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Running title: Thermophilic Cellobiose 2-Epimerase from
Rhodothermus marinus

**Biochemical Characterization of a Thermophilic Cellobiose
2-Epimerase from a Thermohalophilic Bacterium, *Rhodothermus
marinus* JCM9785**

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Abbreviations: CE, cellobiose 2-epimerase; RaCE, *Ruminococcus
albus* NE1 CE; BfCE, *Bacteroides fragilis* NCTC9343 CE; EcCE,
Eubacterium cellulosolvens NE13 CE; AGE, *N*-acetylglucosamine
2-epimerase; RmCE, *Rhodothermus marinus* JCM9785 CE; HPLC,
high performance liquid chromatography; TFA, trifluoroacetic
acid; TLC, thin layer chromatography; MALDI-TOF MS, matrix
assisted laser desorption ionization-time of flight mass

1 spectrometry

2

1 Cellobiose 2-epimerase (CE) reversibly converts glucose residue
2 to mannose residue at the reducing end of β -1,4-linked
3 oligosaccharides. It efficiently produces epilactose carrying
4 prebiotic properties from lactose, but the utilization of known CEs
5 is limited due to thermolability. We focused on thermophilic
6 *Rhodothermus marinus* JCM9785 as a CE producer, since a CE-like
7 gene was found in the genome of *R. marinus* DSM4252. CE activity
8 was detected in the cell extract of *R. marinus* JCM9785. The
9 deduced amino acid sequence of the CE gene from *R. marinus*
10 JCM9785 (RmCE) was 94.2% identical to that from *R. marinus*
11 DSM4252. The N-terminal amino acid sequence and tryptic peptide
12 masses of the native enzyme matched those of RmCE. The
13 recombinant RmCE was most active at 80°C at pH 6.3, and stable in
14 a range of pH 3.2-10.8 and below 80°C. In contrast to other CEs,
15 RmCE demonstrated higher preference for lactose over cellobiose.

16
17 **Key words:** cellobiose 2-epimerase; epilactose; *Rhodothermus*
18 *marinus*; N-acetylglucosamine 2-epimerase

1 Cellobiose 2-epimerase (CE; EC 5.1.3.11) reversibly converts
2 glucose residue to mannose residue at the reducing end of
3 β -1,4-linked oligosaccharides such as cellobiose and lactose to
4 produce 4-*O*- β -D-glucosyl-D-mannose and
5 4-*O*- β -D-galactosyl-D-mannose (epilactose) respectively. It was
6 first found in the culture fluid of an anaerobic ruminal bacterium,
7 *Ruminococcus albus*.¹⁾ It is a unique enzyme that catalyzes
8 hydroxy-stereoisomerism at the C-2 position of a non-modified
9 sugar through a proton abstraction/addition mechanism,²⁾ because
10 almost all carbohydrate epimerases act on activated sugars bearing
11 nucleotidyl, phosphoryl, or acyl groups.
12 The gene encoding CE has been isolated from the genomic DNA of
13 *R. albus* NE1,³⁾ and the enzymatic characteristics of *R. albus* CE
14 (RaCE) were investigated with a recombinant enzyme produced in
15 *Escherichia coli*.⁴⁾ RaCE is most active at neutral pH values and
16 relatively low temperature (30°C). It epimerizes not only
17 cellobiose but also lactose, cellotriose, and cellotetraose,
18 although it strictly recognizes the β -1,4-glycosidic linkage at the
19 reducing end part of substrates.⁴⁾

20 Since the amino acid sequence of RaCE was elucidated, two
21 other CE genes from anaerobic bacteria, *Bacteroides fragilis*
22 NCTC9343 (BfCE) and *Eubacterium cellulosolvens* NE13 (EcCE),
23 have been cloned and the enzymes characterized.^{5,6)} The deduced
24 amino acid sequences of BfCE and EcCE are 39% and 46% identical
25 to RaCE respectively. These enzymes displayed somewhat
26 differences in substrate specificity from RaCE. BfCE showed
27 higher affinity towards cellobiose and lactose than RaCE.⁵⁾ EcCE
28 exhibited higher preference for cellobiose over lactose than

1 RaCE.⁶⁾ Recently, a protein encoded by the BF0772 gene of *B.*
2 *fragilis* NCTC9343 upstream of the CE gene (BF0774) was found to
3 catalyze the phosphorysis of 4-*O*- β -D-mannosyl-D-glucose to
4 produce α -D-mannose-1-phosphate and glucose.⁷⁾ Hence it has been
5 postulated that CE is involved in the metabolism of mannan, where
6 CE produces 4-*O*- β -D-mannosyl-D-glucose from mannobiose
7 supplying a substrate for phosphorysis.⁷⁾

