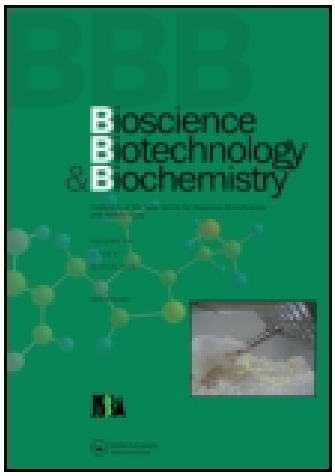

Bioscience, Biotechnology, and Biochemistry
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/tbbb20

Microbial Production of Glycyrrhetic Acid 3-O-Mono-β-D-Glucuronide from Glycyrrhizin by Cryptococcus magnus MG-27

Takashi Kuramoto[a], Yoko Ito, Mayumi Oda[a], Yukiyoshi Tamura[a] & Sumio Kitahata[ab]
[a] Research Laboratories, Maruzen Pharmaceuticals Co., Ltd., 22-1 Sanada, Takanishi-cho, Fukuyama, Hiroshima 729-01, Japan
[b] Osaka Municipal Technical Research Institute, 1-6-50, Morinomiya, Jyoto-ku, Osaka 536, Japan
Published online: 12 Jun 2014.

To cite this article: Takashi Kuramoto, Yoko Ito, Mayumi Oda, Yukiyoshi Tamura & Sumio Kitahata (1994) Microbial Production of Glycyrrhetic Acid 3-O-Mono-β-D-Glucuronide from Glycyrrhizin by Cryptococcus magnus MG-27, Bioscience, Biotechnology, and Biochemistry, 58:3, 455-458, DOI: 10.1271/bbb.58.455

To link to this article: http://dx.doi.org/10.1271/bbb.58.455

Biosci. Biotech. Biochem., **58** (3), 455–458, 1994

Microbial Production of Glycyrrhetic Acid 3-*O*-Mono-β-D-Glucuronide from Glycyrrhizin by *Cryptococcus magnus* MG-27†

Takashi KURAMOTO, Yoko ITO, Mayumi ODA, Yukiyoshi TAMURA, and Sumio KITAHATA*

Research Laboratories, Maruzen Pharmaceuticals Co., Ltd., 22–1 Sanada, Takanishi-cho, Fukuyama, Hiroshima 729–01, Japan

**Osaka Municipal Technical Research Institute, 1–6–50, Morinomiya, Jyoto-ku, Osaka 536, Japan*

Received May 31, 1993

It was found that *Cryptococcus magnus* MG-27, a yeast isolated from soil, selectively hydrolyzed the terminal β-glucuronyl linkage of glycyrrhizin (1) to yield glycyrrhetic acid 3-*O*-mono-β-D-glucuronide (MGGR, 3), a potent sweetener (relative sweetness to sucrose: ×941). The conditions for cultivation of this yeast with the maximum hydrolytic activity as well as the reaction conditions of 1 with the cells for the maximum yield of 3 were investigated. Based on the results, strain MG-27 was cultivated with a medium composed of 1.0% 1, 1.0% glucose, 0.5% Polypepton, and 0.3% yeast extract at 28°C for 24 h. The reaction of the resulting cells and 1 at pH 5.7 at 45°C for 48 h afforded 3 in a yield of 95%.

Glycyrrhizin (**1**), the well-known sweet saponin of licorice, has been used as a food-additive (relative sweetness to sucrose (RS): ×170) and as a medicine. Recently, in the study on the sweetness of glycosides of glycyrrhetic acid (**2**, the aglycone of **1**), it was disclosed that 3-*O*-mono-β-D-glucuronide (MGGR, **3**) of **2** showed potent sweetness (RS: ×941),[1] which is promising as a new sweetener.[2] It was also observed that the preventive effect of **3** against two-stage carcinogenesis was stronger than that of **1** and **2**.[3]

Previously, **3** has been synthesized by Koenigs-Knorr type glycuronylation of a methyl ester of **2**[1,4] or by hydrolysis of **1** with a lysosomal β-glucuronidase from animal liver.[5] For the selective hydrolysis of **1**, we have reported the isolation and characterization of an enzyme named glycyrrhizin hydrolase from *Aspergillus niger* GRM-3 that selectively hydrolyzes the 3-*O*-β-glucuronide linkage of **1** to produce **2** and glucuronobiose (β-D-glucuronyl-(1→2)-D-glucuronic acid) in a high yield without formation of glucuronic acid.[6]

To produce **3** from **1** on an industrial scale, we have screened microorganisms for activity to hydrolyze the terminal glucuronyl unit of **1** selectively, and isolated a yeast from soil that produced **3** from **1**.

