



<b>Title</b>	Practical Preparation of Epilactose Produced with Cellobiose 2-Epimerase from Ruminococcus albus NE1
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1 Note

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3 1) Running title: Practical Preparation of Epilactose

4 2) Practical preparation of epilactose produced with cellobiose 2-epimerase from  
5 *Ruminococcus albus* NE1

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14 6) Abbreviations: CE, cellobiose 2-epimerase; RaCE, *R. albus* CE

15

1 Abstract

2 A practical purification method for a non-digestible disaccharide, epilactose  
3 (4-*O*- $\beta$ -galactosyl-D-mannose) was established. Epilactose was synthesized from lactose  
4 by cellobiose 2-epimerase and purified by the following procedures: 1) removal of  
5 lactose by crystallization, 2) hydrolysis of lactose by  $\beta$ -galactosidase, 3) digestion of  
6 monosaccharides by yeast, and 4) column chromatography with Na-form cation  
7 exchange resin. Epilactose of 91.1% purity was recovered in 42.5% yield.

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10 Key words: epilactose, cellobiose 2-epimerase, practical preparation

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1           Epilactose (4-*O*-β-galactosyl-D-mannose) is a rare oligosaccharide, and is found in  
2 heated and alkali treated cow milk.<sup>1,2)</sup> This disaccharide is highly resistant to rat  
3 intestinal enzymes, and helps the strong proliferation of the human bifidobacteria  
4 *Bifidobacterium bifidum*, *Bifidobacterium longum*, *Bifidobacterium breve*,  
5 *Bifidobacterium adolescentis*, and *Bifidobacterium catenulatum*.<sup>3)</sup> Epilactose promotes  
6 Ca adsorption in the small intestine of rats more so than fructooligosaccharides, which  
7 are also known to enhance Ca adsorption.<sup>4)</sup> Recently, it was demonstrated that  
8 epilactose increased paracellular Ca adsorption in the small intestine through the  
9 induction of myosin light chain phosphorylation *via* myosin light chain kinase- and  
10 Rho-associated kinase-dependent mechanisms.<sup>4)</sup>

11           Cellobiose 2-epimerase (CE; EC 5.1.3.11) catalyzes the 2-epimerization of the  
12 glucose residue at the reducing end of cellobiose and produces  
13 4-*O*-β-glucosyl-D-mannose. CEs were found in some anaerobic bacterium such as the  
14 ruminal strain *Ruminococcus albus* NE1,<sup>5)</sup> *Eubacterium cellulosolvens*,<sup>6)</sup> and  
15 *Bacteroides fragilis* NCTC9343.<sup>7)</sup> CE is also able to act on lactose to produce  
16 epilactose.<sup>3)</sup> However, studies on the properties of epilactose are limited by the low  
17 yields obtained by preparative TLC<sup>3)</sup> and HPLC<sup>8)</sup> used to purify the sugar after  
18 enzymatic synthesis. It takes a lot of effort to obtain even several gram of epilactose by  
19 these methods. Herein, we report a practical purification method of epilactose, which is  
20 suitable for scale up for industrial purposes.

21           The reaction of CE with lactose was performed using recombinant *R. albus* CE  
22 (RaCE) produced by *Escherichia coli* as described previously.<sup>3)</sup> One U of CE was  
23 defined as the enzyme amount that forms 1 μmol of epilactose from 20 mM lactose in 1  
24 minute at 30°C in 100 mM sodium phosphate buffer (pH 7.5). RaCE (3,000 U) was  
25 added to 3 kg lactose monohydrate (Kanto Chemical Co., INC.; Tokyo, Japan)  
26 suspended in 15 L of 5 mM potassium phosphate buffer (pH 7.0). The reaction mixture  
27 was stirred and incubated at room temperature for 48 h. After the reaction, lactose was  
28 completely dissolved. The production of epilactose reached plateau, and 26.5% of

1 lactose was converted to epilactose. It indicated that 795 g of epilactose was produced.  
2 The content of epilactose was measured by high performance liquid chromatography  
3 under following conditions: Sample concentration, 5% (w/v); injection volume, 10  $\mu$ L;  
4 column, Sugar SP0810 ( $\phi$ 8.0 x 300 mm; Shodex, Tokyo, Japan); column temperature,  
5 80°C; eluent, water; flow rate, 0.8 mL/minute; detection, refractive index. Authentic  
6 epilactose was purchased from Sigma (St. Louis, Missouri, USA) and used as standard.

