode and Ag/AgCl as reference electrode. $K_3Fe(CN)_6$ acted as a redox mediator between the NADH (reduced) and the Pt electrode. After incubation period was over the electrodes were inserted into each well with particular concentration in turn with a potential applied at +800 mV with respect to the reference electrode[9]. The electrode was stabilized before every run and background solution current (a steady current when test sample was absent) was deducted with the zero adjustment. After every amperometric measurement obtained, electrodes were washed properly with distilled water. Time period of 5 min was fixed as response time to get a steady amperometric signal output.

Optimization of working variables: To determine optimum working conditions of the electrochemical assay procedure, effect of pH of the detection solution (sodium citrate buffer) on the amperometric response was evaluated over 6.5 to 8.5 range, incubation temperature of the reaction mixture was varied from 15^0C to 40 ^{0}C and the incubation time with the mediator was varied from 5 min to 30 min. All the experiments were performed in triplicate and statistical analysis done (significance level p value < 0.05).

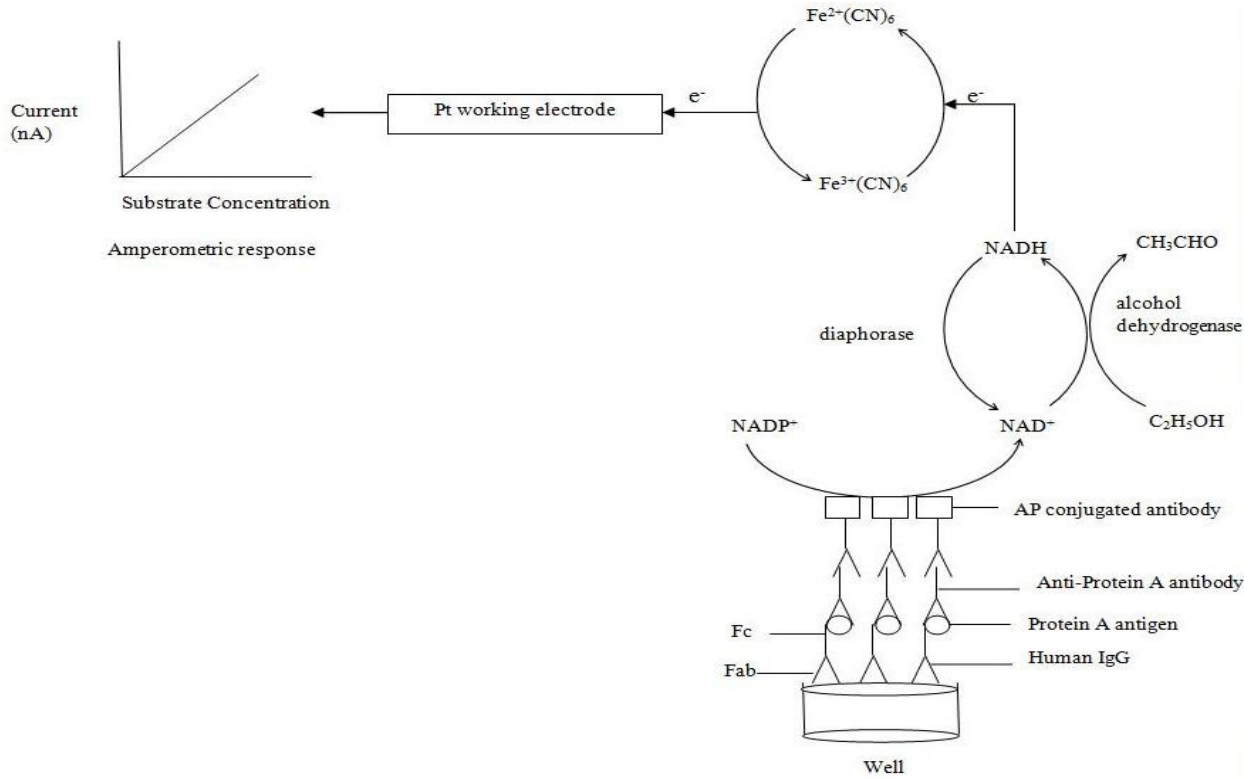

Figure- 1
Schematic representation of the mechanism for amperometric response obtained against protein A bearing *S.aureus*[9]

Results and Discussion

SDS-PAGE analysis of *S.aureus* NCIM 2602 cell wall protein: After estimation of the total protein content of cell wall by Lowry method, the protein antigen sample was loaded to the wells of 7.5% gel. Figure- 2 shows the band obtained for protein extract from *S.aureus* NCIM 2602 varied between 86-47 kDa, it goes well with the molecular size of standard cowan I SpA, suggesting the bands represent SpA only.

