Application of Avidin−Biotin Technology and Adsorptive Transfer Stripping Square-Wave Voltammetry for Detection of DNA Hybridization and Avidin in Transgenic Avidin Maize

Michal Masarik,[†] Rene Kizek,[†,‡] Karl J. Kramer,[§] Sabina Billova,[†] Marie Brazdova,[†] Jan Vacek,[†] Michele Bailey,[⊥] Frantisek Jelen,*,[†] and John A. Howard[⊥]

Institute of Biophysics, Academy of Sciences of the Czech Republic, Kralovopolska 135, CZ - 612 65 Brno, Czech Republic, Deparment of Chemistry and Biochemistry, Mendel University of Agriculture and Forestry, Zemedelska 1, 613 00 Brno Czech Republic, Grain Marketing and Production Research Center, Agricultural Research Service, U.S. Department of Agriculture, Manhattan, Kansas 66502, and ProdiGene, Inc., 101 Gateway Boulevard, College Station, Texas 77845

The proteins streptavidin and avidin were electrochemically detected in solution by adsorptive transfer stripping square wave voltammetry (AdTS SWV) at a carbon paste electrode (CPE). AdTS SWV was used to quantify biotinylated oligonucleotides, DNA hybridizations, and avidin in extracts of transgenic avidin maize. The detection limits of denatured and native streptavidin were 6 pM and 120 nM, respectively. The results demonstrated that streptavidin/avidin AdTS SWV is a sensitive and specific method for quantifying DNA and proteins in biological samples such as foods and tissue extracts, including genetically modified crops (avidin maize) and other plants in neighboring fields.

The glycoprotein chicken egg white avidin and its nonglycosylated homologue streptavidin, which is produced by the soil bacterium *Streptomyces avidinii*, have become important functional proteins owing to their extensive usage in affinity-based separations and diagnostic assays as well as for a variety of other applications that have collectively become known as avidin−biotin technology.[1,2] Both proteins form tetramers of comparable molecular weight (64 kDa) and structure and are very stable.[3,4] Their quarternary structure is composed of four identical subunits, each having a high affinity (dissociation constant of $\sim 10^{-15}$ M)[3,4] for the vitamin biotin. This interaction, which is one of the strongest associations known between a protein and its ligand, can be utilized in various fields of avidin−biotin technology, including immunohistochemistry,[5] electron microscopy,[6] enzyme-linked immunoassay,[7] and DNA hybridization.[1,2] In the latter case, biotin is used as a tag for a nucleic acid nonradioactive probe in biomedical and clinical applications.[8]

Because of avidin's ability to sequester biotin from a diet or another environment, it is toxic not only for certain microorganisms but for other kinds of organisms as well. Previously, it was reported that avidin is toxic to a large spectrum of lepidopteran, coleopteran, and dipteran pests of grains, fruits, and vegetables.[9−11] For the purposes of recombinant avidin production and biological protection of grain, a transgenic avidin corn plant was developed.[12,13] Routinely, for analytical purposes involving genetically modified organisms (GMOs), determination of recombinant protein levels is performed by an immunological method such as ELISA.[12] However, because of the potential high sensitivity and specificity of an electrochemical method, we were interested in developing an alternative electrochemical method for measurement of recombinant protein levels and have utilized avidin maize as a model GMO for that purpose.

In this work, we utilized square-wave voltammetry (SWV) in combination with an adsorptive transfer stripping (AdTS) technique for measuring nanogram quantities of streptavidin and avidin in solution at the surface of a solid carbon electrode. This method measures oxidative signals generated from Trp (W) and Tyr (Y) residues in proteins. We constructed protein-based amperometric bioelectrodes by incorporating a protein into a carbon paste matrix

* Corresponding author. Phone: 420541517213. Fax: 420 541211293. E-mail: jelen@ibp.cz.
† Academy of Sciences of the Czech Republic.
‡ Mendel University of Agriculture and Forestry.
§ U.S. Department of Agriculture.
⊥ ProdiGene, Inc..
(1) Wilchek, M.; Bayer, E. A. *Anal. Biochem.* **1988,** *171,* 1−32.
(2) Wilchek, M.; Bayer, E. A. *Methods Enzymol.* **1990,** *184,* 746.
(3) Green, N. M. *Methods Enzymol.* **1990,** *184,* 51−67.
(4) Green, N. M. *Adv. Protein Chem.* **1975,** *29,* 85−133.
(5) Bayer, E. A.; Safars, M.; Wilchek, M. *Anal. Biochem.* **1987,** *161,* 262−271.

(6) Harmon, F. R.; Berger, M.; Beegen, H.; Wood, H. G.; Wrigley, N. G. *J. Biol. Chem.* **1980,** *255,* 262−271.
(7) Germani, Y.; deRocqunigny, H.; Guesdon, J. L. *J. Immunol. Methods* **1992,** *146,* 25−32.
(8) Klevan, L.; Gebeyehu, G. *Methods Enzymol.* **1990,** *184,* 561−584.
(9) Levinson, H. Z.; Bergman, E. D. *J. Insect Physiol.* **1959,** *3,* 293−305.
(10) Morgan, T. D.; Oppert, B.; Czapla, T. H.; Kramer, K. J. *Entomol. Exp. Appl.* **1993,** *69,* 97−108.
(11) Markwick, N. P.; Christeller, J. T.; Docherty, L. C.; Lilley, C. M. *Entomol. Exp. Appl.* **2001,** *98,* 59−66.
(12) Hood, E. E.; Witcher, D. R.; Maddock, S.; Meyer, T.; Baszczynski, C.; Bailey, M.; Flynn, P.; Register, J.; Marshall, L.; Bond, D.; Kulisek, E.; Kusnadi, A.; Evangelista, R.; Nikolov, Z.; Wooge, C.; Mehigh, R. J.; Herman, R.; Kappel, W. K.; Ritland, D.; Li, C. P.; Howard, J. *Mol. Breeding* **1997,** *3,* 291−306.
(13) Kramer, K. J.; Morgan, T. D.; Throne, J. E.; Dowell, F. E.; Bailey, M.; Howard, J. A. *Nat. Biotechnol.* **2000,** *18,* 670−674.

