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Function, structure, and application of microbial enzymes
related to CE-MGP pathway

(微生物由来CE-MGP経路関連酵素の機能，構造ならび
に応用に関する研究)

Hokkaido University Graduate School of Agriculture
Division of Bio-systems Sustainability Doctor Course

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ABBREVIATIONS

AGE, *N*-acetylglucosamine 2-epimerase

AKI, aldose-ketose isomerase

BfMGP, MGP from *Bacteroides fragilis* NCTC9343

BSA, bovine serum albumin

CE, cellobiose 2-epimerase

CpCE, CE from *Chitinophaga pinensis* NBRC15968

CsCE, CE from *Caldicellulosiruptore saccharolyticus* DSM8903

DfCE, CE from *Dyadobacter fermentans* ATCC700827

DtCE, CE from *Dictyoglomus turgidum* DSM6724

EcAKI, yihS protein from *Escherichia coli*

EcCE, CE from *Eubacterium cellulosovens* NE13

ESI MS, Electrospray Ionization Mass Spectrometry

FjCE, CE from *Flavobacterium johnsoniae* NBRC14942

Gal β 1-4Fru, 4-*O*- β -D-galactosyl-D-fructose

Glc β 1-4Fru, 4-*O*- β -D-glucosyl-D-fructose

Glc-Man, 4-*O*- β -D-glucosyl-D-mannose

$\Delta\Delta G$, the change of Gibbs' free energy

HaCE, CE from *Herpetosiphon aurantiacus* ATCC23779

HEPES-NaOH, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid-NaOH

HPLC, high performance liquid chromatography

IPTG, isopropyl β -D-thiogalactoside

NCBI, National Center for Biotechnology Information

NMR, nuclear magnetic resonance

Man-Glc, 4-O- β -D-mannosyl-D-glucose

Man β 1-4Fru, 4-O- β -D-mannosyl-D-fructose

Man1P, α -D-mannosyl phosphate

Man₂, β -(1 $\rightarrow$ 4)-mannobiose

MES-NaOH, 2-(N-morpholino)ethanesulfonic acid-NaOH

MGP, 4-O- β -D-mannosyl-D-glucose phosphorylase

MI, mannose isomerase

MOP, β -1,4-mannooligosaccharide phosphorylase

MPI, mannose-6 phosphate isomerase

PCR, polymerase chain reaction

PhCE, CE from *Pedobacter heparinus* NBRC12017

Pi, inorganic phosphate

RaCE, CE from *Ruminococcus albus* NE1

RaMGP, MGP from *Ruminococcus albus* NE1

RmCE, CE from *Rhodothermus marinus* JCM9785

PMM, phosphomannomutase

SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SdCE, CE from *Saccharophagus degradans* ATCC43961

SeAKI, yihS protein from *Salmonella enterica*

SlCE, CE from *Spirosoma linguale* ATCC33905

StCE, CE from *Spirochaeta thermophila* DSM6724

TfMI, MI from *Thermobifida fusca* MBL10003

TtCE, CE from *Teredinibacter turnerae* ATCC39867

WT, wild type

CHAPTER I. General introduction

I-1. Metabolism of β -mannan and its metabolic enzymes from bacteria

Plant β -mannan is a major component of hemicellulose in the plant cell wall. It has been classified to 4 subfamilies containing linear mannan, galactomannan, glucomannan, and glucogalactomannan¹ (Fig. I.1). Its main chain is formed by β -(1 $\rightarrow$ 4)-linked D-mannosyl residues or a combination of β -(1 $\rightarrow$ 4)-linked D-mannosyl and D-glucosyl residues (glucomannan)¹. Branches of single α -(1 $\rightarrow$ 4)-linked D-galactosyl residues are present in the backbones of β -mannan. The 2-OH or 3-OH groups of some D-mannosyl and D-glucosyl residues in the main chain are acetylated. Several microbial enzymes degrade β -mannan¹⁻³ such as mannan endo-1,4- β -mannosidase (β -mannanase, EC 3.2.1.78), β -mannosidase (EC 3.2.1.25), and β -glucosidase (EC 3.2.1.21). β -Mannanase is an endo-acting enzyme that hydrolyzes internal β -(1 $\rightarrow$ 4)-mannosidic linkages to produce oligosaccharides. β -Mannosidase and β -glucosidase catalyze exo-hydrolysis of the non-reducing end β -(1 $\rightarrow$ 4)-mannosidic and β -(1 $\rightarrow$ 4)-glucosidic linkages of the resulting oligosaccharides, respectively. α -Galactosidase (EC 3.2.1.22) removes the galactosyl branches to enhance the degradation of β -mannan. Acetyl mannan esterase (EC 3.1.1.6) contributes to the deacetylation of β -mannan.

A novel degradation pathway of β -(1 $\rightarrow$ 4)-mannooligosaccharides, the CE-MGP pathway (Fig. I.2) has been proposed in the intestinal and ruminal anaerobes *Bacteroides fragilis* and *Ruminococcus albus* NE1^{4, 5}. In this pathway, cellobiose 2-epimerase (CE, EC 5.1.3.11), that catalyzes the interconversion of D-mannose residues at the reducing end of β -(1 $\rightarrow$ 4)-linked disaccharides to D-glucose residues, epimerizes β -(1 $\rightarrow$ 4)-mannobiose (Man₂) to β -(1 $\rightarrow$ 4)- β -D-mannosyl-D-glucose (Man-Glc). Man-Glc is then phosphorylated to α -D-mannosyl phosphate (Man1P) and D-glucose by 4-O- β -D-mannosyl-D-glucose phosphorylase (MGP, EC 2.4.1.281). In addition to MGP, *R. albus* NE1 produces an intracellular β -1,4-mannooligosaccharide phosphorylase (MOP; RaMP2)⁵ that catalyzes sequential phosphorylation of β -(1 $\rightarrow$ 4)-mannooligosaccharides longer than Man₂ to liberate Man1P from their non-reducing end (Fig. I.2).

I-2. MGP

MGP is considered as a key enzyme for the CE-MGP pathway. Based on the amino acid sequences, MGP and MOP are both classified into glycoside hydrolase family 130⁶ together with β -1,4-D-mannosyl-*N*-acetyl-D-glucosamine phosphorylase⁷ and unidentified human gut bacterium mannoside phosphorylase that catalyze the phosphorolysis of β -D-mannosyl-(1 $\rightarrow$ 4)- β -*N*-acetyl-D-glucosaminyl-(1 $\rightarrow$ 4)-*N*-acetyl-D-glucosamine⁸. In GH family 130, MGPs are classified into subgroup GH130_1⁶, while the other known enzymes are into GH130_2^{7, 8}. Nakae *et al.*⁹ determined the three-dimensional structures of *B. fragilis* NCTC9343 MGP (BfMGP) in its apo-form and in complexes with Man-Glc and Man1P. The catalytic domain comprises a five-bladed β -propeller fold. A conserved Asp residue (Asp131) located in proximity to the scissile glycosidic oxygen is predicted as the general acid catalyst. However, this Asp is too far to donate a proton directly to the glycosidic oxygen, and hence a proton relay mechanism via the 3-OH of the mannosyl residue at the non-reducing end was proposed⁹.

Even though MGPs found thus far are originated from anaerobic bacteria^{4, 5}, many putative MGP genes are distributed also to aerobes, including *Rhodothermus marinus*, which was first isolated from submarine alkaline hot springs in Iceland. *R. marinus* is an obligatory aerobic, moderately halophilic, thermophilic Gram-negative bacterium¹⁰, and it is known to have many thermophilic glycoside hydrolases, including endo- β -1,4-xylanase (EC 3.2.1.8)¹¹, xylan 1,4- β -xylosidase (EC 3.2.1.37)¹², cellulase (EC 3.2.1.4)¹³, endo-1,3(4)- β -glucanase (EC 3.2.1.6)¹⁴, chitinase (EC 3.2.1.14)¹⁵, β -mannanase¹⁶, trehalase (EC 3.2.1.28)¹⁷, α -glucosidase (EC 3.2.1.20)¹⁸, pullulanase (EC 3.2.1.41)¹⁸, and α -amylase (EC 3.2.1.1)¹⁸. Recently, it was found that *R. marinus* produced a CE (RmCE) and the enzyme was subsequently characterized in detail¹⁹.

I-3. CE and related enzymes

CE is distributed to aerobic and anaerobic bacteria including *R. albus* NE1⁵, *R. marinus* JCM9785¹⁹, *B. fragilis* NCTC9343⁴, *Eubacterium cellulosovens* NE13²⁰, *Flavobacterium johnsoniae* NBRC14942, *Pedobacter heparinus* NBRC12017, *Dyadobacter fermentans* ATCC700827, *Herpetosiphon aurantiacus* ATCC23779, *Saccharophagus degradans* ATCC43961, *Spirosoma linguale* ATCC33905, *Teredinibacter turnerae* ATCC39867, and *Chitinophaga pinensis* NBRC15968²¹. Because RmCE is fully stable at high temperature and prefers lactose to cellobiose unlike other

enzymes, it is an attractive enzyme for producing the prebiotic disaccharide epilactose²² from lactose, and its possible application for the continuous production of epilactose was immobilized RmCE prepared on a resin²³.

Recently, the crystal structures of *R. albus* CE (RaCE) and RmCE were determined^{24, 25}. The overall structures of RaCE and RmCE form an (α/α)₆ barrel fold comprising six inner helices and six outer helices oriented in an anti-parallel manner. The structures of catalytic domains of the CEs are similar to those of *N*-acetylglucosamine 2-epimerase (AGE)^{26, 27} and aldose-ketose isomerase (AKI)²⁸. Not only the overall fold, but the possible catalytic residues are conserved. His243 and His374 in RaCE, and His259 and His390 in RmCE, predicted from the structural analysis, correspond to those of AGE and AKI²⁴⁻²⁸. From this view points, CE is a member of AGE superfamily²⁴. The three-dimensional structure of mannose isomerase (MI) is not available, but MI from *Thermobifida fusca*²⁹ shows high sequence identity to AKIs from *Escherichia coli* and *Salmonella enterica*²⁸, and MI is also included in this superfamily. Although the known AGE superfamily enzymes have significantly similar structures (Fig. I.4), their reaction and substrate specificities are diverse^{25, 27, 28}. AGE and most CE catalyze only reversible epimerization (some CEs catalyze both epimerization and isomerization³⁰⁻³²). AKI catalyzes both the isomerization and epimerization. MI catalyzes only the isomerization. CE is active towards disaccharide substrates, whereas AGE, AKI, and MI use only monosaccharides as substrates. AGE acts on substrates having acetoamide group at the C2 position of substrate: *N*-acetyl-D-glucosamine (GlcNAc) and *N*-acetyl-D-mannosamine (ManNAc)^{26, 27}. Phylogenetic analysis of AGE superfamily proteins clearly divides these enzymes into different groups. AGE superfamily includes many function-unknown proteins with low sequence identity with any known enzymes, which are possible candidate enzymes with novel functions (Fig. I.5).

I-4. Purpose of this study

In this study, enzymatic characterization, and structure-function relationship of microbial enzymes related to the CE-MGP pathway are described together with application of these enzymes. First, biochemical characteristics of putative MGP-like protein from *R. marinus* (Rmar_2440) and Marme_2490 from *M. mediterranea*, a function unknown AGE superfamily protein, will be presented in Chapter II and Chapter IV, respectively. Second, function of amino acid residues of RmCE important for disaccharide specificity

will be analyzed by site-directed mutagenesis in Chapter III. Finally, Chapter V shows the colorimetric quantification method for Man-Glc and Man₂ for demonstrating kinetic studies of enzymes involved in the CE-MGP pathway.

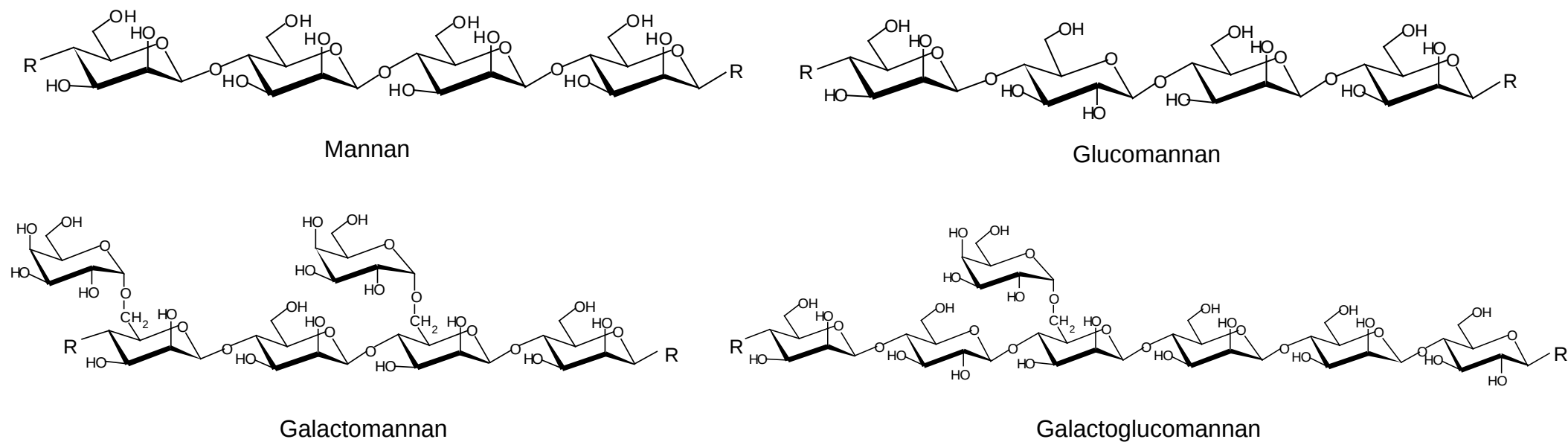


Fig. I.1. Structures of β -mannan

β -Mannan is a component of hemicelluloses in cell wall. β -Mannan has 4 different forms including β -(1 $\rightarrow$ 4)-linked D-mannosyl residues (linear β -mannan), combination of glucosyl residues and mannosyl residues (glucomannan), and branches of single α -(1 $\rightarrow$ 6)-linked D-galactosyl residues which are present in the backbones of β -mannan (galactomannan/galactoglucomannan).

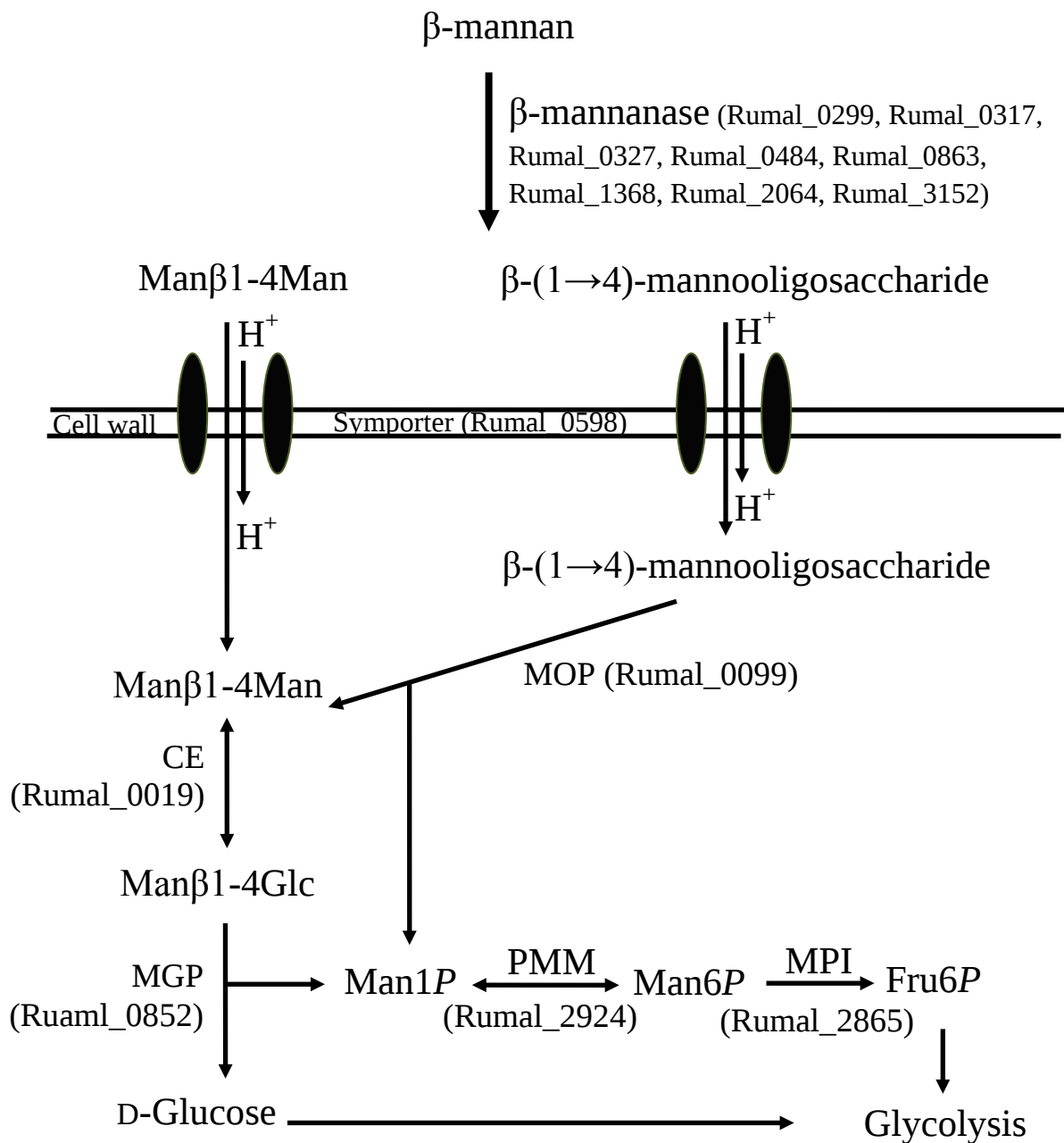


Fig. I.2. The CE-MGP pathway involved in β -mannan metabolism of *R. albus* NE1

The oligosaccharides longer than Man β 1-4Man generated by hydrolysis of β -mannan are degraded to Man β 1-4Man through phosphorolysis catalyzed by β -1,4-mannooligosaccharide phosphorylase (MOP). Man β 1-4Man is epimerized to Man β 1-4Glc, which is then phosphorolyzed to Man1P and D-glucose by Man-Glc specific MGP. The phosphomannomutase (PMM) and mannose-6 phosphate isomerase (MPI) convert Man1P to Fru6P which are metabolized through glycolysis.

