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The Measurement of the Isoflavone Daidzein by Time Resolved Fluorescent Immunoassay: a Method for Assessment of Dietary Soya Exposure

F. Kohen,[1]* S. Lichter,[1] B. Gayer,[1] J. DeBoever[2] and L. J. W. Lu[3]

[1]Department of Biological Regulation, The Weizmann Institute of Science, Rehovot 76100, Israel; [2]Department of Obstetrics and Gynaecology, University Hospital, De Pintelaan 185, B-9000 Ghent, Belgium and [3]Department of Preventive Medicine and Community Health, The University of Texas Medical Branch, Galveston, TX 77555-1110, U.S.A.

We report a novel method for the measurement of urinary daidzein that is suitable for assessment of dietary soya exposure. The method incorporates the following features: (i) a highly specific monoclonal antibody to daidzein (clone 4E4) raised through the 7 position of daidzein and (ii) a europium labeled ovalbumin daidzein conjugate. In the present format, dilute urine samples of subjects who ingested soy milk are hydrolyzed with β-glucuronidase for 30 min on rabbit anti-mouse coated plates. Afterwards, the specific monoclonal antibody to daidzein, clone 4E4, and europium labeled ovalbumin daidzein conjugate are added. After 1 h incubation, the wall bound fluorescence of europium is measured by time resolved fluorescence and is inversely proportional to the concentration of daidzein over the range 0.1–10 ng daidzein/well. The method demonstrates good sensitivity, precision and comparability with the chemical method GC-FID. Unlike the chemical method, the present immunoassay technique for daidzein is applicable for the measurement of large amounts of samples in epidemiological studies for the assessment and monitoring of human exposure to soya food.

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INTRODUCTION

There is substantial epidemiological evidence that dietary intake of soybeans is associated with an overall low cancer mortality rate of hormone dependent cancer in the Third World and Asian population. Soybeans contain several products (inositols, the Bowman-Birk protease inhibitor, phytosterols and isoflavones) that have been shown to suppress carcionogenesis *in vivo* [1,2]. The major isoflavones in soybeans are daidzein and genistein which have been reported to exhibit anti-estrogenic and anti-proliferative effects and to induce differentiation in a number of biological systems [3]. However, their anticancer mechanism in humans is unknown due in part to lack of sensitive and economical method to assess human exposure. Current analytical methods for assessing flavonoids rely on chromatographic procedures which require labor intensive sample preparation to carry out analysis, and therefore impractical for large scale monitoring of human exposure [4].

An alternative and economical method to monitor human exposure to isoflavones is to develop specific and sensitive non-isotopic methods where large amounts of samples can be processed reliably and fast. There have been some reports in the past on the preparation of polyclonal antibodies to the phytoestrogens [5–7]. To our knowledge there have been no papers on the generation of specific monoclonal antibodies to the phytoestrogens. Using the hybridoma technology we describe here the generation, characterization and use of a specific monoclonal antibody for the measurement of daidzein

*Correspondence to F. Kohen. Tel: 972 8 934 2763; Fax: 972 8 934 4116; e-mail: lhkohenf@weizmann.weizmann.ac.il.
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Abbreviations: daidzein: 4′,7-dihydroxyisoflavone; daidzin: 7-glucoside-4-hydroxyisoflavone; formononetin: 7-hydroxy-4-methoxy–isoflavone; equol: 4′,7-isoflavandiol; genistein: 4′,5,7-trihydroxyisoflavone; genistin: 4′,5-dihydroxy-7-glucoside–isoflavone.

levels in human urine after soya digestion and in soya products.

MATERIALS AND METHODS

Reagents

Isoflavones were purchased from Indofine Chemical (Somerville, NJ). Freund's adjuvant, pristane, β-glucuronidase (Type IX, G7396) and mouse antibody isotyping reagents were purchased from Sigma Chemical (St. Louis, MO). Sepharose-Protein A was purchased from Pharmacia (Uppsala). Keyhole Limpet Hemocyanin (KLH) was obtained from CalBiochem (La Jolla, CA). The labeling reagent, *N*-1(*p*-isothiocyanatophenyl) diethylenetriamine-N^1,N^2,N^3-tetraacetate (DTTA) chelated with Eu + + was kindly provided by Dr Ilkka Hemmilä (Wallac Oy, Turku, Finland).

