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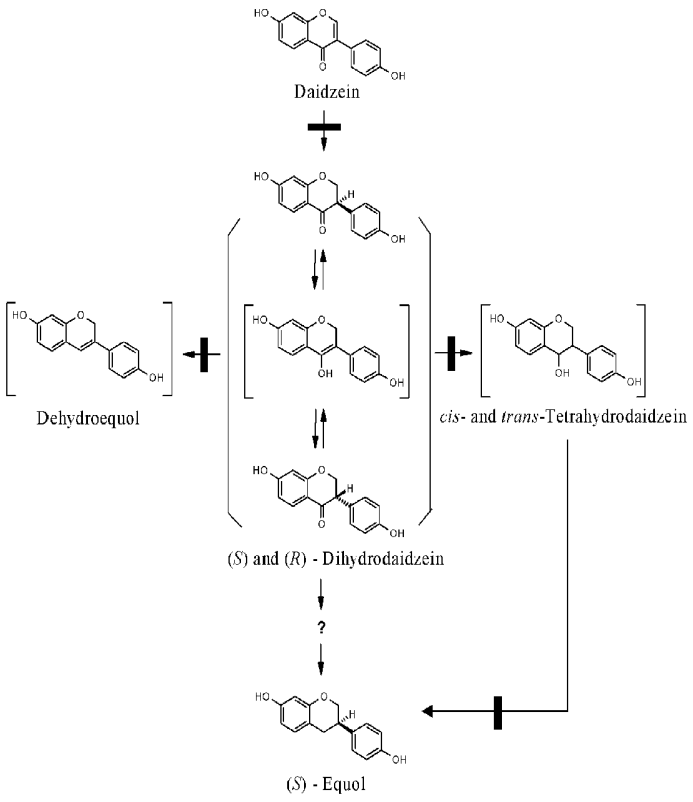
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[Continued on next page]

(54) Title: NOVEL MICROORGANISM CAPABLE OF METABOLIZING DIHYDRODAIDZEIN TO EQUOL



(57) Abstract: The present invention provides a novel microorganism capable of metabolizing dihy- drodaidzein into equol. More particularly, the invention provides Eggerthella sp. microorganism capable of metabolizing dihydrodaidzein into equol under anaerobic conditions. The novel microorganism according to the invention is capable of metabolizing DHD into equol and it thus enables the production of equol from DHD. Also, compositions comprising the microorganism and DHD can be used for prevention or treatment of climacteric diseases especially, osteoporosis, and they can be used as antioxidants, anticancer agents, antimutagens, etc.

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Description

NOVEL MICROORGANISM CAPABLE OF METABOLIZING DIHYDRODAIDZEIN TO EQUOL

Technical Field

[1] The present invention relates to a novel microorganism capable of metabolizing dihydrodaidzein into equol. More particularly, the invention relates to novel *Eggerthella* sp. microorganism capable of metabolizing dihydrodaidzein into equol under anaerobic conditions.

Background Art

[2] Isoflavones have been known to have various physiological activities including anticancer (Adlercreutz, H. et al., *Lance*t 339:1233, 1992; Hirano, T. et al., *Life Sci.* 55:1061-1069, 1994), antimutation (Hartman, P. E., and D. M. Shankel. *Environ. Mol. Mutagen.* 15:145-18, 1990), antioxidation (Jha, H. C. et al., *Biochem. Pharmacol.* 34:1367-1369, 1985) and antiproliferation of tumor cells (Hirano, T. et al., *Res. Commun. Chem. Pathol. Pharmacol.* 64:69-78, 1989). Thus, recently, interests in isoflavone ingestion are increasing. The major components of isoflavones are daidzein, genistein and their glycosides, i.e., daidzin and genistin and universally exist in leguminous plants, for example, soybeans.

[3] Generally, isoflavones contained in foods exist in their glycoside forms where sugar is coupled and isoflavones ingested via diet are metabolized by intestinal microorganisms before absorption. According to Joannou, G. E. et al., *J. Steroid Biochem. Molec. Bio*l. 54:167-184, 1995, it is suggested that daidzein is metabolized into equol via two estimated intermediates such as dihydrodaidzein and tetrahydrodaidzein or alternatively, equol is induced from dehydroequol which is another available metabolite of tetrahydrodaidzein or daidzein (see FIG. 1). This suggestion is supported by the fact that dihydrodaidzein, tetrahydrodaidzein, equol and O-desmethylangolensin are detected as metabolites of daidzein in human urines (Chang, Y. C., and M. G. Nair. *J. Natr. Proc.* 58:1892-1896, 1995; Joannou, G. E. et al., *J. Steroid Biochem. Molec. Biol.* 54:167-18, 1995; Kelly, G. E. et al., *Clin. Chim. Acta.* 223:9-12, 1993).

[4] Furthermore, the isoflavones metabolized via above pathways have been known to have higher activities than those existing in foods. Researches proposed that equol has much stronger antioxidation activities than genistein or daidzein which is contained in high amount in soybeans. Accordingly, in order to increase physiological activities of isoflavones, the roles of intestinal microorganisms have become important.

[5] It was reported that equol, which has been known as being most effective among

the isoflavone metabolites, is produced from the culturing of plant proteins and human fetal bacteria (Setchell, K. D. R. et al., *Am. J. Clin. Nutr.* 40:569-578, 1984). Besides, it was disclosed that daidzein is metabolized into dihydrodaidzein (DHD) and equol and genistein is metabolized into dihydrogenistein by culturing daidzein and genistein individually with human fetal bacteria under anaerobic conditions (Chang, Y. C., and M. G. Nair. *J. Natr. Proc.* 58:1892-1896, 1995).

Disclosure of Invention

Technical Problem

[6] Therefore, it is an object of the present invention to provide a microorganism capable of metabolizing dihydrodaidzein into equol.

[7] Also, it is another object of the invention to provide a method of producing equol, using the microorganism.

[8] Also, it is another object of the invention to provide a composition comprising the microorganism and isoflavone.

Technical Solution

[9] In order to achieve the aforementioned objects, the present invention provides a microorganism capable of metabolizing dihydrodaidzein into equol.

[10] Also, the invention provides a method of producing equol, using the microorganism.

[11] Also, the invention provides a composition comprising the microorganism and isoflavone.

[12] The inventors have completed the invention by isolating a novel strain having capacity of metabolizing dihydrodaidzein into equol from human excretion as a result of screening strains being capable of producing equol from isoflavones, and identifying it.

