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FORMATION OF 6,7,4′-TRIHYDROXYISOFLAVONE (FACTOR 2) FROM SOYBEAN SEED ISOFLAVONES BY BACTERIA ISOLATED FROM TEMPE

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Key Word Index—*Brevibacterium epidermidis*; *Micrococcus luteus*; *Microbacterium aborescens*; tempe; biotransformation; *O*-demethylation; hydroxylation; isoflavones; glycitein; daidzein; 6,7,4′-trihydroxyisoflavone.

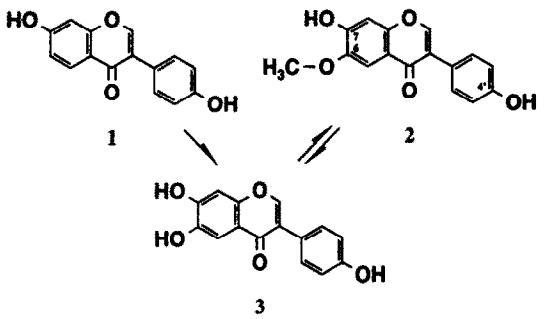
Abstract—The tempe-producing bacteria *Brevibacterium epidermidis* and *Micrococcus luteus* transformed the soybean isoflavone glycitein to 6,7,4′-trihydroxyisoflavone ('factor 2'). A third tempe-producing bacterium, *Microbacterium aborescens*, converted the soybean isoflavone daidzein to factor 2 and glycitein. The products of these transformation reactions were elucidated by spectroscopic techniques.

INTRODUCTION

Tempe is a fermented soybean food from Indonesia, mainly produced by fungi of the genus *Rhizopus*. Recent data [1], however, showed that numerous bacterial species are also involved in tempe production. For fermentation the soybeans are soaked, dehulled, cooked and incubated with microbial inocula for two days. In unfermented soybean seeds the isoflavones genistein, daidzein (**1**) and glycitein (**2**) predominantly occur as isoflavone glucosides. During tempe fermentation the isoflavone aglycones are liberated from the conjugates [2]. Furthermore, during fermentation the 6,7,4′-trihydroxyisoflavone ('factor 2') (**3**), until now only isolated from fermented soybeans, also accumulates [2, 3]. A pronounced antioxidative activity of this compound has been shown [3–6] and the 6-hydroxy group seems to be essential for this high antioxidative activity [7]. The general assumption holds that fungi of the genus *Rhizopus* are responsible for the formation of **3**. Using soybean isoflavones as substrate we have now screened numerous isolates of *Rhizopus*, yeasts and bacteria, which were all isolated from soybean soaking water and tempe samples [1] for their ability to form **3**. As substrates for the microbial transformations leading to **3**, daidzein (**1**) and glycitein (**2**) were used.

RESULTS AND DISCUSSION

During the screening experiments with the tempe-derived microorganisms we could show that the *Rhizopus* and the yeast isolates were not able to transform either **1** or **2** [8]. However, two different bacterial strains metabolized **2**, forming **3** by an *O*-demethylation reaction. These bacteria were determined as *Brevibacterium epidermides* and *Micrococcus luteus*. Another bacterium, isolated from Indonesian tempe samples containing factor 2 (**3**), transformed daidzein (**1**) yielding factor 2 (**3**) by hydroxylation in position 6 and glycitein (**2**) by a 6-*O*-methylation reaction of factor 2 (**3**). This organism was identified as *Microbacterium aborescens*. The isoflavones were extracted from suspension cultures of the bacteria, separated by HPLC and assayed by UV spectroscopy. Using UV shift data obtained with diagnostic substances [9], GC-MS data, as well as cochromatography, **3** and **2** were identified.



In the UV spectrum of **3** the absorption maximum at 324 nm in methanol showed a shift to 337 nm upon the addition of dry NaOAc–H_3BO_3 and to 341 nm upon addition of $AlCl_3$. These data suggested the presence of either 6,7- or 7,8-*ortho*-dihydroxyl groups. Lack of a shift upon the addition of $AlCl_3$–HCl showed the absence of a 5-hydroxyl group [9]. The shift of the maximum at 324 nm to 346 nm upon the addition of dry NaOAc suggested the presence of a free 7-hydroxyl group [9]. For GC-MS analysis the TMSi derivative of **3** was prepared. The peak at m/z 486 $[M]^+$ (100) indicates that compound

3 is a trihydroxyisoflavone. The identity of 3 was established by HPLC with reference compounds.

