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ESTABLISHMENT OF *SALMONELLA* STRAIN EXPRESSING CATALYTICALLY ACTIVE HUMAN UDP-GLUCURONOSYLTRANSFERASE 1A1 (UGT1A1)

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Summary

Human uridinediphosphate-glucuronosyltransferase 1A1 (UGT1A1) was expressed in *Salmonella typhimurium* TA1535 cells by transfection of the cells with plasmids carrying the UGT1A1 cDNA. UGT1A1 cDNA was isolated by a polymerase chain reaction from human liver total RNA and was inserted into the pSE420 plasmid, linked to the *trc* promoter and terminator. The plasmid thus constructed was introduced into *Salmonella* TA1535 cells. The expression of human UGT1A1 protein was confirmed by Western blot analysis. The maximal expression was observed at 24 h after the addition of isopropyl-β-D-thiogalactopyranoside, an inducer. However, the bilirubin conjugation activity of the membrane fraction from the *Salmonella* cells was not detectable. When a β-glucuronidase inhibitor such as saccharic acid 1,4-lactone, glycyrrhizin or 1-naphtyl-β-D-glucuronide was added to the reaction mixture, the bilirubin conjugation activity of the human UGT1A1 was detected. When geniposide was added to the reaction mixture, the bilirubin conjugation activity of UGT1A1 was not seen. Taking these results into account, the established *Salmonella* strain possesses the β-glucuronidase activity. Since the β-glucuronidase activity of the *Salmonella* was lower than that of *E. coli*, it was concluded that *Salmonella* seemed to be a good host to express UGT protein. This is the first study to demonstrate the establishment of a bacterial strain expressing native human UGT protein showing catalytic activity.

Key Words: human UGT1A1, heterologous expression, *Salmonella typhimurium*, β-glucuronidase inhibitor, saccharic acid 1,4-lactone

Glucuronidation is one of the major drug biotransformation reactions. The transfer of glucuronic acid from uridinediphosphate (UDP)-glucuronic acid (UDPGA) to an aglycon is catalyzed by UDP-glucuronosyltransferase (UGT). The human UGT superfamily is composed of families; two of the families have been identified as having the ability to catalyze the oxidation of foreign chemicals (1). The human UGT gene contains coding information for the production of more than 10 known microsomal membrane bound isozymes in man. The UGT1 family is known to be

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responsible for the conjugation of endogenous and exogenous chemicals such as bilirubin, imipramine and phenolic compounds (2-10, 23-25), while the UGT2 family is involved in the metabolism of steroids and xenobiotics including many drugs such as ibuprofen, ketoprofen and naproxen (11-22). Human UGT1A1 is one of the major isozymes in the UGT1 family and is expressed in the human liver, but not in the kidney. The UGT1A1 is also called HUG-Br1 because of its capacity to conjugate bilirubin. However, little is known regarding the role of UGT1A1 in conjugation of xenobiotics.

The use of enzyme preparations expressed in heterologous expression systems has become more popular for the examination of enzymes involved in drug metabolism in humans, partly because the preparation possessing the same properties can be supplied constantly. Thus, efforts made in recent years have realized the expression of several UGT isoforms in cultured mammalian cells (2-8, 10-22). In previous studies, COS-1 cells (2,4, 12, 15, 17), COS-7 cells (6, 10-11, 14, 16, 20, 22, 25), HEK293 cells (5, 18-19, 21, 24), V79 cells (3, 7-8, 13, 23) were used to express human UGT isoforms. Moreover, *Spodoptera frugiperda* cells infected with the baculovirus harboring a plasmid carrying rabbit UGT2B13 produced high level of UGT protein (26). Recently, heterologous expression systems using bacteria such as *Escherichia coli* (*E. coli*) and *Salmonella* were established to express many enzymes including cytochrome P450s (27-32). These systems have some advantages compared to other expression systems in terms of the relatively high expression level of protein with a short period of incubation, ease of use and low cost to maintain. Therefore, if a bacterial strain expressing human UGT1A1 is successfully established, then it will become a useful tool to analyze and characterize the metabolism by this isozyme. Although there are three reports on the expression of UGT protein in *E. coli* cells (33-34), catalytic activity has been observed only in the case of applying UGT2B4 protein purified from *E. coli* cells (35). There has been no report on the detection of catalytic activity of UGT protein expressed in intact *E. coli* cells or membrane fraction prepared from *E. coli* cells for unknown reasons. We noticed the fact that most of the *E. coli* strains possessed β-glucuronidase in cytosol fraction (36-43), which was expected to cause the deconjugation of the glucuronide produced by the UGT enzyme. It was reported that *Salmonella typhimurium* strains did not possess the β-glucuronidase (44-46). Thus, it seemed possible that the glucuronides formed by the UGT expressed in the *Salmonella* cells could be detected. In the present study, we established a new genetically engineered *Salmonella* strain expressing human UGT1A1 and detected the activity with bilirubin as a substrate. We detected the catalytic activity of UGT1A1 only in the presence of chemicals known as inhibitors of β-glucuronidase.

