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Biotransformation of isoflavones by *Aspergillus niger*, as biocatalyst

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Abstract

Biotransformation of the isoflavones, daidzein (**1**), 7,4′-dimethoxyisoflavone (**2**) and 7,4′-3 diacetoxyisoflavone (**3**), by *Aspergillus niger* was investigated. Compound **1** was hardly four metabolized by *A. niger*, while compound **2** was transformed to 6-hydroxy-7,4′-5 dimethoxyisoflavone (**4**) and daidzein (**1**) and compound **3** was transformed to daidzein (**1**). This suggested that **2** was converted to **4** by oxidation at the C-6 position and to **1** by demethylation of methoxy groups at the C-7 and C-4′ positions. Compound **3** was converted to **1** by hydrolysis at the C-7 and C-4′ positions.

Keywords: Biotransformation; Biocatalyst; *Aspergillus niger*; Isoflavone

1. Introduction

Microbial transformation possesses the advantage of proceeding under mild conditions and with high regio and enantioselectivity. In the course of our work, we have studied the biotransformation of lignans and terpenoids by fungus and insects as a biocatalyst [1–5], for example, biotransformation of the lignan, (+)-eudesmin, by *Aspergillus niger*. (+)-Eudesmin was metabolized and transformed to (+)-de-4′-*O*-methyleudesmin and (+)-pinoresinol by oxidation at the para position [6].

Flavonoids are widely distributed in the plant kingdom and the human dietary intake of these natural products is estimated to be about 1 g per day of mixed flavonoids [7]. Various flavonoids are reported to possess antihaemolytic [8], antioxidative [8], antifungal [9], estrogenic [10] and antiumor [11] activities. Biotransformation of isoflavonoids by mammals has been reported [12,13]. Mimura et al. reported the biotransformation of daidzin, an isoflavone glycoside, by *A. niger* and showed that it was converted to daidzein and 8-hydroxydaidzein [14]. Farooq and Tahara reported the biotransformation of formononetin to daidzein by Fusarium pmhferatum through de-methylation at the para position [15]. Tolleson and Doerge reported the biotransformation of biochanin A by human liver to genistein and 3′,4′,5,7-tetrahydroxyisoflavone [16]. However, there is no report of the biotransformation of daidzein (**1**), 7,4′-dimethoxyisoflavone (**2**) and 7,4′-diacetoxyisoflavone (**3**) by *A. niger* and we have now studied the microbial transformation of these compounds.

2. Materials and methods

2.1. General procedure

IR spectra were determined with a JASCO FT/IR-470 plus Fourier transform infrared spectrometer. Nuclear magnetic resonance (NMR) spectra were obtained with a JEOL FX 500 (500.00 MHz, ^{1}H; 125.65 MHz, ^{13}C) spectrometer. TLC/FID analysis was carried out using an latroscan TLC-FID system. Quantitative TLC was performed on silica gel-coated rods (CHROMAROD-S III), and the separated compounds were detected with an latroscan MK5 (Iatron Laborcitovies Inc., Tokyo, Japan). The conditions for analysis were as follows; H_2 flow rate 160 ml/min, air flow rate 2.0 l/mm and scan speed 30 mm/min. The peak area was integrated with an latrocorder TC-II. EI-MS spectra were obtained on a JEOL the Tandem MS station JMS-700 TKM.

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2.2. Isolation of daidzein

Soyaflabon E (powder of *Glycin max*, purchased from FUJI Oil Co. Ltd.) (1 kg) was hydrolyzed with 1 N HCl for 6 h and filtrated. The filtrate was extracted with EtOAc and evaporated under reduced pressure. Daidzein (**1**) (2.0 g) was isolated by silica gel column chromatography from EtOAc extract (120 g). Methyl ether of daidzein (7,4′-dimethoxyisoflavone (**2**)) was obtained by reaction with CH_3I and Ag_2O for 3 h. Acetyl esters of daidzein (7,4′-diacetoxyisoflavone (**3**)) was obtained by reaction with Ac_2O and pyridine for 5 h.

