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RESEARCH ARTICLE

Trace Determination of DNA in an Amaryllidaceae Plant, *Narcissus tazetta* by Square-wave Stripping Voltammetry based on Guanine Signal

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Trace Determination of DNA in an Amaryllidaceae Plant, Narcissus tazetta by Square-wave Stripping Voltammetry based on Guanine Signal

Summary

This study is, to our knowledge, the first application of electrochemical DNA biosensor for the quantification of DNA in plant extracts. The DNA biosensor was assembled by immobilizing the double-stranded fish sperm DNA on the surface of a single-use pencil graphite electrode. Square-wave voltammetric method with the baseline correction was carried out to evaluate the oxidation signal of the guanine base. The experimental variables such as solution pH, buffer concentration, immobilization time, stirring speed, and square-wave parameters were optimized. The extremely low detection limit (0.36 ng/mL) was coupled to a good surface-to-surface reproducibility (a relative standard deviation of 8.4% for 7 repetitive measurements of 40 ng/mL). The renewable and low-cost DNA biosensor developed in this study using pencil graphite electrode was applied to the determination of DNA isolated from an Amaryllidaceae plant, Narcissus tazetta.

Key Words: *Electrochemical DNA biosensor, pencil graphite electrode, square-wave stripping voltammetry, plant samples, Narcissus tazette subsp. tazetta.*

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Kare-dalga Sıyırma Voltametrisi ile Guanin Sinyali üzerinden Amaryllidaceae Bitkisi, Narcissus tazetta'da İz DNA Analizi

Özet

Bu çalışma, bilgimize göre, bitki özütlerinden DNA tayini için elektrokimyasal DNA biyosensörünün ilk uygulamasıdır. DNA biyosensörü, tek kullanımlık kalem grafit elektrot yüzeyinde çift iplikçi balık spermi DNA'sının tutturulması ile oluşturulmuştur. Guanin bazının yükseltgenme sinyalini değerlendirmek için zemin düzeltmesi yapılmış kare-dalga voltametrik yöntem kullanılmıştır. Çözelti pH'si, tampon derişimi, tutturma süresi, karıştırma hızı, kare dalga parametreleri gibi deneysel değişkenler optimize edilmiştir. Oldukça düşük gözlenebilme sınırı (0.36 ng/mL), yüzey tekrarlanabilirliği (40 ng/mL derişiminin ardışık yedi ölçümü sonucunda bağıl standart sapma değeri, %8.4) ile birleştirilmiştir. Bu çalışmada kalem grafit elektrotu kullanarak geliştirilen yenilenebilir ve ucuz DNA biyosensörü, bir Amaryllidaceae bitkisi olan Narcissus tazetta'dan izole edilen DNA'nın miktar tayinine uygulanmıştır.

Anahtar Kelimeler: *Elektrokimyasal DNA biyosensörü, kalem grafit elektrot, kare-dalga sıyırma voltametrisi, bitki örnekleri, Narcissus tazette subsp. tazetta*

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INTRODUCTION

The bioactive compounds of Amaryllidaceae species, which is one of the largest families of ornamental bulbous plants, are known to possess a wide variety of biological activities including antitumor, antiviral, cytotoxic, anti-inflammatory, antinociceptive, anticholinergic and DNA-binding activities (1). In view of the potential biopharmacological importance of this family, it is worthwhile to determine the amount of DNA in its species for future studies and potential biotechnological applications perhaps in biochemistry, medicine and genetics. The largest genus of Amaryllidaceae, *Narcissus* is naturally grown as well as cultivated in some parts of Turkey (2).