Methods

Materials

Bilirubin was obtained from Wako Pure Chemicals (Osaka, Japan), saccharic acid 1,4-lactone, 1-naphtyl-glucuronide and *p*-nitrophenyl-β-D-glucuronide from Sigma Chemical (St. Louis, MO), UDPGA from Yamasa-Syoyu (Choshi, Japan), respectively. Human UGT1A1 western blotting kit (WB-UGT1A1) and pooled human liver microsomes H161 were purchased from Gentest (Woburn, MA). Baicalin, baicalein, glycyrrhizin and geniposide were kind gifts from Tsumura Pharmaceutical Company (Tsukuba, Japan). All other chemicals and solvents were of the highest grade commercially available. The expression plasmid pSE420 was obtained from Invitrogen (Carlsbad, CA).

Isolation of human UGT1A1 cDNA

cDNA coding for UGT1A1 was obtained from human liver total RNA by the reverse transcriptase (RT)-polymerase chain reaction (PCR) method. PCR was performed dividing the whole sequence into two parts. The N-terminal 748 nucleotides were amplified using two specific primers. The

sense primer corresponded to the nucleotides from –12 to 8 of UGT1A1, where the nucleotide 1 corresponded to the first nucleotide of the start codon ATG; 5'-AGCAAAGGCGCCATGGCTGT-3'. The underlined nucleotide sequence was the naturally occurred *Nco* I site. The antisense primer corresponded to the nucleotides from 717 to 736, 5'-CCTGGACAGTCACCTCTCTC-3'. The C-terminal portion of UGT1A1 cDNA was amplified by using the sense primer that corresponded to the nucleotides from 677 to 696, 5'-TTTATTCCCCGTATGCAACC-3', and the antisense primer that corresponded to nucleotides from 1635 to 1654, 5'-TGGAAATGACTGGGAATGG-3'. The nucleotide sequence of the amplified two PCR fragments were confirmed by DNA sequencer ABI PRISM™ 377 (Perkin Elmer). These fragments were ligated using an internal *Eco* RI site at nucleotide 850.

Construction of expression plasmid

The *Hind* III site was introduced by site-directed mutagenesis at the stop codon of UGT1A1 cDNA. The antisense primer corresponding to nucleotides from 1602 to 1625, 5'TTACCTTATTTCCCACAAGCTTCT-3', was used to introduce the *Hind* III restriction site (underlined nucleotides). The UGT1A1 cDNA was ligated into the pSE420 expression plasmid at the sites of *Nco* I and *Hind* III. The *trc* promoter was linked to the top of cDNA coding for UGT1A1, and a transcriptional terminator was ligated to the end of the cDNA. *Salmonella* TA1535 cells were transformed with the pSE/UGT1A1 plasmid, which was modified by *Salmonella typhimurium* LB5000 cells (R^-, M^+; (47)).

Determination of the expression of human UGT1A1 in Salmonella cells

Salmonella TA1535 cells transformed with a plasmid DNA were grown overnight at 37℃ in Luria-Bertani (LB) medium containing 50 μg/mL ampicillin. A 1 mL aliquot was used to inoculate 100 mL of modified Teriffic Broth (TB) media (48) in a 500 mL flask. When the absorbance of the culture medium measured at 600 nm reached 0.5, induction of the *trc* promoter was initiated by the addition of 1.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The mixture was allowed to incubate at 37℃ for 8 to 60 h with vigorous shaking (120 rpm). Cells were harvested and resuspended in 10 mL of 100 mM Tris-acetate buffer (pH 7.6) containing 0.5 M sucrose and 0.5 mM ethylendiaminetetraacetic acid (EDTA), and were frozen at –80℃ until use. The expression of UGT1A1 protein in the *Salmonella* was confirmed by Western blot analysis with *Salmonella* whole cells. The protein concentration was determined by the method of Lowry et al. (49). Sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed with a 10 % acrylamide gel according to the method of Laemmli (50). The separated proteins were transferred onto an Immobilon membrane (Waters-Millipore, Bedford, MA). Immunoblot detection was performed using human UGT1A1 Western blotting kit according to the instruction of the manufacture. The existence of UGT1A1 protein was visualized by staining with 3'-diaminobenzidine.