Advantageous Effects

[13] The novel microorganism of the invention is capable of metabolizing DHD into equol and it thus enables the production of equol from DHD. Also, compositions comprising the microorganism and DHD can be used for prevention or treatment of climacteric diseases especially, osteoporosis, and they can be used as antioxidants, anticancer agents, antimutagens, etc.

Brief Description of the Drawings

[14] FIG. 1 shows metabolic pathway from daidzein into equol in humans.

[15] FIG. 2 shows HPLC analysis results of the microorganism incubated in media containing dihydrodaidzein

[16] FIG. 3 shows EI-MS analysis results of the metabolites of dihydrodaidzein produced by the microorganism of the present invention.

[17] FIG. 4 shows CSP HPLC analysis results of equol biosynthesized from dihydrodaidzein by the microorganism of the present invention.

[18] FIG. 5 shows CD analysis results of equol biosynthesized from dihydrodaidzein by the microorganism of the present invention.

[19] FIG. 6 is a hypothetic metabolic pathway from dihydrodaidzein to equol by the microorganism of the present invention.

Mode for the Invention

[20] The invention is further described in detail.

[21] The microorganism of the invention is a strain capable of metabolizing dihydrodaidzein (DHD) into equol, isolated from human excretion.

[22] The isolation and identification of the microorganism according to the invention were carried out by the following procedures. After human excretion was diluted sequentially, it was incubated on agar plates and about 1000 colonies were thus obtained. In order to select colonies capable of metabolizing daidzein or DHD from the above colonies, each colony was incubated anaerobically in media containing daidzein or DHD and analyzed using HPLC. As a result, a peak which seems to be a metabolite of DHD was detected in one of the microorganisms incubated in media containing DHD. The product regarded as metabolite of DHD was identified as equol by EI-MS and ^{1}H and ^{13}C NMR analysis (see Examples 2 and 3).

[23] The microorganism isolated above was identified as *Eggerthella* sp. by the analysis of the nucleotide sequences of its 16S rDNA (ribosomal DNA) and named SNU2-Ren2H1. The above microorganism was deposited under deposition number KCCM-10490 with Korean Culture Center of Microorganisms, which is an international depository under Budapest treaty, on April 24, 2003.

[24] SNU2-Ren2H1 (KCCM-10490) according to the present invention has the following properties.

[25] First, microorganism SNU2-Ren2H1 according to the invention is capable of metabolizing DHD into equol. More particularly, the microorganism according to the invention is characterized by producing S-type equol only from DHD under anaerobic conditions (see Example 5).

[26] Second, microorganism SNU2-Ren2H1 according to the invention is not capable of metabolizing daidzein into equol. In an embodiment of the invention, after the microorganism of the invention was incubated in growth media containing daidzein, metabolic products were analyzed. As a result, no peaks which were assumed to be metabolites of daidzein were detected (see Example 2). From this experiment, it was verified that the microorganism of the invention has no capacity of metabolizing daidzein into equol.

[27] Third, the microorganism according to the invention does not produce tetrahydrodaidzein and dehydroequol which are intermediate metabolites in the metabolism of daidzein into equol (see Examples 2 and 3).

[28] Fourth, the microorganism according to the invention does not exhibit stereoselectivity for R-DHD or S-DHD. In other words, the microorganism of the invention is capable of metabolizing both R and S-type DHD into S-type equol.

[29] Based on the above properties, as to the metabolic pathway of DHD into equol by the microorganism of the invention, it was hypothesized that the microorganism produces a stereoselective Racemase for conversion of R-type DHD into S-type DHD, thereby converting DHD into its S-type, which is further metabolized into S-type equol via unknown intermediates by enzymes capable of metabolizing S-type DHD into S-type equol (see FIG. 6).

[30] The microorganism capable of metabolizing DHD into equol according to the present invention can be used to produce equol. Accordingly, the invention provides a method of producing equol comprising inoculating microorganism SNU2-Ren2H1(KCCM-10490) of the invention into media containing dihydrodaidzein and incubating it under anaerobic conditions.

[31] The media used above is not limited to specific ones and it refers to conventional culture media containing suitable carbon sources, nitrogen sources, amino acids and/or vitamins. For the carbon sources, there can be used glucose, molasses, lactose, sucrose, maltose, dextrin, starch, mannitol, sorbitol or glycerol. Preferably, glucose or molasses is used. For the nitrogen sources, there can be used inorganic nitrogen sources such as ammonia, ammonium chloride and ammonium sulfate, or organic nitrogen sources such as peptone, NZ-amine, beef extract, yeast extract, corn steep liquor, casein hydrolysate, fish or its decomposition products and dehydrated soybean cake or its decomposition products. Preferably, yeast extract or corn steep liquor is used. For the inorganic compounds, there can be used kalium monohydrogen phosphate, kalium dihydrogen phosphate, magnesium sulfate, ferrous sulfate, manganese sulfate or calcium carbonate, and if necessary, vitamins or auxotrophic bases can be further added. As examples of commercially available, non-specific growth media, there can be mentioned BHI, TSB, Bacto Cooked Meat Medium, and Schaedler Anaerobe Broth. The BHI media was used in the Examples of the invention.

[32] The incubation of the microorganism according to the invention can be carried out under anaerobic conditions, preferably, 5 ~ 10 % CO_2, 5 ~ 15 % H_2 and 75 ~ 90 % N_2 and more preferably, 5 % CO_2, 10 % H_2 and 85 % N_2 at 37 ℃ for 24 hours.

[33] Besides, for the incubation of the microorganism according to the invention, it is preferable that media has an initial pH of 6.0 ~ 8.0 and preferably 6.0 ~ 7.0.

[34] After incubation, equol can be isolated from the culture media and purified using

conventional methods known in the pertinent art. For example, membrane filtration, fractional precipitation, crystallization or column chromatography can be used. The isolated and purified equol can be further processed into dietary or medicinal forms.

[35] Further, the invention provides a pharmaceutical or dietary composition comprising the microorganism of the invention and DHD. The microorganism can be added thereto in the form of its lyophilized strain, or this lyophilized strain can be added after treatment with a suitable coating agent. The DHD can be chemically synthesized using methods known in the pertinent art or can be obtained from daidzein by microorganisms.

[36] The composition of the invention can further comprise elements accelerating maintenance or proliferation of microorganisms. For example, it can further comprise galactosylsucrose, soybean-oligosaccharide, lactulose, lactitol or fructo-oligosaccharide.