8 The amino acid sequence of *R. albus* CE (RaCE) shows relatively
9 weak similarity to *N*-acetylglucosamine 2-epimerases (AGEs) from
10 porcine kidney and *Anabaena* sp. CH1, whose three-dimensional
11 structures are available.³⁾ Both AGEs are dimeric enzymes, while
12 CE is monomeric. Each monomer of the AGE is composed of an
13 (α/α)₆ barrel fold.^{8,9)} A soaking study of porcine AGE crystal with
14 *N*-acetylglucosamine revealed that His382, His248, Glu251, and
15 Arg60 are situated at the nearest position to bound
16 *N*-acetylglucosamine or a mixture of *N*-acetylglucosamine and
17 *N*-acetylmannosamine, which was not precisely identified.⁸⁾ The
18 two corresponding His residues of *Anabaena* AGE were envisaged
19 to serve as the general acid and the general base catalysts in proton
20 abstraction/addition at the C2 position by Lee et al., because these
21 residues were about 8 Å apart and can ideally act as symmetric
22 acid/base catalysts in reversible epimerization.⁹⁾ Secondary
23 structure prediction suggested that RaCE is also formed by an
24 (α/α)₆ barrel as AGEs, and two putative catalytic His residues are
25 observed at the equivalent positions.¹⁰⁾ These conserved His
26 residues of RaCE were confirmed to be essential for the catalytic
27 activity by site-directed mutagenesis,¹⁰⁾ suggesting that CE and
28 AGE share the essential structure of the active site, including

1 catalytic amino acids.

2 Epilactose, an epimerized product from lactose by CE, has
3 beneficial potential for use as a prebiotic food material, because it
4 is highly resistant to rat intestinal enzymes and strongly
5 proliferates human bifidobacteria.⁴⁾ Additionally, epilactose
6 significantly promotes calcium and iron adsorption in the small
7 intestine of rats.¹¹⁻¹³⁾ In spite of the beneficial biological functions
8 of epilactose, utilization of CE for the industrial production of
9 epilactose is significantly restricted due to the low thermal
10 stability of known CEs.⁴⁻⁶⁾

11 Analysis of the complete genome sequence of *Rhodothermus*
12 *marinus* DSM4252, a thermohalophilic bacterium, revealed that it
13 has a CE-like gene, ACY49317, whose deduced amino acid
14 sequence is 32-46% identical to those of known CEs, although
15 ACY49317 was assigned to be an AGE.¹⁴⁾ In this study, we focused
16 on *R. marinus* JCM9785, which is closely related to *R. marinus*
17 DSM4252, as a thermophilic CE-producing bacterium, and assessed
18 its CE activity in a cell-free extract. Next, a gene homologous to
19 ACY49317 of *R. arinus* DSM4252 was cloned from the genome of *R.*
20 *marinus* JCM9785, and the recombinant enzyme encoded by the
21 gene was characterized.

24 **Materials and Methods**

26 *Bacterial strain and plasmid.* *R. marinus* JCM9785 was provided
27 by the Japan Collection of Microorganisms, Riken BioResource
28 Center (Saitama, Japan), which is participating in the National

Bioresource Project of Ministry of Education, Culture, Sports,
Science and Technology, Japan. *E. coli* DH5 α and *E. coli* BL21
(DE3)-CodonplusTM RIL (Stratagene; La Jolla, CA) were used in
the propagation of plasmid DNA and the production of a
recombinant protein respectively. A plasmid vector of pET-22b
(Novagen; Darmstadt, Germany) was used in the expression of the
R. marinus JCM9785 (RmCE) gene in *E. coli*.

Detection of CE activity in the cell free extract of R. marinus
JCM9785. *R. marinus* JCM9785 was aerobically cultivated in 3 L of
Marine Broth 2216 (Becton, Dickinson; Sparks, MD), supplemented
with 0.2 % w/v konjak glucomannan (Rheolex; Shimizu Chemical;
Hiroshima, Japan) and 0.02 % w/v antifoamer (KM72; Shin-Etsu
Chemical; Tokyo) for 24 h at 70°C. The cells were harvested by
centrifugation and resuspended in about 200 mL of 10 mM
potassium phosphate buffer (pH 7.0, buffer A). The suspended cells
were disrupted by sonication using Astrason ultrasonic processor
XL (Misonix; Farmingdale, NY), and then the cell debris was
removed by centrifugation. The resulting extract was used in the
assay of CE activity, as follows: Cell-free extract (0.5 mL) was
mixed with an equal volume of 200 mM lactose in 50 mM sodium
phosphate buffer (pH 7.0), and incubated at 40°C for 48 h. Then the
reaction mixture was analyzed by high performance liquid
chromatography (HPLC) under the following conditions: injection
volume, 10 μ L; column, Sugar SP0810 (ϕ 8.0 x 300 mm; Shodex,
Tokyo); column temperature, 80°C; eluent, water; flow rate, 0.8
mL/min; detection, refractive index. Authentic epilactose (Sigma,
St. Louis, MO) was used as standard. The reaction product was

1 purified by HPLC for further analysis.

