Isolation of Bacterial Strains, Which Hydrolyze Glycyrrhizin and Produce Glycyrrhezic Acid, from Soil

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We isolated eight bacterial strains which could hydrolyze glycyrrhizin to glycyrrhezic acid. The bacterial strains were identified as three strains of *Pseudomonas saccharophila*, two of *Plesiomonas* sp., one of *Pseudomonas stutzeri*, one of *Klebsiella pneumoniae* subsp. *ozaenae* and one of *Kluyvera ascorbata*. Their capacity for the conversion of glycyrrhizin to glycyrrhezic acid was assayed by high performance liquid chromatography. *P. saccharophila* 11 was the most effective among the eight strains. Then, β -glucuronidase, which is responsible for hydrolysis of glycyrrhizin, activity was assayed with *p*-nitrophenyl- β -D-glucuronide as a substrate. *P. saccharophila* 11 showed the highest β -glucuronidase activity among the eight strains. This indicates that *P. saccharophila* 11 may be useful for production of glycyrrhezic acid from glycyrrhizin by industrial fermentation.

Keywords — glycyrrhizin; glycyrrhezic acid; β -glucuronidase; hydrolysis; bioconversion

Introduction

Glycyrrhezic acid is used in the preparation of drugs for detoxification. The production of glycyrrhezic acid has been carried out through the hydrolysis of glycyrrhizin. Glycyrrhizin consists of one molecule of glycyrrhezic acid and two molecules of glucuronic acid, conjugated through β -glycosidic linkage. Thus, hydrolysis of glycyrrhizin gives the two molecules of glucuronic acid and glycyrrhezic acid. This reaction has been carried out under acid conditions in the presence of hydrochloric acid. However, hydrolysis with an acid is not so efficient and side products are produced. On the other hand, β -glucuronidase hydrolyzes β -glucuronide compounds. Thus, it is considered that β -glucuronidase hydrolyzes glycyrrhizin to glycyrrhezic acid. β -Glucuronidases from *Escherichia coli* or *Helix pomatia*, are available from commercial suppliers. The *E. coli* β -glucuronidase gene (*uidA*) has been genetically elucidated,¹⁻⁴⁾ and used as a reporter gene for plants and fungi.⁵⁻⁹⁾ The β -glucuronidase of *Helix pomatia* converts glycyrrhizin to glycyrrhezic acid. However, it is expensive when one considers its use for the industrial production of glycyrrhezic acid from glycyrrhizin. Therefore, if a bacterial strain which produces extracellular β -glucuronidase

can be isolated, it could be used to hydrolyze glycyrrhizin and thus to efficiently produce glycyrrhezic acid. Indeed, it was reported that some strains of anaerobic enteric bacteria, *Pep-
tostreptococcus intermedius*, *Ruminococcus* sp. and *Eubacterium* sp., could convert glycyrrhizin to glycyrrhezic acid through the hydrolysis of β -glycosidic bonds.^{10,11)} However, these strains are strictly anaerobic, hence it is considered that they are difficult to use for production of glycyrrhezic acid at industry scale.

In this paper, we describe the isolation of bacterial strains that are able to hydrolyze glycyrrhizin, and their identification and characterization.

Materials and Methods

Bacterial Strains — Bacterial strains with the ability to grow on the minimal medium supplemented with glycyrrhizin were isolated from soil. Soil samples were collected in Chiba prefecture.

Media — Nutrient broth and agar were used for the growth of the isolated bacterial strains. NCE medium¹²⁾ (no carbon-no citrate E medium) supplemented with 0.65% of glycyrrhizin was used for the selection of bacterial strains which are able to hydrolyze glycyrrhizin and for

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quantitative analysis of the hydrolysis of glycyrrhizin and the production of glycyrrheic acid. NCE agar supplemented with 0.65% of glycyrrheic acid. NCE agar supplemented with 0.65% of glycyrrhizin was used for the selection of strains able to hydrolyze glycyrrhizin. The medium and agar plates were incubated at 27 °C for several days.

Chemicals — *p*-Nitrophenyl- β -D-glucuronide (*p*NPG), 2-mercaptoethanol and 2-amino-2-methyl-1,3-propanediol were purchased from Nakarai Chemicals Co., Ltd., and glycyrrhizin from Sigma Co. Glycyrrheic acid was kindly provided from Tokiwa Phytochemical Co., Ltd.

