Development of Bioluminescent Enzyme Immunoassay for *S*-Equol Using Firefly Luciferase and Its Application to the Assessment of Equol-Producer Status

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In this study, we developed a specific bioluminescent enzyme immunoassay (BLEIA) for *S*-equol, employing firefly luciferase as a labeling enzyme, as an alternative to HPLC methods. Satisfactory correlation (r=0.992) was shown when this *S*-equol BLEIA was compared with HPLC. The cross-reactivity with *R*-equol as its diastereoisomer is <5%, and that with daidzein, which is the substrate of equol, is 0.02%. Frequencies of Japanese equol producers determined using two distinct approaches were compared: a threshold value for urinary *S*-equol concentration of 232 ng/ml gave frequencies of 32% of men and 19% of women. These values correspond to the results for $\log_{10}$-transformed urinary *S*-equol to daidzein ratio threshold of −1.75, namely, 34% of men and 19% of women. When the changes in concentration of urinary equol and daidzein were measured after ingestion of isoflavone, the maximum concentration (C_{max}) of urinary equol appeared after 9.6 h of isoflavone consumption; this C_{max} was 2 h later than that for daidzein. The *S*-equol BLEIA documented in this study is expected to be an important tool for the assessment of equol producer status and demonstration of the bioavailability of isoflavone.

Key words *S*-equol; bioluminescent enzyme immunoassay; equol producer; firefly luciferase

Equol [7-hydroxy-3-(4′-hydroxyphenyl)-chroman] is a metabolite produced *in vivo* from the soy isoflavone daidzein by the action of enterobacteria. It is known to be estrogenic, so exposure to equol could have significant biological effects on humans. Epidemiological studies suggest that it might be beneficial in the prevention of many diseases, including breast cancer,[1] prostate cancer,[2,3] and osteoporosis.[4] The production of equol in humans varies: only 30—50% of any population group can produce equol after ingestion of soy foods, and they are called equol producers.[4] It is thought that certain bacteria in the intestinal microflora are greatly involved in equol bioconversion. Recently, bacteria that convert daidzein to equol were isolated from human feces (ex. *Lactocossus garvieae*).[5—7] A clinical trial intended to identify soy health benefits is taking place, using *S*-equol. Ishiwata *et al.* reported that *S*-equol supplement improved mood-related symptoms in premenopausal/postmenopausal equol non-producers.[8] Jackson *et al.* reported using pure synthetic *S*-equol for the clinical indications of vasomotor symptoms (VMS, hot flashes) in women and benign prostatic hyperplasia (BPH, enlarged prostate) in men, as well as osteoporosis.[9]

The major methods used today for the determination of equol are high-performance liquid chromatography (HPLC) and liquid chromatography-mass spectrometry (LC-MS).[10] These methods are useful for basic study that measures multiple molecules, such as equol, daidzein and genistein, and they show excellent specificity. However, these methods require sample extraction and have relatively low throughput, but the measurement of a large number of samples is necessary for epidemiological study to examine the evidence that the clinical efficacy of isoflavones in humans depends on the production of an enterobacterial metabolite, equol. As an alternative method, enzyme immunoassay is the most useful. Brouwers *et al.* reported a time-resolved fluoroimmunoassay (TR-FIA) method that uses rabbit polyclonal antibodies.[11] Talbot *et al.* reported successful development of monoclonal antibodies and applied them to a TR-FIA method.[12] As a result, a large number of samples have become measurable.

Equol, unlike the soy isoflavones daidzein and genistein, has a chiral center, and therefore it can occur as two distinct diastereoisomers, *R*-equol and *S*-equol. When equol is chemically synthesized, it is the (±) equol that is usually obtained. *S*-Equol has a high affinity for estrogen receptor β (K_i= 0.73±0.2 nmol/l), whereas *R*-equol is relatively inactive (K_i=15.4±1.3 nmol/l). The exclusive product of human enterobacterial synthesis from soy isoflavones is *S*-equol.[13] Therefore, a measurement system that can specifically measure *S*-equol is anticipated in clinical research. However, conventional methods have drawbacks with regard to their specificity for *S*-equol.