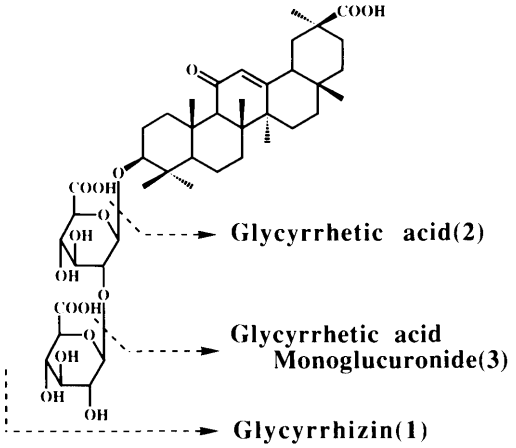

Fig. 1. Structures of Glycyrrhetic Acid (**2**) and Its Glucuronides.

Materials and Methods

Chemicals. Glycyrrhizin (**1**) used in this study was extracted and purified by our company (purity: higher than 78%), and put on the market by Wako Pure Chemical Industries, Ltd.; Code No 075-02171. Glucuronobiose was prepared from **1** by the method reported previously.[6]

Conditions of TLC. On an HP-TLC plate "Silica gel $60F_{254}$" (Merck Co., Ltd.), developed with AcOEt–MeCOEt–HCOOH–H_2O (9:3:3:1), and colored by heating at 110°C after spraying 10% H_2SO_4.

High pressure liquid chromatography (HPLC). Simultaneous analysis of **1** and **3** is possible by HPLC; column: Wakopak Wakosil $10C_{18}$ (Wako Pure Chemical Industries Ltd.) 4.6 mm × 250 mm, mobile phase: 2% AcOH–CH_3CN (55:45), flow rate: 1.0 ml/min, at 40°C, detection: UV 254 nm. However, for accurate measurements, different conditions were used in HPLC analysis of **1** and **3**, respectively. HPLC of **3** was done under the following conditions: column: YMC AQ-302 (S-5 120A ODS, YMC Co., Ltd.) 4.6 mm × 150 mm, mobile phase: 2% AcOH–CH_3CN (4:3), flow rate: 1.5 ml/min, at 42°C, detection: UV 254 nm. Conditions of HPLC for **1** is as follows: column: μ-Bondapak C-18 3.9 mm × 300 mm, mobile phase: 2% AcOH CH_3CN (20:11), flow rate: 2 ml/min, at room temperature, detection: UV 254 nm.

Monitoring of cell growth. Cell growth was monitored by analysis of cell mass by OD at 660 nm.

*Hydrolysis activity (**3** producing activity).* In the experiments on cultivation of strain MG-27 for investigation of the effects of carbon and nitrogen sources as well as a study of the course of the cultivation, the hydrolysis activity was measured under the following conditions. The cells obtained by centrifugation (10,000 rpm) of a culture broth (5 ml) in each experiment were used for the hydrolysis of **1** to **3**. A mixture of 50 mg of **1** and the cells in 1.0 M acetate buffer (pH 5.5, 5 ml) was incubated at 40°C for 1 h with stirring. The content of **3** in a 1-ml sample was measured by HPLC, and the activity was defined as the produced amount of **3** per hour per ml under the assay conditions (mg(**3**)/ml/h).

Analysis of glucose. The mutarotase GOD method for glucose, a CII-Test-Wako (Wako Pure Chemical Industries Ltd.) was used.

NMR and FBMS. ^{1}H-NMR was recorded in C_5D_5N with a JEOL JNM GX-400 spectrometer at 400 MHz. FBMS was recorded with a JEOL JMS SX-102 spectrometer.

Results and Discussion

*Screening of microorganisms for production of **3** from **1***

Twenty soil samples were collected around the factory of

† An oral presentation of this paper was made at the Annual Meeting of Agricultural Chemical Society of Japan (March 30–April 2, 1990).