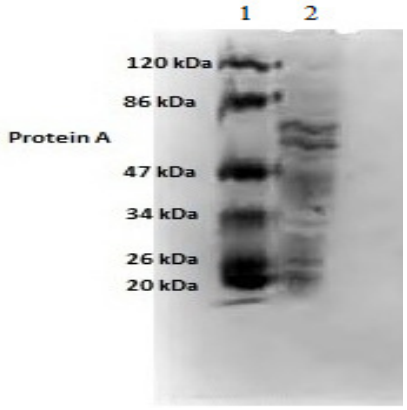

Figure- 2
SDS-PAGE (7.5% Gel) of cell wall extract from *Staphylococcus aureus* NCIM 2602. Lane 1 shows marker; lane 2, protein extract from *S.aureus* NCIM 2602

Optimization study: The pH of the detection solution containing the protein antigen affects the antigen-antibody reaction, thus the effect of pH values varying from 6.5 to 8.5 on the electrochemical response was studied in this work. Figure- 3a shows current response gradually increased with gradual increase in pH from 6.5 to 7.5. However, further increase in pH value resulted in sharp decrease of current response. Thus pH 7.5 was found to be optimum and used throughout the experiment.

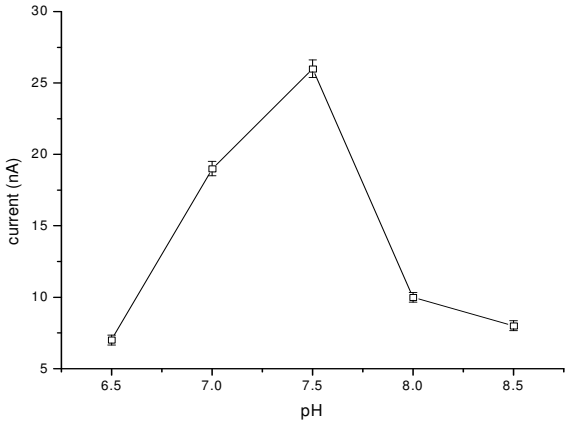

Figure- 3a
Effect of pH of the detection solution on electrochemical response

The effect of different incubation temperatures of the reaction mixture on the amperometric response was investigated from 15^0C to 40^0C of temperature shown in figure- 3b. It was found that the current response increased upto 30^0C, after which the response decreased, indicating that the temperature beyond 30^0C destroyed the immunocomplex so formed. Therefore 30^0C was found to be favorable as the incubation temperature.

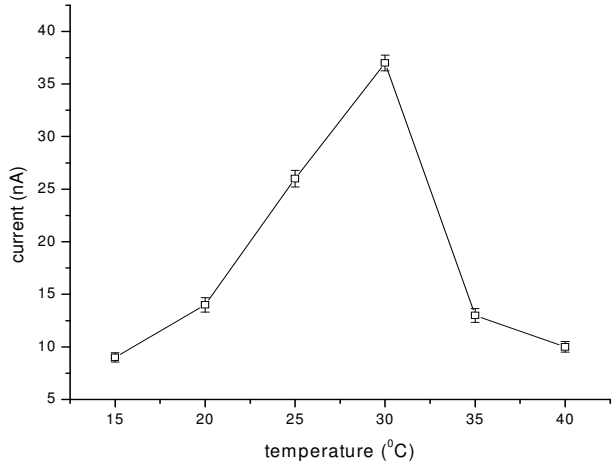

Figure- 3b
Effect of incubation temperature of the reaction mixture on electrochemical response

The incubation time also influenced the electrochemical response for pathogen detection. Figure- 3c shows that the current response gradually increased with increase in incubation time upto 20 min. Longer incubation time did not cause further increase in response current indicating that the specific binding of antigen and antibody has reached equilibrium. Thus the optimum incubation period was set at 20 min for incubation with the redox mediator.

