Lékařská fakulta Masarykovy univerzity v Brně
ÚSTAV PATOLOGICKÉ FYZIOLOGIE

Avidin jako potenciální biopesticid a metody jeho analýzy

Rigorózní práce

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Prohlašuji, že jsem pracoval samostatně a použil pouze literaturu uvedenou v seznamu.

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Seznam použitých zkratek

AdTSV – adsorptivní přenosová rozpouštěcí voltametrie (adsorptive transfer stripping voltammetry)

a.c.V – voltametrie se superponovaným střídavým napětím (alternating current voltammetry)

AdTS DPV – adsorptivní přenosová rozpouštěcí diferenční pulzní voltametrie

CPSA – chronopotenciometrická rozpouštěcí analýza (chronopotentiometric stripping analysis)

CPE – uhlíková pastová elektroda (carbon paste electrode)

CMPE – uhlíková modifikovaná pastová elektroda (carbon modified paste electrode)

CV – cyklická voltametrie

DNA – deoxyribonukleová kyselina (deoxyribonucleic acid)

DPV – diferenční pulzní voltametrie

ELISA – enzymově spřažené imunochemické stanovení (enzyme linked immunosorbent assay)

HMDE – visící rtuťová kapková elektroda (hanging mercury drop electrode)

HPLC – vysokoúčinná kapalinová chromatografie

HPLC-DAD – vysokoúčinná kapalinová chromatografie s detekcí diodového pole

FIA – průtoková injekční analýza

FIA-ED – průtoková injekční analýza s elektrochemickou detekcí

NMR – nukleární magnetická rezonance

PCR – polymerázová řetězová reakce (polymerase chain reaction)

PGE – elektroda z pyrolytického grafitu (pyrolytic graphite electrode)

RNA – ribonukleová kyselina (ribonucleic acid)

QCM – křemenné krystalové mikrováhy (quartz crystal microbalance)

SCE – nasycená kalomelová elektroda (saturated calomel electrode)

SWV – voltametrie s pravoúhlým střídavým napětím (square-wave voltammetry)

UV – ultrafialová oblast spektra elektromagnetického záření

1. ÚVOD

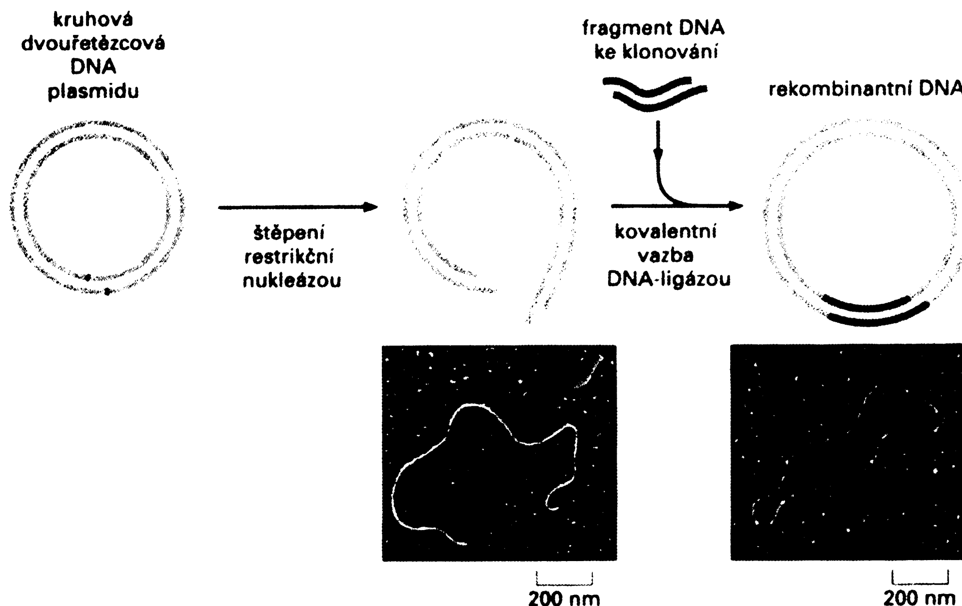
Předkládaná rigorózní práce řeší téma detekce avidinu izolovaného z různých druhů rostlin, do kterých byl vložen gen pro tento protein a dále se zabývá možností využít avidin jako potenciální biopesticid. Je totiž známo, že jednak v průběhu růstu, ale také po sklizení a uskladnění některých hospodářsky významných plodin jako je kukuřice, rýže a brambory jsou tyto napadány celou řadou hmyzích škůdců (*Coleoptera*, *Lepidoptera*, *Diptera*), kteří mohou způsobit velké ekonomické škody.

Avidin je glykoprotein tetramerní povahy, který má velmi silnou afinitu k vitamínu H, biotinu. Tím, že dojde k vazbě mezi avidinem a biotinem, ztratí biotin svou fyziologickou funkci. A právě biotinová deficience může u všech organismů způsobit zakrnělý vzrůst a v případě hmyzích škůdců i smrt. S tímto záměrem byly vytvořeny a vyprodukovány některé geneticky modifikované plodiny, které v sobě nesou gen pro expresi avidinu. Je samozřejmě velmi důležité a nezbytné v těchto transgenních rostlinách tuto expresi kontrolovat, aby nepřesáhla koncentraci, která by mohla být škodlivá pro člověka. Kromě běžně používané techniky jakou je ELISA byla před časem vypracována i metodika využívající elektrochemického stanovení (strept)avidinu v extraktu semen transgenní kukuřice.

Avidin se zdá potenciálně velmi vhodným proteinem pro konstrukci transgenních plodin z důvodu své netoxičnosti k životnímu prostředí. Je však také zapotřebí přísně kontrolovat hladinu tohoto proteinu a elektrochemické metody mohou sloužit jako elegantní nástroj pro detekci avidinu nebo obecně transgenních proteinů a částečně či úplně, např. ve formě biosenzorů, nahradit stávající techniky.

2. LITERÁRNÍ PŘEHLED

Příprava rekombinantních molekul DNA a klonovaných genů je základem genového inženýrství, které se zabývá vytvářením umělých kombinací genů nebo přípravou pozměněných nebo zcela nových genů a jejich zaváděním do genomu organizmů s cílem rekonstruovat jejich genetickou výbavu (Obr. 1). Gen upravený metodami genového inženýrství a přenášený do nového hostitelského organismu se označuje jako transgen. Organismus, jehož genom obsahuje stabilně začleněný transgen, se označuje jako transgenní, nebo též geneticky modifikovaný organismus (GMO) (Alberts, Johnson et al. 2002; Primrose and Twyman 2001; Šmarda, Doškař et al. 2005; Rosypal 2000; Rosypal 2002; Schnepf, Crickmore et al. 1998).



Obr. 1 Příprava rekombinantní DNA pomocí inserce fragmentu DNA do bakteriálního vektoru. Převzato z (Alberts, Johnson et al. 2002).

2.1 TRANSGENNÍ ROSTLINY

Pro přenos cizorodých genů do rostlinných buněk existuje řada účinných způsobů. K nejpoužívanějším patří přenos založený na přirozené schopnosti půdních bakterií rodu *Agrobacterium* napadat poraněné rostliny a přenášet do nich část své vlastní genetické informace. Některé kmeny těchto bakterií obsahují tzv. Ti-plazmid, jehož část označovaná jako T-DNA se přenáší z bakterií do rostlinných buněk, kde se začleňuje do náhodných míst jejich genomu (Rosypal 2002). Obsahuje dva základní typy genů:

a) geny odpovědné za tvorbu fytohormonů, které v rostlinných buňkách navozují dediferenciaci rostlinných pletiv a jejich transformaci na krčkové nádory
b) geny, které kódují syntézu opinů, což jsou deriváty zásaditých aminokyselin, především argininu a lyzinu a agrobakterie jich využívají jako zdroj uhlíku, dusíku a energie (Rosypal 2000).

Z fyzikálních metod se pro přenos genů používá zejména:
Biolistická metoda – při níž se molekuly DNA nejdříve navážou na povrch mikroskopických částic kovu (většinou zlatých), které se pak pomocí speciálního zařízení nastřelují přímo do rostlinných buněk, protoplastů nebo meristémových pletiv. Předností této metody oproti jiným je možnost přenášet geny též do genomu chloroplastů a ovlivňovat tak procesy fotosyntézy (Li, Qu et al. 1993).

Lipofekce – při ní se molekuly DNA nejdříve obalí vrstvou syntetických lipidů za vzniku částic podobných lipozomům. Ty pak snadno proniknou dovnitř protoplastů v důsledku schopnosti tukových látek splývat s buněčnými membránami dalších buněk (Šmarda, Doškař et al. 2005).
Kromě výše uvedených fyzikálních metod se k přenosu genů do rostlin používá též elektroporace, mikroinjekce DNA přímo do buněk nebo makroinjekce DNA do rostlinných pletiv.

2.1.1 Cíle transgenoze u rostlin

Hlavním cílem genetických modifikací u rostlin je ovlivnit především ty vlastnosti, které zvyšují jejich výnosy nebo vedou k vyšší kvalitě plodů a semen. Transgenoze se proto zaměřuje především na přípravu rostlin odolných k hmyzím škůdcům, virovým,

bakteriálním a houbovým chorobám, herbicidům používaným k jejich ochraně před plevely a nepříznivým biotickým podmínkám prostředí (sucho, mráz, zasolení půd).
V současné době jsou transgenní odrůdy připraveny již u mnoha druhů kulturních rostlin, zejména obilovin, kukuřice, rýže, sóji a některých druhů zeleniny. Řada transgenních odrůd byla připravena též u řepky olejné jako průmyslově důležitého druhu a byly připraveny též transgenní dřeviny (smrk, topol etc.) (Wenck, Quinn et al. 1999; Scheffler and Dale 1994; Husken and Dietz-Pfeilstetter 2007; Herschbach and Kopriva 2002).

2.1.2 Průmyslově významné transgenní rostliny

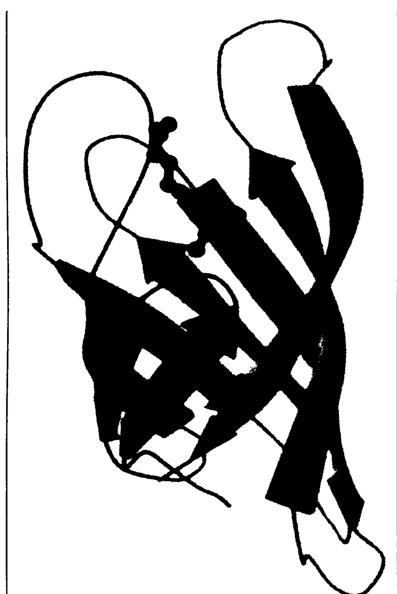
Transgenní rostliny slouží jako zdroj nových surovin pro průmysl. Např. transgenní odrůdy řepky olejné s pozměněným složením zásobního oleje v semenech slouží jako surovina pro výrobu mýdel, mazadel a nylonu (Scarth and Tang 2006; Murphy 1999). Z oleje řepky lze vyrábět bionaftu, která je biologicky degradovatelná a jejímž spalováním vznikají méně toxické sloučeniny. Velmi perspektivní je též využití některých transgenních druhů rostlin při přípravě materiálů s novými vlastnostmi (biodegradovatelné plasty apod).

Transgenní rostliny by mohly být rovněž velmi vhodnými producenty cizorodých látek s farmakologickými účinky, např. antigenů působících jako vakcíny pro imunizaci vůči bakteriálním a virovým chorobám. Syrová zelenina, v níž se tvoří povrchový antigen viru hepatitidy B, indukuje v zažívacím traktu tvorbu protilátek a navozuje u konzumentů určitý stupeň imunity vůči této chorobě (Kumar, Ganapathi et al. 2007; Cramer, Boothe et al. 1999). Asi nejvýznamnějším objevem roku 2005 v oblasti transgenních plodin je tzv. „zlatá rýže“ nesoucí v sobě gen pro produkci provitaminu A (Paine, Shipton et al. 2005).
Významným přínosem pěstování transgenních rostlin z ekologického hlediska je podstatné snížení chemizace v zemědělství. Umožňuje snižovat používání pesticidů nebo nahradit stávající typy herbicidů takovými, které mají krátkou životnost a jsou šetrnější k prostředí (Rosypal 2000; Rosypal 2002).

2.2 Avidin

Avidin je bazický tetramerní glykoprotein o molekulové hmotnosti 66 000, který je možné izolovat z vaječného bílku ptáků, plazů a obojživelníků (Green 1975). Každá ze čtyř identických podjednotek avidinu je tvořena osmi antiparalelními β-listy (Green 1975;

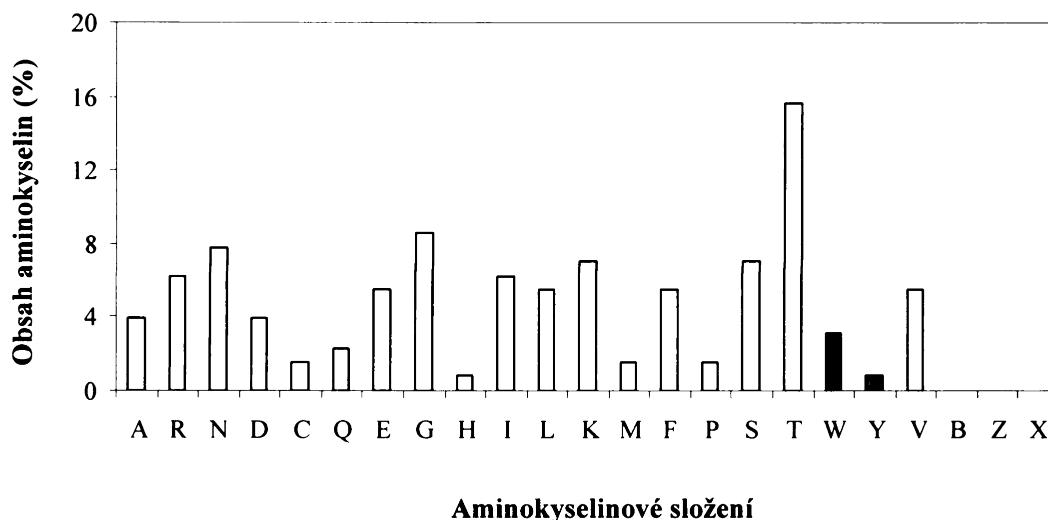
Rosano, Arosio et al. 1999), které udělují proteinu strukturu ve tvaru soudku (Obr. 2). Uvnitř tohoto „soudku“ se nachází vazebné místo pro biotin (vitamín H), který se váže k avidinu s mimořádně velkou afinitou ($K_d \sim 10^{-15}$ M) (Green 1963; Green 1975). Avidin a biotin tak dávají společně vzniknout velmi pevnému spojení, které patří mezi vůbec nejsilnější známé nekovalentní interakce proteinu s ligandem. Významný podíl na tvorbě komplexu avidin-biotin mají hydrofobní interakce mezi biotinem a aminokyselinami přítomnými ve vazebném místě pro biotin (Trp 70, Trp 97, Trp 110 a Phe 79) (Obr. 3). Kromě těchto interakcí se vazby biotinu k avidinu účastní také hydrofilní interakce prostřednictvím rozsáhlé sítě vodíkových můstků a flexibilní smyčka avidinu, která přiléhá k vazebnému místu pro biotin a omezuje tak jeho přístupnost pro rozpouštědlo (Lindqvist and Schneider 1996; Clarkson, Batchelder et al. 2001).



Obr. 2 Schéma terciární struktury monomeru avidinu v komplexu s biotinem. Převzato z (Flower 2007).

β-listy avidinu jsou vyznačeny tyrkysově, jejich spojení zeleně. Biotin je představován barevným kuličkovým modelem.

Podobnou hodnotou disociační konstanty pro biotin, jakou má avidin, se vyznačuje také streptavidin, avidinu strukturně a funkčně podobný protein produkovaný bakteriálním kmenem *Streptomyces avidinii* (Green 1990). Streptavidin na rozdíl od avidinu není glykosylovaný, ani bazický, neobsahuje žádnou sirnou aminokyselinu (zatímco součástí struktury avidinu je jedna disulfidická vazba Cys-S-S-Cys a dva methioninové zbytky) a ve vazebném místě pro biotin má o jeden tryptofanový zbytek více (Trp 79, Trp 92, Trp 108 a Trp 120) (Lindqvist and Schneider 1996).



Obr. 3 Aminokyselinové složení avidinu. Barevně jsou vyznačeny elektroaktivní aminokyseliny tryptofan (W) a tyrosin (Y).

Oba proteiny mají schopnost snižovat koncentraci volného biotinu v prostředí. Mohly by tak mít funkci látek, které inhibují bakteriální růst a tak vedou k selekční výhodě (Kurzban, Gitlin et al. 1989; Pugliese, Coda et al. 1993).

2.2.1 Interakce strept(avidinu) s biotinem

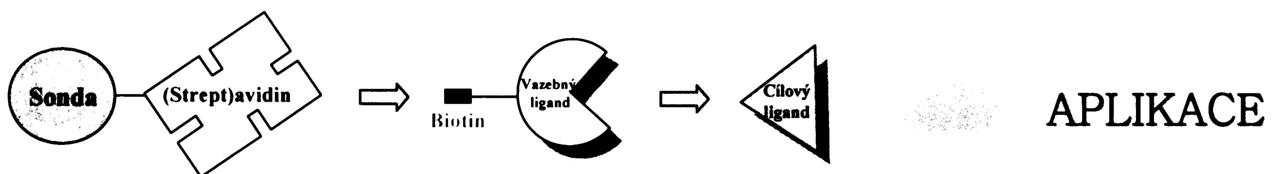
Vazba biotinu ke strept(avidinu) významně ovlivňuje vlastnosti obou proteinů. Avidin i streptavidin se vyznačují vysokou odolností proti působení vysokých teplot (Donovan and Ross 1973), extrémních pH (Green 1963) i velkých koncentrací denaturačních a organických činidel (Sano, Vajda et al. 1998). Interakce s biotinem tuto již tak vysokou stabilitu ještě umocňuje. Např. rozdíl v denaturačních teplotách samotného avidinu a komplexu avidin-biotin činí v rozmezí hodnot pH 7-9 (téměř bez ohledu na typ pufru a/nebo iontovou silu) 47 °C ($t_{m\ avidin}$ = 85 °C, $t_{m\ avidin\text{-}biotin}$ = 132 °C) (Donovan and Ross 1973; Pugliese, Coda et al. 1993; Gonzalez, Argarana et al. 1999). Vazba biotinu do vazebného místa strept(avidinu) také chrání přítomné tryptofanové zbytky před oxidací nejrůznějšími činidly (Spande and Witkop 1967).

2.2.2 Využití interakce strept(avidin)-biotin

Kvůli všem výše zmíněným vlastnostem a kvůli možnému všestrannému využití za současného zachování jednotných experimentálních podmínek je systém strep(avidin)-biotin v poslední době hojně používán v biotechnologických aplikacích (Obr. 4).

Aplikace systému strept(avidin)-biotin je založena na vazbě biotinem modifikované nízko- nebo vysokomolekulární látky k nativnímu nebo reportérovou skupinou značenému (strept)avidinu a na následné detekci této skupiny. Reportérovou skupinou však může být značen také sám biotin (Wilchek and Bayer 1988). Detekce reportérové skupiny může být prováděna v závislosti na jejím charakteru nejrůznějšími způsoby: imunodetekcí (Heitzmann and Richards 1974; Rappuoli, Leoncini et al. 1981), elektronovou mikroskopií (Spiegel, Skutelshy et al. 1982), DNA hybridizací (Wilchek and Bayer 1990), elektrochemicky (Athey, Ball et al. 1993) atd.

Systém strept(avidin)-biotin je využíván dále v separačních metodách (afinitní chromatografie), při lokalizaci antigenů (afinitní cytochemie, buněčná cytometrie, bloting), při hybridizacích, v diagnostice (imunodetekce, PCR), v biosenzorech atd. (Fuccillo 1985; Wilchek and Bayer 1988; Bayer and Wilchek 1990; Schetters 1999).



Obr. 4 Obecné schéma (strept)avidin-biotinové technologie.

