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Site-directed mutagenesis of possible catalytic residues of cellobiose 2-epimerase from *Ruminococcus albus*

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Section Microbial and Enzyme Technology

Abstract Cellobiose 2-epimerase (EC 5.1.3.11) from *Ruminococcus albus* (RaCE) catalyzes the reversible epimerization of cellobiose and lactose to 4-*O*- β -D-glucopyranosyl-D-mannose and 4-*O*- β -D-galactopyranosyl-D-mannose (epilactose). Based on the structure-based amino acid sequence alignment with *N*-acetyl-D-glucosamine 2-epimerases (EC 5.1.3.8) from porcine kidney and *Anabaena* sp. CH1 and on the computer-aided model building of the tertiary structure of RaCE, we performed site-directed mutagenesis of possible catalytic residues in the enzyme, and the mutants were expressed in *Escherichia coli* cells. It was found that R52, H243, E246, W249, M251, W304, E308, H374, and M378 were absolutely required for the activity of RaCE. F114 and W303 (and possibly R377) also contributed to catalysis. These possible catalytic residues protruded into or near the active-site cleft surrounded by the inner α -helices in the predicted $(\alpha/\alpha)_6$ core barrel structure of RaCE.

Keywords Cellobiose 2-epimerase • *Ruminococcus albus* • Homology modeling • Site-directed mutagenesis • Epilactose • Prebiotics

Introduction

Ruminococcus albus is considered to be one of the main cellulolytic bacteria in the rumen, and it secretes various cellulolytic enzymes extracellularly (for instance, Ohmiya et al. 1987). Tyler and Leatherwood (1967) found the first known cellobiose 2-epimerase (CE; EC 5.1.3.11), which catalyzes the reversible epimerization between cellobiose and 4-*O*- β -D-glucopyranosyl-D-mannose (Glc-Man), in the culture broth of *R. albus* 7 (ATCC 27210^T). Recently, we cloned the genes for CEs from *R. albus* NE1 (RaCE) and *Eubacterium cellulosolvens* NE13 and characterized the enzymatic properties of the recombinant enzymes (Ito et al. 2007; Ito et al. 2008; Taguchi et al. 2008). CE catalyzes a hydroxyl stereoisomerism at C-2 of the reducing terminal glucose moiety of cello-oligosaccharides and lactose. We reported that a product from lactose, 4-*O*- β -D-glactopyranosyl-D-mannose (epilactose), had potential for use as a prebiotic. It possesses several health-promoting properties: stimulation of bifidobacteria growth, facilitation of calcium absorption, reduction of plasma cholesterol, and suppression of the conversion of primary bile acids to carcinogenetic secondary bile acids (Ito et al. 2008; Nishimukai et al. 2008; Watanabe et al. 2008). Hence, it is expected that CE can increase the value of lactose, cheese whey, and milk by creating novel milk products with prebiotic properties.

Despite extensive trials, we have not yet succeeded in obtaining crystals of RaCE for X-ray crystallographic analysis. To facilitate understanding of the catalytic feature, we identified possible catalytic residues required for the formation of Glc-Man and epilactose by site-direct mutagenesis.

Materials and methods

Site-directed mutagenesis

The chromosomal DNA of *R. albus* NE1 was isolated by the procedure of Murray and Thompson (1980). *Escherichia coli* JM109 for plasmid preparation and sequencing and *E. coli* BL21 (DE3) for recombinant RaCE expression were grown aerobically in Luria-Bertani broth. The RaCE gene (accession no. AB301953) was amplified by PCR, cloned into a pBluescript II SK(+) vector, and then subcloned into the *Nde*I/*Xho*I site of pET-23a, yielding construct pCE23a (Ito et al. 2008).

