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FORMATION OF POLYHYDROXYLATED ISOFLAVONES FROM THE ISOFLAVONES GENISTEIN AND BIOCHANIN A BY BACTERIA ISOLATED FROM TEMPE

KLAUS KLUS and WOLFGANG BARZ*

Institut für Biochemie und Biotechnologie der Pflanzen, Westfälische Wilhelms-Universität, 48143 Münster, Hindenburgplatz 55, Germany

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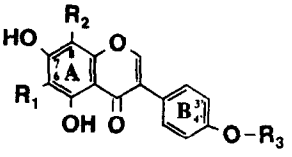
Key Word Index—*Micrococcus*; *Arthrobacter*; biotransformation; tempe; hydroxylation; polyhydroxyisoflavones; biochanin A; genistein.

Abstract—Two tempe-derived bacterial strains identified as *Micrococcus* or *Arthrobacter* species were shown to transform the 5-hydroxyisoflavones biochanin A and genistein to polyhydroxylated isoflavones by hydroxylation reactions at positions C-6 and C-8. Both strains transformed genistein to 5,6,7,4′-tetrahydroxyisoflavone and 5,7,8,4′-tetrahydroxyisoflavone and biochanin A to 4′-methoxy-5,7,8,-trihydroxyisoflavone, whereas only strain I converted biochanin A to 4′-methoxy-5,6,7-trihydroxyisoflavone. The structures of these transformation products were elucidated by spectroscopic techniques.

INTRODUCTION

Tempe is a traditional Indonesian food produced from soybeans by fermentation mainly with fungi of the genus *Rhizopus*. Recent data [1, 2], however, showed that several bacterial species may also be involved in tempe production. For fermentation the soybeans are cooked and dehulled. In traditional tempe fermentation processes the cooked soybeans are soaked overnight, where a bacterial acidification occurs. For industrial tempe fermentation the cooked soybeans are acidified with lactic acid. After the soaking process the soybeans are cooked again and incubated with microbial inocula for 2 days.

In unfermented soybean seeds the isoflavones predominantly occur as isoflavone glucosides and acylglucosides [3, 4]. During tempe fermentation the isoflavone aglycones are liberated from the conjugates and accumulate in the tempe product [5, 6]. Using the soybean 5-deoxyisoflavones daidzein, glycitein and factor 2 (6,7,4′-trihydroxyisoflavone) as substrates in incubation assays with tempe-derived microorganisms, it has been shown that several *Rhizopus* strains and yeast isolates [1] failed to transform these compounds by hydroxylation or demethylation reactions [7, 8]. However, subsequent studies [7] on the metabolism of these soybean 5-deoxyisoflavones with three tempe-derived bacteria had shown that isolates



(**1**)biochanin A : R_1, R_2, = H R_3 = Me
(**2**)genistein R_1, R_2, R_3 = H
(**3**)6-hydroxybiochanin A R_1 = OH R_2 = H R_3 = Me
(**4**)8-hydroxybiochanin A R_1 = H R_2 = OH R_3 = Me
(**5**)6-hydroxygenistein R_1 = OH R_2, R_3, = H
(**6**)8-hydroxygenistein R_1, R_3, = H R_2 = OH

of *Brevibacterium epidermides* and *Micrococcus luteus* readily transformed glycitein forming factor 2. *Microbacterium arborescens* metabolized daidzein producing both glycitein and factor 2 or transformed factor 2 forming glycitein. Five other tempe-derived bacterial strains classified as *Micrococcus* or *Arthrobacter* species were shown to transform the soybean 5-deoxyisoflavones forming five different polyhydroxylated isoflavones by one *O*-demethylation reaction at C-6 and by hydroxylation reactions at C-6, C-8 and C-3′ [8].

We have now investigated two of these five bacterial isolates (strain I and III) for their ability to metabolize the chickpea seed 5-hydroxyisoflavone biochanin A (**1**) and the soybean seed 5-hydroxyisoflavone genistein (**2**). The results show that polyhydroxylated isoflavones are being formed which were isolated and structurally elucidated.