Nedávno byla zveřejněna práce, ve které autoři využívají inteinu (protein splicing element), tedy jakéhosi proteinového intronu, k biotinylaci proteinů jak *in vivo* tak *in vitro* (Lue, Chen et al. 2004), a následné vazbě takto biotinylovaných proteinů na avidinem pokrytou destičku. Tímto způsobem imobilizované proteiny jsou připraveny pro proteinové „arrays", případně pro další aplikace.

2.2.3 Avidin jako potenciální biopesticid

Jednou z cest, jak dosáhnout vyšší produktivity při pěstování zemědělských plodin je zabránit ztrátám způsobeným hmyzími škůdci jak při pěstování, tak během skladování. Tradičním způsobem boje proti těmto ztrátám je používání pesticidů. Jejich nadměrné a neodborné používání často vede ke kontaminaci životního prostředí, otravě necílových organismů a přítomnosti jejich reziduí v potravinách. Pomocí metod genetického inženýrství byly připraveny rostliny vykazující možnou odolnost i k hmyzím škůdcům. Nejznámějším zásahem způsobujícím tuto rezistenci je produkce *cry* proteinu původem z bakterie *Bacillus thuringiensis*, nazývaného též Bt toxin, který činí rostliny odolnými k škůdcům z řádů brouků (*Coleoptera)* a motýlů (*Lepidoptera)* (Schnepf, Crickmore et al. 1998). Od roku 1994 jsou pro komerční použití pěstovány různé odrůdy takto upravené kukuřice, bavlny, rajčat a brambor. Kromě *cry* proteinu může být pro tento účel využita řada jiných makromolekul, například avidin, streptavidin nebo lektiny. V jejich případě, ale ještě nejsou dostupné všechny potřebné informace pro to, aby rostliny s těmito transgeny byly využívány pro komerční produkci.
Kramer et al. připravili rostliny (Hood, Witcher et al. 1997; Kramer, Morgan et al. 2000) exprimující avidin. Následovaly další rostliny, které byly tímto způsobem modifikovány, např. tabák, jablka, rýže, kukuřice, brambory (Burgess, Malone et al. 2002; Yoza, Imamura et al. 2005). Zároveň bylo v mnoha experimentech prokázáno, že rostliny transformované tímto vektorem způsobují úhyn širokého spektra rostlinných škůdců z řádů brouků, motýlů a dvoukřídlých (Burgess, Malone et al. 2002; Yoza, Imamura et al. 2005; Zhu, Adamczyk et al. 2005). Je to způsobeno tím, že avidin resp. streptavidin způsobí u těchto organismů biotinovou deficienci (vazba (strept)avidin-biotin) což vyvolá zakrnělý růst až smrt.

2.3 Studium biologicky významných látek pomocí elektrochemických metod

2.3.1 Typy pracovních elektrod

Kromě běžně používaných metod v analýze proteinů (jako jsou metody elektroforetické, chromatografické případně studium pomocí rentgenové krystalografie nebo NMR

spektroskopie) se v dnešní době rychle rozvíjejí metody elektrochemické jako nástroje pro studium proteinů.

K analýze biologicky významných látek, mezi které proteiny bezesporu patří, je zapotřebí zvolit vhodné pracovní elektrody. V současné elektrochemické analýze jsou nejčastěji využívány zejména elektrody stacionární vyrobené z různých materiálů (Paleček 1983; Bard and Faulkner 2000). Nejčastěji jsou pro své dobré elektrické vlastnosti, snadnou manipulovatelnost a jednoduchou obnovitelnost povrchu používány uhlíkové elektrody a kovové elektrody, zejména visící rtuťová kapková elektroda (HMDE) (Wang 1994).

2.3.1.1 Rtuťové a amalgamové elektrody

HMDE je elektroda, která má dokonale hladký a obnovitelný povrch, což je velmi výhodné při práci s biomakromolekulami, kdy se velmi často využívá jejich silných adsorpčních schopností na této elektrodě. HMDE je polarizovatelná k negativnějším potenciálům než ostatní kovové elektrody (díky vysokému přepětí vodíku na rtuti) a umožňuje tak sledování dějů probíhajících za přítomnosti biopolymerů ve velmi negativních oblastech potenciálů proti nasycené kalomelové elektrodě (Markušová 2000). K této elektrodě jsou vztaženy všechny dále uvedené hodnoty potenciálu. Z dalších elektrochemicky vhodných kovů (např. Ag, Au, Pt) se připravují pevné elektrody, pro něž je charakteristické méně dokonalé (ve smyslu reprodukovatelnosti) a pracnější obnovování jejich pracovního povrchu (Wang 1994).

V posledních letech se objevuje snaha nahradit při konstrukci elektrod kapalnou rtuť jiným materiálem, který by si však uchoval jedinečné vlastnosti rtuti (např. vysoké přepětí vodíku a široký potenciálový rozsah v katodické oblasti). Tomuto kritériu se nejvíce přibližují pevné amalgámové elektrody (Jelen, Yosypchuk et al. 2002; Yosypchuk and Novotny 2002; Yosypchuk and Novotný 2002). Jedná se o slitiny rtuti s jedním nebo více kovy, které se snadno zhotovují, jsou levné a kde stabilita elektrod dosahuje několik týdnů. Nejčastěji používaným amalgámem je slitina rtuti se stříbrem, která je pro svoji naprostou netoxicitu aplikována dlouhá léta ve stomatologii (Mikkelsen and Schroder 2003).

2.3.1.2 Uhlíkové elektrody

Uhlíkové elektrody jsou při studiu biomakromolekul vhodným doplněním elektrod rtuťových, neboť jsou polarizovatelné do oblasti kladných potenciálů (až na +1 V na rozdíl

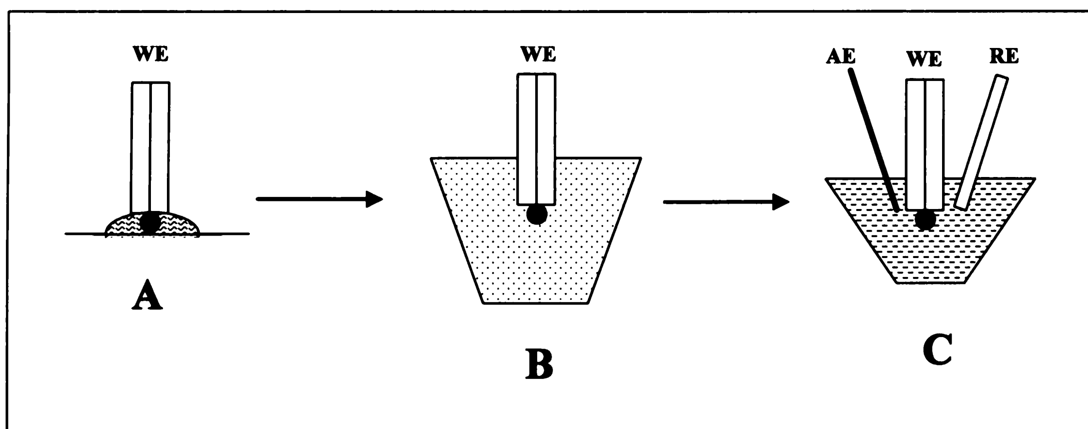
od HMDE, kterou lze polarizovat jen asi k +0.2 V), čímž se rozšiřuje využitelná potenciálová oblast a možnost elektrochemického studia těchto látek. Povrch uhlíkových elektrod se obnovuje obvykle mechanicky, odstraněním použité vrstvy elektrodového materiálu (broušením, sloupáváním) a další mechanickou případně elektrochemickou úpravou nově vzniklé povrchové vrstvy materiálu (např. leštění nebo elektrochemická příprava elektrody). Tyto kroky rozhodují z velké části o reprodukovatelnosti měření. Způsob aktivace povrchu uhlíkových elektrod závisí jednak na vlastním materiálu elektrody, jednak na typu analýzy, pro niž je elektroda určena. Obvykle se uhlíkové elektrody aktivují chemicky nebo elektrochemicky (vystavením dostatečně pozitivním nebo negativním potenciálům ve vhodných prostředích) (Beilby, Sasaki et al. 1995; Chi, Göpel et al. 1997). Nejběžněji používané typy uhlíkových elektrod jsou: uhlíková pastová elektroda (carbon paste electrode, CPE); elektroda z pyrolytického grafitu (pyrolytic graphite electrode, PGE, highly oriented pyrolytic graphite electrode, HOPGE) a dále pak elektrody ze skelného uhlíku nebo z uhlíkového vlákna (Wang 2000). V poslední době se stále častěji využívají uhlíkové elektrody modifikované uhlíkovými nanotrubičkami (carbon-nanotube-modified carbon electrodes) pro citlivější stanovení DNA (Wang, Kawde et al. 2004) nebo i bílkovin (Wang, Li et al. 2002).

2.3.2 Elektrochemické metody

Jestliže bychom měli zmínit elektrochemické metody, které se v dnešní době nejvíce využívají ke studiu biologicky významných látek, vyplyne nám, že to jsou převážně techniky voltametrické a chronopotenciometrické, totiž metody, které sledují faradaické a adsorpčně – desorpční děje na elektrodách. Techniky, které jsou citlivé k tenzametrickým dějům jsou využívány při studiu adsorbce nukleových kyselin (Paleček 1983; Paleček 1996; Fojta and Paleček 1997; Fojta 2002; Fojta 2004; Kucharikova, Novotny et al. 2004) a peptidů (Miller 1971; Rishpon and Miller 1975; Barker, Gleria et al. 1990; Sadik and Cheung 2001). Bylo zjištěno, že proteiny a nukleové kyseliny jsou na elektrodových površích adsorbovány tak pevně, že je možné elektrodu s adsorbovanou biomakromolekulou přenést do jiného prostředí (např. čistého základního elektrolytu, kde dochází k vlastnímu elektrochemickému stanovení), aniž by došlo k podstatné změně elektrochemického signálu vlivem ztráty biopolymeru z povrchu elektrody (Kolthoff and

Kihara 1977; Paleček 1986). Tento princip byl rozpracován technikou adsorptivní přenosové rozpouštěcí voltametrie (adsorptive transfer stripping voltammetry, AdTSV) (Paleček and Postbieglová 1986; Paleček 1988; Paleček, Jelen et al. 1993), která umožnila snížení spotřeby analyzovaného vzorku o několik řádů a podstatně zjednodušila a zrychlila elektrochemickou analýzu.

Na obrázku 5 je uvedeno zjednodušené schéma adsorptivní přenosové rozpouštěcí techniky. Pracovní elektroda (WE – working electrode) je ponořena do studovaného roztoku a tento studovaný analyt je adsorbován na povrch elektrody po určitou dobu (t_A – čas akumulace) (**A**). Po uplynuté době je elektroda omyta ve vodě, případně v základním elektrolytu (**B**). Omytá elektroda je přenesena do měřící nádobky (**C**), ve které je základní elektrolyt, pomocná elektroda (AE – auxiliary electrode), referenční elektroda (RE – reference electrode) a kde probíhá samotné elektrochemické (voltametrické nebo chronopotenciometrické) měření.



Obr. 5 Schéma adsorptivní přenosové rozpouštěcí techniky.

2.3.2.1 Voltametrie s pravoúhlým střídavým napětím

Hlavní výhodou voltametrie s pravoúhlým střídavým napětím (square-wave voltammetry, SWV) je její rychlost, která radikálně snižuje dobu analýzy. U SWV je na lineárně měnící se potenciálovou rampu superponováno střídavé pravoúhlé napětí s volitelnou amplitudou a frekvencí (tyto parametry určují rychlost posunu potenciálu,

citlivost a rozlišovací schopnost metody). Proud je odečítán během celého cyklu dvakrát, vždy na konci půlcyklu. Sledovaný signál je rozdílem odečtených proudů (Wang 2000).

2.3.2.2 Chronopotenciometrická rozpouštěcí analýza

V elektroanalytické chemii jsou pro svoji citlivost hojně využívány metody pracující s předchozím elektrolytickým nebo adsorpčním nahromaděním stanovované látky na povrch elektrody a s následným rozpouštěním takto vzniklého depozitu. K měření elektrického signálu během rozpouštěcího procesu se vedle voltametrie používá také chronopotenciometrie, při níž se sledují časové změny potenciálu měrné elektrody v závislosti na koncentraci analyzované látky. Nejčastěji je při této metodě sledován průběh ***dt/dE*** proti ***E***, a to jak při rozpouštěcí tak i adsorpční chronopotenciometrii (Kalvoda 2002).

2.3.3 Elektrochemie proteinů a peptidů

Elektrochemické metody byly velmi úspěšně aplikovány při analýze proteinů (Brdička 1933; Paleček 1983; Armstrong 2002; Heyrovský 2004) a našly uplatnění v celé řadě oborů včetně biochemie, farmacie, lékařství a částečně i v onkologickém výzkumu (Homolka 1971). Z dvaceti jedna aminokyselin, které obvykle tvoří primární strukturu proteinů, se redoxní aktivitou za běžně používaných podmínek vyznačují pouze tři z nich. Jedná se o aminokyseliny cystein/cystin, tyrosin a tryptofan. Voltametrická nebo chronopotenciometrická analýza proteinů, které neobsahují redox-aktivní prostetické skupiny pro přímé elektrochemické stanovení, je omezena na studium jejich adsorpčních/desorpčních vlastností a měření oxidace tyrosinu (Y) a tryptofanu (W) na uhlíkové elektrodě (Cai, Rivas et al. 1996; Wang, Rivas et al. 1996; Tomschik, Havran et al. 1998), nebo na reakce proteinů obsahující zbytky cysteinu/cystinu se rtuťovou elektrodou za tvorby vazby Hg-S (Havran, Billova et al. 2004), dále pak redukce disulfidické vazby, katalytické vylučování vodíku v roztoku obsahující kobalt na rtuťové elektrodě (tzv. Brdičkova reakce) (Paleček 1983; Honeychurch and Ridd 1996). Opomenuta nemůže být ani katalytická prenatriová vlna (Heyrovský and Babicka 1930; Heyrovský and Kuta 1965), která nevyžaduje zbytky cysteinu/cystinu, ale její použití je omezeno jejím výskytem v oblasti velmi negativních potenciálů (Obr. 6).

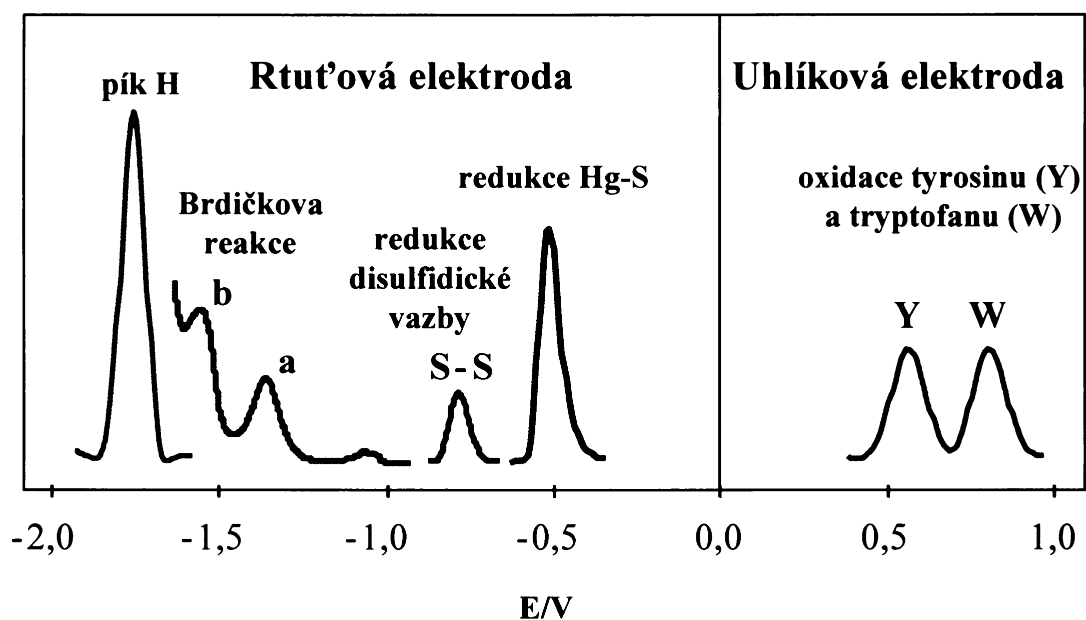
Základem řady reakcí peptidů a proteinů na rtuťové elektrodě jsou skupiny -SH a -S-S- ve zbytcích cysteinu, resp. cystinu. Atomy síry v těchto aminokyselinách mají velkou afinitu ke rtuti a velmi ochotně s ní interagují (Heyrovský, Mader et al. 1994; Havran, Billova et al. 2004). V závislosti na podmínkách mohou vznikat sloučeniny charakteru cystin/cystein-thiolátu rtuťného/rtuťnatého, jejichž redoxní změny na HMDE se projevují voltametrickými píky. Voltametrický pík se podle zvolených podmínek vyskytuje přibližně při potenciálu -0.4 až -0.6 V je přisuzován redukci thiolátu rtuťnatého a negativnější signál v oblasti -0.7 V je přisuzován přímé redukci cystinu (Stankovich and Bard 1977; Heyrovský, Mader et al. 1994). Kromě toho, že takovým způsobem reagují volné aminokyseliny, se i cystin/cysteinové zbytky v peptidech a bílkovinách na HMDE projevují obdobným způsobem (Stankovich and Bard 1977; Stankovich and Bard 1978). Potenciál a velikost signálu jsou závislé na obsahu těchto aminokyselin v molekule, na jejich přístupnosti povrchu elektrody, dále na velikosti molekuly a na jejím redoxním stavu. Redukční pík v oblasti -0.6 V poskytují látky obsahující jak S-S vazby, tak obvykle i látky se skupinami -SH. Od roku 1996 byla metodika elektrochemického studia těchto dějů rozšířena o chronopotenciometrickou rozpouštěcí analýzu (CPSA) (Honeychurch and Ridd 1996).

Ve třicátých letech minulého století bylo objeveno, že bílkoviny poskytují na rtuťové elektrodě dva typy katalytických signálů. Jedná se o katalytické vylučování vodíku při potenciálech pozitivnějších, než jaké jsou potenciály vylučování složek základního elektrolytu. Prvním z nich je Brdičkova reakce (Babička and Heyrovský 1930; Brdička 1933) a druhým z nich je prenatriová vlna (Babička and Heyrovský 1930; Brdička 1936; Heyrovský and Kuta 1965).

Brdičkova reakce je katalytická reakce bílkovin v amoniakálních pufrech obsahujících ionty kobaltu (Co^{2+}, $[Co(NH_3)_6]^{3+}$ (Brdička 1933)). Přítomnost bílkovin v těchto prostředích způsobuje potlačení katalytického maxima kobaltu (díky povrchové aktivitě bílkovin a peptidů) a vznik jedné nebo dvou polarografických vln (nebo voltametrických píků) v oblasti potenciálů -1.2 až -1.5 V. Bylo prokázáno, že tuto reakci způsobují sulfidické skupiny (bílkovin, peptidů i jednodušších látek) ve spojení s komplexy iontů kobaltu (shrnuto v (Brdička, Březina et al. 1965; Heyrovský and Kuta 1965). Brdičkova reakce byla po určitou dobu využívána v medicíně jako citlivá metoda na stanovení bílkovin v tělních tekutinách a pro diagnostiku některých onemocnění (Heyrovský and Kuta 1965).