Determination of nucleic acids is of great importance in molecular biology, biotechnology and other areas (3,4). The commonly used methods of DNA quantification include: (i) assessment of band intensities on an agarose gel, (ii) absorbance measurements by UV

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spectrophotometry, and (iii) fluorescent dye measurement with different DNA-binding dyes. Measurement of absorbance has been the most common and simplest laboratory technique to calculate nucleic acid concentrations for about 65 years. The absorbance at 260 nm of 1 in a 1 cm path-length cuvette approximately equals a DNA concentration of 50 ng/µL for double stranded DNA (dsDNA) and 40 ng/µL for single stranded DNA (ssDNA) and RNA. However, absorbance methods are limited in sensitivity due to the high level of background interference (limit of quantification approximately 250 ng/mL in traditional spectrophotometers) (5). Furthermore, A260 measurements do not distinguish between dsDNA, ssDNA and RNA. Despite the fact that DNA quantification based on fluorescent dyes like SYBR-green is very sensitive, fluorometric methods are not well suited if the origin of DNA is uncertain, because all kinds of DNA from animals, plants, bacteria, etc, are detected in the same sample (6,7). The DNA quantification methods such as slot blot hybridization method, AluQuant and quantitative polymerase chain reaction (qPCR) measure DNA concentration indirectly in a sample. Since qPCR, sometimes referred to as real-time PCR, is the most accurate, precise and efficient method, it is becoming more widely used (8).

Modern electroanalytical techniques have been proven to be very useful in this area (9,10). Compared with other methods, electrochemical techniques are characterized by simplicity, cheapness, and fast detection, and they require a small amount of sample, thus offering advantages over commonly used assays (both biological and chemical). Using the stripping voltammetry based on monitoring of the guanine oxidation has paved the way for increasing the role of electroanalysis in modern DNA diagnostics and nucleic-acid research (11-13). It is well known that between the four nucleobases, guanine has the highest electron density and lowest oxidation potential, making it the preferred target (14). In a DNA electrochemical sensor, DNA (a probe) is immobilized on a working electrode to create a recognition element. The immobilization amount of the DNA probe will directly influence the accuracy, sensitivity, selectivity, and life of the DNA electrochemical sensors. Among the variety of working electrodes, traditional gold and carbon disc electrodes have been widely used to assemble a DNA biosensor (15). Screen-printed transducers have also been proposed for facilitating on-site DNA testing (16). Recently, the attractive performance of renewable pencil electrodes, which compares favorably with that of conventional carbon electrodes, has been reported (17,18). Moreover, pencil biosensors offer a promising alternative for micro-fabricated (screen-printed) electrodes (17).

To the best of our knowledge, no literature has been recorded for the determination of DNA in plant extracts using DNA-electrochemical biosensors. In this paper, a disposable biosensor based on the immobilization of dsDNA on the surface of a pencil graphite electrode was developed in conjunction with a square-wave stripping voltammetric method for the quantitative determination of DNA present at the trace levels in an Amaryllidaceae plant, namely, *Narcissus tazetta* ssp. *tazetta*.

MATERIALS and METHODS

Electrochemical Biosensor Analysis

Instrumentation. Electrochemical measurements were taken using a µAutolab type III electrochemical analysis system running GPES 4.9 software (Eco Chemie, The Netherlands). The electrode system consisted of the pencil working electrode, a silver/silver chloride (3 M sodium chloride) reference electrode (Model RE-1, BAS, USA) and a platinum counter electrode. The preparation of pencil graphite electrode was carried out as described earlier (19). In brief, a mechanical pencil, Model T 0.5 (Rotring, Germany), was used as a holder for the pencil lead (Tombo, Japan), which was purchased from a local bookstore. All leads had a total length of 60 mm and a diameter of 0.5 mm. Electrical contact to the lead was achieved by wrapping a metallic wire around the metallic part of the pencil. A total of 10 mm of lead was immersed in solution per measurement. Such length corresponds to an active electrode area of 15.9 mm^2. Each measurement was performed using a new pencil surface in a homemade 5-mL glass cell containing 4 mL of solution. Before use, the glass cell was soaked in 3 M nitric acid, and rinsed several times with water and acetate buffer. The convective transport was provided by a magnetic stirrer.

Materials. Native ds fish sperm DNA (dsDNA) was purchased from Sigma and was used as-received. 0.1 g/L stock solution of dsDNA was prepared by dissolving DNA in TE buffer [10 mM Tris-hydrochloride, 1 mM ethylenediaminetetraacetic acid (EDTA), pH adjusted to

8.0], and stored at +4°C. More dilute solutions of DNA were prepared by dilution of the stock solution with selected supporting electrolytes. Acetate (pH 4.8), phosphate (pH 7.4 and 12) and Britton-Robinson (pH 3 and pH 7.4) buffer solutions containing 20 mM sodium chloride, were used for the supporting electrolyte. All the chemical reagents used were of analytical grade and doubly distilled water was used throughout.