The measurement of urine samples is known to be influenced by the matrix effect. The extraction of equol from urine reduces this influence, but it results in relatively low throughput. Dilution of sample for measurement reduces the influence of the matrix effect, but it causes a decrease of sensitivity. Therefore, in this study, we overcame this decrease of sensitivity by developing a bioluminescent enzyme immunoassay (BLEIA) using firefly luciferase as a labeling enzyme, with a bridge heterogeneous combination of antiserum and enzyme-labeled antigen. BLEIA, with firefly luciferase used as a labeling enzyme, has a high sensitivity because firefly luciferin-luciferase bioluminescence has high quantum yield (41.0±7.4%).[14] The different bridge haptenic derivatives used for the immunogen and the enzyme-labeled antigen (bridge heterogeneous combination) are more sensitive

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than a homologous combination.[15)]

Thus, we report the development of an assay system specific for *S*-equol using firefly luciferase as a labeling enzyme, namely, a bridge heterogeneous immunoassay method.

Experimental

Materials *R*,*S*-Equol was obtained from LC Laboratories (Woburn, MA, U.S.A.). *S*-Equol was obtained from Toronto Research Chemicals Inc. (Ontario, Canada). β-Glucuronidase Type III from Ampullaria was obtained from Nippon Biotest Laboratories Inc. (Tokyo Japan). Charcoal stripped human serum was purchased from Scantibodies Laboratory, Inc. (Santee, CA, U.S.A.).

Daidzein, Genistein and adenosine-5′-monophosphate (AMP) were purchased from Sigma (St. Louis, MO, U.S.A.). Streptavidin (SA) was procured from MP Biomedicals, LLC (Solon, Ohio, OH, U.S.A.). Ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid disodium salt dehydrate (EDTA·2Na) and 2-morpholinoethanesulfonic acid monohydrate (MES) were obtained from Dojindo Laboratories (Kumamoto, Japan). Goat anti-rabbit immunoglobulin G (IgG) was prepared in our laboratory. Magnetic particles (Dynabeads M280, Tosyl-activated) were obtained from Invitrogen Corp. (Carlsbad, CA, U.S.A.). Biotinylated luciferase (bL248) was supplied by Kikkoman (Chiba, Japan). Other chemicals were of analytical reagent grade.

Subjects A total of 68 men and women between the ages of 26 to 53 years (mean±S.D.: 41±7 years) participated in this study. The selected subjects were in good health and had typical Japanese dietary habits. They fasted for 12 h and the dietary record prior to fasting was not taken. Bioavailability of equol was investigated after ingestion of SOYJOY® Raisin Almond (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan), containing 19 mg of isoflavone/30 g bar. Urine samples were collected before and after consumption of isoflavone. The curves for urinary daidzein and equol were constructed and postprandial maximum concentration (C_{max}) was determined. The study protocol was reviewed and approved by the Human Investigation Review Committee of Eiken Chemical Co., Ltd.

Preparation of *R*-Equol and *S*-Equol The preparations of *R*-equol and *S*-equol using β-cyclodextrin stationary phase liquid chromatography column under reversed-phase conditions were based on the method of Muthyala *et al.*[16)] Two milligrams of *R*,*S*-equol was dissolved in 1 ml of 0.1% formic acid (aq)/acetonitrile (65 : 35). Then, aliquots were applied to a CYCLOBOND I2000 RSP β-cyclodextrin-*R*,*S*-hydroxypropyl ether chiral stationary phase (column 250 mm×4.6 mm, 5 μm particle size, Astec, NJ, U.S.A.) and eluted with 0.1% formic acid (aq)/acetonitrile (65 : 35). Injection volume was 20 μl, and separations were performed at ambient temperature. The flow rate was 0.75 ml/min and the UV spectra of the peaks were recorded at 280 nm. Excellent separation (Rs=2.4) of the two enantiometric forms of racemic equol was obtained under this condition. Peak 1 (retention time, 19.7 min) and peak 2 (retention time, 21.8 min) represented *S*-equol and *R*-equol, respectively. These were used for evaluation of cross-reactivity.