[37] The composition of the invention can be formulated and administered by various dosage forms and methods. This can be prepared by mixing an effective amount of the microorganism of the invention and DHD with carriers which are conventionally employed in pharmaceutical or dietary fields. For the carriers, for example, there are binders, lubricants, disintegrants, excipients, solubilizers, dispersants, stabilizers, suspension agents, pigments or flavors. The composition of the invention can be formulated into various dosage forms according to conventional methods. Dose can be suitably adjusted according to absorption rate, deactivation rate and excretion rate of an active component in body, age, gender and conditions of patients, and seriousness of diseases to be cured.

[38] The composition of the invention can be provided as a pharmaceutical composition in the form of solutions, emulsions, granules, powders, capsules, or tablets.

[39] When the composition of the invention is manufactured in the form of foods, it can be formulated in the form of drinks, dairy products, fermented milks, powders, capsules or tablets. In processing the composition of the invention, it would be preferable to avoid heating or pressurization because it contains microorganism.

[40] The composition of the invention, if necessary, can further comprise various kinds of additives exhibiting nutrition supply effects. For example, there can be vitamins, zinc or selenium.

[41] The composition of the invention can be used for prevention or treatment of climacteric diseases especially, osteoporosis, and it can be also used as antioxidants, anticancer agents, antimutagens, etc.

[42] The invention is further described by way of Examples in detail. It should be understood, however, that the following examples are intended to illustrate the invention more fully without limiting the scope of the invention.

[43]

[44] EXAMPLE 1

[45] Chemical Synthesis of Dihydrodaidzein

[46] Dihydrodaidzein (DHD), which is used as a substrate to select microorganisms having activities of metabolizing isoflavone into equol, was chemically synthesized.

[47] Chemical synthesis of DHD was carried out by modifying known catalyst transition hydrogenation (Krishnamurty and Sathyanarayana, *Synth. Commu.* 16:1657-1663, 1986). 1 mM daidzein (Indofine Co.) dissolved in 20 ml methanol was added to round-bottom flask containing 0.255 g of 10% Pd/C (Aldrich Co.) and 0.252 g of ammonium sulfate (Junsei, Co.). The reaction mixture was refluxed at 65 ℃ for 2 hours. The solutions were monitored with HPLC to see the production of DHD. After the reaction was complete, the reaction mixtures were cooled and filtrated with 0.45 □ filter to remove Pd/C. Thereafter, they were concentrated using a sample concentrator (SpeedVac AES1010, ThermoSavant, New York, N.Y.). The obtained concentrates were dissolved in methanol and purified with Sephadex LH20 (Amersham Pharmacia Biotech, Amersham, U.K.). The specimens purified by LH20 column were concentrated using sample concentrator (SpeedVac AES1010). The obtained concentrates were re-dissolved in 100% methanol and isolated using Thermo Prep-HPLC (ThermoSeparation Products). The reaction products were analyzed using EI-MS and NMR spectrometers. EI-MS was measured using JMS-AX50510A Mass Spectrometer (JEOL, Co. Ltd. Japan) at positive mode (EI^+). Source temperature was 250 ℃ and ionization voltage was 70 eV. Electron multiplier of 1.2 Kv was used. ^1H and ^{13}C NMR spectrums at acetone-d_6 were measured at 400 MHz using Jnm-La NMR Spectrometer (JEOL, Co. Ltd. Japan) at temperature of 296 K. For ^1H NMR analysis, 16 transients were measured along with 3 seconds relaxation delay using 32 K data point. 45 ℃ Pulse was 5.6 seconds and had an area of 400 MHz. ^{13}C NMR was carried out over an area of 100 MHz while collecting 64K data point. 45 ℃ Pulse was 5 seconds. Every calculation was conducted on silicon graphic INDY R4400 work station using MSI software (San Diego, LA).

[48] As a result, daidzein was converted into DHD at conversion rate of 71% by catalyst transition hydrogenation. EI-MS and ^1H and ^{13}C NMR spectrums were the same as the spectrums of DHD which had been previously reported (Chang, Y. C., and M. G. Nair. *J. Natr. Proc.* 58:1892-1896, 1995; Wahala, K., A. Salakka, and H. Adlercreutz. *Proc. Soc. Exp. Biol. Med.* 217:293-29, 1998). As predicted, EI-MS of the synthesized compound showed molecular ion peak of $[M+H]^+$ corresponding to $C_{15}H_{12}O_4$ (DHD) at m/z 256. Other ion peaks of the synthesized compound were 137(100), 120(52), 91(36), 65(16). NMR data of the chemically-synthesized DHD are as follows:

[49] ^1H-NMR ($(CD_3)_2CO$, 400 MHz):δ7.33(1H, d, J=8.8Hz, H-5), 7.12(2H, d, J=8.5Hz,

H-2',6'), 6.78(2H, d, J=8.5Hz, H-3',5'), 6.57(1H, dd, J=8.8Hz, 2.2Hz, H-6), 6.39(1H, d, J=2.2Hz, H-8), 4.61(2H, m, H-2), 3.84(1H, m, H-3).

[50] ^{13}C-NMR ($(CD_3)_2CO$, 100 MHz): 191.0(C-4), 164.9(C-8a), 164.3(C-7), 157.4(C-4'), 130.5(C-2', 6'), 130.0(C-5), 127.8(C-1'), 116.0(C-3', 5'), 115.2(C-4a), 111.2(C-6), 103.3(C-8), 72.5(C-2), 51.6(C-3).

[51]

[52] EXAMPLE 2

[53] Isolation of Microorganism Capable of Metabolizing DHD into Equol

[54] Human excretion specimens obtained from healthy females were collected in 5 ml BHI liquid media which was covered with 2 ml of sterilized mineral oil. The excretion specimens in the BHI liquid media were diluted sequentially from 10^{-1} to 10^{-8}. 100 □of each diluted solution was smeared on BHI agar plate and incubated in an anaerobic chamber (5 % CO_2, 10 % H_2 and 85 % N_2) at 37 ℃ for 24 hours. Colonies more than 1000 were isolated and stored on new BHI agar plates.

[55] In order to select positive pools having capacity of metabolizing DHD into equol, 100 pools containing 10 single colonies randomly gathered were incubated in BHI liquid media each containing 100 □ of daidzein or DHD which was chemically synthe sized in Example 1 under anaerobic conditions for 5 days. The culture media (200 □) was extracted with 1□ of ethyl acetate. Then, it was concentrated using automatic environment SpeedVac AES1010 (ThermoSavant, New York, N.Y.). The concentrates were dissolved in 200 □ of acetonitriles and 10 □ of the obtained solution was analyzed with HPLC.

