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Novel process for producing 6-deoxy monosaccharides from L-fucose by coupling and sequential enzymatic method

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We biosynthesized 6-deoxy-L-talose, 6-deoxy-L-sorbose, 6-deoxy-L-gulose, and 6-deoxy-L-idose, which rarely exist in nature, from L-fucose by coupling and sequential enzymatic reactions. The first product, 6-deoxy-L-talose, was directly produced from L-fucose by the coupling reactions of immobilized D-arabinose isomerase and immobilized L-rhamnose isomerase. In one-pot reactions, the equilibrium ratio of L-fucose, L-fuculose, and 6-deoxy-L-talose was 80:9:11. In contrast, 6-deoxy-L-sorbose, 6-deoxy-L-gulose, and 6-deoxy-L-idose were produced from L-fucose by sequential enzymatic reactions. D-Arabinose isomerase converted L-fucose into L-fuculose with a ratio of 88:12. Purified L-fuculose was further epimerized into 6-deoxy-L-sorbose by D-allulose 3-epimerase with a ratio of 40:60. Finally, purified 6-deoxy-L-sorbose was isomerized into both 6-deoxy-L-gulose with an equilibrium ratio of 40:60 by L-ribose isomerase, and 6-deoxy-L-idose with an equilibrium ratio of 73:27 by D-glucose isomerase. Based on the amount of L-fucose used, the production yields of 6-deoxy-L-talose, 6-deoxy-L-sorbose, 6-deoxy-L-gulose, and 6-deoxy-L-idose were 7.1%, 14%, 2%, and 2.4%, respectively.

[**Key words:** L-Fucose; 6-Deoxy-L-talose; 6-Deoxy-L-sorbose; 6-Deoxy-L-gulose; 6-Deoxy-L-idose; D-Arabinose isomerase; L-Rhamnose isomerase; D-Allulose 3-epimerase; L-Ribose isomerase; D-Glucose isomerase]

Deoxy sugars are the derivatives of monosaccharides, wherein one or more hydroxyl groups of the common monosaccharides have been substituted with a hydrogen atom. Most of them are categorized as rare sugars owing to their rarity in nature according to the definition given by the International Society of Rare Sugars (ISRS) (1). Deoxy sugars, which are present in small amounts in a wide variety of organisms, generally participate in natural products, including antibiotics (2), antifungals (3), anticancer agents (4), antitumor agents (5), and anticoagulant agents (6). Some deoxy sugars are also used in clinical research to elucidate pathological processes (7,8). Although deoxy sugars exhibit extremely interesting and useful properties, their functions and characteristics have not been investigated owing to their rarity. Therefore, the mass production of deoxy sugars should be achieved to extensively elucidate their interesting properties.

To establish a potential and environmental friendly procedure for biosynthesizing rare monosaccharides, we have constructed an Izumoring strategy involving a combination of several microbial enzymes (1,9). All hexoses, pentoses, tetroses, and their alditols are linked through a series of enzymatic reactions (1,9). Microbial enzymes comprising an Izumoring strategy include some alditol dehydrogenases, aldose reductases, aldose isomerases, and ketose 3-epimerases such as D-tagatose 3-epimerase (DTE) and D-allulose 3-epimerase (DAE). These enzymes have a broad substrate specificity whereby one enzyme can recognize and catalyze multiple monosaccharides, which enables the possibility of transformation of various monosaccharides from their starting monosaccharides (10–12).

Noticeably, L-RhI and L-fucose isomerase (L-FI) can catalyze the conversion between various pairs of common monosaccharides as well as the typical deoxy monosaccharide substrates L-rhamnose (6-deoxy-L-mannose) and L-fucose (6-deoxy-L-galactose), respectively (10,13). Therefore, we hypothesized that other enzymes involving an Izumoring strategy, which have the highest activities toward their corresponding common monosaccharides, are able to recognize deoxy monosaccharides and effectively catalyze their transformation. We constructed a deoxy-Izumoring strategy in a way similar to the Izumoring strategy based on the results of previous studies and our experiences. Using a combination of chemical and enzymatic methods, all 12 of the 5-deoxyhexoses and 10 of the sixteen 1- and 6-deoxyketohexoses were produced in our laboratory (14,15). In addition, we recently reported that D-arabinose isomerase (D-AI), DTE, L-ribose isomerase (L-RI), and L-arabinose isomerase (L-AI) could act on 6-deoxy monosaccharides (16). These enzymes were applied to produce 6-deoxy-L-glucose, 6-deoxy-L-allose, and 6-deoxy-L-altrose from L-rhamnose via two intermediates, L-rhamnulose (6-deoxy-L-fructose) and 6-deoxy-L-allulose (16). Although allulose of 6-deoxy-L-allulose was previously called psicose, ISRS has recommended the use of the name allulose rather than psicose (Allulose-psicose nomenclature usage: http://isrs.kagawa-u.ac.jp/image/AlluloseNAME-2.pdf).