Synthesis Labeling Antigen (*S*-Equol-carboxypropylether (CPE)-SA-bL) In the presence of 14 mg of potassium carbonate, 10 mg of *S*-equol was dissolved in 40 μl of *N*,*N*′-dimethyl formamide (DMF), 5.8 μl of 4-bromo-*n*-butyric acid (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) was added, and the reaction mixture was stirred for 4 h at 25 °C. The pH of this solution was set below 2.0 using 6 mol/l hydrochloric acid. The mixture was completely extracted three times with ethyl acetate. Then, after 3 ml of distilled water (DW) was added, the *S*-equol-CPE present in the aqueous solution was completely washed. The organic phase was dried over anhydrous sodium sulfate. Thin-layer chromatography (TLC) of the organic phase developed with 5% methanol/chloroform as the solvent indicated the appearance of monocarboxypropyl ether and dicarboxypropyl ether spots in addition to the spots at the starting points. The starting material and dicarboxypropyl ether were removed using this TLC. Then, 500 μl of methanol was added; this solution was saponified using 78 μl of 8 mol/l sodium hydroxide. The reaction mixture was stirred for 20 min at 50 °C and 3 ml of DW was added. The pH of this solution was set below 2.0 using 6 mol/l hydrochloric acid. The mixtures were completely extracted three times with ethyl acetate and then washed three times with DW. The organic phase was dried over anhydrous sodium sulfate. *S*-Equol-CPE was recrystallized from benzene. *S*-Equol-CPE (2.8 mg) was added to 28 μl of dimethyl sulfoxide (DMSO). Then, 2.1 mg of *N*-hydroxysuccinimide (NHS) and 2.1 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, hydrochloride (EDC) were added, and the reaction mixture was stirred at 25 °C for 60 min. The *S*-equol-CPE-NHS was extracted with ethyl acetate after addition of DW, and the organic phase was dried over anhydrous sodium sulfate. The solvent was then evaporated at reduced pressure, and 0.96 mg/ml of the residue was incubated in 14.46 mg/ml streptavidin (SA) and 0.05 mmol/l carbonate bicarbonate buffer (pH 9.6) for 30 min at room temperature. The crude *S*-equol-CPE-SA conjugate was then purified on a Sephadex G-25 fine gel filtration column (GE Healthcare U.K. Ltd., Little Chalfont, England). The fractions containing SA were collected. A 25 μl aliquot of *S*-equol-CPE-SA solution (10.2 mg/ml) was added to 70 μl of 8.24 mg/ml bL248 and 8.26 ml of conjugate storage buffer (0.1 mol/l phosphate buffer (PB), 0.4 mol/l sodium chloride, 2 mmol/l EDTA·2Na, 5 mmol/l AMP, 0.2% bovine serum albumin (BSA), 0.02% casein and 0.05% sodium azide). The solution was incubated at 25 °C for 60 min. This conjugate solution (*S*-equol-CPE-SA-bL) was diluted to 100 nmol/l with conjugate storage buffer and stored at 4 °C.

Synthesis of Immunogens (*R*,*S*-Equol-carboxymethylether (CME)-BSA) *R*,*S*-Equol-carboxymethylether (CME)-BSA was synthesized using the same method except for a change of SA-bL to BSA and the following reaction of derivative synthesis by a method similar to that for *S*-equol-CPE-SA-bL. *R*,*S*-Equol-CME was prepared from bromoacetic acid methyl ester (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan). In the presence of 225 mg of potassium carbonate, 150 mg of *R*,*S*-equol dissolved in 600 μl of DMSO and 60 μl of bromoacetic acid methyl ester was added, and the reaction mixture was stirred for 2.5 h at 25 °C.

Production of Polyclonal Antibodies Immunogen was prepared by diluting *R*,*S*-equol-CME-BSA conjugate with an equal volume of saline. Five rabbits were subcutaneously administered with the *R*,*S*-equol-CME-BSA conjugate (500 μg per rabbit) emulsified in complete Freund's adjuvant at 3-week intervals. A total of nine immunizations were given to each rabbit. Sera were collected using a standard procedure. The cross-reactivity, IC_{20}, was tested with anti-equol antiserum.

The cross-reactivity were calculated using the following formula with the result of a 20% inhibition test (IC_{20}): cross-reactivity (%)=(ng/ml of compound at IC_{20})/(ng/ml of cross-reacting *S*-equol at IC_{20})×100.