Assay of catalytic activity for human UGT1A1 expressed in Salmonella cells

The bilirubin glucuronidation was assayed by the electrophotometrical determination of metabolites as described elsewhere with minor modifications (51-52). The enzyme assay was carried out with a membrane fraction prepared from genetically engineered *Salmonella* cells carrying human UGT1A1 cDNA. The membrane fraction was prepared as reported by Sandhu et al. (53). The protein concentration of the membrane fraction was determined by the methods as described above. To exert catalytic activity of UGT1A1 expressed in the membrane fraction, lipid bilayer of the membrane fraction was solubilized by treating with cholic acid in a mixture consisting of 7.04 mg of the membrane protein/mL, 0.4% cholic acid and 0.2 M potassium-phosphate buffer (pH 7.6) at 0℃ for 40 min in a final volume of 500 μL. Subsequently, a 100 μL aliquot was treated with digitonin, for further solubilization of lipid bilayer of the membrane fraction, in a reaction mixture consisting of 4.40 mg protein/ mL, 0.188 M Tris-malic

acid-22.5 mM $MgCl_2$ buffer (pH 7.7) and 2.64 mg digitonin/mL (0.60 mg digitonin/mg protein) at 0℃ for 4 h in a final volume of 160 μL. Incubations with bilirubin were performed under dim red light. Incubation mixture consisted of 100 mM Tris-malic acid-12 mM $MgCl_2$ buffer (pH 7.7), 5 mM UDPGA, 0.5 mM bilirubin and 2.35 mg/mL of solubilized membrane protein in a final volume of 300 μL. When necessary, various concentrations of a β-glucuronidase inhibitor such as saccharic acid 1,4-lactone, glycyrrhizin, 1-naphtyl-glucuronide or *p*-nitrophenyl-β-D-glucuronide was added to the incubation mixture (54-56). Reaction was initiated by the addition of UDPGA. Incubation was carried out at 37℃ for 15 min. The reaction was terminated by the addition of ice-cold glycin-HCl buffer (pH 2.6). After the diazo-coupling reaction, bilirubin glucuronide was determined by measuring the absorbance at 530 nm due to the formation of mono- and di-bilirubin glucuronide converted to dipyrrolic azo derivatives. All assays were carried out in duplicate.

Assay of β-glucuronidase activity in Salmonella cells

The β-glucuronidase activity probably contaminated in the membrane fraction prepared from *Salmonella* cells was measured using baicalin and *p*-nitrophenyl-β-D-glucuronide as substrates. The β-glucuronidase activity for *p*-nitrophenyl-β-D-glucuronide in the membrane fraction prepared from established *Salmonella* cells was compared with that of *E. coli* DH5α cells. The membrane fraction from *Salmonella* cells or *E. coli* cells was treated with detergents in the same manner as mentioned in the bilirubin glucuronidation assay. After the pretreatment of the membrane fraction with detergents sequentially, incubations were carried out at 37℃ for 30 min. The reaction mixture for the assay of baicalin deglucuronidation consisted of the same components as described for bilirubin glucuronidation assay except that UDPGA was not added. The reaction was terminated by the addition of an equal volume of 50% methanol. Analysis of the metabolite, baicalein, was performed by the method provided by Tsumura Pharmaceutical Company (Tsukuba, Japan). Briefly, the analysis of the metabolite was carried out by a high-performance liquid chromatography (HPLC) (Tosoh, Tokyo, Japan) equipped with a TSK-GEL ODS-80TM column (4.6×150 mm; 8 nm; 5 μm Tosoh; Tokyo, Japan) and a TSK ODS-80TM guard column (3. 2×15 mm). Metabolite was detected by an electron chemical detector EC-8020 (Tosoh, Tokyo, Japan). The oxidation potential was 450 mV. The mobile phase was a mixture of 1% phosphoric acid, methanol, and tetrahydrofuran at the ratio of 50:40:4. The column temperature was 50℃ and the flow rate was 1.0 mL/min. The reaction mixture for the assay of the β-glucuronidase activity with *p*-nitrophenyl-β-D-glucuronide as a substrate consisted of 50 mM Tris-malic acid-12 mM $MgCl_2$ buffer (pH 7.7), 1.76 mg/mL of the solubilized membrane fraction from *Salmonella* cells or *E. coli* cells and the various concentrations of *p*-nitrophenyl-β-D-glucuronide in the final volume of 500 μL. The reaction was terminated by the addition of 1.0 mL of 0.3 M NaOH. *p*-Nitrophenol formation was measured by the increase in the absorbance at 420 nm. Assays were carried out in duplicate.