Procedure. Unless mentioned otherwise, all measurements were performed by pre-treating the electrode surface at +1.40 V for 60 s in an acetate buffer solution (0.25 M, pH 4.8) containing 20 mM sodium chloride, without stirring. The biosensor was developed by immobilizing DNA at a fixed potential of +0.50 V for 420s with 400 rpm stirring in the same buffer solution containing different concentrations of dsDNA. The following washing step involved dipping the electrode in a fresh acetate buffer solution for 5 s. The square-wave voltammetric curves were then recorded (using a step potential of 12 mV, amplitude of 25 mV and frequency of 25 Hz) in a potential range (+0.45)-(+1.40) V in the same supporting electrolyte. Anodic measurements of DNA were based on measurements of the oxidation peak of the guanine peak at about +1.0 V. The experimental procedure is illustrated in Figure 1. Before evaluation, the stripping curve data were treated using the baseline correction and filter commands of the GPES 4.9 software. All experiments were carried out at the room temperature of the laboratory.

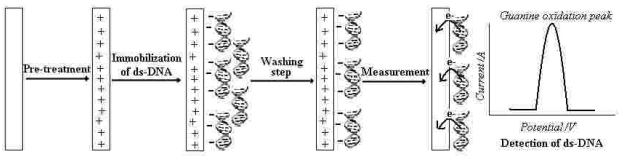

Figure 1: Schematic representation of experimental procedure at pencil graphite electrode.

Plant DNA Isolation Procedure

Plant material. Bulbs of *Narcissus tazetta* ssp. *tazetta* were collected in May 2007 from their natural habitat in Van, Turkey. Specimens were kept at the Herbarium of the Department of General Biology, Faculty of Science and Letters, Yüzüncü Yıl University, Van, Turkey.

Preparation of plant extracts. The chemicals and reagents used in the isolation of DNA were of analytical grade. Water was doubly distilled. The DNA extraction procedure was modified from the method reported previously (20). Dried leaf material of the plant was crushed in liquid nitrogen and kept in deep freezer until use. The frozen powder (1 g) was transferred to a 15 -mL polypropylene tube with 5 mL of isolation buffer (100 mM Tris-hydrochloride, 20 mM EDTA, 1.4 M sodium chloride, 2% (w/v) cetyltrimethylammonium bromide (CTAB) and 2% (v/v) β-mercaptoethanol, pH 8.0). Polyvinylpolypyrrolidone (100 mg/g leaf) was then added to the homogenate and subsequently thawed at +60°C for 25 min and then kept at room temperature. The sample was subjected to 6 mL chloroform-isoamyl alcohol (24:1, v/v) extraction and mixed by inversion. After centrifugation at 6000 rpm for 15 min, the resulting supernatant was transferred to a fresh sterile tube, treated with one-third volume of 5 M sodium chloride, and shaken slightly. Cold (-20°C) ethanol (95%, v/v) was added to the sample in a 2:1 volume ratio, stored at +4oC for 2 h and centrifuged at 5000 rpm for 5 min. The supernatant was removed, the DNA pellet subsequently rinsed with cold (-20°C) ethanol (70%, v/v), centrifuged at 5000 rpm for 5 min and incubated at +37°C for 15 min. The mixture was occasionally mixed by inverting the tube every 15 min during incubation. Crude DNA solutions were obtained by re-suspending the resulting pellets in 300 µL of TE buffer. Further purification of DNA was achieved by digesting the RNA with 3 µL of RNase (10 mg/mL) at +37°C for 30 min.

DNA quality. The purity of the extracted genomic DNA samples was evaluated by the absorbance ratio of A260/A280 using a Shimadzu Model UV-1700 spectrophotometer with a 1 cm quartz cell. DNA purity and integrity were also assessed by electrophoresis (85 V, 30 min) on 1% (w/v) agarose gel (Thermo Electron) stained with 10 mg/mL ethidium bromide in 1xTBE (Tris base, boric acid, 0.5 M EDTA, pH 8) gel buffer. A DNA molecular weight marker (GeneRuler™ DNA Ladder Plus, MBI Fermentas) was included on each gel. The procedure was carried out in a Thermo EC320 mini-cell horizontal gel system. The gels were photographed with a digital camera under ultraviolet light (Vilber Lourmat, France). All determinations were performed in triplicate.