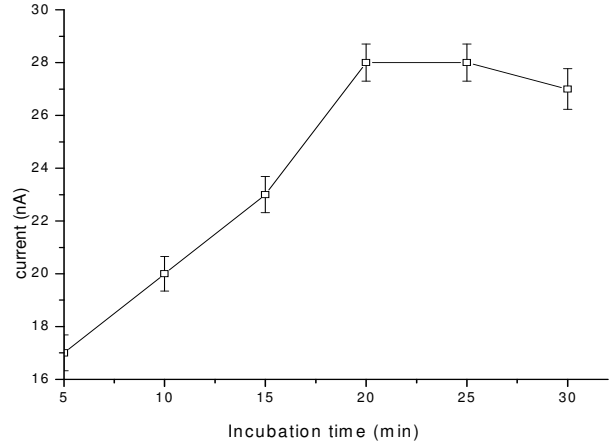

Figure- 3c
Effect of incubation time with $K_3Fe(CN)_6$ mediator on electrochemical response

Amplified Electrochemical Immunoassay: Applying the amplified sandwich enzyme linked immunoassay method for electrochemical detection was quite reproducible. Protein A from pure culture of *S.aureus* NCIM 2602 could be reliably quantified at 5µg/ml and the detection limit was at the level of 10 CFU/ml. Figure- 4a shows the electrochemical response had a linear relation with increasing concentration of protein A (R^2 = 0.96). Ferricyanide mediator was readily reduced by the NADH produced and reoxidized at the Pt electrode surface subsequently. After the electrodes were dipped in the reaction mixture polarization of the electrodes occurred leading to an initial increase in current output.

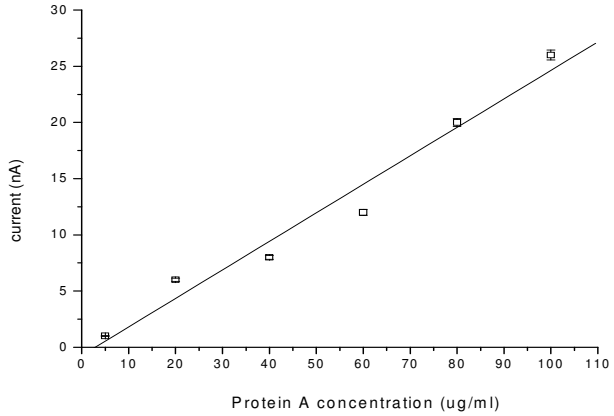

Figure- 4a
Linear Calibration plot corresponding to current responses for different protein A concentration by the amplified electrochemical immunoassay

Figure-4b represents the quantification of pure culture of protein A bearing strain *S. aureus* NCIM 2602. R^2 value of 0.98 shows a good linearity between current generated and logarithmic scale of colony count of *S.aureus*. The detection was quite sensitive at the level of 10 CFU/ml and hence quite reliable.

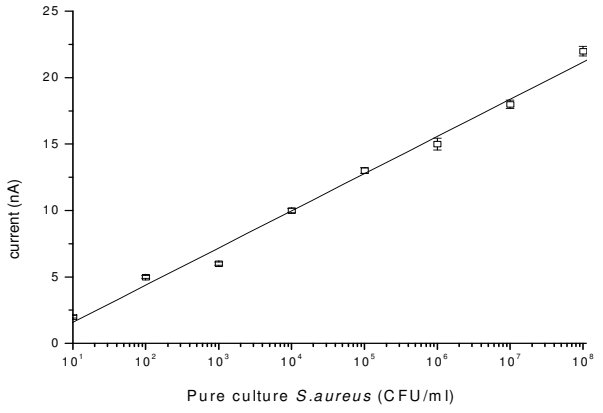

Figure- 4b
Relationship between final current generated and pure culture of *S. aureus* NCIM 2602 by the amplified electrochemical immunoassay

Application to food samples: Food samples inoculated with *S.aureus* NCIM 2602 showed good linear range in figure-5, achieving detection limits approximately 10 CFU/ml. The linearity obtained for inoculated milk (R^2 = 0.98), cheese (R^2 = 0.97) and goat meat (R^2 = 0.98) is comparable with the response obtained from pure culture strain, suggesting that there was no significant interference by the food constituents.

Figure-6 shows a linear relationship between current generated and concentration of protein A from *S. aureus* in naturally contaminated milk sample (R^2 = 0.97). The milk sample was found contaminated with *S.aureus* even when kept in the refrigerator at 4^0C for one day and could be detected with same sensitivity applying amplified electrochemical immunoassay.

