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Biotin–avidin amplified enzyme-linked immunosorbent assay for determination of isoflavone daidzein

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Abstract

A biotin–avidin amplified enzyme-linked immunosorbent assay (BA-ELISA) method was developed and optimized for the determination of a weakly estrogenic isoflavone daidzein in serum, urine and *Puerariae radix*. Specific polyclonal antibody was produced against daidzein by immunization of rabbits with a conjugate of 7-*O*-(carboxymethyl)-daidzein and bovine serum albumin (BSA). The polyclonal antibody showed specific recognition of daidzein, while cross-reactivities to coumarin, 4-hydroxycoumarin, phenol, and other isoflavones such as puerarin and rutin were all lower than 1%. The linear range of daidzein calibration curve was 0.1–1000 ng mL^{-1}. The detection limit was found to be 0.04 ng mL^{-1}, and the intra-assay and inter-assay coefficients of variation were 7 and 16%, respectively. Human serum and urine samples were spiked with known amounts of daidzein and measured by the established BA-ELISA. Recoveries were between 91 and 107%. Daidzein in *P. radix* was determined by the BA-ELISA method and HPLC method, and the content of daidzein was determined to be 0.0219 and 0.0194%, respectively. The results indicated that there was a good agreement between the two methods. The established method is very useful for monitoring daidzein in biological samples and traditional Chinese medicine.

Keywords: Daidzein; Polyclonal antibody; Biotin–avidin amplified ELISA; Serum sample; Urine sample; *Puerariae radix*

1. Introduction:

Isoflavones are a widespread group of natural products, and their biochemical and pharmacological properties have been studied and reported recently [1,2]. In particular, daidzein, as a major isoflavone, has shown antigiardial activity [3], antioxidant action and potential anti-diabetic properties [4,5]. Daidzein exists widely in soy foods, traditional Chinese medicines such as *Puerariae radix*, and human fluids. Therefore, the analysis of this compound is of prime importance.

Traditionally, chromatographic methods combined with other chemical analytical technologies have been applied to the analysis of daidzein in foods [6–8], traditional Chinese medicine [9–11], serum [12,13], and urine [12,14]. The common disadvantage of these methods is the time-consuming sample pretreatment. For GC–MS detection, a tedious derivatization process is often required. Compared with GC–MS, HPLC requires fewer steps for sample preparation and analysis, and demands less technician time and less expensive instrument, but shows less sensitivity and specificity. However, both methods are not suitable for screening purposes in large quantities. Immunoassay is the method of choice for

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large-scale studies of daidzein at lower levels, because it can be applied directly to the analysis of complex samples and enables high throughput.

P. radix is a commonly used Chinese herb, which exerts sedative and antipyretic actions and is often used to treat influenza, wrist stiffness and headache [15]. *P. radix* and its medical preparations are also used as clinical medicine to treat coronary heart disease, myocardial infarction and hypertension [16]. Daidzein is an important isoflavone in *P. radix*. The determination of daidzein in *P. radix* was not easy because of the complexity of components in the derived extract. The application of immunoassay was expanded for the direct analysis of daidzein in complex traditional Chinese medicines because of its good specificity, sensitivity and high throughput. The group of Bennetau–Pelissero has established ELISA method for the analysis of daidzein in food stuffs and human fluids. A daidzein–protein conjugate was synthesized by introducing an ethylene carboxyl group at C2 carbon for conjugating with BSA [17]. The detection limit is 10 ng mL^{-1} [18,19].

In this study, we established a biotin–avidin amplified enzyme-linked immunosorbent assay (BA-ELISA) for determination of isoflavone daidzein in serum, urine and *P. radix*. 7-*O*-(Carboxymethyl)-daidzein was synthesized to prepare the complete antigen and produce a specific polyclonal antibody against daidzein. A high titer of antiserum was obtained, and a BA-ELISA was established for sensitive and specific determination of daidzein. Compared with reported ELISA methods [18–20], the present method has higher sensitivity. For the determination of daidzein in *P. radix*, little matrix effect was observed in this study. At the same time, an HPLC method was established to determine daidzein in *P. radix*. The results indicated that there is good agreement between the two methods.