RESULTS and DISCUSSION

Optimization of the DNA Response

Electrochemical initial work involved the comparison of

the effect of two different voltammetric wave-forms (differential pulse and square-wave) upon the stripping responses of the DNA. Figure 2 compares stripping voltammograms at pencil graphite electrode obtained by differential pulse (DPV) and square-wave (SWV) voltammetry. Each technique yielded a well-defined oxidation peak at around +1.01 V (DPV) and +1.05 (SWV). Owing to its peak shape, high sensitivity, fast response and favorable signal-to-background characteristics, the SW mode was employed throughout this study.

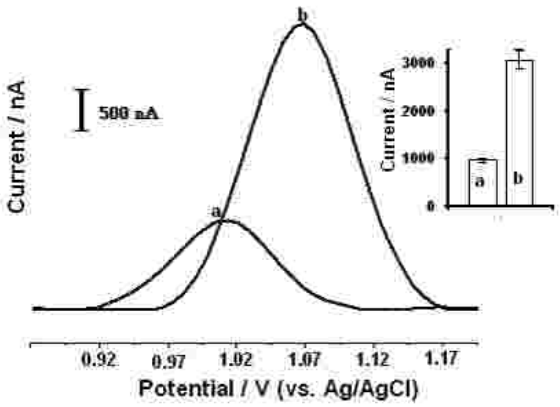

Figure 2: Stripping voltammograms after baseline correction obtained by differential pulse (a) and square wave (b) voltammetric techniques. Inset: The results were also demonstrated with histomograms. Solution: 0.5 M acetate buffer (pH 4.8) containing 5 µg/mL dsDNA and 20 mM NaCl. DPV detection conditions: step potential, 8 mV; pulse amplitude, 50 mV; scan rate, 16 mV/s. SWV detection conditions: frequency, 25 Hz; step potential, 8 mV; pulse amplitude, 20 mV.

Further optimization of the analytical signal focused on studying the effect of solution conditions, such as pH and buffer concentration. The influence of pH upon the stripping response was examined in various supporting electrolytes, such as acetate (pH 4.8), phosphate (pH 7.4 and 12) and Britton-Robinson (pH 3 and 7.4) buffer solutions containing 20 mM sodium chloride. Figure 3A details the variation in the stripping current peak of 5 µg/mL dsDNA as a function of pH. The response increased rapidly up to pH 4.8 and decreased rapidly above this pH value. This profile indicated that the medium at pH 4.8 offers the most favorable performance. When the experiments were carried out at the same pHs using different buffer solutions, it was observed that the stripping response was nearly similar. All the measurements were made in acetate buffer since this is the best medium for performing this kind of measurement (21). The effect of buffer concentration on stripping response was also investigated for a solution containing 5 µg/mL dsDNA in acetate buffer (pH 4.8) over the 0.025-0.5 M range. The corresponding plot of the measured peak current against concentration of acetate buffer is detailed in Figure 3B. The stripping signal increased slowly with the acetate buffer concentration up to 0.25 M and leveled off for more concentrated solutions. Further studies were conducted using an acetate buffer concentration of 0.25 M at pH 4.8.

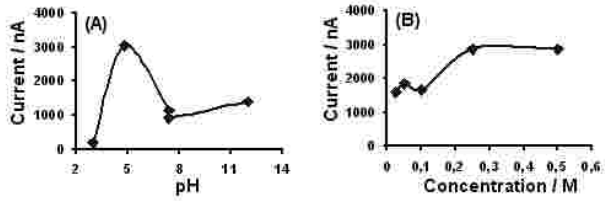

Figure 3: Effects of pH in different supporting electrolytes (A) and concentration of acetate buffer (B) on the stripping response of 5 µg/mL dsDNA. SWV detection conditions, as in Fig. 2. Each point is the mean value of three measurements.