Prenatriová vlna je signál vylučování vodíku katalyzovaného bílkovinami a peptidy v oblastech o 100 až 250 mV pozitivnějších než jsou potenciály vylučování vodíku z pufru v nepřítomnosti iontů kobaltu, niklu či jiných těžkých kovů. Tento jev byl poprvé pozorován v roztocích chloridu sodného, který obsahoval stopy bílkovin krevního séra (Babička and Heyrovský 1930; Brdička 1936). Velmi podobné katalytické proudy vodíku jako u proteinů byly pozorovány v přítomnosti některých alkaloidů, pyridinu a dalších látek. Průběh tohoto děje je velice závislý na složení, koncentraci a pH elektrolytu, i na koncentraci aktivní látky (Brdička 1936; Kolthoff, Yamashita et al. 1975). Přestože bylo navrženo několik mechanismů této reakce, nebylo dosud zcela jasně prokázáno, které funkční skupiny jsou za katalýzu vylučování vodíku zodpovědné. Předpokládá se však, že jde o skupiny obsahující kvartérní dusík a o sirné skupiny. Jak již bylo řečeno, díky tomu, že se vyskytuje ve velice negativních potenciálech i díky špatné měřitelnosti jak voltametrickými tak i polarografickými metodami, nenalezla v minulosti mnoho uplatnění (Heyrovský and Kuta 1965). Avšak díky aplikaci CPSA by příslušné elektrodové děje mohly nalézt analytické využití. Tato metoda poskytuje v oblastech kolem -1.6 až -1.9 V dobře měřitelný signál, který byl nazván píkem H, a který je možné využít pro velmi citlivé stanovení peptidů a proteinů (Tomschik, Havran et al. 1998; Tomschik, Havran et al. 2000; Kizek, Trnkova et al. 2001; Trnková, Kizek et al. 2002).

Na uhlíkových elektrodách dochází k oxidaci tyrosinu (Tyr) a tryptofanu (Trp) (Brabec 1980; Brabec and Mornstein 1980; Brabec and Mornstein 1980). Dále také histidinu, cysteinu, cystinu a methioninu, avšak oxidační signály těchto aminokyselin nenalezly patřičné analytické využití (Sequaris 1992). Obě aromatické aminokyseliny, tedy tyrosin a tryptofan, ať volné, nebo vázané v polypeptidovém řetězci, poskytují v neutrálních nebo mírně kyselých prostředích oxidační signály v potenciálech kolem +0.6 V pro tyrosin a asi +0.8 V vs. nasycené kalomelové elektrodě (SCE) pro tryptofan. Dobře rozlišené signály oxidace bílkovin osahujících Tyr a Trp byly získány na grafitových elektrodách s využitím CV nebo DPV (Brabec and Mornstein 1980; Brabec 1982). V posledních letech byla ke sledování oxidačních procesů některých peptidů na CPE úspěšně využita CPSA (Cai, Rivas et al. 1996; Wang, Rivas et al. 1996). Oxidace dalších zmíněných aminokyselin (histidin, cystein, cystin, methionin) probíhá v neutrálním prostředí u pozitivnějších potenciálů (asi +1.0 V u cysteinu, +1.2 V u histidinu, +1.2 až +1.3 V u cystinu a methioninu) (Sequaris 1992).



Obr. 6 Přehled oxidačních a redukčních signálů, které poskytují peptidy a proteiny na HMDE a CPE.

3. CÍLE RIGORÓZNÍ PRÁCE

Tato rigorózní práce je zaměřena na možnost využití avidinu jako biopesticidu a na stanovení avidinu v transgenních rostlinách pomocí elektrochemických a elektromigračních metod. Jejím cílem je ukázat, že kromě běžných metod analýzy avidinu jakým je např. ELISA technika to jsou především metody elektrochemické, jejichž aplikace v detekci transgenních proteinů může být velice vhodná a výhodná.

Pro tuto práci byly stanoveny následující dílčí cíle:

1. Aplikovat nové elektroanalytické metody ke studiu biotechnologicky významných proteinů jakými jsou avidin a streptavidin a navržení možnosti jejich využití v biologickém výzkumu.

2. Konstrukce modifikované pastové elektrody jako potenciálního biosensoru.

3. Otestovat další techniky ve stanovení avidinu z transgenních rostlin jakou je např. chip-based kapilární elektroforéza EXPERION.

4. EXPERIMENTÁLNÍ ČÁST

Experimentální část předkládané rigorózní práce je přiložena ve formě tří publikací.

Přehled publikací

Příloha A

Kizek R., Masařík M., Kramer K. J., Potěšil D., Bailey M., Howard J. A., Klejdus B., Mikelová R., Adam V., Trnková L., Jelen F.:
An analysis of avidin, biotin and their interaction at attomole levels by voltametric and chromatographic techniques.
ANALYTICAL AND BIOANALYTICAL CHEMISTRY 381: 1167-1178, 2005

Příloha B

Petrlová J., Masařík M., Potěšil D., Adam V., Trnková L., Kizek R.:
Zeptomole detection of streptavidin using carbon paste electrode and square-wave voltammetry.
ELECTROANALYSIS 19(11): 1177 – 1182 , 2007

Příloha C

Křížková S., Hrdinová V., Adam V., Burgess E.P.J., Kramer K.J., Masařík M., Kizek R.:
Utilization of chip-based capillary electrophoresis for avidin determination in transgenic tobacco and its advantages over standard gel electrophoresis and voltammetry.
CHROMATOGRAPHIA - odesláno srpen 2007

4.1 Shrnutí výsledků

Příloha A

Tato publikace navazuje na naši první studii, týkající se detekce avidinu resp. streptavidinu a rozšiřuje možnost stanovení těchto proteinů v transgenních rostlinách.Práce popisuje využití avidin-biotinové technologie a square wave voltametrie v kombinaci s adsorptivní rozpouštěcí přenosovou technikou pro stanovení samotného avidinu s použitím uhlíkové pastové elektrody, a detekci avidinu v transgenní rostlině kukuřice (*Zea mays*), do které byl vnesen gen pro avidin.

Nejprve bylo provedeno základní elektrochemické stanovení avidinu paralelně se stanovením ultrafialového absorpčního spektra. Elektrochemicky byla naměřena koncentrační závislost i závislost výšky oxidačního signálu tyrosinu a tryptofanu na době akumulace na elektrodovém povrchu.

Dále bylo využito myšlenky Wanga et al. (Wang and Lin 1988), který před časem publikoval práci, ve které přidáním banánového extraktu do uhlíkové pasty vytvořil biosenzor pro elektrochemickou detekci dopaminu. Vědomi si toho, že (strept)avidin-biotin technologie je v dnešní době naprosto nepostradatelným nástrojem v celé řadě biochemických a molekulárně biologických aplikací, jsme do uhlíkové pasty přidali avidin a vytvořili tak modifikovanou uhlíkovou pastovou elektrodu (CMPE). Takto modifikovaná pastová elektroda může sloužit jako např. biosenzor pro detekci hybridizace DNA, případně může být využita k řadě dalších aplikací. S takto modifikovanou elektrodou jsme provedli základní elektrochemické stanovení (kalibrační závislosti) a následně jsme využili různých přídavků extraktu z transgenní rostliny do uhlíkové pasty pro průkaz avidinu.

V další části této práce jsme využili HPLC s detekcí pomocí diodového pole ke stanovení biotinu. Tato technika je běžně užívanou pro přímé stanovení biotinu v biologických vzorcích a nás zajímalo, zda s její pomocí můžeme dosáhnout srovnatelných výsledků jako u metod elektrochemických.

Na základě výsledků z elektrochemického stanovení avidinu jsme se na závěr pokusili stanovit tento protein pomocí průtokové injekční analýzy, kde nám jako detektor sloužily průtokové cely obsahující 8 uhlíkových pracovních elektrod.

Příloha B

V této práci jsme srovnávali výhodnost avidinu a streptavidinu pro avidin-biotinovou technologii z hlediska citlivosti stanovení s použitím square wave voltametrie. V našich předchozích experimentech jsme zjistili, že streptavidin dává přibližně 100 krát vyšší elektrochemickou odezvu než avidin za stejných koncentrací i dalších experimentálních podmínek. Využili jsme proto dva přístupy. Jednak jsme prováděli měření streptavidinu v elektrochemické nádobce a poté jsme prováděli měření pomocí přenosové rozpouštěcí techniky. V obou případech se nám jako optimální doba akumulace na elektrodový povrch jevil čas 10 minut. Po této době již elektrochemické signály klesaly. Limity detekce byly stanoveny následovně. U adsorptivní přenosové techniky to bylo 30 amol/L a u nepřenosové techniky 0.3 amol/L.

Příloha C

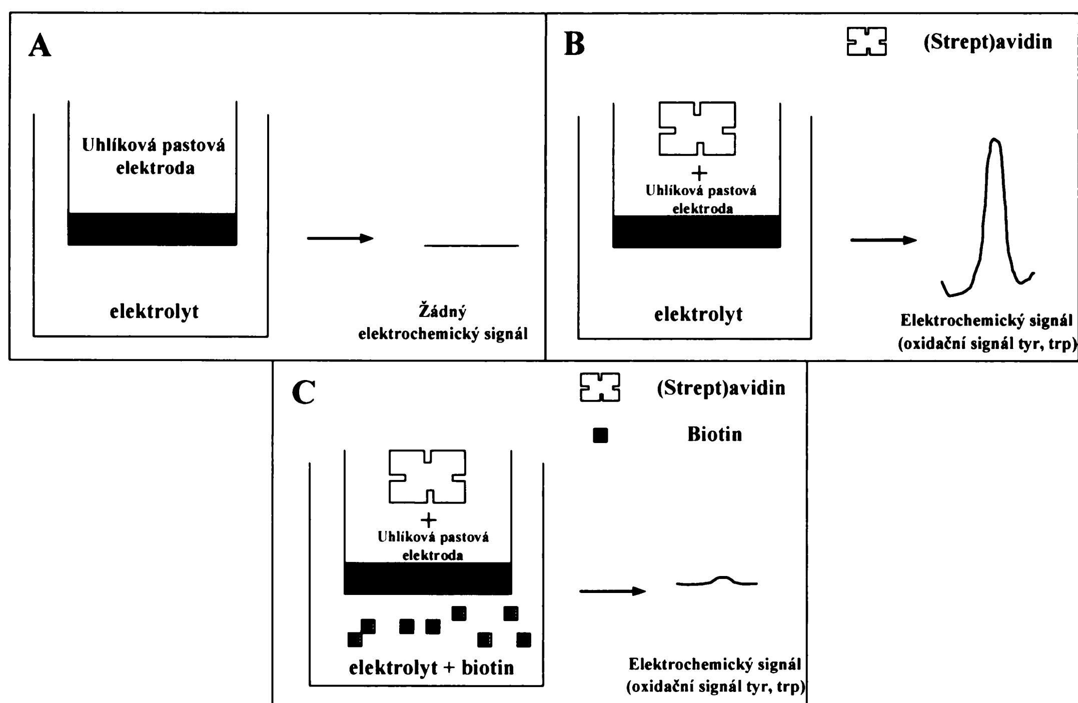
Transgenní rostliny obsahující gen pro avidin jsou potenciální nástrojem pro zajištění odolnosti proti různým druhům hmyzích škůdců. Je to díky silné vazbě avidinu na biotin a tím dochází k inaktivaci tohoto vitamínu a hmyzí škůdci hynou. V této práci jsme srovnávali tři techniky stanovení avidinu z transgenních rostlin tabáku. První z nich byla nová „chip-based“ kapilární elektroforéza EXPERION, další klasická SDS-PAGE elektroforéza a poslední technikou byla square wave voltametrie s použitím uhlíkové pastové elektrody. Zjistili jsme, že automatizovaná kapilární elektroforéza je rychlejší a reprodukovatelnější než ostatní dvě metody. Co se týče citlivosti je velmi srovnatelná s voltametrickou technikou. Velkou nevýhodou je však delší příprava vzorku a cena stanovení. Obsah avidinu měřený ve vzorcích transgenního tabáku s použitím kapilární elektroforézy EXPERION se pohyboval v rozmezí od 15 do 377 ng na mg jednotlivých rostlin, což bylo ve velice dobré shodě s ostatními dvěmi metodami.

5. DISKUZE

Výhodou biopesticidů, mezi které můžeme zařadit i avidin, je jejich netoxičnost pro jiné organismy a také to, že neznečišťují životní prostředí. Bývají specifické vůči hostiteli, což je příznivé z perspektivy integrované ochrany proti škůdcům. Je ale velmi důležité kontrolovat jejich koncentraci, aby nedošlo k jejich nadměrné produkci, což by mohlo vést ke značným komplikacím (Kramer, Morgan et al. 2000).

Metody, které jsou využívány pro detekci proteinů, mezi které patří polyakrylamidová gelová elektroforéza, dvourozměrná gelová elektroforéza, hmotnostní spektrometrie, rentgenová strukturní analýza, NMR a řada dalších, jsou na jednu stranu velice výkonné a robustní, na druhé straně je k těmto analýzám potřeba finančně velmi nákladné přístrojové vybavení. Téma, které řeší tato rigorózní práce, tedy možnost využití elektrochemických metod v analýze proteinů, využívaných jako biopesticidy je z finančního hlediska i z hlediska rozvoje samotné elektrochemické analýzy velice zajímavé a potřebné.

Práce se zabývá vývojem nových technik pro detekci avidinu jako možného biopesticidu a streptavidinu pomocí elektrochemických, elektromigračních a chromatografických technik. Oba proteiny, jak avidin tak i streptavidin, jsou komerčně dostupné, strukturně velice podobné a elektrochemicky aktivní (Havran, Billova et al. 2004). Liší se jednak tím, že avidin je glykoprotein a streptavidin cukernou složku neobsahuje, a také jiným obsahem cysteinových residuí v molekulách proteinů. Dále je z literatury zřejmé, že oba dva tyto proteiny mají velkou afinitu k vitamínu biotinu (Green 1975; Green 1990), která z nich činí potenciální biopesticidy. Je totiž známo, že vyvázání biotinu avidinem u hmyzích škůdců vede k jejich smrti (Kramer, Morgan et al. 2000).
Interakce mezi (strept)avidinem a biotinem je jednou z nejsilnějších vazeb mezi proteinem a ligandem, které jsou v přírodě vůbec známy (Green 1975; Green 1990). Tato skutečnost nás vedla k myšlence použít tuto interakci pro sledování množství avidinu v transgenních rostlinách a jednak sestrojit jednoduchý sensor založený na (strept)avidin-biotinové technologii, tak jak je znázorněno na Obr. 7.



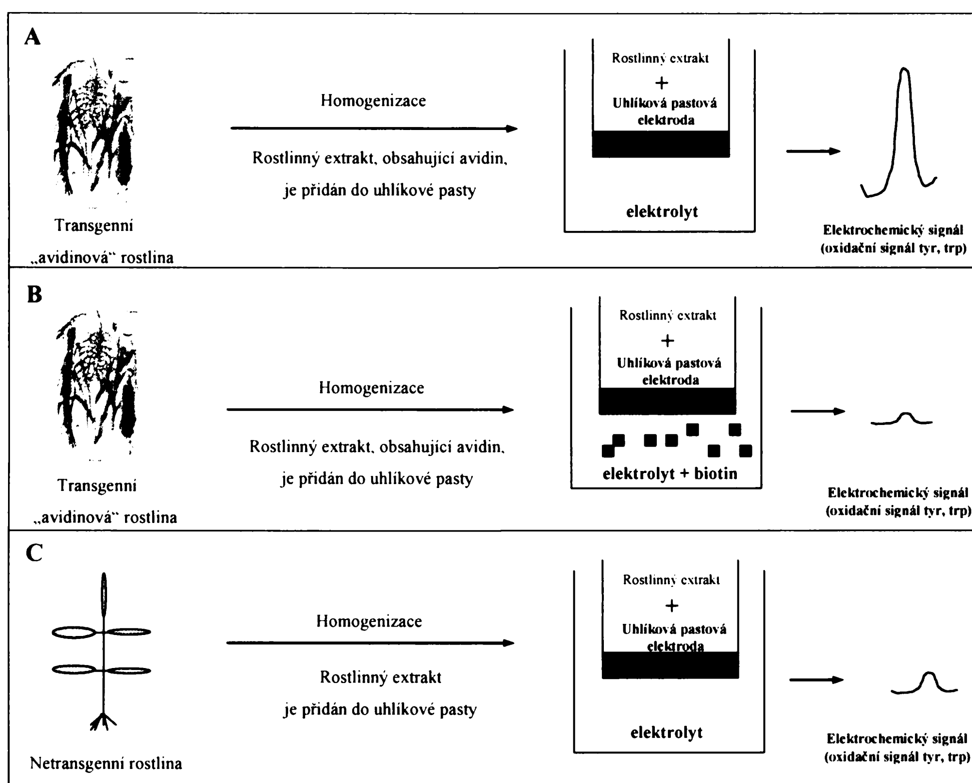
Obr. 7 Schématické znázornění elektrochemické detekce (strept)avidinu pomocí CPE a CMPE a její interakce s biotinem.

Využíváme faktu, že samotná CPE neposkytuje v základním elektrolytu žádný signál (Obr. 7A). Pokud do pasty přidáme (strept)avidin a s touto elektrodou provedeme měření, získáme elektrochemický signál, který odpovídá oxidaci tyrosinových a tryptofanových zbytků v molekule proteinu (Obr. 7B). A pokud k takto modifikované elektrodě přidáme biotin dojde k velmi výraznému poklesu (v podstatě k vymizení) signálu (Obr. 7C). To si vysvětlujeme tím, že biotin specificky reaguje s molekulou (strept)avidinu v uhlíkové pastě a znemožní přístup některým tyrosinovým resp. tryptofanovým zbytkům k povrchu elektrody a tak zabrání jejich oxidaci. Podobné výsledky byly získány i na HMDE v Brdičkově soluci, kde titrací avidinu biotinem dochází ke snížení obou katalytických píků (Havran, Billova et al. 2004).

Takto modifikovaná elektroda se dá použít např. pro detekci hybridizace DNA, kde se paralelně sleduje signál (strept)avidinu a signály, které pocházely z oxidačních reakcí nukleotidových bází (adenin, guanin) DNA (Masarik, Kizek et al. 2003).

Další oblastí, které se tato práce zabývá, je detekce avidinu z transgenních rostlin (kukuřice a tabáku). Izolaci proteinu jsme provedli tak, jak je popsáno v (Hood, Witcher et al. 1997). Vyizolovaný protein jsme kontrolovali pomocí denaturační gelové elektroforézy s SDS (SDS-PAGE) a pomocí imunochemických metod (western blot). Elektrochemickou detekci

jsme provedli jednak na povrchu pastové elektrody a jednak tím způsobem, že vyizolovaný extrakt obsahující avidin jsme přidali do uhlíkové pasty. Celá procedura je opět znázorněna na Obr. 8. Nejprve byl extrakt z transgenní rostliny přidán do uhlíkové pastové elektrody a změřen v základním elektrolytu. Elektrochemická detekce poskytla pěkně vyvinutý oxidační signál tyrosinu a tryptofanu (Obr. 8 A). Následně byla provedena adsorpce biotinu na povrch modifikované uhlíkové elektrody (případně byl biotin přidán do roztoku základního elektrolytu) a bylo opět provedeno měření. Jak je patrné z Obr. 8 B došlo k výraznému poklesu signálu, ne však k úplnému jeho vymizení. Zbytkový signál přičítáme nedokonalému procesu extrakce. V případě rostliny, která neobsahuje gen pro expresi avidinu (Obr. 8C) jsme dostali signál, který svou výškou odpovídá Obr. 8B. Podobné výsledky byly získány i z měření s vysokoúčinnou kapalinovou chromatografií s detekcí pomocí diodového pole (HPLC-DAD) a průtokovou injekční analýzou s elektrochemickou detekcí. Výsledky z elektrochemických a chromatografických stanovení, byly srovnávány s ELISA technikou a byly ve velice dobré shodě.



Obr. 8 Znázornění elektrochemické detekce avidinu z transgenní rostliny.

Poslední částí této práce byla detekce avidinu z transgenní tabákové rostliny pomocí kapilární elektroforézy využívající čipové technologie – EXPERION. Jedná se o poloautomatický systém, který může nahradit klasickou gelovou polyakrylamidovou

elektroforézu. Nejprve jsme opět provedli extrakci různých tabákových rostlin dle Hood at al. a extrakty jsme poté podrobili jednak elektrochemické analýze a také SDS-PAGE a EXPERION analýze a sledovali jsme množství avidinu v tabákových rostlinách. Z naměřených dat vyplývá, že výhody kapilární elektroforézy jsou jednak v rychlosti stanovení, opakovatelnosti a částečně i sensitivitě. Oproti metodám elektrochemickým však jsou však cenově velice nákladné a také velikostně neodpovídající.