Assay buffer and wash solution were prepared as described previously [8]. Enhancement solution was purchased from Gamidor (Savyon).

Preparation of 7-O-carboxymethyl ether daidzein

Sodium (0.21 g), cut into small pieces, was added to a round bottom flask containing *n*-propanol (8 ml). After the dissolution of sodium, daidzein (98 mg) was added. The reaction mixture was refluxed for 15' and bromoacetic acid (0.6 g) was subsequently added. A precipitate was formed immediately, and the color of the reaction changed gradually from yellow to brown. The reaction mixture was refluxed for 3 h, and cooled. The reaction was then stopped by acidification with 5 N HCl, and the reaction mixture was extracted into chloroform. The chloroform layer was then evaporated, and the residue was chromatographed on Silica gel 60. Elution with $CHCl_3$:methanol:acetic acid (89.7:10:0.3) yielded the 7-*O*-carboxymethyl ether derivative of daidzein (II) (20 mg), which exhibited an R_f of 0.5 in the system of $CHCl_3$:methanol:acetic acid (59.7:40:0.3). The ^1H NMR spectrum of this daidzein derivative in DMSO showed the following signals: δ: 8.34 (1H, s, 1-H), 7.96 (1H, d, $\mathcal{J}$ = 2 Hz, 5-H), 7.48 (2H, d, $\mathcal{J}$ = 1 Hz, 2'H and 6'H), 6.927 (3H, dd, 6-H, 3'H and 5'H), 6.88 (1H, d, $\mathcal{J}$=5 Hz, 8-H) and 4.6 (2H, s, O–CH_2–).

Preparation of macromolecular conjugates of daidzein

Carboxymethyl ether daidzein (II) was conjugated to KLH and ovalbumin via a two step reaction as described previously [9]. In the first step of the synthesis the reactive isoflavone derivative (compound II, 3.6 mg) was dissolved in dry dioxane (200 μl) and *N*-hydroxysuccinimide (1.9 mg) and carbodiimide (4 mg) were added to the reaction mixture. After an overnight reaction at room temperature, urea was formed and the supernatant was analyzed by TLC for the presence of the active ester, which showed an R_f of 0.95 in the solvent system of $CHCl_3$:MeOH:HOAc (74.5:25:0.25) for the *N*-hydroxy-succinimide ester derivative of daidzein. The active ester of daidzein derivative was then used in the next step without further purification. KLH (10 mg) was dialyzed twice against PBS, pH 8.0. To this solution (1 ml), 50 μl of 1 M sodium phosphate were added, followed by dropwise addition of the active ester (100 μl). The reaction mixture was stirred for 2 h at room temperature and then was dialyzed overnight against phosphate buffered saline, pH 7.4 (PBS) at 4°C and stored at −20°C until use. For the preparation of the daidzein ovalbumin conjugate, ovalbumin (10 mg) was dissolved in 1 ml of 50 mM sodium phosphate, pH 8 and 100 μl of the active ester of daidzein was added. The reaction mixture was stirred for 2 h at room temperature, dialyzed overnight against PBS and stored at −20°C until use.

Preparation of rabbit anti-mouse IgG-coated microtiter plates

Rabbit anti-mouse IgG was adsorbed at acidic pH onto the walls of microtiter plates as described previously [8].

Preparation of carboxymethyl ether daidzein europium labeled ovalbumin conjugate

Dissolve 1 mg of the europium-chelated labeling reagent (DTTA) into 500 μl of double-distilled water, and add 150 μl of this solution to the carboxymethyl ether ovalbumin conjugate of daidzein (2 mg) in 50 mM carbonate buffer (1 ml). Stir the reaction mixture overnight at 4°C, and purify the labeled protein by gel filtration on Sephadex G25M (eluent: 50 mM Tris–HCl buffer, pH 7.75).

