Biotransformation of Fructose to Allose by a One-Pot Reaction Using *Flavonifractor plautii* D-Allulose 3-Epimerase and *Clostridium thermocellum* Ribose 5-Phosphate Isomerase

Tae-Eui Lee, Kyung-Chul Shin, and Deok-Kun Oh*

Department of Bioscience and Biotechnology, Konkuk University, Seoul 05029, Republic of Korea

**Introduction**

D-Allose is a potential medical sugar because it has anticancer, antihypertensive, anti-inflammatory, antioxidative, and immunosuppressant activities. Allose production from fructose as a cheap substrate was performed by a one-pot reaction using *Flavonifractor plautii* D-allulose 3-epimerase (FP-DAE) and *Clostridium thermocellum* ribose 5-phosphate isomerase (CT-RPI). The optimal reaction conditions for allose production were pH 7.5, 60°C, 0.1 g/l FP-DAE, 12 g/l CT-RPI, and 600 g/l fructose in the presence of 1 mM Co²⁺. Under these optimized conditions, FP-DAE and CT-RPI produced 79 g/l allose for 2 h, with a conversion yield of 13%. This is the first biotransformation of fructose to allose by a two-enzyme system. The production of allose by a one-pot reaction using FP-DAE and CT-RPI was 1.3-fold higher than that by a two-step reaction using the two enzymes.

**Keywords:** Allose, allulose, fructose, *Clostridium thermocellum* ribose 5-phosphate isomerase, *Flavonifractor plautii*, D-allulose 3-epimerase
using fructose was first demonstrated by a two-enzyme system, in which fructose was converted to allulose by DAE from *F. plautii* (FP-DAE), and allulose was subsequently converted to allose by RPI from *C. thermocellum* (CT-RPI). As a result, allose and allulose were simultaneously produced from fructose.

**Materials and Methods**

**Bacterial Strains, Plasmid, and Culture Conditions**

The genomic DNA of *F. plautii* ATCC 29863 (ATCC, USA) and *C. thermocellum* ATCC 27405, *Escherichia coli* ER2566 (New England Biolab, UK), and pET15b plasmid (Novagen, USA) were used as the sources of DAE and RPI R132E variant genes, host cells, and expression vector, respectively. The genes of FP-DAE and CT-RPI were cloned as described previously [10, 21]. A colony of *E. coli* expressing FP-DAE or CT-RPI was selected and inoculated into 5 ml of Luria-Bertani (LB) medium and cultivated at 37°C with shaking at 200 rpm overnight. This seed was transferred into a 2 L Erlenmeyer flask containing 500 ml of LB medium supplemented with 20 mg/ml kanamycin and cultivated at 37°C with shaking at 200 rpm. Induction for enzyme expression was started at the optical density at 600 nm of the culture of 0.8 with the addition of 0.1 mM isopropyl-β-D-thiogalactopyranoside. The culture was further grown for 16 h at 16°C with shaking at 150 rpm.

**Enzyme Purification**

Recombinant cells were harvested from the culture broth by centrifugation at 12,000 ×g for 20 min at 4°C, and then washed twice with 0.85% NaCl. The washed cells were resuspended in 50 mM phosphate buffer (pH 8.0) containing 300 mM KCl and 10 mM imidazole. The resuspended cells were disrupted for 2 min using a sonicator on ice. Unbroken cells and cell debris were removed by centrifugation at 12,000 ×g for 20 min at 4°C, and the supernatant obtained was applied to an immobilized nickel-nitrilotriacetic acid affinity chromatography cartridge (Bio-Rad, USA) previously equilibrated with 50 mM phosphate buffer (pH 8.0). The bound protein was eluted, using the same buffer that contained a linear gradient from 10 to 500 mM imidazole, at a flow rate of 1 ml/min. The eluate was applied to a Bio-Gel P-6 desalting cartridge (Bio-Rad) previously equilibrated with 50 mM Tris-HCl buffer (pH 7.5), and eluted with the same buffer at a flow rate of 1 ml/min. The active fractions were collected and dialyzed. The resulting solution was used as the purified enzyme. All purification steps using the cartridges were carried out at 4°C with a Profinia protein purification system (Bio-Rad).