The surface of the working electrode needs to be conditioned before the immobilization of the DNA. As reported in the literature (21), the application of high potentials in acidic media seems to increase the hydrophilic properties of the electrode surface through the introduction of oxygenated functionalities accomplished with an oxidative cleaning. We applied a fixed potential of +1.40 V for 60 s in acidic media (acetate buffer, pH 4.8) to pre-treat the electrode surface.

The attention was next turned to the effect of parameters of the dsDNA immobilization time and stirring speed. Figure 4A displays the influence of dsDNA immobilization time upon the stripping response by applying a potential of +0.5 V for solution containing 5 µg/mL dsDNA. The response remained constant between 180 and 360 s then increased up to 420 s, and more slowly up to 600 s. Subsequent experiments were conducted with 420 s immobilization time. Figure 4B examines the influence of the stirring speed in the range 300-600 rpm for solution containing 5 µg/mL dsDNA. Since the experimental data showed that maximum peak height is at 400 rpm, this value was utilized for all further measurements.

Final optimization of the analytical signal centered upon varying the SW parameters such as the frequency, step potential and pulse amplitude (not shown). The effect of frequency was studied in the range 10 to 80 Hz. The peak

heights for 5 µg/mL dsDNA increased with SW frequency due to the increase in the effective scan rate but the peak shape was distorted at frequencies higher than 25 Hz. This was attributed to the greater contribution of the capacitive current at higher frequencies. The influence of step potential was investigated between 6 and 14 mV. The peak height increased up to 12 mV because the effective scan rate was increased, but at higher values of step potential, the peak heights decreased. The analytical signal was dependent on the pulse amplitude even if this parameter seems to be less important than the frequency. Pulse amplitude was examined in the range from 5 to 30 mV. Peak heights increased upon increase in the pulse amplitude. However, the peak shape became wider at higher than 25 mV. Thus, frequency of 25 Hz, step potential of 12 mV and pulse amplitude of 25 mV was selected for all subsequent work.

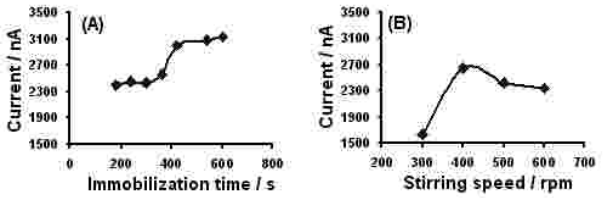

Figure 4: Effect of immobilization time (A) and stirring speed (B) on the stripping response. Solution: 0.25 M acetate buffer (pH 4.8) containing 5 µg/mL dsDNA and 20 mM NaCl. SWV detection conditions, as in Fig. 2. Each point is the mean value of three measurements.

Characterization of the Analytical Performance

The proposed DNA biosensor on pencil graphite electrode offered well-defined concentration dependence. Figure 5A displays stripping voltammograms recorded for increasing dsDNA concentrations over the 10-50 ng/mL range (a-e). The peak current increased proportionally with the DNA concentration to yield a highly linear calibration plot (Figure 5B), ip (nA) = 13.4 C (ng/mL) + 77.8 (correlation coefficient, 0.992, n=3). The limit of detection was 0.36 ng/mL, which was estimated on the basis of the signal-to-noise characteristics (S/N=3). A series of seven successive measurements for 40 ng/mL DNA yielded a relative standard deviation of 8.4%.

Analysis of Plant Extracts

Since the large quantities of coexisting substances in plant samples, such as proteins, ions, etc., may interfere with determination of nucleic acids, in addition to which RNA interferes with the determination of DNA, it is necessary to separate DNA from the interfering substances and prepare DNA extraction solutions for further analysis. Before submitting the plant extracts for quantification of DNA by the proposed voltammetric method, the quality of extracted genomic DNA was assessed by spectrophotometry and gel electrophoresis. The DNA preparations were 100-times diluted with TE solution before checking the DNA quality with UV spectrophotometry. A standard solution of extracted DNA gave a ratio of ultraviolet absorbance at 260 and 280 nm, (A260/A280) of 1.82 (results not shown), indicating that the DNA was sufficiently free from protein contamination (22). Additionally, to check the DNA quality, the extracted samples were analyzed by gel electrophoresis (Figure 6).