2. Experimental

2.1. Materials and instrumentation

Daidzein was purchased from Lancaster Chemical Co. (USA). Puerarin and rutin were obtained from China National Institute of the Control of Pharmaceutical and Biological Products. Coumarin and 4-hydroxycoumarin were purchased from Tianjin Tiantai Chemical Co. (China). Phenol and ethyl bromoacetate were obtained from Beijing Chemical Co. (China). Bovine serum albumin (BSA), *N*-hydroxy-succinimide (NHS), 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide HCl (EDC), Freund's complete adjuvant (FCA), Freund's incomplete adjuvant (FIC), ovalbumin (OVA) and 3,3′,5,5′-tetramethybenzidine (TMB) were purchased from Sigma Chemical Co. (USA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG, biotinylated goat anti-rabbit IgG and avidin–HRP were obtained from Beijing Biotinge Biotech Co. (China).

Stock solutions of daidzein, puerarin, rutin, coumarin, 4-hydroxycoumarin and phenol were prepared with dimethyl sulfoxide (DMSO). The working solutions were made freshly before use by diluting the stock solutions with 0.01 mol L^{-1} phosphate buffered saline (PBS).

The human urine was obtained from a healthy volunteer. The human serum was purchased from Beijing Biotinge Biotech Co. (China). The *P. radix* was purchased from Tongrentang Pharmaceutical Group Co. (China).

Microwell plates were obtained from Nunc (Denmark) and a Bio-Rad model 550 microplate reader (Tecan, Austria) was used to measure the optical density of the ELISA results. A Cary UV–vis spectrometer (Varian, USA) was used to measure the absorbance of the protein solution.

HPLC was performed on an HP G1311A Quat Pump System equipped with an HP G1315 diode array detector and an HP G1328A manual injector (Hewlett Packard, USA). Ultrasound-assisted extractions were performed with a Transsonic SB3200 apparatus (50 KHz, 120 W, Binengxin, China). Avanti J-25 high-speed refrigerated centrifuge (Beckman, USA) was used for centrifugation. Methanol was HPLC grade, and redistilled water was used.

2.2. Preparation of complete antigen

2.2.1. Synthesis of 7-O-(carboxymethyl)-daidzein

Potassium iodide (60 mg, 0.36 mmol) and 3% (w/v) aqueous potassium hydroxide (0.8 mL) were added to a solution of daidzein [I] (100 mg, 0.39 mmol) in acetane (3 mL) and *N*,*N*-dimethylfromanide (1.5 mL) mixed with ethyl bromoacetate (90 μL). The reaction was left to proceed in the dark for 24 h at room temperature. After extraction from the aqueous phase with ethyl acetate, the crude ester was chromatographed on silica gel with 100:5 chloroform–methanol. The ester [II] (35 mg, 0.1 mmol) was treated with sodium hydroxide (2 mol L^{-1}, 0.5 mL) in methanol (5 mL) for 2 h. The mixture was acidified to pH 5.5 with glacial acetic acid, diluted with water (10 mL) and extracted with ethyl acetate. The organic layer, dried over sodium sulfate, was reduced to dryness under vacuum. The residue (26 mg) was hapten [III]. The structural formulas of the synthesized derivatives are given in Fig. 1.