Preparation of monoclonal anti-daidzein antibodies

The KLH conjugate of daidzein was used as an immunogen (50 μg/mouse) to immunize female CD2 mice (age: 2 months). Subsequently, two booster injections were given using the daidzein-KLH conjugate in incomplete Freund's adjuvant. After 2 months of immunization, the antibody titre was checked using rabbit anti-mouse IgG-coated plates, prepared as described previously [8], europium-labeled ovalbumin daidzein conjugate as a label, and time-resolved fluorescence as an end-point. Three months after the initial immunization, the spleen cells of two mice which showed high titre of antibodies to daidzein were fused with a mouse myeloma cell line (NSO, kindly provided by Dr Milstein, Cambridge) using the hybridoma technique of Köhler and Milstein [10], with the modifications introduced in our laboratory [11]. The culture supernatants of growing hybridomas were screened for antibody activity using rabbit anti-mouse IgG-coated plates and europium-labeled ovalbumin daidzein conjugate. Two hybridomas (clone #4E4 and Clone #2F11) secreting

antibodies against daidzein were selected. These hybdridoma cell lines were propagated *in vitro* as culture supernatant and *in vivo* as ascites in pristane primed mice. Both clones belonged to the IgG_1 class.

Characterization of antibodies

A competitive time-resolved fluorescence immunoassay method was used to detect specific antibodies to daidzein. Cross reactivity (%) was calculated as $(100x)/y$ where x is the mass of daidzein and y the mass of heterologous isoflavone required to produce 50% inhibition of the binding of europium labeled daidzein conjugate.

Immunoassay procedure

A time resolved fluorescence immunoassay was developed for the measurement of daidzein from urine. Urine from subjects who ingested soya food was diluted with assay buffer 1:100 and 1:1000 and a stock solution of β-glucuronidase of 5000 u/ml assay buffer was prepared. Diluted urine samples (10 μl) were then hydrolyzed with β-glucuronidase (20 μl) for 30 min on anti-mouse IgG coated plates. Anti-daidzein antibody (10 μl/well, 100 ng), and europium labeled ovalbumin daidzein conjugate (10^6 cps, 100 μl) were added to the wells containing hydrolyzed urine samples and to the wells containing daidzein standards in 100 μl of buffer. Buffer (70 μl) was added to the wells containing the samples in order to adjust the volume of the reaction to 200 μl. The plates were incubated for 1 h at room temperature, washed and processed for time resolved fluorescence as described previously [12]. The daidzein concentration of each well was calculated directly from the curve.

Urine sample collection and analysis of daidzein by gas chromatography

The urine samples used for validating time resolved fluorescence immunoassay have been previously analyzed by a gas-chromatography flame ionization detection method (GC-FID) as described [4]. The protocol used in this study has also been described [13].

RESULTS

Structure of antigen

Reaction of daidzein (I) with excess bromoacetic acid in the presence of sodium in *n*-propanol gave the 7-*O*-carboxymethyl ether derivative of daidzein (II) in 25% yield (Fig. 1). Support for the formation of 7-addition product was obtained by examination of the NMR spectrum of II and by comparing it to the published NMR spectrum of the 7-*O*-glucoside derivative of daidzein, namely daidzin [14]. Moreover, clone 4E4 did not react with formononetin, the 4'-methoxy

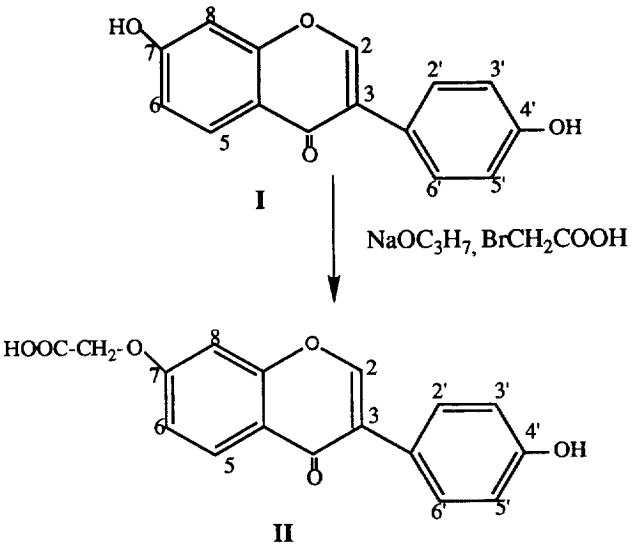

Fig. 1. Synthetic scheme for the preparation of the 7-carboxymethyl ether of daidzein.

derivative of daidzein (see Table 1), indicating that the site of conjugation of compound II was through the 7 position and not through the 4' position. In addition, one of the clones selected (2F11) recognized daidzin, the 7-glucoside derivative of daidzein, as well as daidzein (see Table 1), suggesting that the epitopes near the site of conjugation to KLH are masked. The present data indicates that the specificity of the antibodies are directed towards the ring containing the free 4'-OH position, therefore, the addition of the carboxymethyl group occurred through the 7-position of daidzein.