2 To identify the monosaccharide component of the reaction
3 product, purified product was hydrolyzed with trifluoroacetic acid
4 (TFA) and the resulting monosaccharides were analyzed by thin
5 layer chromatography (TLC). Twenty-five μL of 4 M TFA and an
6 equal volume of 0.4% w/v reaction product were mixed and this
7 was kept at 100°C for 3 h. Then the mixture was dried *in vacuo* and
8 the residue was dissolved in 25 μL of water. One μL of the
9 hydrolysate was spotted on a TLC plate (Silica Gel 60 F₂₅₄; Merck;
10 Darmstadt, Germany) and developed in a solvent system of
11 2-propanol/1-butanol/water (2:2:1; v/v). The chromatogram was
12 visualized using a detection reagent (ethanol/sulfuric acid/acetic
13 acid/anisaldehyde, 92:3:2:3; v/v). Glucose (Kanto Chemical;
14 Tokyo), mannose (Sigma), galactose (Wako Pure Chemical; Osaka,
15 Japan), and fructose (Nacalai Tesque; Kyoto, Japan) were used as
16 standards.

17
18 *Cloning of RmCE gene.* Cells of *R. marinus* JCM9785 from 1 mL
19 of culture fluid were suspended in 0.132 mL of 0.5 N NaOH and this
20 was boiled for 10 min. Then the resulting mixture was neutralized
21 by adding 32 μL of 2 M Tris-HCl buffer (pH 7.0) and used as the
22 template in PCR to amplify the RmCE gene. PCR was performed
23 using KOD Plus DNA polymerase (Toyobo; Osaka, Japan), and the
24 following primers designed on the basis of the sequence up- and
25 downstream of the ACY49317 gene from *R. marinus* DSM4252:
26 5'-TGCTGGATTACGTCATGCACACG-3' (antisense orientation)
27 and 5'-CTGCAGCAGCAGGTGATCGGAC-3' (sense orientation).
28 Dimethylsulfoxide at 2% v/v was added to the PCR mixture to

1 facilitate amplification of the DNA fragment with a GC-rich
2 template. The sequence of the amplified PCR product was analyzed
3 using a GenomeLabTM Dye Terminator Cycle Sequencing Kit
4 (Beckman Coulter; Brea, CA) and an automatic DNA sequencer,
5 CEQ 2000XLTM (Beckman Coulter). Then *Nde*I and *Xho*I sites were
6 introduced into the 5'- and 3'-termini of the RmCE gene
7 respectively by PCR to clone into an expression vector, pET22b.
8 The sequences of the primers used were as follows: 5'-
9 AAACCATATGGTGAGCACGGAGACCATC-3' (sense, *Nde*I site
10 underlined) and 5'-AAACTCGAGCTACCGGGATCGAACGTG-3'
11 (antisense, *Xho*I site underlined). The genomic DNA was used as
12 the template. The amplified DNA fragment and pET22b were
13 digested with *Nde*I and *Xho*I (both from Takara; Kyoto, Japan) and
14 connected using DNA Ligation Kit Mighty Mix (Takara). The
15 transformation of *E. coli* and preparation of plasmid DNA were
16 carried out by Hanahan's method¹⁵⁾ and the alkaline lysis method
17 respectively.¹⁶⁾ The amplified DNA fragment was verified by
18 sequencing as well.