2.2.2. Preparation of daidzein–BSA conjugate

The hapten (7 mg) was dissolved in DMSO (1 mL), and NHS (3.5 mg) and EDC (5.9 mg) were subsequently added. This solution (A) was stirred for 2 h at room temperature. BSA solution was prepared by dissolving 45 mg of BSA solution in 4 mL of 0.1 mol L^{-1} $NaHCO_3$ (pH adjusted to 7.0). To this solution, solution A was added slowly in droplets under stirring. The reaction was performed for 2 h and the obtained solution was dialyzed against 0.01 mol L^{-1} PBS at 4 °C for 24 h. After 6 changes of the PBS, the solution was dialyzed against pure water for 48 h with 10 changes of water. Finally, the solution was lyophilized and the white crystal obtained was stored at −20 °C [21]. The

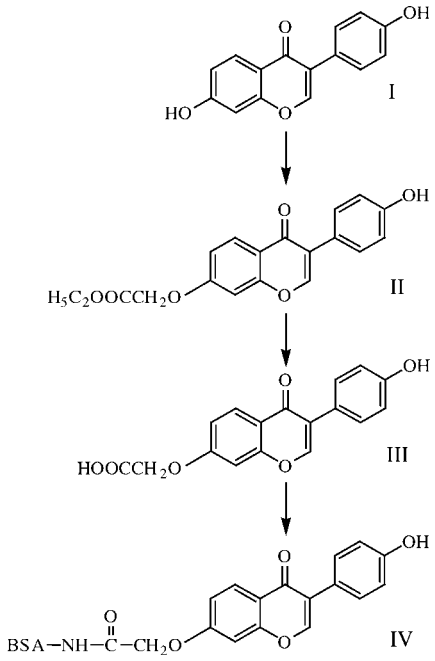

Fig. 1. Synthesis of the complete antigen of daidzein.

molar ratio of daidzein–BSA conjugate was determined by MALDI MS.

The daidzein–OVA conjugate was prepared in the same way as the daidzein–BSA.

2.3. Production and purification of the polyclonal antibody

Three New Zealand white rabbits (2–3 kg) were used for the immunization [21]. For each rabbit, a total of four injections of 4 mg of daidzein–BSA conjugate in Freund's adjuvant were performed at days 0, 11, 22, 33 before final bleeding at day 45. The first injection of 1.5 mg of the antigen was emulsified with FCA. The later three booster injections were emulsified with FIC and contained 0.5, 1 and 1 mg of the antigen, respectively. The titer of antiserum was determined with ELISA using daidzein–OVA coated onto the microplate.

The obtained antibody was purified according to a modified caprylic acid-saturated ammonium sulfate (SAS) method [22]. To 2 ml of antiserum diluted with 6 mL of acetate buffer (0.06 mol L^{-1}, pH 4.8), 200 μL of caprylic acid was added dropwise under gentle stirring. The formed turbid solution was slowly stirred for 30 min and then centrifuged at 12,000 rpm for 30 min at room temperature. The precipitates were discarded and 0.1 mol L^{-1} PBS was added to the supernatant at a volume ratio of 1:10. The solution was adjusted to pH 7.4 with 1.0 mol L^{-1} NaOH and cooled to 4 °C. To this solution, SAS (pH adjusted to 7.4) of the same volume was added dropwise under stirring. The obtained solution was allowed to stand for 2 h and then centrifuged at 12,000 rpm for 30 min at 4 °C. The supernatant was discarded and the precipitates were dissolved in 2 mL 0.01 mol L^{-1} PBS (pH 7.4). The solution was dialyzed against 0.01 mol L^{-1} PBS until no sulfate ion could be detected in the dialysis solution and then stored at −20 °C.

2.4. Titration level determination of antiserum and purified antibody

The daidzein–OVA conjugate was absorbed in the wells of polystyrene microtiter strips. The wells were coated overnight at 4 °C with 100 μL daidzein–OVA conjugate at different concentrations in carbonate buffer (0.05 mol L^{-1}, pH 9.6). After being washed three times with 350 μL PBST (0.01 mol L^{-1} PBS containing 0.05% (v/v) Tween 20), the wells were blocked with 200 μL of 5% (v/v) non-fat milk powder in 0.01 mol L^{-1} PBS for 2 h at 37 °C. After being washed three times with PBST, the wells were incubated with 100 μL of 10-fold serially diluted antiserum or purified antibodies for 1 h at 37 °C. Then the wells were washed three times and 100 μL of 1:1000 diluted HRP-conjugated goat anti-rabbit IgG were added and incubated for 1 h at 37 °C. After being washed three times with PBST and twice with pure water, the wells were filled with 100 μL of 60 μg mL^{-1} TMB in DMSO dissolved in citrate phosphate buffer (pH 6.0) containing 450 ppm of H_2O_2. After reaction for 15 min at room temperature, 50 μL of 2 mol L^{-1} H_2SO_4 was added to stop the enzyme reaction. The absorbance of each well was determined with the Bio-Rad microplate reader at 450 nm.

