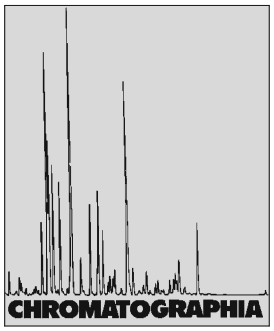
# Chip-Based CE for Avidin Determination in Transgenic Tobacco and Its Comparison with Square-Wave Voltammetry and Standard Gel Electrophoresis

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## Abstract

Avidin transgenic plants are a potential tool for providing resistance against various species of insect pests due to the sequestration of vitamin H (biotin) in the plant from the insect pests. In this project we compared three techniques for avidin determination in transgenic tobacco plants, a novel chip-based capillary electrophoretic method (Experion), classical polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE) and a square wave voltammetric method using a carbon paste electrode. We determined that the automated chip-based capillary electrophoretic method is rapid, sensitive and the results obtained are well repeatable. The avidin content measured in transgenic tobacco leaves using chip-based capillary electrophoresis varied from 15 to 854 ng per mg of fresh mass depending on the individual plant.

## Keywords

Chip-based capillary electrophoresis
Avidin, transgenic tobacco plants
Voltammetry
SDS-PAGE

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## Introduction

Protection of various agricultural crops against insect pests that cause severe economic damage is an issue being researched worldwide [1–3]. One of many possibilities to achieve higher productivity in agricultural crops is to use pesticides for crop protection. It is in common knowledge that pesticide residues can contaminate the environment, food and many of them are poisonous to humans and other organisms [4].

One way to reduce the use of harmful pesticides is to exploit the methods of genetic engineering. One of the most successful applications of genetic engineering is the utilization of recombinant endotoxins of *Bacillus thuringiensis* (Bt) [2, 5, 6]. Hood et al. in 1997 [5] and Kramer et al. in 2000 [2] described the commercial production and analysis of avidin in maize. The susceptibility of this crop to stored-product insects had not been examined until the latter paper, but today it is clear that avidin maize is highly resistant toward many species of


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stored-product insect pests like lesser grain borer (*Rhyzopertha dominica*), sawtoothed grain beetle (*Oryzaephilus surinamensis*), red flour beetle (*Tribolium castaneum*), confused flour beetle (*Tribolium confusum*), flat grain beetle (*Cryptolestes pusillus*), Indianmeal moth (*Plodia interpunctella*), Mediterranean flour moth (*Anagasta kuehniella*), warehouse beetle (*Trogoderma variabile*), which exhibit 95–100% mortality at >100 ppm avidin concentrations [2]. So far in addition to maize, transgenic avidin expressing tobacco [7], rice [8], and apple [9] have been developed.

Avidin is a glycosylated basic protein with isoelectric point of 10–10.5 composed of four subunits with a tetrameric relative molecular mass of about 67,000. Each subunit contains one binding site with high affinity for biotin (vitamin H, *cis*-hexahydro-2-oxo-1-*H*-thieno-[3,4]-imidazoline-4-valeric acid) with a dissociation constant $K_d = 10^{-15}$ M. This interaction exhibits one of the highest known affinities between a protein and its ligand, and can be utilized for various fields of avidin-biotin technology including immunohistochemistry, electron microscopy, DNA hybridization and biosensors. In nature avidin occurs as a minor component of bird, reptile and amphibian egg white and probably plays a role in protecting embryos by biotin sequestration [10]. Hereby it can interfere with biotin-containing enzymes, which are involved in carboxylation, decarboxylation and transcarboxylation reactions [2].

The most commonly used techniques for detection and evaluation of avidin-biotin interactions are ELISA, fluorimetry, and several electrochemical methods [11–16]. In our previous papers we proposed alternative methods for avidin detection in transgenic plants. With a carbon paste electrode and suitable conditions for electrochemical measurement, we were able to detect zeptomole levels of the protein in 5 µL sample volume [17]. In comparison with the ELISA technique, the electrochemical determination exhibited good agreement, but it was much more sensitive [17, 18].