[56] Analysis HPLC profiles were measured using Varian ProStar HPLC(Varian, Walnut Creek, C.A.) equipped with Waters ODS C18 columns (particle size 5 □, 4.6 × 250 mm, Fullerton, C.A.) and PDA (photo diode array) detector. For mobile phase, there were used 10% acetonitrile solution (A) and 90% acetonitrile solution (B) buffered with 0.1% acetic acid. Elution programs were as follows. 30% of B solution was eluted for 15 min. and increased in a straight line to 50%, which was then eluted for 10 min. and increased in a straight line to 70%, which was then eluted for 10 min. Flow rate was As a result, a peak which presumptively seemed to be a major metabolite of DHD was detected at 16.3 min. in one of the microorganisms incubated in media containing DHD (FIG. 2), and the metabolite of DHD showed maximum absorbance at 280 nm. In case of the microorganisms incubated in media containing daidzein, however, there were no peaks regarded as metabolites of daidzein.

[57]

[58] EXAMPLE 3

[59] Analysis of Metabolite of DHD by Isolated Microorganism

[60] The microorganism isolated in Example 2 was incubated in a 500 □-flask containing

200 □ of BHI liquid media for 2 days. On the second day, DHD stock solution (solution of 40 mM concentration dissolved in N,N-dimethylformamide) prepared in Example 1 was added to the culture media until its final concentration became 0.8 mM. The incubation of the microorganism was carried out in an anaerobic chamber (5 % CO_2, 10 % H_2 and 85 % N_2) at 37 ℃ successively for one week.

[61] After the incubation was complete, the culture media was extracted three times with an equal volume of ethyl acetate and concentrated with an evaporator (Eyela New Rotary Vacuum Evaporator NE, Company, Tokyo, Japan). The dried metabolite was re-dissolved in 100% methanol and purified using Recycling Preparative LC-918 (Anal. Ind. Co., Tokyo, Japan). The purified metabolite was identified by EI-MS and ^{1}H and ^{13}C NMR analysis. EI-MS and ^{1}H and ^{13}C NMR analysis was carried out by the same methods as used in Example 1.

[62] As a result, EI-MS spectrum of the metabolite of DHD produced by the microorganism showed molecular ion peak of [M+H]+ at m/z 242, which corresponds to molecular formula of equol ($C_{15}H_{14}O_3$) (FIG. 3). Other ion peaks were 120(100), 123(82), 135(27), 107(28), 91(14), which correspond to EI-MS spectrum analysis results of equol that had been previously known (Alda, J. O. et al., *Biochem. Biophys. Res. Commu.* 221:279-28, 1996). Accordingly, it was verified that the metabolite of DHD produced by the microorganism of the invention was equol.

[63] ^{1}H and ^{13}C NMR analysis results with regard to the metabolite of DHD produced by the microorganism of the invention were the same as the analysis results of equol that had been previously reported (Adlercreutz, H. et al., *Clin. Chim. Acta.* 158:147-154, 1986; Alda, J. O. et al., *Biochem. Biophys. Res. Commu.* 221:279-285, 1996; Chang, Y. C., and M. G. Nair. *J. Natr. Proc.* 58:1892-1896, 1995). Accordingly, it was verified that the metabolite of DHD produced by the microorganism of the invention was equol.

[64] ^{1}H-NMR($(CD_3)_2CO$, 400MHz): 2.91(m, 2H, H-4), 3.06(m, 1H, H-3), 3.92(m, 1H, H-2), 4.17(m, 1H, H-2), 6.27(d, 1H, J=2.4 Hz, H-8), 6.35(dd, 1H, J=8.3Hz, H-5), 6.82(d, 2H, J=8.5Hz, H-3'), 7.14(d, 2H, J=8.5Hz, H-6'), 8.08(s, 1H, OH), 8.22 (s, 1H, OH).

[65] ^{13}C-NMR ($(CD_3)_2CO$, 100MHz): 32.65(C-4), 38.79(C-3), 71.57(C-2), 103.59(C-8), 108.77(C-6), 114.04(C-4a), 116.20(C-3',5'), 129.21(C-2',6'), 130.99(C-5), 133.42(C-1'), 155.97(C-8a), 157.13(C-7), 157.51(C-4').

[66]

[67] EXAMPLE 4

[68] Identification of the Isolated Microorganism

[69] The microorganism which was verified to have biotransformation ability of converting DHD into equol in Example 3 was identified by analysis of 16S rDNA gene

sequences. The microorganism was inoculated into 5 ml of BHI liquid media and incubated in an anaerobic chamber for 2 days. 1 □ of the culture media was centrifuged at 15,000 × g for 5 min. After the supernatant was removed, 30 □ of NaOH(0.05 N) was added to the tube. In order to extract genomic DNA, the tube was placed in boiling water for 15 min. 16S rDNA from chromosomal DNA of the microorganism was amplified by PCR using primers 27F(AGAGTTTGATCMTGGCTCAG, SEQ. ID. NO. 1) and 1492R(GGYTACCTTGTTACGACTT, SEQ. ID. NO. 2). In the above primer sequences, M refers to A or C and Y refers to T/U or C. The PCR program for amplification was conducted as follows. The reaction was carried out at 94 ℃ for 5 min., the cycle of 94 ℃ for 1 min., 55 ℃ for 30 sec. and 72 ℃ for 1 min and 30 sec. was repeated 29 times and then, the final elongation was carried out at 72 ℃ for 10 min. The PCR products amplified from the genomic DNA were purified using Accu Prep gel purification kit (Accu. Chem. Sci. Corp. Westbury, N.Y.). The PCR products of 16S rDNA were ligated and transformed using ToPo-cloning kit (Invitrogen, Carlsbad, C.A.). The obtained nucleotide sequences (1449 bp) were analyzed using ABI 377 Xl Upgrade DNA Sequencer (Perkin Elmer, Boston, M.A.) and program supplied by the manufacturers. The sequences of 16S rDNA analyzed above were subject to homology test with BLAST.

[70] As a result, the microorganism of the invention showed 94% homology with Eggerthella sp. Microorganisms. 16S rDNA gene (1449 bp) sequence of the above microorganism was registered with GenBank (Reg. No. AY310748, SEQ. ID. NO. 3), named SNU2-Ren2H1, and deposited under deposition number KCCM-10490 with Korean Culture Center of Microorganisms (KCCM).