2.5. Specificity tests and determination of daidzein in serum and urine by BA-ELISA

The specificity of the polyclonal antibody was investigated by testing with daidzein, puerarin, rutin, coumarin, 4-hydroxycoumarin and phenol. The wells of microtiter plates were coated with 100 μL of 1.0 mg L^{-1} daidzein–OVA and blocked in the same way as above. Standard solutions (0–100,000 mg L^{-1}, 50 μL per well) of one of the competitive compounds were added together with 50 μL of the 1:5000 diluted purified antibody solution and incubated for 1 h at 37 °C. Then the wells were washed three times and 100 μL of 1:1000 diluted biotinylated goat anti-rabbit IgG were added and incubated for 1 h at 37 °C. Subsequently the wells were washed three times and 100 μL of the 1:200 diluted avidin–HRP was added and incubated for 45 min at 37 °C. The following steps were similar to those of the titration level determination of antiserum (Section 2.4).

The recoveries of the assay were determined by spiking human serum and urine samples with known amount of daidzein and comparing with the spiked standard diluent controls.

2.6. Determination of daidzein in P. radix by BA-ELISA

P. radix was ground into powder and dried for 4 h at 50 °C. *P. radix* powder (10 g) was extracted with 300 ml methanol

in ultrasonic bath for 30 min. The supernatant liquid was separated from the solid phase by centrifugation for 10 min at a speed of 10,000 rpm. The solution was quantitatively transferred into a flask and evaporated under vaccum, and 1.239 g extract was obtained. *P. radix* extract (25 mg) was dissolved in 10 mL methanol, then it was diluted to a suitable concentration with 0.01 mol L^{-1} pH 7.4 PBS for determination by BA-ELISA.

2.7. Determination of daidzein in P. radix by HPLC

Satisfactory separation and determination of daidzein was obtained with a Diamonsil C_{18} (150 × 4.6 mm, i.d. 5 μm, Dikma, Beijing, China) connected with ZORBAX Ext-C_{18} guard column (12.5 × 2.1 mm, 4-Pack) at 25 °C eluted at a flow rate of 0.5 mL min^{-1}. The mobile phase was 50:50 (v/v) methanol:1% acetic acid. Absorbance was monitored at 250 nm. Each solution (20 μL) was injected for analysis.

3. Results and discussion

3.1. Daidzein–BSA derivative

Since daidzein is not capable of initiating an immune response itself, it needs to be conjugated with a protein to form a complete antigen. Covalent attachment of hapten (III) proceeded via the amino groups of lysine (ε-amino group) residues present in the BSA and yielded a very stable compound. MALDI MS enabled rapid characterization of the daidzein-BSA derivative. The difference between average molecular weight of the conjugate and standard BSA resulted in a mean hapten density of 9.8 per mole BSA.

3.2. Optimization of immunoassays

The optimal concentration for daidzein–OVA as the coating ligand was determined by coating daidzein–OVA in different concentrations. Fig. 2 showed the chequerboard titration results for the antiserum. The optimal concentration was found to be 1 μg mL^{-1}. Based on the definition of titer as the antiserum dilution required to bind 50% of a small, given amount of labeled antigen, the titers of the antiserum and purified antibody were found to be 1:20,000 and 1:30,000, respectively. The dilution of purified antibody used in the following study was 1:10,000, and the concentration was 1.81 μg mL^{-1}.