**Enzyme Reaction**

The enzyme reaction was conducted after treatment with 1 mM EDTA at 4°C for 1 h. Unless otherwise stated, the reaction was performed in 50 mM Tris-HCl buffer (pH 7.5) containing 600 g/l D-fructose or 250 g/l D-allulose, 0.1 g/l FP-DAE and/or 12 g/l CT-RPI in the presence of 1 mM Co²⁺ at 60°C in a 100 ml flask containing 10 ml of the reaction solution with shaking at 200 rpm for 2 h. To determine the buffer type and the presence or absence of Co²⁺ of the one-pot reaction by FP-DAE and CT-RPI at pH 7.5 and 60°C, the reactions were performed with 600 g/l D-fructose in 50 mM piperazine-N,N’-bis(2-ethanesulfonic acid) (PIPES) and Tris-HCl buffers in the presence and absence of 1 mM Co²⁺. In order to confirm whether Co²⁺ affects the stability of CT-RPI, the reaction was performed in 50 mM Tris-HCl buffer (pH 7.5) containing 50 mM D-allulose and 1 g/l CT-RPI in the presence or absence of Co²⁺ at 60°C for 10 min.

**Optimization of Reaction Conditions for the Production of Allose from Fructose**

The concentration of FP-DAE for the production of allulose from fructose was varied from 0.025 to 0.4 g/l to determine the optimal enzyme concentration. The reactions were performed in 50 mM Tris-HCl buffer (pH 7.5) containing 600 g/l fructose at 60°C for 2 h. The optimal concentration of CT-RPI for the production of allose from allulose was determined by varying the concentration of CT-RPI from 4 to 40 g/l. The reactions were performed in 50 mM Tris-HCl buffer (pH 7.5) containing 250 g/l allulose in the presence of 1 mM Co²⁺ at 60°C for 2 h.

The effect of the ratio of FP-DAE to CT-RPI on the production of allose from fructose was evaluated by the enzymatic reactions of FP-DAE and CT-RPI at the ratios of 0.24 to 0.20 g/l. The reactions were performed in 50 mM Tris-HCl buffer (pH 7.5) containing the two enzymes and 600 g/l fructose in the presence of 1 mM Co²⁺ at 60°C for 2 h. The optimal concentrations of the two enzymes required for the production of allose from fructose were determined by varying the concentration of FP-DAE from 0.025 to 0.15 g/l and the concentration of CT-RPI from 3 to 18 g/l at the optimal concentration ratio of 0.1:12. The reactions were performed in 50 mM Tris-HCl buffer (pH 7.5) containing 600 g/l fructose in the presence of 1 mM Co²⁺ at 60°C for 2 h.

**Production of Allose from Fructose by One-Pot and Two-Step Reactions**

The one-pot time-course reactions for the production of allose from fructose via allulose by the two enzymes were investigated under the optimized conditions. The reactions were performed at 60°C in 50 mM Tris-HCl buffer (pH 7.5) containing 0.1 g/l FP-DAE, 12 g/l CT-RPI, and 600 g/l fructose in the presence of 1 mM Co²⁺ for 2 h. In the two-step reactions, allulose production was performed with 0.1 g/l FP-DAE and 600 g/l fructose at pH 7.5 and 60°C in the presence of 1 mM Co²⁺ for 2 h. After the reaction, FP-DAE was removed from the reaction solution using a centrifugal filter (Ultracel-10; Merck, USA). For allose production, 12 g/l CT-RPI was added to the enzyme-free reaction solution and incubated for 2 h.

**Analytical Methods**

The concentrations of fructose, allulose, and allose were analyzed...
by Bio-LC (ICS-3000; Dionex, USA) using a CarboPac PA1 column and HPLC (Agilent 1100; Agilent, USA) with a refractive index detector using an SP-0810 column. The PA1 and SP-0810 columns were eluted at 30°C with 200 mM NaOH at a flow rate of 1 ml/min and at 80°C with water at a flow rate of 1 ml/min, respectively.