Isolation of Bacterial Strains Which Hydrolyze Glycyrrhizin — One gram of soil was mixed with 10 ml of NCE medium supplemented with 0.65% of glycyrrhizin. The mixture was incubated at 27°C for several days with shaking. The culture which showed bacterial growth was diluted 100-fold with NCE medium supplemented with 0.65% of glycyrrhizin and then incubated at 27 °C. This step was repeated two times. Then the culture was plated onto NCE agar supplemented with 0.65% of glycyrrhizin and incubated at 27 °C. A single colony was purified on the same agar plate two times. The single colony was inoculated into 10 ml of NCE medium supplemented with 0.65% of glycyrrhizin and then incubated with shaking. The culture was subjected to high performance liquid chromatographic (HPLC) analysis for the quantitation of glycyrrhizin and glycyrrheic acid.

Quantitative Analysis of Glycyrrhizin and Glycyrrheic Acid — A culture which had been grown in NCE medium supplemented with 0.65% of glycyrrhizin was centrifuged and the supernatant was collected (sample S). The pellet was dried under vacuum and then dissolved in methanol (sample P). HPLC was carried out on an ODS column (Capcell Pak C18, 4.6 $\times$ 250 mm; Shiseido Co., Ltd.). Glycyrrhizin was eluted with H₂O-acetonitrile-acetic acid = 60:40:1 (v/v/v) at the flow rate of 1.0 ml/min and glycyrrheic acid with H₂O-acetonitrile-acetic acid = 20:80:1 (v/v/v) at the flow rate of 1.5 ml/min. Retention times of glycyrrhizin and glycyrrheic acid are 8.0 and 4.75 min, respectively. Sample S and sample P were subject-

ed to assay for glycyrrhizin and glycyrrheic acid. Glycyrrhizin and glycyrrheic acid were detected by the absorption at 254 nm and were quantitatively analyzed by using calibration lines obtained with authentic samples of glycyrrhizin (0.002 — 10 mg/ml) and glycyrrheic acid (0.002 — 5 mg/ml).

Preparation of Crude β -Glucuronidase Extract — A crude β -glucuronidase extract was obtained according to the method of Novel *et al.*¹³⁾ An overnight culture in nutrient broth was diluted 100-fold with NCE medium supplemented with 0.65% of glycyrrhizin and then incubated at 27 °C with shaking until the OD₆₀₀ reached 0.5. The culture was centrifuged at 10000 rpm for 10 min and the supernatant was collected as the extracellular fraction (sample S). The pellet was washed with 20 mM sodium phosphate buffer (pH 7.1) and the cells were suspended in the same buffer. The cell suspension was adjusted to 0.1 to 0.6 mg dry weight/ml, treated with toluene (1:25 to 1:50, v/v) and then shaken at 27 °C for 30 min. This sample was used as the intracellular fraction (sample P).

Assay for β -Glucuronidase Activity — β -Glucuronidase activity was assayed according to the method described by Novel *et al.*¹⁾ The assay was performed in a mixture comprising 20 mM sodium phosphate buffer (pH 7.1), 0.1 M 2-mercaptoethanol, 1 mM *p*NPG and the enzyme sample. The reaction was started by the addition of the sample, followed by incubation at 27, 30 or 37°C for 30 min. The reaction was stopped by the addition of 2-amino-2-methyl-1, 3-propanediol (final concentration, 0.5 M) and the absorbance at 405 nm was measured with a Hitachi Model 100-10 spectrophotometer. One unit of β -glucuronidase activity was expressed as the amount of enzyme which liberated 1 nmol of *p*-nitrophenol per min. Specific activity was expressed as units per mg of dry weight.

Identification of the Bacterial Species — Identification of the isolated bacteria was carried out according to Bergey's Manual of Systematic Bacteriology.¹⁴⁾

Results

Isolation of Bacterial Strains Which Hydro-

Fig. 1. HPLC Patterns of the Extracellular and Intracellular Fractions of *P. saccharophila* A1

(A) HPLC pattern of the extracellular fraction eluted with H_2O -acetonitrile-acetic acid = 60:40:1. (B) HPLC patterns of extracellular (S) and intracellular (P) fractions eluted with H_2O -acetonitrile-acetic acid = 20:80:1. Peaks 1 and 2 correspond to glycyrrhizin and glycyrrheic acid, respectively. t_R , retention time.

lyze Glycyrrhizin

For the isolation of bacteria which can grow in the minimal medium supplemented with glycyrrhizin, each of the one hundred soil samples collected was mixed with 10 ml of NCE medium supplemented with 0.65% of glycyrrhizin followed by shaking at 27 °C for 1 to 3 d. Bacterial growth was observed in 3 soil samples.