2.3. Microorganism and culture conditions

Spores of *A. niger* NBRC 4414 (purchased from NITE Biological Resource Center, Chiba, Japan) maintained on nutrient agar slants at 10 °C were inoculated into the autoclaved culture medium (50 ml in a 200 ml conical flask): 1.5% saccharose, 1.5% glucose, 0.5% polypeptone, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% KCl, 0.1% K_2HPO_4, 0.001% $FeSO_4 \cdot 7H_2O$ and distilled water; pH 7.2. The culture was maintained for 2 days in the incubator (28 °C under shaking). Mycelia were then transplanted to the culture medium (15 ml in a 50 ml Petri dish) and incubated for 36–48 h (until mycelia occupied 60–80% of the surface area of a culture medium) under the static conditions. Daidzein (**1**) (100 mg) was dissolved in 2 ml of DMSO and added to the culture medium (corresponding to 35–40 mg of substrate per Petri dish). Petri dishes were incubated at 28 °C under static situation, together with two controls, which contained either mycelia with medium or substrate dissolved in DMSO with medium. 7,4′-Dimethoxyisoflavone (**2**) (100 mg) and 7,4′-diacetoxyisoflavone (**3**) (100 mg) were also dissolved in DMSO and added to the culture medium in the same way.

2.4. Time-course of substrates and metabolic products

Cultivation media from Petri dishes were acidified to pH 2 with 1 N HCl and extracted EtOAc several times at various intervals. Metabolic products of each substrate were confirmed by preliminary examination. EtOAc extracts (10 mg) were dissolved in 1 ml of CH_2Cl_2 and 5 μl of each solution applied to silica gel-coated rods. The rods were dried and then developed in chloroform-acetone (1:1) to a distance of 10 cm from the point of application. The ratio of the substrate and metabolites were quantified and is shown in Figs. 1 and 2.

2.5. Incubation of daidzein (*1*)

Daicizein (**1**) (100 mg) was incubated with *A. niger* as described above. Eight days of incubation afforded no detectable metabolite. Compound **1** was recovered unchanged from culture medium.

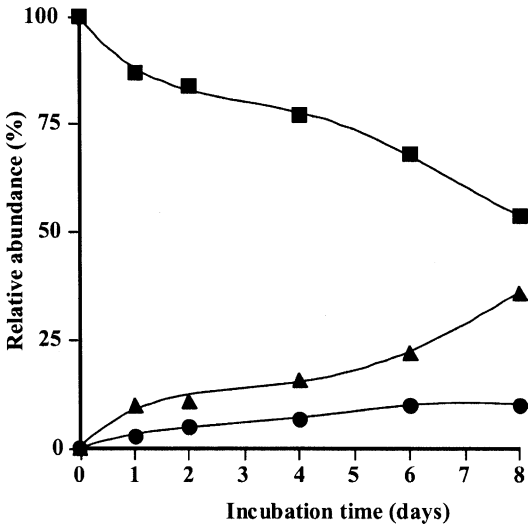


Fig. 1. Time-course of 7,4′-dimethoxydaidzein (**2**) metabolism and 6-hydroxy-7,4′-dimethoxyisoflavone (**4**) and daidzein (**1**) formation after incubation with *A. niger*: (■) 7,4′-dimethoxydaidzeiri (**2**); (▲) 6-hydroxy-7,4′-dimethoxyisoflavone (**4**); (●) daidzein (**1**).

2.6. Isolation of metabolic products of 7,4′-dimethoxyisoflavone (*2*)

7,4′-Dmethoxyisoflavone (**2**) (100 mg) was incubated with *A. niger* as described above. After 8 days cultivation, the culture medium was collected and acidified to pH 2 with 1 N HCl and saturation with NaCl. The culture was then extracted with EtOAc several times, the EtOAc extract (178 mg) dried (Na_2SO_4) and evaporated under reduced pressure. The EtOAc extract was chromatographed on silica gel repeatedly and metabolic products **4** (36 mg) and **1**(10 mg) were isolated.