Z předchozích stránek tedy vyplývá, že elektrochemické metody mají zcela jistě své místo v analýze biopesticidních proteinů a mohou se stát dalším nástrojem ke zkoumání jejich distribuce v rostlinných tělech.

6. ZÁVĚR

V práci byla soustředěna pozornost na studium avidinu izolovaného z různých typů transgenních rostlin, do kterých byl vložen gen pro tento protein. V souvislosti s tím se dále tato práce zabývala možností využít avidin jako potenciální biopesticid. Metody, které jsme při studiu využívali byly založeny na elektrochemické, elektroforetické a chromatografické analýze.

Výsledky je možno shrnout do následujících bodů:

- Avidin a streptavidin jsou proteiny, které jsou oba elektrochemicky aktivní na povrchu uhlíkové elektrody a poskytují oxidační signály tyrosinu a tryptofanu.

- Bylo prokázáno, že přidáním (strept)avidinu do uhlíkové pasty, poskytuje takto modifikovaná uhlíková elektroda signál a (strept)avidin v ní neztrácí schopnost vázat biotin.

- Ukázali jsme, že SWV je alternativní metodou pro detekci avidinu v transgenních rostlinách, kde ve srovnání s jinými metodami vykazovala velice dobrou shodu.

- Navrhli jsme novou techniku pro detekci avidinu z transgenních rostlin založenou na poloautomatické kapilární elektroforéze EXPERION.

7. SUMMARY

Electrochemical analysis of proteins was successfully applied in biochemistry, pharmacy and medicine and particularly in clinical oncology research for several decades in the middle of the 20th century. Later the attention of electrochemists turned to direct electrochemistry of a limited number of redox-active center-containing proteins and the potentialities of the electrochemical methods as tools for protein analysis in molecular biology and biomedicine were neglected. At present electrochemistry of proteins is mainly oriented toward direct electrochemistry of redox-active center-containing proteins offering fast reversible electrochemical processes at solid electrodes. Voltammetric or chronopotentiometric analysis of proteins not containing any redox center for direct electrochemistry is limited to the studies of their adsorption/desorption behavior and measurements of oxidation of tyrosine and tryptophan residues at carbon electrodes or reactions involving cystine/cysteine residues such as the formation of the Hg-S bond, reduction of the disulfidic group or the so-called Brdicka's catalytic hydrogen evolution in cobalt containing solutions at mercury electrodes. Voltammetric analysis of proteins at carbon electrodes was not very sensitive yielding at relatively high protein concentrations only poorly developed inflections at the voltammetric curves. In 1996 we showed that the constant current chronopotentiometric stripping analysis with sophisticated baseline correction is capable to produce well developed oxidation peaks of tyrosine and tryptophan residues in peptides at nanomolar concentrations. The so-called polarographic presodium wave of proteins (obtained with mercury dropping electrodes), which does not require cystine or cysteine residues, was little used for analytical purposes because it appeared at too negative potentials very close to the background discharge and its shape was poorly developed and difficult to measure.

The aim of this thesis is to show that avidin is best suited macromolecule for biopesticides and that except rutinely used methods in protein analysis such as two-dimensional gel electrophoresis, mass spectrometry, X-ray crystalography or nuclear magnetic resonance spectroscopy, electrochemical methods are very suitable and useful methods in protein analysis.

Presented work describes application of new electroanalytical methods in the study of biotechnologicaly important proteins such as avidin and streptavidin and their utilisation in biological research. 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. Both proteins form tetramers of comparable molecular weight (64 kDa) and structure, and are very stable. Their quarternary structure is composed of four identical subunits, each having a high affinity 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, electron microscopy, enzyme-linked immunoassay, and DNA hybridization. In the latter case biotin is used as a tag for a nucleic acid nonradioactive probe in biomedical and clinical applications. Both proteins were electrochemically detected in solution by AdTS SWV at CPE. AdTS SWV was used to quantify avidin in extracts of transgenic avidin maize and tobacco. The results demonstrated that streptavidin/avidin AdTS SWV is the 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.

It follows from the presented results that electrochemical methods might be successfully applied in protein analysis and could broaden the spectrum of methods used in proteomics, genomics and biomedicine.

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9. SOUBOR PUBLIKACÍ

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ORIGINAL PAPER

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An analysis of avidin, biotin and their interaction at attomole levels by voltammetric and chromatographic techniques

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Abstract The electroanalytical determination of avidin in solution, in a carbon paste, and in a transgenic maize extract was performed in acidic medium at a carbon paste electrode (CPE). The oxidative voltammetric signal resulting from the presence of tyrosine and tryptophan in avidin was observed using square-wave voltammetry. The process could be used to determine avidin concentrations up to 3 fM (100 amol in 3 μl drop) in solution, 700 fM (174 fmol in 250 μl solution) in an avidin-modified electrode, and 174 nM in a maize seed extract. In the case of the avidin-modified CPE, several parameters were studied in order to optimize the measurements, such as electrode accumulation time, composition of the avidin-modified CPE, and the elution time of avidin. In addition, the avidin-modified electrode was used to detect biotin in solution (the detection limit was 7.6 pmol in a 6 μl drop) and to detect biotin in a pharmaceutical drug after various solvent extraction procedures. Comparable studies for the detection of biotin were developed using HPLC with diode array detection (HPLC-DAD) and flow injection analysis with electrochemical detection, which allowed biotin to be detected at levels as low as 614 pM and 6.6 nM, respectively. The effects of applied potential, acetonitrile content, and flow rate of the mobile phase on the FIA-ED signal were also studied.

Keywords Avidin · Biotin · Avidin–biotin technology · Square-wave voltammetry · Modified electrode · High-performance liquid chromatography · Diode array detection · Electrochemical transfer technique · Carbon paste electrode · Drug analysis

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Introduction

Biotin (*cis*-hexahydro-2-oxo-1-H-thieno-[3,4]-imidazoline-4-valeric acid), commonly known as vitamin H, is involved in the metabolism of amino acids and carbohydrates in organisms [1]. A low biotin intake has been reported to result in serious biochemical disorders in animal organisms, such as a reduced carboxylase activity, inhibition of protein and RNA syntheses, reduced antibody production, and other metabolic abnormalities [1–3]. Therefore, it is very important to monitor biotin levels in biological fluids as well as in food and food supplemental products, which constitute the main source of biotin for humans. A few analytical methods have been developed for the direct detection of biotin in pharmaceutical preparations, including high performance liquid chromatographic procedures coupled with different types of detectors (diode array, mass, electrochemical, and others) [2, 4–7].

Avidin, a minor constituent of egg white from reptiles, amphibians and birds, is a glycosylated and positively charged protein (at neutral pH) which usually forms tetramers [8, 9]. There are only a few papers that describe the direct detection of avidin, and those deal primarily with the levels in eggs. On the other hand, avidin combines selectively with biotin. The vitamin has a very high affinity to avidin (dissociation constant of 10^{-15} M), and this interaction has been utilized in many types of avidin-biotin technologies, such as immunohistochemistry, electron microscopy, enzyme-linked immunoassay, DNA hybridization, and construction of biosensors [10–18]. In an application that makes use of avidin-biotin binding, it is essential to establish methods for detecting and evaluating the interaction. The most commonly-used methods for detecting and evaluating avidin-biotin interactions are ELISA, fluorimetry, and some electrochemical methods [19–27].

To ensure that sufficient food is available for an expanding human population, it is necessary to exploit a number of new technologies that may help to increase production of agricultural foodstuffs. This production is heavily suppressed by bacterial or viral diseases, and also damage caused by insect pests. These undesirable processes can be prevented by using chemical compounds such as pesticides [28]. Use of these compounds, however, can result in environmental contamination, and they can also enter into the food chain. New molecular biology techniques are helping researchers to utilize natural biocidal compounds produced by plants that can protect them against pests [29, 30]. Recently, it was reported that avidin is toxic to a broad spectrum of lepidopteran, coleopteran, and dipteran pests of grains, fruits, and vegetables [31–34]. For the purposes of recombinant avidin production and biological protection of grain, a transgenic avidin corn plant was therefore developed.

There is much evidence demonstrating that a carbon paste electrode (CPE) is a suitable tool for use in biosensors [16, 35–47]. Moreover, carbon paste electrodes can be easy modified by adding different substances in order to increase sensitivity, selectivity and rapidity of determination [14, 40, 48, 49]. We primarily focused on utilization of these electrodes (CPE and modified CPE) in combination with square-wave voltammetry and adsorptive transfer stripping techniques for measuring nanogram quantities of avidin.

In this paper we describe the preparation of avidin- and avidin extract-modified CPEs for sensitive avidin detection in solution and in transgenic maize plants. We also describe the detection of biotin by an electrochemical method that utilizes the strong avidin–biotin interaction. In addition, we optimized and applied hyphenated analytical techniques for the detection of avidin, biotin and avidin-biotin interactions. We used high performance liquid chromatography coupled with a diode array detector (HPLC-DAD) to detect biotin, and flow injection analysis (FIA) with electrochemical detection (ED) to detect avidin and/or avidin-biotin interactions.

Experimental

Chemicals

Avidin, biotin, trifluoroacetic acid, carbon powder, sodium chloride, sodium citrate, hydrochloric acid, sodium carbonate, sodium acetate, acetic acid, and mineral oil were purchased from Sigma Aldrich Chemical Corp. (St. Louis, USA). HPLC-grade acetonitrile and methanol (>99.9%; v/v) were from Merck (Darmstadt, Germany). Flavone and all other reagents of ACS purity were purchased from Sigma Aldrich. Solutions were prepared using ACS water from Sigma Aldrich. The stock standard solutions of avidin and biotin 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 filters (MetaChem, Torrance, CA, USA) prior to HPLC separations.

Measuring pH and absorption spectra

The pH was measured using a WTW inoLab Level 3 instrument (Weilheim, Germany), controlled by a personal computer program (MultiLab Pilot; Weilheim, Germany). The pH electrode (SenTix H, pH 0–14/0–100 °C/3 mol l^{-1} KCl) was regularly calibrated using a set of WTW buffers (Weilheim, Germany). UV absorption spectra of avidin were determined using a diode array spectrophotometer (Hewlett-Packard model 8452A).

Electrochemical measurements

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, an Ag/AgCl/3 M KCl reference electrode, and a platinum wire counter electrode. Acetate buffer (0.1 M CH_3COOH + 0.1 M CH_3COONa, pH 4.0) was used as the supporting electrolyte. Adsorptive transfer stripping square wave voltammetry (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) and a moving average baseline correction (peak width = 0.05 mV) of the GPES software.

Preparation of CPE and avidin-modified CPE

The carbon paste (about 0.5 g) was made of graphite powder (Aldrich) and mineral oil (Sigma; free of DNase,

RNase, and protease). The ratio of the graphite powder and mineral oil was tested (see "Results" and "Discussion" sections). This paste was housed in a Teflon body with a 2.5-mm-diameter disk surface. Prior to measurements, the electrode surface was renewed by polishing with a soft filter paper. The surface was then ready to take measurements in a sample volume of 3–6 μl. The avidin-modified CPE was prepared in the same way as described above, with the addition of avidin to the CPE.

Flow injection analysis with CoulArray electrochemical detector

The flow injection analysis with electrochemical detection (FIA-ED) system consisted of two solvent delivery pumps operating over a range of 0.001–9.999 ml min^{-1} (Model 582 ESA Inc., Chelmsford, MA, USA), a reaction loop of 1 m length, and a CoulArray electrochemical detector (Model 5600A, ESA, USA). The electrochemical detector included two low volume flow-through analytical cells (Model 6210, ESA, USA). Each analytical cell consisted of four carbon porous working electrodes, palladium electrodes as reference electrodes, and carbon auxiliary electrodes. The detector and the column were thermostated. Temperatures under 30 °C were obtained using an air-conditioner (ET9, Italy). The sample (5 μl) was injected manually using a glass syringe (Hamilton, USA).

High-performance liquid chromatography coupled with diode array detection

An HP 1100 liquid chromatographic system (Hewlett Packard, Waldbronn, Germany) was equipped with a vacuum degasser (G1322A), a binary pump (G1312A), an autosampler (G1313A), a column thermostat (G1316A), and a UV-VIS diode array detector (model G1315A) working at 190–690 nm. ChemStation software (Rev. A 08.01) controlled the whole liquid chromatographic system. Spectra were recorded in the range of 190–400 nm (SBW 100 nm). Biotin was separated on an Atlantis dC18 reversed-phase chromatographic column (150×2.1 mm, 3 μm particle size, Waters Corp. Milford, USA) in an isocratic mode with methanol–0.01% trifluoroacetic acid in Milli-Q water (35:65). The flow rate was 1.5 ml min^{-1}. The temperature of the column and detector was set to 35 °C. The autosampler injection volume was 5 μl.

Avidin maize sample and its preparation

The construction of plasmids used to transform the maize with the chicken avidin gene, the transformation itself, tissue culture, and the generation of avidin-expressing transgenic plants were all performed as described previously [50]. Briefly, 10 g of transgenic or non-transgenic (control) maize kernels were ground for 1 min in a coffee grinder. The resulting powder 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% (v/v) Tween-20. The extraction mixture was centrifuged at 16,000*g* for 15 min (Jouan MR 23i) at 4 °C. The supernatant was removed and filtered through four layers of cheesecloth. The filtrate was then centrifuged at 14,500*g* for 15 min at 4 °C. The supernatant was recovered and the pH adjusted to 10.5, and then centrifuged at 14,000*g* for 30 min (Eppendorf 5402) at 4 °C [14, 50]. The final supernatant was recovered and subsequently used to prepare the modified CPE.

Extraction procedures for the pharmaceutical drug

Pharmaceutical drug used

Tablets of the pharmaceutical drug Biotin (Nature's Bounty, Inc., USA) were used. An Ika A11 basic grinder (IKA Werke GmbH and Co., Staufen, KG, Germany) was used for homogenization of the tablets.

Accelerated solvent extraction procedure

An accelerated solvent extraction procedure [30, 51] was performed in a PSE (Applied Separations, USA) pressurized solvent extractor. The homogenized (0.500 ± 0.005 g) pharmaceutical drug Biotin was placed in a small filter paper envelope and sprinkled with 3.0 g SPE-ed matrix, 1.5 g florisil (15.5% MgO, 84% SO_2, 0.5% Na_2SO_4; pH 8.5; 60–100 μm particle size) and 3.0 g Ottawa sand (Allentown, PA, USA). Flavone was used as an internal standard and was pipetted onto the top of the sample. The mixture was placed into a 10 ml stainless steel extraction cell and extracted under controlled conditions in one step: pre-heating period (5 min), the solvent was 50% methanol; temperature 100 °C, pressure 150 bar; two extraction cycles (5 min), 90 s using pressurized nitrogen. The final extracts were collected in 40 ml glass vials. The obtained extract was filtered through a 0.45 μm Teflon membrane filter (MetaChem, Torrance, CA, USA) prior to injection into the HPLC system.

Water extraction

Sonication of the homogenised sample (0.500 ± 0.005 g) was performed at laboratory temperature for 15 min on a K5 Sonicator (Slovakia) at 38 kHz and 150 W. Water (10 ml) was used as an extraction solvent. Flavone was used as an internal standard and was pipetted onto the top of the sample before sonication. The obtained extract was filtered through a Teflon disc filter (0.45 μm, 13 mm diameter, Alltech Associates, Deerfield, IL, USA) prior to injection into the HPLC system.

Phosphoric acid extraction

The homogenization of the pharmaceutical drug sample (0.500 ± 0.005 g) was performed at laboratory temperature for 5 min with stirring at 250 rpm. The extraction solvent (10 ml) was an aqueous solution of phosphoric acid at a final concentration of 1.5% (v/v) [6]. The sample was sonicated for 15 min. Flavone was used as an internal standard and was pipetted onto the top of the sample before stirring. The obtained extract was filtered through a Teflon disc filter (0.45 μm, 13 mm diameter, Alltech Associates, Deerfield, IL, USA) prior to injection into the HPLC system.

Accuracy, precision and recovery

Accuracy, precision and recovery of the biotin were evaluated with homogenates (pharmaceutical drug) spiked with standards. Before an extraction, 100 μl of biotin standard (various concentrations from 10 ng ml^{-1} to 100 ng ml^{-1}), 100 μl water and 100 μl flavone were added to the pharmaceutical sample. The precision (coefficient of variation; %CV) of the intra-day assay was measured in six homogenates. Inter-day precision was determined by analyzing six homogenates over a five-day period. Homogenates were assayed blindly and biotin concentrations were derived from the calibration curves. Accuracy was evaluated by comparing the estimated concentration with the known concentrations of biotin. Calculation of accuracy (%Bias), precision (%CV) and recovery was expressed as described in [52, 53].

Statistical analysis

STATGRAPHICS software (Statistical Graphics Corp, USA) were used for statistical analyses. Results are expressed as mean ± SD unless stated otherwise. A value of $p < 0.05$ was considered significant.

Results and discussion

Adsorptive transfer stripping voltammetry of avidin at CPE

Avidin is an important protein that contains a variety of amino acids in its structure (Fig. 1A). From an electrochemical point of view, only tyrosine (Y) and tryptophan (W) have been found to be electroactive using a variety of electrodes [54, 55]. Square-wave voltammetric analysis at solid carbon electrodes is not very sensitive and yields only poorly developed signals. However, by using a CPE and sophisticated baseline correction, we obtained well-defined voltammetric signals for both Y and W at 0.78 and 0.92 V vs. Ag/AgCl/3 M KCl, respectively (Fig. 1C). The peaks were obtained at 100 μg ml^{-1} (~7 μM) of avidin by using the adsorptive transfer stripping square-wave voltammetry technique. A scheme showing the transfer technique is provided in Fig. 1B. The technique is based on the strong adsorption of avidin on the electrode surface and subsequent electrode transfer to a washing solution and then to an electrolyte that does not contain any avidin in bulk solution, where electrochemical measurements are performed. An advantage of using this approach is that only a small amount of sample is required (6 and/or 3 μl drops) per voltammetric analysis. These electrochemical transfer techniques have been previously described in detail in [56–58].

We determined that the peak height was linearly dependent on the time of avidin accumulation up to a time of approximately 2 min, after which the height decreased (Fig. 1D). It should be noted that our measurements were performed without any stirring; when the solution is stirred, the same coverage saturation should be reached in a shorter accumulation time under the same experimental conditions. To determine the dependence of peak height on avidin concentration, we varied the avidin concentration from 0 to 100 μg ml^{-1}. The dependence was found to be linear over the studied concentration range, with $R^2 = 0.9978$ (Fig. 1E). The limit of detection (3×S/N ratio criterion) was about 500 pg ml^{-1} (~3 fM). The reproducibility of the method was checked ($n = 5$) by performing determinations of 6 and 3 μl drops of avidin (1 μg ml^{-1}) at an accumulation time of 120 s, with the relative standard deviations (RSD) found to be only 6.9 and 9.8%, respectively.

Electrochemical behavior of avidin in modified CPE

Avidin in CPE

Avidin-modified CPE was prepared by simply mixing a desired quantity of avidin into a conventionally-prepared CPE composed of graphite powder and mineral oil (Fig. 2A). Compared to CPE, avidin-modified CPE exhibited similar voltammetric signals for Y and W (for comparison see Figs. 1C and 2A). When the amount of carbon powder (at constant concentration of avidin of 10 μg ml^{-1}) was changed in the paste mixture, we observed the highest voltammetric signal at a powder/oil ratio of 70/30 (w/w) (Fig. 2B). This result is in good agreement with other studies of unmodified CPE [14, 40, 59] and indicates that the avidin-modified CPE possesses electrochemical properties similar to those of the CPE. We also investigated whether avidin was washed out from the paste by keeping the modified electrode in the electrolyte solution. The voltammetric signal decreased by about 15% over an incubation time of 20 min (Fig. 2C). From these results, it is clear that an accumulation time of just 2 min would not markedly affect the peak height of avidin.