[71]

[72] EXAMPLE 5

[73] Analysis of Isomer of Equol Biosynthesized by Strain SNU2-Ren2H1 of the Invention

[74] Isomers of equol biosynthesized by the microorganism of the invention in above Example 3 were analyzed. Equol is an isomeric compound because it has an asymmetric carbon at C-3 position of isoflavone carbon chains. The above biosynthesized equol was dissolved in ethanol and then used as specimen. For analysis of enantiomers of equol, the specimen was analyzed with chiral stationary-phase HPLC (CSP HPLC) (Sumi Chiral OA-7000, particle size 5 □, 4.6 × 250 nm, Sumika Chem. Osaka, Japan). For mobile phase, 40% acetonitrile dissolved in 20 mM potassium phosphate buffer (pH 3.0) was used. Isocratic elution program was carried out for 35 min. using the mobile phase, flow rate was adjusted to 1 □/min, and UV spectrums of peaks were recorded within ranges of 200 to 400 nm. Specific rotation of the specimen was measured using Polarimeter Autopol (Rudolph, N. J.). CD (Circular

dichroism) spectrums were measured using Jasco J-715 Spectrometer (Jasco, Corp. Tokyo, Japan) and melting point was measured using TA gauge (DSC Q-1000, City, D.E.). DSC (Differential scanning calorimeter) analysis was carried out in the range of from -70 ℃ to 300 ℃ at heating rate of 10 ℃/min.

[75] As a result of analysis of equol biosynthesized in Example 3 with chiral stationary-phase HPLC, one peak was detected (FIG. 4). Specific rotation of the biosynthesized equol is $[\alpha]D^{24} = -23.0°$ It was known that $[\alpha]_D$ value of S-form equol is negative ($[\alpha]_{548}$ = -21.5° at chloroform) (Buckingham, J., Dictionary of Natural Products, 1st ed. Chapman and Hall, London, 1994). Accordingly, it was determined that the equol biosynthesized by strain SNU2-Ren2H1 of the invention was S-type. CD spectrums of S-type equol dissolved in ethanol exhibited positive and negative cotton effects in the information range of 200 300 nm (FIG. 5). S-type equol exhibited high positive cotton effects at 237 nm and negative cotton effects at 285 nm. Melting point of S-type equol was 190.8 ℃. This was similar to the melting point of S-type equol isolated from heartwoods of *Millettia pendula*, which was reported as 189 ~ 190.5 ℃. Accordingly, it was verified that all of the equol biosynthesized by the strain of the invention, SNU2-Ren2H1, was S-type equol.

Industrial Applicability

[76] The novel microorganism of the invention is capable of metabolizing DHD into equol and it thus enables the production of equol from DHD. Also, compositions comprising the microorganism and DHD can be used for prevention or treatment of climacteric diseases especially, osteoporosis, and they can be used as antioxidants, anticancer agents, antimutagens, etc.

Sequence Listing

[77] Related seqence list was added as apendix file.

Claims

[1] An *Eggerthella* sp. microorganism capable of metabolizing dihydrodaidzein into equol (Deposition Number KCCM-10490).

[2] The microorganism of claim 1 wherein the equol is S-type.

[3] A method of producing equol comprising inoculating the microorganism of claim 1 into media containing dihydrodaidzein and incubating it under anaerobic conditions.

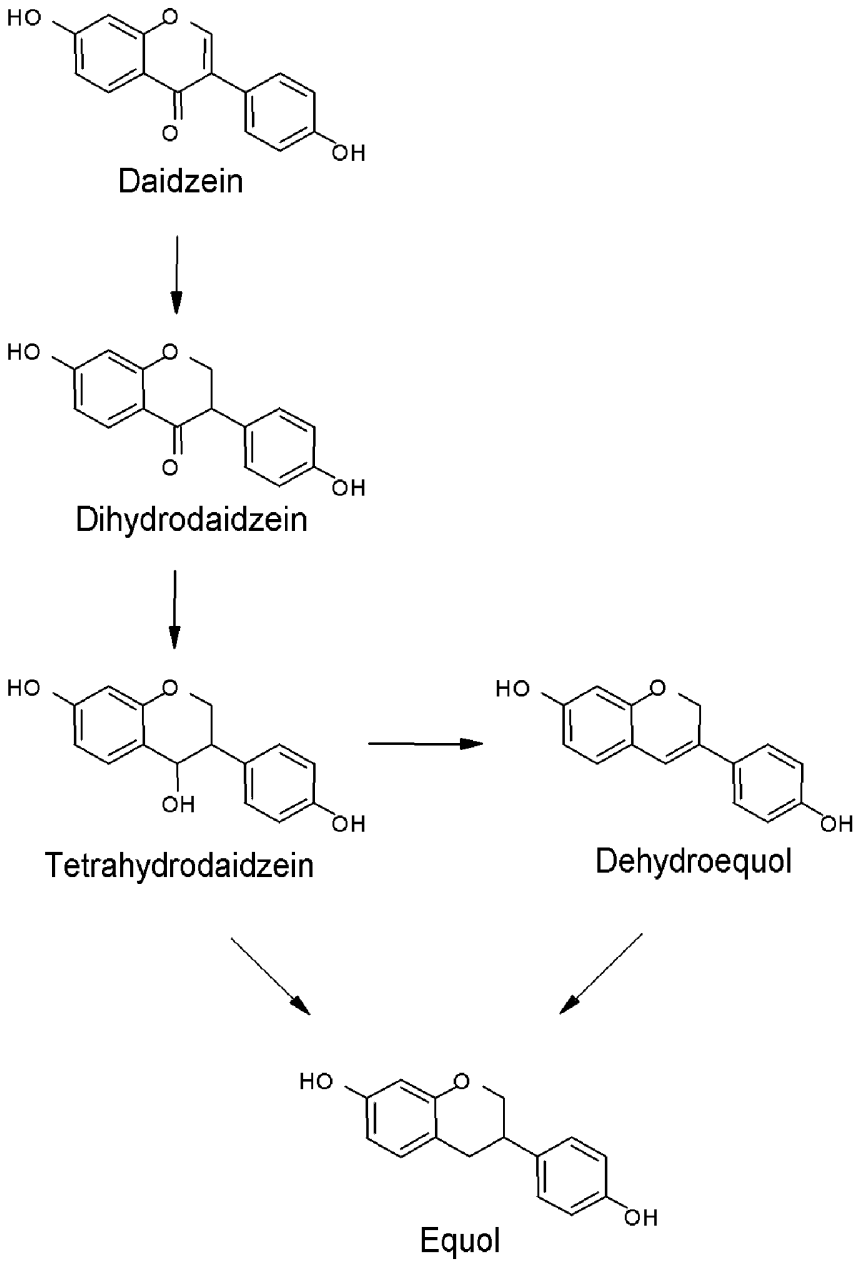
[4] The method of producing equol of claim 3 wherein the media is BHI.