Preparation of Goat Anti-rabbit IgG-Immobilized Magnetic Particles The goat anti-rabbit IgG was immobilized on magnetic particles (Dynabeads M280, Tosyl-activated). The suspension of magnetic particles (4 ml×100 mg/ml) was washed four times with 20 ml of distilled water. Then, the suspension of magnetic particles was washed two times with 20 ml of 0.05% Tween 20 in 0.1 mol/l PB, pH 7.4. Additionally, the magnetic particles were washed two times with 20 ml of 0.1 mol/l carbonate buffer (pH 10.0). The magnetic particles were re-suspended with 20 ml of 0.1 mol/l carbonate buffer (pH 10.0) containing goat anti-rabbit IgG (0.1 mg/ml), followed by mixing at 37 °C for 18 h. After removal of the supernatant, 0.5% Lipidure 206 (NOF Corp., Tokyo, Japan) was added and mixed at 37 °C for 60 min. The suspension was washed with 20 ml of 0.1 mol/l PB (pH 7.5), after which it was washed with 20 ml of 0.01% TritonX-100 in 1 mol/l sodium chloride, followed by mixing at 37 °C for 30 min. Upon completion, the suspension was washed four times with 20 ml of bead buffer (0.05 mmol/l MES, 0.15 mol/l sodium chloride, 0.5% Purebright (NOF Corp., Tokyo, Japan), pH 6.0). The suspension containing goat anti-rabbit IgG-immobilized magnetic particles was diluted to 2 mg/ml with bead buffer and stored at 4 °C until use.

Competitive Bioluminescent Enzyme Immunoassay (BLEIA) for *S*-Equol This assay method was based on the utility of firefly luciferase as a labeling enzyme and a competitive immunoassay procedure. Each urine specimen was diluted 11-fold with diluents (charcoal stripped human serum) as a dilute sample. Following transfer of 20 μl of the dilute sample to a polystyrene test tube (12×75 mm), 50 μl of deconjugation buffer (3% β-glucuronidase in 0.1 mol/l acetate buffer, pH 5.5) was added to the test tube and mixed for 2 s. The mixture was incubated at 25 °C for 30 min. After the incubation, 50 μl of *S*-equol-CPE-SA-bL, 20 μl of goat anti-rabbit IgG-immobilized magnetic particles, and 50 μl of anti-equol rabbit antiserum were added to the mixture and incubated at 25 °C for 15 min, then washed with 0.5 ml of 0.05% Tween 20 in PBS (repeated four times). Luciferin-luciferase reaction and luminescence measurement were performed as reported previously.[17)] Most of the equol in human urine exists as a conjugate, such as glucuronide, sulfoglucuronide, and sulfate.[18)] Therefore, the result is shown in ng/ml, equivalent to deconjugated aglicon.

Competitive Enzyme-Linked Immunosorbent Assay (ELISA) for Daidzein To each well of goat anti-rabbit IgG-immobilized plate, 20 μl of standard (11—3300 ng/ml) in charcoal stripped human serum or urine specimen, diluted 11-fold with diluents (charcoal stripped human serum), was added. Then, 50 μl of daidzein-labeled horseradish peroxidase (HRP) in deconjugation buffer (6% β-glucuronidase in 0.1 mol/l acetate buffer, pH 5.5) was added and mixed well. The mixture was incubated for 30 min at 25 °C. Additionally, 50 μl of anti-daidzein rabbit antiserum in 0.1 mol/l phosphate

buffer, 0.15 mol/l sodium chloride, 0.5% bovine serum albumin, and 0.01% Tween 20 were added and mixed well. The mixture was then incubated for 1 h at 25 °C. After incubation, the plate was washed with 350 μl of 0.025% Tween 20 in 0.3 mmol/l phosphate buffer and 100 mmol/l sodium chloride, pH 7.5 (repeated four times). After washing, 100 μl of ELISA substrate solution (0.05% hydrogen peroxide, 2.2 mg/ml *o*-phenylenediamine, 50 mmol/l citrate buffer, pH 4.1) was added to each well and the mixture was allowed to react for 30 min at 25 °C. The enzymatic reaction was stopped with 100 μl of 1.5 mol/l sulfuric acid. The color intensities of the wells were determined using a spectrophotometer at 492 nm.