In the present work we evaluated chip-based capillary electrophoresis for avidin analysis in transgenic tobacco plants (*Nicotiana tabacum*) and compared this technique with two other methods, standard gel electrophoresis and voltammetry. Tobacco plants are useful model plant for genetic engineering of plants and the methods for its transformation via the used vectors are routinely used and well handled. Therefore we choose this model in our experiments.

## Experimental

### Chemicals

Avidin, carbon powder, mineral oil and other chemicals were purchased from Sigma-Aldrich Chemical Corp. (St. Louis, USA). Solutions were prepared using water from Sigma Aldrich in ACS purity. Stock standard solutions of avidin at 1 µg $mL^{-1}$ were prepared and stored in the dark at 4 °C. All solutions were filtered through a 0.45 µm Teflon membrane filter (MetaChem, Torrance, CA, USA).

### Biological Samples

Transgenic tobacco plants (*Nicotiana tabacum* cv. Samsun) were obtained from the Horticulture and Food Research Institute of New Zealand. 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 [19]. As controls, nontransformed plants were used. All plants were grown in a containment glasshouse at approximately 28 °C. Following harvest, leaves from identified plants were stored in −20 °C.

### Sample Preparation

Approximately 0.1 g of the frozen plant tissue (leaves) was homogenized using mortar and liquid nitrogen, and extracted for 4 h at 4 °C with constant stirring in buffer (5:1, *w*/*v*) containing 50 mM sodium carbonate (pH 11), 500 mM EDTA, and 0.05% (*v*/*v*) Tween-20. The homogenate was centrifuged at 16,000 × *g* for 15 min at 4 °C (Eppendorf, Type 16F6-38 rotor, Germany), supernatants were removed and centrifuged at 14,500 × *g* for 15 min at 4 °C, the pH was adjusted up to 10.5 with 1 M NaOH, and then the mixture was centrifuged at 11,000 rpm for 30 min to remove precipitated proteins [5]. The protein concentration in plant samples was determined according to the method of Bradford [20]. The prepared samples were stored at −20 °C.

### Electrochemical Determination of Avidin

Electrochemical measurements were performed using a CHI440A instrument (CH Instruments, Austin, USA). The three-electrode system consisted of a carbon-paste working electrode (CPE), an Ag/AgCl/3 M KCl reference electrode, and a platinum wire counter electrode. Acetate buffer (0.2 M $CH_3COOH$ + 0.2 M $CH_3COONa$ in ratio 9:2 (*v*/*v*), pH 4) was used as the supporting electrolyte. Square wave voltammetry (SWV) was performed using the following parameters: initial potential = 0.1 V, end potential = 1.5 V, amplitude = 25 mV, step potential = 5 mV, and frequency = 260 Hz. All experiments were carried out at 25 °C. The raw data were treated using the Savitzky and Golay filter, level 4 (included in GPES software). GPES software supplied by EcoChemie (Utrecht, Netherlands) was employed for smoothing and baseline correction (peak width = 0.05). Other details can be found in Kizek et al. [10].

### Preparation of Carbon Paste Electrode

The carbon paste (about 0.5 g) was made of 70% graphite powder (Sigma-Aldrich) and 30% mineral oil (*w*/*w*) (Sigma-Aldrich; 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 with a soft filter paper in preparation for measurement of a sample volume of 5 µL.