19
20 *Purification of RmCE from the cell-free extract of R. marinus*
21 *JCM9785*. *R. marinus* JCM9785 was aerobically cultivated in 15 L
22 of culture medium. Bacterial cells harvested by centrifugation were
23 resuspended in 1 L of buffer A and disrupted by sonication. The
24 cell debris was removed by centrifugation. Then ammonium sulfate
25 was added to the supernatant to 90% saturation, and the sample was
26 stored at 4°C overnight. Precipitated protein, recovered by
27 centrifugation, was dissolved in 200 mL of buffer A and dialyzed
28 against buffer A. The resulting sample was applied onto a

Toyopearl DEAE-650M column (ϕ 5 x 42 cm; Tosoh, Tokyo) equilibrated with buffer A. The non-adsorbed fraction was obtained by thorough washing with buffer A. This column chromatographic procedure was repeated 2 times, but enzyme activity was detected only in the non-adsorbed fractions. Next, ammonium sulfate was added to the collected sample up to 0.5 M and the sample was subjected to Toyopearl Butyl-650M (Tosoh) column chromatography, where a column (ϕ 2.6 x 35 cm) was equilibrated with buffer A containing 0.5 M ammonium sulfate. After the non-adsorbed protein was eluted completely, the adsorbed protein was eluted by a descending linear gradient of ammonium sulfate from 0.5 to 0 M (total elution volume, 1 L). The active fractions were pooled and concentrated up to 5 mL by ultrafiltration with Amicon Ultra-15 (Millipore; Billerica, MA). Then the concentrated sample was applied to a Sephacryl S-200 column (GE Healthcare; Uppsala, Sweden; ϕ 2.6 x 87 cm) equilibrated with buffer A containing 0.1 M NaCl. Column chromatography was performed at flow rate of 0.3 mL/min. The pooled active fractions were dialyzed against buffer A, and were used as the final enzyme preparation. The purity of the enzyme preparation was confirmed by SDS-PAGE by Laemmli's method,¹⁷⁾ in which a commercially produced molecular size marker (Invitrogen; Carlsbad, CA) was used, and the gel was stained with Rapid CBB Kanto (Kanto Chemical). The protein concentration was determined by the Bradford method.¹⁸⁾ Bovine serum albumin (Bio-Rad; Hercules, CA) was used as a standard protein.

N-Terminal sequence analysis of RmCE. Purified RmCE at 11 μ g

1 was subjected to SDS-PAGE and the separated protein was
2 transferred to a polyvinylidene difluoride membrane
3 (Immobilon-P^{SQ}; Millipore) by electroblotting using a semi-dry
4 blotting apparatus.¹⁹⁾ The band of RmCE, detected by staining with
5 Coomassie Brilliant Blue R-250, was cut off from the membrane
6 and applied to N-terminal sequence analysis using a protein
7 sequencer, Procise 492cLC (Applied Biosystems; Foster City, CA).

8
9 *Production of recombinant RmCE in E. coli.* To produce
10 recombinant RmCE, a single colony of *E. coli* transformant
11 harboring the expression plasmid was inoculated with 3 mL of
12 Luria-Bertani broth containing 100 µg/mL of ampicillin, and
13 cultured at 30°C overnight. Then 0.2 mL of the culture fluid was
14 transferred to a fresh medium at 200 mL and this was cultured as
15 well until A_{600} reached 0.5. Then 0.2 mL of 100 mM isopropyl
16 1-thio-β-D-galactoside (final concentration, 0.1 mM) was added to
17 induce the production of the recombinant protein, and the culture
18 fluid was further incubated at 16°C for 24 h. The cells were
19 collected by centrifugation and resuspended in 20 mL of 20 mM
20 sodium phosphate buffer (pH 7.0). They were disrupted by
21 sonication as described above, and the cell debris was removed by
22 centrifugation. The resulting supernatant was incubated at 70°C for
23 30 min and centrifuged to remove aggregated protein. Then
24 supernatant obtained was dialyzed against buffer A and used for
25 enzyme characterization.

26
27 *Enzyme assay.* A reaction mixture at 1 mL consisting of an
28 appropriate concentration of the enzyme, 100 mM lactose, and 30

1 mm sodium phosphate buffer (pH 7.0) was incubated at 60°C for 10
2 min. The enzymatic reaction was stopped by adding 0.2 mL of 0.1 N
3 HCl and successive boiling for 3 min. Then 0.5% w/v sorbitol at
4 0.3 mL was added to the mixture as an internal standard, and HPLC
5 was performed under the conditions mentioned above to measure
6 the epilactose produced. A standard curve was obtained with a peak
7 area of varying concentrations of authentic epilactose relative to
8 sorbitol. One U of enzyme activity was defined as the amount of
9 enzyme that forms 1 μ mol of epilactose from lactose in 1 min under
10 these conditions.