**Results and Discussion**

**Optimization for the Conversion of Fructose to Allose by a One-Pot Reaction Using FP-DAE and CT-RPI**

DAE is an enzyme that catalyzes the reversible epimerization of C3-OH in fructose to make allulose. RPI is an enzyme that catalyzes the reversible isomerization of the aldose allulose to the ketose allose. The maximum activity of FP-DAE was observed at pH 7.0 in PIPES buffer and 65°C in the presence of 1 mM Co²⁺ [21]. The effect of the metal ions Mn²⁺, Ni²⁺, Co²⁺, Mg²⁺, and Fe²⁺ on the activity of FP-DAE was evaluated, and the activity was highest in the presence of Co²⁺ and no enzymatic activity was found for the reaction without metal ions. The maximum activity of CT-RPI was observed at pH 7.5 in Tris-HCl buffer and 80°C in the absence of metal ions [11]. The half-lives of FP-DAE at 60°C and 65°C were 2.2 and 0.7 h, respectively, and the half-lives of CT-RPI at 60°C and 65°C were 13 and 8.2 h, respectively. These results indicate that the optimal temperature in the two-enzyme reaction of 2h is 60°C owing to the instability of CT-RPI. The specific activity of FP-DAE for fructose was approximately 16-fold higher than that of CT-RPI for allulose, indicating that the reaction pH of CT-RPI for allose production is more critical than that of FP-DAE. Thus, the reaction pH in the two-enzyme system was adjusted to 7.5 as the optimal pH of CT-RPI. The specific activity of FP-DAE for fructose was approximately 16-fold higher than that of CT-RPI for allulose, indicating that the reaction pH of CT-RPI for allose production is more critical than that of FP-DAE. Thus, the reaction pH in the two-enzyme system was adjusted to 7.5 as the optimal pH of CT-RPI. To determine the buffer type and the presence or absence of Co²⁺ of the one-pot reaction at pH 7.5 and 60°C, the effects of buffer type (PIPES or Tris-HCl buffer) and Co²⁺ on the production of allose from fructose by FP-DAE and CT-RPI were investigated. The maximum production of allose was observed at Tris-HCl buffer in the presence of Co²⁺ (Fig. S1). The addition of Co²⁺ to CT-RPI did not affect enzyme activity, but the half-life of CT-RPI without Co²⁺ at 60°C was 1.1-fold higher than that with Co²⁺ (Fig. S2). Thus, the addition of Co²⁺ to one-pot reaction using FP-DAE and CT-RPI had little effect on the production of allose from fructose.

On the basis of these results, the optimum reaction conditions of pH, temperature, and metal ions for the conversion of fructose to allose by the two-enzyme system were determined as pH 7.5 in Tris-HCl buffer, 60°C, and 1 mM Co²⁺. The highest concentration of fructose used in the production of allulose by 0.38 g/l FP-DAE was 750 g/l [21]. The concentration of FP-DAE used for the conversion of fructose to allose under the optimized conditions was 120-fold higher than that of CT-RPI. Thus, a high concentration of CT-RPI (46 g/l) should be used in the one-pot reaction of FP-DAE and CT-RPI using 750 g/l fructose. The high concentrations of CT-RPI and fructose caused imperfect mixing due to high viscosity. The maximal concentration of fructose used to mix the reaction solution perfectly was 600 g/l, which was used for allose production.

![Fig. 1. Effects of the concentrations of *Flavonifractor plautii* D-allulose 3-epimerase (FP-DAE) (A) and *Clostridium thermocellum* ribose 5-phosphate isomerase (CT-RPI) (B) on the production of allulose and allose, respectively. Data represent the means of three separate experiments, and error bars represent the standard deviations.](image-url)
Optimization of the Ratio of FP-DAE to CT-RPI for the Conversion of Fructose to Allose

The optimal concentration of FP-DAE for allulose production using 600 g/l fructose was determined by varying the concentration of FP-DAE from 0.025 to 0.4 g/l (Fig. 1A). Allulose production increased with increasing enzyme concentration up to 0.2 g/l FP-DAE. However, above 0.2 g/l FP-DAE, the production reached a plateau. Therefore, the optimal enzyme concentration for allulose production was 0.2 g/l FP-DAE.