## Electromigration Methods

### *Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis*

Electrophoresis was performed according to Laemmli [21] using a Biometra maxigel apparatus with gel dimension of 17 × 18 cm (Biometra, Germany). First 10% (*w*/*v*) running, then 5% (*w*/*v*) stacking gel was poured, the gels were prepared from 30% (*w*/*v*) acrylamide stock solution with 1% (*w*/*v*) bisacrylamide concentration; the polymerization of the running gel was carried out at room temperature for 1 h and 30 min for the stacking gel. Prior to analysis the samples were mixed with sample buffer containing 5% (*v*/*v*) 2-mercaptoethanol in a 1:1 ratio. The samples were boiled for 2 min, then loaded onto a gel in 20 µL aliquots. For determination of relative molecular mass ($M_r$), the protein ladder "Precision plus protein standards" from Bio-Rad was used. The electrophoresis was run at 150 V with cooling by tap water (approx. 7 °C) until the front dye reached the bottom of the gel. To compare the avidin amount, 5,000 ng of avidin was run as a standard in one lane. Coomassie blue staining of the gels was performed according to Diezel et al. [22]. After staining, the gel was scanned and analyzed using Biolight software (Vilber-Lourmat, Germany).

### *Experion System*

Analyses on an automated microfluidic Experion electrophoresis system (Bio-Rad) were carried out according to the manufacturer's instructions with supplied chemicals (Experion Pro260 analysis kit, Bio-Rad). Each sample was diluted with water to the same protein concentration of 300 µg mL$^{-1}$, 4 µL aliquots were then mixed with 2 µL of reducing sample buffer, and after 4 min of boiling, 84 µL of water was added. After priming of the chip with gel and gel-staining solution in the priming station, the mixture (6 µL) was loaded into sample wells. The Pro260 Ladder included in the kit was used as a standard.

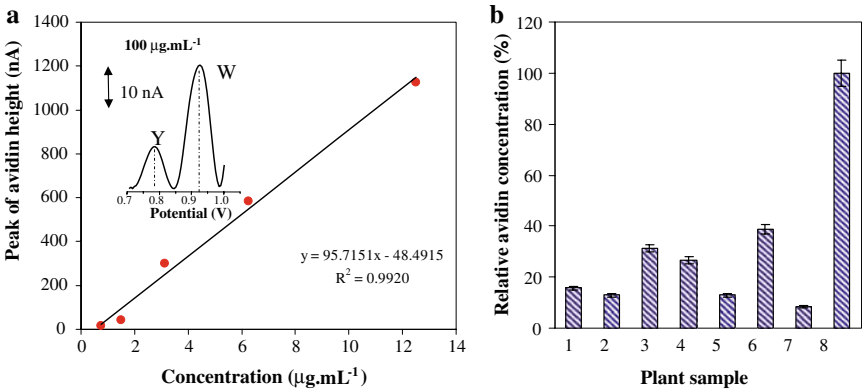


**Fig. 1.** Electrochemical behaviour of avidin on CPE surface. Calibration curve of avidin obtained by square wave voltammetry, in inset—electrochemical signals of avidin. Protein was adsorbed from 5 µL drop of solution at the carbon paste electrode followed by electrode washing and transfer into the electrolytic cell with blank supporting electrolyte—sodium acetate, pH 4.0. SWV was performed under following conditions: frequency 260 Hz, initial potential 0.1 V, end potential 1.5 V, step potential 5 mV, amplitude 25 mV, time of accumulation 60 s (**a**). Relative avidin concentrations in analyzed samples were obtained by square wave voltammetry. The peak of 100% height corresponds to the highest avidin positive tobacco extract (**b**)

### *Accuracy, Precision and Recovery*

Accuracy, precision and recovery of avidin were evaluated with homogenates (tobacco plant tissues) spiked with the standard by Experion. Before sample preparation, 100 µL avidin standards and 100 µL water were added to tobacco tissue samples. Homogenates were assayed blindly and avidin concentration was derived from the calibration curve. Accuracy was evaluated by comparing estimated concentrations with known concentrations of avidin. The spiking of avidin was determined as a standard measured without presence of real sample. Calculation of accuracy (%Bias), precision (%C.V.) and recovery was carried out as indicated by Causon [23], Bugianesi et al. [24], and Vanatta and Coleman [25].