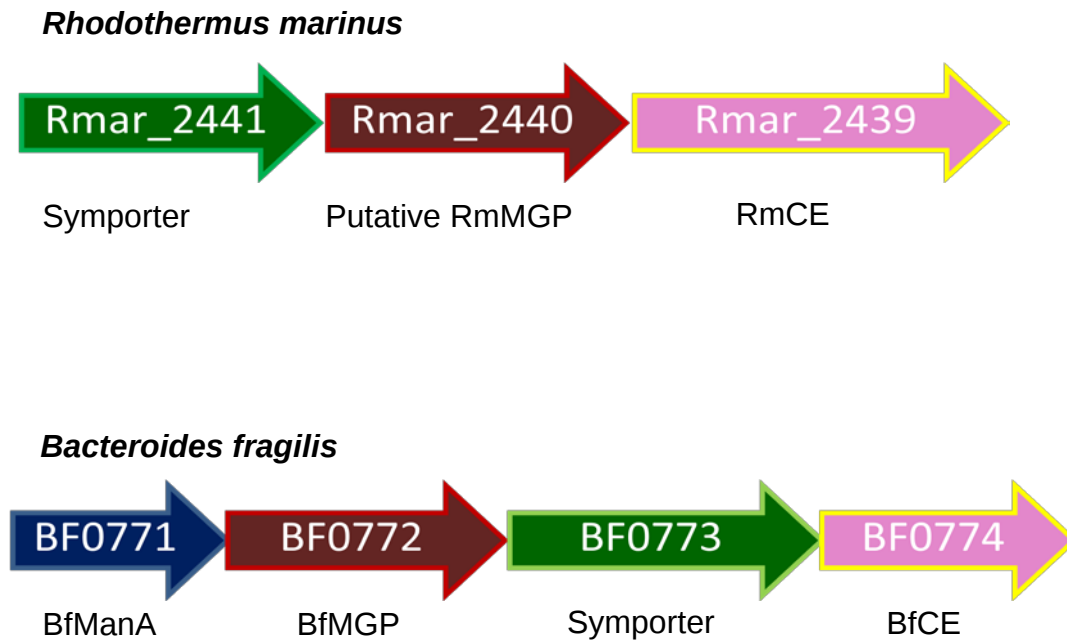


Fig. I.3. The comparison of gene clusters from *R. marinus* and *B. fragilis*.

Four *B. fragilis* genes, BF0771-BF0774 constitute an operon. *BF0771* is coding β -mannanase, *BF0772* is coding MGP, *BF0773* is coding putative symporter, and *BF0774* is coding CE. This cluster is responsible for β -mannan metabolic pathway, *R. marinus* possesses a similar organized gene cluster, Rmar_2441-Rmar_2439 in its genome. The putative MGP from *R. marinus*, RmMGP (*Rmar_2440*), is encoded upstream the RmCE gene (*Rmar_2439*). Putative transporter gene (*Rmar_2441*) is also found in this gene cluster.

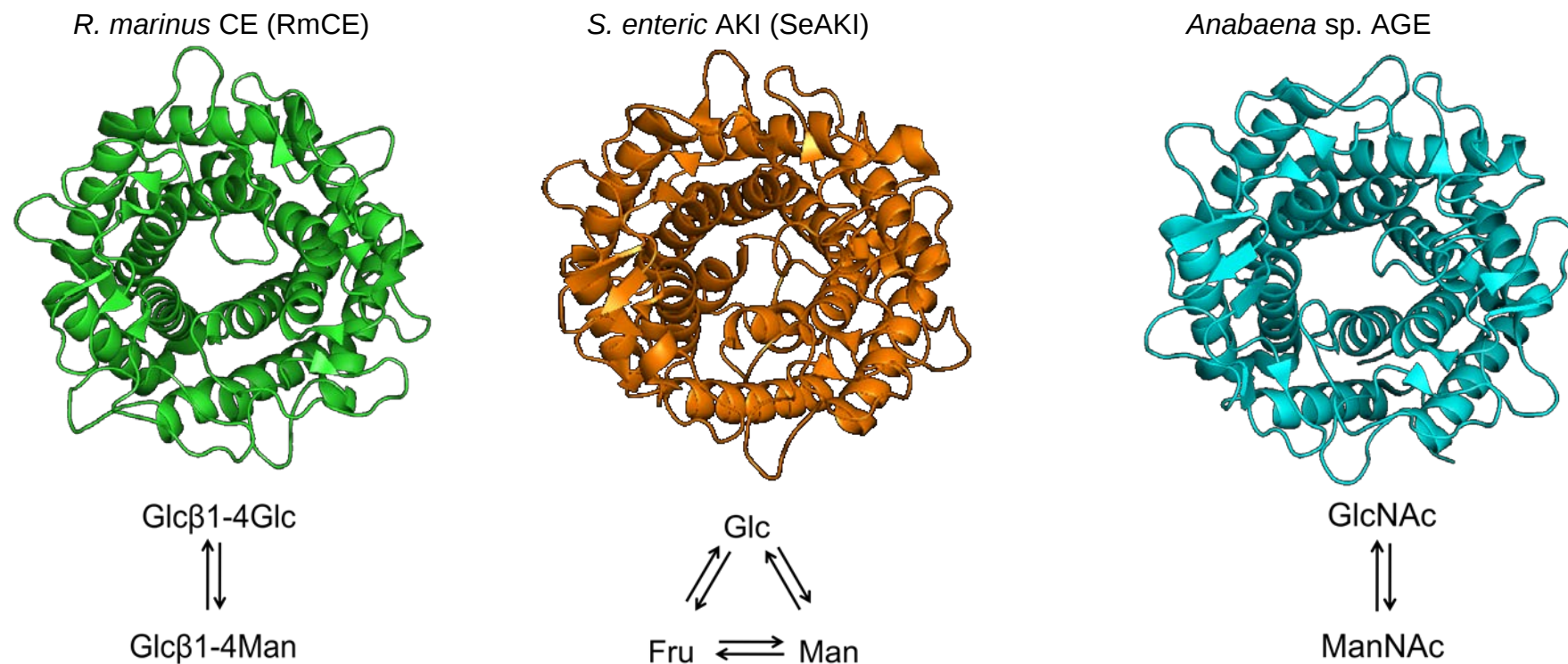


Fig. I.4. Structures and reactions of AGE superfamily enzymes.

Structures of cellobiose 2-epimerase from *R. marinus* (RmCE) (Protein Data Bank code 3WKG), aldose–ketose isomerase (AKI) from *S. enterica* (2ZBL), and acetylglucosamine 2-epimerase (AGE) from *Anabaena* sp. (2GZ6) are shown. The enzymes are formed by similar $(\alpha/\alpha)_6$ -barrel folds, although the amino acid sequence similarity of these enzymes is low. The reactions catalyzed by these enzymes are shown below the structures.

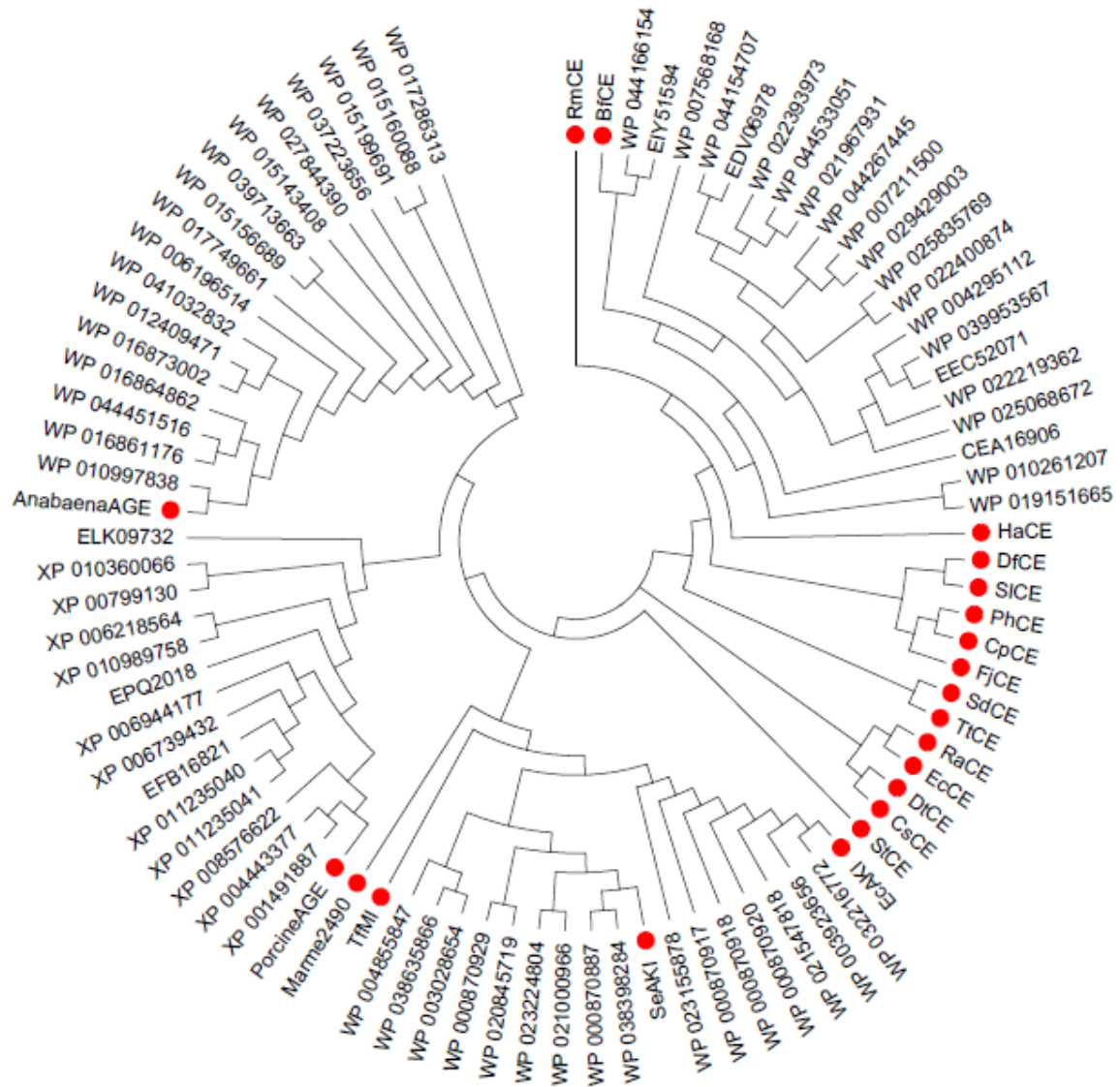


Fig. I.5. Phylogenetic tree of AGE superfamily enzymes.

CE from several bacteria strain; *R. marinus* JCM9785, RmCE; *B. fragilis* NCTC9343, BfCE; *Herpetosiphon aurantiacus* ATCC23779, HaCE; *Dyadobacter fermentans* ATCC700827, DfCE; *Spirosoma linguale* ATCC33905, SICE; *Pedobacter heparinus* NBRC12017, PhCE; *Chitinophaga pinensis* NBRC15968, CpCE; *Flavobacterium johnsoniae* NBRC14942, FjCE; *Saccharophagus degradans* ATCC43961, SdCE; *Teredinibacter turnerae* ATCC39867, TtCE; *R. albus* NE1, RaCE; *Eubacterium cellulosovens* NE13, EcCE; *Dictyoglomus turgidum* DSM6724, DtCE; *Caldicellulosiruptore saccharolyticus* DSM8903, CsCE; *Spirochaeta thermophila* DSM6724, StCE. AKI from *E. coli*, EcAKI; AKI from *S. enteric*, SeAKI; AGE from porcine kidney, PorcineAGE; AGE from *Anabaena* sp., AnabaenaAGE. D-Mannose isomerase from *Thermobifida fusca* MBL10003, TfMI. Unknown protein from *Marinomonas mediterranea*, Marme_2490.

CHAPTER II. Characterization of a thermophilic MGP from *R. marinus*

II-1. Introduction

R. marinus R-10^T is an obligatory aerobic, moderately halophilic, thermophilic Gram-negative bacterium. Analysis of the *R. marinus* genome revealed that a putative MGP (RmMGP) is encoded by Rmar_2440, which is located upstream of Rmar_2439 (coding CE gene)²². Amino acid sequence of RmMGP is 68% and 61% identical to those of *B. fragilis* NCTC9343 MGP (BfMGP)⁴ and *R. albus* NE1 MGP (RaMGP)⁵, respectively. Three genes encoding glycoside hydrolase family 26 β -mannanase (Rmar_0016 from *R. marinus*, ManA¹⁶, BF0771 from *B. fragilis*, ManA⁴, and Ruma1_0484 a putative β -mannanase⁵ from *R. albus*) are also present, implying that *R. marinus* R-10^T possesses the CE-MGP pathway similar to those of *B. fragilis* NCTC9343 and *R. albus* NE1. In this chapter, the change of activities of β -mannanase, CE, and MGP of *R. marinus* cultured in the presence and absence of konjak glucomannan and the enzymatic properties of recombinant Rmar_2440 protein produced in *E. coli* are presented.

II-2. Materials and methods

II-2-1. Bacterial strain

R. marinus R-10^T (ATCC43812) was supplied from the American Type Culture Collection (Manassas, VA, USA).

II-2-2. Culture conditions and protein extraction

R. marinus was cultured in 50 mL of Marine Broth 2216 (Becton Dickinson, Sparks, MD, USA) with (2 mg/mL) or without glucomannan (Shimizu Kagaku, Hiroshima, Japan) at 50°C for 60 h. Ten mL of culture broth was harvested every 12 h, and the cells were harvested by centrifugation at $5,400 \times g$ for 10 min at 4°C. β -Mannanase activity in the culture supernatant was assayed. The bacteria were suspended in 1 mL of 10 mM potassium phosphate buffer (pH 7.0), and disrupted by sonication using an Ultrasonic Disruptor UD-201 (Tomy, Tokyo, Japan). Cell debris was removed by centrifugation at

19,000 × g for 5 min at 4°C. Protein concentrations and activities of CE, MGP, and β-mannanase were measured as described below.

II-2-3. Preparation of an *Rmar_2440* expression plasmid

Rmar_2440 was amplified using the polymerase chain reaction (Table II.1&2) with the primer pair as follows: 5'-AATGCCATATGGGAAGTGCGAATGGCACCGAC-3' (*Nde*I site underlined), 5'-TTAACTCGAGTCACGGGCGCTTCAGCAGTT-3' (*Xho*I site underlined), and Primestar HS DNA polymerase (Takara Bio, Otsu, Japan). *R. marinus* ATCC43812 genomic DNA was used as template¹⁸. The amplified DNA fragment was cloned into the *Eco*RV site of pBluescript II SK (+) vector (Stratagene, La Jolla, CA, USA). A DNA fragment prepared by double-digestion with *Nde*I and *Xho*I was inserted into the *Nde*I and *Xho*I sites of pET23a (Novagen, Darmstadt, Germany). The DNA sequence of the inserted region was analyzed using an Applied Biosystems 3130 Genetic Analyzer (Life Technologies, Carlsbad, CA, USA).

II-2-4. Production and purification of recombinant RmMGP

II-2-4-1. Production of recombinant RmMGP

E. coli BL21(DE3) transformed with the *Rmar_2440* expression plasmid was cultured in 1 L of Luria-Bertani (LB) medium containing 50 µg/mL ampicillin at 37°C until the absorbance at 600 nm reached 0.6. Protein expression was induced by adding 1 mL of 0.1 M isopropyl β-D-thiogalactoside (IPTG) to the culture medium (final concentration of 0.1 mM), and the incubation was continued at 18°C for 24 h with vigorous shaking. Bacterial cells were harvested by centrifugation at 8,400 × g at 4°C for 10 min, re-suspended in 40 mL of 20 mM 2-(*N*-morpholino)ethanesulfonic acid (MES)-NaOH buffer (pH 7.0), and disrupted using sonication. Cell-free extract was prepared by centrifuging the suspension of the disrupted cells at 13,000 × g at 4°C for 10 min.

II-2-4-2. Toyopearl DEAE-650M column chromatography

The supernatant from disrupted cell suspension was subjected to anion exchange chromatography using a Toyopearl DEAE-650M column (i.d. 3.0 × 10.5 cm, Tosoh, Tokyo, Japan). After washing the column thoroughly with 20 mM MES-NaOH

buffer (pH 7.0), the adsorbed proteins were eluted with a linear gradient of NaCl from 0 to 0.5 M (total elution volume was 250 mL).

II-2-4-3. Toyopearl Butyl-650M column chromatography

Active fractions from anion exchange chromatography were pooled and applied on a Toyopearl Butyl-650M column (i.d. 3.0×10.5 cm, Tosoh) equilibrated with 10 mM MES-NaOH buffer (pH 7.0) containing 300 g/L ammonium sulfate. Non-adsorbed proteins were completely eluted with the same buffer, and the adsorbed proteins were eluted using a descending linear gradient of ammonium sulfate from 300 to 0 g/L (total elution volume was 250 mL).

II-2-4-4. Sephacryl S-300 column chromatography

The active fractions from Toyopearl Butyl-650M column chromatography were pooled and concentrated to 3 mL using a Vivaspin 20 (nominal molecular weight limit 30,000, Sartorius, Göttingen, Germany) and subjected to Sephacryl S-300 column chromatography (i.d. 1.6×67 cm, GE Healthcare, Uppsala, Sweden). The column was eluted with 10 mM MES-NaOH buffer (pH 7.0) containing 0.2 M NaCl at a flow rate of 0.3 mL/min. The purity of the fractions was confirmed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and highly purified fractions were collected. The purified enzyme was dialyzed against 10 mM MES-NaOH buffer (pH 7.0) and stored at -80°C .

II-2-5. Protein assay

The protein concentration of the cell-free extract was measured by the Bradford method³⁴ with bovine serum albumin (BSA; Nacalai Tesque, Kyoto, Japan) as the standard. The protein concentrations of column chromatography fractions were determined using the UV method³⁵ assuming an extinction coefficient of 1.0 mg/mL of protein equal to 1.00. The concentration of the purified enzyme was calculated from each free amino acid concentrations based on the protein sequence information after protein was hydrolyzed in acid (6 M HCl at 110°C for 24 h). Amino acids concentrations were

measured using the ninhydrin colorimetric method with a JLC-500/V (JEOL, Tokyo, Japan)³⁶.

II-2-6. Enzyme activity assays

II-2-6-1. MGP activity

The reaction mixture (50 μ L) contained 2 mM Man-Glc, 100 mM sodium phosphate buffer (pH 6.5), 4 mM MES-NaOH buffer (pH 6.5), 0.2 mg/mL BSA, and an appropriate concentration of enzyme. After incubation at 50°C for 10 min, the reaction was stopped by adding 100 μ L of 2 M Tris-HCl buffer (pH 7.0) and incubated immediately at 100°C for 3 min. Liberated D-glucose was measured using a Glucose CII test (Wako Pure Chemical Industries, Osaka, Japan). Man-Glc was prepared as described previously⁵. Enzyme was diluted with 20 mM MES-NaOH buffer (pH 6.5) containing 1 mg/mL BSA. One unit (U) of enzyme activity was defined as the amount of enzyme that formed 1 μ mol of glucose in 1 min under these conditions.

II-2-6-2. β -Mannanase activity

The reaction mixture (100 μ L) containing 1 mg/mL glucomannan, 25 mM sodium phosphate buffer (pH 6.0), and enzyme was incubated at 60°C for 10 min. The reducing sugar produced was quantified using the Somogyi-Nelson method³⁷ with 0–1 mM D-mannose as standard. One U of enzyme activity was defined as the amount of enzyme amount that produced 1 μ mol of reducing sugar in 1 min.

II-2-6-3. CE activity

The reaction mixture (100 μ L) containing 100 mM β -1,4-mannobiose (Man₂; Megazyme, Wicklow, Ireland), 20 mM potassium phosphate buffer (pH 7.0), and enzyme was incubated at 50°C for 1 h, and the reaction was stopped by incubation at 100°C for 5 min. The amount of the reaction product Man-Glc was measured using high-performance liquid chromatography (HPLC) under the conditions as follows: injection volume, 5 μ L; columns, two tandem Sugar SP0810 columns (i.d. 8.0 $\times$ 300 mm $\times$ 2; Shodex, Tokyo, Japan), column temperature, 70°C; eluant, water; flow rate,

0.5 mL/min; detection, refractive index. One U of enzyme activity was defined as the amount of enzyme that produced 1 μ mol of Man-Glc in 1 min.

