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

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

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

Cellobiose 2-epimerase (CE; EC 5.1.3.11) reversibly converts glucose residue to mannose residue at the reducing end of β-1,4-linked oligosaccharides such as cellobiose and lactose to produce 4-*O*-β-D-glucosyl-D-mannose and 4-*O*-β-D-galactosyl-D-mannose (epilactose) respectively. It was first found in the culture fluid of an anaerobic ruminal bacterium, *Ruminococcus albus*.[1] It is a unique enzyme that catalyzes hydroxy-stereoisomerism at the C-2 position of a non-modified sugar through a proton abstraction/addition mechanism,[2] because almost all carbohydrate epimerases act on activated sugars bearing nucleotidyl, phosphoryl, or acyl groups.

The gene encoding CE has been isolated from the genomic DNA of *R. albus* NE1,[3] and the enzymatic characteristics of *R. albus* CE (RaCE) were investigated with a recombinant enzyme produced in *Escherichia coli*.[4] RaCE is most active at neutral pH values and relatively low temperature (30°C). It epimerizes not only cellobiose but also lactose, cellotriose, and cellotetoraose, although it strictly recognizes the β-1,4-glycosidic linkage at the reducing end part of substrates.[4]

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

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

The amino acid sequence of *R. albus* CE (RaCE) shows relatively weak similarity to *N*-acetylglucosamine 2-epimerases (AGEs) from porcine kidney and *Anabaena* sp. CH1, whose three-dimensional structures are available.[3] Both AGEs are dimeric enzymes, while CE is monomeric. Each monomer of the AGE is composed of an $(\alpha/\alpha)_6$ barrel fold.[8,9] A soaking study of porcine AGE crystal with *N*-acetylglucosamine revealed that His382, His248, Glu251, and Arg60 are situated at the nearest position to bound *N*-acetylglucosamine or a mixture of *N*-acetylglucosamine and *N*-acetylmannosamine, which was not precisely identified.[8] The two corresponding His residues of *Anabaena* AGE were envisaged to serve as the general acid and the general base catalysts in proton abstraction/addition at the C2 position by Lee et al., because these residues were about 8 Å apart and can ideally act as symmetric acid/base catalysts in reversible epimerization.[9] Secondary structure prediction suggested that RaCE is also formed by an $(\alpha/\alpha)_6$ barrel as AGEs, and two putative catalytic His residues are observed at the equivalent positions.[10] These conserved His residues of RaCE were confirmed to be essential for the catalytic activity by site-directed mutagenesis,[10] suggesting that CE and AGE share the essential structure of the active site, including

catalytic amino acids.

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

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

Materials and Methods

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

Bioresource Project of Ministry of Education, Culture, Sports, Science and Technology, Japan. *E. coli* DH5α and *E. coli* BL21 (DE3)-Codonplus^{TM} 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 μL; column, Sugar SP0810 (ϕ8.0 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

purified by HPLC for further analysis.

To identify the monosaccharide component of the reaction product, purified product was hydrolyzed with trifluoroacetic acid (TFA) and the resulting monosaccharides were analyzed by thin layer chromatography (TLC). Twenty-five μL of 4 M TFA and an equal volume of 0.4% w/v reaction product were mixed and this was kept at 100°C for 3 h. Then the mixture was dried *in vacuo* and the residue was dissolved in 25 μL of water. One μL of the hydrolysate was spotted on a TLC plate (Silica Gel 60 F_{254}; Merck; Darmstadt, Germany) and developed in a solvent system of 2-propanol/1-butanol/water (2:2:1; v/v). The chromatogram was visualized using a detection reagent (ethanol/sulfuric acid/acetic acid/anisaldehyde, 92:3:2:3; v/v). Glucose (Kanto Chemical; Tokyo), mannose (Sigma), galactose (Wako Pure Chemical; Osaka, Japan), and fructose (Nacalai Tesque; Kyoto, Japan) were used as standards.

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

facilitate amplification of the DNA fragment with a GC-rich template. The sequence of the amplified PCR product was analyzed using a GenomeLab™ Dye Terminator Cycle Sequencing Kit (Beckman Coulter; Brea, CA) and an automatic DNA sequencer, CEQ 2000XL ™ (Beckman Coulter). Then *Nde*I and *Xho*I sites were introduced into the 5'- and 3'-temini of the RmCE gene respectively by PCR to clone into an expression vector, pET22b. The sequences of the primers used were as follows: 5'-AAACATATGGTGAGCACGGAGACCATC-3' (sense, *Nde*I site underlined) and 5'-AAACTCGAGCTACCGGGATCGAACGTG-3' (antisense, *Xho*I site underlined). The genomic DNA was used as the template. The amplified DNA fragment and pET22b were digested with *Nde*I and *Xho*I (both from Takara; Kyoto, Japan) and connected using DNA Ligation Kit Mighty Mix (Takara). The transformation of *E. coli* and preparation of plasmid DNA were carried out by Hanahan's method[15] and the alkaline lysis method respectively.[16] The amplified DNA fragment was verified by sequencing as well.