Amino acid replacements were performed using a QuikChange site-directed mutagenesis kit (Stratagene, USA) by following the manufacturer's protocol. PCR was performed using *Pfu* polymerase (Promega, USA) with pCE23a as the template. For instance, the primers used for R52→Ala (R52A) mutation were 5'-GGGTGTTATACTGCATTCGGCGATACTGTGGTTC-3' and 5'-GAACCACAGTATCGCCGAATGCAGTATAACACCC-3'. The primers for the R52K mutation were 5'-GGGTGTTATACTGCATTCGAAGATACTGTGGTTC-3' and 5'-GAACCACAGTATCTTCGAATGCAGTATAACACCC-3' (the underlined sequences indicate mutated codons). Other enzymes with mutations at specific positions were created in a similar way with appropriate primers. Incorporation of the desired mutation in all the mutant enzyme sequences and the absence of other PCR errors were verified. To produce mutant proteins, the resulting plasmids were introduced into competent *E. coli* BL21 (DE3) cells.

Purification of wild-type and mutant proteins of RaCE

The transformants were grown at 37°C in 3 l of Luria-Bertani broth supplemented with ampicillin (100 µg/ml) to an A_{600} of 0.6. Expression of CE was induced with isopropyl β -D-galactosyl pyranoside at 0.1 mM. After induction, the cells were grown at 16°C for 24 h before harvest. They were collected by centrifugation and sonicated in 20 mM sodium phosphate buffer (pH 6.3) on ice. The centrifugal supernatant was successively applied to columns of Q-Sepharose FF (GE Healthcare, USA) and hydroxyapatite (Seikagaku Kogyo, Japan) (Ito et al. 2008). All the purified recombinant mutants, including the wild-type enzyme, migrated as single band on 10% SDS-PAGE (Laemmli 1970), and all showed molecular masses of approximately 43.7 kDa.

Enzyme assays

The composition of the reaction mixture was the same as described previously (Ito et al. 2008). Briefly, the reaction mixture in a total volume of 100 µl containing 100 mM sodium phosphate buffer (pH 7.5), 20 mM cellobiose (or lactose), and enzyme was routinely incubated at 30°C for defined lengths of time. The rates of formation of Glc-Man from cellobiose or epilactose from lactose were measured in a Sugar SP0810 HPLC column (Shodex, Japan) with maltitol as the internal standard. One unit (U) of enzyme activity was defined as the amount of enzyme that formed 1 µmol of Glc-Man or epilactose per min. The K_m , V_{max} , and k_{cat} values of recombinant RaCE were estimated by a double reciprocal plot method. The catalytic efficiency toward each substrate was expressed as k_{cat}/K_m ($s^{-1} \text{ mM}^{-1}$). The protein concentration was determined by the method of Bradford (1976) with bovine serum albumin as a reference standard.

Structure-based alignment and homology modeling of RaCE

The secondary structure assignment of RaCE was carried out by the Kabsch-Sander method (Kabsch and Sander 1983). Structure-based alignment of the amino acid sequence of RaCE (accession no. BAF81108) was done with those of *N*-acetyl-D-glucosamine 2-epimerase (AGE) from porcine kidney (pAGE; PDB code 1fp3) (Itoh et al. 2000) and that from *Anabaena* sp. CH1 (aAGE; PDB code 2gz6) (Lee et al. 2007). A model of the tertiary structure of RaCE built with computer assistance was constructed based on the X-ray crystallographic structure of pAGE. Before modeling, the sequences where insertion and deletion occurred were manually aligned. All data sets were processed on a Silicon Graphics Octane2 workstation using the Insight II/Discover software package (Accelrys Inc., USA). The modeled structure was confirmed using the 3D-ID and ProStat programs. The figures were prepared using DS Visualizer (Accelrys Inc.).

Results and Discussion

Secondary structure prediction and structure-based multiple alignment of RaCE

To allow accurate homology modeling of RaCE and to select the possible catalytic residues for site-directed mutagenesis, we performed structure-based alignment of the amino acid sequence of RaCE with those of pAGE and aAGE, which have a known structure. As shown in Fig. 1, the sequence of RaCE was found to consist of 12 long

α -helices ($\alpha 1$ through $\alpha 12$) and 10 short β -strands, essentially similar to pAGE and aAGE. The even-numbered α -helices form the active-site cleft in a modeled structure of RaCE (see Fig. 2a). Among them, the strictly conserved residues were L49, R52, and W55 from $\alpha 2$, F114 and A118 from $\alpha 4$, G242, H243, E246, A245, and W251 from $\alpha 8$, W303, W304, E308, and Y317 from $\alpha 10$, and H374 and R377 from $\alpha 12$ (in RaCE numbering). Given increasingly numerous bacterial genomes, the CE family enzymes are phylogenetically distant from but connected to the AGE family enzymes (Senoura et al. in press). The sequence of RaCE had no homology to the other heterogonous carbohydrate-active epimerases reported to date (Ito et al. 2007).