II-2-7. Effects of pH and temperature on the activity and stability of RmMGP

II-2-7-1. Optimum pH and temperature

Enzyme activity was determined over a pH range of 3.1–9.7 and a temperature range of 30–100°C. To determine the optimum pH, reaction mixtures containing 74.8 nM RmMGP, 2 mM Man-Glc, 10 mM sodium phosphate buffer (pH 6.5), and 100 mM reaction buffer [sodium citrate buffer, MES-NaOH buffer, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES)-NaOH buffer, and glycine-NaOH buffer with pH values of 3.1–6.4, 6.5–6.9, 7.3–8.1, and 8.2–9.7, respectively] were incubated at 50°C for 10 min. The concentrations of D-glucose were measured as described above. To determine the optimum temperature, a reaction mixture containing 150 nM RmMGP, 2 mM Man-Glc, 10 mM sodium phosphate buffer (pH 6.5), and 100 mM MES-NaOH buffer (pH 6.5) was incubated at 30–90°C for 10 min. The concentrations of D-glucose were measured as described in II-2-6-1.

II-2-7-2. The pH and temperature stability

The pH stability and thermostability of RmMGP were evaluated by determining residual activity after the pH and heat treatments, respectively. To determine enzyme stability as a function of pH, mixtures (100 μ L) containing 3.74 μ M RmMGP and 80 mM buffers described in II-2-7-1 (pH 3.1–9.7) were incubated at 4°C for 24 h. To determine enzyme stability as a function of temperature, mixtures (40 μ L) containing 93.5 nM RmMGP, 12.5 mM sodium phosphate buffer (pH 6.5), and 125 mM MES-NaOH buffer (pH 6.5) were incubated at 30–90°C for 20 min. The enzyme was considered stable under conditions that retained 90% of the activity before the treatment.

II-2-8. Kinetic analysis of MGP

Kinetic parameters for the phosphorolysis of Man-Glc were determined from reaction rates at various concentrations of Man-Glc and inorganic phosphate (Pi). The

reaction mixture (50 μ L) containing 37 nM RmMGP, 1–20 mM Man-Glc, 1–10 mM sodium phosphate, 100 mM MES-NaOH buffer (pH 6.5), and 0.2 mg/mL BSA was incubated at 50°C for 10 min, and D-glucose was measured as described in II-2-6-1. The kinetic parameters were calculated by fitting the reaction rates to the equation for a sequential bi-bi mechanism³⁸ as follows:

$$v = k_{\text{cat}}[A][B]/(K_{\text{iA}}K_{\text{mB}} + K_{\text{mB}}[A] + K_{\text{mA}}[B] + [A][B])$$

(A = Man-Glc, B = Pi)

Non-linear regression was carried out using Grafit version 7.0.2 (Erithacus Software Ltd., West Sussex, UK).

II-2-9. Acceptor specificity of RmCE in the synthetic (reverse phosphorolysis) reaction

Twenty-five μ L of a reaction mixture containing enzyme, 10 mM Man1P, 10 mM acceptor substrate, 54 mM MES-NaOH buffer (pH 6.5), and 0.2 mg/mL BSA was incubated at 50°C for 10 min. The enzyme reaction was stopped by adding 62.5 μ L of 100 mg/mL ascorbic acid solution, and inorganic phosphate was measured following the method published by Lowry and Lopez³⁹. Man1P (dicyclohexylamine salt) was synthesized from D-mannose and ATP using *N*-acetylhexosamine 1-kinase⁴⁰. The acceptor substrates were as follows: D-glucose, D-mannose, D-allose, D-xylose, 1,5-anhydro-D-glucitol, methyl α -D-glucoside, and methyl β -D-glucoside (Wako Pure Chemical Industries); 6-deoxy-D-glucose and cellobiose (Sigma, St. Louis, MO, USA); D-glucosamine (Tokyo Chemical Industries, Tokyo, Japan); D-glucitol and *N*-acetyl-D-glucosamine (Nacalai Tesque); and Man₂. Apparent kinetic parameters were determined by fitting the reaction rates at varying concentrations of acceptor substrates (1.25–40 mM) and 10 mM Man1P to the Michaelis-Menten equation.

II-2-10. Estimation of molecular mass of RmCE using gel filtration column chromatography

One hundred μ L of 0.42 mg/mL RmMGP was subjected to gel filtration column chromatography under the conditions as follows: column, Superose 12 10/300 GL (i.d. 1.0 x 30 cm, GE Healthcare, column was connected with ÄKTA Purifier); buffer, 20 mM MES-NaOH buffer (pH 7.0) containing 0.2 M NaCl; flow rate, 0.5 mL/min; detection,

absorbance at 280 nm. The gel filtration molecular mass standards (Bio-Rad, Hercules, CA, USA) were used to generate a calibration curve.

II-3. Results and discussions

II-3-1. CE, MGP, and β -mannanase activities of *R. marinus* cultured in the presence or absence of β -mannan

Growth of *R. marinus* and production of the β -mannan-metabolizing enzymes in the presence (2 mg/mL) or absence of glucomannan were compared (Fig. II.1). Growth was more abundant in the presence of glucomannan as carbon source than in its absence. The absorbance at 600 nm of the culture broth with glucomannan after 60 h was higher than that of the control by a factor of approximately two. Production of extracellular β -mannanase in the presence of glucomannan reached its maximum in the early phase of cell growth (36 h). The β -mannanase activity at 36 h was 0.087 U/mL, which was higher by a factor of 37 than that in the absence of glucomannan. The β -mannanase activity in the presence of glucomannan was maintained until 48 h but decreased significantly by 60 h. Intracellular MGP and CE activities of cells grown in the presence of glucomannan were significantly higher than the control. The maximum activities of intracellular MGP and CE in cells cultured in the presence of glucomannan were higher by factors of 6.1 (60 h) and 34 (48 h), respectively, than the control.

II-3-2. Preparation of an *Rmar_2440* expression plasmid

The PCR was performed with primers designed based on the nucleotide sequence of *Rmar_2440* and the genomic DNA of *R. marinus* as template. The PCR product was 1,203 bp (Fig. II.2.a). The native amplified DNA fragment was cloned into the *EcoRV* site of pBluescript II SK (+) vector. The native DNA fragment obtained by the double-digestion with *NdeI* and *XhoI* was inserted into the *NdeI* and *XhoI* sites of pET23a expression plasmid (Fig. II.2.b).

II-3-3. Production and purification of recombinant RmMGP

From *E. coli* cells proliferating in 1 L culture, cell-free extract was prepared by sonic disruption. The purification of enzyme was carried out in 3 steps including Toyopearl DEAE-650M, Toyopearl Butyl-650M, and Sephacryl S-300 column chromatographies.

Cell-free extract contained RmMGP as a major protein (Fig. II.3). The cell-free extract was applied to Toyopearl DEAE-650M column. The MGP activity was eluted by 0.1-0.3 M NaCl (Fig. II.4). The SDS-PAGE analysis revealed that the 45.3 kDa protein (RmMGP) was found in the fractions corresponding to the active fraction (Fig. II.5). The fractions 107-127 (105 mL, total protein 69.5 mg, specific activity 1.5 U/mg) were pooled. Ammonium sulfate was added up to 300 g/L for Toyopearl Butyl-650M column chromatography. The sample was applied to the column, and the MGP activity was eluted by 170-50 g/L ammonium sulfate (Fig. II.6). The SDS-PAGE analysis revealed that the 45.3 kDa protein (RmMGP) was found in the fractions 135-158 (Fig. II.7). But MGP activity was detected from fractions 139-146 (Fig. II.6). Pool sample (37 mL, total protein 63.3 mg, specific activity 7.6 U/mg) was dialyzed against ammonium sulfate in 20 mM MES-NaOH buffer (pH 7.0), and concentrated to 3 mL for the gel filtration column chromatography. It was applied to a Sephacryl S-300 column, and the MGP activity was detected in the fractions 45-56, 12.5 mL (Fig. II.8) corresponding to active fractions on SDS-PAGE (Fig. II.9). The fractions 45-56 were collected and dialyzed against 20 mM MES-NaOH buffer (pH 7.0). The sample was concentrated by ultrafiltration. The purification is summarized in Table II.3. The yield of purified enzyme was 10.6 mg, specific activity was 8.6 U/mg, and total activity was 45.9 U. The recovery of the activity was 21.9% in the purification.

The recombinant enzyme catalyzed the phosphorolysis of Man-Glc. The specific activity to 2 mM Man-Glc and 100 mM Pi was 7.17 U/mg at pH 6.5 and 50°C. The molecular mass of RmMGP was estimated to be 45.3 kDa by SDS-PAGE (Fig. II.10.a), which is consistent with 45,295.67 Da, calculated from its amino acid sequence including the 1st Met, and is close to those of BfMGP⁴ and RaMGP⁵. Under non-denaturing conditions, the molecular mass of RmMGP was estimated to be 222 kDa by gel filtration (Fig. II.10.b), indicating that RmMGP forms a homopentamer, while RaMGP and BfMGP form homodimers and homoexamers, respectively^{4, 5}.

II-3-4. Effects of pH and temperature on enzyme activity and stability

RmMGP activity was the highest at pH 6.5, which is similar to those of other MGPs^{4, 5} (Fig. II.11.a) and was stable at pH 5.5–8.3 (4°C for 24 h). Activity was the highest at 75°C, which is similar to those of the other *R. marinus* enzymes¹¹⁻¹⁸. RmMGP retained greater than 90% of the activity when incubated for 20 min at temperatures up to

80°C (Fig. II.11.b). The optimum temperature of RmMGP is higher than those of the other MGPs, which are most active at 50°C^{4, 5}. The Pro residue content of RmMGP (8.3%) is approximately twice as high as those of BfMGP (4.9%) and RaMGP (3.6%). The higher content of Pro residues is also observed in *R. marinus* CE enzyme, RmCE, compared with those of other CEs with mild optimum temperatures¹⁹. Pro residues situated in loops connecting adjacent secondary structures may account for the high thermostability of *Bacillus thermoglucosidasius* KP1006 oligo-1,6-glucosidase⁴¹, and may contribute to the rigidity and thermostability of RmMGP as well.

II-3-5. Kinetic analysis of the phosphorolysis of Man-Glc

The initial velocities of phosphorolysis of Man-Glc at various concentrations of Pi and Man-Glc were determined (Fig. II.12). The curves obtained by plotting $1/v$ versus $1/[\text{Man-Glc}]$ at various Pi concentrations were linear and intersected at the same point. These data indicate that RmMGP catalyzed the phosphorolysis of Man-Glc through a sequential bi-bi mechanism involving the formation of a ternary complex as observed for RaMGP and other inverting carbohydrate phosphorylases^{5, 6, 42-48}. The calculated kinetic parameters are as follows: $k_{\text{cat}} = 20.5 \pm 0.1 \text{ s}^{-1}$, $K_{\text{mA}} = 0.994 \pm 0.051 \text{ mM}$, $K_{\text{mB}} = 1.07 \pm 0.03 \text{ mM}$, and $K_{\text{iA}} = 5.78 \pm 0.43 \text{ mM}$ (A, Man-Glc; B, Pi).

II-3-6. Acceptor specificity of the synthetic reaction

Acceptor specificity of the synthetic reaction (reverse phosphorolysis) was determined by measuring initial reaction rates at the same concentrations (10 mM) of Man1P and various substrates. D-Glucose, methyl β -D-glucoside, 1,5-anhydro-D-glucitol, 6-deoxy-D-glucose, and D-xylose served as acceptors. No synthetic activity was detected in the presence of D-allose, D-mannose, methyl α -D-glucoside, cellobiose, D-glucosamine, *N*-acetyl-D-glucosamine, and β -(1 $\rightarrow$ 4)-mannobiose. The apparent kinetic parameters for acceptors were determined from reaction rates at various concentrations of acceptors and 10 mM Man1P (Table II.4). The value of $k_{\text{cat(app)}}/K_{\text{m(app)}}$ of RmMGP for 6-deoxy-D-glucose is similar to that of D-glucose, although for the synthetic reaction of *R. albus* MGP (RaMGP), monodeoxygenation at the 6-OH position results in large decreases of $k_{\text{cat(app)}}/K_{\text{m(app)}}$ caused by an increase of $K_{\text{m(app)}}$ ⁵. Because $k_{\text{cat(app)}}/K_{\text{m(app)}}$ of RaMGP for D-xylose is 12% of that for 6-deoxy-D-glucose⁵, methylene group of acceptor substrates is important for activity. In other words, the 6-OH group of D-glucose is less important in

RmMGP compared with in RaMGP. However, for RmMGP, $k_{\text{cat(app)}}/K_{\text{m(app)}}$ for D-xylose is 36% of that for 6-deoxy-D-glucose. These findings indicate that the 6-OH and C-6 methylene groups of acceptor substrates are less important for the recognition of acceptor substrate by RmMGP compared with RaMGP.

In the complex of BfMGP and Man-Glc, hydrophobic interactions with the C-6 methylene group of the reducing-end D-glucose residue are provided by Phe214 and Ile215⁹. The corresponding residues are conserved in RmMGP and RaMGP judging from the sequences. Therefore, the difference in synthetic activity towards the 6-OH derivatives of D-glucose between RmMGP and RaMGP may be attributed to the differences in indirect interactions with the acceptors. In contrast to RaMGP, RmMGP exhibited synthetic activity towards the 1-OH glucose derivatives 1,5-anhydro-D-glucitol and methyl β -D-glucoside. Because neither absence of the 1-OH group or modification of this OH group resulted in complete loss of synthetic activity, the 1-OH group of the acceptor substrate is less important for the synthetic activity of RmMGP compared with RaMGP. In the BfMGP structure, Arg94 forms a hydrogen bond with the 1-OH group of the β -D-glucose residue of Man-Glc⁹, and this Arg is conserved in the sequence of RaMGP and RmMGP. The synthetic activity of RmMGP towards methyl β -D-glucoside suggests that the orientation of Arg101, corresponding to BfMGP Arg94, differs from that of BfMGP, because the space in the acceptor binding site accommodates the β -linked methyl group.

Table II.1. The reaction mixture composition of PCR mixture.

Component	Volume (μL)
Genomic DNA (100 ng/μL)	1
5×buffer for Primestar HS DNA polymerase	10
2 mM dNTP	4
10 μM sense primer	1
10 μM antisense primer	1
Primestar HS DNA polymerase	0.5
Water	32.5
Total volume	50

Table II.2. The reaction conditions of PCR.

Steps	Conditions	
Pre-denaturation	98°C, 1 min	
Denaturation	98°C, 10 s	} 30 cycles
Annealing	55°C, 15 s	
Extension	72°C, 1 min	
Final extension	72°C, 5 min	

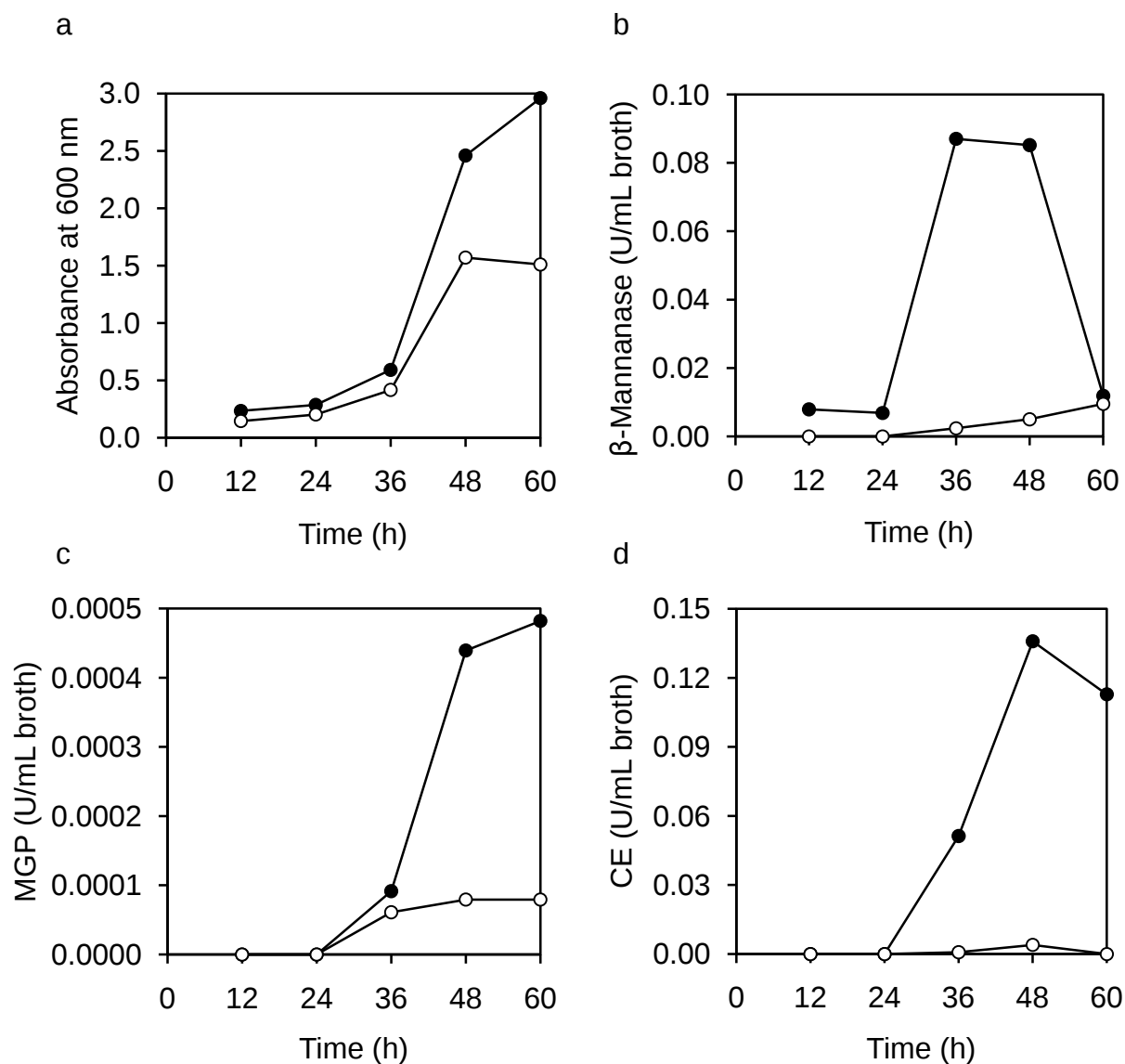


Fig. II.1. Growth curve of *R. marinus* R-10^T and production of β -mannan-metabolizing enzymes.

Growth of *R. marinus* and production of β -mannan-metabolizing enzymes in the presence (black circles) and absence (white circles) of glucomannan as a carbon source were analyzed, respectively. a, Growth curve; b, Extracellular β -mannanase activity; c, intracellular MGP activity; d, intracellular CE activity.