The UV spectrum of 2 with an absorption maximum at 319 nm in methanol [10] did not show a shift upon the addition of either dry NaOAc–H_3BO_3 or $AlCl_3$. These data suggested the absence of 6,7- or 7,8-*ortho*-dihydroxyl and 5-hydroxyl groups [9]. Addition of dry NaOAc followed by a shift of the absorption maximum at 319 nm in MeOH to 347 nm detected a free 7-hydroxyl group. GC-MS analysis with the TMSi derivative of 2 showed peaks at m/z 428 $[M]^+$ (100), m/z 413 $[-Me]^+$ (23) and m/z 398 $[M-H_2CO]^+$ (42) indicated that 2 is a dihydroxymonomethoxyisoflavone; 2 comigrated with glycitein.

In incubation experiments (48 hr) with *Brevibacterium epidermides* and *Microbacterium luteus*, 2 (5×10^{-5} M) was transformed to 30% resulting in the formation of 3. In suspension of *Microbacterium aborescens* the conversion of 1 (5×10^{-5} M) produced both 3 (40% yield) and 2 (15% yield) within 48 hr. Formation of both 3 and 2 only occurred in the absence of sucrose. In sucrose-containing medium (1%) conversion of 1 only led to the accumulation of 3 whereas 2 was *O*-demethylated to 3. Isoflavone conversion reactions with *Microbacterium aborescens* were not induced by substrate as shown by appropriate preincubation and control experiments. *O*-Demethylation or hydroxylation reactions are often the first step in flavonoid metabolism by microorganisms [11–17]. In that way 6-methoxy- and 6-deoxyisoflavones have been isolated from culture filtrate of *Streptomyces* species [15]. The aforementioned bacteria will now be investigated concerning their role in isoflavone metabolism during the various stages of tempe production.

EXPERIMENTAL

Bacteria. Brevibacterium epidermides (ID92-332), *Micrococcus luteus* [1] and *Microbacterium aborescens* (ID92-333) were cultivated on Merck Standard I nutrient agar and for incubation experiments for 15 hr in 100 ml Merck Standard I nutrient broth. Identification of bacteria was performed by Deutsche Sammlung für Mikroorganismen, Braunschweig, F.R.G.

Mineral medium. Na_2HPO_4 (22 mM), KH_2PO_4 (7.4 mM), $(NH_4)_2SO_4$ (7.6 mM), $MgSO_4$ (0.8 mM), $Ca(NO_3)_2$ (0.2 mM), $FeSO_4$ (0.035 mM) and micronutrients according to Pfennig and Lippert were used [18].

Incubation experiments. Prior to incubation the bacteria were washed twice with 200 ml KPi buffer (0.05M, pH 7.5). After centrifugation (10000 *g*, 15 min) 100 mg bacteria (fr. wt) were inoculated in 5 ml mineral medium and 50 μl substrate solution (DMSO–MeOH, 1:10) was applied to the bacterial culture. Substrate concn was 5×10^{-5} M. The cultures were incubated in culture tubes (200 × 16 mm) in an orbital shaker at 200 rpm, 30°.

Isolation of compounds. After 2–48 hr of incubation the transformation products were extracted twice with 5 ml EtOAc, the solvent evapd to dryness and residual material resolved in 150 μl MeOH. HPLC was conducted on RP 18 columns under isocratic conditions (1.5% HOAc–acetonitrile, 39:11, flow rate 0.8 ml min^{-1}) with a Waters chromatograph attached to a photodiode array detector for recording UV spectra.

Substrates. Compound 1 was obtained from Roth, Karlsruhe, 2 and 3 were a gift of Dr H. C. Jha, Bonn.

Analytical methods. UV spectra of the metabolites were measured in MeOH and after the addition of diagnostic reagents [7]. GC-MS measurements were performed using a Varian GC 3400 through a capillary column HP5 (25 m), with 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).