Homology model building of tertiary structure of RaCE

Like the two AGEs, the modeled structure of RaCE has an $(\alpha/\alpha)_6$ -barrel with six outer helices ($\alpha 1$, $\alpha 3$, $\alpha 5$, $\alpha 7$, $\alpha 9$, and $\alpha 11$) facing the solvent and six inner helices ($\alpha 2$, $\alpha 4$, $\alpha 6$, $\alpha 8$, $\alpha 10$, and $\alpha 12$) forming an active-site cleft, as shown in Fig. 2a. It also contains five short anti-parallel β -sheets. The α_6/α_6 -fold architecture of RaCE is homologous to those of cellulose-degrading enzymes such CelA (PDB code 1cem), CelD (1clc), end/exocellulase E4 (Sakon et al. 1997), and alkaline endoglucanase N257 (Shirai et al. 2008), and the two AGEs as well, although their types of catalytic reactions are different.

Mutation of ionizable residues

The conserved residues with ionizable side-chains (R52, H243, E246, E308, H374, and R377) were the first targets for site-directed mutagenesis because these residues

protrude into the active-site pocket (Fig. 2b, left), and some of them must serve as acid/base catalysts. We substituted possible catalytic residues for other residues by site-directed mutagenesis: R52 to Ala (R52A), His (R52H), and Lys (R52K); H243 to Ala (H243A), Arg (H243R), and Lys (H243K); E246 to Ala (E246A) and Gln (E246Q); E308 to Ala (E308A), Asp (E308D), and Gln (E308Q); H374 to Ala (H374A), Lys (H374K), and Arg (H374R); and R377 to Ala (R377A) and His (R377H). The kinetic parameters of these mutants toward cellobiose and lactose are summarized in Tables 1 and 2. Mutants with R52A, R52K, R52H, H243A, H243R, E246A, E246Q, E308A, E308D, E308Q, H374A, H374K, and H374R completely lost the activity toward cellobiose and lactose, indicating that R52, H243, E246, E308 and H374 are absolutely required for catalysis. Itoh et al. (2000) suggested that R60 (R52), H248 (H243), E251 (E246), and H372 (H374) played an important role in the reaction of pAGE base on X-ray crystallographic analysis, and Lee et al. (2007) identified R57 (R52), H239 (H243), E308 (E308), and H378 (H374) as critical residues and E242 (E246) and R375 (R377) as essential residues in aAGE by site-directed mutagenesis (the numbers in parentheses indicate the corresponding residues in RaCE). Mutant with R377H exhibited no activity but one with R377A retained 43% of wild-type enzyme toward cellobiose and 23% toward lactose, suggesting that the conserved R377 may not be essential for catalysis in RaCE.

The 2-epimerization reaction involves a proton-abstracting step (Amein and Leatherwood 1969; Lee et al. 2007), and RaCE is active over the pH range 6–9.5 but not at pH 5.0–5.5 (data not shown). These results suggest that the crucial active-site residues possess a pK_a value greater than 5, such as His (pK_a = around 6.3) rather than Glu and Asp (pK_a = around 4.1). Therefore, the conserved H243 and H374 residues were assumed to be the key acid/base catalysts. R52, E246, and E308 (and possibly

R377) may assist the catalysis of RaCE.

Mutation of aromatic residues

It is generally accepted that Trp, Tyr, and Phe residues around an active site play a role in substrate binding in carbohydrate-metabolizing enzymes. In fact, RaCE activity was inhibited by *N*-bromosuccinimide (Ito et al. 2008). When the conserved F114 was replaced with Ala, the resulting F114A mutant was found to show a very low specific activity toward cellobiose and lactose. The reduced activity was accompanied by an increase in the K_m value and a decrease in the k_{cat} value drastically, when cellobiose was used as a substrate. We did not replace Y317 in the $\alpha 8$ element because it is buried deep inside the enzyme molecule.