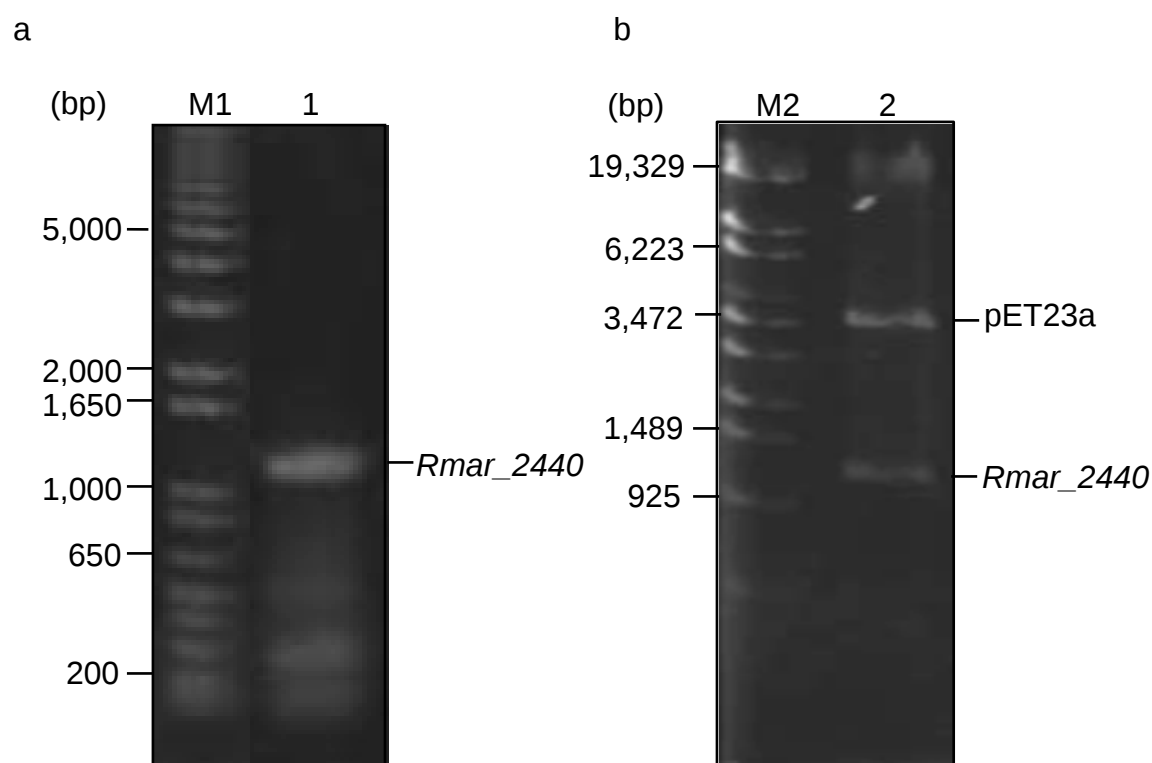


Fig. II.2. Electrophoretic analysis of PCR of *R. marinus* and electrophoretic analysis of expression plasmid of *Rmar_2440*.

a, Result of PCR. M1, 1 kb Plus DNA ladder; 1, *Rmar_2440* PCR product; b, expression plasmid of *Rmar_2440* was digested by *Nde*I and *Xho*I. M2, λ -EcoT14I marker; 2, expression plasmid of *Rmar_2440* was digested by *Nde*I and *Xho*I.

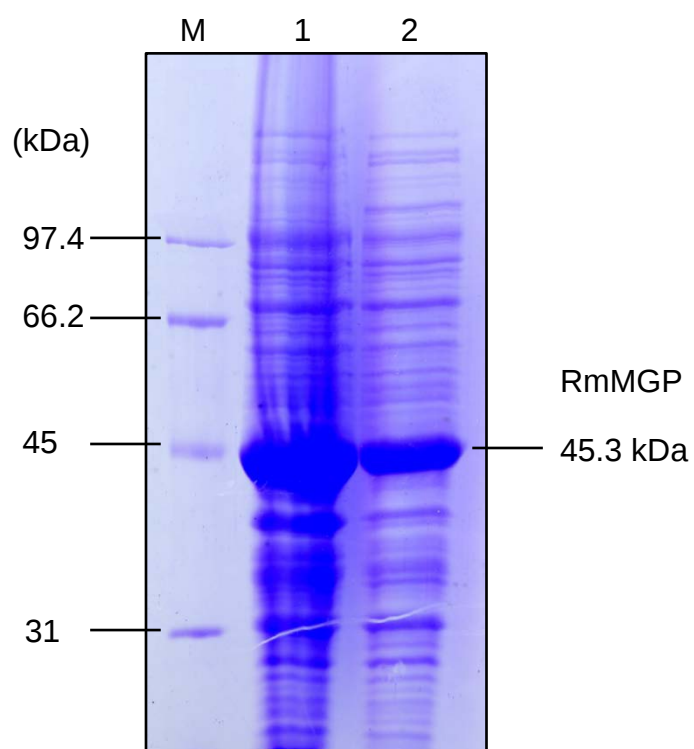


Fig. II.3. SDS-PAGE analysis of cell-free extract of *E. coli* transformant producing recombinant RmMGP.

SDS-PAGE was performed with 10% polyacrylamide gel, and protein was detected by the Coomessie Brilliant Blue staining. M, size marker; 1, cell-free extract; 2, a ten-fold dilution of the cell-free extract.

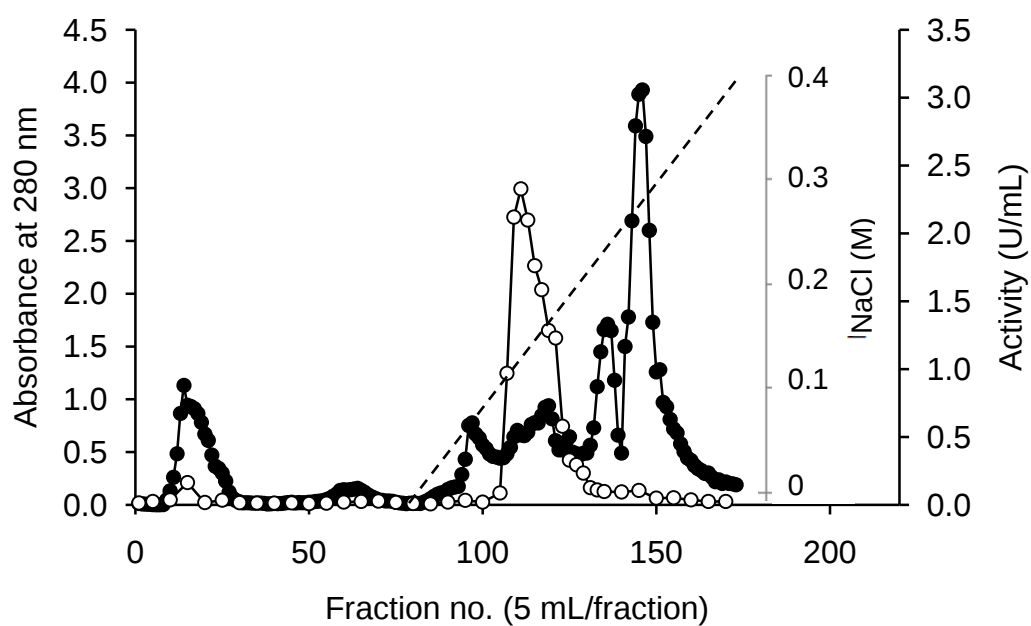


Fig. II.4. Toyopearl DEAE-650 M column chromatography of recombinant RmMGP.

Enzyme activity (-○-), absorbance at 280 nm (-●-), NaCl (---). Toyopearl DEAE-650M column (i.d. 3.0×10.5 cm) was equilibrated with 20 mM MES-NaOH buffer (pH 7.0). The elution was done by a linear gradient of NaCl from 0 to 0.5 M. The fractions number 107-127, 105 mL were pooled.

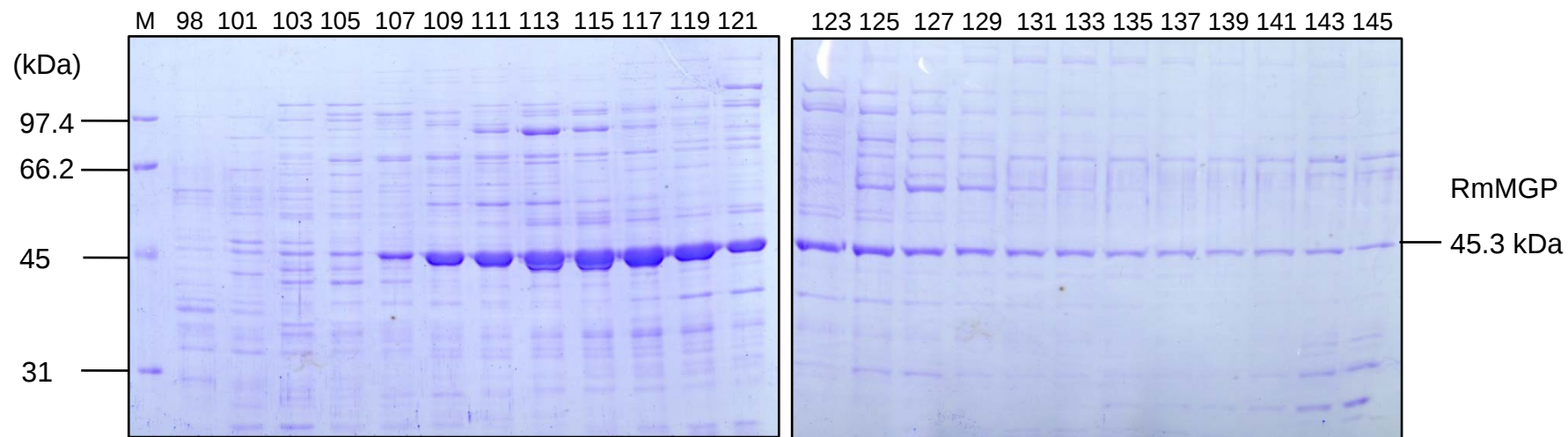


Fig. II.5. SDS-PAGE analysis of adsorbed proteins containing RmMGP in anion exchange chromatography.

Adsorbed proteins were eluted with 0-0.5 M NaCl (see Fig. II.4). Active fractions containing of RmMGP activity were investigated by SDS-PAGE, and the fractions number 107-127, 105 mL were pooled. SDS-PAGE was performed on 10% polyacrylamide gel, and protein was detected by the Coomessie Brilliant Blue staining. M, size marker (2 μ L was loaded); 98-145, fraction number (10 μ L was loaded).

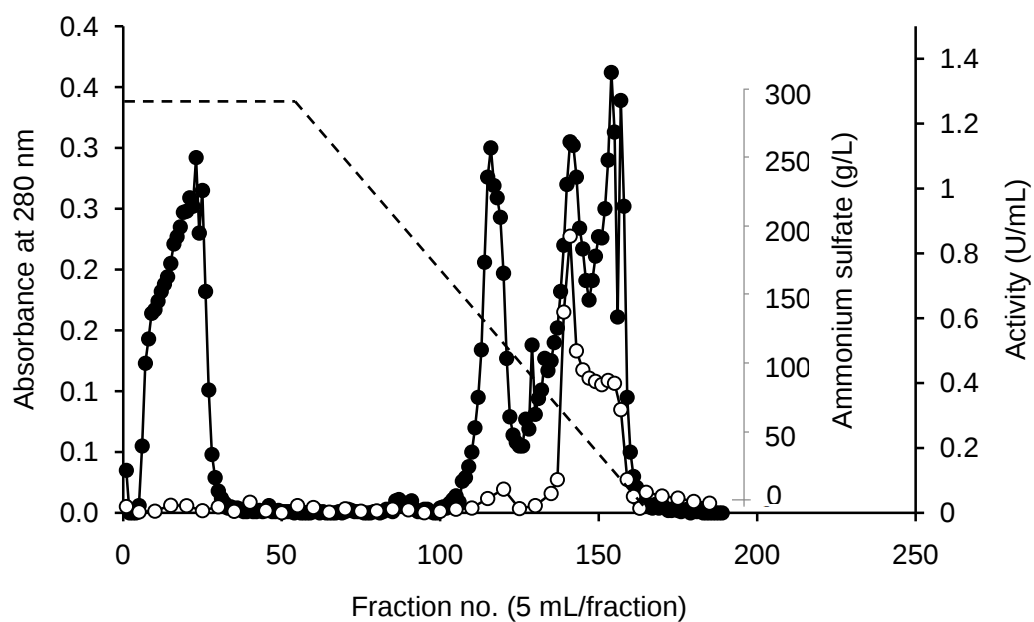


Fig. II.6. Toyopearl Butyl-650M column chromatography of RmMGP.

Enzyme activity (-○-), absorbance at 280 nm (-●-), ammonium sulfate (---). Toyopearl Butyl-650M column (i.d. 3.0×10.5 cm) was equilibrated with 20 mM MES-NaOH buffer (pH 7.0) containing 300 g/L ammonium sulfate. Elution was done by a linear gradient of ammonium sulfate from 300 to 0 g/L. The fractions 139-146, 37 mL were pooled.

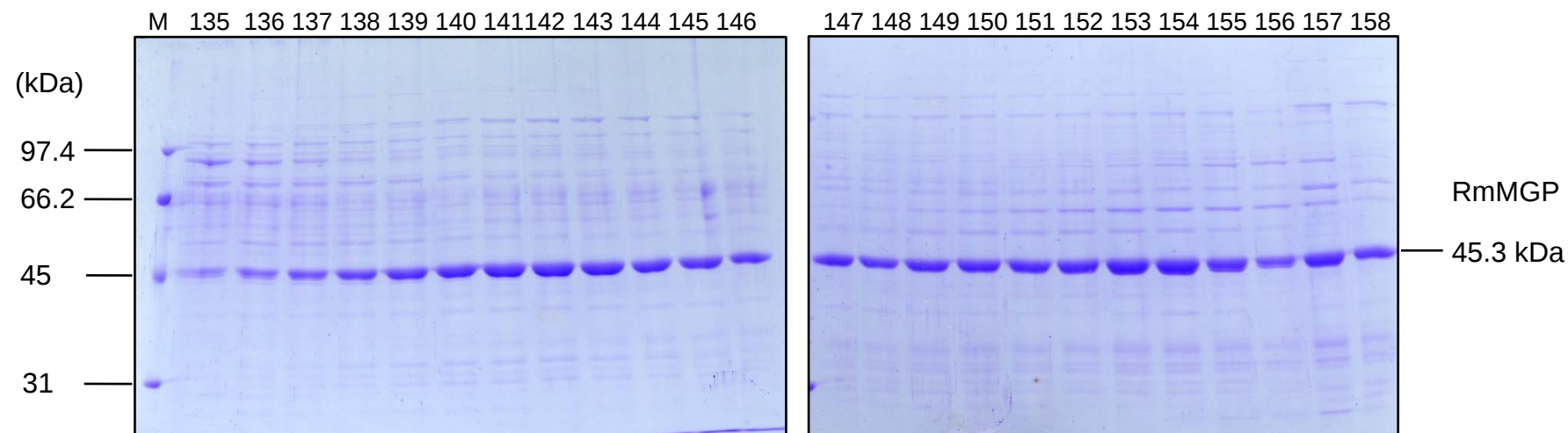


Fig. II.7. SDS-PAGE analysis of adsorbed proteins containing RmMGP in Toyopearl Butyl-650M column.

Adsorbed protein was eluted with a linear gradient of 300-0 g/L ammonium sulfate (see Fig. II.6). Active fractions containing RmMGP activity were investigated by SDS-PAGE. The fractions 139-146, 37 mL were pooled and applied to the next column. SDS-PAGE was performed with 10% polyacrylamide gel, and protein was detected by the Coomessie Brilliant Blue staining. M, size marker (2 μ L was loaded); 135-158, fraction number (10 μ L was loaded).

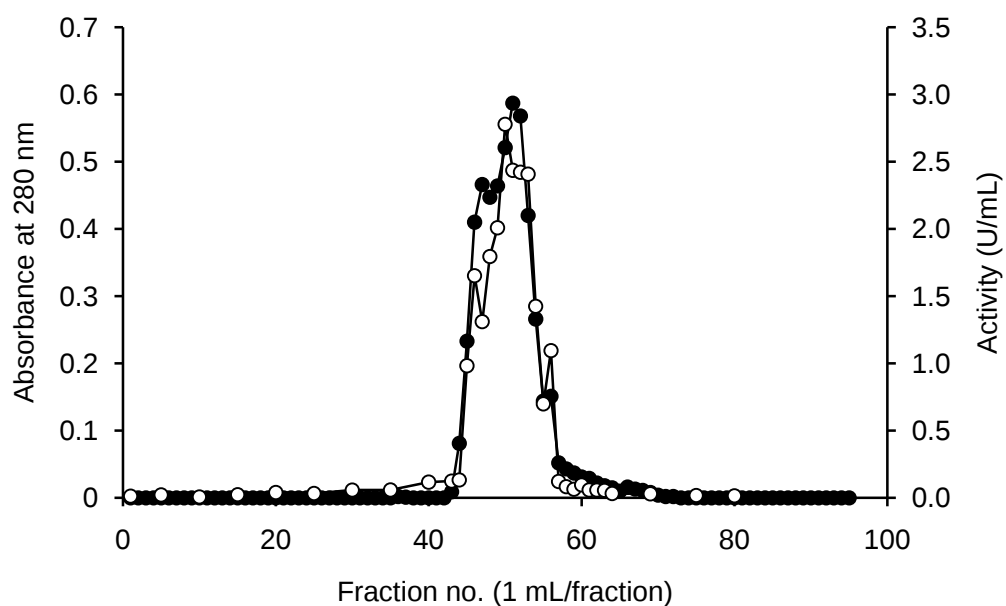


Fig. II.8. Sephacryl S-300 column chromatography of recombinant RmMGP.

Enzyme activity (-o-), absorbance at 280 nm (-●-). Sephacryl S-300 column(i.d. 1.6×67 cm) was equilibrated with 10 mM MES-NaOH buffer (pH 7.0) containing 0.2 M NaCl. The column chromatography was carried out at a flow rate of 0.3 mL/min. The fractions 45-56, 12.5 mL were pooled.

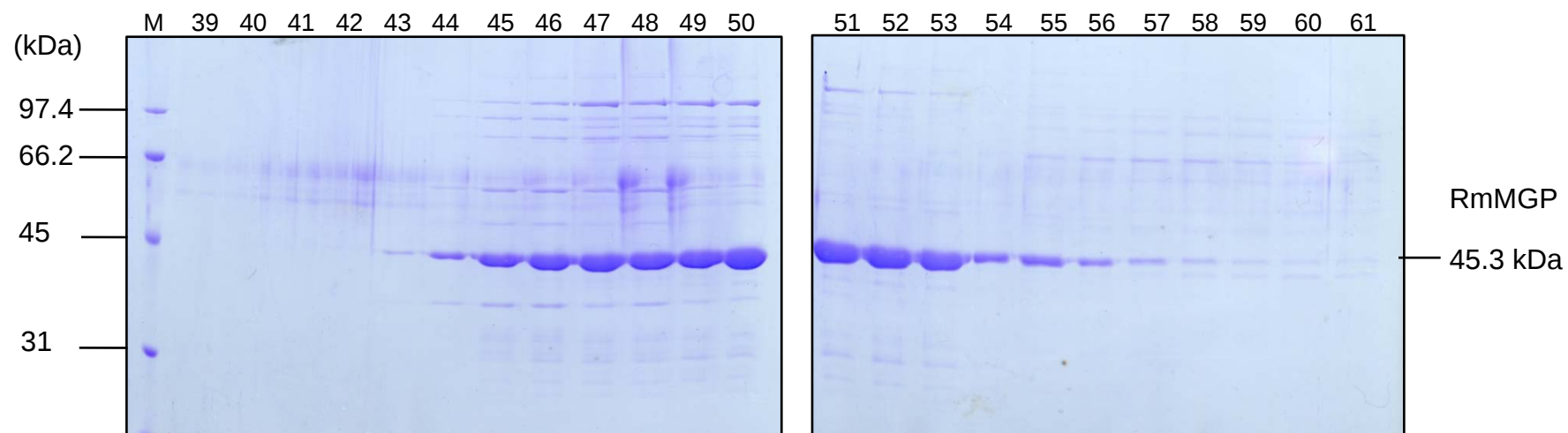


Fig. II.9. SDS-PAGE analysis of active fractions containing RmMGP in Sephacryl S-300 column chromatography.

Proteins were eluted with 10 mM MES-NaOH buffer (pH 7.0) containing 0.2 M NaCl at a flow rate of 0.3 mL/min (see Fig. II.8). Active fractions containing of RmMGP activity were investigated by SDS-PAGE, and the fractions 45-56, 12.5 mL were pooled, and dialyzed against 10 mM MES-NaOH buffer (pH 7.0). SDS-PAGE was performed with 10% polyacrylamide gel, and protein was detected by Coomessie Brilliant Blue staining. M, size marker (2 μ L was loaded); 39-61, fraction number (10 μ L was loaded).