HPLC Determinations HPLC analysis was done using a modified protocol of Franke's methods at SRL, Inc. (Tokyo, Japan).[19]

Results and Discussion

Development of BLEIA for *S*-Equol Upon determination of the standard curve for *S*-equol BLEIA in accordance with the aforementioned protocol, a measurable range of 110 to 8910 ng/ml was evident, displaying good linearity. Intra-assay coefficients of variation (CV) for 565—3679 ng/ml equol in human urine were within the range of 3.3—4.4%, $n=10$. The inter-assay CV of 592—3543 ng/ml equol in human urine ranged between 9.4—10.4%, $n=10$. The recovery test was performed *via* addition of one volume of antigen to four volumes of urine. Recovery was 102±3—129±4% (mean±S.D., $n=3$) in all samples. This *S*-equol was found to show cross-reactivity to *R*-equol of 4.83%, and dehydroequol and dihydrodaidzein, which are metabolic intermediates of equol, showed values of 0.40% and 0.21%, respectively. Cross-reactivity of daidzein, which is a substrate of equol, was only 0.02%. Other similar molecules such as estrogen showed less than 0.03% cross-reactivity (Table 1). Equol concentration, which was measured by employing the current BLEIA method, was compared with that determined with HPLC. Satisfactory correlation ($r=0.992$, $y=1.07x-303$, $n=22$) was evident. Therefore, basic validation of the immunoassay was achieved; intra- and inter-assay reproduction and correlation coefficient were excellent.

Equol Producer Status of Japanese Population We examined the equol producer status of the Japanese population using *S*-equol BLEIA. The result of equol producer status by *S*-equol BLEIA was compared to the equol to daidzein ratio. Urine specimens from 68 Japanese subjects, who had fasted for 12 h, but without the dietary record prior to fasting being taken, were analyzed by *S*-equol BLEIA and daidzein ELISA (Figs. 1a, b). The over-the-range specimens were re-measured after dilution. This data was grouped by gender and in ascending order of equol concentration. The ratio of equol to daidzein was calculated, transformed, and expressed as $\log_{10}$.[20]

The approach to define equol producers as those with an urinary equol concentration >232 ng/ml (960 nmol/l)[12] corresponds to another approach that defines equol producers as those with $\log_{10}$ urinary equol to daidzein concentration ratio >-1.75.[21] Within the limitations of the small sample size of this study, the results of classification of male volunteers were as follows. On the basis of their urinary equol concentration, 32% (15 of 47) of the male volunteers were classified as equol producers. On the basis of the $\log_{10}$ urinary equol to daidzein concentration ratio, the proportion of equol producers among male volunteers was 34% (16 of 47). On the other hand, the proportion of female volunteers classified as equol producers was 19% (4 of 21) in both classification methods, namely, urinary equol concentration and $\log_{10}$ urinary equol to daidzein concentration ratio. There was good agreement in the classification of equol producers between the two approaches: that is, 94% (44 of 47) of male and 90% (19 of 21) of female subjects were assigned the same classification. In terms of the frequency of equol producers in this study, male (32% or 34%) were higher than female (19%). This result corresponds to the reports of Morton *et al.* (58% of men and 38% of women with a serum equol concentration >20 nmol/l)[21] and Setchell and Cole (65% of men, 28% of women).[20] However, the results from our study are generally lower than those observed by Morton *et al.*[21] and Setchell and Cole,[20] even though the samples in both studies were collected from Japanese subjects. The difference in results is thought to be due to a variation of the subject population.

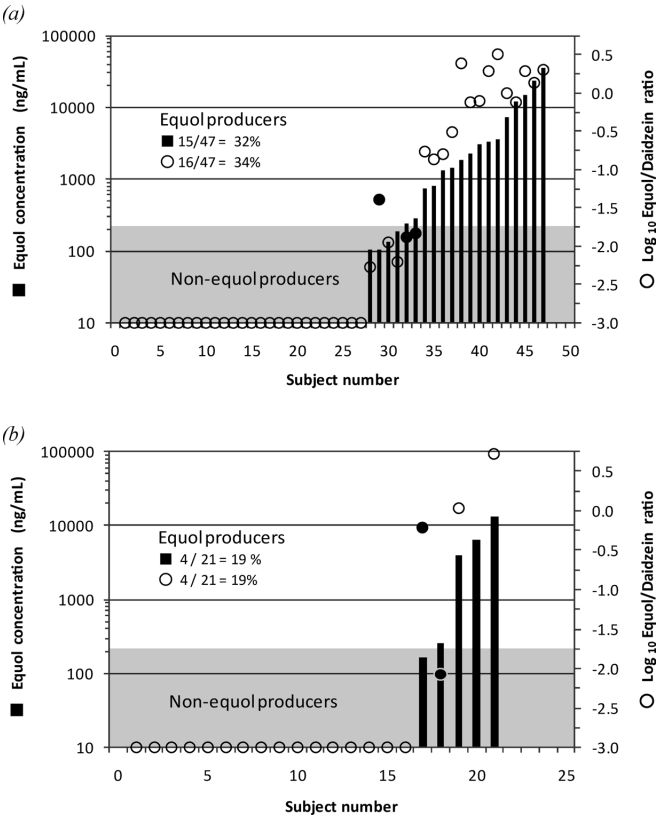

Fig. 1. Equol Producer Status of Japanese Men (a) and Women (b) Based on Two Approaches