and detected chemicals that interact with the protein. A similar approach was used for construction of a banana tissue−carbon electrode for detection of dopamine.[14] Sensor fabrication was accomplished simply by mixing a desired quantity of the protein into a conventionally prepared carbon paste electrode made of graphite powder and mineral oil. Here, we demonstrate the application of these modern voltammetric methods to nucleic acid research and for detection of avidin extracted from transgenic maize.

EXPERIMENTAL SECTION

Apparatus and Electrochemical Procedures. Electrochemical measurements were performed using an Autolab analyzer (EcoChemie, The Netherlands) in connection with a VA-Stand 663 (Metrohm, Zurich, Switzerland). The electrode system consisted of a carbon-paste working electrode, a Ag/AgCl/3 M KCl reference, and a platinum wire counter electrode. Acetate buffer (0.1 M CH_3COOH + 0.1 M CH_3COONa, pH 5.0) was used as a supporting electrolyte. AdTS SWV was performed using the following parameters: initial potential = 0.1 V, end potential = 1.3 V, amplitude = 25 mV, step potential = 5 mV, and frequency = 200 Hz. All experiments were carried out at 25 °C. The raw data were treated using the Savitzky and Golay filter (level 2)[15] and a moving average baseline correction (peak width = 0.05 mV) of the GPES software.[16]

Preparation of Carbon Paste and Modified Carbon Paste Electrodes. The carbon paste was made of 70% graphite powder (Aldrich) and 30% mineral oil (Sigma; free of DNase, RNase, and protease). This paste was housed in a Teflon body having a 2.5-mm-diameter disk surface. Prior to measurements, the electrode surface was renewed by polishing it with soft filter paper. Then, the surface was ready for measurement of a sample in a volume of 6 μL. The streptavidin-CPE was prepared in the same way as described above, with the exception of adding a solution of 10 μg/mL of streptavidin to the CPE.

Chemicals. Biotin, carbon powder, Tween-20, 2-mercapto-ethanol, sodium dodecyl sulfate, glycerol, Tris(hydroxymethyl)-aminomethane, sodium chloride, sodium citrate, hydrochloric acid, sodium carbonate, sodium acetate, acetic acid, EDTA, and mineral oil were purchased from Sigma Aldrich Chemical Corp. (St. Louis, MO). Calf thymus (CT) DNA isolation and thermal denaturation were carried out as previously described.[17] Oligonucleotides (ODN) 5'bio-(TTC)$_{12}$, 5'bio-TGC TTT TCG GAG ACA TGC CTA GAC ATG CCT (ODN-I), and the complementary (GAA)$_{12}$, AGG CAT GTC TAG GCA TGT CTC CGA AAA GCA (ODN-II) were synthesized by the Department of Functional Genomics and Proteomics (Masaryk University, Brno, Czech Republic). The nucleic acid concentration and the UV absorption spectra of streptavidin and avidin were determined using a diode array spectrophotometer (Hewlett-Packard model 8452A) in a temperature-controlled cell. Solutions were prepared using ACS water from Sigma Aldrich.

Streptavidin (STV). The amino acid sequence of STV with all Ys (Tyr) and Ws (Trp) in bold font is shown below:
DPSKDSKAQVSAAEAGITGT**WY**NQLGSTFIVTAGADG-

ALTGT**Y**ESAVGNAESR**Y**VLTGR**Y**DSAPATDGSGTA-LG**W**TVA**W**KNN**Y**RNAHSATT**W**SGQ**Y**VGGAEARIN-TQ**W**LLTSGTTEANA**W**KSTLVGHDTFTKVKPSAASIDA-AKKAGVNNGNPLDAVQQ (Y and W = 6; MW = 16 kDa).

Avidin. PGLSARKCSLTGK**W**TNDLGSNMTIGAVNSRGEFT-GT**Y**ITAVTATSNEIKESPLHGTENTINKR TQPTFGFTVN-**W**KFSESTTVFTGQCFIDRNGKEVLKTM**W**LLRSSVNDIG-DD**W**KATRVGINIFTRLRTQKE (Y = 1; W = 4; MW = 14 kDa).

Preparation of DNA Hybrid in Solution. One hundred μg/mL ODN-bio was mixed with 100 μg/mL complementary ODN in 50 mM phosphate buffer pH 7.0, 0.3 M NaCl. Solutions were heated (Thermomixer, Eppendorf) to 80 °C for 10 min followed by slow cooling to room temperature for 1 h.

Avidin Maize. The construction of plasmids used for transformation of maize with the chicken avidin gene, transformation, tissue culture, and generation of avidin-expressing transgenic plants were as described previously.[12]

Extraction of Avidin from Transgenic Maize. A 10-g portion of transgenic or nontransgenic (control) maize seed was ground for 1 min in a coffee grinder. The meal was extracted for 1 h at 4 °C with constant stirring in 50 mL of a buffer containing 50 mM sodium carbonate (pH 11.0), 500 mM NaCl, 5 mM EDTA, and 0.05% Tween-20. The extraction mixture was centrifuged at 16000g for 15 min (Jouan MR 23i) at 4 °C. The supernatant was removed and filtered through 4 layers of cheesecloth. The filtrate was then centrifuged at 14500g for 15 min at 4 °C. The supernatant was recovered, and the pH was adjusted to 10.5, then centrifuged at 14000g for 30 min (Eppendorf 5402) at 4 °C.[12]

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS−PAGE). Samples (5−10 μg of protein in 10 μL) were diluted with one-half volume of sample buffer [9% w/v sodium dodecyl sulfate (SDS), 30% w/v glycerol, 2% w/v 2-mercapto-ethanol, and 0.2% w/v bromphenol blue in 62.5 mM Tris−HCl buffer, pH 6.8]. Slab gel SDS−PAGE was performed using 5% polyacrylamide stacking gels and 10% separating gels. Gels were usually stained with Coomassie Brilliant Blue R-250[18] to visualize the proteins.