Table II.3. Purification of recombinant RmMGP from 1 L fermentation of *E. coli* culture.

Procedure	Total Protein (mg)	Volume (mL)	Total Activity (U)	Specific Activity (U/mg)	Recovery (%)	Purification (-fold)
Cell-free extract	912*	40	210	0.309	100	1
DEAE Toyopearl650M	69.5**	105	104	1.50	49.5	4.85
Butyl Toyopearl650M	8.33**	37	63.3	7.60	30.1	24.6
Sephacryl S-300	10.6***	12.5	45.9	8.60	21.9	27.8

*Protein concentration was measured by the Bradford method.

**Protein concentration was measured at absorbance 280 nm assuming an extinction coefficient of 1.0 mg/mL of protein equal to 1.00.

***Protein concentration was measured by amino acid analysis.

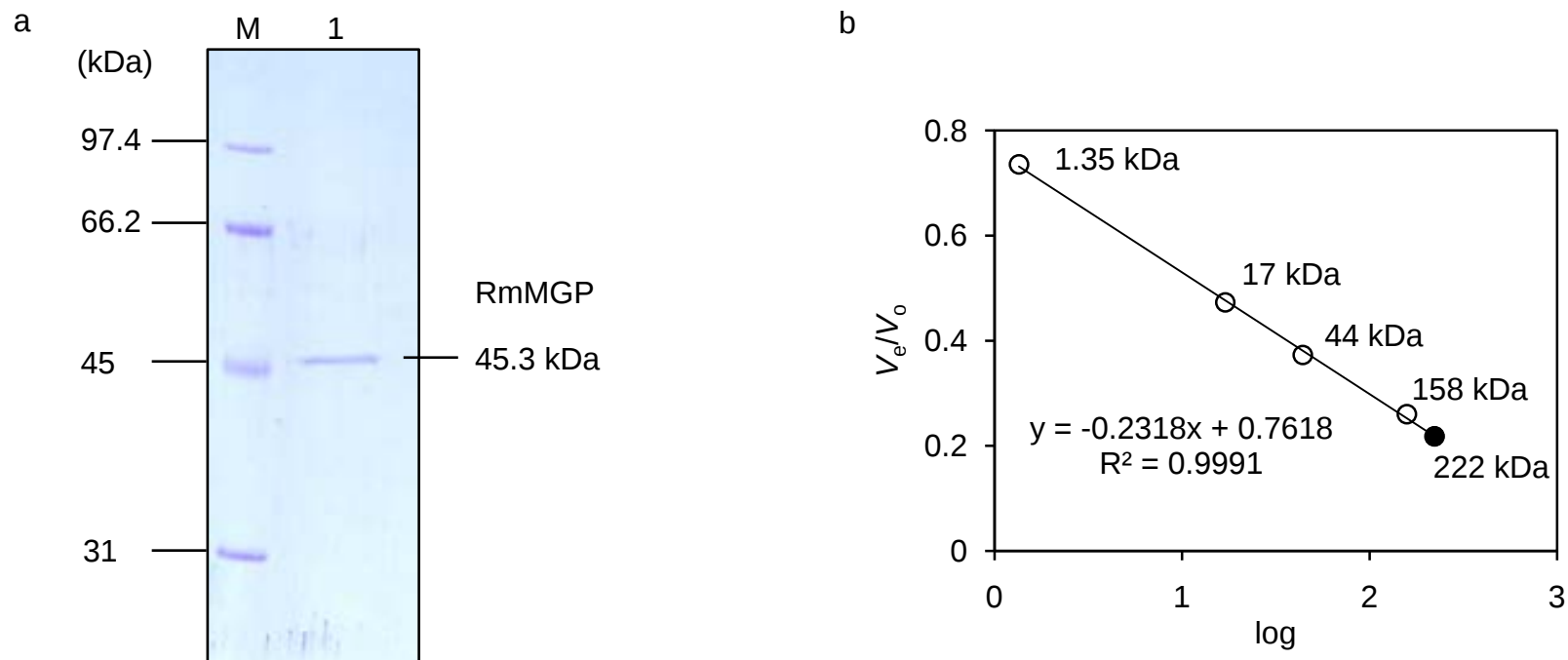


Fig. II.10. SDS-PAGE analysis of purified RmMGP and molecular mass of RmMGP.

a, SDS-PAGE analysis of purified RmMGP; M, size marker; 1, purified RmMGP (1 μ g). Molecular masses of standard proteins are indicated on the left. The proteins were stained with Coomassie Brilliant Blue. b, Molecular mass of RmMGP on gel filtration column; standard protein (-○-), RmMGP (-●-). Plotting the logarithms of the standard molecular masses versus their respective V_e/V_o values produces a linear calibration curve (V_e is the elution volume and V_o is the void volume).

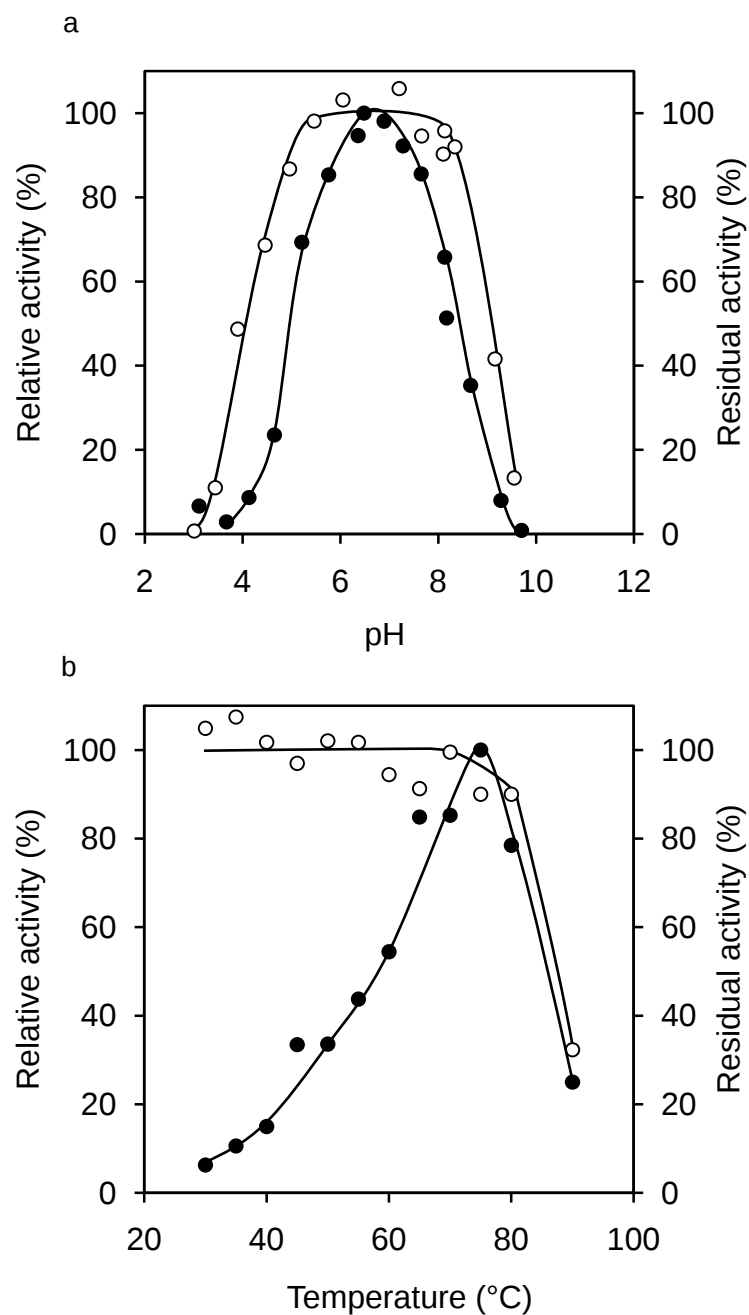


Fig. II.11. Effects of temperature and pH on enzyme activity and stability.

MGP activity was measured at various pH values (a, closed circles) and temperature (b, closed circles) to determine the optimal conditions (reaction time was 10 min). Stability was evaluated with remaining activity after pH (a, open circles) and heat treatment (b, open circles). The enzyme was incubated at various pH values at 4°C for 24 h and at various temperatures at pH 6.5 for 20 min.

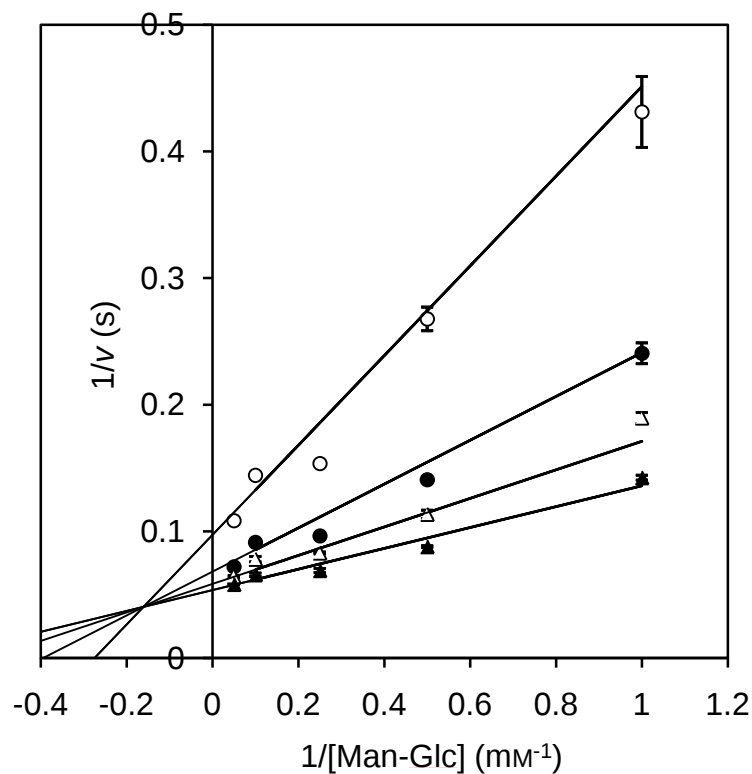


Fig. II.12. Double-reciprocal plots for the phosphorolysis of Man-Glc by recombinant RmMGP.

The initial velocities for phosphorolysis of Man-Glc with various concentrations of Man-Glc and Pi were determined. Concentrations of Pi were 1 (open circles), 2.5 (filled circles), 5 (open triangles), and 10 mM (filled triangles). Data represent the mean $\pm$ standard deviation (error bars) for three independent experiments.

Table II.4. Comparison of apparent kinetic parameters for synthetic reactions between RmMGP and RaMGP

Substrate	RmMGP			RaMGP ⁴		
	$k_{\text{cat(app)}}$ (s ⁻¹)	$K_{\text{m(app)}}$ (mM)	$k_{\text{cat(app)}/K_{\text{m(app)}}$ (s ⁻¹ mM ⁻¹)	$k_{\text{cat(app)}}$ (s ⁻¹)	$K_{\text{m(app)}}$ (mM)	$k_{\text{cat(app)}/K_{\text{m(app)}}$ (s ⁻¹ mM ⁻¹)
1,5-Anhydro-D-glucitol	1.62 ± 0.18	47.5 ± 7.6	0.0341	N.D.	N.D.	N.D.
6-Deoxy-D-glucose	42.3 ± 3.7	65.7 ± 9.0	0.644	177 ± 21	119 ± 18	1.49
D-Glucose	29.6 ± 0.7	42.1 ± 1.1	0.703	126 ± 1	25.9 ± 0.1	4.86
Methyl β-D-glucoside	2.02 ± 0.26	58.0 ± 8.9	0.0348	N.D.	N.D.	N.D.
D-Xylose	N.D.	N.D.	0.229*	N.D.	N.D.	0.180

Apparent kinetic parameters were determined from the reaction rates at 1.25-40 mM an acceptor and 10 mM Man1P.

Values are mean ± SD for three independent experiments.

N.D., not determined.

*determined from the slope of *s-v* plot due to very high $K_{\text{m (app)}}$ value.

CHAPTER III. Site-directed mutational analysis of amino acid residues in CE from *R. marinus* important for disaccharide specificity

III-1. Introduction

The AGE superfamily enzymes, CE, AGE, and AKI, share similar catalytic domain formed by an (α/α)₆ barrel and the catalytic site, but their substrate specificities are significantly different from each other. Especially, only CE acts on disaccharide substrates, but the other enzymes do not. CEs catalyze the epimerization of those disaccharides at the reducing-end part. The complex structure of RmCE with Glc-Man suggests the amino acid residue candidates responsible for the disaccharide specificity of CEs. Trp385 of RmCE, located at the entrance of the catalytic center, stacks onto the pyranose ring of non-reducing end glycosyl residue, and Ser185 forms a hydrogen bond with O4 atom of the same glycosyl residue in the CE complex structure (Fig. III.1). These Trp and Ser residues are conserved in CEs^{24, 25}. Furthermore, Asp188 of RmCE forms a hydrogen bond with O6 atom at non-reducing end part of substrate, and many CEs have Asp at the corresponding position²⁵. In this chapter, amino acid residues involved in disaccharide specificity of CE are described. Through the comparison of activity of RmCE and RaCE to monosaccharide and disaccharide substrates, and the site-directed mutagenesis of Asp188 and Trp385 of RmCE, the significance of the residues for disaccharide specificity is clarified.

III-2. Materials and methods

III-2-1. Preparation of expression plasmids for RmCE mutant enzymes

Nucleotide substitution for the site-directed mutagenesis of RmCE was done by PCR using Primestar max DNA polymerase and primers as follows:

D188A_F, sense, 5'-GCCAAAGCCGCACCCGAGCCGCGCAGT-3';

D188A_R, antisense, 5'-ACTGCGCGGCTCGGGTGCGGCTTTGGC-3';

W385A_F3, sense, 5'-GACTTCGCGAAGGGACCCTACCACAAC-3';

W385A_R3, antisense, 5'-GTTGTGGTAGGGTCCCTTCGCGAAGTC-3'.

The contents of the reaction mixture and reaction conditions are summarized in Table III.1 and Table III.2. The expression plasmid for the wild type¹⁹ was used as the template.

III-2-2. Purification of CEs

III-2-2-1. RaCE

III-2-2-1-1. Production

Recombinant RaCE was produced in *E. coli*. The *E. coli* transformant was cultured in 3 L of LB broth containing 50 µg/mL ampicillin. When absorbance at 600 nm reached 0.6 in the incubation at 37°C, protein expression was induced with 0.1 mM IPTG. The induction culture was done at 16°C for 20 h. Bacterial cells were harvested by the centrifugation at $8,400 \times g$ at 4°C for 10 min, re-suspended in 40 mL of 10 mM sodium phosphate buffer (pH 6.7), and disrupted by sonication (Duty cycle, 60%; output control, 4; time, 1 min/500 mL culture for 10 times). Cell-free extract was obtained by centrifugation at $13,000 \times g$ at 4°C for 10 min, and subjected to column chromatographic procedures described below.

III-2-2-1-2. Purification

III-2-2-1-2-1. Toyopearl DEAE-650M column chromatography

The cell-free extract was subjected to anion exchange column chromatography using a Toyopearl DEAE-650M column (i.d. 3.0×23 cm) pre-equilibrated with 10 mM sodium phosphate buffer (pH 6.7). The non-adsorbed proteins were washed with the buffer (total wash buffer volume was 500 mL), and the adsorbed proteins were eluted by a linear gradient of NaCl from 0 to 0.5 M in the buffer (total elution volume was 250 mL).

III-2-2-1-2-2. Toyopearl Butyl-650M column chromatography

To the collected fractions, ammonium sulfate was added up to 30% saturation, then the protein sample was loaded to a Toyopearl Butyl-650M column, (i.d. 3.0×10.5 cm) equilibrated with 10 mM sodium phosphate buffer (pH 6.7) containing 176 g/L ammonium sulfate (30% saturation). Non-adsorbed protein was completely eluted with the buffer ammonium sulfate containing, and the adsorbed protein was eluted using a linear gradient of 176 – 0 g/L ammonium sulfate (total

elution volume was 250 mL). The fractions containing RaCE were collected, and dialyzed against 5 mM sodium phosphate buffer (pH 7.0). The protein was concentrated by vivaspin and confirmed the purity by SDS-PAGE. The purified enzyme was stored at -20°C in the presence of 50% glycerol.

III-2-2-2. RmCE and mutant enzymes

III-2-2-2-1. Production

RmCE and its mutants were produced in *E. coli* host cells and purified as described⁴. Here is a description in detail. The recombinant RmCE was produced in *E. coli* BL21(DE3) in 3 L of LB containing 50 $\mu\text{g/mL}$ ampicillin. The expression of protein was induced by adding 0.1 mM IPTG when absorbance at 600 nm reached 0.6, and the culture broth was further incubated at 16°C for 20 h with vigorous shaking. Bacterial cells were harvested, re-suspended in 30 mL of 10 mM sodium phosphate buffer (pH 8.5), disrupted, and centrifuged at $13,000 \times g$ at 4°C for 10 min. The cell-free extract obtained was incubated at 70°C for 30 min and centrifuged at $13,000 \times g$ at 4°C for 10 min to remove *E. coli*-derived proteins (this heat treatment was avoided for the mutant enzymes). The supernatant was applied through 2 column chromatographies as follows¹⁹.

III-2-2-2-2. Purification

III-2-2-2-2-1. Toyopearl DEAE-650M column chromatography

The obtained cell-free extract containing 10 mM sodium phosphate buffer (pH 7.0) was subjected to anion exchange column chromatography using a Toyopearl DEAE-650M column, (i.d. 3.0×23 cm) equilibrated with 10 mM sodium phosphate buffer (pH 7.0). The RmCE was eluted with the buffer and obtained as non-adsorbed protein.

III-2-2-2-2-2. Toyopearl Butyl-650M column chromatography

The collected fractions, ammonium sulfate was added up to 0.5 M saturation, then the protein sample was loaded to a Toyopearl Butyl-650M column (i.d. 3.0×23 cm) equilibrated with 10 mM sodium phosphate buffer (pH 7.0)

containing 0.5 M ammonium sulfate. Non-adsorbed protein was eluted with the linear gradient of 0.5-0 M ammonium sulfate. The fractions containing RmCE were pooled, dialyzed against 10 mM Tris-HCl buffer (pH 8.5), and concentrated by ultrafiltration. The purity of purified protein was investigated using SDS-PAGE, and the sample was stored at -20°C in the presence of 50% glycerol.

III-2-3. Protein assay

The protein concentrations of the cell-free extract, the column chromatography fractions, and the purified enzymes were measured as described in Chapter II-2-5.

III-2-4. TLC analysis of reaction product from various substrates

The reaction mixture (200 μL) containing 100 mM substrate (cellobiose, maltose, lactose, and D-glucose), 20 mM sodium phosphate buffer (pH 7.0), and 15 μM RaCE/2.8 μM RmCE, was incubated at 37°C (RaCE) or 60°C (RmCE) for 24 h. The sample was desalted with Amberlite MB4 (Roam and Hass, Philadelphia, PA, USA). The product was analyzed on TLC using 2-propanol/1-butanol/water (2:2:1 v/v/v) and anisaldehyde-sulfuric acid reagent [acetic acid/sulfuric acid/anisaldehyde (100:2:1, v/v/v)] as a developing solvent and detection reagent, respectively. The samples TLC plate was developed twice in described developing solvent.