Characterization of monoclonal antibodies to daidzein

The culture supernatant derived from two hybridomas, 4E4 and 2F11, were further characterized for specificity by a competitive time resolved fluorescence immunoassay procedure. Clone 4E4 cross reacted 100% with daidzein, showed minimal cross-reaction (1–2%) with daidzin, dihydrodaidzein and genistein and showed almost no cross reaction with structurally related flavones and isoflavones (see Table 1). On the other hand, clone 2F11 recognized better daidzin, the 7-*O*-glycoside derivative of daidzein, than daidzein, reacted 100% with genistin, showed minimal cross reaction (2%) with dihydrodaidzein and genistein and showed almost no cross-reaction with related compounds (Table 1).

Calibration curves

Calibration curve for daidzein was generated using clone 4E4 as the specific antibody and for daidzin using clone 2F11 as the specific antibody and the conditions described in Section 2. Sensitivity, calculated as the least amount of daidzein or daidzin significantly different from zero at 95% confidence limits (i.e. mean for zero - 2SD) was 0.5 ng daidzein/

Table 1. Specificity of various monoclonal antibodies to daidzein as determined by time resolved fluorescence (FIA)

Compound	Cross-reaction (%), Clone #	
	4E4	2F11
Daidzein	100	100
Daidzin	2	200
Dihydrodaidzein	2	2
Genistein	1	2
Genistin	<0.01	100
Equol	<0.01	<0.01
Formonometin	<0.01	<0.01
Apigenin	<0.01	<0.01
Naringenin	<0.01	<0.01
7,4′-dihydroxyflavone	<0.01	<0.01
Quercetin dihydrate	<0.01	<0.01
Biochanin A	<0.01	<0.01
Heavy chain class:	IgG_1	IgG_1

ml [see Fig. 2(A)] and 0.25 ng daidzin/ml [see Fig. 2(B)].

Reliability of the assay

Within-assay and between-assay precision were estimated by measuring daidzein levels in urine samples of a subject on soya diet using as antibody clone 4E4 and the conditions described in Section 2. The within-assay and between-assay coefficient variation were 5–8% and 6–10%, respectively. The recovery of daidzein from soy-milk containing 1.68 μg daidzein/ml ranged from 80 to 90%. In order to check linearity, five urine samples containing daidzein concentrations between 5 to 15 μg/ml were first hydrolyzed and then diluted serially 1:50, 1:100 and 1:200, and the daidzein levels were measured using time resolved fluorescence. Assay of these 15 dilutions showed a linear correlation between the expected and the observed values for daidzein ($r = 0.94$).

Correlation with gas chromatography

Urinary levels of daidzein in one subject were determined during one month of daily soymilk ingestion on five separate occasions initially 2 days after being placed on a low soya diet (e.g. without prior soya exposure) and again after additional 12, 13, 25 and 26 days of 12 oz of soymilk per meal for three meals. In all instances daidzein excretion in urine exhibited time dependence as determined by a solid phase extraction followed by GC-FID [4, 13] and time resolved fluorometric immunoassay (FIA) using the monoclonal antibody to daidzein clone 4E5, and the conditions described in Section 2. The concentration of urinary daidzein in a total of 60 samples collected from this subject as determined by FIA (x) and as measured by the chemical method (y) showed good correlation characteristics. The linear regression equation was $y = 0.868x - 0.105$ with a correlation of $r = 0.924$. Figure 3 shows the time course of daidzein excretion in the urine of this subject after first soymilk ingestion as determined by FIA or GC-FID.