Four conserved Trp residues (W55, W249, W303, and W304) were replaced with Phe individually. As the result, mutants with W249F and W304F completely lost their activities toward cellobiose and lactose (Tables 1 and 2). This result may reflect the fact that the two Trp residues are in close proximity to the above-mentioned crucial residues in the active-site pocket surrounded by the inner α -helices (see Fig. 2b, right). The specific activities toward cellobiose and lactose of the mutant with W303 were 11% and 1.9%, respectively, and the K_m values were increased greatly with a drastic decrease in k_{cat}/K_m values, suggesting that W303, together with the neighboring W304, also facilitates substrate binding. In contrast, the specific activities toward cellobiose and lactose of the mutant with W55F retained more than 80% of those of the wild-type enzyme although their K_m values were increased very greatly.

Early studies demonstrated that Trp residues were involved in the reaction of cellulases from fungi and bacteria (Pettersson 1968; Hurst et al. 1977; Clarke 1987;

Ozaki and Ito 1991). Further, in the cases of an acid endoglucanase from *Bacillus* (Kawaminami et al. 1994), CelD (1clc) from *Clostridium thermocellum* (Joy et al. 1992), and a cellobiohydrolase from *Trichoderma reesei* (Rouvinen et al. 1990), some Trp residues are suggested to be involved in substrate binding from X-ray crystallographic studies. Therefore, F114, W249, and W304 (and W303, if any) may be important for this role rather than direct involvement in the 2-epimerization reaction.

Mutation of sulfur-containing residues

Sulfhydryl inhibitors such as 4-chloromercuribenzoate, iodoacetamide, and heavy metal ions inhibit the activity of RaCE (Ito et al. 2008). Although the activities of mutants with C255S, C371S, and C379S were reduced significantly, they were still inhibited by the sulfhydryl inhibitors. Both C255 and C379 residues are buried inside the enzyme molecule (data not shown), and thus the substitution with Ser might cause the conformational change–induced reduction of enzyme activity. Further, C371 is located far from the possible catalytic residues. These results exclude the possibility that RaCE is a thiol enzyme, and the sensitivity to heavy metal ions might be the result of interaction with Trp residues required for catalysis (Pettersson 1968; Hurst et al. 1977).

RaCE is very sensitive to H₂O₂ at a concentration lower than 1 mM, suggesting that certain Met residues are readily inactivated by oxidants and air (Saeki et al. 2000; Hagihara et al. 2001; Hagihara et al. 2003; Nonaka et al. 2004). Besides the two Met residues at the N-terminus, RaCE has 10 Met residues at positions 62, 95, 96, 106, 179, 212, 251, 270, 378, and 380 (Fig. 1). Some of these residues must be changed by non-oxidizable residues for the industrial synthesis of prebiotic epilactose.

Unexpectedly, mutants with M251T and M378T were found to be inactive forms (Table

1). Although we have no idea at present why the M251T mutant lost the activity, it is noteworthy that M378 is spatially near the possible critical residue H374 (see Fig. 2b). This may imply that RaCE favors a CH₃S group over an OH group for catalysis. The other mutants with Met→Thr substitution retained about 11–51% activity of wild-type enzyme and had K_m values 130% to 1,060% of the wild-type enzyme (Table 1). They had not acquired oxidative stability against H₂O₂, indicating that the inactivation is due to the oxidation of other oxidizable residues.

A hydropathy plot of the sequence of RaCE was drawn according to the equation of Hopp and Woods (1981). As far as the plot reflects the actual active-site cleft, H243, W249, H374, H377 and M378 are located at the entrance, and the other possible catalytic residues are in the cleft. In particular, R52 and F114 are located in a deep and hydrophobic bottom (data not shown).

Finally, the possible catalytic residues identified in this study are included in the (α/α)₆-barrel concentric structure of RaCE, as shown in Fig. 2b. The deep active-site cleft is likely to have the crucial catalytic residues and substrate-binding sites that specifically recognize the reducing terminal glucose or mannose of β -1,4-linked disaccharide moieties of oligosaccharides.