* Author to whom correspondence should be addressed.

RESULTS AND DISCUSSION

During the screening experiments with two tempe-derived bacterial strains (strains I and III) which formed polyhydroxylated isoflavones by *O*-demethylation and hydroxylation reactions when the soybean 5-deoxyisoflavones daidzein, factor 2 and glycitein were used as substrates [8], transformed the 5-hydroxyisoflavones biochanin A (**1**) and genistein (**2**) yielding the four polyhydroxylated isoflavones (**3**) to (**6**).

The two gram-positive isolates possess short rod (strain I) to coccoid cells (strain III), form no spores, show a light yellow pigmentation and were tentatively identified as *Micrococcus* or *Arthrobacter* species [8]. As documented by the chemical structures of the metabolites (see below) the bacterial strains each catalyze aromatic hydroxylation reactions of **1** and **2** at C-6 and/or C-8. Hydroxylation of **1** in position 6 led to the formation of 4′-methoxy-5,6,7-trihydroxyisoflavone (**3**) and hydroxylation in position 8 resulted in the formation of 4′-methoxy-5,7,8-trihydroxyisoflavone (**4**). In the culture medium of strain I both **3** and **4** accumulated when biochanin A was used as substrate, whereas strain III only produced compound **4**. Hydroxylation of **2** in position 6 gave rise to 5,6,7,4′-tetrahydroxyisoflavone (**5**) and this product was formed by both strains I and III. During several incubation experiments with the two bacterial strains a second metabolite (**6**) accumulated in the culture medium in such low concentrations that a definitive structural elucidation of this compound was not possible. The UV spectrum of **6** which was identical with published data of 5,7,8,4′-tetrahydroxyisoflavone [9] and the higher hydrophilicity in comparison with **2** leads to the hypothesis that 8-hydroxygenistein had been isolated.

The isoflavones were extracted from suspension cultures of the bacteria, separated by HPLC and assayed by UV-spectroscopy. Our HPLC analyses showed that the bioconversion products **3** to **6** were not further metabolized within the experimental period of 48 hr and that degradation of the isoflavone skeleton did not occur. Using UV shift data obtained with diagnostic substances [10], GC mass spectrometric procedures, as well as cochromatography, **3** to **5** were unequivocally identified.

A characteristic feature of all metabolites was a free 7-hydroxyl group (positive NaOAc-induced UV-shift). The two absorption maxima in the short wavelength band II in the UV spectra of **3** at 239 and 290 nm and of **5** at 238 and 274 nm in methanol upon the addition of $AlCl_3$ causes complexes of the aluminium cations with either 6,7- or 7,8-*ortho*-dihydroxyl groups and with the C-5 hydroxyl groups and the neighbouring ketone of the C-rings of these compounds. The $AlCl_3$–HCl induced bathochromic shifts of bands II in the UV spectra of **3** and **5** from 270 to 2874 nm (**3**) and from 270 to 281 nm (**5**) suggested the presence of free 5-hydroxyl groups [10]. From GC mass spectral analyses the TMSi derivatives of **3** and **5** were prepared. The peak at m/z 516 $[M]^+$ indicates that compound **3** is a monomethoxytrihydroxyisoflavone. The GC mass spectral analysis of the TMSi derivative of **5** showed that the molecular mass (m/z 574$[M]^+$) represents a tetrahydroxyisoflavone. 8-hydroxyl groups can be excluded in **3** and **5**, because the UV spectrum of 8-hydroxygenistein with an absorption maximum at 284 nm in methanol upon addition of $AlCl_3$ [9] differs from the UV spectrum of **5** in the presence of $AlCl_3$. Furthermore, the UV data of **3** did not match with published data of 8-hydroxybiochanin A [11]. Decrease of absorption in the UV spectrum of **5** in methanol upon addition of NaOAc with time was observed, which could, however, only be shown for 6-hydroxygenistein [10]. During HPLC compound **3** co-migrated with 4′-methoxy-5,6,7-trihydroxyisoflavone (6-hydroxybiochanin A) and compound **5** with 5,6,7,4′-tetrahydroxyisoflavone (6-hydroxygenistein).