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The current study and the previous report (16) are serial experiments under the main objectives of (i) to elucidate that microbial aldose isomerases and DTE-family enzyme, which their typical substrates are common monosaccharides, are able to catalyze the transformation of 6-deoxy monosaccharides, and (ii) to produce various 6-deoxy monosaccharides from commercially available substrate using combination of microbial enzymatic reactions. Here, we describe a methodology for producing four types of 6-deoxyhexoses: 6-deoxy-L-talose, 6-deoxy-L-sorbose, 6-deoxy-L-gulose, and 6-deoxy-L-idose. L-Fucose was converted to these deoxy monosaccharides by several microbial enzymes that were discovered and produced in our laboratory together with the commercial enzyme D-glucose isomerase (DGI) from *Streptomyces murinus*. The recombinants of D-AI from *Bacillus pallidus* 14a (17), L-RhI from *Pseudomonas stutzeri* LL172 (18), and L-RI from *Acinetobacter calcoaceticus* LR7C (19) were overexpressed in *Escherichia coli*. Only wild-type DAE was produced from *Shinella* sp. NN-6 (unpublished data). The overall procedure to biosynthesize these deoxy monosaccharides from L-fucose is summarized in Fig. 1. In addition, production of 6-deoxy-L-gulose and 6-deoxy-L-idose using microbial enzymatic reactions has never been published before. To the best of our knowledge, the present study is the first report described the enzymatic production of these rare 6-deoxy monosaccharides. Moreover, we have recently found that DAE from *Shinella* sp. NN-6 and commercial DGI are able to catalyze the transformation of 6-deoxy monosaccharides effectively.

MATERIALS AND METHODS

Chemicals L-Fucose was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). L-Rhamnose, D-arabinose, and L-ribose were purchased from Sigma (St. Louis, MO, USA). D-Allulose was prepared in our laboratory by a method previously described (20). Bacto tryptone and Bacto yeast extract were purchased from Becton, Dickinson (Sparks, MD, USA). All other chemicals and culture media used were from Wako Pure Chemical Industry (Osaka, Japan) and were of reagent grade.

Bacterial strains and culture conditions The bacterial strains used in this study were recombinant *E. coli* JM 109 harboring the D-AI gene of *B. pallidus* 14a (DDBJ (DNA Data Bank of Japan) accession no. AB429010; 109-BpDAI) and recombinant *E. coli* KRX (Promega, Madison, MI, USA) harboring the L-RhI gene of *P. stutzeri* LL172 (DDBJ accession no. AB121136; KRX-PsLRhI) and the L-RI gene of *A. calcoaceticus* LR7C (DDBJ accession no. AB062121; KRX-AcLRI). These bacteria were transferred from stock culture to Luria agar (LA) (Becton, Dickinson, Franklin Lakes, NJ, USA) supplemented with 100 μg/mL of ampicillin, and were grown routinely at 30 °C for 24 h. *Shinella* sp. NN-6 was grown on Tryptic soy agar (Becton, Dickinson) at 30 °C for 24 h.

Overexpression of recombinant enzymes and production of DAE The cultivation of 109-BpDAI and the expression of D-AI were conducted by a method previously described (16). KRX-PsLRhI was cultivated in LB medium supplemented with 100 μg/mL of ampicillin and incubated at 37 °C with shaking at 250 rpm. In contrast, KRX-AcLRI was cultivated in super broth (SB) medium (16) supplemented with 100 μg/mL of ampicillin and incubated at 30 °C. After the cell cultures reached an OD_{660} of 0.5—0.6, enzyme expression was induced by the addition of 1 mM IPTG. The cell cultures were incubated continuously at 25 °C with shaking at 250 rpm for a total of 16—18 h.

For DAE production, *Shinella* sp. NN-6 was cultivated in mineral salt (MS) medium [0.26% $(NH_4)_2SO_4$, 0.24% KH_2PO_4, 0.56% K_2HPO_4, 0.01% $MgSO_4 \cdot 7H_2O$, and 0.05% yeast extract] supplemented with 1% D-allulose. The cell culture was incubated at 30 °C with shaking at 200 rpm for 48 h.

