POLISH JOURNAL OF FOOD AND NUTRITION SCIENCES
Pol. J. Food Nutr. Sci. 2003, Vol. 12/53, SI 2, pp. 61-63

ELECTROCHEMICAL GENOSENSORS FOR DETECTION OF *L. MONOCYTOGENES* AND GENETICALLY-MODIFIED COMPONENTS IN FOOD

Marta Ligaj[1], Tomasz Oczkowski[1], Joanna Jasnowska[1], Wojciech Grzegorz Musiał[2], Marian Filipiak[1]*

[1]Chair of Biochemistry and Microbiology, Faculty of Commodity Sciences, Poznań University of Economics, Poznań, Poland;
[2]Department of Biotechnology, Adam Mickiewicz University, Poznań, Poland

Key words**:** carbon paste electrode, DNA biosensors, GM food, hybridization, intercalators, *L. monocytogenes*

An electrochemical method for analysing sequence-specific fragments of genes from *Listeria monocytogenes* and genetically-modified components in food is presented. This method was applied for the detection of listeriolysin (*hlyA*) gene, present only in pathogen strains of *L. monocytogenes* and phosphotransferase neomycine (*nptII*) gene coding for neomycin/kanamycin resistance, which is often utilized for the production of transgenic plants. The analytical procedure consisted of four steps: single-stranded DNA (ssDNA) probe immobilization at the controlled potential of +0.5 V on the surface of carbon paste electrode (CPE); hybridization between the immobilized probe and the target complementary sequence in the sample; association of double-stranded (dsDNA) binding compounds (daunomycin and methylene blue), and amperometric detection by square-wave voltammetry.

INTRODUCTION

Electrochemical transducers combined with a nucleic acids layer produce a new kind of affinity biosensors. One of their interesting possible applications is the testing of food for the detection of some analytes interacting with DNA, like carcinogens, mutagenic pollutants, antibiotics, heavy metals, *etc*. The DNA-trapped compounds can either be detected directly if they are electroactive or *via* changes in electrochemical DNA signal [Wang *et al.*, 1996a, b; Marazza *et al.*, 1999a]. The DNA-based biosensors may also be used for the detection of sequence-specific deoxyribonucleic acid fragments [Palecek *et al.*, 2002a, b; Wang & Kawde, 2002; Wang *et al.*, 2003; Pividori *et al.*, 2001]. DNA hybridization sensors use ssDNA oligonucleotide probe immobilized on the electrode surface. Such modified electrode is immersed in the investigated solution with target DNA and when the sequence of probe and target DNA are complementary, DNA duplex is formed at the electrode surface. Hybridization may be detected by some indicators, usually DNA binding intercalators associating selectively with dsDNA structure. Typical indicators are daunomycin, methylene blue, metal coordination complexes (osmium, ruthenium or cobalt complexes with bipyridile or phenanthroline), acridine dyes, benzamide dyes, ferrocene derivatives, *etc*. [Marazza *et al*., 1999b, 2000; Kara *et al*., 2002; Rodriguez *et al*., 1990; Millan *et al*., 1993].

In this paper, some preliminary results on two DNA genosensors (hybridization sensors) useful in food analysis were reported. One biosensor was destined for detection of *L. monocytogenes* – a typical example of food pathogens. The second biosensor was designed for the detection of some genetically-modified (GM) components in foods. The majority of GM plants have been transformed with constructs containing the P-35S promoter (CaMV) and T-35S (CaMV) or T-nos (*Agrobacterium tumefaciens*) terminators, genes coding for resistance to ampicillin (*bla*) or neomycin/kanamycin (*nptII*) antibiotics (as marker gene). Consequently, detection of these target sequences is a simple way of screening for genetically-modified material [Holst-Jensen et al., 2003]. In our experiments, *nptII* gene fragment was detected as one out of the "screening genes". The hybrids were detected with the use of electrochemically-active intercalators, daunomycin for *hlyA* and methylene blue for *nptII* genes.