Western Blot. The gels were blotted onto nitrocellulose transfer membranes Protran R (Schleicher and Schuell, Germany) in 1× blotting buffer (48 mM Tris, 39 mM glycine buffer, 0.037% SDS, 20% methanol) at 50 V using an electro blotting system (BioRad).[19] The membrane was blocked with 5% nonfat milk in 1 × PBS (150 mM NaCl, 10 mM KCl, 5 mM Na_2HPO_4, 2 mM NaH_2PO_4), and the streptavidin or avidin was detected using a 1 mg/mL primary avidin polyclonal antibody (diluted 1:100) and a secondary antibody anti-mouse IgG (peroxidase conjugate; diluted 1:3000). The complex was visualized with the ECL Detection System (Amersham Pharmacia Biotech). Chemiluminescent signals were detected using autoradiographic film (AGFA, Belgium).

ELISA Method. Rabbit antibodies specific for avidin were prepared as described in Hood et al.[12] and Kramer et al.[13] ELISAs were typical sandwich-type in which the microtiter plates were coated with rabbit anti-avidin (custom serum from Bethyl Laboratories, Montgomery TX). The avidin protein was captured overnight and reacted with goat anti-avidin (Vector Laboratories,

(14) Wang, J.; Lin, M. S. *Anal. Chem.* **1988**, *60*, 1545−1548.
(15) Bromba, M. U. A.; Ziegler, H. *Anal. Chem.* **1981**, *53*, 1583−1586.
(16) *Autolab Electrochemical Instuments*; Eco Chemie BV: Utrecht, 1997.
(17) Palecek, E. *Bielectrochem. Bioenerg.* **1981**, *8*, 469−477.

(18) Bayer, E. A.; Ehrlich-Rogozinski, S.; Wilchek, M. *Electrophoresis* **1996**, *1996*, 1319−1324.
(19) Sambrook, J.; Fritsch, E. F.; Maniatis, T. *Molecular Cloning. A Laboratory Manual*, 2nd ed.; Cold Spring Harbor: New York, 1989.
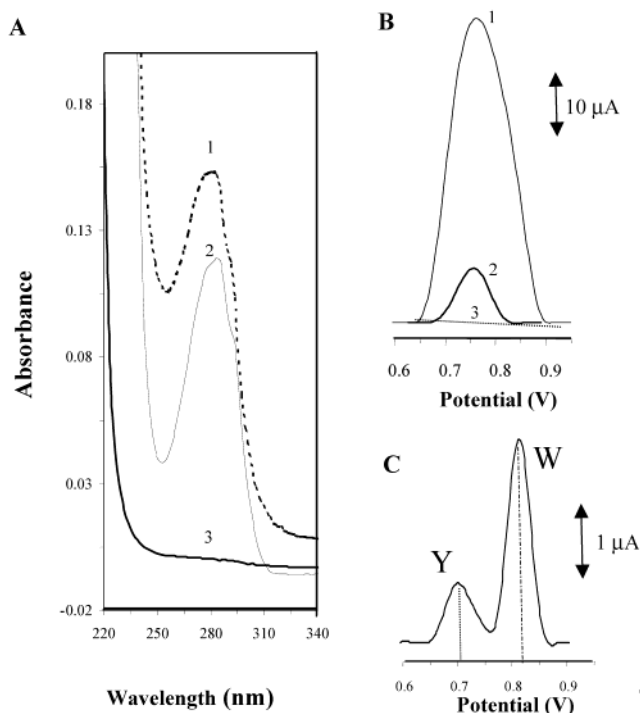
Figure 1. (A) Ultraviolet absorption spectrum of streptavidin in 50 mM phosphate buffer, pH 7.0, measured in a 1-cm path length cuvette. (1) Denatured streptavidin (50 μg/mL, 99 °C, 30 min). (2) Native streptavidin (50 μg/mL, 25 °C). (3) Reference sample: 50 mM phosphate buffer, pH 7.0. Adsorptive transfer stripping square-wave voltammograms (AdTS SWV) of streptavidin at concentration of (B) 50 μg/mL and (C) 1 μg/mL adsorbed from 50 mM phosphate buffer, pH 7.0, adsorption time (t_A) of 120 s, background electrolyte of 0.2 M acetate buffer, pH 5.0. The AdTSV SWV was performed using an initial potential of +0.1 V, ending potential of +1.7 V, amplitude of 25 mV, step potential of 5 mV, and frequency of 200 Hz. (1) Denatured streptavidin (50 μg/mL, 99 °C, 30 min). (2) Native streptavidin (50 μg/mL, 25 °C). (3) Background electrolyte: 0.2 M acetate buffer, pH 5.0. Y refers to the electrochemical response due to tyrosine and W, to tryptophan. The raw data were treated using the Savitzky and Golay filter (level 4) and polynomial baseline correction of the Autolab GPES software.
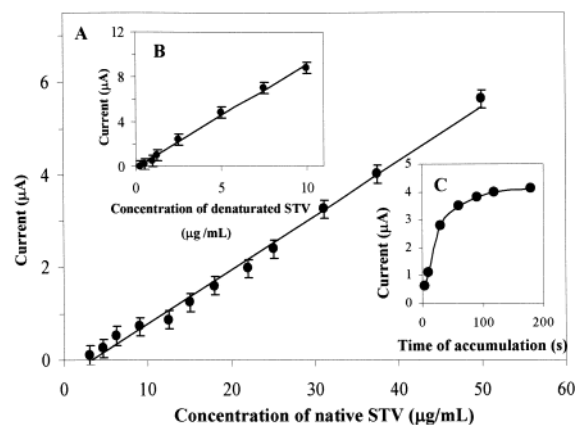