Preparation of immobilized D-AI and L-RhI KRX-PsLRhI and 109-BpDAI grown in the culture media described above were harvested by centrifugation at 10,000 × *g* for 10 min at 4 °C. To prepare the immobilized enzymes, each recombinant strain was treated using a method previously described (16). D-AI and L-RhI were immobilized on Chitopearl beads BCW 2510 (Fuji Spinning, Tokyo, Japan) at 4 °C using a modified version of a previously reported method (21). The resulting of them were designated as IE-BpDAI and IE-PsLRhI, respectively.

Preparation of toluene-treated bacterial cells possessing LRI and DAE activity Cell pellets of *Shinella* sp. NN-6 and KRX-AcLRI were collected by centrifugation at 10,000 × *g* for 10 min at 4 °C. They were resuspended and washed twice with 50 mM Tris—HCl buffer (pH 8.0) and 50 mM glycine—NaOH buffer (pH 9.0), respectively. The each cell suspension was treated with toluene using the method previously described (16).

Enzyme assay and the determination of equilibrium ratio D-Arabinose, L-rhamnose, and L-ribose were used as substrates to determine the activities of D-AI, L-RhI, and L-RI, respectively. The reaction mixtures were incubated for 10 min at 55 °C for D-AI, 45 °C for L-RhI, and 30 °C for L-RI following the previously reported method (16). The amount of ketose and reducing sugar were determined by the cysteine—carbazole method (22) and the Somogyi—Nelson method (23,24), respectively. To determine the DAE activity, the method was modified using the DTE assay in the previous report (16). The reaction mixture was incubated at 60 °C for 10 min and then the reaction was stopped by boiling for 5 min D-Allulose and D-fructose were measured using high-performance liquid chromatography (HPLC). Unless otherwise stated, all of the deoxy sugars were analyzed by HPLC as described previously (16). One unit of each enzymatic activity was defined as 1 μmol of product formed within 1 min under the aforementioned conditions. The time course changes of conversion ratios between each substrate and each product were analyzed by HPLC.

Production of 6-deoxy monosaccharides and investigation of equilibrium ratios 6-Deoxy monosaccharides including 6-deoxy-L-talose, 6-deoxy-L-sorbose, 6-doxy-L-gulose, and 6-deoxy-L-idose were produced from the starting material, L-fucose. The microbial enzymatic reaction was conducted in a 500-mL Erlenmeyer flask containing 100 mL of reaction mixture. The reaction condition of

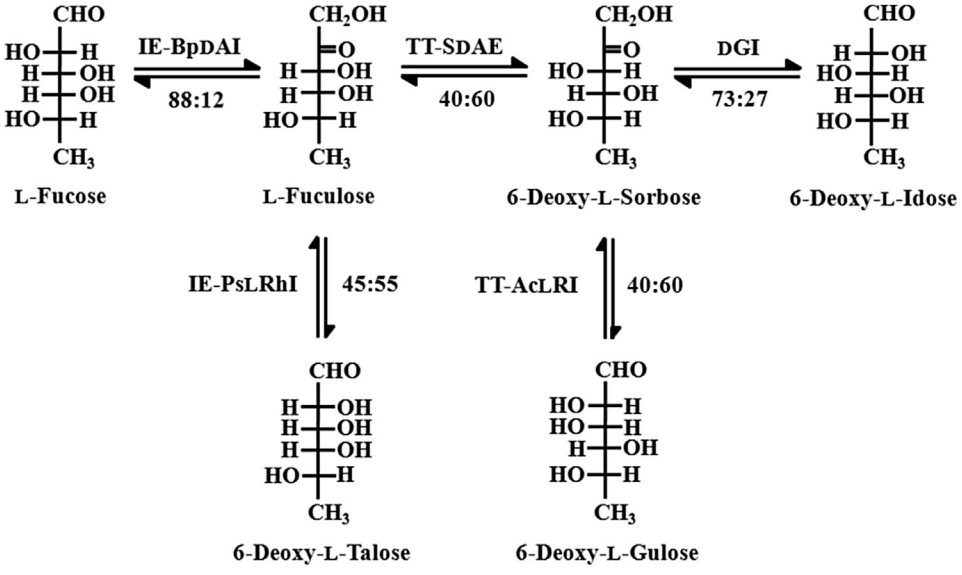

FIG. 1. Summary of overall procedures for producing 6-deoxy-L-talose, 6-deoxy-L-sorbose, 6-deoxy-L-gulose, and 6-deoxy-L-idose from L-fucose. 6-Deoxy-L-talose was produced in a one-pot reactions using IE-BpDAI and IE-PsLRhI. L-Fuculose and 6-deoxy-L-sorbose were prepared from L-fucose by sequential reactions using IE-BpDAI and TT-SDAE. 6-Deoxy-L-sorbose was further isomerized by TT-AcLRI and DGI to produce 6-deoxy-L-gulose and 6-deoxy-L-idose, respectively (16).