[5] The method of producing equol of claim 3 wherein the incubation is carried out under conditions of 5 ~ 10 % CO_2, 5 ~ 15 % H_2 and 75 ~ 90 % N_2.

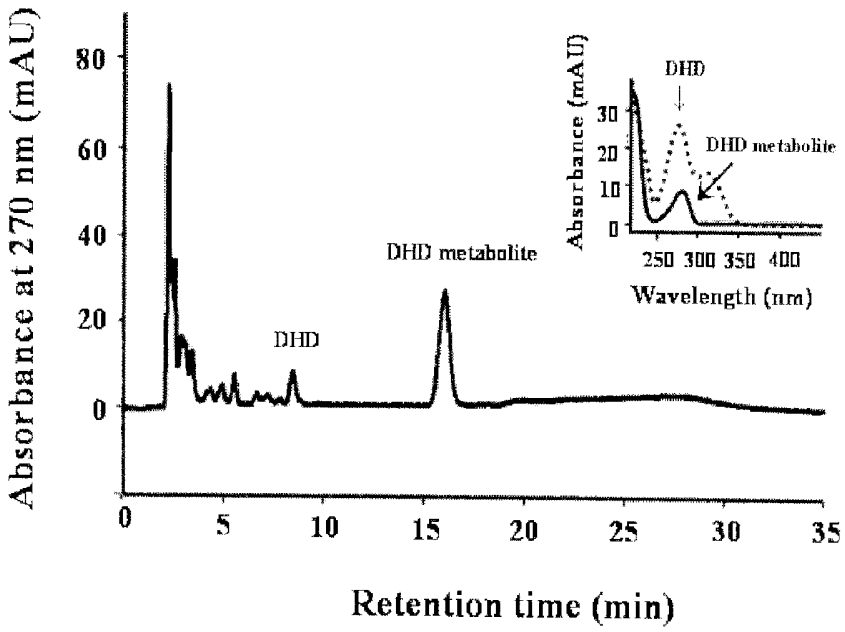
[6] A dietary composition comprising the microorganism of claim 1 and dihydrodaidzein.

[7] A pharmaceutical composition comprising the microorganism of claim 1 and dihydrodaidzein.

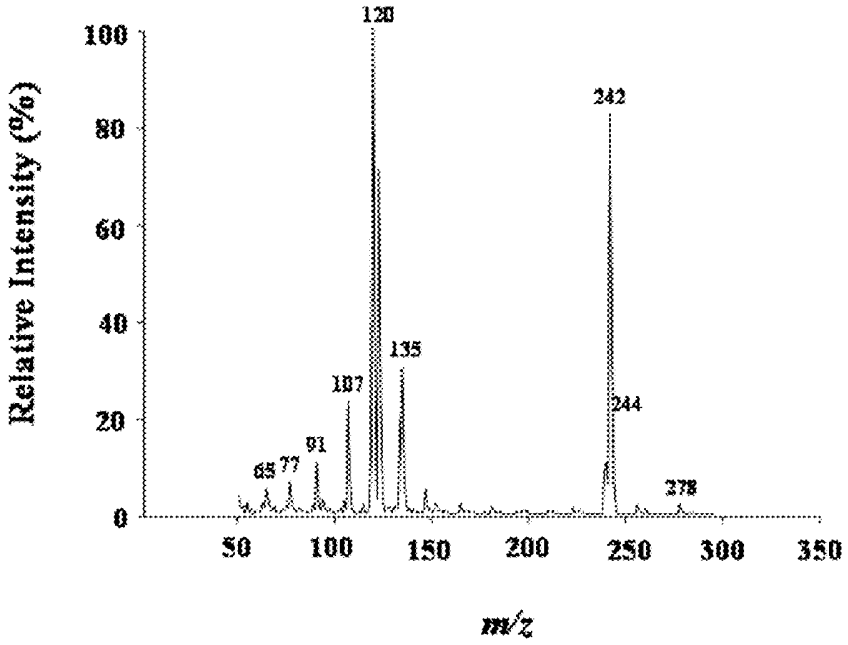
[Fig. 1]


HO
O
O
OH
Daidzein
HO
O
O
OH
Dihydrodaidzein
HO
O
OH
OH
Tetrahydrodaidzein
HO
O
OH
Dehydroequol
HO
O
OH
Equol

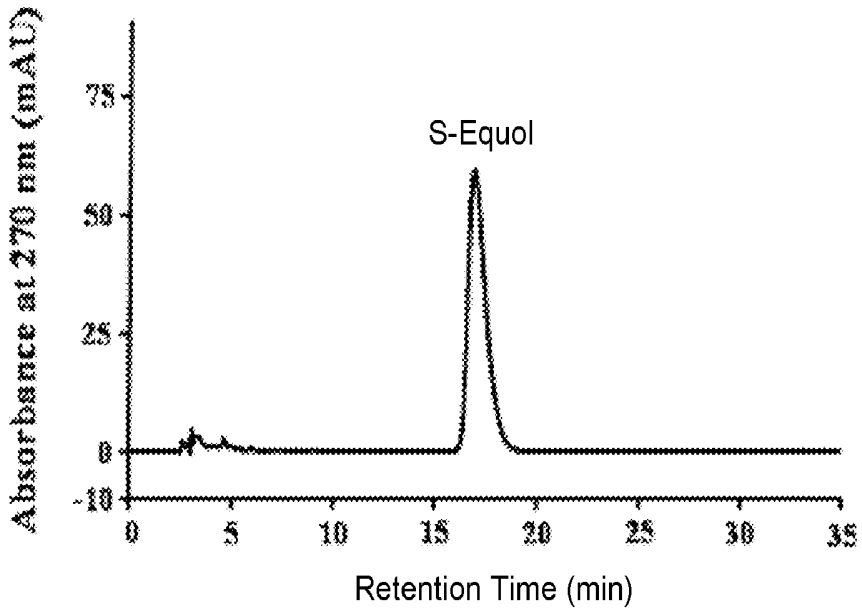
[Fig. 2]


