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Mass Production of D-Psicose from D-Fructose by a Continuous Bioreactor System Using Immobilized D-Tagatose 3-Epimerase

KEI TAKESHITA, AKIHIRO SUGA, GORO TAKADA, AND KEN IZUMORI*

Department of Biochemistry and Food Science, Faculty of Agriculture, Kagawa University, Miki-cho, Kagawa 761-0795, Japan

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An improved process for the mass production of D-psicose from D-fructose was developed. A D-fructose solution (60%, pH 7.0) was passed at 45°C through a column filled with immobilized D-tagatose 3-epimerase (D-TE) which was produced using recombinant *Escherichia coli*, and 25% of the substrate was converted to D-psicose. After epimerization, the substrate, D-fructose, was removed by treatment with baker's yeast. The supernatant was concentrated to a syrup by evaporation under vacuum and D-psicose was crystallized with ethanol. Approximately 20 kg of pure crystal D-psicose was obtained in 60 d.

[**Key words:** D-psicose, D-tagatose 3-epimerase, bioreactor]

Rare sugars are assumed to exhibit characteristics. Arnold *et al.* reported that a rare aldo-hexose, D-allose, substantially inhibited segmented neutrophil production and lowered platelet counts without other detrimental clinical effects (US pat. no. 5620960, 1997). Furthermore, they suggested that D-allose may be used in the treatment of myeloid leukemia, but it is not abundant in nature and is difficult to prepare by chemical methods. Previously, we reported an enzymatic method of D-allose production from D-psicose using L-rhamnose isomerase (1). In this paper, we report an improved method for the large-scale production of D-psicose from D-fructose which is a raw material of D-allose production (Fig. 1). D-Psicose has previously been produced by chemical methods. Bilik and Tihlarik prepared D-psicose from D-fructose by catalytic action of molybdate ions in an acidic aqueous solution (2). E. J. McDonald synthesized it from 1,2:4,5-di-*o*-isopropylidene-β-D-fructopyranose in three chemical steps (3), while L. W. Doner prepared D-psicose by boiling D-fructose in ethanol and triethylamine (4). All these chemical methods are laborious and inefficient in terms of the large-scale production of D-psicose. We previously reported that we could produce D-psicose from galactitol, D-tagatose or D-talitol by microbial cell reaction (5). However, galactitol, D-tagatose and D-talitol are also comparatively rare in nature to use as substrates. In addition, we reported on the small-scale production of D-psicose from D-fructose using D-tagatose 3-epimerase (D-TE) from *Pseudomonas cichorii* ST-24 (6). In this process, many steps were required to obtain pure crystal D-psicose. Recently, we have succeeded in expressing the D-TE gene in *Escherichia coli* JM105 which allows the production of large amounts of the enzyme (7). As a result of cloning and expression experiments, the amount of enzyme produced by the recombinant *E. coli* was about 100 times greater than that produced by *P. cichorii* ST-24.

In this study, we improved the method of D-psicose production from D-fructose using immobilized D-TE, and established a continuous and effective method for the mass production of D-psicose. (a) Partially purified D-TE produced by the recombinant *E. coli* JM105 was immobilized to construct a bioreactor (7). (b) D-Fructose at a high concentration (60%) was used as the substrate, and no buffer was used in any step of the process. (c) To remove D-fructose from the reaction mixture, we fermented the D-fructose using baker's yeast instead of using column chromatography. (d) We found that the ethanol crystallization method was very effective and convenient for obtaining pure crystal D-psicose from the unpurified syrup containing D-psicose.

D-TE was partially purified from *E. coli* JM105 grown in 2 *l* of NZC medium in a 2.5-*l* jar fermentor under the same conditions as reported previously (7). The partially purified D-TE (14,000 U) was immobilized on Chitopearl beads BCW 2510 (200 g, wet weight) by ionic binding and almost 90% of the enzyme activity was retained. A water-jacketed column (3.5 × 45 cm) was filled with this immobilized enzyme and the substrate solution (60% D-fructose, pH adjusted to 7.0 by NaOH without using buffer) was passed through the column for epimerization. The reaction was continued at a flow rate of 2.0 ml/min for 60 d at 45°C. According to the results of high-performance liquid chromatography (HPLC, Nihonbunko HPLC 880 PU liquid chromatograph, Shimadzu RID-6A refractive index detector, Shimadzu CR-6A chromatopac and Hitachi HPLC column GL-611), it was confirmed that 25% of the D-fructose was transformed to D-psicose without production of by-products. Using this continuous bioreactor, we could epimerize approximately 0.4 kg of D-fructose to D-psicose per day, and the reaction mixture was then subjected to the next step which was removal of D-fructose and crystallization of D-psicose.

To remove D-fructose, the reaction mixture was treated with baker's yeast in a 40-*l* glass jar under aerobic conditions. The reaction was carried out at 28°C and pH 7.0 (adjusted with NaOH) in a suspension (30 *l*) containing 20% sugar (D-fructose+D-psicose) and 1.5 kg of baker's yeast. The amount of ketose in the reaction mixture was determined by the cysteine-carbazole method (8). As a result of treatment with baker's yeast the ketose concentration decreased rapidly and 25% of the ketose remained after 24 h. HPLC analysis showed that D-fructose was degraded completely by the yeast and D-psicose remained. A small peak that was thought to be

*Corresponding author.