MATERIAL AND METHODS

Fragments of *hlyA* and *nptII* genes preparation. Strains of *L. monocytogenes* used for isolation of hlyA gene were a kind gift from the Chair of Microbiology and Biotechnology, Agricultural University in Poznań. For DNA isolation from *L. monocytogenes*, AT Biotechnology kit was used. The target (fragment of *hlyA* gene) was amplified by PCR reaction. The amplification was performed in a DNA Thermal Cycler (PTC-100, MJ Research) using primers with the sequence: 5'CTGCAAGTCCTAAGACGCCA and 5'GTCACTCATCTCCGTGGTATACTA. Each amplification reaction was carried out in a 25 µL final volume containing: 0.2 U *Taq* DNA polymerase, 5 ng of bacterial DNA, 1x*Taq* buffer solution (Finnzymes OY), 5 mmol/L $MgCl_2$, 200 µmol/L of each deoxynucleoside triphosphate and 2 µmol/L of each primer. The amplification was performed during 30 cycles (95°C for 30 s, 60°C for 30 s, 72°C for 30 s) after the first denaturation at 95°C for 5 min. The PCR product (115 base pairs) had the sequence: 5'CTGCAAGTCCTAAGACGCCAATCGAAAAGAAA-

*Author's address for correspondence: Marian Filipiak, Chair of Biochemistry and Microbiology, Faculty of Commodity Sciences, Poznań University of Economics, Al. Niepodległości 10, 60-967 Poznań, Poland; tel.: (48 61) 856 95 36; e-mail: marian.filipiak@ae.poznan.pl

CACGCGGATGAAATCGATAAGTATATACAAGGATTG-GATTACAATAAAAACAATGTATTAGTATACCACGGA-GATGCAGTGAC. For the detection of *hlyA* gene from *L. monocytogenes*, 27-oligonucleotide probe with the sequence: 5'GATTTCATCCGCGTGTTTCTTTTCGAT was used.

A fragment of *nptII* gene (92-mer with the sequence: 5'CGAGGCCGCGATTAAATTCCAACATGGATGCT-GATTTATATGGGTATAAATGGGCTCGCGATAATGTC-GGGCAATCAGGTGCGACAATCTAT), which was used as the target, was a kind gift of the Department of Biotechnology, Adam Mickiewicz University in Poznań. The ssDNA probe for the detection of *nptII* gene was 21-mer: 5'GACTA-AATATACCCATATTTA.

All stock solutions were prepared using deionized and autoclaved water. Daunomycin, methylene blue, other chemicals, calf thymus DNA, DNA probes and primers were obtained from Sigma.

Procedures and measurements. Square wave voltammetric experiments were performed with a μAutolab electrochemical analyzer (Eco Chemie, The Netherlands) controlled by the GPES 4.8 software. Analytical procedure was performed in 2.5 mL cylindrical glass cell. A three-electrode system consisted of a carbon paste working electrode (CPE), an Ag/AgCl reference electrode and a platinum wire counter electrode. The carbon paste made of 70:30 g/g graphite powder/mineral oil (Sigma) was prepared in Teflon body, giving a 1 mm diameter disk surface. A copper wire inserted into the carbon paste provided the electrical contact. Before each use, the carbon electrode surface was polished on a mat glass to obtain smooth surface.

The CPE was pretreated by applying +1.7 V for 1 min in 0.2 mol/L acetate buffer solution (pH 4.7) with the addition of 0.01 mol/L KCl**.** The ssDNA specific probes for *hlyA* or *nptII* genes (5 μg/mL) were immobilized on CPE surface by applying a potential of +0.5 V for 2 min in a stirred acetate buffer solution. The electrode was then rinsed with 2xSSC buffer solution (300 mmol/L sodium chloride, 30 mmol/L sodium citrate; pH 7.4) for 10 s. The CPE electrode with immobilized DNA probe was immersed for 2 min into 2XSSC buffer solution containing the target DNA (*hlyA* or *nptII* genes at the concentration of 1 μg/mL). In this period, the hybridization between the DNA in the sample and the probe on a CPE occurred. After hybridization, the electrode was rinsed with 0.02 mol/L Tris-HCl buffer solution (pH 7.4) for 10 s. The hybridization reaction was performed with the samples containing the targets *hlyA* or *nptII*, respectively and in a solution containing noncomplementary sequences (calf thymus DNA). Daunomycin and methylene blue were chosen as indicators of hybridization reaction. The CPE electrode was immersed into a stirred 10 μmol/L daunomycin solution (for the *hlyA* gene) or 20 mmol/L MB solution (for the *nptII* gene) in Tris-HCl buffer for 2 min and washed for 5 s. The voltammetric measurements were performed with square wave voltammetry (SWV). The raw data were treated using the Savitzky and Golay filter (level 2) of the GPES software, followed by the moving average baseline correction, using a "peak width" of 0.01.

All experimental procedures were carried out at room temperature.