Results

Expression of human UGT1A1 in Salmonella TA1535 cells

The expression of human UGT1A1 protein in the *Salmonella* cells was examined by Western blot analysis using the *Salmonella* whole cells. The expression of UGT1A1 protein is shown in Fig. 1. Antibodies raised to the human UGT1A1 cross-reacted with the UGTs present in liver microsomes from humans and recombinant UGT1A1 obtained by baculovirus expression system included in the WB-UGT1A1 kit. (Fig. 1). *Salmonella* cells transformed with the pSE420 plasmid did not show any signals of the expression of UGT1A1 (Fig. 1). Introduction of the pSE/UGT1A1 plasmid into *Salmonella* cells resulted in the expression of the UGT1A1 protein. After the addition of IPTG, the expression level of the UGT1A1 protein gradually increased, and the maximal expression level was obtained at 24 h after the addition of the inducer (data not shown). Thus, we harvested the *Salmonella* cells 24 h after the addition of IPTG for the following studies.

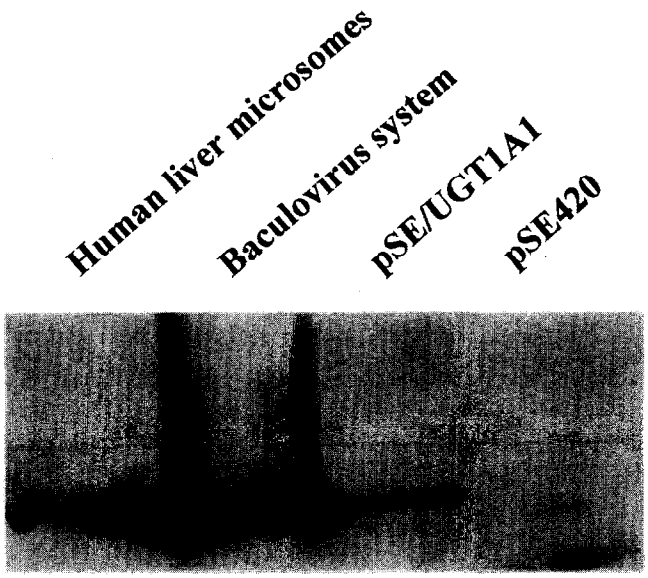

Fig. 1
Western blot analysis for the human UGT1A1 expressed in the genetically engineered *Salmonella* cells. One hundred μg protein of *Salmonella* whole cells transformed with the pSEUGT1A1 plasmid was applied. Proteins were separated by SDS-PAGE and detected by immunobloting with rabbit anti human UGT1A1. UGT1A1 protein expressed in human liver microsomes H161 and recombinant UGT1A1 protein obtained by baculovirus system included in the WB-UGT1A1 kit were applied as positive controls. *Salmonella* cells transformed with the pSE420 plasmid were applied as a negative control.

Catalytic activity of human UGT1A1 expressed in the membrane fraction of Salmonella cells
To examine the catalytic activity of human UGT1A1 expressed in the established genetically engineered *Salmonella* cells, the membrane fraction of *Salmonella* TA1535 cells transformed with the pSE/UGT1A1 or the pSE420 was prepared and provided as an enzyme source (53). As shown in Fig. 2, bilirubin conjugation activity was not observed when bilirubin was incubated with the membrane fraction prepared from *Salmonella* cells carrying the pSE/UGT1A1 or the pSE420. When bilirubin was incubated in the presence of saccharic acid 1,4-lactone, a potent β-glucuronidase inhibitor (54-55), the bilirubin conjugation activity appeared. The UGT1A1 activity increased with the concentration of saccharic acid 1,4-lactone added to the mixture. The activity was not seen using the membrane fraction prepared from *Salmonella* cells carrying the pSE420. This is the first to demonstrate the expression of UGT protein showing catalytic activity without purificatin and reconstitution of the UGT protein with an appropriate lipid. The bilirubin conjugation activity of the UGT1A1 expressed in human liver microsomes was also examined by the same method as the case of UGT1A1 expressed in the *Salmonella* membrane fraction. As was expected, the bilirubin conjugation activity was observed in the presence of 5 to 40 μM saccharic acid 1,4-lactone or in the absence of the β-glucuronidase inhibitor. The activity was 199 pmol/min/mg protein. The effects of other chemicals known as β-glucuronidase inhibitors were also examined and compared with that of saccharic acid 1,4-lactone. As shown in Fig. 3, glycyrrhizin and 1-naphthyl-β-D-glucuronide also exerted effects to apparently produce the bilirubin conjugation activity of human UGT1A1 expressed in the membrane fraction of the established *Salmonella* cells harboring pSE/UGT1A1.