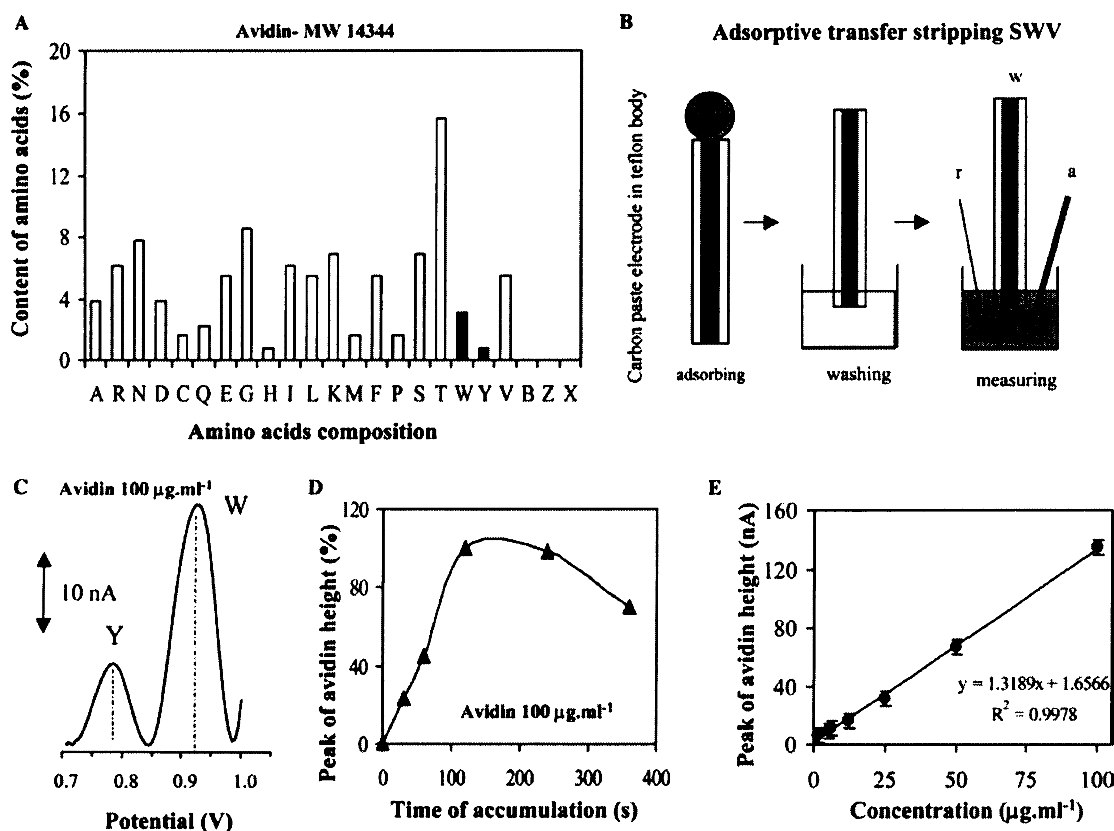

Fig. 1 **A** Amino acid composition of avidin; MW: 14,344 Da. **B** Adsorptive transfer stripping (AdTS) technique used with the carbon paste electrode (CPE). **C** Voltammogram of avidin (100 µg ml^{-1}; $t_A = 120$ s) obtained by CPE. The voltammetric signal corresponds to the oxidation of Trp (Y) and Tyr (W) residues in the avidin molecule. **D** Dependence of AdTS SWV peak height of avidin on time of accumulation. **E** Dependence of AdTS SWV avidin peak height on avidin concentration. All measurements were performed in acetate buffer (pH 4). The SWV method was used with the following parameters: initial potential = 0.1 V, end potential = 1.3 V, step potential = 5 mV, amplitude = 25 mV, frequency = 200 Hz

As expected, the voltammetric signal was dependent on the amount of avidin present in the avidin-modified CPE. A linear increase in peak height was observed as the concentration of avidin in the CPE increased up to an avidin concentration of 500 µg ml^{-1}, when a fresh CPE surface was prepared for each measurement (RSD = 3.4%, $n = 3$). The calibration curve for the concentration range examined was linear, with a regression line of $y = 1.00x + 9.04$ ($R^2 = 0.996$) (Fig. 1D). The lowest concentration at which the voltammetric signal was observable under our experimental conditions was 10 ng ml^{-1} of avidin in the avidin-modified CPE (700 fM; RSD = 3.4%, $n = 3$). We assume that the different avidin detection limits obtained by CPE versus CMPE might be due to different arrangements of the analyte in active electrode surface-adsorption versus incorporation.

Determination of avidin in transgenic avidin maize extract using avidin extract-modified CPE

Modern genetic engineering technologies make it possible to prepare plant species that produce a number of value-added proteins (such as monoclonal antibodies, antigens for vaccines, and so on) [31, 50, 60–64]. Transgenic maize that produces avidin as an insecticidal agent against insect pests, was recently prepared [31, 32, 50]. In our previous work [14], we mixed the plant seed extract obtained from transgenic maize with carbon powder. Then we prepared the avidin extract-modified CPE. The height of the resulting electrochemical signal corresponded to the concentration of avidin present in the maize plant sample. In the present study, we determined how the avidin peak height changes depending on the amount of maize plant tissue extract added to the carbon powder (each avidin-modified CPE was used only for one measurement; RSD = 7.8%, $n = 3$). After the plant seed homogenization step, the CPE was modified by the plant extract and the voltammetric response was recorded. We were able to observe well-developed square-wave voltammetric signals in all tested samples (inset in Fig. 2E).

A relatively easy, rapid and easily applicable electrochemical procedure for avidin detection in transgenic seeds was developed. For analytical purposes we constructed a calibration curve showing a linear relationship between the avidin concentration and the volume of a

Electrochemical behaviour of avidin in carbon paste

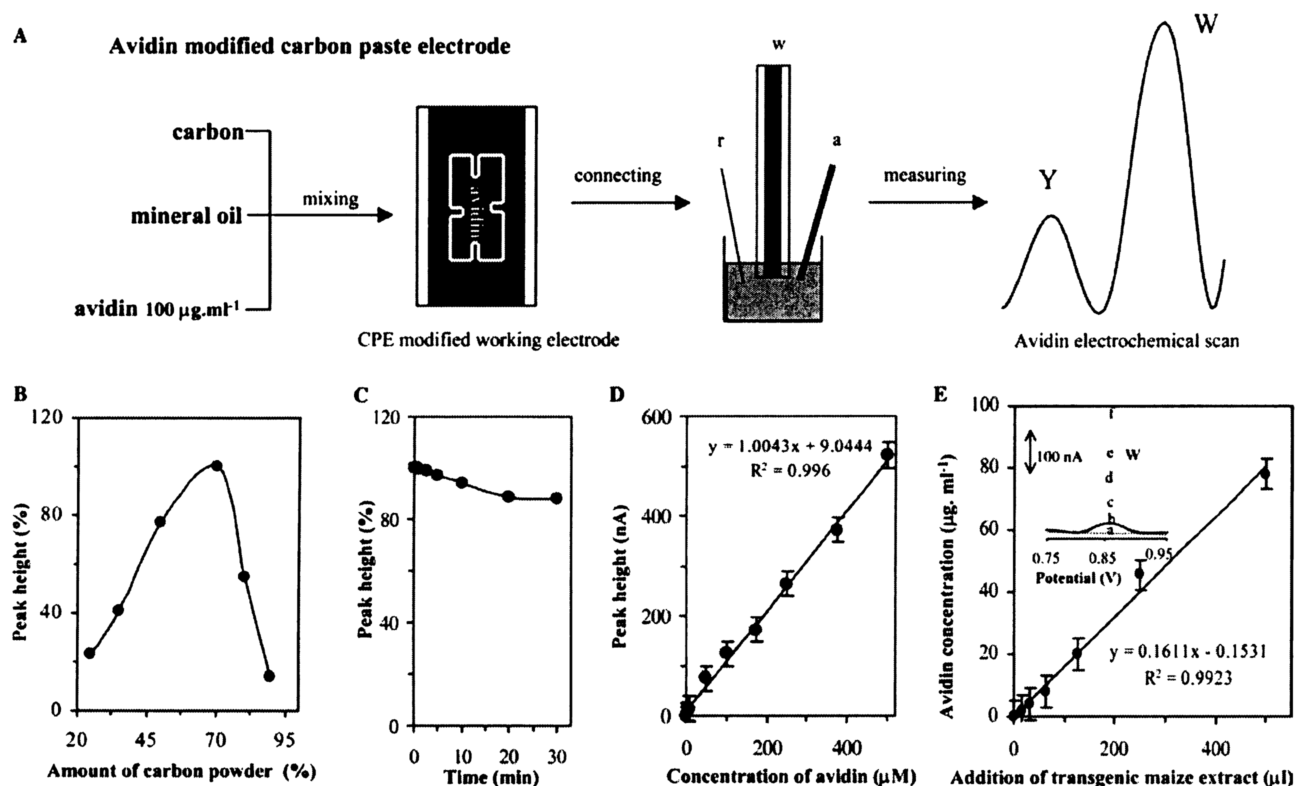

Fig. 2 Electrochemical behavior of the avidin-modified CPE. **A** Scheme of avidin-modified CPE: mixing, connection and resulting SW voltammogram of avidin. **B** Influence of amount of carbon powder in the carbon paste. **C** Dependence of avidin-modified CPE SWV peak height on time that the electrode was immersed in the background electrolyte. **D** Dependence of AdTS SWV avidin peak height on avidin concentration (avidin in CPE). **E** Dependence of AdTS SWV avidin peak height on avidin concentration in transgenic maize extract (inset: voltammograms of transgenic maize extract in modified CPE at different amounts of extract in CPE: a, 15.6; b, 31.3; c, 62.5; d, 125; e, 250; f, 500 μl; dotted line is background electrolyte). A peak height of 100% represents 100 μg ml^{-1} avidin concentration in avidin-modified CPE. Other experimental conditions are the same as in Fig. 1

transgenic maize extract ($y = 0.1611x - 0.1531$; $R^2 = 0.9923$) (Fig. 2E). The peak height of the voltammetric signal increased linearly with as the amount of transgenic seed extract added into the avidin-modified CPE was increased from 15.6 μl to 500 μl. The lowest detectable volume of the plant extract was about 15 μl, which corresponds to 2.5 μg ml^{-1} of avidin in the sample. When we tested a non-transgenic avidin plant, only a very small signal was observed (curve *a* in Fig. 2E, inset). To compare the avidin signal from the transgenic seeds to that from the non-transgenic ones, we prepared a set of extract samples from the commercial maize food (farina, grits, and grout). Voltammetric signals obtained from these samples were very low and their heights were about 2–8% relative to the signal obtained from the transgenic sample extract (data not shown). These results were in good agreement with our previously published results [14]. This procedure can be used to analyse other transgenic products that contain avidin, such as avidin apple, tobacco and rice [33, 65, 66]

Avidin-modified CPE in the presence of biotin

Study of the interaction between avidin and biotin

The interaction between avidin and biotin is one of the strongest known associations between a protein and its ligand [10, 67]. Because of the extremely strong interaction of this complex, avidin–biotin technology is used in various experimental techniques, including electrochemical ones [21–25, 35, 68, 69]. To evaluate the properties of an avidin-modified electrode in the presence of biotin, we added biotin to the background electrolyte and followed the voltammetric response of avidin. We kept the amount of avidin in the avidin-modified CPE and the accumulation time constant (10 μg ml^{-1}; 120 s) and changed the concentration of biotin in the electrochemical cell from 0.5 μM to 14 μM. We observed that the voltammetric signal of avidin decreased with increasing concentration of biotin. The observed decrease in avidin signal after addition of biotin is probably caused by the fact that the avidin–biotin complex formed could mask some of the electroactive tryptophans and tyrosine of avidin. The electrochemical signal of avidin was nearly undetectable at 10 μM biotin (Fig. 3A:a). The shape of the response

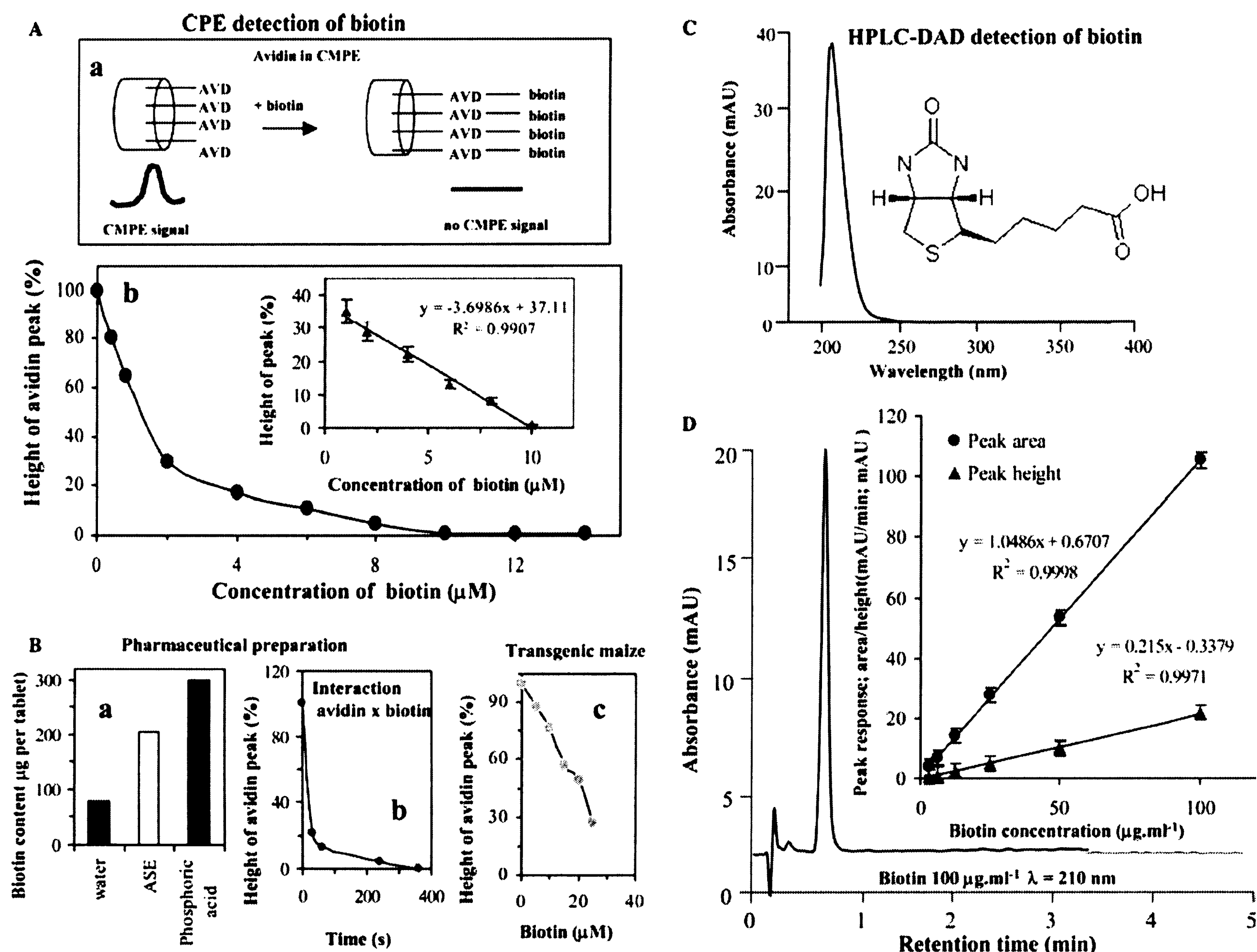

Fig. 3 Interaction of avidin contained in avidin-modified CPE with biotin. **A** (a) Scheme of avidin-modified CPE interaction with biotin present in solution and resulting voltammetric signal. (b) Dependence of AdTS SWV avidin peak height on concentration of biotin in solution; inset: the same dependence for 2–10 μM concentration of biotin in solution. **B** Avidin-modified CPE determination of biotin in a pharmaceutical preparation. (a) The pharmaceutical samples containing biotin were prepared by three different extraction procedures (for other details, see the "Experimental" section and Table 1). 100% of biotin represents 300 μg g^{-1} in one tablet. (b) Dependence of AdTS SWV avidin peak height on time of interaction with biotin. (c) Dependence of AdTS SWV avidin peak height (CPE electrode modified by plant seed extract obtained from transgenic maize) on biotin concentration. Peak height of 100% represents 100 μg ml^{-1} avidin in avidin-modified CPE. Other experimental conditions were the same as in Fig. 1. **C** HPLC diode array (HPLC-DAD) spectrophotometric measurements of biotin. Chemical structure and UV spectrum of biotin. **D** HPLC-DAD chromatogram of biotin (100 μg ml^{-1}); inset: dependence of peak height and/or area on biotin concentration. Detection wavelength was 210 nm. For other chromatographic conditions, see the "Experimental" section

curve was exponential ($y = 80.2\ e^{-0.36x}$). For analytical purposes we attempted to divide the concentration interval into two parts in order to be able to apply a titration curve. The first part (concentration of biotin = 0–2 μM) was strictly linear ($y = -42.73x + 99.92$; $R^2 = 1$). The second part (concentration of biotin = 2–10 μM) had the following parameters: $y = -3.70x + 37.11$; $R^2 = 0.9907$; $n = 5$; RSD = 5.9% (see in inset Fig. 3A,B).

Detection of biotin by avidin-modified CPE

Biotin is also known as vitamin H (Fig. 3) [1]. The determination of biotin in pharmaceutical preparations and/or food samples is relatively difficult. The most commonly used biotin detection methods are based on using high performance liquid chromatography with UV detection or on using microbiological techniques [5, 20]. We tested the SWV voltammetric method for avidin detection by indirectly determining the biotin in a pharmaceutical drug. The preparation of the sample was a very important factor for accurate and precise analysis. In the case of biotin detection, we selected three different extraction procedures: water extraction, accelerated solvent extraction (ASE) and extraction using phosphoric acid (for additional details, see the "Experimental" section) (Fig. 3B:a). Recoveries and amounts of biotin extracted by these procedures are given in Tables 1 and 2. We selected the extraction by phos-

Table 1 Biotin detected in a pharmaceutical drug using three procedures based upon the use of avidin-modified CPE or HPLC-ED

	Avidin-modified CPE	HPLC -DAD
Extraction procedure[c]	Found μg per tablet[a,b]	Found μg per tablet[a,b]
Water	80 ± 4	76 ± 3
ASE	205 ± 6	209 ± 5
Phosphoric acid	299 ± 4	303 ± 7

[a]Declared biotin amount in one tablet is 300 μg
[b]Amount expressed as mean ± SD ($n=6$)
[c]Extraction procedures are described in the "Experimental" section

phoric acid as the best extraction procedure since it gave the highest amount of extracted biotin. The reproducibility of the procedure was determined using six repetitive analyses of representative samples over five days (Table 3). The biotin amounts measured were in good agreement with the declared values.

Electrochemical indirect determination of biotin using the avidin-modified CPE (10 μg ml^{-1}) was performed at an avidin–biotin interaction time of 120 s with constant stirring (1,430 rpm). We observed that the electrochemical signal of avidin rapidly decreased after the avidin–biotin interaction occurred at the CPE surface (Fig. 3B:b). In addition we obtained similar results with the interaction between biotin and a plant avidin extract-modified CPE (Fig. 3B:c). This simple testing method can be used for rapid, low-cost and selective determinations of avidin in plants and other types of materials.

Direct detection of biotin by HPLC with diode array detection

The most common method used for the direct determination of biotin is high performance liquid chromatography (HPLC) with diode array detection or fluorescence detection [1, 4–6, 20, 70]. On the basis of our determinations of biological compounds by high performance liquid chromatography with diode array detection (HPLC-DAD) published previously [70], we selected the same analytical method for the detection of biotin. The UV spectrum of biotin presented in Fig. 3C clearly shows that, in order to obtain a high signal-to-noise ratio, the detection should be performed using a wavelength that is a low as is possible. Hence, the detection was performed at $\lambda = 210$ nm during the HPLC experiments. Detection at wavelengths below $\lambda = 210$ nm resulted in background noise that was too high due to the presence of methanol in the mobile phase (cut-off at $\lambda = 200$ nm). A typical HPLC-DAD chromatogram of biotin (100 μg ml^{-1}) is shown in Fig. 3D. The dependence of peak area and/or height on biotin concentration is shown in the inset to Fig. 3D; the equations for the obtained bisectors were $y = 1.0486x + 0.6707$; $R^2 = 0.9998$ for peak area and $y = 0.2150x - 0.3379$; $R^2 = 0.9971$ for peak height, respectively ($n = 5$; SD = 2.5%). For direct HPLC-DAD biotin detection in a pharmaceutical drug, we tested three different extraction procedures as described above. The results are summarized in Tables 1 and 2.