The 0.8% (v/v) gelatin in 0.01 mol L^{-1} PBS and 5% (v/v) non-fat milk powder in mol L^{-1} PBS were compared as blocking solutions. The milk powder in PBS showed better blocking effect because of its lower blank value, so it was selected as the blocking solution in the following experiments.

The reaction time of biotin and avidin was tested in order to improve the sensitivity. The reaction time of 20, 30, 45, and 60 min were investigated, respectively. Finally, the reaction time of biotin and avidin was selected to be 45 min because of the best sensitivity.

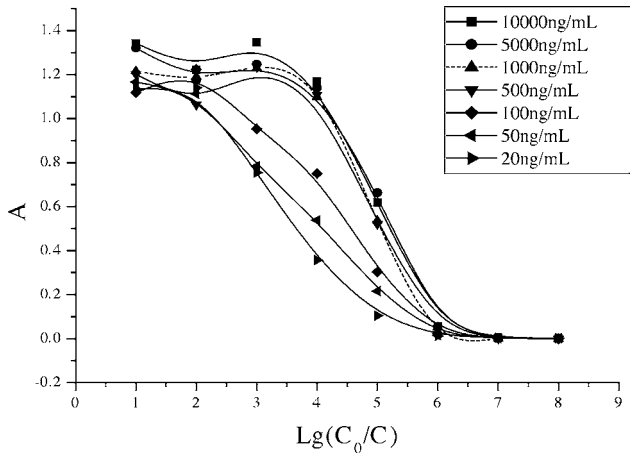

Fig. 2. Chequerboard titration results of the antiserum. The microtiter plate was coated with 100 μL of daidzein–OVA at different concentrations (20, 50, 100, 500, 1000, 5000, and 10,000 ng mL^{-1}) in 50 mmol L^{-1} pH 9.6 carbonate buffer. For each concentration level of the coated antigen, the antiserum was 10-fold serially diluted and added to the well. The titer was determined as the antiserum dilution corresponding to 50% of the maximum absorbance.

3.3. Analytical performance of BA-ELISA

3.3.1. Calibration graph

The logit-log algorithm was used to establish the linear regression [23]. The linear range was found to be between 0.1 and 1000 ng mL^{-1}. The equation was $\ln[A/(A_0 - A)] = 2.968 - 1.095 \log C_{daid}$ ($R^2 = 0.9899$, $n = 5$) (A, absorption value; A_0, the most absorption value; C_{daid}, concentrations of daidzein). The detection limit was 0.04 ng mL^{-1} [24]. The intra-assay and inter-assay coefficients of variation were 7 and 16%, respectively. The coefficients of variation were acceptable for ELISA method.

3.3.2. Specificity

To investigate the specificity of this antibody, three isoflavones (daidzein, puerarin, and rutin), two coumarins (coumarin and 4-hydrocoumarin), and phenol were used. The dose–response curves of the selected compounds are shown in Fig. 3. This clearly shows that with the increasing of the concentration of the competitive compounds, the antibody–antigen reaction was inhibited by the hapten (daidzein), while the other compounds had little influence on the reaction. According to the 50% replacement method [25], the cross reactions of the other five compounds were all lower than 1%. For the analysis of most actual samples such as human serum and urine samples, and *P. radix*, puerarin has just little disturbance for the determination of daidzein because the concentation of puerarin is always less than 100 times of that of daidzein. If the concentration of puerarin is more than 100 times of that of daidzein, puerarin would be a significant interferent.

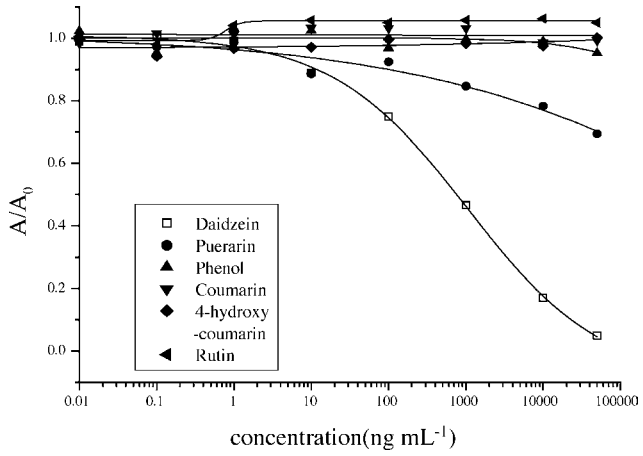

Fig. 3. Dose–response curves of the selected compounds based on BA-ELISA.