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Legends to figures

Fig. 1 Structure-based amino acid sequence alignment of RaCE with two AGEs of known structure. The amino acid sequence of RaCE (accession no. BAF81108) was aligned with those of pAGE (PDB code 1fp3) and aAGE (PDB code 2gz6). Long α -helices in red and short β -strands in blue are the elements of pAGE and aAGE. The corresponding elements in the RaCE sequence, which were predicted by the method of Kabsch and Sander (1983), are underlined, and the α -helices are labeled with $\alpha 1$ through $\alpha 12$. Strictly conserved residues are indicated by stars beneath the three sequences.

Fig. 2 Ribbon stereo-diagram representation of a model structure of RaCE. **(a)** The model structure of RaCE was constructed using the crystal structure of pAGE as a template. The secondary structure elements, α -helices ($\alpha 1$ through $\alpha 12$), β -strands, and loops and coils are colored red, cyan, and green, respectively. **(b)** The possible catalytic residues identified by site-directed mutagenesis are shown in the CPK representation on the ribbon diagrams (right and left) of the model structure of RaCE.

Table 1 Kinetic values for cellobiose of RaCE and its mutant proteins

Mutation	Spec act (U mg ⁻¹)	k_{cat} (s ⁻¹)	K_{m} (mM)	$k_{\text{cat}}/K_{\text{m}}$ (s ⁻¹ mM ⁻¹)
Wild type	51.0 (100)	63.8	13.8	4.62
R52A	ND*	—***	—	—
R52K	ND	—	—	—
R52H	ND	—	—	—
W55F	42.0 (84)	215	127	1.69
M62T	10.9 (21)	30.3	53.8	0.58
M95T	12.3 (24)	33.4	56.2	0.61
M96T	8.73 (17)	43.8	121	0.37
M106T	5.60 (11)	29.7	128	0.23
F114A	1.15 (2.3)	4.64	55.6	0.08
M179T	6.30 (12)	36.9	146	0.25
M212T	6.35 (13)	31.5	20.1	1.57
H243A	ND	—	—	—
H243R	ND	—	—	—
H243K	TR**	—	—	—
E246A	TR	—	—	—
E246Q	ND	—	—	—
W249F	ND	—	—	—
M251T	ND	—	—	—
M270T	9.00 (18)	14.4	18.0	0.80
W303F	5.99 (11)	46.4	108	0.43
W304F	ND	—	—	—
E308A	ND	—	—	—
E308D	ND	—	—	—
E308Q	ND	—	—	—
H374A	ND	—	—	—
H374K	ND	—	—	—
H374R	ND	—	—	—
R377A	12.1 (43)	26.6	36.8	0.72
R377H	ND	—	—	—
M378T	1.40 (2.7)	5.16	83.7	0.06
M380T	25.8 (51)	40.0	34.3	1.17

ND*, no activity; TR**, trace activity (<0.2%); —***, not determined.

Table 2 Kinetic values for lactose of RaCE and its mutant proteins

Mutation	Spec act (U mg ⁻¹)	k_{cat} (s ⁻¹)	K_{m} (mM)	$k_{\text{cat}}/K_{\text{m}}$ (s ⁻¹ mM ⁻¹)
Wild type	28.4 (100)	52.1	33.0	1.58
R52A	ND*	—***	—	—
R52K	ND	—	—	—
R52H	ND	—	—	—
W55F	22.7 (80)	78.3	72.3	1.08
F114A	0.44 (1.6)	1.79	95.2	0.019
H243A	ND	—	—	—
H243R	ND	—	—	—
H243K	TR**	—	—	—
E246A	TR	—	—	—
E246Q	ND	—	—	—
W249F	ND	—	—	—
W303F	0.53 (1.9)	4.04	145	0.028
W304F	ND	—	—	—
E308A	ND	—	—	—
E308D	ND	—	—	—
E308Q	ND	—	—	—
H374A	ND	—	—	—
H374K	ND	—	—	—
H374R	ND	—	—	—
R377A	6.60 (23)	20.9	73.3	0.285
R377H	ND	—	—	—

ND*, no activity; TR**, trace activity (<0.2%); —***, not determined.