Figure 2. Dependence of AdST SWV peak height of streptavidin (peak W) on its concentration and accumulation time. Concentration curve of (A) native and (B) thermally denatured (99 °C, 30 min) streptavidin. (C) Dependence on time of accumulation of thermally denatured streptavidin (1 μg/mL) in range of 0−200 s. Other conditions were the same as described in Figure 1B.
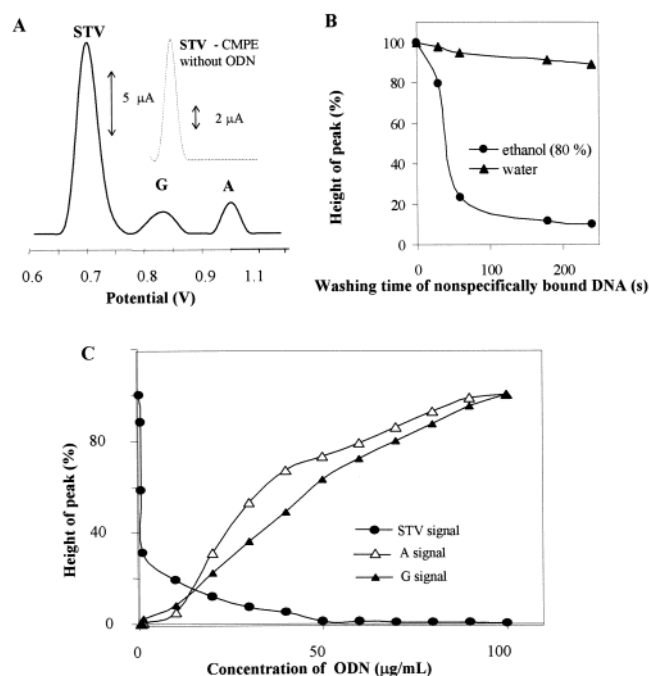

Figure 3. Use of streptavidin-modified carbon paste electrode (CMPE) for detection of biotinylated oligonucleotides. (A) Square wave voltammogram of ODN-I (1 μg/mL) at modified electrode. Biotinylated ODN was adsorbed on the modified electrode for 120 s, and the electrode was washed with 80% ethanol for 180 s and transferred into background electrolyte and analyzed. Dashed line curve, background electrolyte. (B) Washing procedure of nonspecifically bound DNA and binding of biotinylated oligonucleotides (ODN) on streptavidin-modified carbon paste electrode. Nonbiotinylated oligonucleotide (10T + GAA GAA GAA), concentration = 100 μg/mL, was adsorbed onto the modified carbon paste electrode for 120 s, and then nonspecific DNA was washed off the electrode with 80% ethanol. Results are presented as height of peak G. (C) Dependence of peak height of ODN-I on its concentration. Concentration of streptavidin in the carbon paste electrode = 10 μg/mL; signal obtained at this concentration corresponds to 100% peak height.

Burlingame, CA) and then with anti-goat alkaline phosphatase conjugate (Jackson Immunoresearch, West Grove, PA). The alkaline phosphatase activity was detected by reaction with *p*-nitrophenyl phosphate as substrate (Sigma, St. Louis, MO) at 405 nm.

RESULTS AND DISCUSSION

Determination of Streptavidin at CPE. The electrochemical behavior of streptavidin (STV) was investigated by square wave voltammetry on a carbon paste electrode using the adsorptive transfer stripping (AdTSV) technique. This technique is based on the immobilization of the analyte in the form of a small drop of solution on CPE (or carbon modified-paste electrode (CMPE)), followed by washing and detection steps in a cell containing a background or blank electrolyte. Details about the AdTSV were published elsewhere.[20,21] Proteins or peptides containing Y or W residues in their amino acid sequences are known to be electro-

(20) Palecek, E.; Postbieglova, I. *J. Electroanal. Chem.* **1986**, *214*, 359−371.
(21) Tomschik, M.; Havran, L.; Palecek, E.; Heyrovsky, M. *Electroanalysis* **2000**, *12*, 274−279.