DISCUSSION

Daidzein is a major isoflavone in soya that has been shown to be anti-estrogenic and anti-proliferative [1]. It has also been reported that dietary intake of soybeans containing isoflavonoid phytoestrogens may contribute to the relatively low incidence of hormone dependent cancers in the Third World and Asian population. The ability to assess human exposure is critical in understanding anti-cancer mechanism of soya consumption. Current analytical methods for studying the intakes, bioavailability and metabolism of dietary isoflavonoids rely mainly on GC-MS (gas

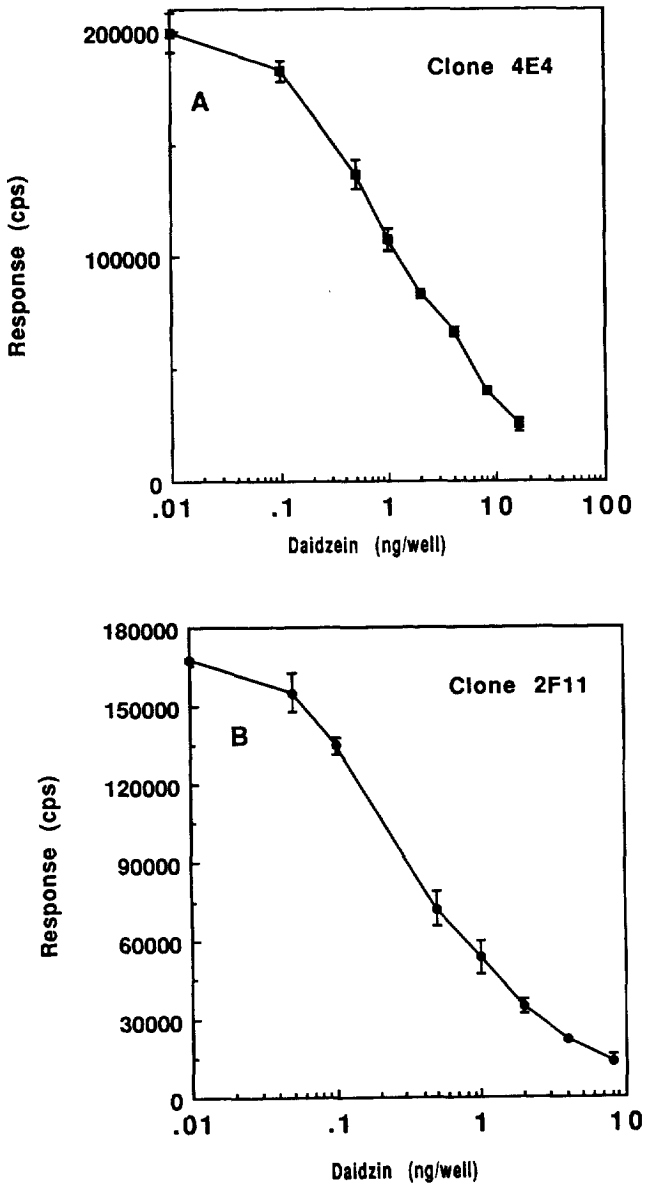

Fig. 2. Calibration curves for daidzein (A) and daidzin (B) using the conditions described in Section 2.