7 The pH value of this solution was adjusted to pH 4.0 to avoid isomerization of  
8 epilactose and heated to 80°C to stop the enzymatic reaction. Then, the mixture was  
9 concentrated to about 60% (w/v) *in vacuo* and stored at 4°C for 1 day to crystallize the  
10 unreacted lactose. The crystallized lactose was removed by filtration and the filtrate was  
11 recovered. The lactose was washed by several hundred mL of ice-cold water. This  
12 filtrate was combined with the first filtrate (5 L in total), and using a differential  
13 refractometer (Atago, Co., Ltd.; Tokyo, Japan), the solution was found to contain 15.8%  
14 (w/w) of carbohydrate. The specific gravity was approximated by 1.0, and the measured  
15 value was identified as the carbohydrate concentration. It indicates that the recovered  
16 sample included 790 g of solid material. The crystallization of lactose is effective for  
17 the purification of epilactose, and increased the content of epilactose to 70.3%.  
18 Produced epilactose of 555 g (69.8% of produced epilactose) was recovered and the  
19 purity of the epilactose was increased 2.6-fold.

20 The concentration of carbohydrate was adjusted to 10% (w/v) with water. Then,  
21 1/100 vol of 1 M potassium phosphate buffer (pH 6.0) and 12,600 U of *Bacillus*  
22 *circulans*  $\beta$ -galactosidase (16 U per gram solid; Amano Enzyme Inc.; Nagoya, Japan)  
23 were added. One U of  $\beta$ -galactosidase was defined as the enzyme amount to liberate 1  
24  $\mu$ mol *o*-nitrophenol from 20 mM *o*-nitrophenyl  $\beta$ -galactoside in 1 minute at 40°C in 50  
25 mM sodium acetate buffer (pH 6.0). The reaction mixture was incubated at 53°C for 3.5  
26 h. Under these conditions, 57% and 16% of lactose and epilactose was hydrolyzed,  
27 respectively, when authentic sample was used as a substrate. The lactose in the sample  
28 was preferentially hydrolyzed to galactose and glucose and the content of lactose

1 decreased from 29.8% to 5.3%. On the other hand, epilactose was slightly hydrolyzed  
2 by this enzyme. After the reaction, mannose content was increased up to 4.0% of total  
3 carbohydrate. The reaction mixture was incubated at 80°C for 30 minutes to stop the  
4 reaction and then the mixture was cooled down to 30°C.

5 Baker's yeast (15 g) (Kirin Kyowa Foods Ltd.; Tokyo, Japan) was added and  
6 stirred at 30°C overnight to digest monosaccharides. After yeast treatment, only  
7 monosaccharides, galactose and glucose, were successfully consumed and glycerol  
8 (3.8% of the solid material) was produced by the yeast. The mixture was centrifuged  
9 (7,000xg, 4°C, 5 minutes) and a supernatant was obtained. Then, several g of active  
10 carbon (Kirin Kyowa Foods Co. Ltd.; Tokyo, Japan) was added and incubated at 80°C  
11 for 30 minutes. The mixture was filtered with a diatomite filter and deionized by ion  
12 exchange column chromatography using Amberlite MB-4 (Rohm and Haas Co.;  
13 Philadelphia, PA, USA). The deionized mixture was filtered using a 0.45 µm membrane  
14 filter and concentrated *in vacuo*. A concentrate of 900 mL was obtained and the  
15 concentration of carbohydrate was 56.6% (w/v; specific gravity was 1.27). The solid  
16 material in the obtained sample was 647 g and the content of epilactose was 83.9%,  
17 indicating that 543 g of epilactose (68.3% of produced epilactose) was recovered.

18 Column chromatography using Na-form cation exchange resin, UBK530 (Mitsubishi  
19 Chemical Co.; Tokyo, Japan), which is often utilized in industrial processes, was  
20 performed. The conditions of chromatography were as follows: sample concentration,  
21 50% (w/v); injection volume, 113 mL; column, UBK530 (φ28.0 x 550 mm x 4); column  
22 temperature, 80°C; flow rate, 11.3 mL/minute; fraction volume, 15 mL. Epilactose and  
23 glycerol were successfully separated using column chromatography, and the purity of  
24 epilactose was increased up to 93.3% (Fig. 1). The fractions containing more than 90%  
25 epilactose (fractions 45-57) were collected. The content of epilactose from the pooled  
26 fractions was 91.1% and the total carbohydrate was 32.4 g, indicating that 62.2% of  
27 epilactose applied to the column was collected. Based on this result, the total yield of  
28 purified epilactose from the reaction mixture of CE was estimated to be 42.5% (Table 1).