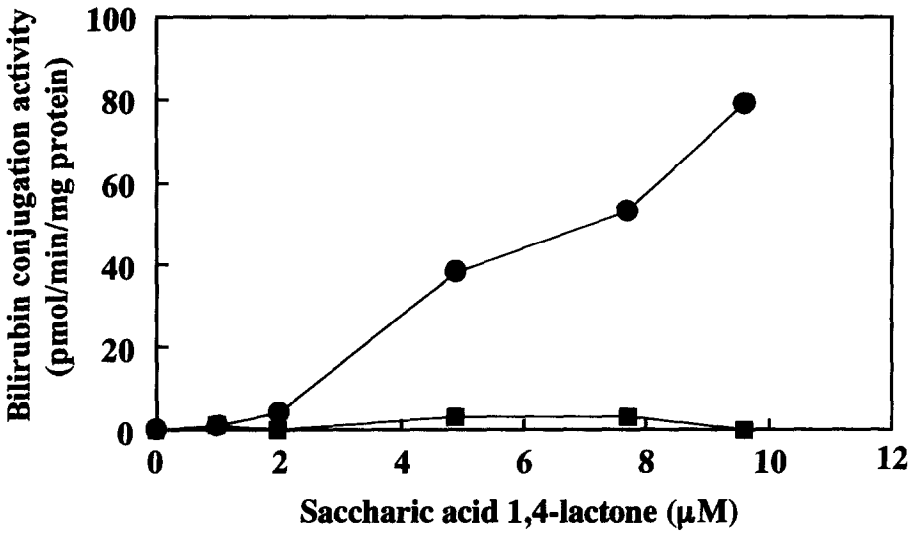

Fig. 2
Bilirubin conjugation activity of human UGT1A1 expressed in the membrane fractions of *Salmonella* cells carrying pSE/UGT1A1 or pSE420. The bilirubin conjugation activity of human UGT1A1 was determined in the presence or absence of saccharic acid 1,4-lactone. The concentrations of bilirubin and UDPGA were 500 μM and 5mM, respectively. Assays were carried out in duplicate at each inhibitor concentration and the mean values were shown. ●, *Salmonella* cells carrying pSE/UGT1A1; ■, *Salmonella* cells carrying pSE420.

The bilirubin conjugation activity increased depending on the concentration of the inhibitor. The activity was not seen with the membrane fraction from *Salmonella* cells carrying pSE420 (data not shown). The effect of saccharic acid 1,4-lactone was more pronounced than glycyrrhizin and 1-naphthyl-β-D-glucuronide. The maximal activity of UGT1A1, 79 pmol/min/mg protein, was obtained with saccharic acid 1,4-lactone at a concentration of 9.6 μM. The bilirubin conjugation activity of human UGT1A1 was observed only when the concentration of *p*-nitrophenyl-β-D-glucuronide was higher than 1.6 mM and the value was 5.7 pmol/min/mg protein (data not shown). The effects of β-glucuronidase inhibitors on the bilirubin conjugation activity was seen in the following order; saccharic acid 1,4-lactone > glycyrrhizin > 1-naphthyl-β-D-glucuronide > *p*-nitrophenyl-β-D-glucuronide. We also analyzed the effects of geniposide, a glycoside, expecting that this compound might not inhibit β-glucuronidase (56). As was expected, the bilirubin conjugation activity of UGT1A1 did not appear when the glycoside was added to the incubations. It was reported that the *Salmonella typhimurium* did not possess β-glucuronidase activity toward *p*-nitrophenyl-β-D-glucuronide (44-46). The results mentioned above suggest the existence of β-glucuronidase in the established *Salmonella* cells, the derivative of the TA1535 cells. Our results also suggest that the cytosolic β-glucuronidase may be contaminated to the membrane fraction prepared from the established *Salmonella* cells (57). Therefore, the presence of β-glucuronidase activity of the *Salmonella* cells was examined in the subsequent study.