11 The enzymatic characteristics of RmCE were investigated with
12 an *E. coli* recombinant enzyme sample. Optimum pH and
13 temperature were evaluated by measuring the initial reaction
14 velocities under the conditions of the enzyme assay modified in
15 terms of reaction pH and temperature respectively. To identify the
16 optimum pH, 20 mM Britton-Robinson buffer (pH 3-10; composed
17 of an acidic mixture of 20 mM acetic acid, 20 mM phosphoric acid,
18 and 20 mM glycine, the pH value of which was adjusted with NaOH)
19 was used as the reaction buffer. In the analysis of the optimum
20 temperature, the reaction rate at 50-100°C was measured.

21 pH and temperature stabilities were assessed by measuring
22 residual activity under standard conditions following the pH and
23 temperature treatments. pH treatment was performed by incubating
24 150 μ L of enzyme solution (2.2 mg/mL) in 20 mM Britton-Robinson
25 buffer (pH 3.0-12.0), 50 mM KCl-HCl (pH 2.0), and 100 mM
26 KCl-KOH (pH 13.0) for 24 h at 4°C. For temperature treatment, 0.5
27 mL of 0.2 mg/mL of the enzyme in 20 mM sodium phosphate buffer
28 (pH 7.0) was incubated at 50-100°C for 30 min, and then

1 immediately cooled on ice to stop the temperature treatment. The
2 activity before the treatment was considered to be 100% of residual
3 activity.

4 The kinetic parameters of RmCE for cellobiose and lactose were
5 determined from the initial reaction rates toward varying
6 concentrations of substrates (10-100 mM) by non-linear regression
7 to the Michaelis-Menten equation. The reaction conditions, except
8 for the substrate concentration, were the same as the standard
9 conditions described above.

10
11 *TLC analysis of the reaction products with various sugars.* The
12 reaction mixtures of RmCE containing various sugars were
13 analyzed by TLC as well. A reaction mixture consisting of 0.8
14 µg/mL of RmCE, 100 mM substrate described below, and 20 mM
15 potassium phosphate buffer (pH 6.5) was incubated at 60°C for 24 h.
16 Glucose, galactose, lactose, epilactose, cellobiose (Sigma),
17 cellotriose (Seikagaku; Tokyo), mannobiose (Megazyme; Wicklow,
18 Ireland), gentiobiose (Wako Pure Chemical), maltose (Nihon
19 Shokuhin Kako; Tokyo), laminaribiose (Seikagaku), sophorose
20 (Sigma), mannose, and *N*-acetylmannosamine (Nacalai Tesque)
21 were tested as substrates.

22 23 24 **Results**

25
26 *Detection of CE activity in the cell-free extract of R. marinus*
27 *JCM9785*

28 The cell-free extract of *R. marinus* JCM9785 was incubated with

1 lactose, and the reaction mixture was analyzed by HPLC. A peak
2 with the same retention time as authentic epilactose was detected
3 in the reaction mixture. The hydrolysate of purified product with
4 TFA showed two spots with the same mobility and color as mannose
5 and galactose on TLC analysis (not shown), indicating that the
6 glucose residue at the reducing end of lactose was enzymatically
7 converted to mannose residue. Thus *R. marinus* JCM9785 produced
8 a CE as predicted based on the high sequence similarity of
9 ACY49317 protein from *R. marinus* DSM4252 to known CEs.

11 *Production of recombinant RmCE in E. coli*

12 A gene homologous to the ACY49317 gene from *R. marinus*
13 DSM4252 was obtained from the genome of *R. marinus* JCM9785 by
14 PCR. The deduced amino acid sequence of the gene obtained was
15 94.2% identical to the ACY49317 protein (Fig. 1). In order to
16 confirm that the cloned gene encoded RmCE, native RmCE was
17 purified from the cell-free extract of *R. marinus* JCM9785 (Fig. 2a),
18 and the N-terminal amino acid sequence and the masses of the
19 tryptic peptides of the purified enzyme were analyzed. The
20 N-terminal amino acid sequence of the purified native RmCE,
21 Glu-Thr-Ile-Pro-Asp-Val-Arg-Arg-Leu, analyzed by Edman
22 degradation, was included in the deduced amino acid sequence of
23 the cloned gene (Fig. 1). Additionally, the peptide masses of the
24 tryptic peptides of the native RmCE, analyzed by MALDI-TOF-MS,
25 matched the theoretical values with 27.2% coverage of the entire
26 amino acid sequence (Fig. 1, Supplemental Fig. 1, and
27 Supplemental Table 1; see *Biosci. Biotechnol. Biochem.* Web site).
28 These results unambiguously indicate that the cloned gene encoded

RmCE in *R. marinus* JCM9785.