1 It was very difficult to achieve higher purity of epilactose with this system because of  
2 the lactose remaining after the  $\beta$ -galactosidase reaction. Therefore, it was thought that  
3 complete hydrolysis of lactose by  $\beta$ -galactosidase is required to achieve higher purity of  
4 epilactose. However, longer  $\beta$ -galactosidase treatment results in low yield due to  
5 degradation of epilactose. So  $\beta$ -galactosidase more specific to lactose was required. In  
6 this study, we established a practical purification method for epilactose, which can be  
7 scaled up for use in industrial processes. This method should prove useful for providing  
8 purified epilactose for further biological studies.

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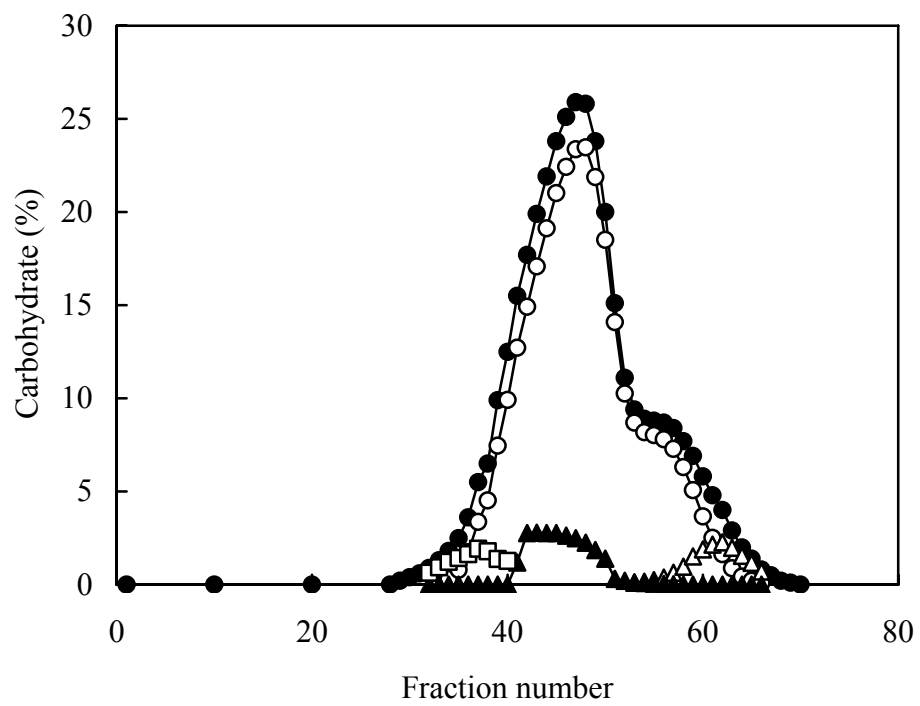
Table 1. Summary of the purification of epilactose.

Procedure	Total sugar (g)	Epilactose (g)	Purity (%)	Yield (%)
Reaction mixture of CE <sup>*</sup>	3,000	795	26.5	100
Crystallization of lactose	790	555	70.3	69.8
Hydrolysis of lactose	790	557	70.5	70.1
Yeast treatment	647	543	83.9	68.3
UBK530 column chromatography	371 <sup>**</sup>	338 <sup>**</sup>	91.1	42.5

<sup>\*</sup>, cellobiose 2-epimerase; <sup>\*\*</sup>, estimated value based on the yield from the UBK530 column chromatography with a portion of the sample purified.



Fig. 1. Saburi et al.



## Figure legends

Fig. 1. Elution pattern of UBK530 column chromatography.

Sample concentration, 50% (w/v); injection volume, 113 mL; column, UBK530 ( $\phi$ 28.0 x 550 mm x 4); column temperature, 80°C; flow rate, 11.3 mL/minute; fraction volume, 15 mL. The concentration of carbohydrate was measured with a differential refractometer. ●, total carbohydrate; ○, epilactose; ▲, lactose; △, glycerol; □, unknown.