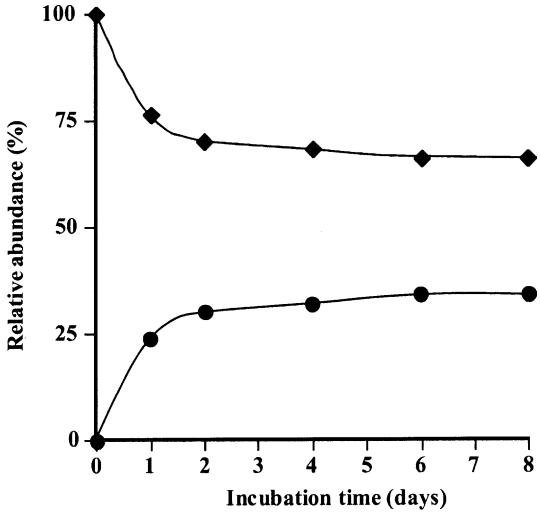


Fig. 2. Time-course of 7,4′-diacetoxydaidzein (**3**) metabolism and daidzein (**1**) formation after incubation with *A. niger*: (◆) 7,4′-diacetoxydaidzein (**3**); (●) daidzein (**1**).

Table 1
^{1}H NMR spectral data for isoflavone (**2** and **3**) and metabolic products (**4** and **1**) (δ, TMS, in $CDCl_3$ at 500 MHz)

H	1	2	3	4
1	–	–	–	–
2	8.24 (1H, s)	7.92 (1H, s)	8.54 (1H, s)	7 94 (1H, s)
3	–	–	–	–
4	–	–	–	–
5	7.97 (1H, d, $J = 8.8$)	8.21 (1H, d, $J = 8.8$)	8.20 (1H, d, $J = 8.8$)	7.64 (1H, s)
6	6.93 (1H, dd, $J = 2.2$, 8.8)	6.98 (1H, dd, $J = 2.3$, 8.8)	7.32 (1H, dd, $J = 2.0$, 8.8)	–
7	–	–	–	–
8	6.85 (1H, d, $J = 2.2$)	6.85 (1H, d, $J = 2.3$)	7.55 (1H, dt, $J = 2.0$)	6.89 (1H, s)
2′	7.39 (2H, dt, $J = 2.4$, 9.2)	7.50 (2H, dt, $J = 2.3$, 9.3)	7.65 (2H, dt, $J = 2.3$, 9.1)	7.42 (2H, di, $J = 2.0$, 8.6)
3′	6.18 (2H, di, $J = 2.4$, 9.2)	6.97 (2H, dt, $J = 2.3$, 9.3)	7.20 (2H, dt, $J = 2.3$, 9.1)	6.89 (2H, dt, $J = 2.0$, 8.6)
4′	–	–	–	–
5′	6.18 (2H, dt, $J = 2.4$, 9.2)	6.97 (2H, dt, $J = 2.3$, 9.3)	7.20 (2H, dt, $J = 2.3$, 9.1)	6.89 (2H, dt, $J = 2.0$, 8.6)
6′	7.39 (2H, dt, $J = 2.4$, 9.2)	7.50 (2H, dt, $J = 2.3$, 9.3)	7.65 (2H, dt, $J = 2.3$, 9.1)	7.42 (211, dt, $J = 2.0$, 8.6)
OMe	–	3.84 (3H, s)	–	3.99 (3H, s)
	–	3.91 (3H, s)	–	4.00 (3H, s)
OAc	–	–	2.29 (3H, s)	–
	–	–	2.34 (3H, s)	–

Coupling constants in Hz.

2.7. Isolation of metabolic products of 7,4′-diacetoxyisoflavone (3)

7,4′-Diacetoxyisoflavone (**3**) (100 mg) was incubated with *A. niger* as described above. After 8 days cultivation, the culture medium was collected. It was treated in the same way as that from 7,4′-diacetoxyisoflavone (**3**). The EtOAc extract (90 mg) was chromatographed on silica gel repeatedly and metabolic product **1** (30 mg) was isolated.