Fig. 1

Next, the cloned gene was expressed in *E. coli* to produce recombinant RmCE. This was successfully produced in the soluble fraction of the *E. coli* cell (Fig. 2b). The total enzymatic activity produced in 200 mL of culture medium was 1,050 U, estimating that 12 mg of recombinant enzyme was produced based on the specific activity of the purified recombinant enzyme. Heat treatment of the cell-free extract at 70°C for 30 min was effective in the purification of recombinant RmCE. The resulting enzyme sample showed a single band on SDS-PAGE (Fig. 2b), and its specific activity was 87.5 U/mg.

Fig. 2

Effects of temperature and pH on the activity and stability of RmCE

The effects of temperature and pH on the activity and stability of recombinant RmCE was investigated. The activity of RmCEs significantly elevated with increasing temperature up to 80°C (Fig. 3a). At 80°C, 2.5-fold higher activity was shown than at 50°C. Similar activity was observed at 90°C, but it was decreased at 100°C. The thermal stability of RmCE was assessed by measuring residual activity after heat treatment for 30 min. Residual activity over 90% of the original activity was shown below 80°C, whereas very low and no residual activity was observed at 90°C and 100°C respectively (Fig. 3a).

The pH profile of the RmCE reaction was elucidated. The highest activity of RmCE was evident at pH 6.3 (Fig. 3b). Enzyme activity gradually decreased with increasing pH values over pH 6.3. Ninety percent of activity relative to the maximum activity (pH 6.3) was

1 shown at pH 9.6, where no non-enzymatic epimerization occurred
2 (data not shown). Reaction rates at pH values higher than 9.6 were
3 not measured due to significant non-enzymatic epimerization.
4 Enzymatic activity dropped remarkably at acidic pH, and very low
5 activity was observed at pH 3.3, although the enzyme was fully
6 stable at that pH value. RmCE demonstrated the widest stable range
7 of pH values among CEs known to date,⁴⁻⁶⁾ retaining the original
8 activity of RmCE in a range of 3.2 to 10.8 after 24 h of incubation
9 at 4°C (Fig. 3b).

11 *Substrate specificity of RmCE*

12 The reaction products of various carbohydrates were analyzed by
13 TLC to assess the substrate specificity of RmCE (Fig. 4). The
14 reaction products of lactose, epilactose, cellobiose, mannanobiose,
15 and cellotriose were detected though the amount of reaction
16 product from cellotriose was small, whereas glucose, galactose,
17 mannose, sophorose, laminaribiose, gentiobiose, and maltose did
18 not serve as substrates for RmCE. In addition, RmCE was inactive
19 toward *N*-acetyl mannosamine, a substrate of AGE. Similarly to all
20 known CEs,⁴⁻⁶⁾ RmCE is highly specific to oligosaccharides linked
21 by the β -1,4-glycosidic linkage, and not active towards
22 monosaccharides.

Fig. 4

23 Next, the kinetic parameters of RmCE for cellobiose and lactose
24 were determined from the initial velocities for varying
25 concentrations of substrates (Table 1). RmCE showed similar K_m
26 values for the two substrates, while the k_{cat} value for lactose (111
27 s⁻¹) was 1.4-fold higher than that of cellobiose (80.8 s⁻¹), which led
28 to a higher preference for lactose over cellobiose, unlike known

1 CEs.⁴⁻⁶⁾

2 The conversion ratios of lactose and cellobiose for epilactose
3 and 4-*O*-D- β -glucosyl-D-mannose respectively after the reactions
4 reached full equilibrium were essentially equal, around 30%, at all
5 the substrate concentrations tested (Supplemental Fig. 2). In the
6 reaction to 100 mM mannobiose and epilactose, the conversion
7 ratios for the epimerized products were 69.5% and 67.9%
8 respectively.

11 Discussion

13 In this study, we characterized a thermophilic CE from *R.*
14 *marinus* JCM9785. Multiplealignment of the amino acid sequence
15 of RmCE with those of known CEs (RaCE, BfCE, and EcCE), aAGE,
16 and pAGE suggested that RmCE has a similar structure. RmCE was
17 predicted to consist of 12 α -helices to establish an (α/α)₆ barrel
18 structure, like comparable enzymes (Fig. 1). All the amino acid
19 residues important for catalysis, which have been confirmed by
20 site-directed mutagenesis studies,^{9,10)} were completely conserved
21 in RmCE. It is assumed that His259 and His390 of RmCE,
22 corresponding to the putative catalytic His residues of RaCE,
23 porcine AGE, and *Anabaena* AGE,⁸⁻¹⁰⁾ play roles as catalytic amino
24 acid residues. Consistently with this assumption, the pK_a value of
25 side chain of His is about 6.3, and RmCE is active in a range of pH
26 6-9 but not on the acidic side, although it was fully stable at such
27 pH values.