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

Toyopearl DEAE-650M column (ϕ5 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 (ϕ2.6 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; ϕ2.6 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 μg

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

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

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

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

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

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

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

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

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

Results

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

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

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

Production of recombinant RmCE in E. coli

A gene homologous to the ACY49317 gene from *R. marius* DSM4252 was obtained from the genome of *R. marinus* JCM9785 by PCR. The deduced amino acid sequence of the gene obtained was 94.2% identical to the ACY49317 protein (Fig. 1). In order to confirm that the cloned gene encoded RmCE, native RmCE was purified from the cell-free extract of *R. marinus* JCM9785 (Fig. 2a), and the N-terminal amino acid sequence and the masses of the tryptic peptides of the purified enzyme were analyzed. The N-terminal amino acid sequence of the purified native RmCE, Glu-Thr-Ile-Pro-Asp-Val-Arg-Arg-Leu, analyzed by Edman degradation, was included in the deduced amino acid sequence of the cloned gene (Fig. 1). Additionally, the peptide masses of the tryptic peptides of the native RmCE, analyzed by MALDI-TOF-MS, matched the theoretical values with 27.2% coverage of the entire amino acid sequence (Fig. 1, Supplemental Fig. 1, and Supplemental Table 1; see *Biosci. Biotechnol. Biochem.* Web site). 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 70ºC 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

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

Substrate specificity of RmCE

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

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

CEs.[4-6] Table 1

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

Discussion

In this study, we characterized a thermophilic CE from *R. marinus* JCM9785. Multiplealignment of the amino acid sequence of RmCE with those of known CEs (RaCE, BfCE, and EcCE), aAGE, and pAGE suggested that RmCE has a similar structure. RmCE was predicted to consist of 12 α-helices to establish an $(\alpha/\alpha)_6$ barrel structure, like comparable enzymes (Fig. 1). All the amino acid residues important for catalysis, which have been confirmed by site-directed mutagenesis studies,[9,10] were completely conserved in RmCE. It is assumed that His259 and His390 of RmCE, corresponding to the putative catalytic His residues of RaCE, porcine AGE, and *Anabaena* AGE,[8-10] play roles as catalytic amino acid residues. Consistently with this assumption, the pK_a value of side chain of His is about 6.3, and RmCE is active in a range of pH 6-9 but not on the acidic side, although it was fully stable at such pH values.

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

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

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

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

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

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

With high thermal stability and high activity at weakly acidic pH, RmCE is the most useful for the production of epilactose among known CEs. Enzyme reaction at high temperature makes it possible to carry out the CE reaction at high substrate concentrations, simplifying the purification of epilactose from the reaction mixture because the tedious concentration procedure to remove unreacted lactose by crystallization[26] is made easy. In addition, non-enzymatic epimerization, which occurs at alkaline pH and high temperature, can be avoided because RmCE is most active at 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 $(\alpha/\alpha)_6$ 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 Coomasie Brilliant Blue staining. a, SDS-PAGE of the native RmCE purified from the cell-free extract from *R. marinus* JCM9785. One μg 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

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

Fig. 3. Effects of Temperature and pH on RmCE Activity and Stability.

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

Fig. 4. TLC Analysis of the Reaction Product of RmCE for Various Sugars.