Determination of avidin by FIA with electrochemical detection

On the basis of the above-mentioned results obtained via electrochemical detection of avidin and/or biotin, we also wanted to know whether we would be able to detect avidin and/or biotin by flow injection analysis with electrochemical detection. The electrochemical detector included two low-volume flow-through analytical cells containing eight carbon porous working electrodes [71]. Each carbon porous working electrode was connected to two reference and two counter electrodes (Fig. 4Aa).

Influence of the applied potentials of the working electrodes of the CoulArray detector

Next, the optimal potentials of the CoulArray detector's working electrodes were identified so that we could maximize the sensitivity of the determination of avidin. The flow injection analysis conditions utilized were an isocratic mobile phase consisting of 94% 0.02 M phosphate buffer (pH 6.7; solvent A) and 6% acetonitrile (solvent B). The flow rate of the mobile phase was 0.5 ml min^{-1}. The column and detector temperatures were both 25 °C. Potentials of 600, 650, 700, 750, 800,

Table 2 Recovery of biotin from pharmaceutical drug homogenate using modified CPE or HPLC-DAD methods in triplicate ($n=6$)

Detection method	Extraction procedure	Homogenate (μg ml^{-1})[a,b]	Spiking biotin (μg ml^{-1})[a,b]	Homogenate + spiked biotin (μg ml^{-1})[a,b]	Recovery (%)
Modified CPE	Water	5.5 ± 0.2 (3.6)	5.8 ± 0.2 (3.4)	11.8 ± 0.6 (5.1)	104
	ASE	5.6 ± 0.3 (5.4)	5.4 ± 0.4 (7.4)	10.6 ± 0.5 (4.7)	96
	Phosphoric acid	5.1 ± 0.3 (5.9)	5.5 ± 0.3 (5.5)	10.9 ± 0.5 (4.6)	103
HPLC-DAD	Water	5.5 ± 0.4 (7.3)	5.2 ± 0.2 (3.8)	11.2 ± 0.6 (5.4)	105
	ASE	5.3 ± 0.1 (1.9)	5.3 ± 0.4 (7.5)	11.0 ± 0.4 (3.6)	104
	Phosphoric acid	5.4 ± 0.1 (1.9)	5.1 ± 0.1 (2.0)	10.3 ± 0.3 (2.9)	98

[a]Biotin amounts are per one milliliter of diluted solution obtained by extraction
[b]Results expressed as mean ± SD (CV%) ($n=6$)

Table 3 Precision and recovery of biotin for pharmaceutical drug analysis ($n=6$)

Detection method	Extraction procedure	Homogenate (µg ml^{-1})[a,b]	Spiking biotin (µg ml^{-1})[a,b]	Homogenate + spiked biotin (µg ml^{-1})[a,b]	Recovery (%)
Modified CPE	Intra-day ($n=6$)	5.2 ± 0.2 (3.8)	5.7 ± 0.3 (5.3)	10.8 ± 0.3 (2.8)	99
	Inter-day ($n=30$)	5.0 ± 0.4 (8.0)	5.1 ± 0.3 (5.9)	10.4 ± 0.8 (7.7)	103
HPLC-DAD	Intra-day ($n=6$)	5.3 ± 0.1 (1.9)	5.4 ± 0.4 (7.4)	11.0 ± 0.5 (4.5)	103
	Inter-day ($n=30$)	5.5 ± 0.5 (9.1)	5.3 ± 0.4 (7.5)	10.5 ± 0.9 (8.6)	97

[a]Biotin amounts per one milliliter of diluted solution obtained by the phosphoric acid extraction method
[b]Results expressed as mean ± SD (CV%)

850, 900 and 950 mV were applied to the porous graphite working electrodes. According to the results obtained from using the electrochemical detection method (Figs. 1 and 2), we expected the highest electrochemical response from avidin to occur at 900 mV. The full scan of the FIA-ED detection of avidin is shown in Fig. 4A:b. The resulting hydrodynamic voltammogram (the dependence of current response on the cell detector applied potential) is shown in Fig. 4B. A potential obtained from the limit diffusion current area or from the point of the highest current difference and the smallest potential difference is the most suitable for electrochemically detecting the studied substances [72]. The most effective potential (950 mV) was selected from the point of the highest current difference and the smallest potential difference for measurements of avidin (Fig. 4B).

FIA - ED behaviour of avidin

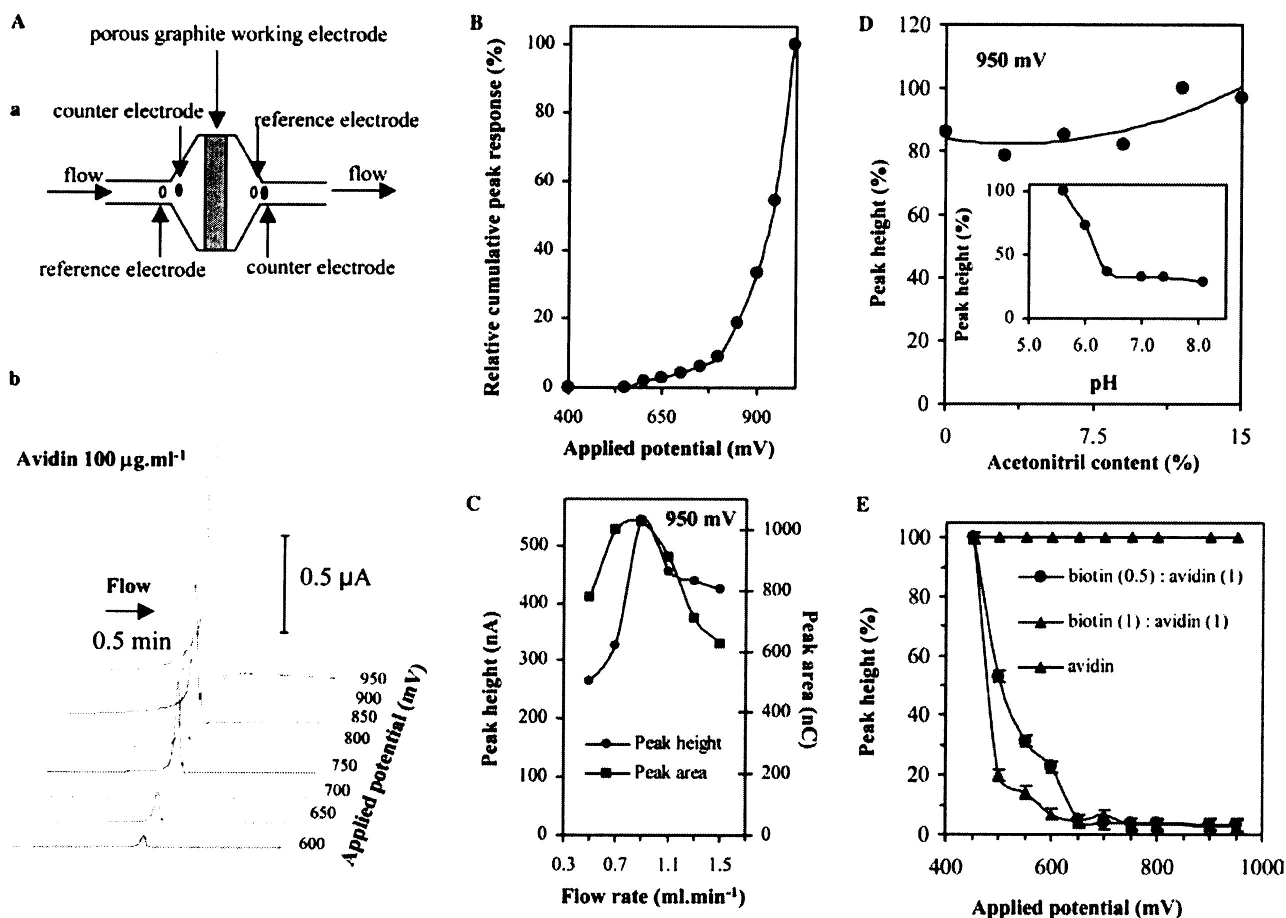

Fig. 4 Analysis of avidin by flow injection analysis with electrochemical detection (FIA-ED). **A** (a) Scheme of the detection cell with the graphite porous working electrode; (b) Full scans at different applied potentials. **B** Hydrodynamic voltammogram of avidin (100 µg ml^{-1}) at applied potentials from 400 mV to 1,000 mV. **C** Influence of the flow rate of the mobile phase. **D** Acetonitrile content in the mobile phase; inset: effect of pH on peak height of avidin. **E** Study of the avidin–biotin interaction by FIA-ED. Peak height of 100% represents 100 µg ml^{-1} avidin concentration. FIA-ED conditions: a mobile phase containing 0.02 M phosphate buffer and acetonitrile was used; column and detector temperature = 25 °C

Influence of mobile phase flow rate

The flow rate of the mobile phase is very important for chromatographic analysis because of its significant influence on the retention time, height and character of the analytical signals from the detected compounds [73]. The effect of different flow rates (0.3, 0.6, 0.9, 1.2 and 1.5 ml min^{-1}) on the avidin peak height was studied (Fig. 4C). According to the height of the electrochemical avidin signals obtained, the most suitable flow rate of the isocratic mobile phase consisting of 94% 0.02 M phosphate buffer (pH 6.7) and 6% acetonitrile was 0.9 ml min^{-1}. The current response of the avidin decreased by up to 85–90% at the highest selected mobile phase flow rates (1.2 and 1.5 ml min^{-1}) in comparison with the current response of avidin obtained at a flow rate of 0.9 ml min^{-1}. This phenomenon is probably caused by a shorter preconcentration time of the solute on the working electrode's surface (Fig. 4C).

Influence of acetonitrile levels and pH of phosphate buffer in the HPLC mobile phase

The organic component of the mobile phase can negatively influence the electrochemical response of a sample [70, 72, 74, 75]. In our experiments, we used the organic solvent of the mobile phase to facilitate chromatographic separation. The levels of acetonitrile (0–15% ACN) used in the mobile phase did not markedly influence the height of the avidin signal (Fig. 4A). The highest response was obtained at 15% acetonitrile in the mobile phase. Inorganic components of the mobile phase can also significantly influence the detection of avidin [70, 72, 74, 75]. We studied the influence of the pH of the phosphate buffer in the range 5.6–8.2. The highest electrochemical signal was obtained at the lowest pH value tested, pH 5.6 (see inset of Fig. 4D).

The most effective FIA-ED parameters

The best FIA-ED detection conditions for avidin were as follows: isocratic mobile phase, 0.02 M phosphate buffer (pH 5.6):acetonitrile in a ratio of 85:15; flow rate, 0.9 ml min^{-1}; column and detector temperature, 25 °C; electrode potential, 950 mV.

Influence of avidin concentration on its electrochemical response

We determined the detection limit of avidin by FIA-ED using the optimized method parameters. The limit was 392 fmol of avidin per injection (5 µl) for a signal-to-noise ratio S/N = 3. The RSD was only about 3.9% ($n = 7$).

Study of avidin-biotin interaction by FIA-ED

There are only a few published methods that are used for biotin detection. Direct electrochemical determination of biotin by FIA-ED with a silver electrode was described in 1986 [7]. We also determined biotin using an optimized FIA-ED technique. The results obtained will be published elsewhere. Besides direct FIA-ED determination of biotin and/or avidin, we used the optimized HPLC-ED technique to study the avidin-biotin interaction at full scan. Before injection of the avidin and biotin into the FIA-ED system, the biotin was mixed with avidin in two molar ratios (avidin: biotin; 1:0.5 and 1:1). It clearly follows from the results shown in Fig. 4E that the electrochemical signal of avidin decreased with increasing biotin concentration. During the electrochemical study of the avidin-biotin interaction we did not observe any signal from biotin, because all molecules of biotin were associated with avidin.

Conclusions

We constructed a protein-modified voltammetric bioelectrode by incorporating a protein into a CPE (avidin-modified CPE), which allows specific analysis of proteins of interest as well as their ligands. This technology offers useful and promising possibilities in the field of biosensor development. Square-wave voltammetry scans oxidative signals generated from Trp and Tyr residues in the proteins. We also demonstrated the application of

Table 4 Limits of detection (LODs for S/N = 3) for avidin and biotin using four analytical methods

Detected compounds	Detection procedure											
	AdTS SWV CPE			SWV Modified CPE			HPLC-UV			FIA-ED		
	pg	amol	fM	ng	fmol	fM	pg	pmol	pM	ng	fmol	pM
Avidin	3 or 1.5[a]	200 or 100[a]	3	2.5[b]	174[b]	700	ND	ND	ND	5.6[c]	390[c]	78
Biotin	ND	ND	ND	1.8	7.6×10^3	2.5×10^3	750	3.0	614	8.1[c]	33.5×10^{3}[c]	6.6×10^3

ND not detected
[a]Detection limits per drop (6 or 3 µl)
[b]Detection limits per 250 µl of modified carbon paste
[c]Detection limits per injection (5 µl)

this pulse voltammetric method for detection of avidin extracted from transgenic maize. In the case of biotin determination, we applied this voltammetric method in conjunction with high performance liquid chromatography with electrochemical detection. The results obtained demonstrate that the method is very sensitive and specific. The limits of detection for the avidin and/or biotin are summarized in Table 4.

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Full Paper

Zeptomole Detection of Streptavidin Using Carbon Paste Electrode and Square-Wave Voltammetry

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Abstract
We compared the suitability of avidin and streptavidin for avidin-biotin technology in view of the sensitivity of the analysis using square-wave voltammetry. We found out during our preliminary experiments that streptavidin gave approximately 100 times higher electrochemical response in comparison with avidin at the same experimental conditions and concentration. Thus, we used two approaches for streptavidin determination – analysis directly in electrochemical cell and analysis by adsorptive transfer technique (AdTS). Ten minutes long accumulation on carbon paste electrode surface was ascertained as optimal in both cases. Limits of detection were 0.3 aM (electrochemical cell) and 30 aM (AdTS).

Keywords: Streptavidin, Avidin, Modified electrode, Electrochemistry, Protein, Carbon paste electrode

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1. Introduction

Avidin, which was discovered in the 1920's, was isolated from the bird egg white [1]. It is alkaline glycoprotein which consists from four subunits with total molecular weight about 66 kDa (Fig. 1A; isoelectric point of the protein is about 9.5) [1]. Few years later after discovering of avidin, a protein, whose sequence is evolutionary related to that of avidin, was isolated from bacteria *Streptomyces avidinii.* This protein was called as streptavidin [2]. Streptavidin forms homotetrameric complexes as avidin with total molecular weight about 60 kDa (Fig. 1B; the isoelectric point is 6.1). There is a difference between the proteins, because streptavidin is not glycosylated unlike avidin [3].

Avidin takes its name from the avidity with which it binds biotin (vitamin H). Due to structural similarity between avidin and streptavidin, streptavidin itself have the affinity to biotin. The vitamin has a very high affinity to avidin/streptavidin (dissociation constant of 10^{-15} M). This interaction has been utilized in many types of avidin-biotin technologies, such as imunohistochemistry, electron microscopy, ELISA, DNA hybridization and construction of biosensors [4–13].

Suggesting of advantageous methods for the analysis of the avidin/streptavidin-biotin interaction is need. Electrochemical sensors and biosensors utilizing various kinds of working electrodes and detection procedures is suitable tool for this purpose [10, 14–29]. One of the most promised working electrodes are carbon paste electrodes (CPE) due their easy-to-use, no toxicity, easily and fast modifications, low cost and high sensitivity [30–39]. On the other, metal electrodes such as gold [40–43] or mercury [8] can be utilized for the analysis of the avidin/streptavidin-biotin interaction. The aim of the work was the utilizing of carbon paste electrode for ultra sensitive determination of streptavidin in the solution and also in the very low sample volume (5 μL drop).

2. Experimental

2.1. Chemicals

Streptavidin (from *Streptomyces avidinii*, essentially salt-free; molecular weight ca. 60 kDa), avidin (from egg white; molecular weight 66 kDa, subunit molecular weight 14 kDa), carbon powder, sodium acetate, acetic acid, and mineral oil were purchased from Sigma Aldrich Chemical Corp. (St. Louis, USA). Solutions were prepared using ACS water from Sigma Aldrich. The stock standard solutions of streptavidin at 16.7 nM were prepared and stored in the dark at 4 °C. All solutions were filtered through a 0.45 μm Teflon membrane filters (MetaChem, Torrance, CA, USA) prior to an electrochemical analysis.

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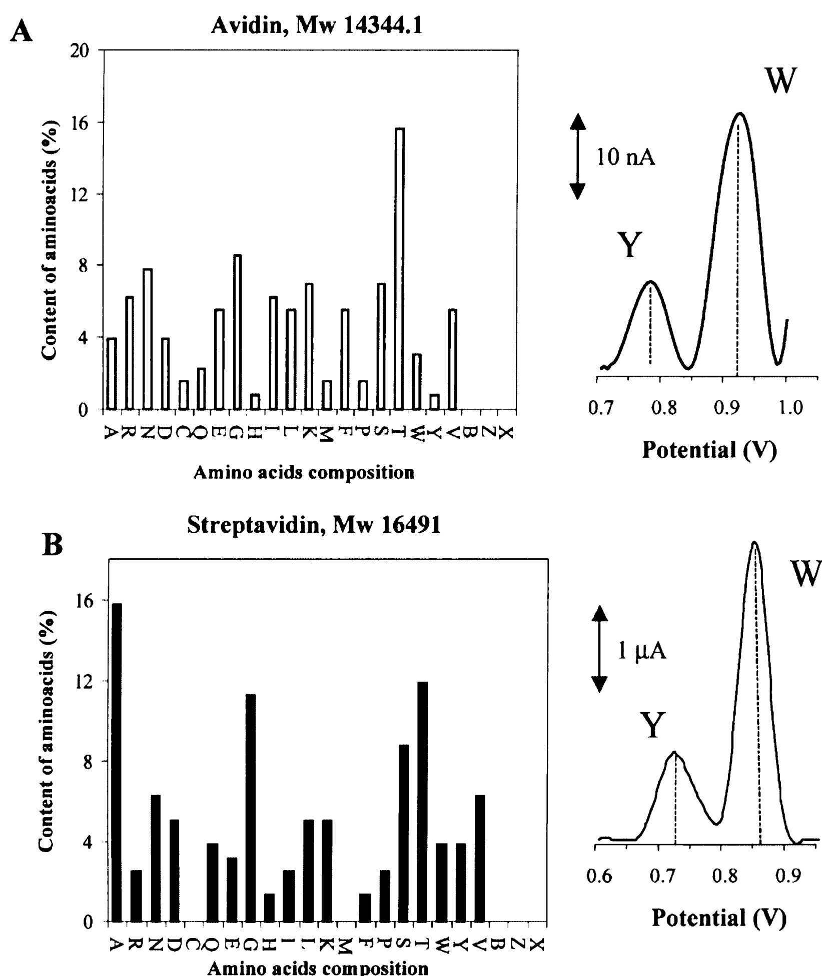

Fig. 1. Amino acids composition of avidin (A) and streptavidin (B). SWV CPE voltammogram of avidin and streptavidin (1.52 and 1.67 µM, respectively; t_A 120 s). The voltammetric signal corresponds to the oxidation of W and Y residues in avidin/streptavidin molecule. All measurements were performed in acetate buffer, pH 4. The square-wave voltammetric method was used with the following parameters: initial potential: 0.1 V, end potential: 1.3 V, amplitude: 25 mV, step potential: 5 mV, and frequency: 200 Hz. M_w corresponds to a subunit of avidin or streptavidin.