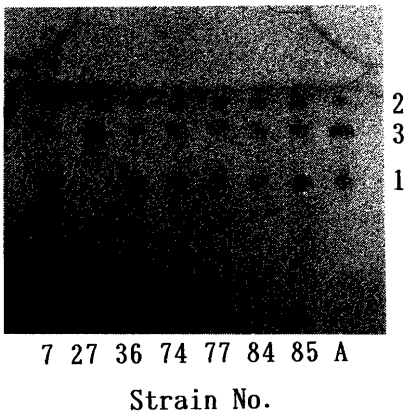

Fig. 2. Thin Layer Chromatograms of Culture Broth in Screening of Microorganisms

Conditions: see Materials and Methods. Lane A, standard samples; **1** = glycyrrhizin, **2** = glycyrrhetic acid, **3** = MGGR.

this company, Mukaihigashi-cho, Onomichi-shi, Hiroshima-ken. About 0.1 g of each soil sample was suspended in 10 ml of sterilized water, and 100 μl of the supernatant was spread on an agar plate which contained **1** as a sole carbon source; 1.0% **1**, 0.3% $NaNO_3$, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, and 1.7% agar (pH 7.0, adjusted with 1.0 N NaOH). The cultivation was done at 30°C for 3–7 days. Typical colonies grown on these plates were further inoculated in a liquid medium consisted of 1.0% **1**, 0.5% yeast extract, 0.3% $NaNO_3$, 0.1% K_2HPO_4, and 0.05% $MgSO_4 \cdot 7H_2O$ at pH 7.0 (adjusted with 1.0 N NaOH) on a reciprocal shaker (110 strokes/min) at 30°C for 7 days. Each culture broth was analyzed by TLC (Fig. 2).

Most of the strains slowly hydrolyzed **1** non-selectively, affording **2** through **3** together with unchanged **1**. Only one strain, named MG-27, hydrolyzed **1** rapidly to give **3** selectively without formation of **2** even after 1 week of cultivation. It was also observed that the activity was mostly detected not in the culture broth but in the cells. Since a microorganism that hydrolyzed **1** to **3** efficiently, was found in this screening, no further screening under other conditions has been done.

Morphological investigation suggested that the strain MG-27 was a kind of yeast. Base on the taxonomical characterization of yeast,[7] morphological, physiological, and biochemical investigation (Table) led to identification of the strain as *Cryptococcus magnus*. We now designated this strain as *C. magnus* MG-27.

Effects of carbon and nitrogen sources on growth and the hydrolysis activity of strain MG-27

It is noteworthy that in the medium without carbon source other than **1**, the growth of the cells was rather slow. With regard to an additional carbon source, strain MG-27 was cultivated in a medium composed of 1.0% **1**, 0.5% Polypepton, 0.3% yeast extract, 0.1% K_2HPO_4, and 0.05% $MgSO_4 \cdot 7H_2O$ with 1% solution of one of the following carbon sources on a reciprocal shaker (110 strokes/min) at 28°C for 3 days; glucuronic acid, glucose, fructose, maltose, sucrose, starch, and glucuronobiose (β-D-glucuronyl-(1→2)-D-glucuronic acid).[6] It was found that addition of the glucuronobiose, the sugar moiety of **1**, afforded the strongest activity. Glucose, fructose, and sucrose also led to fairly strong activity and good cell growth, while glucuronic acid was not effective for the production of the activity or for cell growth. From the economic viewpoint, glucose was used for the cultivation. It was also found that no significant influence on inducing the activity was observed for the amount of **1** up to the concentration of 0.5%.

Table Taxonomic Characteristics of *Cryptococcus magnus* MG-27

Cell morphology and cell size	:	Round-ovoid, 5.0–10.0 × 6.0–15.0 μm
Growth on YM agar	:	Cream colored, smooth
Pseudomycelium	:	−
Ascospore	:	−
Assimilation of carbon compounds		
Positive	:	Glucose, galactose, maltose, sucrose, lactose, cellobiose, inositol, xylose, raffinose, mannitol, starch, succinate, citrate, arabinose, inulin, glycerol
Negative	:	Melibiose, erythritol, galactitol, rhamnose, ribitol
Assimilation of nitrate	:	−
Growth in vitamin-free medium	:	−
Starch formation	:	+
Urease	:	+
Gelatin liquefaction	:	−
Growth on 50% (w/w) glucose-YM agar	:	−
Growth at 37°C	:	−

+, positive; −, negative.

With regard to nitrogen source, strain MG-27 was cultivated in a medium composed of 1.0% **1**, 1.0% glucose, 0.1% K_2HPO_4, and 0.05% $MgSO_4 \cdot 7H_2O$ with one (or a combination) of the following nitrogen sources on a reciprocal shaker (110 strokes/min) at 28°C for 3 days; $NaNO_3$, Polypepton, yeast extract, Trypton, beef extract, and corn steep liquor (at concentration of 0.8%). It was found that corn steep liquor and a mixture of yeast extract and Polypepton were significantly effective for the production of the hydrolysis activity, while beef extract was less effective and no activity was observed for the cultivation with $NaNO_3$ or Polypepton.