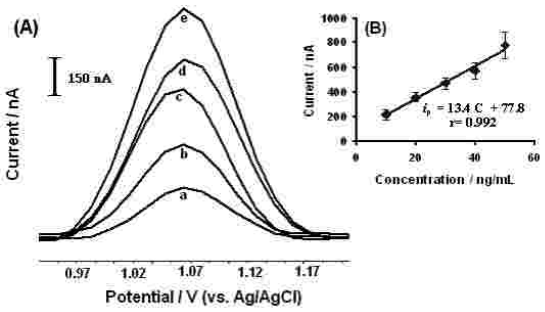

Figure 5: The stripping voltammograms (a) 10, (b) 20, (c) 30, (d) 40, (e) 50 ng/mL dsDNA based on guanine signal (A) and the corresponding calibration plot (B). Solutions: 0.25 M acetate buffer (pH 4.8) containing 20 mM NaCl. SWV detection conditions: frequency, 25Hz; step potential, 12 mV; pulse amplitude, 25 mV.

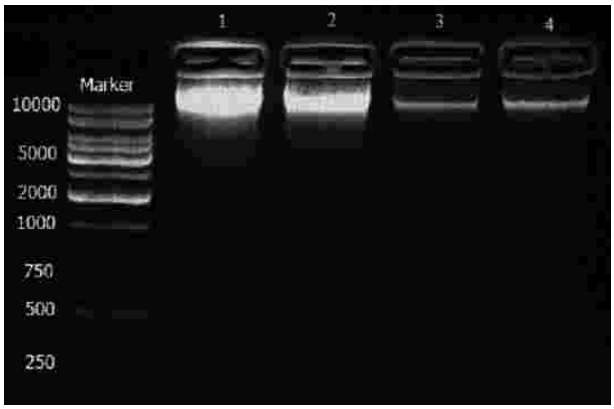

Figure 6: Electrophoretograms of extracted DNA samples. Plant sample weight: 2 g (lanes 1, 2); 1 g (lanes 3, 4).

The findings showed intact genomic DNA bands of high molecular weight (approximately equal to 10,000 base pairs) without RNA contamination. The optimization of the amount of plant tissue per unit volume of extraction buffer is one of the most critical factors in plant DNA isolation procedures (23). According to Krizman et al (24),

the plant tissue amount per volume of extraction buffer has an effect on DNA quality and yield. The isolation buffer is responsible for the lysis of membranes and liberation of DNA from cellular organelles. Thus, the smaller quantity of plant tissue per unit volume would reduce the probability of co-precipitation of contaminants with the DNA pellet. In the case of our study, the tissue amount for good quality genomic DNA was 1 g per 5 mL of isolation buffer, while 2 g of plant tissue amount resulted in a reduction in electrophoretic quality. This finding is in agreement with the postulation of these authors (24).

Finally, the possibility of applying the proposed DNA sensor for the determination of DNA in plant extracts was tested. Appropriate solutions were prepared by taking suitable aliquots of DNA extracts in TE buffer, and diluting with 0.25 M acetate buffer, pH 4.8 containing 20 mM sodium chloride. Each solution was transferred to a glass cell and the voltammetric procedure was followed. The amount of isolated DNA (after 40000-times dilution of the DNA extracts) was found as 22 ± 1.45 ng/mL (n=3) from the equation of linear regression in calibration plot. A recovery test was also performed by analyzing three parallel diluted DNA extracts which were spiked with standard solution of dsDNA at level of 10 ng/mL. A mean recovery of 96.1% was found with a relative standard deviation of 4.9%.

CONCLUSION

The renewable, inexpensive and easy to make DNA biosensor developed in this study using pencil graphite electrode has been demonstrated to be useful for stripping measurements of trace DNA from real samples. The proposed method allows quantification of very low levels of DNA (nanogram scale), and provides a voltammetric alternative that is more sensitive than UV absorbance methods.

The simple and reliable electrochemical detection of DNA is of great importance to the development of modern DNA hybridization chips for the detection of nucleic acids following their chromatographic or electrophoretic separations, or for sensing DNA damage and interactions. In view of the potential bioanalytical importance of the method described in this study, it may have promise for adaptation into automated biosensors for future applications.

ACKNOWLEDGEMENTS

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