RESULTS AND DISCUSSION

The presence of the *hlyA* gene from *L. monocytogenes* was detected using daunomycin solution as an intercalator. The oxidation peak of daunomycin was observed at the potential of +0.65 V and the current of this peak increased when a sample contained *hlyA* gene fragment (Figure 1, upper line). When a sample contained noncomplementary sequences (calf thymus DNA), the catalytic wave at + 0.65 V was lower (Figure 1, lower line), although the current of the peak is generated probably not only by daunomycin adsorbed directly on the porous surface of the carbon electrode but also by some nonspecific adsorption of intercalator on the probe immobilized on the CPE surface [Marazza *et al.,* 2000]. Unfortunately, this process limits the sensitivity of the method; however, the biosensor is able to distinguish complementary and noncomplementary sequences in the investigated sample.

The presence of *nptII* gene, typical of GM food was monitored using 20 mmol/L methylene blue as an indicator generating at SW voltammogram a peak at the potential of –0.4 V. Electrochemical signal of this indicator was decreased when a sample contained a target complementary sequence of *nptII* gene comparing to a sample devoid of this sequence (Figure 2).

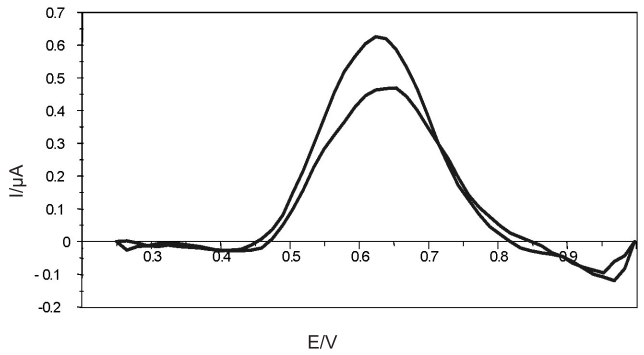

FIGURE 1. Square-wave voltammograms of 10 μmol/L daunomycin at DNA modified CPE after interaction with dsDNA – upper line (*hlyA* gene fragments present in the sample) and with ssDNA – lower line (calf thymus DNA fragments present in the sample).

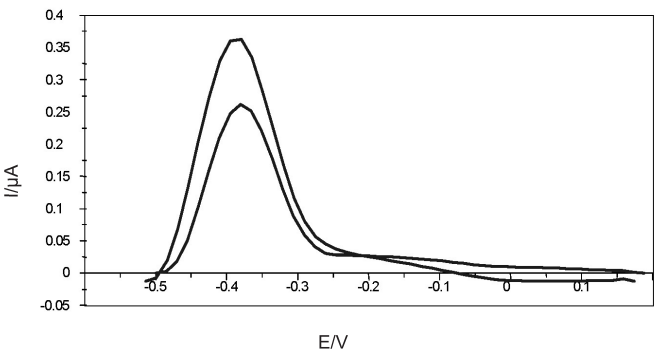

FIGURE 2. Square-wave voltammograms of 20 mmol/L methylene blue at DNA modified CPE after interaction with dsDNA – lower line (*nptII* gene fragments present in the sample) and with ssDNA – upper line (calf thymus DNA fragments present in the sample).

Most of the intercalators generate higher voltammetric peaks after interaction with dsDNA formed as a result of hybridization of probe with a target sequence. The detection of DNA hybridization with the use of MB looks different. Methylene blue reveals high affinity towards the guanine bases in ssDNA immobilized on the carbon electrode which offers an easier access of the guanine bases than dsDNA. Therefore, the accumulation of methylene blue on the electrode results in higher electrochemical peak when ssDNA is present on the electrode surface (Figure 2, upper line). Consequently, hybridization reflects in decreasing MB peak on SW voltammogram (Figure 2, lower line) [Kara *et al*., 2002; Erdem *et al*., 2000].

CONCLUSIONS

It was concluded that the applied indicators both daunomycin and methylene blue gave promising results and can be very helpful in the detection of hybridization with the use of electrochemical biosensors.

DNA biosensors can be applied for the detection of some genes specific to bacterial pathogens and genetically-modified components in foods. Although PCR methods are generally accepted as the most sensitive and reliable methods for the detection of GM-derived material, they are not useful for routine testing because they require skillful technicians and also because many components of food may inhibit the enzymatic reactions and lead to false results [Holst-Jensen *et al.,* 2003; Rijpens *et al.,* 2002]. Simple electrochemical genosensors presenting high specificity and compatibility with modern microfabrication and miniaturization technologies might be a good alternative to PCR methods in DNA diagnostics [Palecek, 2002].

ACKNOWLEDGEMENTS

This work was in part supported financially by KBN (Polish State Committee for Scientific Research), project 3P06T00522.

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