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

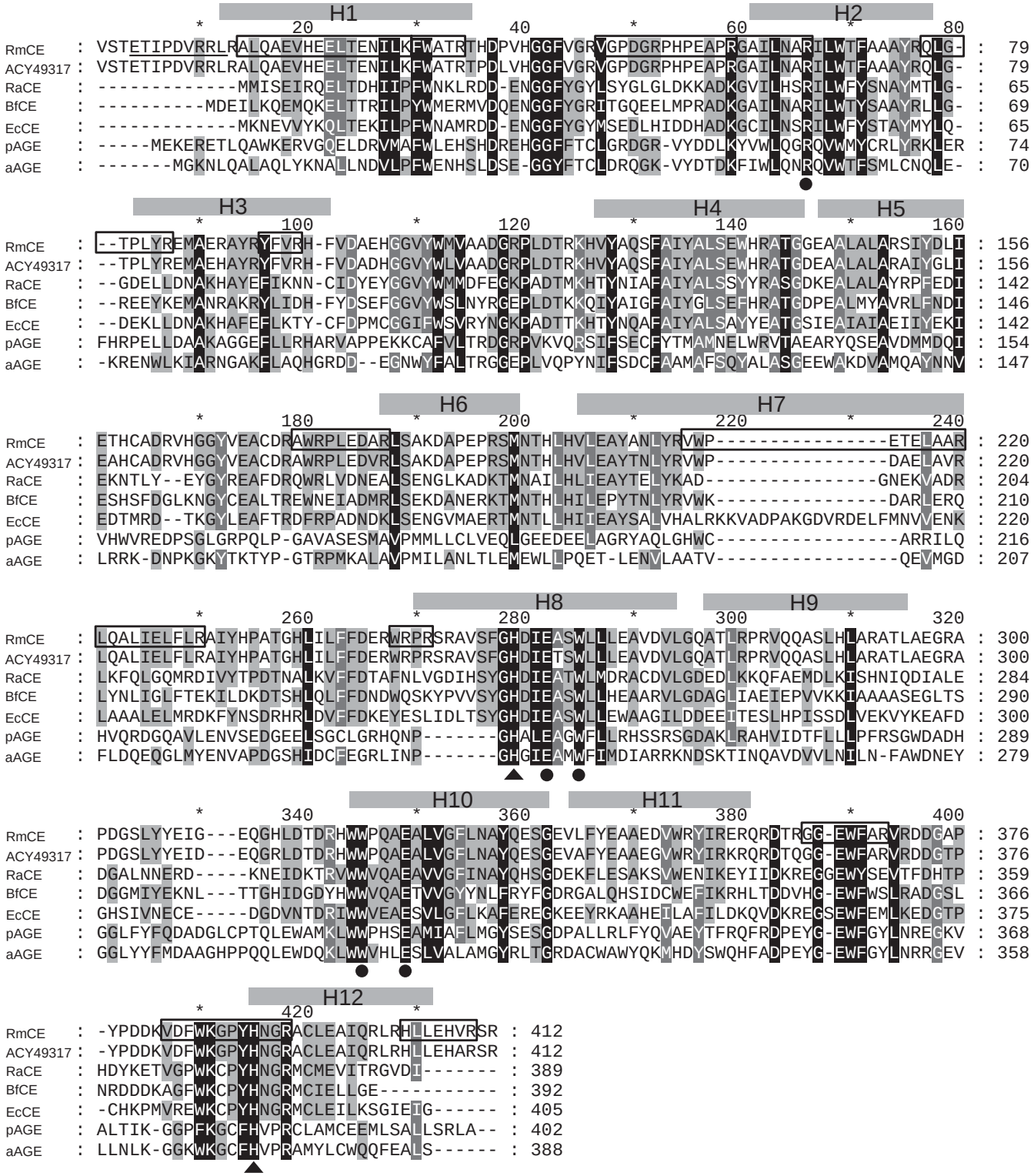


Fig. 1. Ojima, et al

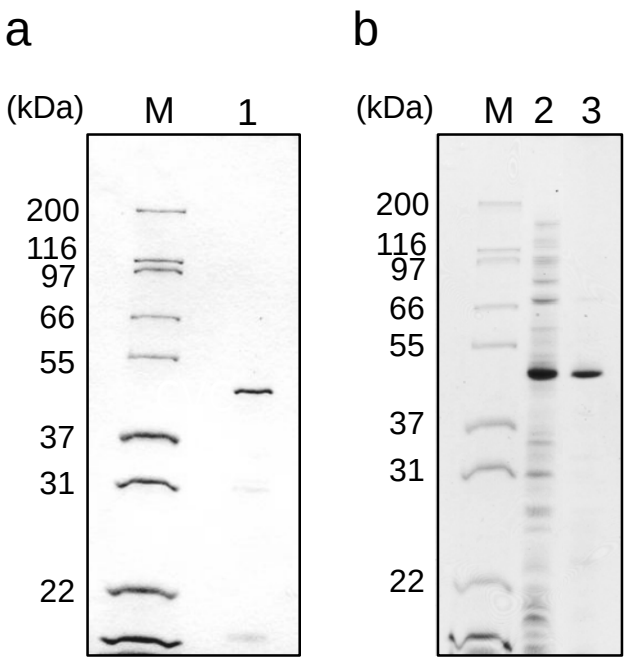
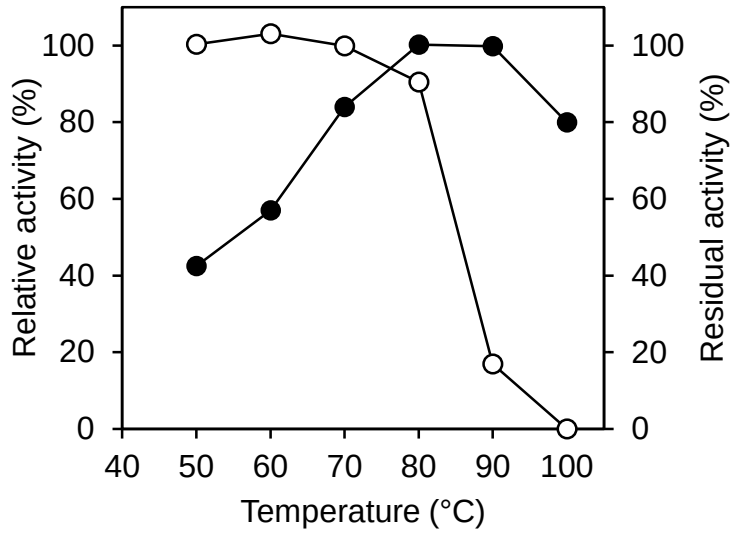