2.8. Structure of isoflavonoids

Compound **1** was isolated as colourless needles: mp 300–302 °C; MS, *m/z* 254 $[M]^+$, 137, 118; IR (KBr) ν_{max} cm^{-1} 3433, 1631; ^{1}H and ^{13}C NMR as shown Tables 1 and 2. Compound **1** was identified as daicizein (7-hydroxy-3-(4-hydroxypheny)-4H-1-benzopyran-4-one) from these spectral data and physical properties.

Compound **2** was isolated as colorless needles: mp 160–161 °C; MS, *m/z* 282 $[M]^+$; IR (KBr) ν_{max} cm^{-1} 1634; ^{1}H and ^{13}C NMR as shown Tables 1 and 2. Compound **2** was identified as 7,4′-dimethoxyisoflavone (7-methoxy-3-(4-methoxyphenyl)-4H-1-benzopyran-4-one) from these spectral data and physical properties.

Compound **3** was isolated as colorless needles: mp 192–193 °C; MS, *m/z* 338 $[M]^+$; IR (KBr) ν_{max} cm^{-1} 1637; ^{1}H and ^{13}C NMR as shown in Tables 1 and 2. Compound **3** was identified as 7,4′-diacetoxyisoflavone (7-acetyl-3-(4-acetylphenyl)-4H-1-benzopyran-4-one) from these spectral data and physical properties.

Compound **4** was isolated as oil; MS, *m/z* 298 $[M]^+$, 253, 132; IR (KBr) ν_{max} cm^{-1} 3420, 1677; ^{1}H and ^{13}C NMR as shown in Tables 1 and 2. Compound **4** was identified as 6-hydroxy-7,4′-dimethoxyisoflavone (6-hydroxy-7-methoxy-3-(4-methoxyphenyl)-4H-1-benzopyran-4-one) from these spectral data and physical properties.

Table 2
^{13}C NMR spectral data for isoflavone (**2** and **3**) and metabolic products (**4** and **1**) (δ, TMS, in $CDCl_3$ at 500 MHz)

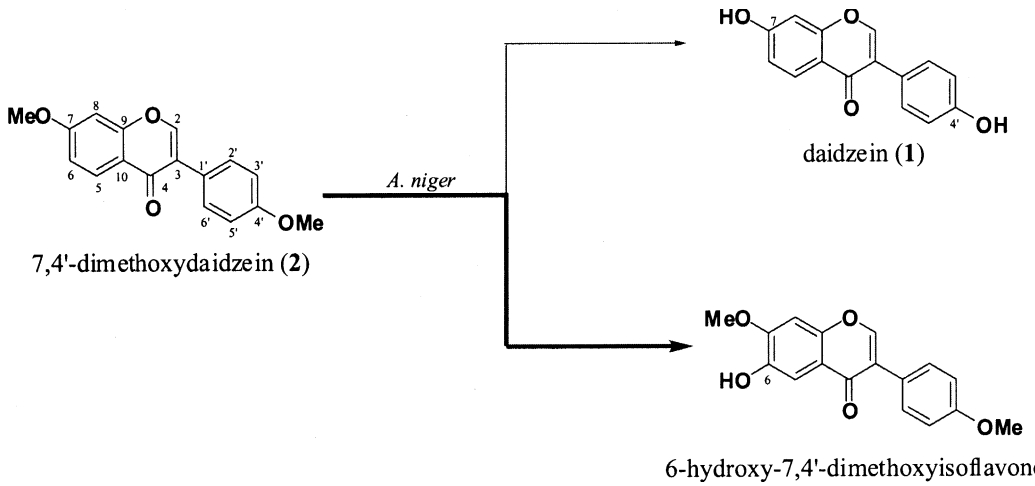
C	1	2	3	4
2	152.3 (CH)	152.9 (CH)	153.3 (CH)	152.5 (CH)
3	122.7 (C)	123.8 (C)	124.9 (C)	122.9 (C)
4	178.6 (C)	174.9 (C)	175.6 (C)	174.6 (C)
5	127.2 (CH)	127.0 (CH)	128.0 (CH)	108.1 (CH)
6	115.1 (CH)	114.6 (CH)	119.7 (CH)	145.3 (C)
7	162.6 (C)	163.8 (C)	156.8 (C)	153.7 (C)
8	102.2 (CH)	100.3 (CH)	111.1 (CH)	100.0 (CH)
8a	157.6 (C)	157.6 (C)	154.8 (C)	151.0 (C)
4a	116.9 (C)	117.9 (C)	122.5 (C)	117.7 (C)
1′	123.9 (C)	124.2 (C)	129.4 (C)	124.6 (C)
2′	129.9 (CH)	130.0 (CH)	130.2 (CH)	130.0 (CH)
3′	115.1 (CH)	113.6 (CH)	121.8 (CH)	113.6 (CH)
4′	157.3 (C)	159.2 (C)	150.9 (C)	159.1 (C)
5′	115.1 (CH)	113.6 (CH)	121.8 (CH)	113.6 (CH)
6′	129.9 (CH)	130.0 (CH)	130.2 (CH)	130.0 (CH)