2.2. pH Measurements

The pH was measured using a WTW inoLab Level 3 instrument (Weilheim, Germany), controlled by a personal computer program (MultiLab Pilot; Weilheim, Germany). The pH-electrode (SenTix H, pH 0–14/0–100 °C/3mol L^{-1} KCl) was regularly calibrated using a set of WTW buffers (Weilheim, Germany).

2.3. Electrochemical Measurements

Electrochemical measurements were performed using an AUTOLAB analyzer (EcoChemie, The Netherlands) in connection with a VA-Stand 663 (Metrohm, Switzerland). The electrode system consisted of a carbon-paste working electrode, 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, pH 4.0) was used as the supporting electrolyte. Square-wave voltammetry (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) and a moving average baseline correction (peak width = 0.05) of the GPES software. Other details can be found the paper by authors Kizek et al. [10].

2.4. Preparation of CPE

The carbon paste (about 0.5 g) was made of 70% graphite powder (Sigma-Aldrich) and 30% mineral oil (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, an electrode surface was renewed by polishing with a soft filter paper. Then, the

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surface was ready for measurement of a sample volume of 5 μL.

2.5. Statistical Analysis

Precision of the measurements expressed as relative standard deviation was evaluated with standard solutions of avidin and/or streptavidin at concentration of 0.15 pM and/or 16.7 fM, respectively. Intraday precision was tested during 9 hours ($n = 4$). Interday precision was tested for four days ($n = 9$). STATISTICA Cz (StatSoft, Inc. (2005), version 7.1.) was used for statistical analyses. Value of $p < 0.05$ was considered significant. Limits of detection are expressed to one significant digit.

3. Results and Discussion

A great attention of number of scientific groups is devoted to the proteomic research now (more than 2000 articles contains proteomic in article titles, keywords, or abstracts according to Web of Science database from years of 2000 to 2006). Utilizing of carbon paste electrodes (CPE) instead of the mercury and solid ones [8, 44, 45] is very promising in the proteomic research. Possibility of CPE modification by various compounds eventually by extracts and homogenates is their excellent property and advantage [35, 36, 38]. The aim of such modification is to achieve maximal possible sensitivity and selectivity of the electrochemical measurement [46].

3.1. Electrochemical Determination of Streptavidin

Recently we published the paper, where we described electrochemical behavior of avidin on CPE by square-wave voltammetry (SWV) [10]. We obtained limit of detection (*LOD*; 3 *S/N*) for avidin 30 pM (100 amol of avidin in 3 μL) using this time-non-consuming technique (the measurement time was up to 3 min including the accumulation time of 120 s) [10]. As for avidin incorporation into the CPE, detection limit expressed as 3 *S/N* for avidin was 0.7 nM (0.2 pmol in 250 μL solution added to 1 g of carbon paste). Relative standard deviation was up to 10%. We also detected biotin by avidin modified CPE with limit of detection 0.2 pmol in 5 μL (3 μM; 3 *S/N*). We also utilized this procedure for biotin analysis in pharmaceutical drug [10].

The sensitivity of the abovementioned determination is insufficient if we want to utilize the technique for, e.g., monitoring of hybridization of DNA using biotinylated nucleotides. Due to this we were faced to a problem of another decreasing of the detection limit. As it is mentioned in Introduction section, streptavidin, the avidin-like protein, also effectively and selectively binds biotin. For that reason we were interested in the issues how do streptavidin behave on the surface of CPE.

During our preliminary experiments we found out that streptavidin gave approximately 100 times higher electrochemical response in comparison with avidin at the same experimental conditions and concentration (16.7 nM). This phenomenon relates probably with the different amino acid composition and also with absence of saccharide in streptavidin molecule, which results in dissimilar orientation of amino acid residues to the surface of the working electrode. More electroactive amino acid residues (tryptophan – W and/or tyrosine – Y) of streptavidin in comparison with avidin are able to be oxidized on the CPE surface and that leads to the higher current response. This presumption confirms the potential shift of peak (Y_{avidin} 770 mV)/($Y_{streptavidin}$ 730 mV) and (W_{avidin} 910 mV)/($W_{streptavidin}$ 860 mV) (Fig. 1). These results show applicability of streptavidin as biological part of a biosensor in avidin-biotin technologies. Due to this we decided to study streptavidin as biotechnologically advantageous protein in comparison with avidin.

Particularly, we investigated changes in streptavidin electrochemical signal with its increasing concentration in the electrochemical cell filled by 2 mL of 0.1 M acetate buffer (pH 4). We obtained well developed signal of streptavidin (with two peaks corresponding to amino acids W and Y) at highest tested concentration (167 nM, accumulation time of 120 s was used). Then, we decreased streptavidin concentration down to 167 fM. At this concentration, the electrochemical response was less noticeable at accumulation time of 120 s. Thus, we investigated the influence of accumulation time on streptavidin signal in order to improve sensitivity of streptavidin analysis. We used very low concentration of streptavidin in electrochemical cell (167 fM) for this purposes and construed dependence of peak height of amino acid W on concentration of compound of interest. The highest signal of amino acid W was observed at 10 min (upper inset in Fig. 2A). We were interested in the issue how much sensitive is our analysis now. Typical Langmuir dependence of W signal height on streptavidin concentration (from 0.65 fM to 330 fM) has been obtained (Fig. 2A). The curve was strictly linear ($y = 8.1627x - 1.8917$; $R^2 = 0.9964$) in the range from 0.65 to 11 fM (lower inset in Fig. 2A). Detection limit of streptavidin (*LOD*; expressed as 3 *S/N*) in the electrochemical cell (2 mL of background electrolyte and streptavidin solution) was 0.3 aM. Other details see in Table 1.

In the following experiments we aimed on detection of streptavidin by adsorptive transfer technique (AdTS) on CPE due to utilizing of the technique for analysis of low volumes of a sample (about 5–10 μL). On the basis of our previous experimental results we choose a low concentration of streptavidin during optimization of accumulation time. Particularly, we choose the same concentration as in the case of analysis performed in electrochemical cell – 167 fM (it corresponds to 835 zmol of the streptavidin in 5 μL). The highest current response was obtained at 10 min. long accumulation time similarly to electrochemical cell determination (upper inset in Fig. 2B). We construed

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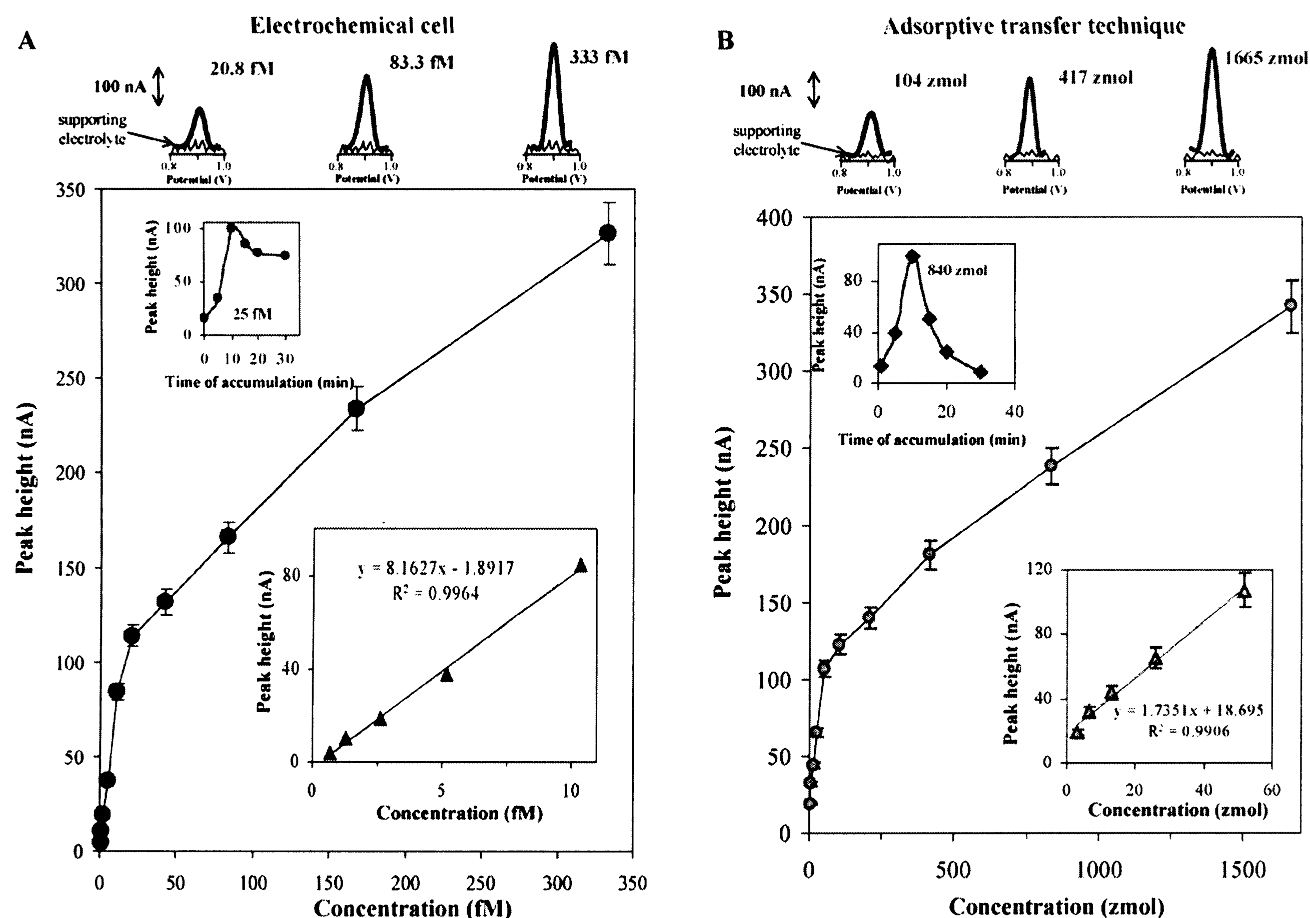

Fig. 2. A) Electrochemical cell. Analysis of streptavidin in the electrochemical cell. Dependence of peak streptavidin height on concentration at accumulation time of 10 min. Inset: dependence of peak height of streptavidin at concentration 167 fM on accumulation time and on streptavidin concentration in the range from 0.65 fM to 11 fM. The signals of streptavidin were baseline corrected and smoothed; the signals of supporting electrolyte are shown without any data treatment. B) Adsorptive transfer technique. Dependence of current response on streptavidin amount in 5 μL at accumulation time of 10 min. Inset: dependence of peak height of streptavidin (840 zmol, sample volume 5 μL) on accumulation time and on its concentrations (from 3 to 60 zmol of streptavidin in 5 μL). The signals of streptavidin were baseline corrected and smoothed; the signals of supporting electrolyte are shown without any data treatment. Other details in Figure 1 and Section 2.

Table 1. Calibration and statistical data for avidin and streptavidin measurement.

Compound of interest	Regression equation	R^2 [a]	*LOD*	[b]	*RSD* [f] (%)	Intraday ($n=4$; %) [g]	Interday ($n=9$;%) [g]
Avidin	$y = 1.3189x + 1.6566$	0.9978	30 pM	100 amol [c]	3.6	4.2	4.6
Streptavidin	[A] $y = 8.1627x - 1.8917$	0.9964	0.3 aM	0.7 zmol [d]	16		
	[B] $y = 1.7351x + 18.695$	0.9906	30 aM	0.2 zmol [e]	15	24	22

[A] Analysis of avidin in electrochemical cell (electrolyte volume 2 mL).
[B] AdTS SWV analysis of avidin.
[a] Regression coefficients.
[b] Limits of detection expressed as 3 *S/N*.
[c] Sample volume of 3 μL.
[d] Measurement was performed in 2 mL of supporting electrolyte containing defined amount of streptavidin.
[e] Sample volume of 5 μL.
[f] Relative standard deviations.
[g] Relative standard deviations of intra- or interday precision evaluated with avidin and/or streptavidin standards at concentration of 0.15 pM and/or 16.7 fM, respectively.

dependence of streptavidin peak height on its concentration in the range from 3 to 1660 zmol at the most optimal accumulation time (sample volume of 5 μL). The curve obtained shown the same manner as for analysis of streptavidin in electrochemical cell (Fig. 2B). The curve was linear ($y = 1.7351x + 18.695$; $R^2 = 0.9906$) in the range from 3 to 60 zmol of streptavidin per 5 μL (lower inset in Fig. 2B). Limit of detection (*LOD*; expressed as 3 *S/N*) of

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streptavidin determined by AdTS SWV was 30 aM (0.2 zmol in 5 μL). Other details are shown in Table 1.

During statistical data treatment, we investigated precision of the technique by five times repeated ($n = 5$), intraday (analysis after 3 hours up to 9; $n = 4$) and interday (analysis after 12 hours up to 96; $n = 9$) measurements. We found out that relative standard deviation (*RSD*) of the measurement is about 16% and/or 15% as for streptavidin determination in 2 mL of background electrolyte (accumulation in electrochemical cell for 120 s) and/or in 5 μL drop (adsorptive transfer technique), respectively (Table 1). The significant difference between *RSD* of the avidin (3.6%) and streptavidin (16%) analysis is probably due to the amount of determined analyte by our sensitive method. The concentration streptavidin is approximately million times lower versus avidin concentration [10]. This difference may result in higher deviations due to adsorption of the analyte on the electrochemical cell surface, test tube etc., which have much more significant effect at studied concentration. This higher relative standard deviation could be minimized e.g. by using of specially coated accessories. Due to an influence of the adsorption, we studied interday precision using on the one hand streptavidin working solution prepared from the stock solution (streptavidin concentration 16.7 nM) exactly before analysis (solution A) and on the other hand working streptavidin solution prepared before the first interday precision measurement (solution B). In the case of precision of interday measurement using solution A, electrochemical signal was equally distributed around the mean value. On the other hand, if we used solution B, marked decrease (about 60%) in the streptavidin current response appeared after the 12 hours. After that the signal decrease was more gradually (about 5%). It clearly follows from these results that adsorption of streptavidin has significant influence on measurement precision.

4. Conclusions

Carbon paste electrodes belong to promising tools for detection of biotechnologically important compounds. Here, we revised and evaluated the limits of detection and experiments, where we determined avidin. We shown that streptavidin gave approximately 100 times higher current response than avidin. Thus, detection limit of streptavidin was about 0.2 zmol per 5 μL, which was lower than as for avidin. We assume that streptavidin could be more suitable than avidin for avidin-biotin technological purposes.

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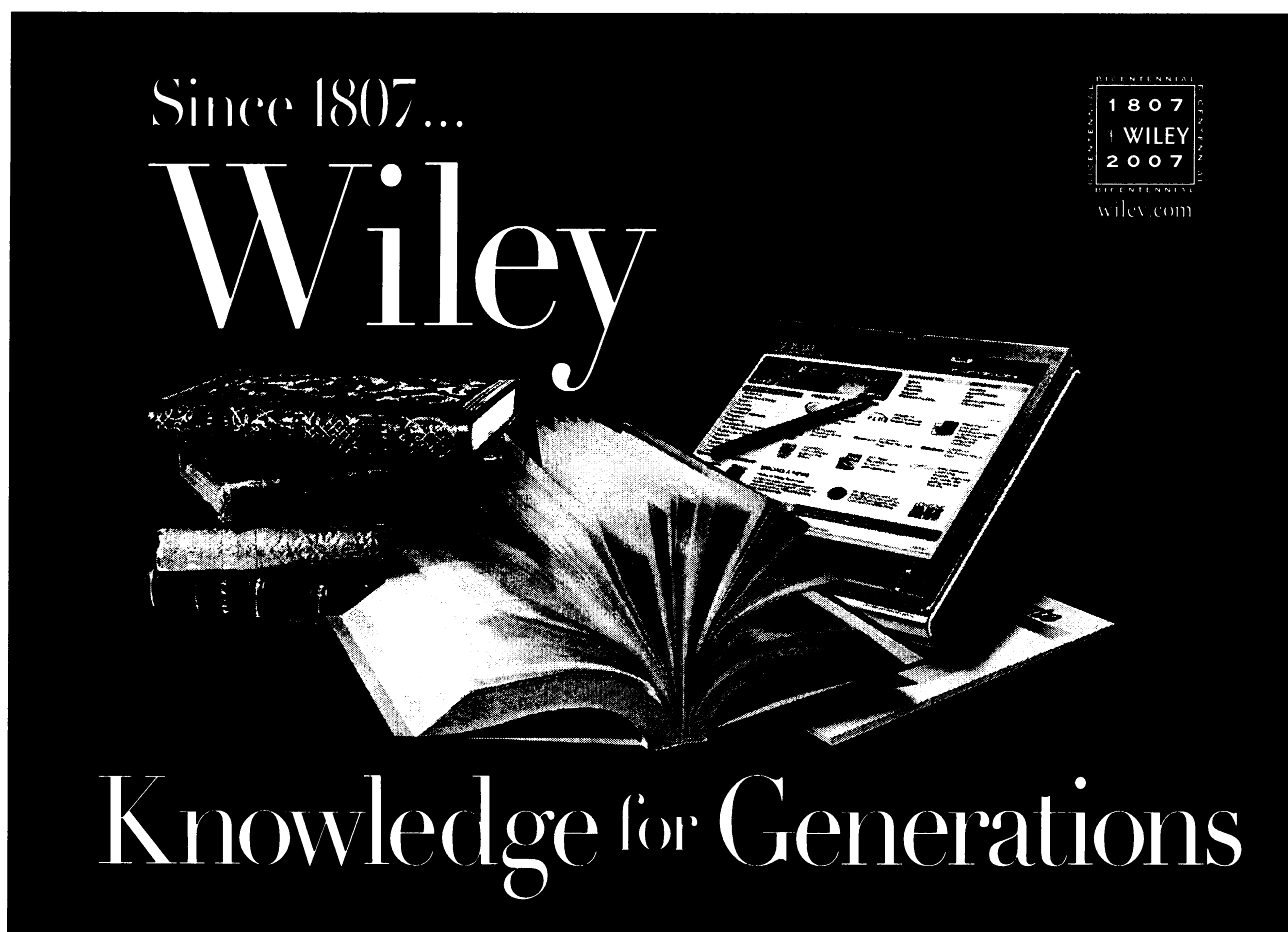

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UTILIZATION OF CHIP-BASED CAPILLARY ELECTROPHORESIS FOR AVIDIN DETERMINATION IN TRANSGENIC TOBACCO AND ITS ADVANTAGES OVER STANDARD GEL ELECTROPHORESIS AND VOLTAMMETRY

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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), SDS-PAGE electrophoretic method and a square wave voltammetric method using a carbon paste electrode. We determined that the automated capillary electrophoretic method is more rapid, sensitive and reproducible than the other methods. The avidin content measured in transgenic tobacco using chip-based capillary electrophoresis varied from 15 to 377 ng per mg depending on the individual plant, a result that was in good agreement with results obtained using the other methods.

Keywords: chip-based capillary electrophoresis, avidin, transgenic tobacco plants, electrochemical methods, SDS-PAGE

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. The pesticide may be a chemical substance, biological agent (such as a virus or bacteria), antimicrobial or disinfectant used against pests including insects, plant pathogens, weeds, birds, mammals, fish, nematodes and microbes that compete with humans for food, spread or are a vector for disease. It is a 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]. This soil dwelling bacterium forms spores and can produce a crystalline protein (*cry*-protein, or delta-endotoxin), which is toxic to certain kinds of insects. The *cry* gene can be cloned into the genome of plant cells and thus the plants are able to express this protein. Since 1994 various types of transgenic plants have been cultivated, e.g. maize, potatoes, tomatoes and cotton. Although *Bt* formulations have been evaluated extensively for controlling insect pests of stored grain, commercially acceptable levels of protection against coleopteran (beetle) pests have not been achieved. In addition, the development of insect resistance to these naturally occurring toxins is a concern, prompting efforts to exploit other insecticidal proteins [6]. Hood et al. in 1997 [7] 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 stored-product insect pests [8]. So far in addition to maize, transgenic avidin expressing tobacco [9], rice [10], and apple [11] have been developed.

Avidin is a glycosylated positively charged protein that is composed of four subunits with a tetrameric molecular weight of about 67 kDa. 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 [12].