Fig. 2. Ojima, et al

a



b

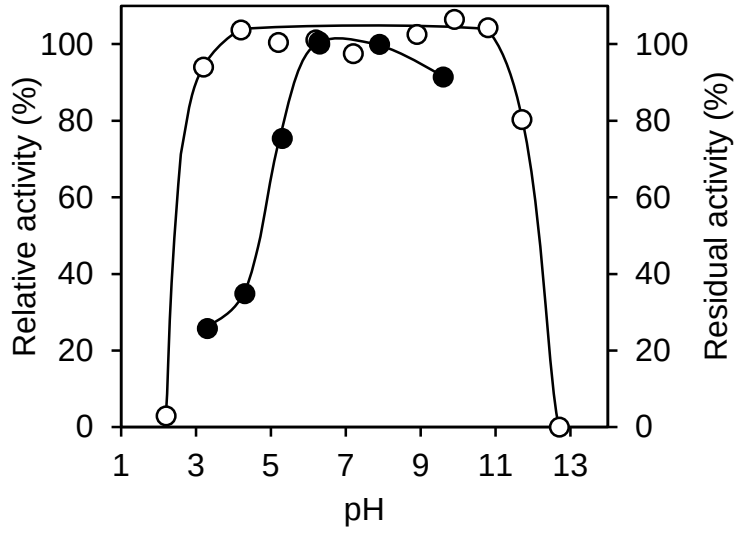


Fig. 3. Ojima, et al

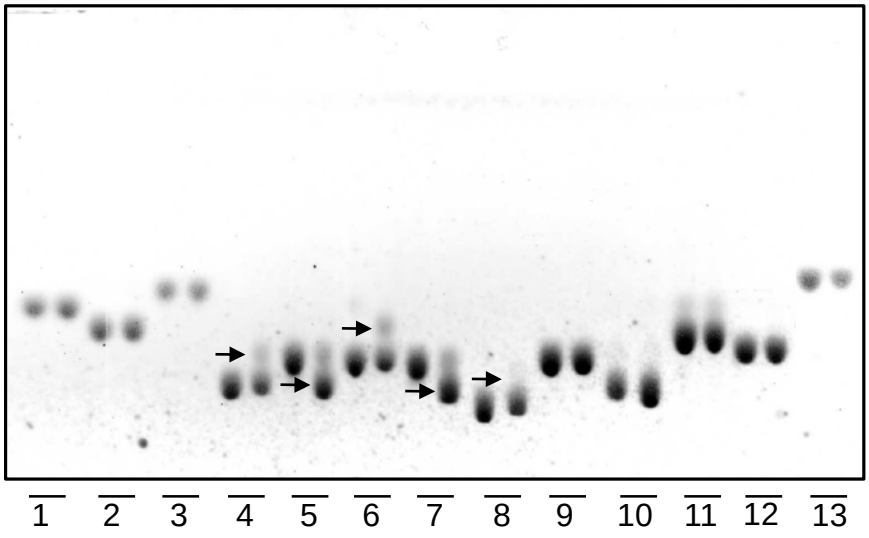


Fig. 4. Ojima, et al

Table 1. Kinetic Parameters of CE for Cellobiose and Lactose

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

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