Absorbance at 270 nm (mAU)
80
60
40
20
0
0
5
10
15
20
25
30
35
DHD
DHD metabolite
Absorbance (mAU)
30
20
10
0
DHD
DHD metabolite
250 300 350 400
Wavelength (nm)
Retention time (min)

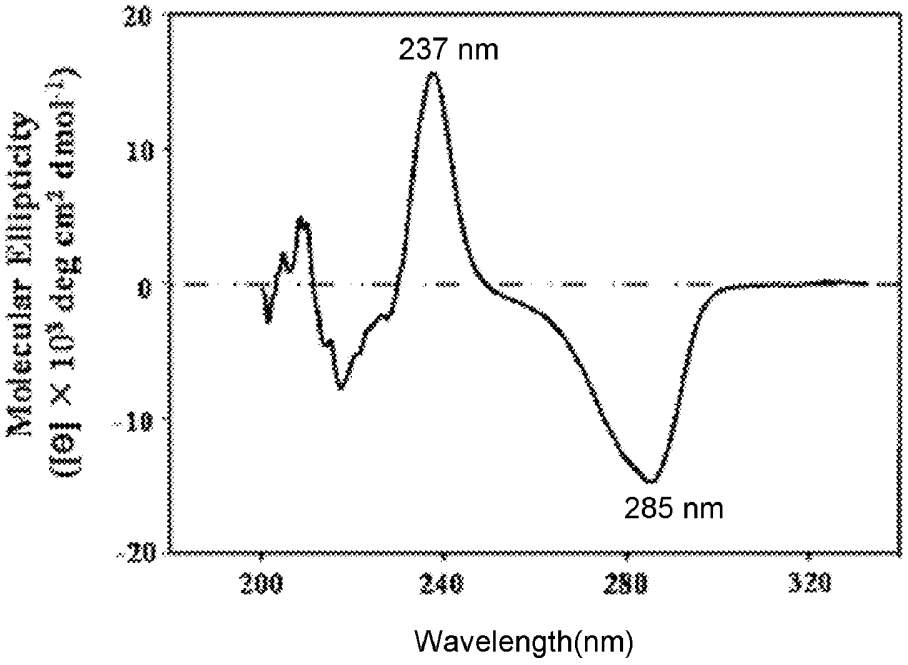
[Fig. 3]


Relative Intensity (%)
100
80
60
40
20
0
120
242
135
187
244
91
77
65
278
50
100
150
200
250
300
350
m/z

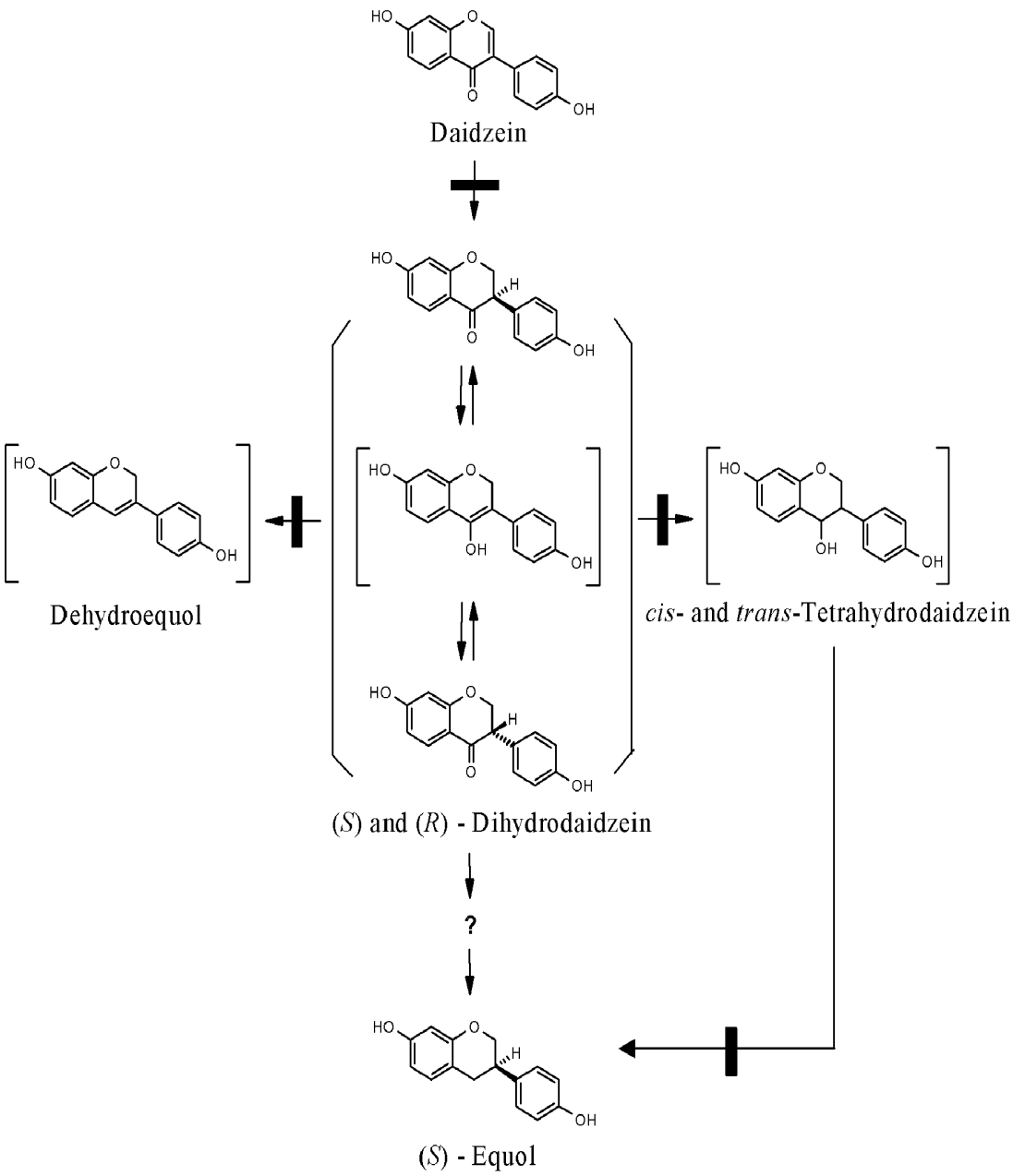
[Fig. 4]


Absorbance at 270 nm (mAU)
75
50
25
0
-10
0
5
10
15
20
25
30
35
S-Equol
Retention Time (min)