## Results and Discussion

### Electrochemical Analysis of Avidin and Its Determination in Transgenic Plants

As it has been shown in many previously published papers [26, 27], tyrosine (Y) and tryptophan (W) residues are responsible for the electroacitivity of a protein at carbon electrodes. Square-wave voltammetric analysis using solid carbon electrodes is very sensitive and yields well-developed signals. However, using a CPE and base line correction of the data, we determined well-defined voltammetric signals for both Y and W at 0.78 and 0.92 V vs. Ag/AgCl/3 M KCl, respectively (Fig. 1a—inset). The peaks were measured at 100 µg mL$^{-1}$ of avidin by using the adsorptive transfer stripping square-wave voltammetry technique. These electrochemical transfer techniques were described in detail previously [28–37]. Based on our experiences with electrochemical detection of avidin and streptavidin [10], we applied adsorptive transfer stripping (AdTS) square wave voltammetry (SWV) in our measurements. The dependence of peak Y height on the frequency resulted in optimal separation of the signal from the background at 260 Hz. The calibration plot in expected concentration range of 0.75–12.5 µg mL$^{-1}$ avidin is shown in Fig. 1. For $t_A$ = 60 s the height of peak Y increased linearly (equation: $y = 95.7151x - 48.4915$, $R^2 = 0.9920$) with the avidin concentration up to 1 µg mL$^{-1}$. Full coverage of the electrode surface was not reached within the protein concentration range studied. The relative standard deviation (RSD) was only about 4%.