III-2-5. Measurement of reaction velocity for a single concentration of cellobiose, maltose, lactose, and D-glucose

The reaction mixture (400 μL), containing 100 mM substrate (cellobiose, lactose, maltose, and glucose), 30 mM sodium phosphate buffer (pH 7.0), and appropriate concentration of enzyme, was incubated at 37°C (RaCE) or 60°C (RmCE) for following time: 5-40 min for cellobiose and lactose, 30-240 min for maltose, and 360-720 min for D-glucose. Fifty microliters of the sample was taken from the reaction mixture, mixed with 25 μL of 0.1 M HCl, and heated at 100°C for 3 min. The reaction product was measured by HPLC. Distilled water was added to the sample up to 100 μL , and the sample was desalted with Amberlite MB4. The sample filtered through membrane with pore size 0.45 μm (Advantech, Tokyo, Japan) was injected. HPLC conditions were as follows: column, SP0810 (i.d. $8.0 \times 300 \text{ mm} \times 2$); column temperature, 70°C ; eluent, water; flow rate, 0.5 mL/min; detection, refractive index; injection volume, 5 μL . As standards, 0-2.5 mM Glc-

Man, Glc α 1-4Man, epilactose, and D-mannose were analyzed in the presence of 50 mM of cellobiose, maltose, lactose, and D-glucose, respectively.

III-2-6. Effects of pH and temperature on activity and stability of RmCE variants

III-2-6-1. Optimum pH and temperature

To determine the optimum pH, the reaction mixture (25 μ L) containing 10 mM Man₂, 30 mM buffer [Britton-Robinson buffer (pH 3.6-11.5), KCl-HCl (pH 2.6-3.1), and KCl-KOH (pH 11.9)]¹⁹, and an appropriate concentration of enzyme was incubated at 60°C for 10 min. The pH values of the reaction mixture were confirmed in mock solutions, which were the same as the reaction mixture but without the substrate and enzyme. To determine optimum temperature, the reaction mixture (25 μ L) containing 10 mM Man₂, 30 mM sodium phosphate (pH 7.0), and appropriate concentration of enzyme was incubated at 30-100°C for 10 min. Man-Glc produced was measured as described in the chapter V.

III-2-6-2. pH and temperature stability

The pH and temperature treatments were carried out under the following conditions: as for the pH treatment, 20 μ L of enzyme (3.87 μ M RmCE wild type/12.4 μ M W385A/14.5 μ M D188A) and 45 mM buffer described above, was kept at 4°C for 24 h; for the temperature treatment, 25 μ L enzyme (enzyme was used as same concentration as pH treatment) in 45 mM sodium phosphate buffer (pH 7.0) was incubated at 30-100°C for 30 min. In the both cases, the residual activity was measured using 10 mM Man₂ as substrate. The reaction mixture (50 μ L), containing 10 mM Man₂, 30 mM sodium phosphate buffer (pH 7.0), and the enzyme, was incubated at 60°C for 10 min. The reactions were terminated by addition of 20 μ L of 0.1 M HCl and heating the sample at 100°C for 3 min. The produced Man-Glc was measured as described in the Chapter V.

III-2-7. Kinetic parameters of RmCE variants for monosaccharide and disaccharide substrates

The kinetic parameters were determined by fitting initial reaction rates toward various concentrations of substrates to the Michalis-Menten equation.

III-2-7-1. Man₂

A reaction mixture (175 μ L) containing 5-25 mM Man₂, 30 mM sodium phosphate buffer (pH 7.0), and an appropriate concentration of enzyme was incubated at 60°C for 2-10 min. Fifty microliters of the sample was taken every 2 min. Twenty five microliters of 0.1 M HCl was mixed, and the sample was heated at 100°C for 3 min. The concentration of Man-Glc was measured as described in Chapter V-II-5.

III-2-7-2. Cellobiose and lactose

A reaction mixture (120 μ L) containing 40-200 mM cellobiose or lactose, 30 mM sodium phosphate buffer (pH 7.0), and an appropriate concentration of enzyme was incubated at 60°C. Fifty microliters of the sample was taken at reaction 5 and 30 min. The reaction was terminated by adding 25 μ L of 0.1 M HCl and heating the sample at 100°C for 3 min. The concentration of Glc-Man or epilactose was determined by HPLC as described in III-2-5. Reaction velocity was calculated from the difference of the product concentrations at reaction 5 and 30 min.

III-2-7-3. D-Mannose

A reaction mixture (250 μ L), containing 50-500 mM D-mannose, an appropriate concentration of enzyme, and 15 mM sodium phosphate buffer (pH 7.0) was incubated at 60°C for 3-12 h. Fifty microliters of the sample was taken from the reaction mixture at appropriate times. The reaction was terminated by adding 25 μ L of 0.1 M HCl and heating the sample at 100°C for 3 min. To neutralize the sample, 50 μ L of 200 mM sodium phosphate buffer (pH 8.0) was added. The sample was centrifuged to remove the aggregated protein at 4°C, 6,500 $\times$ g for 10 min. To measure D-glucose, the supernatant (50 μ L) was mixed with 25.5 μ L of the D-glucose quantification reagent (R-biopharm, Darmstadt, Germany), and the sample was incubated at room temperature for 30 min. Absorbance at 340 nm was measured. The mixture containing 0-1 mM of D-glucose and 50 mM of D-mannose was used as standard.

III-2-8. Preparation of Glc α 1-4Man produced from maltose by RaCE and analysis of the monosaccharide component of the purified product

A reaction mixture (1 mL), containing 100 mM maltose, 30 mM sodium phosphate buffer (pH 7.0), and 5.9 μ M RaCE, was incubated at 60°C for 6 days. The reaction product

was purified by HPLC under the conditions as follows: column, SP0810 (i.d. 8.0×300 mm $\times$ 2); column temperature, 70°C; eluent, water; flow rate, 0.5 mL/min; detection, refractive index; injection volume, 50 μ L. To identify the monosaccharide component of the purified product, 50 μ L of the sample (0.4% w/v) was hydrolyzed in 4 M trifluoroacetic acid (TFA) at 100°C for 3 h¹⁹. The sample was dried *in vacuo* and re-suspended in 50 μ L of water. The monosaccharide was analyzed by HPLC as described in III-2-5.

III-3. Results and discussions

III-3-1. Preparation of the recombinant CE

III-3-1-1. RmCE derivatives

Protein production and purification of the RmCE wild type, W385A, and D188A were done as described previously¹⁹. The recombinant variants were produced in *E. coli* BL21(DE3) in 3 L. The harvested bacteria cells were disrupted and cell-free extract was purified through Toyopearl DEAE-650M and Toyopearl Butyl-650M column chromatographies as described previously⁴. RmCE was not adsorbed on Toyopearl DEAE-650M column, and eluted by 10 mM sodium phosphate buffer (pH 7.0) as observed previously⁴. The active fractions in the Toyopearl Butyl-650M column chromatography were regarded as purified protein after dialysis against 10 mM Tris-HCl buffer (pH 8.5), and concentrated by ultrafiltration. The purified proteins showed a single band on SDS-PAGE (Fig. III.2). Finally, 29.9, 26.5, and 15.3 mg of purified RmCE wild type, W385A, and D188A, respectively, were obtained.

III-3-1-2. RaCE

The recombinant plasmid was prepared as described previously¹⁹. *E. coli* BL21(DE3) cells were harvested from 3 L of the culture and disrupted by sonication. Cell-free extract contained RaCE as a major protein (Fig. III.3) was subjected to Toyopearl DEAE-650M and Toyopearl Butyl-650M column chromatographies. In the Toyopearl DEAE-650M column chromatography, RaCE adsorbed onto the column was eluted by 0.05-0.15 M NaCl (Fig. III.4). The SDS-PAGE analysis revealed that the 44 kDa of RaCE was detected in the fractions 165-183 (Fig. III.5). Pooled fractions (95 mL) were applied to

Toyopearl Butyl-650M column chromatography (Fig. III.6). The fraction containing RaCE was eluted by 100-50 g/L ammonium sulfate corresponding to the fraction 164-182 on SDS-PAGE (Fig. III.7). The pooled fractions (95 mL) were dialyzed against 5 mM sodium phosphate buffer (pH 7.0) and concentrated by ultrafiltration. The resulting sample was concentrated, and 34.3 mg of purified RaCE was obtained. The purity of the protein was confirmed on SDS-PAGE (Fig. III.8).

III-3-2. Analysis of epimerization products from various substrates

Epimerization activity of RaCE and RmCE to cellobiose, lactose, maltose, and D-glucose was examined. These enzymes epimerized not only cellobiose and lactose, but also maltose and D-glucose (Fig. III.9; for D-glucose, see Fig. III.10). In the HPLC analyses, the retention times of the products from cellobiose, lactose, and D-glucose were the same as authentic Glc-Man, epilactose, and D-mannose (Fig. III.10).

The product from maltose by RaCE was purified by HPLC. The purification is shown in Fig. III.11.a&b. The purified disaccharide product (0.4% w/v) was hydrolyzed by 4 M TFA. HPLC analysis indicated that the resulting monosaccharides were D-glucose and D-mannose (Fig. III.11). As CE catalyzes epimerization of reducing-end sugar residue, the product from maltose is regarded as considered to be 4-O- α -D-glucosyl-D-mannose. The reaction product produced from maltose by RmCE was also hydrolyzed to D-mannose and D-glucose by the same method¹⁹.

III-3-3. Epimerization activity of RaCE and RmCE to maltose and D-glucose

Reaction velocities of RaCE and RmCE for maltose and D-glucose were compared with those for cellobiose and lactose. Epimerization activities of RaCE and RmCE to maltose were 0.0211% and 0.642% of those to cellobiose, respectively (Table III.3). Maltose is a disaccharide of two glucose molecules as same as cellobiose, but two glucoses are linked by α -(1 $\rightarrow$ 4)-glycosidic bond. Because of the difference configuration, maltose could not serve as a substrate for CE efficiently. Glycosyl residue at non-reducing end part might be populate irregular/far from amino acid residues binding substrate. The epimerization activities of these enzymes to D-glucose were lower than those to maltose: RaCE and RmCE showed 0.00152% and 0.145% of activity for cellobiose, respectively. Thus, monosaccharide serves as a substrate for CE poorly.

III-3-4. Comparison of substrate specificity between RmCE mutants and wild type

The kinetic parameters of RmCE variants for Man₂, cellobiose, lactose, and D-mannose were compared (Table III.4). In D188A and W385A, $k_{\text{cat}}/K_{\text{m}}$ values decreased, but the $k_{\text{cat}}/K_{\text{m}}$ values for disaccharide substrates were more severely decreased than that for D-mannose. In D188A and W385A, the $k_{\text{cat}}/K_{\text{m}}$ values for Man₂, cellobiose, and lactose were 10-17% and 0.06-0.19% of those of wild type, respectively, while those for D-mannose retained 88% and 5.5%, respectively, the $k_{\text{cat}}/K_{\text{m}}$ value for this monosaccharide did not reduce significantly. These results indicate these two mutant enzymes showed lower preference for disaccharide than wild type, and thus Trp385 and Asp188 are important for high preference for disaccharide-substrates over the monosaccharide in RmCE. The change of Gibbs' free energy, $\Delta\Delta G$ was calculated as a follow⁴⁹; $\Delta\Delta G = -RT \ln [(k_{\text{cat}}/K_{\text{m}})_a/(k_{\text{cat}}/K_{\text{m}})_b]$. The $\Delta\Delta G$ values of W385A and D188A for disaccharide substrates were compared to those of wild type. The $\Delta\Delta G$ values of W385A for disaccharide substrates were 4.1-4.9 kcal.mol⁻¹ to those of wild type, while $\Delta\Delta G$ values of D188A were 1.2-1.5 kcal.mol⁻¹ to those of wild type. These support that the strong interaction between enzyme and substrate at Trp385 and Asp188 residues were lost. As the enzyme-substrate interaction with hydrogen bond at 6-OH position of substrate, D188A was been weakened binding energy, while stacking interaction of W385A was weaker than D188A. Comparing the kinetic parameters for the three kinds of disaccharide substrates, the two mutant enzymes showed similar selectivity for these substrates to wild type. Thus the specificity for disaccharide substrates was not significantly changed by the mutations.

III-3-5. Effects of pH and temperature of RmCE variants

The maximal activity of RmCE wild type, D188A, and W385A were pH 7.1, 7.5, and 7.4, respectively. The wild type enzyme was stable in a range of pH 2.3-12, while both of the mutant enzymes showed narrower stable range (pH 3.6-11) than wild type (Fig. III.12). The optimum temperatures of wild type, D188A, and W385A were 70, 55, and 80°C, respectively, in 10 min reaction. Wild type and W385A were stable at 70°C or lower in 30 min incubation. D188A showed slightly higher heat stability than these enzymes, although this mutant enzyme showed lower optimum temperature than wild type (Fig. III.13).

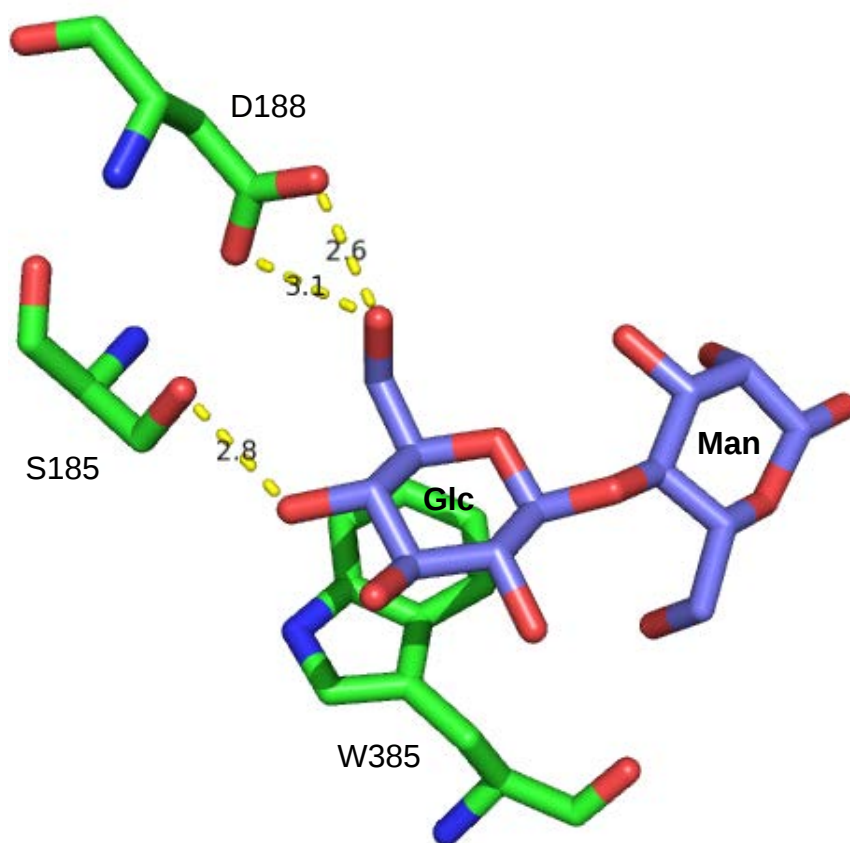


Fig. III.1. Close-up view of substrate binding site of RmCE around non-reducing end part of Glc-Man.

Table III.1. Composition of the reaction mixture of PCR for site-directed mutagenesis.

Component	Volume (μL)
Expression plasmid for RmCE (10 pg/ μL)	1
1 μM sense primer	2
1 μM antisense primer	2
2x Primestar max DNA polymerase	5
Total volume	10

Table III.2. The reaction conditions of PCR for site-directed mutagenesis.

Steps	Conditions	
Denaturation	98°C, 10 s	} 30 cycles
Annealing	55°C, 15 s	
Extension	72°C, 35 s	

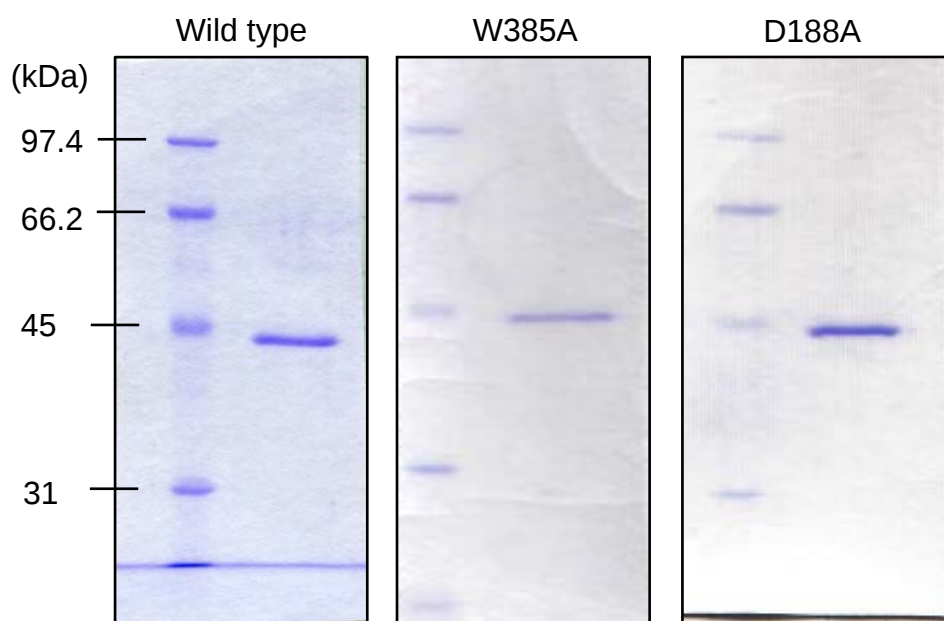


Fig. III.2. SDS-PAGE analysis of purified RmCE variants.

Molecular masses of standard proteins migrated in the left lane and purified enzyme did in the right lane. The proteins were stained with Coomassie Brilliant Blue.

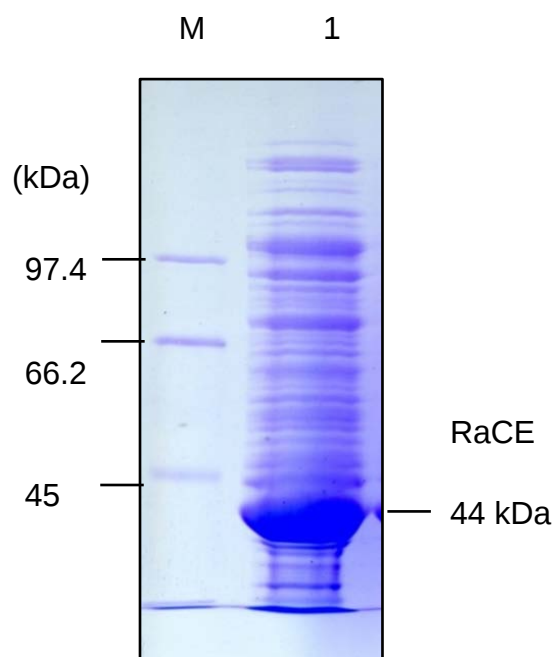


Fig. III.3. SDS-PAGE analysis of the production of recombinant RaCE in *E. coli* cells.

SDS-PAGE was performed on 10% polyacrylamide gel, and protein was stained with the Coomessie Brilliant Blue. One microliter of the cell-free extract was loaded on the gel. M, size markers; 1, cell-free extract of *E. coli* transformant producing recombinant RaCE.