[Fig. 5]


Molecular Ellipticity
([θ] × 10³ deg cm² dmol⁻¹)
20
10
0
-10
-20
237 nm
285 nm
200
240
280
320
Wavelength(nm)

[Fig. 6]


Daidzein
Dehydroequol
(S) and (R) - Dihydrodaidzein
cis- and trans-Tetrahydrodaidzein
?
(S) - Equol

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2005/001285

A. CLASSIFICATION OF SUBJECT MATTER

IPC7 C12N 1/20, A23J 1/14

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7 C12N 1/20, A23J 1/14, A23L 1/20, A61K 35/78

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean Patents and applications for inventions since 1975
Korean Utility models and applictions for Utility models since 1975
Japanese Utility models and application for Utility models since 1975

Electronic data base consulted during the intertnational search (name of data base and, where practicable, search terms used)

eKIPASS, Delphion, PubMed, 'dihydrodaidzein', 'equol', 'Eggerthella sp.)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 1999/007392 A1 (OTSUKA PHARMA CO. LTD.) 18 February 1999 See the whole document.	1-7
A	ATKINSON, C. et al., 'In vitro incubation of human feces with daidzein and antibiotics suggests interindividual differences in the bacteria responsible for equol production' In: J. Nutr., March 2004, Vol.134(3), pp.596-599 See the whole document.	1-7
A	CHANG, Y.C. and NAIR, M.G., 'Metabolism of daidzein and genistein by intestinal bacteria' In: J. Nat. Prod., December 1995, Vol.58(12), pp.1892-1896 See the whole document.	1-7

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 JULY 2005 (18.07.2005)	**18 JULY 2005 (18.07.2005)**

Name and mailing address of the ISA/KR	Authorized officer
Korean Intellectual Property Office 920 Dunsan-dong, Seo-gu, Daejeon 302-701, Republic of Korea	LEE, CHUNG HO
Facsimile No. 82-42-472-7140	Telephone No. 82-42-481-8160

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2005/001285

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of :

   a. type of material
   - [x] a sequence listing
   - [ ] table(s) related to the sequence listing

   b. format of material
   - [ ] on paper
   - [x] in electronic form

   c. time of filing/furnishing
   - [ ] contained in the international application as filed
   - [x] filed together with the international application in electronic form
   - [ ] furnished subsequently to this Authority for the purposes of search

2. [ ] In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

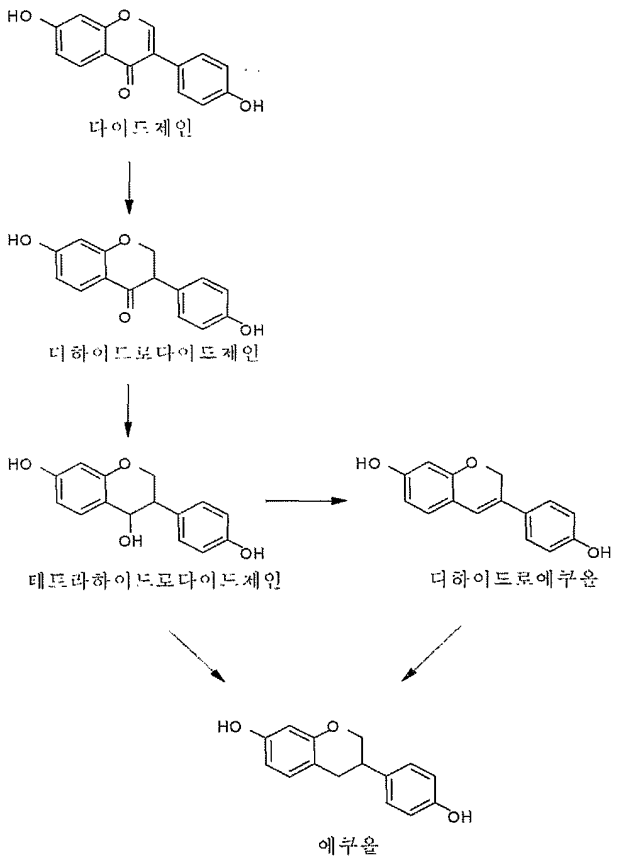
INTERNATIONAL SEARCH REPORT

Information on patent family members

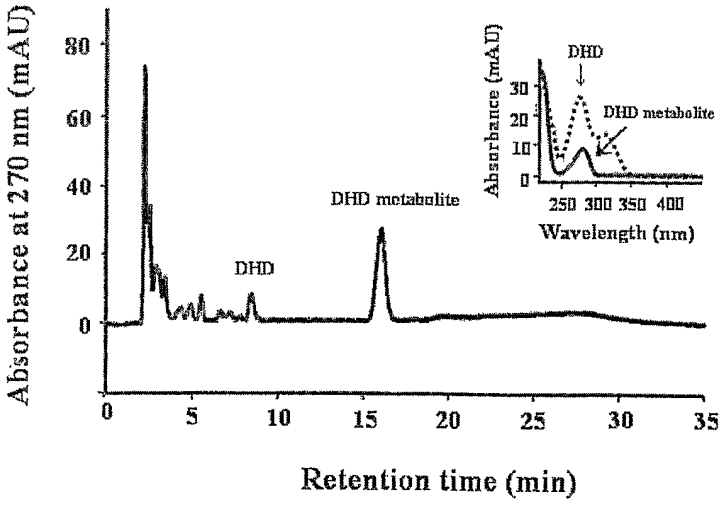
International application No.
PCT/KR2005/001285

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 1999007392 A1	18.02.1999	AU19988463182	01.03.1999
		AU8463198A1	01.03.1999
		CA2298679A1	18.02.1999
		CN1265596	06.09.2000
		CN1265596A	06.09.2000
		EP1025850A1	09.08.2000
		JPW099/07392	18.02.1999
		KR1020010022669	26.03.2001
		TW580517B	21.03.2004
		US06716424	06.04.2004
		US2004141954AA	22.07.2004

[Fig. 1]


HO
O
OH
다이드제인
디하이드로다이드제인
테트라하이드로다이드제인
디하이드로에쿠윤
에쿠윤

[Fig. 2]


Absorbance at 270 nm (mAU)
80
60
40
20
0
DHD
DHD metabolite
0
5
10
15
20
25
30
35
Retention time (min)
Absorbance (mAU)
30
20
10
0
DHD
DHD metabolite
250 300 350 400
Wavelength (nm)