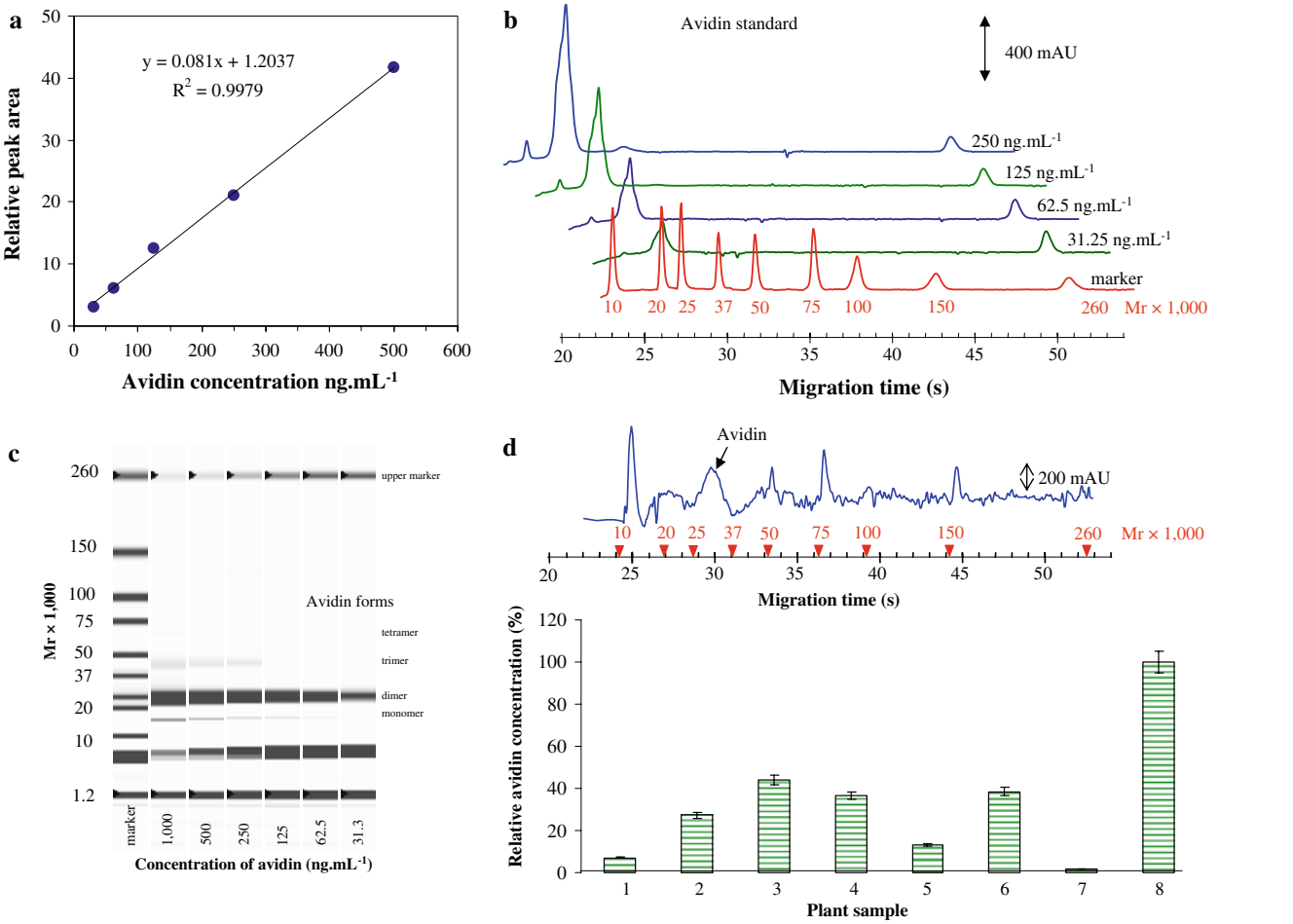


**Fig. 2.** Analysis of avidin in plant samples by Experion automated electrophoresis system. Calibration curve of avidin, the relative peak areas were recounted by the Experion software according to the area of relative molecular mass standard 260 × 1,000 (**a**), different concentrations of avidin standards in comparison with relative molecular mass standard (**b**), virtual gel output from Experion system, different concentrations of avidin standards (**c**), relative avidin concentrations in analyzed samples obtained by Experion system. The peak of 100% height corresponds to the highest avidin positive tobacco extract. Inset—record of real plant sample (**d**)

Under the optimized conditions the amount of avidin in various transgenic tobacco plants in comparison to controls was measured. The relative avidin concentrations of avidin-positive tobacco extracts are shown in Fig. 1b ($n$ = 3, RSD = 5%). For the avidin negative samples, in which the content of electroactive amino acids should be relatively low, only very small signals of 5% compared to avidin positive samples were produced. The calculated content of analyte in the control plants was subtracted from the analyte content in avidin-positive plants as previously described. Using electrochemical methods we determined that avidin levels in transgenic tobacco plants varied from 42 to 503 ng avidin per mg of fresh mass of the plant tissue.

## Determination of Avidin by Experion: Automated Electrophoresis System

Experion is an automated microfluidic electrophoresis system that uses a Caliper Life Sciences innovative LabChip microfluidic separation technology with sensitive fluorescent sample detection. It performs rapid and repeatable analyses of protein, DNA and RNA samples, which allows the analysis of protein or nucleic acids samples within 30 min. The separation, detection and data analysis are performed within a single platform, so the time-consuming steps in classic electrophoretic methods are minimized. Many types of samples, such as bacterial lysates, protein extracts, and chromatography fractions, can be analyzed. In addition to the significant shortening of time required, the chip-based method allows both repeatable and accurate sizing and quantification of the proteins.

After application of different concentrations of an avidin standard on the chip, the highest responses were observed at (25.8 ± 0.6) × 1,000 (migration time = 27.3 ± 0.1 s), which corresponds to the subunit dimer. In the case of the four highest concentrations (125, 250, 500 and 1,000 ng $mL^{-1}$) analyzed, the signals

corresponding to monomer ((16.4 ± 0.4) × 1,000, migration time = 24.9 ± 0.1 s), trimer ((44.7 ± 0.7) × 1,000, migration time = 30.7 ± 0.1 s) and tetramer of subunits ((66.7 ± 0.3) × 1,000, migration time = 34.0 ± 0.1 s), exhibited a slight signal shift with decreasing concentration of the protein to a higher relative molecular mass of about 0.5 (Fig. 2b, c). Considering the fact that the signals for monomer, trimer and tetramer were observed only at the four highest avidin concentrations tested, with each representing approximately 9.5, 7.3, 4.5 and 1.5% of the total signal intensity, respectively, it can be assumed that the absolute majority of the avidin present in plant extracts exists in the form of a subunit dimer. Within the range from 31.25 to 500 ng $mL^{-1}$ concentration, a linear dependence of signal intensity on avidin concentration was obtained as defined by the equation of $y = 0.0810x + 1.2037$ with an $R^2 = 0.9979$ (Fig. 2). The RSD of a measurement was 5.5% and the detection limit was 15.5 ng $mL^{-1}$ with analysis time not higher than 1 min.