It was also found that addition of K_2HPO_4 and $MgSO_4$ is not always necessary for the cell growth and induction of the hydrolysis activity.

Course of the cultivation

Because of the above results, strain MG-27 was cultivated with a medium (2.5 liters) composed of 1.0% **1**, 1.0% glucose, 0.5% Polypepton, and 0.3% yeast extract in a 5-liter jar fermentor (Takasaki Co., Ltd.) at 28°C under aeration (1 vvm) and agitation (250 rpm). The courses of consumption of **1** and glucose as well as the cell growth and the hydrolysis activity, are shown in Fig. 3. In the first stage of the cultivation, glucose was consumed exclusively as a carbon source without consumption of **1**. Just when glucose disappeared in the medium (18 h), assimilation of **1** started with concomitant increase of the hydrolysis activity. The cells with the maximum hydrolysis activity were obtained after cultivation for 24 h.

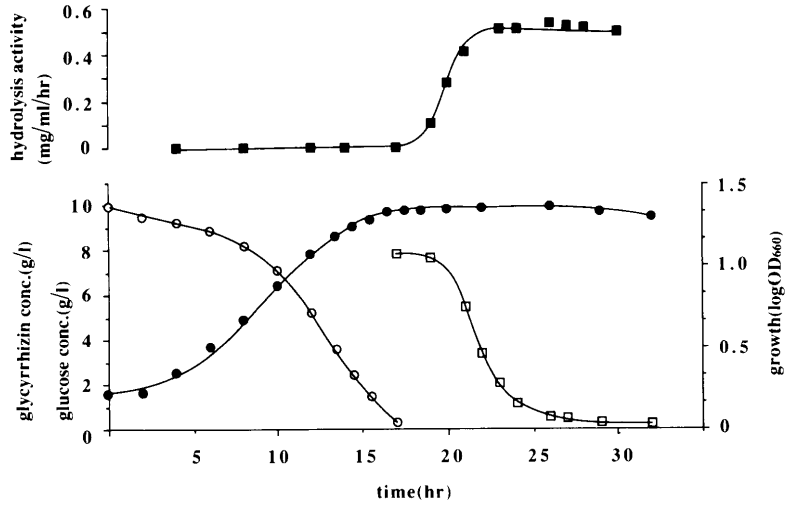

Fig. 3. Course of Cultivation of *Cryptococcus magnus* MG-27

Conditions: see the text. □, glycyrrhizin(1); ○, glucose; ■, hydrolysis activity; ●, growth.

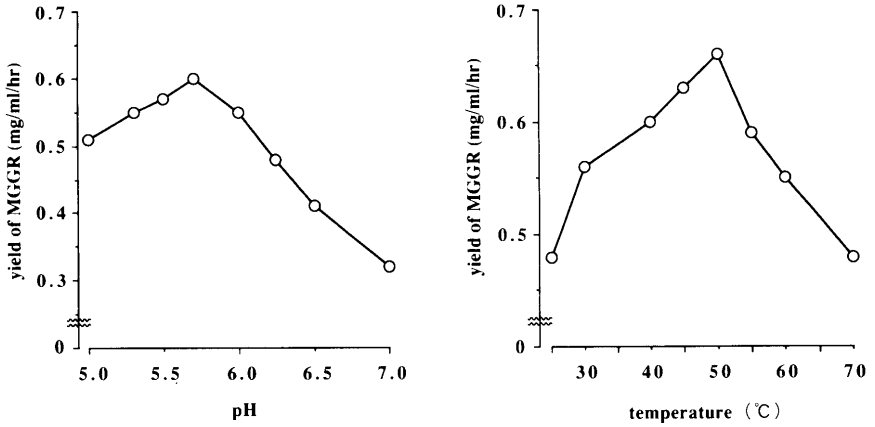

Fig. 4. Effects of pH and Temperature on Production of Glycyrrhetic Acid Mono-β-glucuronide(3) from Glycyrrhizin(**1**) by *Cryptococcus magnus* MG-27.

Conditions: see the text.

Effects of pH and temperature on production of **3**

Strain MG-27 was cultivated under the above conditions for 24 h and the cells were harvested by centrifugation at 10,000 rpm for 10 min then washed with saline solution. The optimum pH and temperature for the production of **3** with the cells were investigated. A mixture consisting of the cells (1 g wet) and 1% solution of **1** (10 ml) in 1.0 M acetate or 1.0 M phosphate buffer solution at various pHs between 5.0 and 7.0, was incubated at 40°C for 1 h. The yield of **3** at each pH was measured by HPLC. It was found that the maximum yield of **3** was observed at pH 5.7 as shown in Fig. 4. A mixture of the cells (1 g wet) and 1% solution of **1** (10 ml) in 1 M acetate buffer at pH 5.7 was incubated at a variety of temperatures (20–65°C) for 1 h. Yield of **3** at each temperature was analyzed by HPLC. It was demonstrated that the maximum production of **3** from **1** was obtained at 50°C as shown in Fig. 4. In consideration of the stability of the cells for recycling, the reaction was done at 45°C.