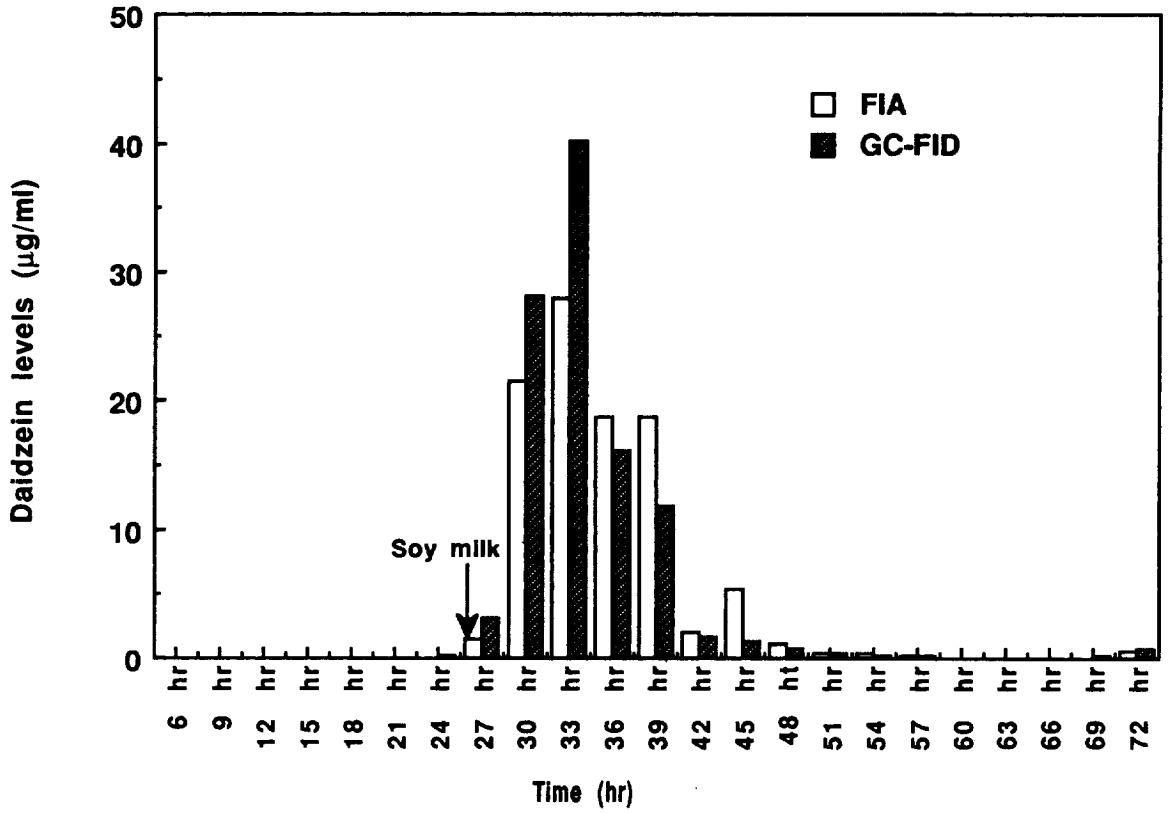

Fig. 3. The measurement of the time course of daidzein excretion in urine in a subject after first soymilk ingestion as determined by time resolved fluorescence immunoassay (FIA) and by GC-FID.

chromatography–mass spectrometry) or HPLC methods. The chemical methods require sample prepurification, followed by analysis by chromatography, and expensive instrumentation and are, therefore, not suitable for large scale screening of biological samples derived from epidemiological studies.

An alternative to the chemical methods are non-isotopic immunoassay techniques which are sensitive, fast and amenable to the measurement of large amounts of samples and are therefore, suitable for epidemiological studies. Indeed in a recent paper, a radioimmunoassay for daidzein using polyclonal antibodies to daidzein raised through position 4'O and an iodinated daidzein derivative has been described [7]. In this study we have reported the isolation and characterization of a highly specific antibody to daidzein (clone 4E4) and of a non-specific antibody (clone 2F11) (see Table 1). These hybridomas were derived from the fusion of the spleen cells of two mice immunized with 7-*O*-carboxymethyl daidzein KLH conjugate with mouse myeloma cells (NSO). Support for the addition of the reactive group bromoacetic acid to position 7 of daidzein was based mainly on the characterization of the specificity of the two clones 4E4 and 2F11. Clone 4E4 did not recognize equol (a metabolite of daidzein) and formonometin, the 4'-methoxy derivative of daidzein (see Table 1) and clone 2F11 recognized daidzin better than daidzein. All these data point out that addition of the carboxymethyl group occurred most probably through position 7 and not 4 since clone 4E4 does not bind the 4' derivative, formonometin.

In this study we have shown that there was a good correlation between the immunoassay method and the solid phase extraction method followed by gas chromatography. We have used equivalent of 1 to 10 μl of urine for the measurement of daidzein by immunoassay whereas the conventional method, e.g., GC-FID requires >1 ml of urine [4]. The immunoassay has a sensitivity of 0.5 ng/ml while GC-FID can measure 0.5 μg/ml urine. In addition, we have used a non-isotopic label. Thus, the present method is quite sensitive (0.5 ng/ml) and is amenable to the rapid measurement of daidzein from urine and is quite suitable for epidemiological studies. To our knowledge this is the first report on the preparation, characterization and use of a specific monoclonal antibody to the isoflavone daidzein.

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