active, yielding anodic signals on carbon electrodes.[22,23] In the case of denatured and native STV at a concentration of 50 μg/mL, the voltammogram showed a broad peak at a potential of ~0.85 V
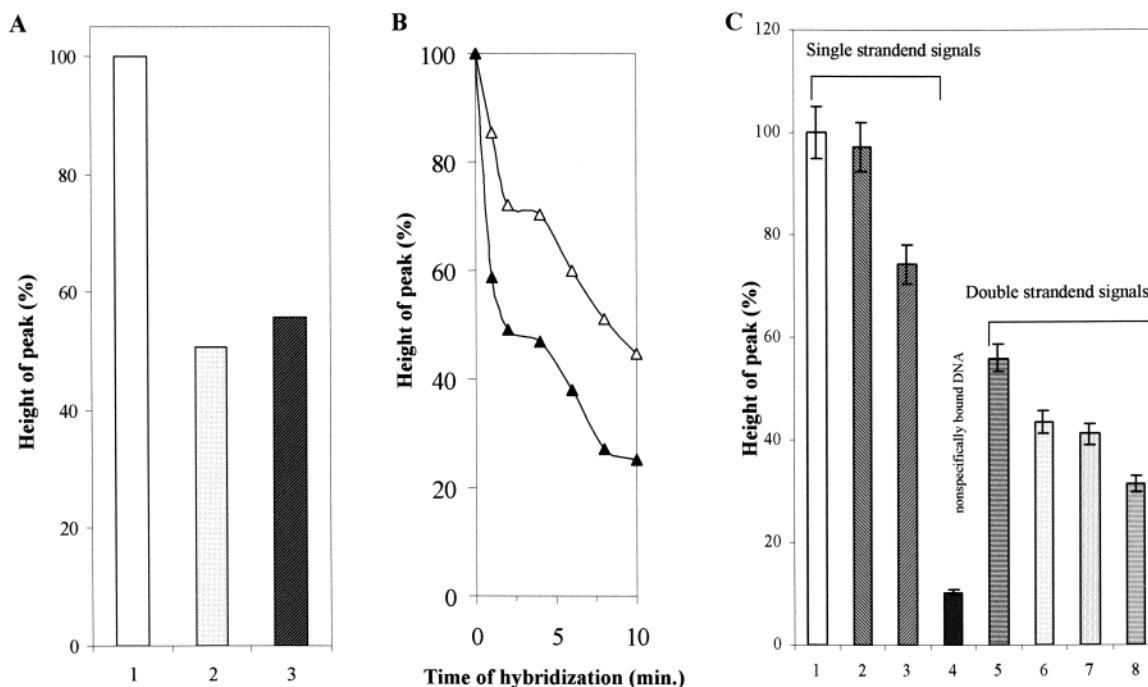
Figure 4. (A) Hybridization of two biotinylated oligonucleotides and their detection using the streptavidin-modified carbon paste electrode (CMPE). First column corresponds to single-stranded biotinylated oligonucleotide, $t_A = 120$ s, followed by washing in 80% ethanol for 180 s and then analysis in the background electrolyte. Second column corresponds to a hybrid mixture that was made in solution (100 μg/mL ODN I + 100 μg/mL ODN II were heated to 80 °C and then slowly cooled), adsorbed at electrode, $t_A = 120$s, followed by washing in 80% ethanol for 180 s and then analysis in the background electrolyte. Third column corresponds to hybridization at the electrode (100 μg/mL ODN I was adsorbed on the electrode for 120 s, then the electrode was washed in water, and finally 100 μg/mL ODN II was adsorbed for 120 s). Electrode was washed in 80% ethanol for 180 s and transferred into the background electrolyte, and then measurements were taken. (B) Hybridization time of two biotinylated ODNs. ODN I (100 μg/mL) was adsorbed on the electrode for 120 s, then the electrode was washed in 80% ethanol, and 100 μg/mL ODN II was adsorbed for various time intervals. The electrode was washed in 80% ethanol for 180 s and transferred into background electrolyte, and then peak G measurements were taken. △, A signal; ▲, G signal. (C) Hybridization of ODN and influence of nonspecific DNA. Single-stranded signals: (1) single-stranded biotinylated ODN at 100 μg/mL; (2) single-stranded biotinylated ODN at 100 μg/mL mixed with nonspecific DNA, ratio 1:1; (3) single-stranded biotinylated ODN at 100 μg/mL mixed with nonspecific DNA, ratio of 1:10; (4) nonspecifically bound DNA; adsorption on modified carbon paste electrode, followed by washing in 80% ethanol, and then measurements were taken. Double-stranded signals: (5) hybrid mixture in solution; (6)hybrid mixture with calf thymus (CT DNA) DNA in ratio of 1:1; (7) 1:10; (8) 1:50; adsorption on modified carbon paste electrode, followed by washing in 80% ethanol. Other conditions were the same as described in Figure 1B. 100% height of peak corresponds to 100 μg/mL ODN-II.

(Figure 1B). The denatured protein exhibited a >5-fold response relative to that of the native protein, indicating that there were substantially more aromatic residues exposed to the electrode surface in the denatured state. At a much lower concentration (1 μg/mL), the response was divided into two well-resolved signals at potentials of 0.70 V (Y) and 0.82 V (W) (Figure 1C). Quite recently, this type of overlapping signal was described and resolved in a study of homogeneous chemical reactions using square-wave voltammetry.[24] A similar analysis could be applied to our system.

Compared to voltammetric measurements, UV spectrophotometry was less sensitive and yielded satisfactory results only at relatively high concentrations of STV. Figure 1A shows the UV absorption spectra of a solution of 50 μg/mL native and denatured streptavidin obtained in phosphate buffer at 25 °C. The denatured protein exhibited only a slightly greater absorption than the native protein.

The concentration−response calibration curve of native STV for a concentration range from 2 to 50 μg/mL under optimal electrochemical conditions is shown in Figure 2A. The calibration curve for the whole concentration range was linear with a regression line of $y = 11.8x - 42$ ($R^2 = 0.994$). For thermally denatured STV at a concentration range from 500 ng/mL to 10 μg/mL (Figure 2B), the corresponding parameters were $y = 9.3x - 1.4$ ($R^2 = 0.995$). Under the described conditions, native and thermally denatured STV can be determined at submicromolar concentrations. The detection limits of the native and denatured STV are ~120 nM and 6 pM, respectively (data not shown). As was expected for an adsorptive stripping technique, the electrochemical response was strongly dependent on the accumulation time. The AdTSV signal for 1 μg/mL STV exponentially increased with accumulation time and was limiting for a current value of 400 nA above an accumulation time of 100 s (Figure 2C). The AdTSV method would be ~2-fold less sensitive for avidin relative to streptavidin because of fewer Y and W residues in the latter protein. We also studied formation of the avidin−biotin complex in solution using both spectrophotometric and electrochemical techniques. Those results will be published elsewhere.