Table 1
Recovery of daidzein in the serum and urine samples by the biotin–avidin amplified enzyme-linked immunosorbent assay

Daidzein (pg per well)	Recovery (%)[a]	
	Serum samples	Urine samples
20	107 ± 6	104 ± 7
200	97 ± 4	102 ± 3
2000	91 ± 7	96 ± 5

[a] The recovery has been determined by spiking daidzein in human serum samples and healthy human serum samples and comparing with spiked standard dilution controls. The data were expressed as mean ± S.D. of 10 experiments.

Table 2
Analysis of daidzein in *P. radix* extract by the biotin–avidin amplified enzyme-linked immunosorbent assay

Dilution	Determined value[a] (ng mL^{-1})	Content[b] (%)	Average content[a] (%)
1:100	42.3 ± 2.4	0.0219 ± 0.0012	
1:1000	4.16 ± 0.12	0.0215 ± 0.0006	0.0219
1:10000	0.43 ± 0.03	0.0222 ± 0.0015	

[a] The data was expressed as mean ± S.D. of 10 experiments.
[b] The content was that of daidzein in *P. radix*.

3.3.3. Determination of daidzein in serum, urine and P. radix

For the serum samples, a 1:40 dilution with 0.01 mol L^{-1} pH 7.4 PBS was necessary prior to addition to the well to obtain quantitative recoveries. The urine samples were also diluted 10-fold with PBS in order to eliminate matrix effect. The recovery results are shown in Table 1.

Daidzein in *P. radix* extract was also determined by this established biotin–avidin amplified enzyme-linked immunosorbent assay. *P. radix* extract (25 mg) was dissolved in 10 mL methanol, and it was diluted to suitable concentration with 0.01 mol L^{-1} pH 7.4 PBS. The results are shown in Table 2.

3.4. Correlation with HPLC method

An HPLC method was applied as a reference method to determine daidzein in *P. radix*. The equation was $Y = -52.996 + 14.655X$ (R^2 = 0.99988, n = 3), and the linear range was between 0.575 to 57.4 μg mL^{-1}. The detection limit was 17 ng mL^{-1}. The content of daidzein in *P. radix* by HPLC method was found to be 0.0194%. For daidzein analysis in *P. radix*, the value (0.0219 ± 0.0011%) by BA-ELISA was similar with the value (0.0194 ± 0.0005%) by HPLC. Relate recovery (%) = [(content found with BA-ELISA)/(content found with HPLC)] × 100% = 113%.

Compared with the HPLC method, the BA-ELISA method has lower detection limit (0.04 ng mL^{-1}), and it offered the advantages of high sample turn over, small sample volume and the potential to be developed into test kits.

4. Conclusions

The described BA-ELISA was applied for the analysis of daidzein in serum, urine, and *P. radix*. It is more sensitive than reported ELISA methods [18–20], with a detection limit of 0.04 ng mL^{-1}. The precision and accuracy showed satisfactory performance. For analysis of daidzein in serum and urine, the recoveries were satisfactory. For the determination of daidzein in *P. radix*, the result obtained by the BA-ELISA method had good agreement to that obtained by the HPLC reference method. It showed that the specificity was excellent because little matrix effect was observed during the analysis of daidzein in complex traditional Chinese medicines. In conclusion, the presented BA-ELISA method is useful for the analysis of daidzein in biological samples and traditional Chinese medicine, especially for high throughput screening. It also provides a useful alternative to conventional chromatographic methods for daidzein analysis.

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