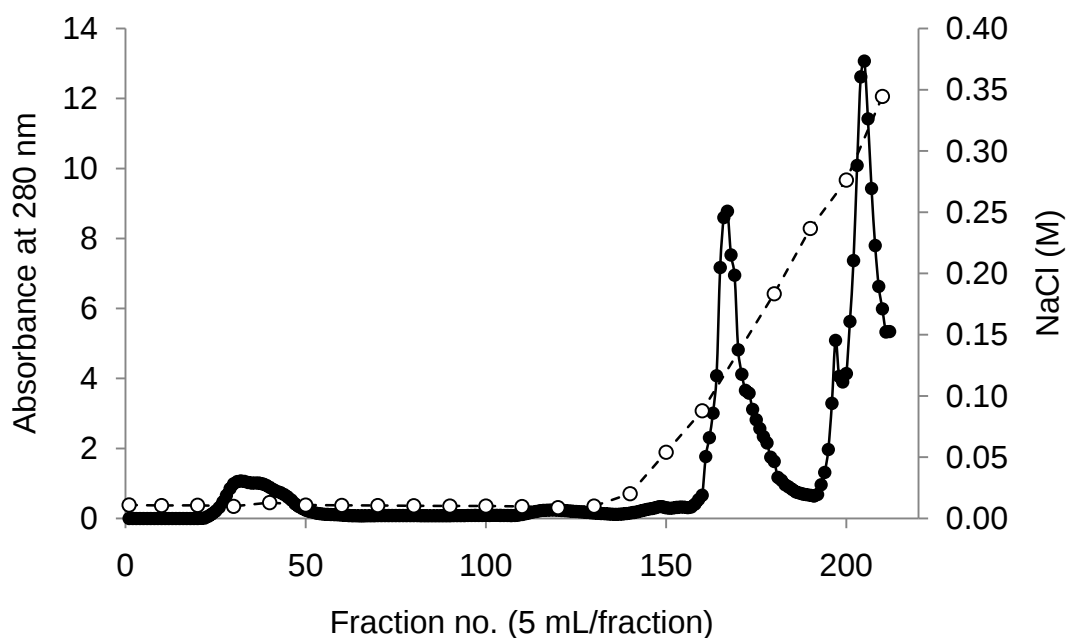


Fig. III.4. Toyopearl DEAE-650M column chromatography of recombinant RaCE.

Toyopearl DEAE-650M column (i.d. 3.0×23 cm) was equilibrated with 10 mM sodium phosphate buffer (pH 6.7). Elution was done by a linear gradient of NaCl from 0 to 0.5 M. The fractions 165-183 containing RaCE were pooled (95 mL). ○, NaCl concentration; ●, absorbance at 280 nm.

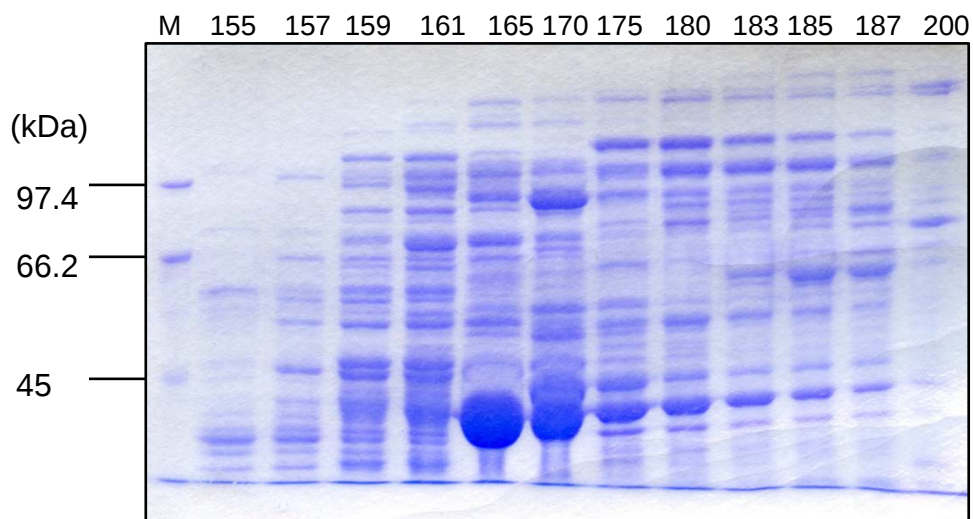


Fig. III.5. SDS-PAGE analysis of absorbed proteins containing RaCE in Toyopearl DEAE-650M column chromatography.

Adsorbed protein was eluted with 0-0.5 M NaCl, and purity of the fractions was investigated on SDS-PAGE. The fractions 165-183 containing RaCE, 95 mL were pooled and applied to the next column. SDS-PAGE was performed with 10% polyacrylamide gel, and protein was stained with the Coomessie Brilliant Blue. M, size markers; 155-200, fraction number (10 μ L was loaded).

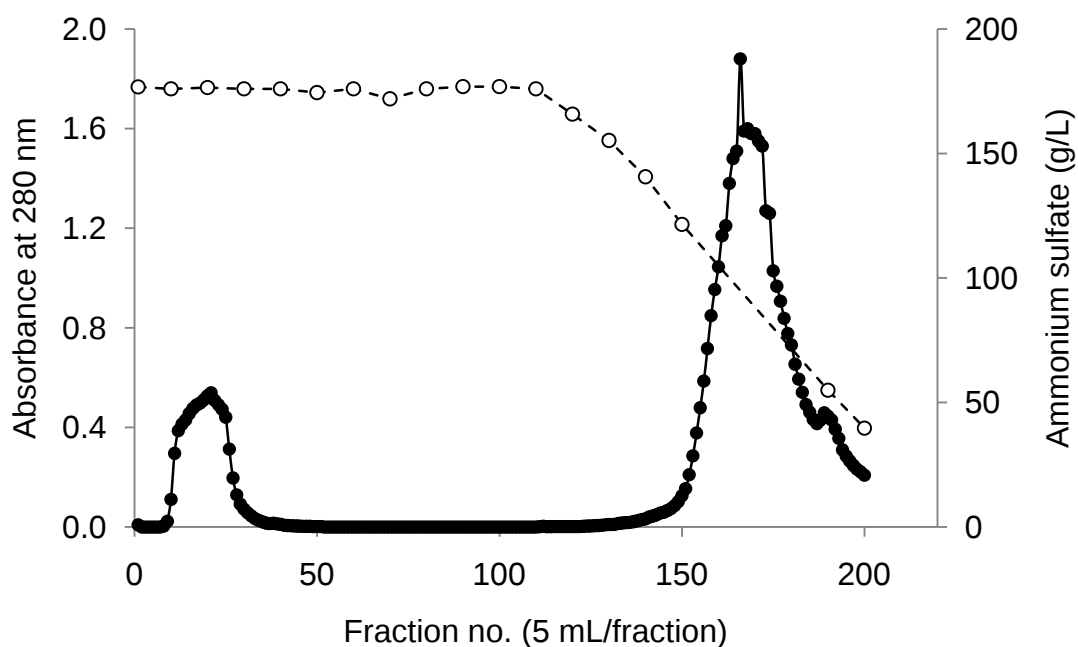


Fig. III.6. Toyopearl Butyl-650M column chromatography of recombinant RaCE.

Toyopearl Butyl-650M column (i.d. 3.0×10.5 cm) was equilibrated with 10 mM sodium phosphate buffer (pH 6.7) containing 176 g/L ammonium sulfate. Elution was done by a linear gradient of ammonium sulfate from 167 to 0 g/L. Fractions 164-182 (95 mL) were pooled. ○, Ammonium sulfate concentration; ●, absorbance at 280 nm.

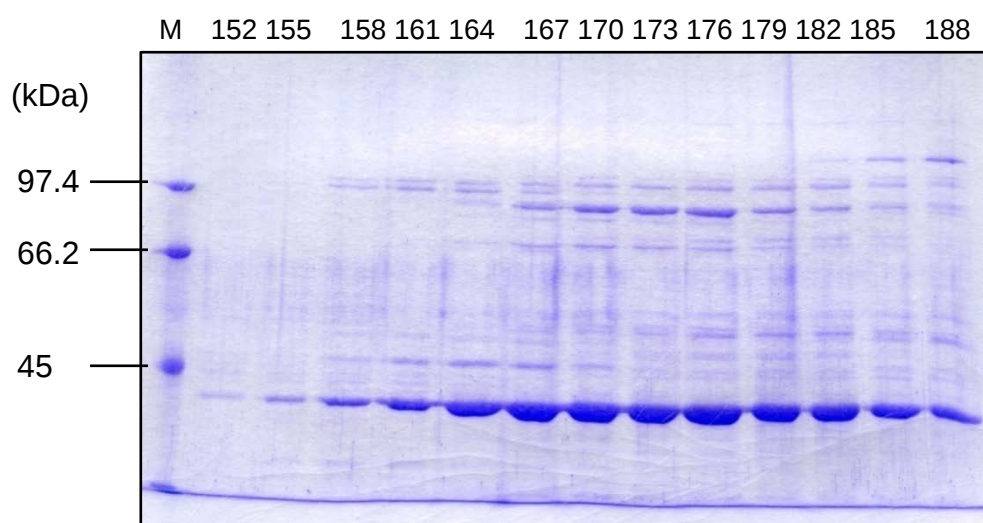


Fig. III.7. SDS-PAGE analysis of fractions of Toyopearl Butyl-650M column chromatography.

Purity of the adsorbed fractions was investigated by SDS-PAGE. Fractions 164-182 (95 mL) were pooled. SDS-PAGE was performed with 10% polyacrylamide gel, and protein was stained with the Coomessie Brilliant Blue. M, size markers; 152-188, fraction number (10 μ L was loaded).

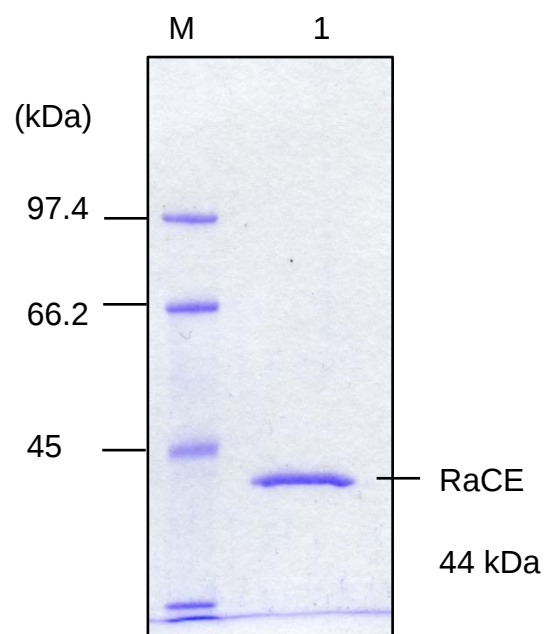


Fig. III.8. SDS-PAGE analysis of purified RaCE.

M, size markers; 1, purified RaCE (1 μ g). Molecular masses of standard proteins are indicated on the left. The proteins were stained with Coomassie Brilliant Blue.

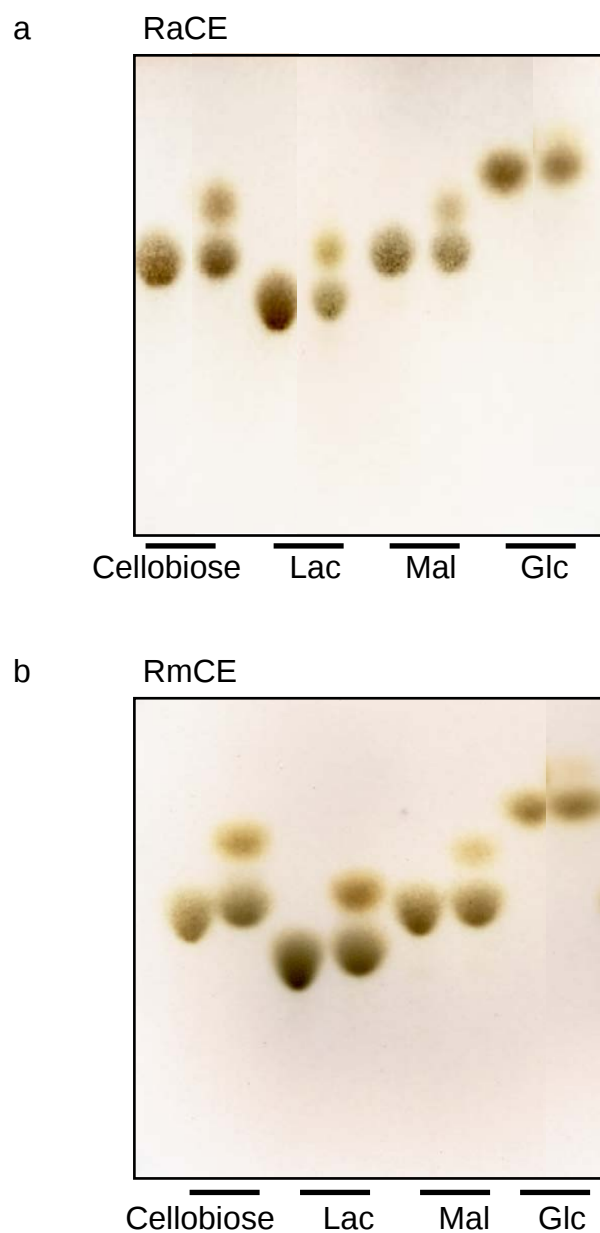


Fig. III.9. TLC analysis of the reaction mixtures of CE with four substrates.

Products from cellobiose, lactose (Lac), maltose (Mal), and D-glucose (Glc) were analyzed by TLC. a, RaCE reactions; b, RmCE reactions. Standard substrates and reaction samples are indicated on the left and right, respectively.

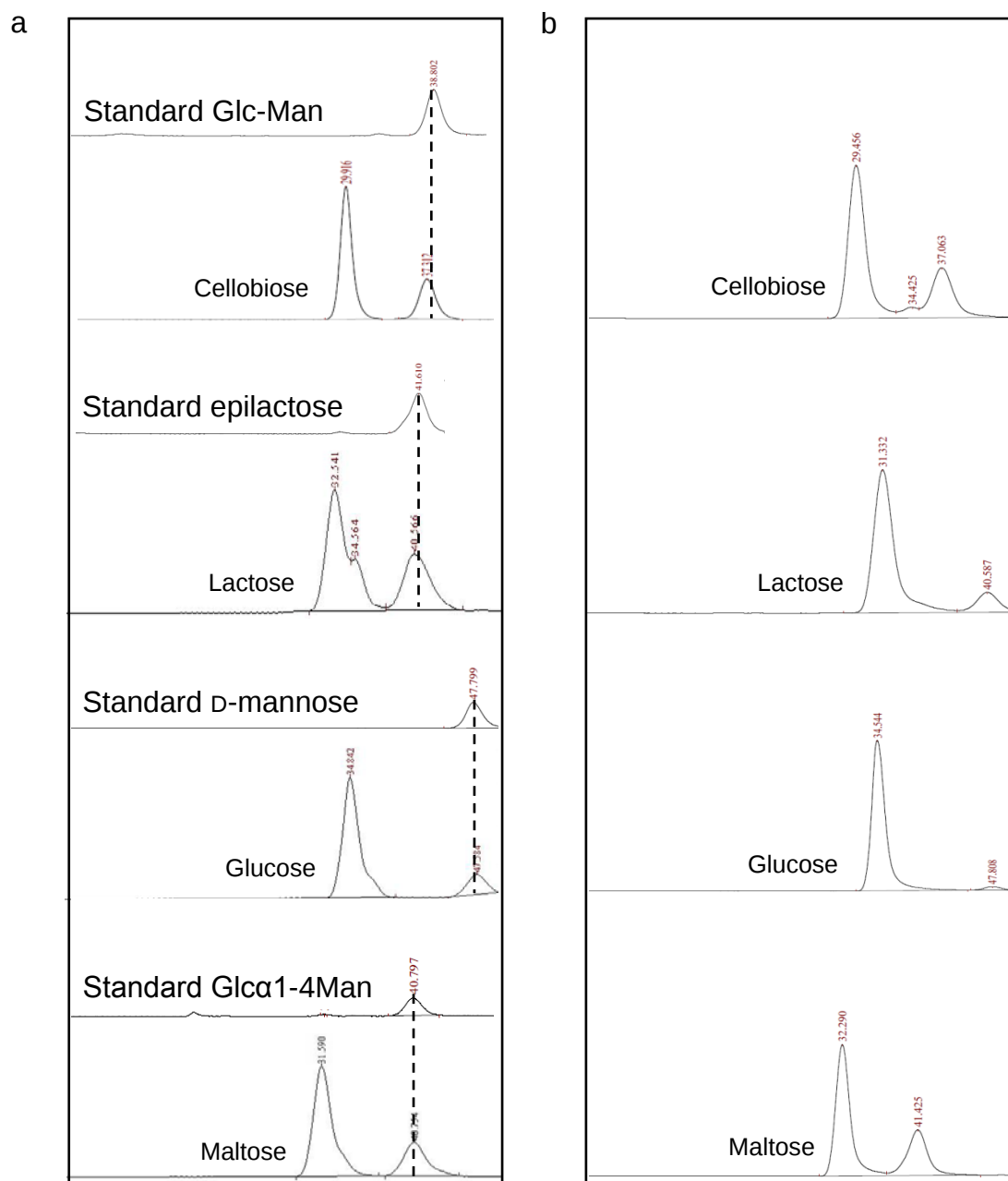


Fig. III.10. HPLC analysis of the reaction mixtures of CE with four substrates.

A reaction mixture containing 100 mM substrate (cellobiose, maltose, lactose, and glucose) and enzyme was incubated at 37°C (RaCE)/ 60°C (RmCE) for 24 h. The products were analyzed by HPLC. a, Reaction products produced by RaCE. b, Reaction products produced by RmCE. HPLC conditions were as follows: column, SP0810 (i.d. 8.0 × 300 mm × 2); column temperature, 70°C; eluent, water; flow rate, 0.5 mL/min; detection, refractive index; injection volume, 5 µL.

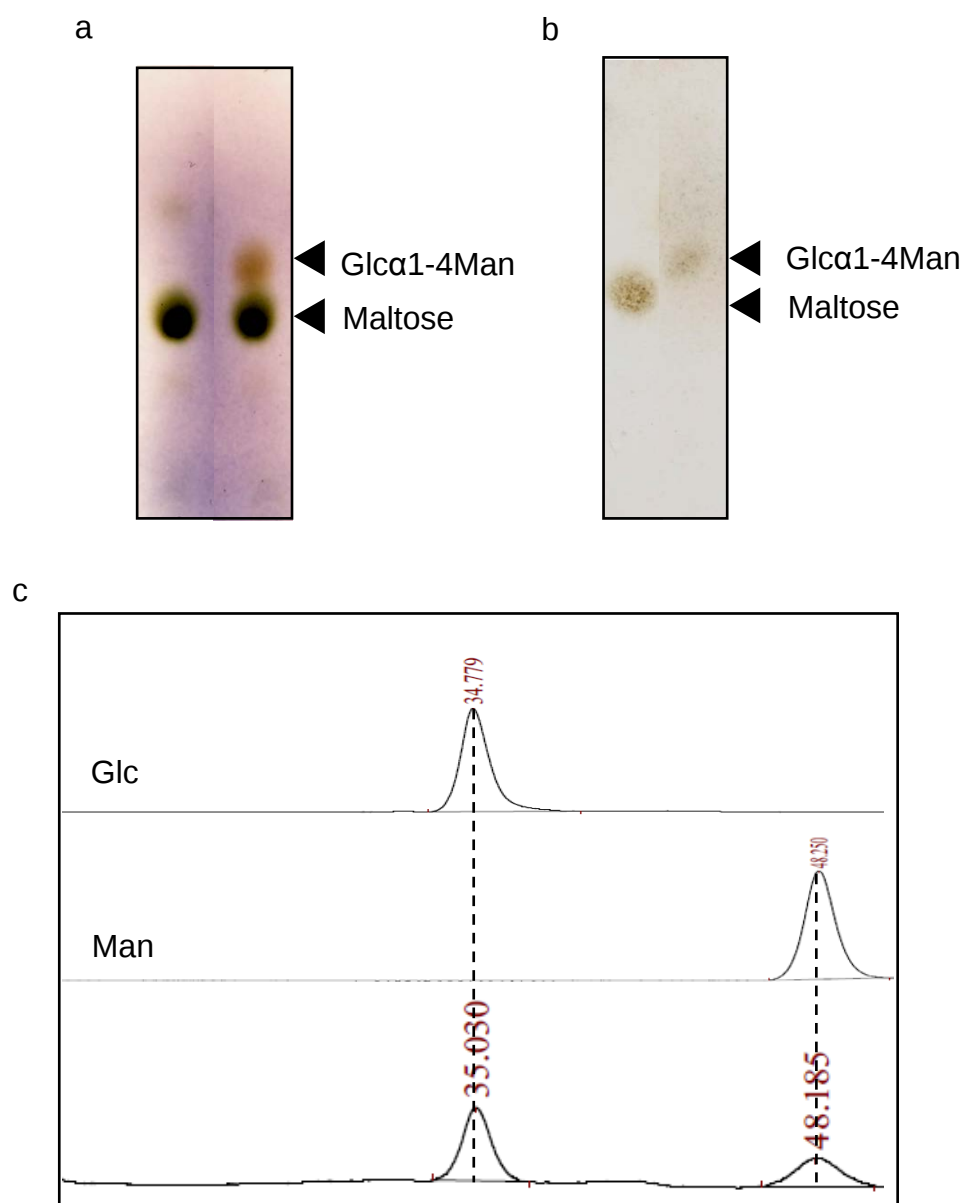


Fig. III.11. Analysis monosaccharide component of maltose-derived reaction product produced by RaCE.