(22) Cai, X. H.; Rivas, G.; Farias, P. A. M.; Shiraishi, H.; Wang, J.; Palecek, E. *Anal. Chim. Acta* **1996**, *332*, 49−57.
(23) Brabec, V.; Mornstein, V. *Biochim. Biophys. Acta* **1980**, *625*, 43−50.
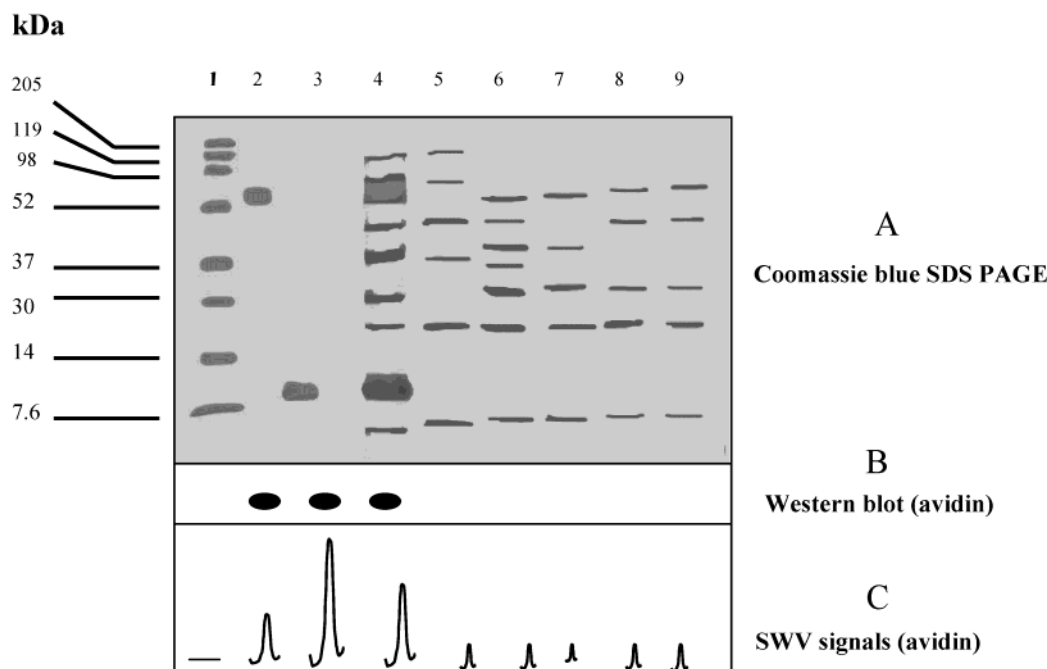(24) Macfic, G.; Atherton, J. H.; Compton, R. G. *Electroanalysis* **2002**, *14*, 479−485.

Figure 5. Comparison of various methods for detection of avidin. (A) Coomassie blue-stained SDS−PAGE. (B) Western blot stained with affinity-purified polyclonal antibody raised against avidin. (C) Corresponding voltammetric (SWV) responses. Lanes: (1) molecular weight standards (kDa): myosin (205), β-galactosidase (119), bovine serum albumin (98), ovalbumin (52.3), carbonic anhydrase (36.8), soybean trypsin inhibitor (30.1), lysozyme (14), aprotinin (7.6); (2) native streptavidin; (3) thermally denatured streptavidin (99 °C, 30 min); (4) 10 μg of avidin-positive maize seed extract; (5) 10 μg of avidin-negative maize seed extract (negative control); (6) 10 μg of avidin-negative corn low-grade flour extract; (7) 10 μg of avidin-negative corn grout extract; (8) 10 μg of avidin-negative corn grits extract, and (9) 10 μg of avidin-negative corn farina extract.

DNA Hybridization at CMPE. The liquid (mercury) electrode is not well suited for the DNA hybridization analysis because of strong hydrophobic interactions of bases in ssDNA with the electrode's surface.[25−27] However, DNA hybridization apparently does proceed satisfactorily at solid electrodes such as gold and carbon. Nonetheless, when working with rather long target DNAs, it can be difficult to avoid nonspecific DNA adsorption.[26,27] To overcome these potential drawbacks, we proposed a new approach in which hybridization was monitored by following the avidin−biotin interaction with electrochemical detection of the hybridization using CMPE as the detection electrode.

The CMPE is specifically able to detect biotinylated DNA, whereas nonspecifically bound DNA can be removed from the electrode's surface using a convenient washing procedure. The efficiency of removing nonspecifically bound DNA from the CMPE is shown in Figure 3B. This AdTSV experiment was performed with ODN at a concentration of 100 μg/mL, which had been adsorbed at CMPE for 2 min and washed with water and 80% ethanol. The results (manifested as peak G in Figure 3A) of this experiment showed that there were only small changes in DNA signals in the case of water rinsing. On the other hand, when 80% ethanol was used instead of water, the rinsing was more effective; the signals decreased dramatically by ∼90% (Figure 3B). This washing procedure was routinely used for subsequent experiments dealing with hybridization. The voltammogram of

STV with a small amount of biotinylated ODN is shown in Figure 3A. When the amount of biotinylated ODN adsorbed on the CMPE was increased, then the STV signal decreased as a result of the saturation of STV with biotin on CMPE. Simultaneously with increasing amount of biotinylated ODN, we observed increasing signals of adenine (A) and guanine (G) in the concentration range of 0−100 μg/mL (Figure 3C). These signals appeared to be due to the oxidation of guanine and adenine in the ODN chain and can be used for analytical purposes.[28] Details about the oxidation mechanism of these purines were reported previously by Palanti et al.[29]