Fig. 1

		α1		α2	
RaCE		M M I S E I R Q E L T D H I I P F W N K L R D D E N G G F Y G Y L S Y G L E L D K K A D K G V I L H S R I L W F Y S N A Y M T L G			65
pAGE		M E K E R E T L Q A W K E R V G Q E L D R V M A F W L E H S H D R E H G G F F T C L G R D G R V - Y D D L K Y V W L Q G R Q V W M Y C R L Y R K L E			73
aAGE		M G K N L Q A L A Q L Y K N A L I N D V L P F W E N H S L D S E - G G Y F T C L D R Q G K V - Y D T D K F I W L Q N R Q V W T F S M L C N Q L E			70
		* * * *		* * * *	
		α3		α4	
RaCE		---G D E L L D N A K H A Y E F I K N N C I D Y E Y - G G V Y W M M D F E G K P A D T M K H T Y N I A F A I Y A L S S Y Y R A S G D K E A L A L A			135
pAGE		R F H R P E L L D A A K A G E F L L R H A R V A P P E K K C A F V L T R D G R P V K V Q R S I F S E C F Y T M A M N E L W R V T A E A R Y Q S E A			147
aAGE		K N - R E N W L K I A R N G A K F L A Q H G R D D E G N - - W Y F A L T R G G E P L V Q P Y N I F S D C F A A M A F S Q Y A L A S G E E W A K D V A			140
		* * *		* * *	
		α5		α6	α7
RaCE		Y R L F E D I E K N T L Y E Y G Y R E A F D R Q W R L V D N E A L S E N G L K A D K T M N A I L H L I E A Y T E L Y K A D G N E K V A D R L K F Q L			209
pAGE		V D M M D Q I V H W V R E D P S G L G R P Q L - - - - - P G A V A S E S M A V P M M L L C L V E Q L G E E D E E L A G R Y A Q L G H W C A R R I L Q			216
aAGE		M Q A Y N N V L R R - K D N P K G K Y T K T Y - - - - - P G T R P M K A L A V F M I L A N L T L E M - E W L L P Q E T L E N V L A A T V Q E V M G D			207
				*	
			α8	α9	
RaCE		G Q M R D I V Y T P D T N A L K V F F D T A F N L V G D I H S Y G H D I E A T W L M D R A C D V L G D E D L K K Q F A E M D L K I S H N I Q D I A L			283
pAGE		H V Q R D - G Q A V L E N V S E D G E - E L S G C L G R H Q N P G H A L E A G W F L L R H S S R S G D A K L R A H V I D T F L L L P F R S C W D A D			288
aAGE		F L D Q E - Q G L M Y E N V A P D G S - H I D C F E G R L I N P G H G I E A M W F I M D I A R R K N D S K T I N Q A V D V V L N I L N F A - W D N E			278
		*	* * * *	*	*
			α10	α11	
RaCE		E D G A L N N E R D K N E I D - - - - - K T R V W W V Q A E A V V G F I N A Y Q H S G D E K F L E S A K S V W E N I K E Y I I D K R E G G E W Y S			351
pAGE		- H G G L F Y F Q D A D G L C P T Q L E W A M K L W W P H S E A M I A F L M G Y S E S G D P A L L R L F Y Q V A E Y T F R Q F R D P - E Y G E W F G			360
aAGE		- Y G G L Y Y F M D A A G H P P Q Q L E W D Q K L W W V H L E S L V A L A M G Y R L T G R D A C W A W Y Q K M H D Y S W Q H F A D P - E Y G E W F G			350
		* * *	* * *	* * * *	
			α12		
RaCE		E V T F D H T P H D Y K E T V G P W K - C P Y H N G R M C M E V I T R G V D I			389
pAGE		Y L N R E G K V A L T I K - G G P F K G C - F H V P R C L A M C E E M L S A L L S R L A			402
aAGE		Y L N R R G E V L L N L K - G G K W K G C - F H V P R A M Y L C W Q Q F E A L S			388
		* * * *			

Fig. 2