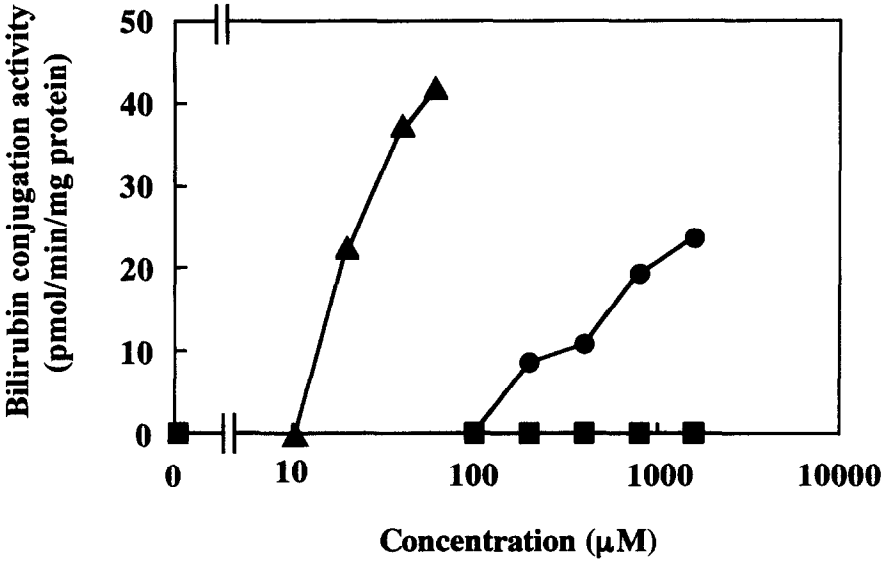

Fig. 3
Bilirubin conjugation activity of human UGT1A1 expressed in the membrane of the *Salmonella* cells carrying pSE/UGT1A1. The bilirubin conjugation activity of human UGT1A1 was determined in the presence or absence of a β-glucuronidase inhibitor. The concentrations of bilirubin and UDPGA were 500 μM and 5mM, respectively. Assays were carried out in duplicate at each inhibitor concentration and the mean values were shown. ▲, glycyrrhizin; ●, 1-naphtyl-β-D-glucuronide; ■, geniposide.

Baicalin deconjugation activity in the established Salmonella cells

Baicalein is the aglycon of baicalin and is formed from baicalin by enzymatic deconjugation of a glucuronic acid. To confirm the existence of the β-glucuronidase in the *Salmonella* cells, the formation of baicalein from baicalin was determined. The same membrane fraction from established *Salmonella* cells expressing human UGT1A1 as those used for bilirubin assay was also used in this study. As shown in Fig. 4, baicalein was formed by the incubation of the membrane fraction with baicalin. The formation of baicalein increased with the amounts of baicalin added to the incubation mixture. The activity was 73 nmol/min/mg protein at a baicalin concentration of 80 μM. Thus, we confirmed the existence of β-glucuronidase in *Salmonella typhimurium* cells, in contrast to the previous findings.

Comparison of β-glucuronidase activity in Salmonella and E. coli cells

Salmonella typhimurium strains are known to lack β-glucuronidase for *p*-nitrophenyl-β-D-glucuronide (44-46), while the above-mentioned results clearly indicated the existence of β-glucuronidase toward baicalin. Thus, it was necessary to examine the possibility that the *Salmonella* cells possessed the β-glucuronidase activity toward *p*-nitrophenyl-β-D-glucuronide. The formation of *p*-nitrophenol from *p*-nitrophenyl-β-D-glucuronide was determined using the membrane fraction from the *Salmonella* and *E. coli* DH5α cells, and compared. Most of the *E. coli* strains are known to possess β-glucuronidase for *p*-nitrophenyl-β-D-glucuronide (36-43).

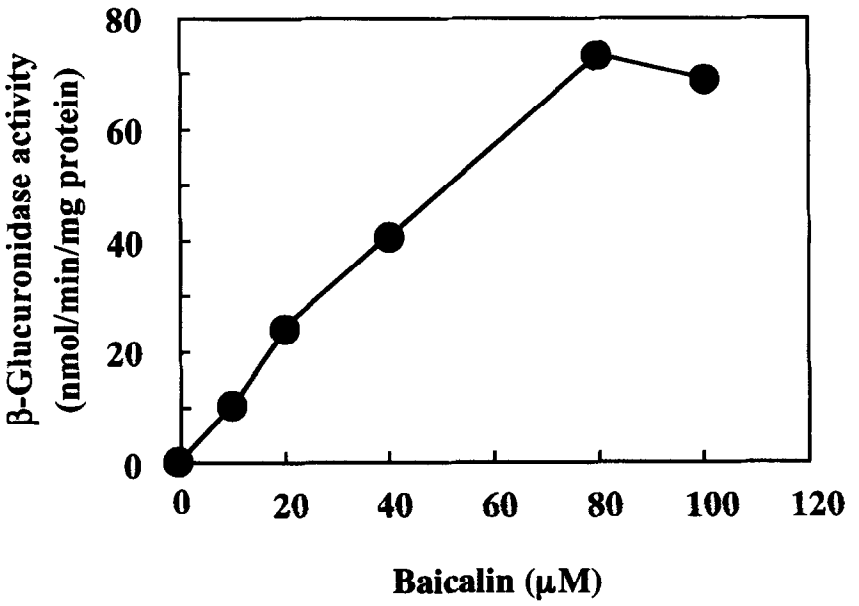

Fig. 4
Baicalin β-glucuronidase activity in the established *Salmonella* strain. Bicalein formation from baicalin in the genetically engineered *Salmonella* cells was analyzed. The membrane fraction of *Salmonella* cells expressing human UGT1A1 was used. Assays were carried out in duplicate at each concentration of baicalin and the mean values were shown.