These cultures were diluted with NCE medium and then shaken at 27 °C. This step was repeated two times. We isolated from the culture eight bacterial strains which could grow on the minimal agar supplemented with 0.65% of glycyrrhizin. On gram staining, all these bacteria were found to be gram negative and rod-shaped. On flagellar staining, six of the eight strains were

TABLE I. Bioconversion of Glycyrrhizin to Glycyrrheic Acid by the Isolated Strains

Strain	Amount of glycyrrhizin ^{a)}	Amount of glycyrrheic acid ^{a)}			Culture days
		Sample S	Sample P	Total	
<i>P. saccharophila</i> A1	1.4	0.3	1.7	2.0	2
<i>P. saccharophila</i> 11	0.01	1.4	2.5	3.9	2
<i>P. saccharophila</i> 18	3.8	—	1.6	1.6	2
<i>K. ascorbata</i> 18	5.8	—	0.3	0.3	2
<i>P. stutzeri</i> 23	5.2	—	1.6	1.6	3
<i>Plesiomonas</i> sp. 23	5.0	0.5	0.9	1.4	3
<i>Plesiomonas</i> sp. 21	4.4	0.1	1.1	1.2	3
<i>K. pneumoniae</i> subsp. ozaenae 31	1.8	0.2	1.6	1.8	3
Medium	6.5	(3.7) ^b			

a) mg/ml.

b) Amount of glycyrrheic acid when glycyrrhizin has been completely hydrolyzed.

found to have polar flagella, the other two having lateral and no flagella, respectively. On physiological and biochemical characterization, these were determined to be three strains of *Pseudomonas saccharophila*, one of *Kluyvera ascorbata*, one of *Pseudomonas stutzeri*, two of *Plesiomonas* sp. and one of *Klebsiella pneumoniae* subsp. *ozaenae*.

Determination of Glycyrrhizin Hydrolysis and Glycyrrhezic Acid Production

The eight strains were grown in NCE medium supplemented with 0.65% of glycyrrhizin, with shaking, at 27 °C for 2 or 3 d. The cultures were centrifuged and the supernatant and extract of pellets were subjected to HPLC for determination of glycyrrhizin and glycyrrhezic acid. A typical chromatogram for *P. saccharophila* A1 is shown in Fig. 1. The peak area of glycyrrhizin in the case of the *P. saccharophila* A1 culture was reduced to 1/4.5 in comparison with control of NCE medium supplemented with 0.65% of glycyrrhizin as a control. And a new peak appeared, with the retention time corresponding to glycyrrhezic acid. These results indicated that *P. saccharophila* A1 hydrolyzed

glycyrrhizin and produced glycyrrhezic acid. The chromatogram also suggested that no appreciable by product was formed in a culture of *P. saccharophila* A1. The HPLC patterns of the cultures of the other seven strains were similar to that in the case of *P. saccharophila* A1, but the extent of hydrolysis was not the same (data not shown). The glycyrrhizin and glycyrrhezic acid determined by HPLC and are shown in Table I. Three strains of *P. saccharophila* hydrolyzed glycyrrhizin and liberated glycyrrhezic acid to different extents *P. saccharophila* 11 most efficiently hydrolyzed glycyrrhizin and produced glycyrrhezic acid. The 2 strains of *Plesiomonas* sp. also hydrolyzed glycyrrhizin and produced glycyrrhezic acid, to different extents, *Plesiomonas* sp. 23 being somewhat effective. Among these eight strains, *P. saccharophila* 11 most efficiently hydrolyzed glycyrrhizin and produced glycyrrhezic acid.

Determination of β -Glucuronidase Activity

Extracellular and intracellular β -glucuronidase were prepared according to the method of Novel *et al.*¹³⁾ Enzyme activity was measured at 27, 30 and 37 °C. The results for *P. saccharophila*

Fig. 2. Effect of Culture Temperature on the Activity of β -Glucuronidase

(A) Extracellular fractions; (B) intracellular fractions. ○, *P. saccharophila* A1; ●, *P. saccharophila* 11; ■, *P. saccharophila* 18; △, *Plesiomonas* sp. 21; ▲, *Plesiomonas* sp. 23. One unit of β -glucuronidase activity is expressed as the amount of enzyme which liberates one nmol of *p*-nitrophenol per min.

