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Outline of Research Result

Functional analysis of cellobiose 2-epimerase and related enzymes and their applications for the efficient synthesis of useful carbohydrates

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Research aims

Isomerization and epimerization of carbohydrates are important reactions not only for carbohydrate metabolism but also for the production of rare sugars from abundantly available sugars. From among carbohydrate isomerases and epimerases, cellobiose 2-epimerase (CE) is the sole enzyme catalyzing the epimerization of oligosaccharides. Structural analysis of CE revealed that it forms a superfamily together with several monosaccharide isomerases and epimerases (Fig. 1).[1)]

This superfamily includes many proteins with unknown functions, and thus novel enzymes may be found among them. In this study, to establish the efficient synthesis of carbohydrates using sugar isomerases and epimerases, mutational analysis of CE and functional analysis of an enzyme with unknown functions were carried out.

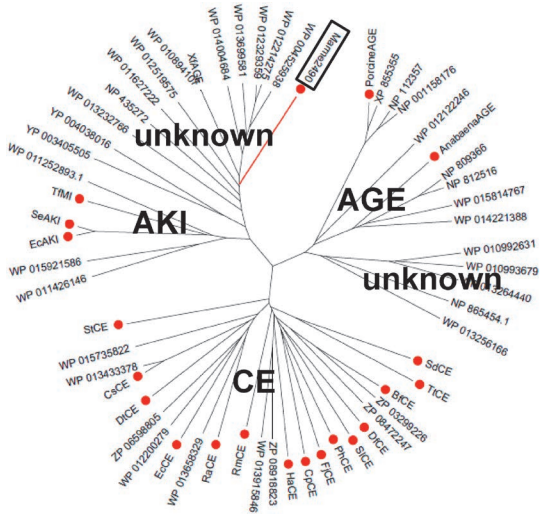

Fig. 1. Phylogenetic tree of CE and related enzymes.
AKI, aldose-ketose isomerase; AGE, acylglucosamine 2-epimerase. Marme_2490 is enclosed in a box.

Methods

1. Analysis of mutant cellobiose 2-epimerase from *Rhodothermus marinus* (RmCE)

Arg66, Tyr124, Asn196, His200, Glu262, Tyr307, His320, and Trp322, which are situated around the substrate-binding site of RmCE, were substituted with Ala using Primestar Mutagenesis Basal Kit (Takara Bio). The mutant enzymes were produced in *Escherichia coli* BL21 (DE3) using the pET22b (Merck)-derived expression plasmid as wild type enzyme.[2)] The mutant enzymes produced were purified to homogeneity by Toyopearl DEAE 650M and Toyopearl Butyl 650M column chromatographies. The purified mutant RmCEs were incubated with sophorose (Glcβ1-2Glc), laminaribiose (Glcβ1-3Glc), cellobiose (Glcβ1-4Glc), and gentiobiose (Glcβ1-6Glc). A reaction mixture (5 μL) containing 11.3 μM enzyme, 20 mM substrate, and 20 mM sodium phosphate buffer (pH 7.0) was incubated at 60°C for 24 h. The reaction products were analyzed on thin layer chromatography (TLC), in which 2-propanol/1-butanol/water (12/3/4) was used as a developing solvent. The chromatogram was visualized with a detection reagent (acetate/sulfate/anisaldehyde=100/2/1).

2. Functional analysis of a protein with unknown functions showing structural similarity to CE

Recombinant Marme_2490 protein from *Marinomonas mediterranea* was produced in *E. coli*, and its biological functions were analyzed. The Marme_2490 gene was obtained by PCR using the genomic DNA of *M. mediterranea* as the template. The obtained gene was cloned into pET23a (Merck), and the recombinant protein harboring His-tag at the C-terminal was produced in *E. coli* BL21 (DE3). The *E. coli* transformant harboring the expression plasmid was cultured in 1 L of LB broth containing 100 μg/mL ampicillin at 37°C, and the protein expression was induced by 0.1 mM isopropyl β-D-thiogalactopyranoside. The induction culture was incubated at 16°C for 20 h. The recombinant protein was purified from the cell-free extract by the Ni-chelating column chromatography. Purified protein was incubated with various carbohydrates (40 mμ) at 37°C. The reaction products were analyzed on TLC, and a developing