28 RmCE acted only on β -1,4-linked oligosaccharide, cellobiose,

1 lactose, epilactose, mannobiose, and cellotriose like other CEs (Fig.
2 4). Although the structures of the epimerized products were not
3 confirmed, the reaction products of the cellooligosaccharides,
4 mannobiose, and epilactose were assumed to be epimerized
5 cellooligosaccharides with a mannose residues at the reducing end,
6 β -1,4-D-mannosyl-D-glucose, and lactose respectively, based on the
7 reaction pattern of RmCE toward lactose and those of known
8 CEs.⁴⁻⁶⁾ Kinetic analysis of RmCE revealed that it is a sole enzyme
9 with a preference for lactose over cellobiose in terms of reaction
10 efficiency (Table 1). At present, the three-dimensional structure of
11 CE has not been elucidated, and the structure-function relationship
12 of CE is not fully understood, although some important amino acid
13 residues for catalysis have been reported.¹⁰⁾ In contrast to AGE,
14 which acts on *N*-acetylmannosamine and *N*-acetylglucosamine, all
15 the CEs reported to date act on β -1,4-linked oligosaccharides and
16 are inert toward monosaccharides, including *N*-acetylglucosamine
17 and *N*-acetylmannosamine. This implies the existence of structural
18 elements for the recognition of the β -1,4-glucosidic linkage at the
19 reducing end of substrate, chain-length of a substrate, and
20 monomer component of the substrate.

21 It is not easy to compare the kinetic parameters of RmCE with
22 those of other known CEs, because the reaction temperature of
23 RmCE was different from those of the others, but RmCE
24 demonstrated 1.4 to 3.4-fold higher k_{cat} for lactose than known CEs.
25 This resulted from the high thermal stability of RmCE, and the
26 enzyme reaction at 60°C cannot have been performed by the other
27 known enzymes due to thermal lability. The high k_{cat} for lactose of
28 RmCE is very attractive for industrial use, since the enzyme

1 reaction in the practical process is thought to be performed at much
2 higher concentrations than the K_m value of lactose, and the k_{cat}
3 value is the sole determinant of the reaction rate per mg of enzyme.

4 RmCE showed maximum activity at 80-90°C and was stable up to
5 80°C. This is the first report of the thermo-tolerance CE. In RmCE,
6 the contents of basic amino acids, Arg and His, are considerably
7 higher than RaCE, whereas the content of Lys of RmCE is lower
8 (Supplemental Table 2). On the other hand, the contents of the
9 acidic amino acids, Glu and Asp, of RmCE are close to and lower
10 than RaCE respectively. It is known that basic amino acids play an
11 important role in thermostability in thermophilic enzymes, because
12 they coordinate the salt-bridge at the surface of the protein and
13 toughen protein structure against high temperature.^{20,21)} Especially,
14 the guanidinium group of Arg is able to form more than one
15 electrostatic bond,²²⁾ and increased Arg content has been
16 correlated with heat stability in thermophilic enzymes.²³⁾ Indeed,
17 the Arg content of RmCE was remarkably higher (2.7-fold) than
18 that of RaCE (Supplemental Table 2). Model structures of RaCE
19 and RmCE constructed using the CPHmodels-3.0 program²⁴⁾
20 suggested that RmCE has significantly higher numbers of Arg
21 residues on the surface of the protein than RaCE does
22 (Supplemental Fig. 3). Arg residues on the surface of RmCE might
23 form salt-bridge interactions with acidic amino acid residues to
24 make the protein structure significantly rigid. The surface negative
25 charges of RmCE are assumed to be canceled by these salt-bridge
26 interactions, providing an explanation for the non-adsorption of
27 RmCE on an anion exchange resin, Toyopearl DEAE-650M, at a
28 higher pH value (7.0) than the theoretical pI (6.3). Comparing the

1 amino acid sequences, more Pro residues were situated in the
2 putative loop connecting α -helix 1 and α -helix 2 of RmCE than the
3 other CEs and AGEs (Fig. 1). Especially, three Pro residues are
4 positioned near the N-terminal of putative α -helix 2, including a
5 crucial Arg66.^{9,10)} These Pro residues might stabilize this loop
6 structure to maintain the structure of the active site at high
7 temperatures as seen for thermostable oligo-1,6-glucosidase.²⁵⁾

8 With high thermal stability and high activity at weakly acidic pH,
9 RmCE is the most useful for the production of epilactose among
10 known CEs. Enzyme reaction at high temperature makes it possible
11 to carry out the CE reaction at high substrate concentrations,
12 simplifying the purification of epilactose from the reaction mixture
13 because the tedious concentration procedure to remove unreacted
14 lactose by crystallization²⁶⁾ is made easy. In addition,
15 non-enzymatic epimerization, which occurs at alkaline pH and high
16 temperature, can be avoided because RmCE is most active at
17 weakly acidic pH.