The $AlCl_3$-induced UV-shift of the absorption maximum of **4** at 268 nm (band II) in methanol could not completely be reverted upon addition of HCl. These data suggested the presence of a free 5-hydroxyl group and free 6,7- or 7,8-*ortho*-dihydroxyl groups [10]. The UV spectrum of **4** in methanol and the exceptional strong 31 nm bathochromic shift of band II upon addition of $AlCl_3$ were identical with the published UV data of 8-hydroxybiochanin A [11]. The GC mass spectral analysis of the TMSi-derivative of **4** indicates that this compound is a monomethoxytrihydroxyisoflavone. **4** co-migrated with 4′-methoxy-5,7,8-trihydroxyisoflavone (8-hydroxybiochanin A) during HPLC.

In incubation experiments (48 hr) with the bacterial strain I biochanin A (**1**) (5×10^{-5} M) was transformed to 60–70% resulting in the formation of **3** (30–60% yield) as well as **4** (10–30% yield). 48 hr after application of **2** (5×10^{-5} M) the culture medium of strain I 65–75% of the substrate was converted to **5** (60–70% yield) and **6** (5% yield). When **1** was used as substrate (5×10^{-5} M) with strain III, only **4** accumulated in the culture medium to 5% yield within the experimental period of 48 hr, whereas **2** as substrate in identical incubation experiments resulted in the formation of **5** (20–25%) and **6** (1% yield). In conclusion, the tempe-derived bacterial strains I and III were able to catalyse two hydroxylation reactions in positions 6 and 8 of the 5-hydroxyisoflavones **1** and **2**. These strains completely failed to hydroxylate the 5-hydroxyisoflavones in position 3′, which in parallel studies on the metabolism of the soybean 5-deoxyisoflavone daidzein, factor 2 and glycitein [8] had been shown to be a prominent reaction performed by these strains.

The elucidated hydroxylation sequences of the 5-deoxy- [8] and 5-hydroxyisoflavones by the 2 tempe-derived bacterial strains represent a wide spectrum of oxidative isoflavone transformations which have so far never been observed with isoflavone metabolizing fungi [12]. Various fungal species (*Fusarium*, *Ascochyta*) only perform one or two oxidative steps.

Hydroxylation and methylation reactions are often the first step in flavonoid metabolism by micro-organism [12, 13]. Examples for the degradation of the isoflavone carbon skeleton are presently only known from fungi [12]. In contrast, the bacterial strains I and III only hydroxylated but failed to degrade the substrates. In this context 8- and 6-methoxyisoflavones with 5-hydroxylgroups and 3′-hydroxygenistein have been isolated from culture filtrate of *Streptomyces* species when growing in a culture medium containing 2% soybean meal [14, 15]. The relevant 3′-hydroxy- and 6- or 8- methoxyisoflavones could not be found in this substrate before inoculation of the bacteria [14, 15]; therefore, it appears possible that the soybean-derived isoflavone genistein had been converted to these compounds. The rapid and quantitatively pronounced conversion of **1** and **2** yielding **3** to **5** by the bacterial strains I and III are recommended for biotechnological production of the difficult to synthesize polyhydroxylated isoflavones.

Future investigations will elucidate the exact role of these bacteria in tempe fermentation with regard to the accumulation of polyhydroxylated isoflavones. Such studies are mandatory in view of the pronounced biological activity and possible nutritional value of these isoflavones for tempe quality as a food product. Various polyhydroxylated isoflavones are known to exhibit antioxidant, anti-inflammatory and anti-allergic activities [5, 16, 17] and to express anti-carcinogenic properties due to the inhibition of protein tyrosine kinases [18, 19].

EXPERIMENTAL

Bacteria. The bacterial strains I and III had been isolated from tempe samples [1] and were cultivated as previously described [7]. The results of assays to identify the isolates were summarized by Klus and Barz [8].

Incubations experiments. The standard incubation assays of bacteria (100 mg fr. wt) with isoflavone substrates (5×10^{-5} M), the composition of the mineral salt medium and the isolation of the transformation products from the medium were essentially as described [7].