Table 1. Kinetic parameters of Marme_2490 and related enzymes

Enzyme	D-Mannose			D-Lyxose			D-Talose		
	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($s^{-1}mM^{-1}$)	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($s^{-1}mM^{-1}$)	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($s^{-1}mM^{-1}$)
Marme_2490	329	16.7	19.7	64.4	42.3	1.52	0.319	27.8	0.0115
EcAKI	25.3	108	0.234	13.5	405	0.033	N.D.	N.D.	N.D.
SeAKI	23.3	134	0.174	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
TfMI	788	115	6.85	63.3	537	0.118	N.D.	N.D.	N.D.

EcAKI, *E. coli* aldose-ketose isomerase; SeAKI, *Salmonella enterica* aldose-ketose isomerase; TfMI, *Thermobifida fusca* MI.

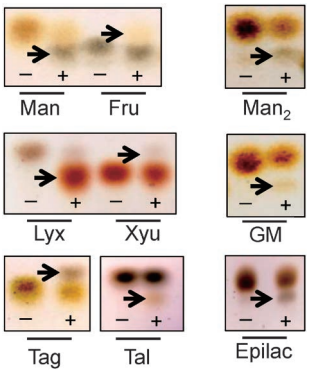

Fig. 2. Screening of substrate of Marme_2490.
Plus and minus indicate with and without enzyme, respectively. Arrows indicate reaction products.

solvent, 2-propanol/1-butanol/water (2/2/1) was used. Reaction products were purified by TLC, and their chemical structures were determined by electrospray ionization mass spectrometry and nuclear magnetic resonance. In the kinetic analysis, reaction rates were determined based on the amount of D-fructose, D-tagatose, and D-xylulose produced from D-mannose, D-talose, and D-lyxose, respectively.

Results

1. Site-directed mutagenesis study of RmCE

Change of substrate specificity through the site-directed mutations at the substrate-binding site was expected; however, besides cellobiose, the mutant enzymes did not show epimerization activity to other glucobioses.

2. Biochemical functions of Marme_2490 from *M. mediterranea*

Recombinant Marme_2490 produced in *E. coli* was incubated with various carbohydrates, and the reaction products were analyzed. This protein showed high isomerization activity to D-mannose, indicating that Marme_2490 is a type of D-mannose isomerase (MI) (Fig. 2). The k_{cat}/K_m value of this enzyme for D-mannose was 2.9–113-fold higher of any other reported MI (Table 1). Marme_2490 exhibited isomerization activity to D-lyxose, similar to other MIs, but in contrast to other MIs, it had isomerization activity to D-talose (4-epimer of D-mannose) and β1-4 linked oligosaccharides. It generated Manβ1-4Fru, lactulose, and cellobiulose from β1-4mannobiose, lactose, and cellobiose, respectively.

Conclusion

In this study, a MI, Marme_2490, with novel substrate specificity was discovered from *M. mediterranea*. Marme_2490 has isomerization activity to disaccharide substrates and D-talose, unlike other reported enzymes. Thus it is considered to be useful for carbohydrate conversion. Molecular basis for the substrate specificity of Marme_2490 is required to be elucidated for better understanding of carbohydrate isomerases and epimerases.

References

1) Fujiwara, T., Saburi, W., Matsui, H., Mori, H., and Yao, M. (2014) Structural insights into the epimerization of β-1,4-linked oligosaccharides catalyzed by cellobiose 2-epimerase, the sole enzyme epimerizing non-anomeric hydroxyl groups on unmodified sugars. *J. Biol. Chem.* **289**: 3405–3415.
2) Ojima, T., Saburi, W., Sato, H., Yamamoto, T., Mori, H., and Matsui, H. (2011) Biochemical characterization of a thermophilic cellobiose 2-epimerase from a thermohalophilic bacterium, *Rhodothermus marinus* JCM9785. *Biosci. Biotechnol. Biochem.* **75**: 2162–2168.