Chemical shifts in ppm; multiplicities were determined by the DEPT pulse sequence.

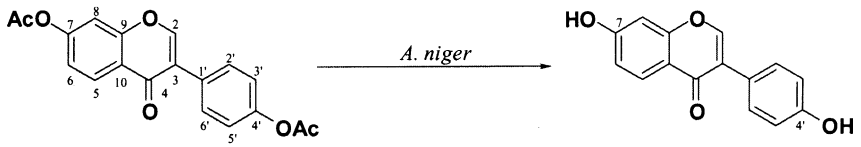
3. Results and discussion

Biotransformation of daidzein (**1**), 7,4′-dimethoxydaidzein (**2**) and 7,4′-diacetoxydaidzein (**3**) by *A. niger* were investigated. Compounds **2** and **3** were transformed to several metabolites, but compound **1** was recovered unchanged.

The transformation of 7,4′-dimethoxyisoflavone (**2**) by *A. niger* was examined. *A. niger* at static cultivation afforded



Scheme 1. A specific oxidation process to daidzein (**1**) and 6-hydroxy-7,4′-dimethoxyisoflavone (**4**) from 7,4′-dimethoxydaidzein (**2**) by *A. niger*.



Scheme 2. A specific hydrolysis process to daidzein in (**1**) from 7,4′-ditoxydaidzein (**3**) by *A. niger*.

4 and **1**. Time-course of the reaction was measured by TLC and TLC/FID analysis (Fig. 1: **1**, R_f = 0.52; **2**, R_f = 0.82; **4**, R_f = 0.66). The starting material was consumed gradually and **4** and **1** produced. Other metabolite such as monomethoxyisoflavone was not found. After 8 days, the reaction mixture was extracted with ethyl acetate and products (**4** and **1**) were isolated by column chromatograph on silica gel.

Compound **4** was determined as 6-hydroxy-7,4′-dimethoxyisoflavone (alfalone) from the following MS, IR and NMR data [17,18]. $C_{17}H_{14}O_5$ from MS analysis; IR: 3418 cm^{-1} (hydroxyl group); NMR: the signal from C-6 proton was disappeared and two methyl group were existed.

Compound **1** was determined as daizein by comparing the MS, IR, and NMR spectrum with the authentic sample [19,20].

The reaction of **2** was illustrated in Scheme 1: metabolite **4** and **1** were produced by oxidation at the C-6 position and at the C-7 and C-4′ positions of **2**, respectively.

The transformation of 7,4′-diacetoxyisoflavone (**3**) by *A. niger* was examined. Compound **3** was metabolized and product **1** generated by static cultivation. The time-courses of each metabolic product were observed by TLC and quantitatively calculated by TLC/FID analysis (Fig. 2). After incubation of **3** (R_f = 0.84) for 1 day, it was metabolized and the amount decreased gradually. Compound **1** (R_f = 0.52) was produced and accumulated for 2 days, and then slowly disappeared. Other metabolite, e.g. mono-acetoxyisoflavone, did not detected by TLC and TLC/FID. Metabolite **1** was identified as daidzein by spectral data. The reaction of **3** was illustrated in Scheme 2: acetyl moieties at the C-7 and C-4′ were hydrolyzed by the microbe and **1** was produced.

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