A1, *P. saccharophila* 11, *P. saccharophila* 18, *Plesiomonas* sp. 21 and *Plesiomonas* sp. 23 are shown in Fig. 2. The β -glucuronidase, both extracellular and intracellular, produced by all five strains showed the highest activity at 37 °C. β -Glucuronidase activity exhibited by the three strains of *P. saccharophila* were apparently related to the capacity of hydrolysis of glycyrrhizin. The extracellular activities of the two *Plesiomonas* strains were also related to the capacity of hydrolysis. However, the activities of the other three strains, i.e., *K. pneumoniae* subsp. *ozaenae* 31, *P. stutzeri* 23 and *K. ascorbata* 18, were hardly detectable.

Discussion

We attempted to isolate bacterial strains which efficiently hydrolyze glycyrrhizin and produce glycyrrheic acid. A desirable strain would be one which uses glucuronic acid produced from glycyrrhizin as a carbon source but not glycyrrheic acid. Eight isolated strains were characterized and identified. We then examined the efficiency of hydrolysis of glycyrrhizin, the production of glycyrrheic acid and the *p*-nitrophenyl β -glucuronidase activity. Three strains out of eight were identified as species to *P. saccharophila*. These three strains showed different efficiencies in the hydrolysis of glycyrrhizin and the production of glycyrrheic acid. Determination of β -glucuronidase activities indicated that the differences are due to the differences of β -glucuronidase activity as measured with *p*NPG. Namely, *P. saccharophila* 11, the most efficient, exhibited the highest glucuronidase activity and *P. saccharophila* 18, the least efficient, exhibited the lowest activity. Despite the differences between them in the production of β -glucuronidase, the ratios of extracellular and intracellular β -glucuronidase were similar, about one-tenth of the intracellular β -glucuronidase being excreted into the culture medium. This indicates that in spite of different levels of β -glucuronidase activity, three *P. saccharophila* strains have similar efficiencies in the secretion of β -glucuronidase. The two strains of *Plesiomonas* sp. show different efficiencies in the hydrolysis of glycyrrhizin and the production of glycyrrhe-

ic acid. The two strains produced similarly low amounts of intracellular β -glucuronidase but very different amounts of the extracellular enzyme. *Plesiomonas* sp. 21 excreted about 50% of the β -glucuronidase activity into the culture medium. On the other hand, *Plesiomonas* sp. 23 excreted only about 10%. However, these differences between the two strains were not reflected in the hydrolysis of glycyrrhizin. With *p*-NPG as a substrate, the activities of the extracellular and intracellular fractions of *K. pneumoniae* subsp. *ozaenae* 31, *P. stutzeri* 23 and *K. ascorbata* 18 were hardly detectable. This suggested that these strains produced a β -glucuronidase that is not able to use *p*NPG as a substrate. It was reported that three anaerobic bacterial strains, *Rumicoccus* sp., *Peptostreptococcus intermedius* and *Eubacterium* sp., could hydrolyze glycyrrhizin to glycyrrheic acid.^{10,11} Since these bacteria are anaerobic cocci, it is considered that they may not be useful for industrial fermentation. On the other hand, our strains are gram-negative, facultatively anaerobic and aerobic rods. Thus, *P. saccharophila* strains isolated by us, are useful for industrial production of glycyrrheic acid from glycyrrhizin at large-scale fermentation.

Eubacterium produces two types of β -glucuronidase. One type hydrolyzes *p*NPG, 4-methylumbelliferyl mono- β -D-glucuronide (MUG), etc., but not glycyrrhizin. The other type hydrolyzes glycyrrhizin. We did not examine whether or not our strains produce two types of β -glucuronidases. We are preparing to investigate this possibility by means of mutation analysis involving Tn 5-induced mutagenesis.

The β -glucuronidase activity of the *P. saccharophila* strains is highest at 37 °C. Thus, if this activity is responsible for the hydrolysis of glycyrrhizin, it is expected that the bioconversion of glycyrrhizin to glycyrrheic acid is most efficient at 37 °C, but *P. saccharophila* grows better at 27 °C rather than 37 °C. Therefore, we will attempt to clone β -glucuronidase gene from the *P. saccharophila* strains into *Escherichia coli*. If this is possible, it is expected that the resultant *E. coli* clones will be more efficient in the bioconversion than the *P. saccharophila* strains.

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