Urinary equol concentrations plotted for the individual subjects are shown with bars. Log_{10}-transformed urinary equol to daidzein ratios for individual subjects are shown with circles. Equol producers were defined by two approaches: those with a $\log_{10}$ urinary equol to daidzein concentration ratio of >-1.75[20] and those with a urinary equol concentration of >232 ng/ml (960 nmol/l).[12] The subjects that gave different results by the two approaches are shown with closed circles.

Postprandial Urine Concentration of Equol and Daidzein after Intake of Isoflavone To demonstrate the bioavailability of daidzein to equol, the urinary equol and daidzein concentration changes were measured after ingestion of isoflavone (Fig. 2). Among the volunteers, a man aged 33 years was selected for his urinary equol concentration of more than 1000 ng/ml. The urinary daidzein concentration increased rapidly 7.7 h after isoflavone consumption. Thereafter, the urine concentration of daidzein decreased until 18.4 h. The urine C_{max} for daidzein after the consumption of isoflavone was 4704 ng/ml. On the other hand, there was a rapid increase in equol concentration in urine at 9.6 h after

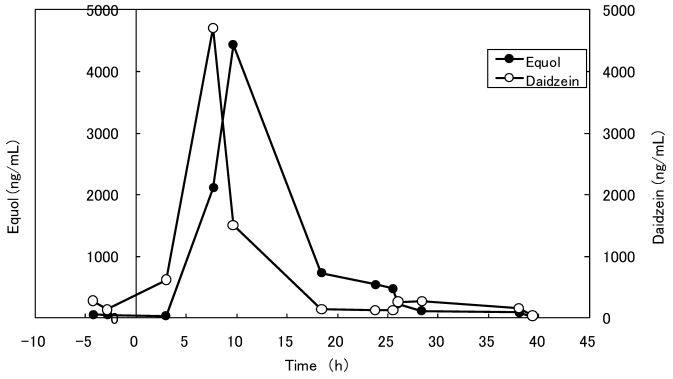

Fig. 2. Postprandial Equol Concentration in Urine after Intake of a Single Bar of SOYJOY (19 mg of Isoflavone)

Table 1. Cross-Reactivity of the Polyclonal Antibody for Equol Based on *S*-Equol BLEIA

Cross-reactivity with	Cross-reactivity
S-Equol	100.00%
R-Equol	4.83%
Dehydroequol	0.40%
Dihydrodaidzein	0.21%
Daidzein	0.02%
Daidzin	0.01%
Genistein	0.02%
Genistin	<0.01%
Glycitein	<0.01%
Glycitin	<0.01%
E2	<0.01%
BiochaninA	0.03%
Formononetin	0.02%
Apigenin	<0.01%
Luteolin	<0.01%

isoflavone consumption. As shown in Fig. 2, the urine concentration of equol decreased until 28.4 h. The urine C_{max} for equol after the consumption of isoflavone was 4433 ng/ml. As such, the time when equol reached C_{max} was 2 h later than that of daidzein. In addition, equol was still detectable for another 10 h after daidzein became undetectable. This delay is thought to be due to the time during which daidzein is metabolized to equol by enterobacteria.[22)]

Conclusion

We developed BLEIA for *S*-equol and applied it to investigate the equol producer status of Japanese subjects and its urine concentration after intake of isoflavone. When this *S*-equol BLEIA was compared with HPLC, satisfactory correlation (r=0.992) was observed. Additionally, this assay method is very convenient because it does not require extraction, and it can be completed in about 1 h. The cross-reactivity with other isoflavones is low, with that for *R*-equol, which is a diastereoisomer that does not exist as a metabolite of daidzein is <5%, and that for daidzein, which is a substrate of equol is 0.02%. The determination of equol producer status of Japanese subjects by this *S*-equol BLEIA corresponded to that using the ratio of equol to daidzein. When urinary equol and daidzein concentration changes were measured after ingestion of isoflavone the time difference between when equol (9.6 h) and when daidzein reached C_{max} (7.7 h) was 2 h.