The most commonly used techniques for detection and evaluation of avidin-biotin interactions are ELISA, fluorimetry, and several electrochemical methods [13-18]. 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 protein. In comparison with the ELISA technique, the electrochemical determination exhibited good agreement, but it was much more sensitive [19, 20].

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.

Experimental

Chemicals

Avidin, carbon powder, mineral oil and other chemicals were purchased from Sigma-Aldrich Chemical Corp. (St. Louis, USA). Solutions were prepared using ACS water from Sigma Aldrich. Stock standard solutions of avidin at 1 μg/mL 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 [21]. 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 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% 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 to 10.5, 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 [22]. The prepared samples were stored at -20°C.

Electrochemical determination of avidin

Electrochemical measurements were performed using a CHI440A instrument (CH Instruments, INC., 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, 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). GPES software supplied by EcoChemie was employed for smoothing and baseline correction (peak width = 0.05). Other details can be found Kizek et al. [23].

Preparation of CPE

The carbon paste (about 0.5 g) was made of 70% graphite powder (Sigma-Aldrich) and 30% mineral oil (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

SDS-PAGE

Electrophoresis was performed according to Laemmli [24] using a Biometra apparatus (Biometra, Germany). First 10% running, then 5% stacking gel was poured; 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%   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 MW, the protein ladder "Precision plus protein standards" from Biorad was used. 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. [25]. 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, 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 diluted priming station sample, the mixture (6 µl) was loaded into sample wells. The Pro260 Ladder included in the kit was used as a standard.

Results and discussion

Improved methodology is always an important analytical goal, especially if those improvements provide solutions to problems of contemporary interest. As we mentioned above, pesticides are very dangerous substances that can contaminate the environment. Therefore, we continue to search for new tools that are harmless and safe to humans and to other organisms. One possible means of protecting plants against insect pests is to transform them with the avidin transgene. The aim of this study was to compare three methods suitable for avidin detection in transgenic tobacco.

Electrochemical analysis of avidin and its determination in transgenic plants

Avidin is an important protein that contains a diversity of amino acids in its structure. From an electrochemical point of view, only tyrosine (Y) and tryptophan (W) have been found to be electroactive using a variety of electrodes [26, 27]. 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 of avidin by using the adsorptive transfer stripping square-wave voltammetry technique. These electrochemical transfer techniques were described in detail previously [28-30]. Based on our experiences with electrochemical detection of avidin and streptavidin [12], we applied adsorptive transfer stripping square wave voltammetry (AdTS 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 of 0.75 – 12.5 μg/mL avidin is shown in Figure 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. Full coverage of the electrode surface was not reached within the protein concentration range studied. The relative standard deviation (RSD) was only about 4 %.

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 28 to 130 ng avidin per mg of plant tissue (Table 1).

Determination of avidin by EXPERION – Automated electrophoresis system

Experion is an automated microfluidic electrophoresis system that uses a combination of Caliper Life Sciences innovative LabChip microfluidic separation technology and sensitive fluorescent sample detection. It performs rapid and reproducible 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 reproducible 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 25.77 ± 0.57 kDa (migration time = 27.29 ± 0.11 s), which corresponds to the subunit dimer. In the case of the four highest concentrations (125, 250, 500 and 1,000 ng/mL) analyzed, the signals corresponding to monomer (16.43 ± 0.37 kDa, migration time = 24.89 ± 0.11s), trimer (44.7 ± 0.69 kDa, migration time = 30.70 ± 0.12 s) and tetramer of subunits (66.68 ± 0.25 kDa, migration time = 33.97 ± 0.04 s), exhibited a slight signal shift with decreasing concentration of the protein to a higher molecular weight of about 0.5 kDa (Fig. 2B and 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 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.

Determination of avidin in transgenic plants

Based on the previous results, the presence and intensity of the signal corresponding to the subunit dimer (peak of molecular weight of 26 ± 2 kDa) 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

nontransformed control plants was observed (Fig. 2D). The average intensity of this peak measured in extracts from the transgenic plants was 7.1 ± 2.1 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 377 ng/mg of plant tissue (Table 1). This large variability can be attributed to different avidin expression levels in individual transgenic plants. This variability was similar to the results obtained in the case of avidin determination using the electrochemical method.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

In the case of SDS-PAGE using reducing conditions, the most intense band corresponding to the monomer of avidin subunit with a molecular weight of 15.8 kDa 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 evaluated by Biolight software. In comparison to the control, the intensity of this band was 9.8 ± 3.0 times higher in all transgenic plants. In one sample this intensity was markedly higher (plant #2 and in three plants lower (plants #5, 6 and 9). To determine the avidin level in transgenic plants, the concentration of proteins with the same mobility quantified in control was subtracted from the concentration determined from the calibration curve. The amount of avidin detected in the transgenic plants ranged from 31.8 to 165.7 ng/mg of plant tissue (Table 1). The relative avidin concentrations detected by this method were similar to those determined by

electrochemical and capillary electrophoretic methods, but the differences in the concentrations found were less significant (Fig. 3).

Comparison of the three methods 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 [31-33], while electromigration methods utilize mobilities of charged proteins after a subsequent colour reaction. 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 10 to 260 kDa in mass. The sensitivity of this kit is comparable to colloidal Coomasie Blue staining of proteins in SDS-PAGE gels. Other advantages of the Experion system are rapidity (each analysis requires only tens of seconds), automation and good repeatability. The result of each analysis provides a logging of individual peaks of proteins and the creation of virtual electrophoretograms.

The three procedures 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 before it was loaded into the Experion chip or SDS-PAGE gel. Therefore, the native avidin was converted to monomeric (SDS-PAGE) and dimeric units (Experion). The reason 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. Electrochemical measurements were carried out with native avidin because it was not necessary to denature it. Compared to electrophoretic methods, the electrochemical method yields lower amounts (~50%). This phenomenon 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. If we compare both electrophoretic methods, the Experion system is more sensitive, faster and reproducible. In comparison with the literature where the values of avidin amount in transgenic tobacco plants were between 70–90 ng per mg of leaves, our results are in relatively good agreement with those obtained by the earlier techniques used (Table 1). The average values for the variation in avidin content for the 8 transgenic plants were 197.9+/-146.1, 84.8+/-38.9, and 66.5+/-34.3 ng/mg plant tissue for the Experion, SDS-PAGE and AdTS SWV methods, respectively. The former method yields the highest values, which indicates the best recovery of avidin of the three methods. In general, insect pests cannot develop and survive feeding on transgenic plants expressing 47 ppm avidin or more [2, 9, 11]. 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 EXPERION is more sensitive, rapid and reproducible than the other methods. This new approach is likely to be suitable tool in detection and quantification of a range of proteins and other biologically important compounds.

Acknowledgement

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Table 1

Content of avidin in transgenic plants determined by three different techniques.

Method Plant sample	SDS-PAGE* ng/mg	Experion* ng/mg	AdTS-SWV* ng/mg
Plant 1	119.9±6.1	60.0±3.1	52.8±2.9
Plant 2	85.9±4.2	343.0±17.4	43.3±2.2
Plant 3	84.9±3.2	377.2±18.0	104.9±4.9
Plant 4	72.7±3.9	313.4±15.6	89.7±4.1
Plant 5	61.7±3.1	113.6±6.1	43.6±2.2
Plant 6	165.7±8.4	329.0±16.2	129.5±6.3
Plant 7	31.8±1.9	15.5±1.3	28.3±0.9
Plant 8	55.8±2.8	31.7±0.9	39.7±2.1

* Mean value +/- standard deviation, n=5.

Captions for figures

Figure 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).

Figure 2.

Analysis of avidin in plant samples by Experion automated electrophoresis system. Calibration curve of avidin (A), different concentrations of avidin standards in comparison with molecular weight 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 10 % height corresponds to the highest avidin positive tobacco extract. Inset – record of real plant sample (D).

Figure 3.

SDS-PAGE analysis of avidin in plant samples. SDS-PAGE gel, L-standards of molecular weight, S-5000 ng of avidin standard, 1-8 – samples of transgenic plants 1-8

respectively, C - control plant (A), relative avidin concentrations in analyzed samples obtained by densitometric analysis of the gel. The 100% peak height corresponds to the highest avidin positive tobacco extract (B).

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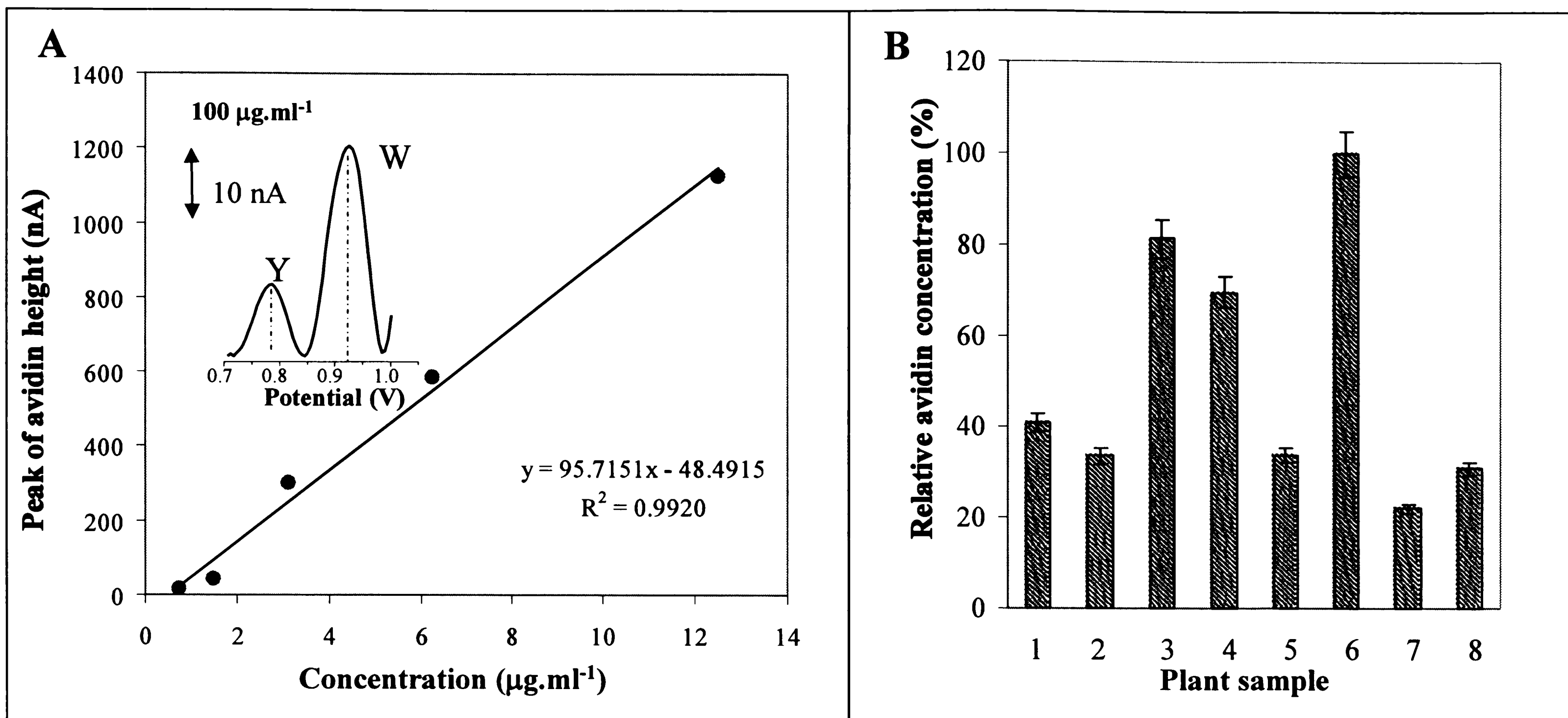

Fig. 1

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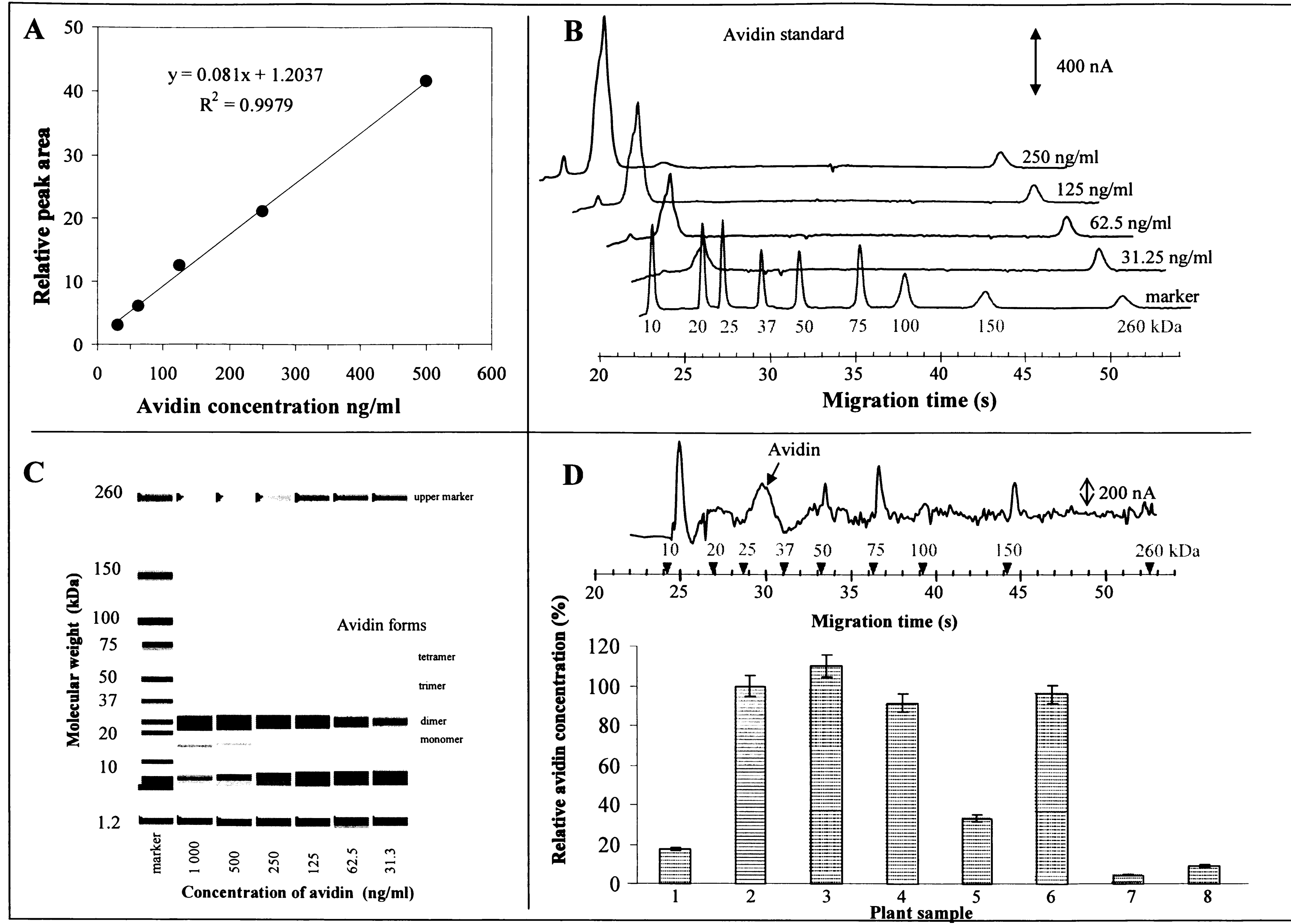

Fig. 2

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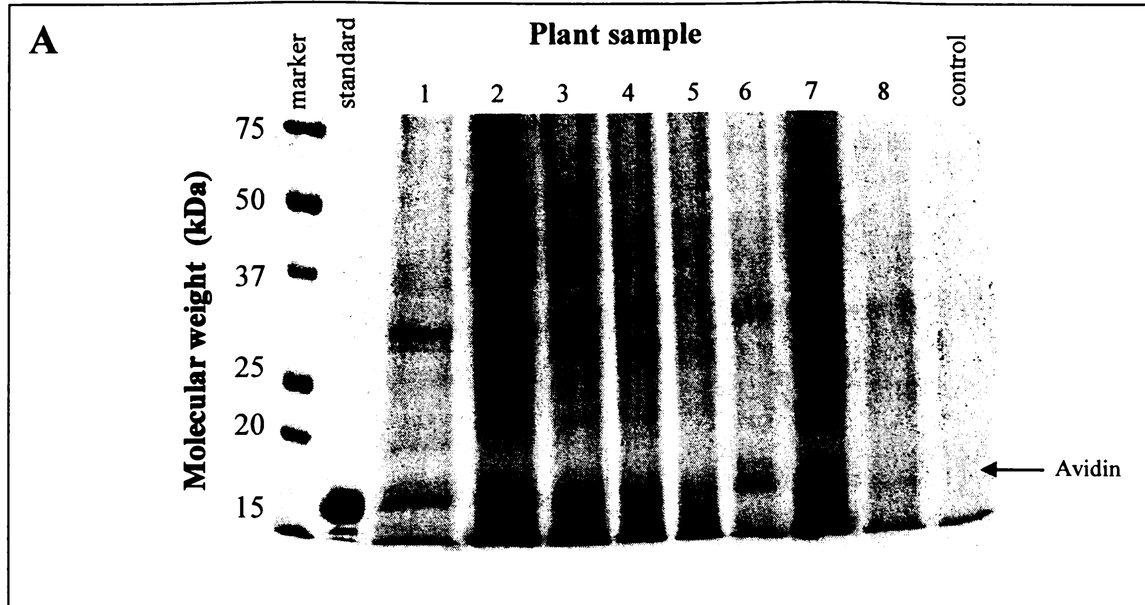

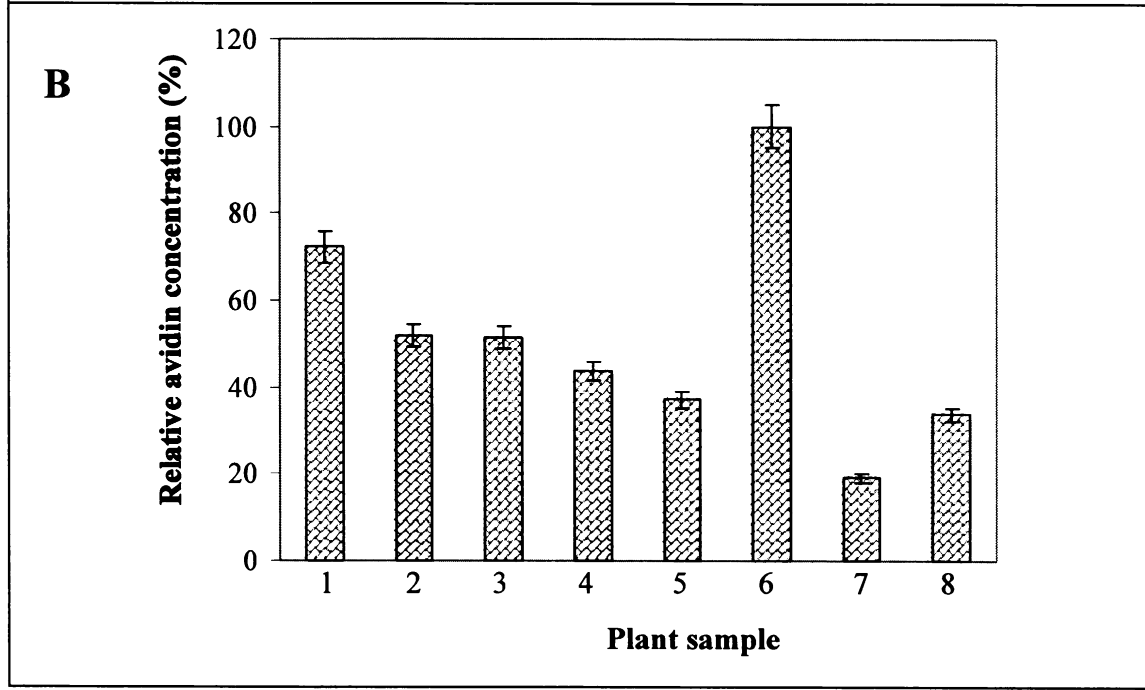

Fig. 3

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