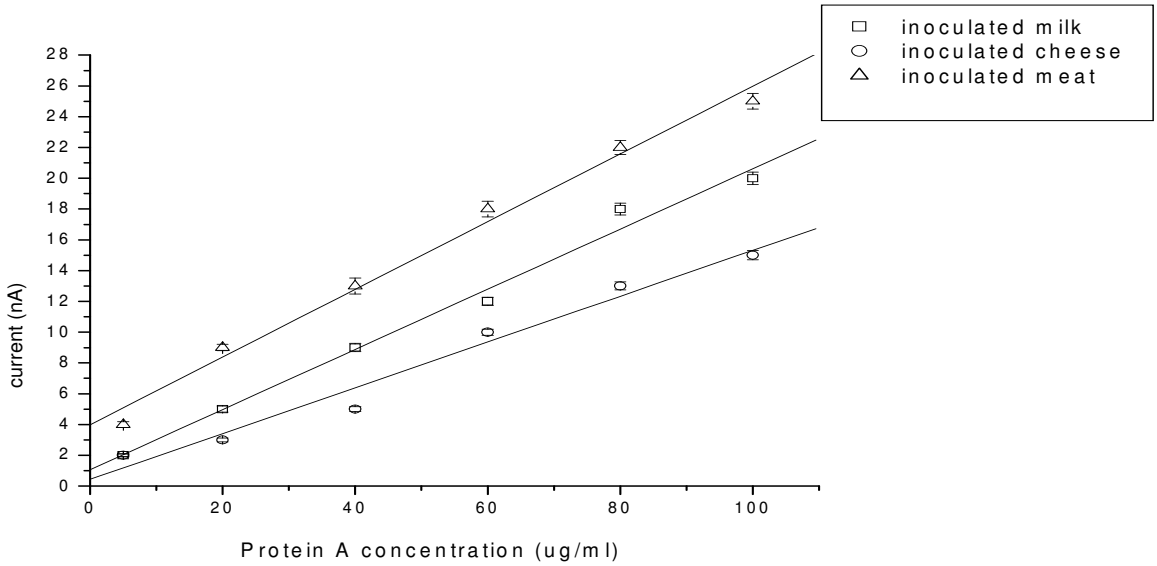

Figure- 5
Relationship between final current generated by the amplified electrochemical immunoassay specific to protein A from *S. aureus* NCIM 2602 inoculated into □ milk, ○ cheese and △ goat meat

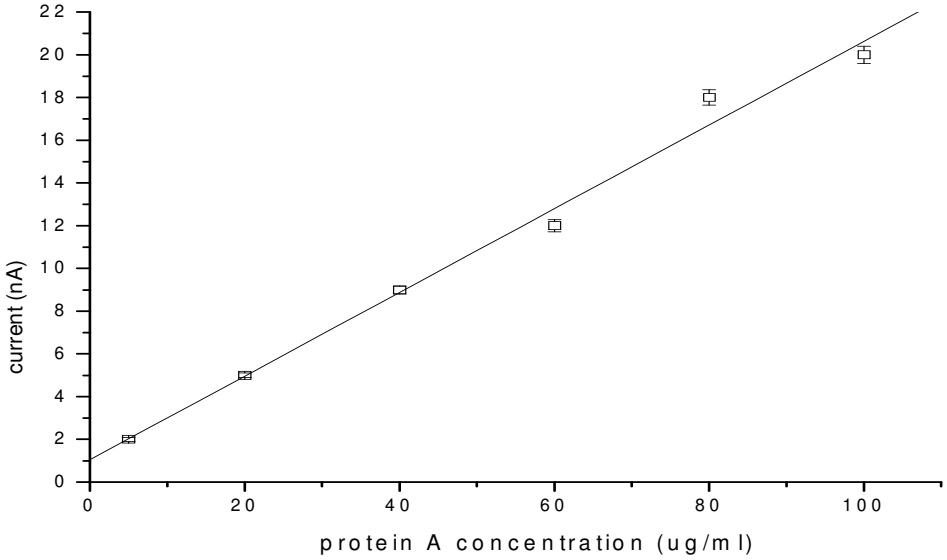

Figure- 6
Relationship between final current generated by the amplified electrochemical immunoassay specific to protein A from *S. aureus* in naturally contaminated milk

Conclusion

In this study, the target antigen isolated from *S.aureus* has been confirmed as protein A by SDS-PAGE for amperometric electrochemical immunoassay. Optimization study further provided a favorable condition for the assay to be carried out more precisely. In all the inoculated food samples of milk, cheese and goat meat as well as naturally contaminated milk nearly same degree of sensitivity has been found. Such detection methods are also applicable to heat processed foods, as target molecule protein A is heat stable. Therefore the electrochemical assay procedure has been proved to be rapid, reliable and reproducible one and can be applied to detect other pathogenic microorganisms by the use of antibodies against specific antigen.

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