In this study, we developed *S*-equol BLEIA that can be used to measure *S*-equol specifically. The *S*-equol BLEIA documented here is expected to be an important tool to assess equol producer status and demonstrate bioavailability.

References

1) Duncan A. M., Merz-Demlow B. E., Xu X., Phipps W. R., Kurzer M. S., *Cancer Epidemiol. Biomarkers Prev.*, **9**, 581—586 (2000).
2) Akaza H., Miyanaga N., Takashima N., Naito S., Hirao Y., Tsukamoto T., Mori M., *Jpn. J. Clin. Oncol.*, **32**, 296—300 (2002).
3) Ozasa K., Nakao M., Watanabe Y., Hayashi K., Miki T., Mikami K., Mori M., Sakauchi F., Washio M., Ito Y., Suzuki K., Wakai K., Tamakoshi A., *Cancer Sci.*, **95**, 65—71 (2004).
4) Setchell K. D., Brown N. M., Lydeking-Olsen E., *J. Nutr.*, **132**, 3577—3584 (2002).
5) Uchiyama S., Ueno T., Suzuki T., *J. Intest. Microbiol.*, **21**, 212—220 (2007).
6) Yokoyama S., Suzuki T., *Biosci. Biotechnol. Biochem.*, **72**, 2660—2666 (2008).
7) Maruo T., Sakamoto M., Ito C., Toda T., Benno Y., *Int. J. Syst. Evol. Microbiol.*, **58**, 1221—1227 (2008).
8) Ishiwata N., Melby M. K., Mizuno S., Watanabe S., *Menopause*, **16**, 141—148 (2009).
9) Jackson R. L., Schwen R., Merchenthaler I., 8th International Symposium on the Role of Soy in Health Promotion and Chronic Disease Prevention and Treatment, Tokyo, November 9—12, 2008.
10) Lundh T. J., Pettersson H., Kiessling K. H., *J. Assoc. Off. Anal. Chem.*, **71**, 938—941 (1988).
11) Brouwers E., L'homme R., Al-Maharik N., Lapcík O., Hampl R., Wähälä K., Mikola H., Adlercreutz H., *J. Steroid Biochem. Mol. Biol.*, **84**, 577—588 (2003).
12) Talbot D. C., Ogborne R. M., Dadd T., Adlercreutz H., Barnard G., Bugel S., Kohen F., Marlin S., Piron J., Cassidy A., Powell J., *Clin. Chem.*, **53**, 748—756 (2007).
13) Setchell K. D., Clerici C., Lephart E. D., Cole S. J., Heenan C., Castellani D., Wolfe B. E., Nechemias-Zimmer L., Brown N. M., Lund T. D., Handa R. J., Heubi J. E., *Am. J. Clin. Nutr.*, **81**, 1072—1079 (2005).
14) Ando Y., Niwa K., Yamada N., Nomoto E. T., Irie T., Kubota H., Ohmiya H., Akiyama H., *Nature Photon.*, **2**, 44—47 (2008).
15) Hosoda H., Kawamura N., Nambara T., *Chem. Pharm. Bull.*, **29**,1969—1974 (1981).
16) Muthyala R. S., Ju Y. H., Sheng S., Williams L. D., Doerge D. R., Katzenellenbogen B. S., Helferich W. G., Katzenellenbogen J. A., *Bioorg. Med. Chem.*, **12**, 1559—1567 (2004).
17) Minekawa T., Ohkuma H., Abe K., Maekawa H., Arakawa H., *Luminescence*, **24**, 394—399 (2009).
18) Adlercreutz H., van der Wildt J., Kinzel J., Attalla H., Wähälä K., Mäkelä T., Hase T., Fotsis T., *J. Steroid Biochem. Mol. Biol.*, **52**, 97—103 (1995).
19) Franke A. A., Custer L. J., *J. Chromatogr. B Biomed. Appl.*, **662**, 47—60 (1994).
20) Setchell K. D., Cole S. J., *J. Nutr.*, **136**, 2188—2193 (2006).
21) Morton M. S., Arisaka O., Miyake N., Morgan L. D., Evans B. A., *J. Nutr.*, **132**, 3168—3171 (2002).
22) Zubik L., Meydani M., *Am. J. Clin. Nutr.*, **77**, 1459—1465 (2003).