As shown in Fig. 5, the *p*-nitrophenol formation was observed in the two bacteria. However, the activity seen in the membrane fraction from *Salmonella* cells was much lower than that seen in the membrane fraction from *E. coli* DH5α cells. The bacterial β-glucuronidase is a soluble protein (57). Both of *Salmonella* and *E. coli* membrane fractions were prepared by the same method described in methods. Therefore, the extent of the contamination of soluble proteins into membrane fraction from both strains of bacteria may be equal. The contamination of soluble β-glucuronidase into the membrane fraction may be reflected the expression level of them in the soluble fraction. Thus, we confirmed that the established *Salmonella* strain derived from *Salmonella* TA1535 possesses low but significant amounts of β-glucuronidase for *p*-nitrophenyl-β-D-glucuronide.

Discussion

Enzymes produced in a heterologous expression system can be used for a wide variety research such as drug metabolism and toxicological studies and structure-function analysis (58).

In general, the expression level of the heterologous protein in mammalian cells has been reported to be low. For example, the expression level of human cytochrome P450 protein in lymphoblast cells was about 50 pmol P450/mg microsomal protein or 10-20 pmol/mg total cell lysate protein (59). Therefore, the bacterial cells have been used as a host to produce large amounts of a

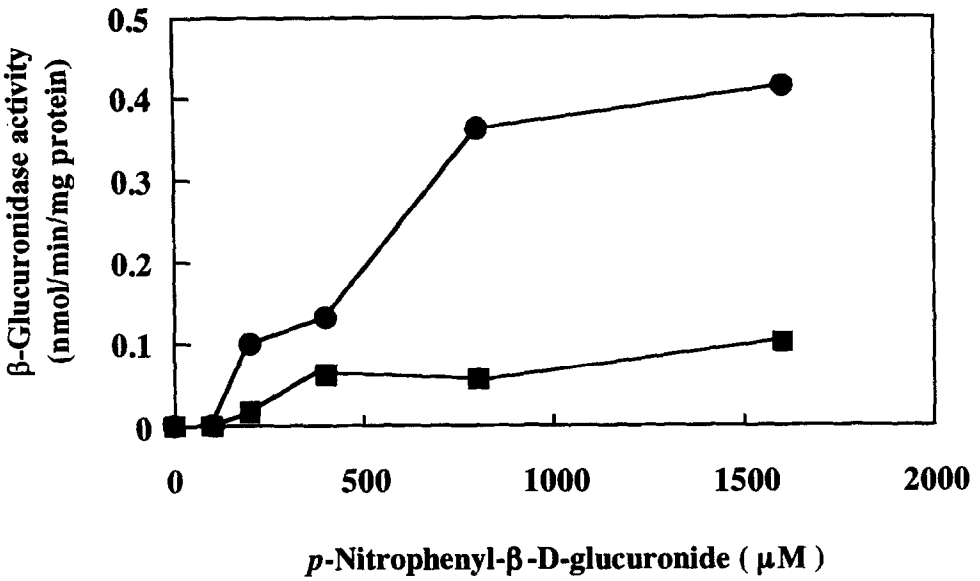

Fig. 5

β-Glucuronidase activity toward *p*-nitrophenyl-β-D-glucuronide in the *Salmonella* cells. *p*-Nitrophenol formation from *p*-nitrophenyl-β-D-glucuronide seen in the genetically engineered *Salmonella* cells was analyzed and compared with that seen in *E. coli* DH5α cells. The membrane fractions of *Salmonella* cells expressing human UGT1A1 and the DH5α cells were used. Assays were carried out in duplicate at each concentration of *p*-nitrophenyl-β-D-glucuronide and the mean values were shown . ●, *E. coli* DH5α cells; ■, *Salmonella* cells expressing human UGT1A1.