6,7,4′-Trihydroxyisoflavone (factor 2) (3). UV λ^{MeOH} nm: 257, 324; $\lambda^{MeOH+NaOMe}$ nm: 258, 348; $\lambda^{MeOH+NaOAc}$ nm: 253, 346; $\lambda^{MeOH+NaOAc-H_3BO_3}$ nm: 249, 334; $\lambda^{MeOH+AlCl_3}$ nm: 251, 3411; $\lambda^{MeOH+AlCl_3-HCl}$ nm: 255, 322. GC-MS 70 eV, m/z (rel. int.): 486 $[M]^+$ (100).

7,4′-Dihydroxy-6-methoxyisoflavone (glycitein) (2). UV λ^{MeOH} nm: 256, 317; $\lambda^{MeOH+NaOMe}$ nm: 258, 344; $\lambda^{MeOH+NaOAc}$ nm: 255, 345; $\lambda^{MeOH+NaOAc-H_3BO_3}$ nm: 317; $\lambda^{MeOH+AlCl_3}$ nm: 258, 315; $\lambda^{MeOH+AlCl_3-HCl}$ nm: 258, 315. GC-MS m/z (rel. int.): 428 $[M]^+$ (100), 413 $[M-Me]^+$ (23), 398 $[M-H_2CO]^+$ (42).

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REFERENCES

1. Baumann, U. (1992) Ph.D. Thesis, University of Münster.
2. Ebata, J., Fukuda, Y., Hirai, K. and Murata, K. (1972) *J. Agric. Chem. Soc. Jpn* **46**, 323.
3. György, P., Murata, K. and Ikehata, H. (1964) *Nature* **203**, 870.
4. Murata, K. (1985) *Asian Symposium on Non-salted Soybean Fermentation* (Aida, K., Seinusuke, U., Murata, K. and Watanabe, T., eds), p. 186. Tsukuba.
5. Murata, K. (1988) *JAOCS* **65**, 799.
6. Murakami, H., Asakawa, T., Terao, J. and Matsushita, S. (1984) *Agric. Biol. Chem.* **48**, 2971.
7. Jha, H. C., Bockemühl, S. and Egge, H. (1990) *Second Asian Symposium on Non-salted Soybean Fermentation* (Hermana, Mien, M. and Karyadi, D., eds), p. 4. Jakarta.
8. Börger-Papendorf, G. (1992) Ph.D. Thesis. University of Münster.
9. Mabry, T. J., Markham, K. R. and Thomas, M. B. (1970) *The Systematic Identification of Flavonoids*. Springer, Berlin.
10. Naim, M., Gestetner, B., Kirson, I., Birk, Y. and Bondi, A. (1973) *Phytochemistry* **12**, 169.

11. Barz, W. and Hösel, W. (1975) in *The Flavonoids* (Harborne, J. B., Mabry, T. J. and Mabry, H., eds), p. 916. Chapman & Hall, London.
12. Barz, W. and Weltring, K. M. (1985) in *Biosynthesis and Biodegradation of Wood Components* (Higuchi, T., ed.), p. 607. Academic Press, London.
13. Van Etten, H. D. (1982) in *Plant Infection* (Asada, Y., Bushnell, W. R., Ouchi, S. and Vance, C. P., eds), p. 315. Springer, Berlin.
14. Van Etten, H. D., Matthews, D. E. and Matthews, P. S. (1989) *Ann. Rev. Phytopathol.* **27**, 143.
15. Mackenbrock, K. and Barz, W. (1983) *Z. Naturforsch.* **38c**, 708.
16. Weltring, K.-M., Mackenbrock, K. and Barz, W. (1982) *Z. Naturforsch.* **37c**, 570.
17. Chimura, H., Sawa, T., Kumada, Y., Naganawa, H., Matsuzaki, M., Takita, T., Hamada, M., Takeuchi, T. and Umezawa, H. (1975) *J. Antibiotics* **28**, 619
18. Pfennig, R. and Lippert, K. L. (1966) *Arch. Microbiol.* **55**, 246.