In general, for DNA hybridization with electrochemical detection, a single-stranded DNA probe was immobilized at the electrode, followed by hybridization with the target DNA, and electrochemical detection was performed using the same electrode.[30] Because of suppression of nonspecific adsorption of DNA on the electrode, it was better to separate hybridization and the determination step using different transducers.[31−33] Duplex formation was usually characterized by a decrease of signal generated from single-stranded ODNs.[31,34−36] For verification of hybridization

(25) Cai, X.; Rivas, G.; Shiraishi, H.; Farias, P.; Wang, J.; Tomschik, M.; Jelen, F.; Palecek, E. *Anal. Chim. Acta* **1997**, *344*, 64−76.

(26) Palecek, E.; Fojta, M. *Anal. Chem.* **2001**, *73*, 74A−83A.

(27) Jelen, F.; Yosypchuk, B.; Kourilova, A.; Novotny, L.; Palecek, E. *Anal. Chem.* **2002**, *74*, 4788−4793.

(28) Wang, J.; Bollo, S.; Paz, J. L. L.; Sahlin, E.; Mukherjee, B. *Anal. Chem.* **1999**, *71*, 1910−1913.

(29) Palanti, S.; Marrazza, G.; Mascini, M. *Anal. Lett.* **1996**, *29*, 2309−2331.

(30) Fojta, M. *Electroanalysis* **2002**, *14*, 1449−1463.

(31) Wang, J.; Danke, X.; Erdem, A.; Polsky, R.; Salazar, M. A. *Talanta* **2002**, *56*, 931−938.

(32) Palecek, E.; Billova, S.; Havran, L.; Kizek, R.; Miculkova, A.; Jelen, F. *Talanta* **2002**, *56*, 919−930.

(33) Palecek, E.; Kizek, R.; Havran, L.; Billova, S.; Fojta, M. *Anal. Chim. Acta* **2002**, *469*, 73−83.

(34) Ozkan, D.; Erdem, A.; Kara, P.; Kerman, K.; Meric, B.; Hassmann, J.; Ozsoz, M. *Anal. Chem.* **2002**, *74*, 5931−5936.
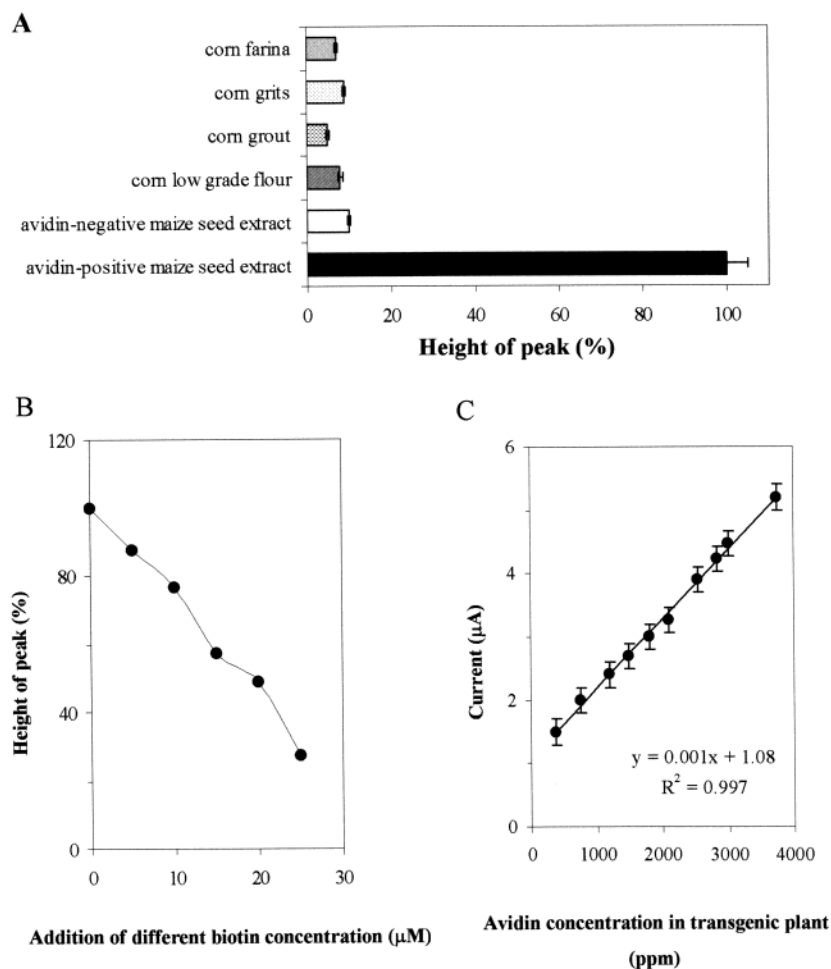
Figure 6. Voltammetric detection of avidin in transgenic maize using SWV. (A) Comparison of various corn flour extracts, avidin-negative maize seed extract, and avidin positive maize seed extract. 100% height of peak corresponds to avidin positive maize seed extract. (B) Effect of the presence of different amounts of biotin added to avidin-positive maize seed extract. (C) Calibration curve of avidin concentration in transgenic maize. Other conditions were the same as in Figure 1B.