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Figure legends

Fig. 1. Multiple-Alignment of Amino Acid Sequences of CEs and AGEs.

The amino acid sequences of CEs and AGEs were aligned using the Clustal W program.²⁷⁾ Black, dark gray, and light gray indicate complete, 80%, and 60% conservation respectively. Gray bars above a sequence represent predicted α -helices forming the catalytic (α/α)₆ barrel. Black triangles and circles show the putative catalytic His residues and the amino acid residues important for catalysis respectively. RmCE, *R. marinus* JCM9785 CE; ACY49317, ACY49317 protein from *R. marinus* DSM4252; RaCE, *R. albus* NE1 CE (Genbank ID: BAF81109); BfCE, *B. fragillis* NCTC9343 CE (Genbank ID: BAH23773.1); EcCE, *E. cellulosolvens* NE13 CE (Genbank ID: BAG68451.1); pAGE, pig AGE (Swiss-Prot ID: P17560.2); aAGE, *Anabaena* sp. CH1 AGE (Genbank ID: ABG57043.1). The N-terminal amino acid sequence of native RmCE and the sequences of tryptic peptides whose masses matched the theoretical values are underlined and boxed respectively.

Fig. 2. SDS-PAGE Analysis of Native and Recombinant RmCE.

SDS-PAGE was performed on a 15% polyacrylamide gel, and protein was detected by Coomassie Brilliant Blue staining. a, SDS-PAGE of the native RmCE purified from the cell-free extract from *R. marinus* JCM9785. One μ g of protein was loaded on the gel. The molecular masses of the size markers are shown on the left. M, size marker; 1, purified native RmCE. b, SDS-PAGE of the

1 recombinant RmCE produced in *E. coli*. 2, Cell-free extract of *E.*
2 *coli* transformant producing recombinant RmCE (5 µg of protein
3 was analyzed). 3, Purified recombinant RmCE (1 µg).

4
5 **Fig. 3.** Effects of Temperature and pH on RmCE Activity and
6 Stability.

7 a, Effect of temperature. Solid and hollow circles indicate
8 relative activity at the indicated temperature and the residual
9 activity after heat treatment for 30 min, respectively. b, Effect of
10 pH. Solid and hollow circles indicate relative activity at the
11 indicated pH and the residual activity after pH treatment for 24 h at
12 4°C respectively.

13
14 **Fig. 4.** TLC Analysis of the Reaction Product of RmCE for Various
15 Sugars.

16 The reaction of RmCE to various sugars was analysed by TLC to
17 elucidate the substrate specificity of RmCE. The left and right
18 lanes for each number indicate the samples before and after the
19 reaction respectively. The reaction products are indicated by
20 arrows. 1, glucose; 2, galactose; 3, mannose; 4, lactose; 5,
21 epilactose; 6, cellobiose; 7, mannobiose; 8, cellotriose; 9, maltose;
22 10, gentiobiose; 11, laminaribiose; 12, sophorose; 13, *N*-acetyl
23 mannosamine.

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Fig. 2. Ojima, et al

a

b

Fig. 3. Ojima, et al

Fig. 4. Ojima, et al

Table 1. Kinetic Parameters of CE for Cellobiose and Lactose

Origin	Cellobiose			Lactose		
	k_{cat} (s ⁻¹)	K_{m} (mM)	$k_{\text{cat}}/K_{\text{m}}$ (s ⁻¹ ·mM ⁻¹)	k_{cat} (s ⁻¹)	K_{m} (mM)	$k_{\text{cat}}/K_{\text{m}}$ (s ⁻¹ ·mM ⁻¹)
<i>R. marinus</i> ^{a)}	80.8	27.2	2.97	111	28.8	3.85
<i>R. albus</i> ^{4) b)}	63.8	13.8	4.62	52.1	33.0	1.58
<i>B. fragilis</i> ^{5) b)}	67.6	3.75	18.0	79.5	6.56	12.1
<i>E. cellulosolvens</i> ^{6) b)}	28.5	11.3	2.52	32.5	72.0	0.451

The reaction velocity at varying substrate concentrations was measured at 60°C and 30°C for a) and b) respectively.