Production and isolation of **3**

Based on these results, the microbial production of **3** from **1** was done as follows. A mixture of **1** (10 g) and the cells (10 g wet) in H_2O (1000 ml, pH 5.7 adjusted with 1.0 N NaOH) was incubated at 45°C with stirring until no starting material (**1**) was detected in the mixture (for 48 h). The mixture was centrifuged at 8000 rpm for 15 min. MeOH (670 ml) was added to the supernatant and the pH was adjusted to 4.0 with 1 N HCl. The solution was absorbed on a column of Diaion HP-20 (500 ml, Mitsubishi Kasei Co., Ltd.) and the column was eluted with 40% MeOH (2500 ml) and then with 90% MeOH (2500 ml) which is sufficient enough to desorb **3** completely. The 90% MeOH eluate was concentrated to remove MeOH and the resulting precipitate was collected by centrifugation to give **3** (7.5 g, yield: 95%). Recrystallization of the product from aqueous MeOH gave completely pure **3** for identification.

Identification of **3** was established by comparison of mp, optical rotation, elemental analysis, and FBMS, UV, and NMR spectra, with those of an authentic sample.[1] Compound **3**: colorless crystals, mp 213–218°C (decomp.), $[\alpha]_D^{20}$ +79.4° (*c* 1.0, MeOH), UV λ_{max} (MeOH) nm (ε): 249 (11,400), FBMS *m/z* 685 (M^++K), 669 (M^++Na), 647 (M^++H), NMR (in C_5D_5N) δ_H 5.20 (1H, doublet, J=7.7 Hz, anomeric proton). Found: C, 65.18; H, 8.55. Calcd. for $C_{36}H_{54}O_{10}\cdot H_2O$: C, 65.04; H, 8.49.

This study is significant for the production of **3** from **1** on an industrial scale.

References

1) K. Mizutani, Y. Tamura, T. Kuramoto, N. Ohtake, S. Doi, M. Nakaura, and O. Tanaka, Abstracts of Papers, the 110th Annual Meeting of Pharmaceutical Society of Japan, Sapporo, August 1990, p. II-183; K. Mizutani, T. Kuramoto, Y. Tamura, N. Ohtake, S. Doi, M. Nakaura, and O. Tanaka, *Biosci. Biotech. Biochem.*, **58**, 554–555 (1994).
2) T. Kuramoto and M. Yamamoto, *Shokuhin Kogyo*, **33**, 46–50 (1990); T. Kuramoto and M. Yamamoto, *New Food Industry*, **33**, 17–20 (1991).
3) K. Mizutani, Biological Activities, Production and Utilization of Chemical Constituents of Licorice, Symposium of Food Phytochemicals for Cancer Prevention, Division of Agric. and Food Chem., 204th ACS Natl. Meeting, Washington D.C., Abstract Part 1, AGFD-188 (1992).
4) C. H. Brieskorn and J. Lang, *Arch. Pharm.*, **311**, 1001–1009 (1978); M. Kanaoka, S. Yano, H. Kato, and T. Nakada, *Chem. Pharm. Bull.*, **34**, 4978–4983 (1986); M. Hirooka, N. Norishima, E. Kaji, Y. Moari, and S. Zen, *Yakugaku Zasshi*, **109**, 544–559 (1989).
5) T. Akao, T. Akao, M. Hattori, M. Kanaoka, K. Yamamoto, T. Namba, and K. Kobayashi, *Biochem. Pharm.*, **41**, 1025–1029 (1991).
6) T. Muro, T. Kuramoto, K. Imoto, and S. Okada, *Agric. Biol. Chem.*, **50**, 689–692 (1986); Y. Sasaki, T. Morita, T. Kuramoto, K. Mizutani, R. Ikeda, and O. Tanaka, *Agric. Biol. Chem.*, **52**, 207–210 (1988).
7) L. Rodringues de Miranda, in "The Yeasts, a Taxonomic Study," 3rd revised and enlarged edition, ed. by N. J. W. Kreger-van Rij, Elsevier, Amsterdam, 1984, pp. 845–872.