Purification of compounds. HPLC was conducted on RP 18 columns with a Waters chromatograph attached to a photodiode array detector for recording UV spectra. For the isolation of transformation products a linear gradient of 12% B (MeCN) to 38% B in (A + B) in 20 min was applied. Solvent A was 1.5% HOAc and the flow rate 0.8 ml min^{-1}.

Substrates. Compounds **1** and **2** was obtained from ROTH, Karlsruhe, F.R.G. Other compounds were from the institute's collection.

Analytical methods. UV absorption spectra of the metabolites were measured in MeOH and after the addition of diagnostic reagents [10]. The spectra were recorded using a KONTRON Uvikon 810 spectrophotometer. GC-MS measurements were performed using a VARIAN GC 3400 equipped with a capillary column HP5 (25 m), set to a temp. gradient from 200° to 300° at a rate of 6° min^{-1} and held for 10 min at 300°; injector temp. of 320°, connected with a FINNIGAN MAT 312 MS (70 eV). For GC-MS analyses polyhydroxyisoflavones were derivatized with *n*-methyl-*n*-trimethylsilylfluoroacetamide (Machery and Nagel) according to the manufacturer's protocol.

5,6,7-Trihydroxy-4′-methoxyisoflavone (6-hydroxybiochanin A) (**3**). UV λ^{MeOH} nm: 245 (sh), 270, 350 (sh); $\lambda^{MeOH+NAOAc}$ nm: 303 (sh), 337 (sh); $\lambda^{MeOH+NaOAc-H_3BO_3}$ nm: 279 (sh), 335 (sh); $\lambda^{MeOH+AlCl_3}$ nm: 239, 249 (sh), 276 (sh), 290, 355; $\lambda^{MeOH+AlCl_3-HCl}$ nm: 245 (sh), 284, 329. GC-MS *m/z* (rel. int.): 516 $[M]^+$ (6), 501 $[M\text{-}Me]^+$ (100), 413 [M-Me-OTMSi]$^+$ (4), 369 [ring A fragment-Me] (2), 73 [TMSi] (3).

5,7,8-Trihydroxy-4′-methoxyisoflavone (8-hydroxybiochanin A) (**4**). UV λ^{MeOH} nm: 268, 310 (sh), 355; $\lambda^{MeOH+NaOAc}$ nm: 290; $\lambda^{MeOH+NaOAc-H_3BO_3}$ nm: 280; $\lambda^{MeOH+AlCl_3}$ nm: 299, 235 (sh); $\lambda^{MeOH+AlCl_3-HCl}$ nm: 284, 319 (sh), 363. The spectral data were confirmed with published data [11]. GC-MS *m/z* (rel. int.): 516 $[M]^+$ (6), 501 $[M\text{-}Me]^+$ (100), 413 [M-Me-OTMSi]$^+$ (11), 369 [ring A fragment-Me] (2), 147 [$Me_3Si\text{-}O = SiMe_2$] (2), 73 [TMSi] (3).

4′,5,6,7-Tetrahydroxyisoflavone (6-hydroxygenistein (**5**). UV λ^{MeOH} nm: 245 (sh), 270, 350 (sh); $\lambda^{MeOH+NaOAc}$ nm: 338 (sh); $\lambda^{MeOH+NaOAc-H_3BO_3}$ nm: 275, 320 (sh); $\lambda^{MeOH+AlCl_3}$ nm: 238, 248 (sh), 274, 295 (sh), 354; $\lambda^{MeOH+AlCl_3-HCl}$ nm: 245 (sh), 281, 329. The spectral data were confirmed with published data [9]. GC-MS 70 eV, *m/z* (rel. int.): 574 $[M]^+$ (5), 559 $[M\text{-}Me]^+$ (100), 471 [M-Me-OTMSi]$^+$ (7), 147 [$Me_3Si\text{-}O = SiMe_2$] (2), 73 [TMSi] (33).

Compound **6**. UV λ^{MeCN} nm: 268, 310 (sh), 350.

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