Based on the previous results, the presence and intensity of the signal corresponding to the subunit dimer (peak of relative molecular mass of (26 ± 2) × 1,000) was measured in the plant protein extracts. The results obtained with plant samples are shown in Fig. 2d. With all of the analyzed plants, an increased intensity of the dimer peak signal compared to non-transformed control plants was observed (Fig. 2d). The average intensity of this peak measured in extracts from the transgenic plants was 7 ± 2 times higher than in control extracts. To determine the amount of avidin in the transgenic plants, the concentration of proteins with the same mobility determined in control plant extracts was subtracted from the concentration determined from the calibration curve. The avidin concentration in the transgenic plants ranged from 15 to 854 ng $mg^{-1}$ of fresh mass of the plant tissue. This large variability can be attributed to different avidin expression levels in individual transgenic plants. It clearly follows from the results obtained that Experion can be used for analysis of avidin in extracts from transgenic tobacco plants. However, the influence of the biological matrix on the avidin responses is considerable with recovery of 90% (Table 1).

**Table 1.** Recovery of avidin spiked in a tobacco tissue sample ($n = 5$)

| | Homogenate (μM)[a] | Spiking (μM)[a] | Homogenate + spiking (μM)[a] | Recovery (%)[b] |
|---|---|---|---|---|
| Avidin | 380 ± 30 (7.8) | 250 ± 11 (4.4) | 570 ± 51 (8.9) | 90 ± 8 |

[a] Avidin concentration; expressed as mean ± SD (C.V.%)
[b] Recovery; expressed as mean ± C.V.%

## Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis

In the case of sodium dodecyl sulphate—polyacrylamide gel electrophoresis (SDS-PAGE) using reducing conditions, the most intense band corresponding to the monomer of avidin subunit with a relative molecular mass of 15.8 × 1,000 was measured. Bands corresponding to other avidin forms were not detected probably due to the denaturing conditions and the high concentration of reducing agent (2.5% in the sample mixture). The optical density of the band with the same mobility as standard avidin monomer was approximately evaluated by Biolight software. In comparison to the control, the intensity of this band was of about 10 times higher in all transgenic plants. In one sample (plant #2) this intensity was markedly higher and in three plants (#5, 6 and 8) lower (Fig. 3a).

## Comparison of the Techniques Used for Avidin Determination in Transgenic Tobacco Plants

Avidin-expressing transgenic plants could be useful in insect control technology due to the toxicity of avidin to many species of insect pests. All three detection techniques used in this study are nonspecific because both electrochemical and electromigration methods demonstrate the presence of any protein. The electrochemical method (AdTS SWV at CPE) utilizes the electroactivity of two amino acids, tyrosine and tryptophan [26, 38, 39], while electromigration methods utilize mobilities of charged proteins after a subsequent colour reaction. Particularly the novel Experion system is based on binding of the specific fluorescent dye to a protein and subsequent detection of fluorescence intensity. The Experion analysis kit can separate and quantify protein samples ranging from relative molecular mass from 10 to 260 × 1,000. The result of each analysis provides a logging of individual peaks of proteins and the creation of virtual electrophoreograms. The sensitivity of this kit is comparable to colloidal Coomassie Blue staining of proteins in SDS-PAGE gels, which has been also used.