on the CMPE, the DNA hybrid was prepared in solution using the following conditions: 100 μg/mL ODN-bio was mixed with 100 μg/mL complementary ODN in 50 mM phosphate buffer pH 7.0, 0.3 M NaCl and heated (Thermomixer, Eppendorf) to 80 °C with subsequent cooling to room temperature (1 h). Figure 4A shows a comparison of duplex formation in solution (column 2) with duplex formation on CMPE surface (column 3) and biotinylated single-stranded ODN (column 1) expressed as the peak height of the oxidation signal. The signal from the biotinylated single-stranded ODN was taken as 100%. We observed a small difference in signals from duplexes formed in solution and on the CMPE. The relative heights of both duplexes were approximately one-half of the height of the biotinylated single-stranded ODN (Figure 4A). We studied the influence of time on the process of hybridization (Figure 4B). We used ODN-I and complementary ODN-II in our hybridization experiments. The relative peak heights were rapidly decreased after mixing of the ODN; after 2 min, the decrease was ~50 or 70%, respectively (Figure 4A and B). In all cases, the adenine oxidation signal of biotinylated single-

stranded ODN was taken as 100%. Figure 4C shows the influence of the presence of nonspecific DNA on specific binding of biotinylated single-stranded ODN and duplex formation on the CMPE. Increasing the concentration of nonspecific ODN led to a gradual decrease of signal from single-stranded ODN A (Figure 4C, columns 1−3), for example, at a 1:10 molar ratio, the signal was only ~20% that of the sample without nonspecific DNA. When duplex DNA was made on the CMPE, hybridization on CMPE was negatively influenced by addition of nonspecific DNA, as well. Increasing the amount of thermally denatured CT DNA in the sample reduced the signal from the duplex; for example, at a 1:50 molar ratio, the signal was only ~10% relative to that of the sample without denatured DNA. Oxidation signals from single-stranded and double-stranded DNAs were probably decreased because of hydrophobic interactions between bases in the denatured DNA and the electrode.

Determination of Avidin in Transgenic Avidin Maize at CPE. Native and denatured STV were compared with maize-derived avidin on a Coomassie blue-stained SDS polyacrylamide gel (Figure 5A), a western blot stained with an affinity-purified polyclonal antibody raised against avidin (Figure 5B), and the AdTS SWV responses (Figure 5C). Native STV behaved as a

(35) Ozkan, D.; Kara, P.; Kerman, K.; Meric, B.; Erdem, A.; Jelen, F.; Nielsen, P. E.; Ozsoz, M. *Bioelectrochemistry* **2002**, *58*, 119−126.
(36) Kara, P.; Ozkan, D.; Kerman, K.; Meric, B.; Erdem, A.; Ozsoz, M. *Anal. Bioanal. Chem.* **2002**, *373*, 710−716.

tetrameric, whereas denatured STV acted like a monomeric one. The avidin-positive maize seed extract (Figure 5A, B, and C, lanes 4), avidin-negative maize seed extract (negative control) (Figure 5A, B, and C, lanes 5), and different corn milling fractions also were subjected to SDS−PAGE. It was clear that avidin was present in the maize seed extract.

We applied an electrochemical technique for the detection of avidin in a transgenic plant seed extract. The electrochemical responses of the avidin-positive maize seed extract and several milling fractions are shown in Figure 6A ($n = 5$, SD = 4.2%). For the avidin negative samples, in which the content of electroactive amino acids should be relatively small, only very small signals of ∼10% were produced. We studied the electrochemical response of the avidin-positive maize seed extract in the presence of different biotin concentrations (Figure 6B). Increasing the concentration of biotin led to a dramatic decrease of avidin signal, which was a verification that the signals measured indeed were due to avidin. For direct determination of avidin in the transgenic seed extract (ng avidin/mg dry weight of sample), we constructed a concentration−response calibration curve (Figure 6C). The response was linear in the concentration range of 500−4000 ppm, with $y = 0.001x + 1.08$ ($R^2 = 0.997$). Quantification of avidin levels in avidin corn meal was performed by both SWV and ELISA analyses. Kernels from the T7 generation of maize plants expressing avidin were used for those experiments.[12] Because of the male-sterile nature of these plants, this seed was heterozygous for the avidin gene; thus, only half of the kernels contain avidin. This grain was dry-milled to produce flour. The avidin content determined using the modified carbon-paste electrode was 1975 ppm, which was in good agreement with the results of ELISA. Two separate extracts were prepared from this flour in phosphate-buffered saline with 0.05% Tween-20 as described in Kramer et al.[13] and analyzed in triplicate by ELISA. We determined that 26.63 ± 3.04% (mean value ± SD, $n = 6$) of the soluble seed protein was avidin, corresponding to ∼1795 ppm dry seed weight, which is in good agreement with the SWV result.

CONCLUSION

In conclusion, we have developed an electrochemical method using the CPE and CPME for the detection of (a) streptavidin/avidin and the biotin complex in solution or at the electrode surface, (b) DNA hybridization, and (c) avidin in transgenic maize. This method coupled with avidin/streptavidin/biotin technology is very sensitive and, when utilized with a washing procedure, is selective against noncomplementary nucleic acids while specific for complementary ones. This approach offers novel possibilities in bioelectrochemical research in connection with protein-supplemented carbon electrodes, and complements immunological and spectroscopic methods previously used for detection of recombinant proteins produced by GMOs.[13,37,38] For example, it could be used to monitor fields surrounding transgenic crops, such as avidin maize, for unintentional contamination of other crops and weedy species.

(37) Kramer, K. J. In *Genetic Transformation in Crop Plants*; Liang, G. H., Skinner, D. Z., Eds.; Haworth Press: New York, 2002; Vol. in press.

(38) Roussel, S. A.; Connie, L.; Hardy, C. L.; Hurburgh, J. C. R.; Rippke, G. R. *Appl. Spectrosc.* **2001**, *55*, 1425−1430.