heterologous protein. On the expression of cytochrome P450 protein in *E. coli* DH5α cells, the expression level was about 500 pmol/mg bacterial membrane protein in the case of CYP2C8 (unpublished data). There are only three reports on the expression of UGT protein in bacterial cells (33-34). Catalytic activity of UGT protein expressed in bacterial cells was detectable only when purified preparation of UGT protein from bacterial cells was employed (35). Despite the efforts to express catalytically active UGT, there is no paper demonstrating catalytic activity of UGT protein expressed in intact bacterial cells or membrane fraction of bacterial cells. Among bacterial strains, *E. coli* is often used as host cells for heterologous expression. However, most of the *E. coli* strains possess β-glucuronidase (36-43). If the UGT is expressed in bacterial strain possessing β-glucuronidase, the conjugates formed in the cells may be deconjugated immediately, and the conjugate may not be detectable. Therefore, *E. coli* cannot be regarded as an appropriate host to express UGT protein. It was reported that only 30-50% of *Salmonella* strains possessed the β-glucuronidase activity (44-46). Furthermore, Minor et al. (44-45) and Massenti et al. (46) reported that all strains of *Salmonella typhimurium* did not exhibit the *p*-nitrophenyl-β-D-glucuronide deconjugation activity. Thus, we employed *Salmonella typhimurium* TA1535 strain as a host to express human UGT1A1. As shown in Fig. 1, we successfully expressed the human UGT1A1 protein in the *Salmonella* TA1535 cells, while no catalytic activity was observed (Fig. 2). Thus, we assumed the existence of the β-glucuronidase in the established *Salmonella* strain. To confirm the hypothesis, we analyzed the bilirubin conjugation activity in the presence of β-glucuronidase inhibitors. Saccharic acid 1,4-lactone is known to be one of the most potent β-glucuronidase inhibitors (54-56). Narita et al. (56) reported that some components extracted from plants, such as glycyrrhizin, baicalin, wogonoside and luteolin-3'-glucuronide, strongly inhibited the β-glucuronidase. As was expected, the bilirubin

conjugation activity of UGT1A1 expressed in the membrane fraction from the *Salmonella* cells appeared when the glycyrrhizin was added to the reaction mixture. In this study, we found that the UGT1A1 protein expressed in the *Salmonella* cells possessed the catalytic activity. This is the first study to demonstrate the establishment of a genetically engineered bacterial strain expressing human UGT1A1 showing catalytic activity without purification and reconstitution of the UGT protein. Furthermore, it could be concluded that *Salmonella* seemed to be a good host to express UGT protein, since the β-glucuronidase activity of the *Salmonella* towared *p*-nitrophenyl-β-D-glucuronide was lower than that of *E. coli* (Fig. 5).

The bilirubin conjugation activity of human UGT1A1 expressed in the membrane fraction of the *Salmonella* cells was compared with that seen with various preparations of human UGT1A1 (Table 1).

TABLE I
Comparison of bilirubin conjugation activity catalyzed by UGT1A1 expressed in the genetically engineered *Salmonella* with that by various UGT1A1 preparations

Source	Enzyme preparations	*Km* (μM)	Activity[a]	Reference
Salmonella cell	Membrane fraction	N.D.[b]	79 (500)	Present
V79 cell	Homogenate	N.D.	400 (122)	(3)
	Homogenate	24	430[c]	(23)
HEK293 cell	Homogenate	9.0, 19	90, 92[c]	(24)
COS-7 cell	Microsomes	N.D.	360 (75)	(25)
Human liver	Microsomes	N.D.	199 (500)	Present
Human liver	Microsomes	N.D.	450 (75)	(25)

[a]Activity (pmol/min/mg protein), Numbers in parentheses are the substrate concentrations (μM).
[b]N.D.; Not determined.
[c]*V*max.

The *V*max value of bilirubin glucuronidation catalyzed by UGY1A1 expressed in the microsomes from mammalian cells was 90-430 pmol/min/mg protein. In the present study, the bilirubin conjugation activity of UGT1A1 expressed in the membrane fraction of the *Salmonella* cells was examined with 500 μM of bilirubin. The activity obtained in this study may be similar to *V*max value, since the *K*m value of bilirubin glucuronidation is lower than 24 μM (23-24). Thus, the bilirubin conjugation activity of the UGT1A1 expressed in the membrane fraction of the *Salmonella* cells was slightly lower than that obtained with other preparations of UGT1A1. The result may be reflected the concentration of UGT1A1 protein in the membrane fraction of the *Salmonella* cells.

The established *Salmonella* cells possessed β-glucuronidase activity toward baicalin, while the cells showed a low β-glucuronidase activity toward *p*-nitrophenyl-β-D-glucuronide (Fig. 4, 5). Baicalin may be a good substrate for the analysis of β-glucuronidase activity in *Salmonella* cells. It has been reported that *Eubacterium* sp. from human intestinal flora possessed two forms of β-glucuronidase (60). One form is responsible for the deconjugation of the glucuronide such as *p*-nitrophenyl-β-D-glucuronide, 4-methylumbelliferyl mono-β-D-glucuronide and phenolphthalein mono-β-D-glucuronide. Another form is involved in the deconjugation of the glycyrrhizin. Thus, it seems likely that there are plural forms of β-glucuronidase (60). The established *Salmonella* strain may express the second type of β-glucuronidase.

Since the β-glucuronidase activity of the *Salmonella* toward *p*-nitrophenyl-β-D-glucuronide was lower than that of *E. coli* (Fig. 5), it could be concluded that *Salmonella* seemed to be a good host to express UGT protein.

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