The maximum enzymatic conversion yields of fructose to allulose and of allulose to allulose were 31–33% [16, 21] and 33% [10], respectively. When equilibriums of fructose to allulose and of allulose to allulose were 32% and 33%, respectively, 600 g/l fructose in the two-enzyme system was theoretically converted to 192 g/l allulose by DAE, which was converted to 63 g/l allose by RPI. After the two-step reaction, the residual fructose and allulose were 408 and 129 g/l, respectively. However, these residue concentrations were not in equilibrium in the one-pot reaction. Thus, the residual fructose was converted to allulose for reaching the equilibrium between fructose and allulose. The concentration of allulose at the equilibrium was 172 g/l because the 32% portion for the concentration of fructose plus allulose (537 g/l), was allulose. As a result, the residue concentrations between allulose (172 g/l) and allose (63 g/l) were deviated from the equilibrium. Thus, the residual allulose was also converted to allose, and the concentration of allose at the equilibrium was 78 g/l because the 33% portion for the concentration of allulose plus allose (235 g/l) was allose. These reactions were continuously performed until reaching the equilibrium, and then the theoretical equilibrium concentrations of fructose, allulose, and allose using 600 g/l fructose in the two-enzyme system were 352, 166, and 82 g/l, respectively. When equilibriums of fructose to allulose and of allulose to allose were 33% and 33%, respectively, the theoretical equilibrium concentrations of fructose, allulose, and allose were 346, 170, and 84 g/l, respectively. The concentration of allulose plus allose was 248 g/l for the conversion yield of fructose to allulose at 32%, and 254 g/l for the conversion yield at 33%. Thus, 250 g/l allulose was used for determining the optimal concentration of CT-RPI.

The effect of CT-RPI concentration on allose production was investigated by varying the concentration of CT-RPI from 4 to 40 g/l (Fig. 1B). Allose production increased with increasing enzyme concentration, reaching a plateau above 24 g/l CT-RPI. Thus, 24 g/l CT-RPI was considered as the optimal enzyme concentration for allose production. Thus, the ratio of FP-DAE to CT-RPI was suggested as 0.2:24 g/l.

The experiments for the determination of the optimal ratio of FP-DAE to CT-RPI should be performed at the ratios ranging from 0:48 to 0.4:0 g/l. However, the mixed solution of 600 g/l fructose and 48 g/l CT-RPI was too viscous. Thus, the biotransformation of fructose to allose was performed by FP-DAE and CT-RPI at the ratios ranging from 0.24 to 0.20 g/l (Fig. 2). The maximal production of allose was observed at the concentration ratio of 0.1:12 g/l as the optimal ratio of FP-DAE to CT-RPI.

Optimization of the Concentrations of FP-DAE and CT-RPI at the Optimal Ratio for the Conversion of Fructose to Allose

The conversion of fructose to allose was conducted with 600 g/l fructose by varying the concentration of FP-DAE from 0.025 to 0.15 g/l and the concentration of CT-RPI from 3 to 18 g/l at the optimal ratio of 1:120, respectively (Fig. 3). Allose production increased with the increasing concentration of enzymes, reaching a plateau above 0.1 g/l FP-DAE and 12 g/l CT-RPI. Thus, the optimal concentrations of FP-DAE and CT-RPI were 0.1 and 12 g/l, respectively.
The thermostability was examined with 0.1 g/l FP-DAE and 12 g/l CT-RPI, and the half-lives at 50°C, 55°C, 60°C, 65°C and 70°C were 15.1, 9.3, 3.3, 0.9 and 0.3 h, respectively.

The thermostability was examined with 0.1 g/l FP-DAE and 12 g/l CT-RPI, and the half-lives at 50°C, 55°C, 60°C, 65°C and 70°C were 15.1, 9.3, 3.3, 0.9 and 0.3 h, respectively.