As we mentioned above, the three techniques employed for avidin determination in transgenic plants are quite different. In the case of electromigration methods, it was necessary to denature the sample thermally and chemically prior to its loading into the Experion chip or SDS-PAGE gel. Therefore, the native avidin was converted to monomeric (SDS-PAGE) and dimeric units (Experion). The reason why avidin was detected as a dimer in the case of the automated electrophoretic system is due to an incomplete denaturation and/or partial renaturation during the dilution and loading of samples into the Experion instrument. The 5–6 unit differences of the expected and obtained molecular mass of dimeric and trimeric avidin forms can be explained by used chip technology. Although the proteins can be quickly separated on the Experion system, sizing results may vary from those obtained using conventional SDS-PAGE (5,299 and 5,423 Bio-Rad bulletin). Electrochemical measurements were carried out with native avidin because it was not necessary to denature it. The electrochemical method well correlates with Experion with correlation coeffi-

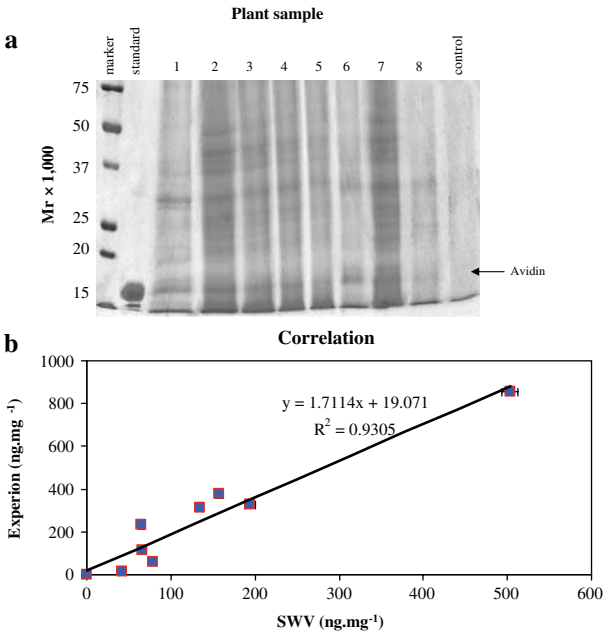


**Fig. 3.** SDS-PAGE analysis of avidin in plant samples. SDS-PAGE gel, standards of relative molecular mass, 5,000 ng of avidin standard, 1–8—samples of transgenic plants 1–8 respectively, control plant (**a**). Correlation of square wave voltammetry (SWV) and Experion method used to determine avidin in tobacco transgenic tissue sample (**b**)

cient higher than 0.9300 (Fig. 3b). However, compared to electrophoretic methods, the electrochemical method yields lower amounts (~40%), Fig. 3b. This difference can be explained by considering features of the different methods of detection. In both of the electromigration methods, the peptidic bond present in all proteins is detected, but the electrochemical determination is based on the detection of only the electroactive amino acids, Tyr and Trp, whose content is markedly increased in avidin relative to other proteins.

In comparison with the literature where the values of avidin amount in transgenic plants were between 70 and 2,500 ng per mg of fresh mass of the tissue, our results are in relatively good agreement with those amounts. In general, insect pests cannot develop and survive feeding on transgenic plants expressing 47 ppm (e.g. ng per mg of fresh mass) avidin or more [2, 7, 9]. Therefore, the data demonstrate that the transgenic tobacco plants expressed sufficient avidin such that they would be resistant to insect attack.

## Conclusions

We have proposed the use of a novel automated electrophoresis system (Experion) for avidin determination in transgenic plants. The technique is based on mobility of charged proteins in an electric field, the binding of fluorescent dye to the protein and subsequent analysis of the fluorescence intensity. We believe that this technology offers useful and promising possibilities in the field of biosensor development. We have also demonstrated the application of Experion for detection of avidin extracted from transgenic tobacco. In the case of avidin determination, we also compared the chip-based capillary electrophoretic method with the SDS-PAGE technique and square wave voltammetric method. Based on the results obtained here, it can be concluded that the square-wave voltammetry and Experion are sensitive, rapid and repeatable tools for quantitative protein analysis. These new approaches are likely to be a suitable tool in detection and quantification of a range of proteins and other biologically important compounds.

## Acknowledgment

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