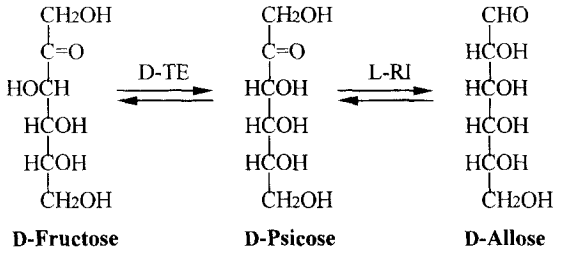

FIG. 1. D-Psicose and D-allose production from D-fructose using D-TE and L-RI (D-TE, D-tagatose 3-epimerase; L-RI, L-rhamnose isomerase).

ethanol was observed on the chromatogram. This peak disappeared following the evaporation step. Following treatment with the baker's yeast, the yeast cells were removed by centrifugation (at 6000 rpm) and the supernatant was concentrated by approximately 80% (Brix) of syrup by evaporation under vacuum at 35°C. To reduce the number of purification and crystallization steps of D-psicose (6), we used ethanol precipitation. The conditions for the crystallization of D-psicose (sugar concentration, ethanol concentration and temperature) were optimized. Based on our results, the same volume of ethanol was added to unpurified D-psicose syrup and stirred gently at room temperature. The D-psicose crystals appeared after 1 d and the supernatant was removed by centrifugation. The yield of crystal D-psicose from the syrup was about 85%. The HPLC retention time (28.4 min), infrared spectrum and specific optical rotation ($\alpha_D = +3.15$) of the isolated crystals were identical to those of standard D-psicose. The ^{13}C-NMR spectrum of the crystal was indistinguishable from that of standard D-psicose. From these findings, the crystal product was identified as pure D-psicose, and about 20 kg of crystal D-psicose was obtained in 60 d.

The improved process of the large-scale production of D-psicose from D-fructose is summarized in Fig. 2. D-TE produced by recombinant *E. coli* was partially purified and immobilized on Chitopearl beads of BCW 2510. A D-fructose solution (60%) was passed through the bioreactor at 45°C, and 25% of the substrate was converted to D-psicose. After removal of the D-fructose by baker's yeast treatment and concentration of the supernatant to a syrup by evaporation under vacuum, the same volume of ethanol was added. The D-psicose crystals appeared after 1 d. In this study, we used D-TE produced by recombinant *E. coli*. However, considering the contamination of the D-TE by pyrogen produced by *E. coli*, it may be beneficial to produce the enzyme using recombinant yeast.

In conclusion, the preparation method of D-psicose described in this paper is simple and cost-effective. The epimerase reaction was continued for 60 d at high substrate concentration (60%) without using buffer, and D-psicose concentration in the reaction mixture eluted after 60 d was same as at the start of the reaction. The half-life of the immobilized D-TE under the reaction conditions used was around 60 d. Accordingly, the D-TE activity in the column after 60 d decreased by 50%. However, under the reaction conditions, the epimerization reaction between D-fructose and D-psicose reached equilibrium even after 60 d, and thus the concentration of D-psicose in the reaction mixture after 60 d was the same as at the start of the reaction. After 70 d of reaction, the concentration of D-psicose in the reaction mixture was about 15%. The high concentration (60%) prevented bacterial contamination during the long reaction period. When a substrate dissolved in buffer is used as the starting material in a bioreactor, the buffer has to be removed before purifying the product. As described above, we did not need to use buffer in any steps for the production of D-psicose, and this facilitates the large-scale production of D-psicose. The use of baker's yeast to remove D-fructose resulted in substantial savings in terms of labor, cost and time. After evaporation, we could easily recover D-psicose crystals by ethanol treatment at a yield of about 85%. The addition of ethanol to the syrup containing D-psicose resulted in crystallization of D-psicose even if the syrup was not pure D-psicose. Therefore, the method described in this paper appears to be applicable to the large-scale production of D-psicose which should allow commercial utilization of this expensive sugar. We believe that this nonmetabolizing sugar can be used as a no-calorie sweetener or as a raw material for the production of various rare sugars (9,10).

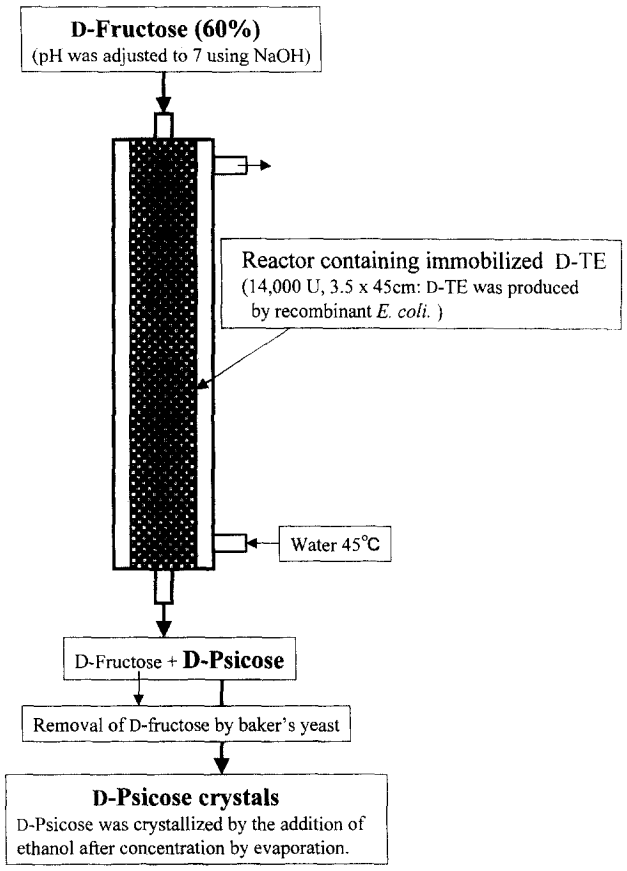

FIG. 2. Continuous production of D-psicose from D-fructose using immobilized D-TE.

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