**Table 1.** Biotransformation of fructose to allose via allulose by microbial enzymes and cells.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Microorganism</th>
<th>Biocatalyst</th>
<th>Fructose (g/l)</th>
<th>Allulose (g/l)</th>
<th>Allose (g/l)</th>
<th>Conversion yield (%)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>DTE</td>
<td><em>Rhodobacter sphaeroides</em></td>
<td>Free enzyme</td>
<td>700</td>
<td>118</td>
<td></td>
<td>17</td>
<td>[15]</td>
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<tr>
<td>DAE</td>
<td><em>Agrobacterium tumefaciens</em></td>
<td>Free enzyme</td>
<td>700</td>
<td>230</td>
<td></td>
<td>33</td>
<td>[16]</td>
</tr>
<tr>
<td></td>
<td><em>Flavonifractor plautii</em></td>
<td>Free enzyme</td>
<td>700</td>
<td>195</td>
<td></td>
<td>28</td>
<td>[17]</td>
</tr>
<tr>
<td></td>
<td><em>Bacillus subtilis</em></td>
<td>Permeabilized cells</td>
<td>750</td>
<td>235</td>
<td></td>
<td>31</td>
<td>[21]</td>
</tr>
<tr>
<td></td>
<td><em>Ruminococcus sp.</em></td>
<td>Free enzyme</td>
<td>700</td>
<td>196</td>
<td></td>
<td>28</td>
<td>[20]</td>
</tr>
<tr>
<td></td>
<td><em>Clostridium sp.</em></td>
<td>Free enzyme</td>
<td>500</td>
<td>125</td>
<td></td>
<td>25</td>
<td>[18]</td>
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<td>RI</td>
<td><em>Pseudomonas stutzeri</em></td>
<td>Free enzyme</td>
<td>100</td>
<td>25</td>
<td></td>
<td>25</td>
<td>[4]</td>
</tr>
<tr>
<td></td>
<td>Immobilized enzyme</td>
<td></td>
<td>500</td>
<td>150°</td>
<td>30°</td>
<td>30°</td>
<td>[3]</td>
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<tr>
<td>RPI</td>
<td><em>Clostridium thermocellum</em></td>
<td>Free enzyme</td>
<td>500</td>
<td>165</td>
<td></td>
<td>33</td>
<td>[11]</td>
</tr>
<tr>
<td></td>
<td><em>C. thermocellum</em> R132E variant</td>
<td>Free enzyme</td>
<td>500</td>
<td>165</td>
<td></td>
<td>33</td>
<td>[10]</td>
</tr>
<tr>
<td>DAE / RPI</td>
<td><em>Flavonifractor plautii</em> / <em>C. thermocellum</em> R132E variant</td>
<td>Free enzymes</td>
<td>600</td>
<td>190</td>
<td>62</td>
<td>10°</td>
<td>This study</td>
</tr>
<tr>
<td>DAE + RPI</td>
<td><em>Flavonifractor plautii</em> + <em>C. thermocellum</em> R132E variant</td>
<td>Free enzymes</td>
<td>600</td>
<td>172</td>
<td>79</td>
<td>29°/13°</td>
<td>This study</td>
</tr>
</tbody>
</table>

DTE, D-Tagatose 3-epimerase; DAE, D-allulose 3-epimerase; RI, L-rhamnose isomerase; RPI, ribose 5-phosphate isomerase.

a Approximate value; * Two-step biotransformation; ° Conversion yield of fructose to allose; † One-pot biotransformation; ‡ Conversion yield of fructose to allulose.

**Fig. 3.** Effects of the concentrations of the *Flavonifractor plautii* D-allulose 3-epimerase (FP-DAE) and *Clostridium thermocellum* ribose 5-phosphate isomerase (CT-RPI) on the production of allose from fructose at the optimal concentration ratio. Data represent the means of three separate experiments, and error bars represent the standard deviations.

**Fig. 4.** Time-course reactions for the biotransformation of fructose (empty square) to allose (filled circle) via allulose (empty circle) under the optimized conditions. Data represent the means of three separate experiments, and error bars represent the standard deviations.

