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 log10-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.5) 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. Lactobacillus garrigueae).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.9) 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 \(( \pm \))-equol that is usually obtained. \( S \)-Equol has a high affinity for estrogen receptor \( \beta \) \((K_i = 0.73 \pm 0.2 \text{ nmol/l})\), whereas \( R \)-equol is relatively inactive \((K_i = 15.4 \pm 1.3 \text{ nmol/l})\). 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 \((41.0 \pm 7.4\%)\).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 immun Assay 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. (San
tee, 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), 5 mmol/l AMP, 0.2% bovine serum albumin (BSA), and 0.02% casein and 0.05% sodium azide. The solution was incubated at 25 °C for 60 min. This conjugate solution ($S$-equol-SA-bL) was diluted to 100 nmol/l with conjugate storage buffer and stored at 4 °C.

Synthesis of Immunogens ($R,S$-Equol-carboxymethylthylether (CME)-BSA)  $R,S$-Equol-carboxymethylthylether (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-CEA-

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 ng/ml) was washed four times with 20 ml of distilled water. Then, the suspension of magnetic particles was washed twice with 20 ml of 0.05% Tween 20 in 0.1 mol/l PB at pH 7.4. Additionally, the magnetic particles were washed twice 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 ng/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% Puraprev (NOF Corp., Tokyo, Japan), pH 6.0). The suspension containing derivative synthesis by a method similar to that for $S$-equol-CEA-

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 poly-styrene 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-CEA-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.17,18 Most of the equol in human urine exists as a conjugate, such as glucuro

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 de-

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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 reproducibility 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}.

The approach to define equol producers as those with an urinary equol concentration >232 ng/ml (960 nmol/l) corresponds to another approach that defines equol producers as those with log_{10} equol urinary equol to daidzein concentration ratio > −1.75. 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)3(3) 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.

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...
isoflavone consumption. As shown in Fig. 2, the urine concentration of equol decreased until 28.4 h. The urine concentration for equol after the consumption of isoflavone was 4433 ng/ml. As such, the time when equol reached $C_{\text{max}}$ 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 $C_{\text{max}}$ (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