**Biotransformation of Fructose to Allose by a One-Pot Reaction Using FP-DAE and CT-RPI under the Optimized Conditions**

The optimal reaction conditions for the biotransformation of fructose to allose by a one-pot reaction using FP-DAE and CT-RPI were pH 7.5, 60°C, 0.1 g/l FP-DAE, 12 g/l CT-RPI.
Co2+ and 600 g/l fructose in the presence of 1 mM Co2+
Under the optimized conditions, the two enzymes converted
600 g/l fructose to 172 g/l allulose and 79 g/l allose for 2 h
(Fig. 4). FP-DAE at 0.1 g/l converted 600 g/l fructose to
190 g/l allulose at pH 7.5 and 60°C in the presence of 1 mM
Co2+ for 2 h. After removing FP-DAE from the reaction
solution, CT-RPI at 12 g/l was added and incubated for 2 h.
As a result, 62 g/l allose was produced from 190 g/l allulose.

The biotransformation of fructose to allulose via allulose
by various enzymes and cells is summarized in Table 1. RPI
from C. thermocellum and C. thermocellum R132E variant
produced 165 g/l allulose from 500 g/l allulose, with a
conversion yield of 33% [10, 11]. This is the highest conversion
yield of allulose to allose. DAE from Agrobacterium tumefaciens
[16] produced 230 g/l allulose from 700 g/l fructose, with a
conversion yield of 33%, which is the highest conversion
yield. The two-step reactions of FP-DAE and CT-RPI using
600 g/l fructose resulted in the production of 128 g/l allulose
and 62 g/l allose, with a total conversion yield of
32%. The two enzymes combined produced 172 g/l allulose
and 79 g/l allose, with a total conversion yield of 42% from
the same concentration of fructose. Therefore, the total
conversion yield and the concentrations of allulose and
allose in the two-enzyme system were increased by 131%,
134%, and 127%, respectively, due to the push-pull effect
caused by the simultaneous reactions of the two enzymes.

In summary, in the present study, the biotransformation
of allose from fructose was first demonstrated by a one-pot
reaction using FP-DAE and CT-RPI. The production of
allulose and allose by the one-pot reaction using the two
enzymes was increased by 134% and 127%, respectively,
compared with that by two-step reactions. Therefore, the
simultaneous production of allulose and allose from fructose
as a cheaper substrate by the two-enzyme system is an
 economical and meaningful process. We will produce
allulose and allose from fructose continuously in a packed-
bed bioreactor containing co-immobilized FP-DAE and
CT-RPI.

Acknowledgments

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

References

isomerase and its use for biotechnological production of

2. Lim YR, Oh DK. 2011. Microbial metabolism and
Biotechnol. 91: 229-235.

3. Morimoto K, Park CS, Ozaki M, Takeshita K, Shimonishi T,
Granstrom TB, et al. 2006. Large scale production of D-allose
from D-psicose using continuous bioreactor and separation

4. Menavuvu BT, Poonperm W, Leang K, Noguchi N, Okada H,
from D-psicose by cross-linked recombinant L-rhamnose
isomerase: separation of product by ethanol crystallization.

5. Poonperm W, Takata G, Okada H, Morimoto K, Granstrom TB,
Izumori K. 2007. Cloning, sequencing, overexpression and
characterization of L-rhamnose isomerase from Bacillus pallidus
1297-1307.

2010. Characterization of a thermophilic L-rhamnose isomerase
from Thermoanaerobacterium saccharolyticum NTOU1. J. Agric.
Food Chem. 58: 10431-10436.

thermophilic L-rhamnose isomerase from Caldicellulosiruptor
saccharolyticus ATCC 43494. J. Agric. Food Chem. 59: 8702-
8708.

Characteristics and kinetic properties of L-rhamnose isomerase
from Bacillus subtilis by isothermal titration calorimetry for

phosphate isomerase converting D-psicose to D-allose from
Thermotoga lettingae TMO. Biotecnol. Lett. 35: 719-724.

production by the R132E mutant of ribose-5-phosphate
Biotechnol. 89: 1859-1866.

2007. Characterization of ribose-5-phosphate isomerase of
Clostridium thermocellum producing D-allose from D-psicose.

12. Hossain A, Yamaguchi F, Matsu T, Tsukamoto I, Toyota Y,
Ogawa M, et al. 2015. Rare sugar D-allulose: potential role
and therapeutic monitoring in maintaining obesity and type


production of D-psicose from D-fructose by a continuous
bioreactor system using immobilized D-tagatose 3-epimerase. 