A reaction mixture containing 100 mM maltose and RaCE was incubated at 37°C for 24 h. a, The product in the reaction mixture was detected on TLC; b, TLC analysis of the purified product by HPLC (SP0810 column); c, HPLC analysis of hydrolysate of the reaction product.

Table III.3. Reaction velocities of RaCE and RmCE for the substrates.

Substrate	RaCE		RmCE	
	Velocity	Relative	Velocity	Relative
	($\mu\text{mol/min}$)/(mg/l)	(%)	($\mu\text{mol/min}$)/(mg/l)	(%)
Cellobiose	158,065 $\pm$ 28,893	100	21,525 $\pm$ 3,542	100
Lactose	28,129 $\pm$ 2,892	17.8	63,294 $\pm$ 6,054	294
Maltose	33.4 $\pm$ 2.21	0.0211	138 $\pm$ 4.19	0.642
D-Glucose	2.40 $\pm$ 0.175	0.00152	31.2 $\pm$ 3.91	0.145

Data are mean $\pm$ S.D. for three independent experiments.

Table III.4. Kinetic parameters of RmCE variants.

Substrate	Enzyme	k_{cat} (s ⁻¹)	K_{m} (mM)	$k_{\text{cat}}/K_{\text{m}}$ (s ⁻¹ mM ⁻¹)	Relative $k_{\text{cat}}/K_{\text{m}}$ (%)
Man ₂	WT	94.7	3.53	26.8	100
	W385A	0.655	40.2	0.0163	0.0608
	D188A	95.5	20.8	4.59	17.1
Cellobiose	WT	78.8	24.1	3.27	100
	W385A	-	-	0.00619	0.189
	D188A	40.7	88.2	0.462	14.1
Lactose	WT	111 ^a	28.8 ^a	3.85 ^a	100
	W385A	-	-	0.00390	0.101
	D188A	86.3	232	0.372	9.66
D-Mannose	WT	3.53	668	0.00528	100
	W385A	0.103	354	0.000291	5.51
	D188A	2.15	462	0.00465	88.1

Data are mean ± S.D. for three independent experiments.

a, Ref.-4.

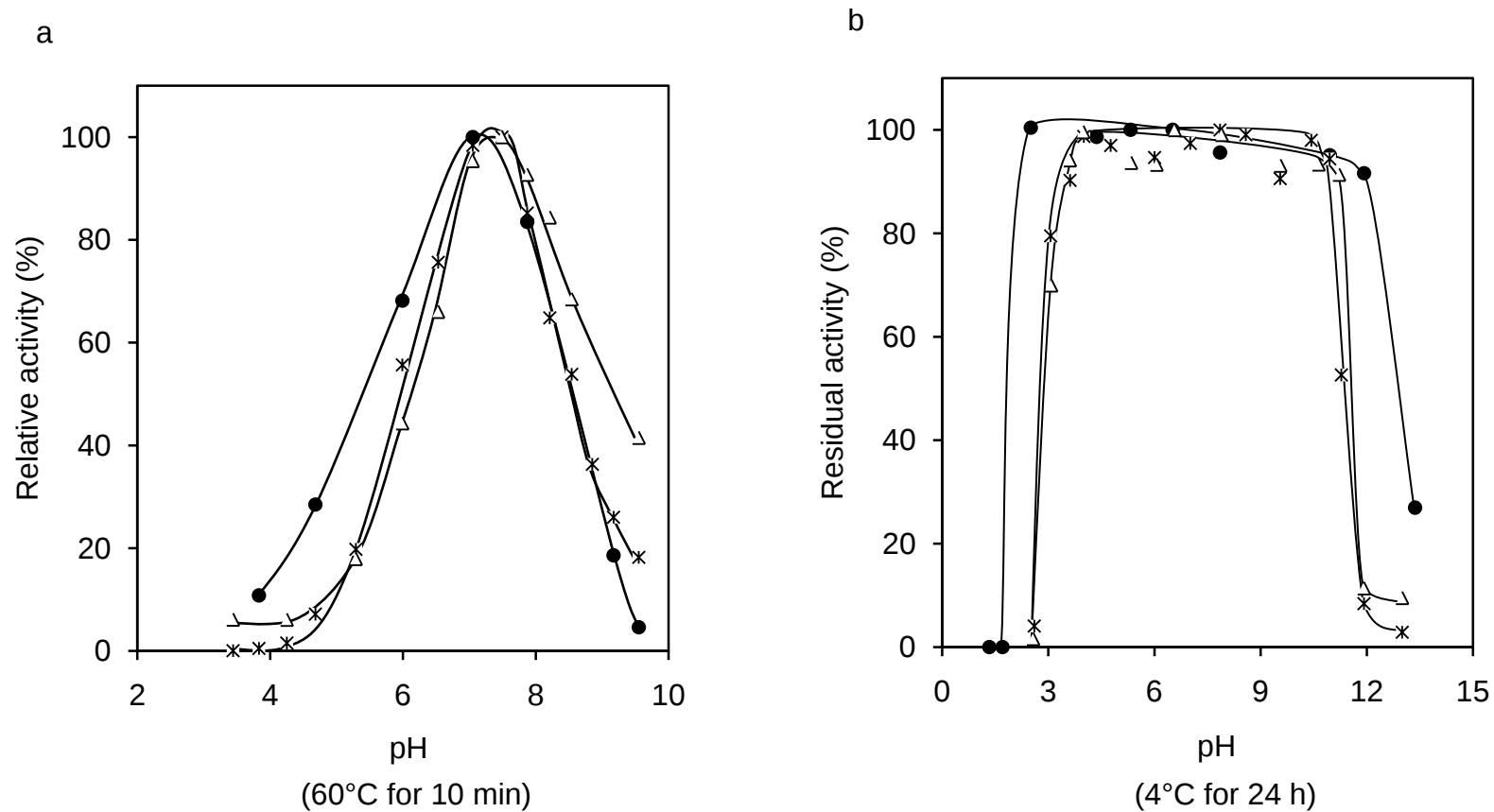


Fig. III.12. Effects of pH on enzyme activity and stability of RmCE variants.

a, Measurement of pH activity; b, pH stability. The epimerization activity of RmCE variants for Man₂ (10 mM) was measured for activity and stability at various pH values. Close circle, RmCE wild type; open triangle, W385A; asterisk, D188A.

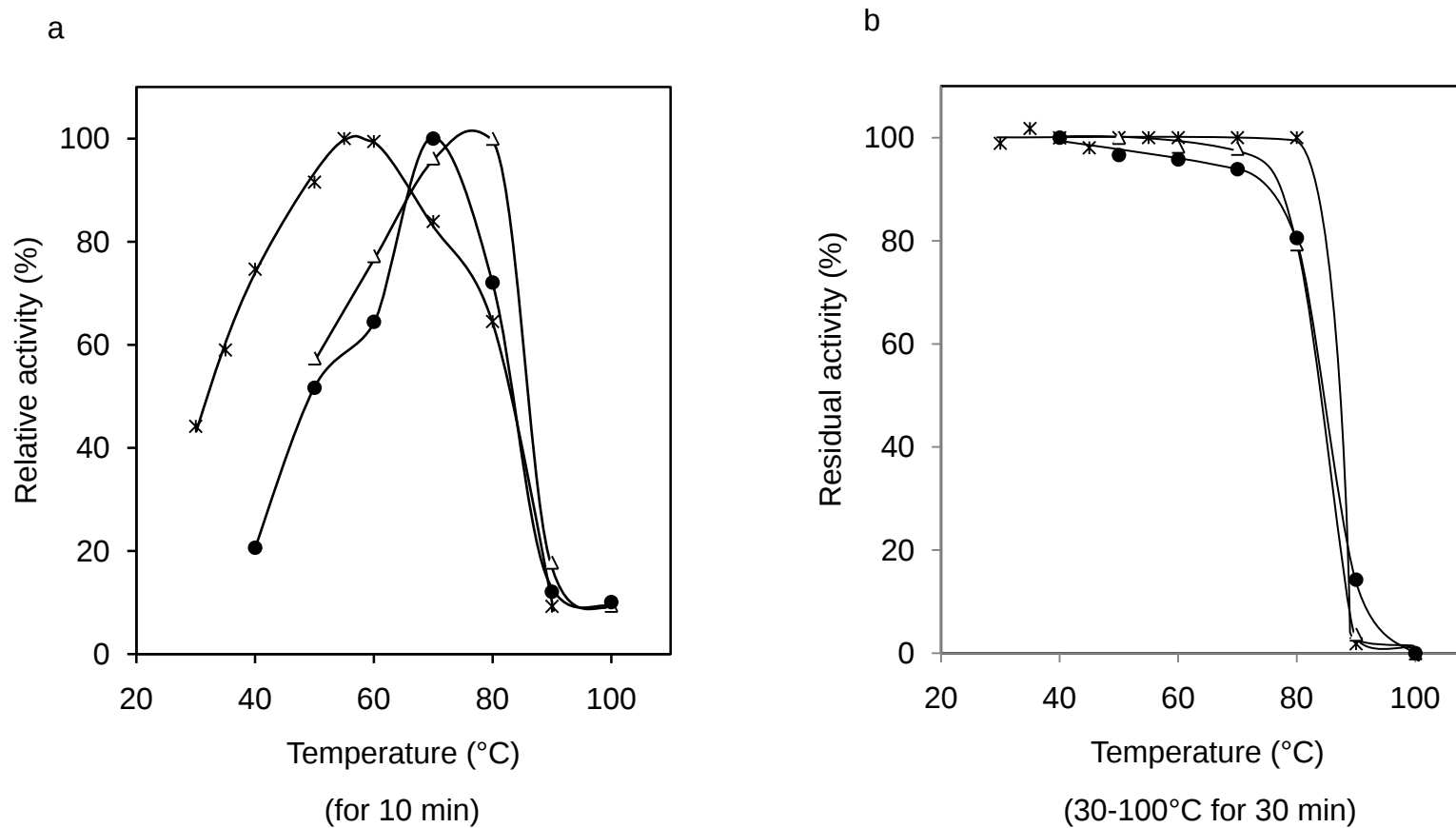


Fig. III.13. Effects of temperature on enzyme activity and stability of RmCE variants.

a, Optimum temperature; b, temperature stability. The epimerization activity of RmCE variants for Man₂ (10 mM) was measured for activity and stability at various temperature values. Close circle, RmCE wild type; open triangle, W385A; asterisk, D188A.

Chapter IV. Biochemical characterization of a function-unknown protein *Marinomonas mediterranea* Marme_2490 belonging to AGE superfamily

IV-1. Introduction

AGE superfamily, in detail as mention in Chapter I, includes AGE, AKI, CE, and MI. In addition to these known enzymes, there are many unknown proteins exhibiting low sequence identity with any known AGE superfamily members. A new type of carbohydrate epimerase/isomerase is expected to be found from such unknown proteins. A putative protein, Marme_2490 [National Center for Biotechnology Information (NCBI) accession number, ADZ91722] from marine bacterium, *Marinomonas meditteranea*, is one of such proteins. Its amino acid sequence is 12-27% identical to the known AGE superfamily enzymes. To characterize the biological function of Marme_2490, recombinant Marme_2490 was produced in *E. coli* and characterized in detail. In this chapter, its characteristics are shown and precisely compared with known enzymes.

IV-2. Materials and methods

IV-2-1. Preparation of the recombinant *M. meditteranea* Marme_2490 and *E. coli* AKI.

M. meditteranea Marme_2490 and *E. coli* *yihS* encoding AKI (EcAKI) were amplified by PCR as described in Chapter II with the primers including restriction sites (*Marme_2490*, *EcoRI* and *XhoI*; *EcAKI*, *NdeI* and *XhoI*, restriction enzyme sites underlined). The stop codon was removed and replaced with the sequence coding His6. The sequences of primers are as follows:

sense-EcoyihS_F: 5'-AATTCATATGAAATGGTTTAACACCCTAAG-3'

antisense-EcoyihS_R: 5'-ATTTCTCGAGTTTCGCATTAATATCCAGCA-3'

sense-Mm2490Ecos: 5'-AAAAAGAATTCATGTCTTACCCTGCATTTG-3'

antisense-Mm2490Xhoa: 5'-AAAAACTCGAGTAAGGTCAGTGTTTCAAT-3'

The PCR products were cloned into the *EcoRV* site of pBluescript II SK (+). The DNA fragment, obtained from the resultant plasmid by the double digestion using the restriction enzymes, was cloned into pET23a at the corresponding sites. The DNA sequence of the

inserted DNA including its franking region was determined using an Applied Biosystems 3130 Genetic Analyzer. *E. coli* BL21(DE3) transformant harboring the expression plasmid was cultured in 1 L of LB medium containing 50 µg/mL ampicillin at 37°C. The protein expression was induced by the addition of 0.1 mM IPTG when absorbance at 600 nm reached 0.6. The induction culture was carried out at 16°C for 20 h.

Bacterial cells were harvested by centrifugation at $8,400 \times g$ at 4°C for 10 min, and re-suspended in 40 mL of 20 mM sodium phosphate buffer (pH 7.0) containing 0.5 M NaCl. The cells were disrupted by sonication, and the sample was centrifuged at $13,000 \times g$ at 4°C for 10 min to obtain cell-free extract. The cell-free extract was applied onto a Ni-chelating Sepharose Fast Flow column (i.d. 3.0×10.5 cm, GE Healthcare) equilibrated with 20 mM sodium phosphate buffer (pH 7.0) containing 0.5 M NaCl. The column was washed with 20 mM sodium phosphate buffer (pH 7.0) containing 0.5 M NaCl and 10 mM imidazole. The adsorbed proteins were eluted by a linear gradient of imidazole from 10 mM to 0.5 M (total elution volume, 200 mL). The purified protein was collected, dialyzed against 10 mM sodium phosphate buffer (pH 7.0), and concentrated by ultrafiltration. The purity of the purified protein was investigated by SDS-PAGE, and stored at -20°C in the presence of 50% glycerol.

IV-2-2. Protein assays

The protein assay was carried out according to Chapter II-2-5. The protein concentrations of the cell-free extract and the column chromatography fractions were measured by the Bradford method and the UV method (absorbance at 280 nm), respectively. The concentration of purified enzyme was calculated from amino acid concentrations after complete acid hydrolysis, which were measured by the ninhydrin colorimetric method.

IV-2-3. TLC analysis of the reaction toward various substrates

The reaction mixture (25 µL) containing 40 mM sugar [D-glucose, D-mannose, D-allose, D-xylose, 1,5-anhydro-D-glucitol, methyl β-D-glucoside, methyl α-D-glucoside, D-tagatose, D-lyxose (Wako Pure Chemical Industries); 6-deoxy-D-glucose, cellobiose, L-rhamnose, D-talose, D-altrose, D-xylulose, D-mannose 6-phosphate (Sigma); maltose, N-acetyl-D-glucosamine, D-glucitol, D-fructose, D-mannitol, D-galactose (Nacalai Tesque); 2-deoxy-D-glucose (Tokyo Chemical Industry), lactose, sucrose, D-arabinose (Kanto

Chemical), epilactose (Nihon Shokuhin Kako), 4-*O*- β -D-galactosyl-D-fructose (Gal β 1-4Fru) (Acros Organics), Man₂, Man-Glc, Glc-Man], 40 mM Tris-HCl buffer (pH 7.4), and 0.4-4.0 μ M Marme_2490/2.8-8.4 μ M EcAKI was incubated at 30°C for Marme_2490 and 37°C for EcAKI for 10 min and 16 h. The reaction was terminated by heating at 100°C for 3 min. The sample (1 μ L) was spotted on silica gel plate, and developed twice using 2-propanol:1-butanol:water (2:2:1, v/v/v). The chromatogram was visualized by spraying a detection reagent [acetic acid/sulfuric acid/anisaldehyde (100:2:1, v/v/v)] and heating the plate.

IV-2-4. Enzyme assay

To determine isomerization activity to D-mannose, the reaction mixture (50 μ L) containing an appropriate concentration of enzyme, 10 mM D-mannose, 40 mM Tris-HCl buffer (pH 7.4), and 0.02 mg/mL BSA was incubated at 30°C for 10 min. The reaction was stopped by heating at 100°C for 3 min. The concentration of D-fructose was determined with D-glucose/D-fructose assay reagent. Authentic D-fructose (0-2.5 mM) containing 10 mM D-mannose was used as standard. One unit of enzyme activity was defined as the amount of enzyme that generates 1 μ mol of D-fructose per min under these conditions.

IV-2-5. Effects of pH and temperature on on activity and stability of Marme_2490

To determine the optimum pH, the reaction mixture (50 μ L) containing 20-40 nM Marme_2490, 10 mM D-mannose, 40 mM buffer (sodium acetate buffer, pH 3.2-6.1; MES-NaOH buffer, pH 5.9-6.9; Tris-HCl buffer, pH 6.5-9.5; Britton-Robinson buffer, pH 10.9-11.7), and 0.2 mg/mL BSA was incubated at 30°C for 10 min. To determine the optimum temperature, the reaction mixture (50 μ L) containing 40 nM Marme_2490, 10 mM D-mannose, 40 mM Tris-HCl buffer (pH 7.4), and 0.2 mg/mL BSA was incubated at 10-50°C for 10 min. The reaction was terminated by heating the sample at 100°C for 3 min. The concentration of D-fructose was measured as described above.

To determine pH stability, a mixture (50 μ L) containing 20 μ M Marme_2490 and 50 mM buffer described above (pH 3.2-11.7) was incubated at 4°C for 24 h. To determine temperature stability, a mixture (50 μ L), containing 20 μ M Marme_2490 and 10 mM Tris-HCl (pH 7.4) was incubated at 10-50°C for 30 min. After the pH and heat treatment, the residual activity to D-mannose was measured as described above.

IV-2-6. Kinetic parameters for monosaccharides

The kinetic parameters for the isomerization of D-mannose, D-talose, and D-lyxose, were determined from the initial reaction rates at various concentrations of the substrates. A reaction mixture (350 μ L) containing Marme_2490, 1-35 mM substrate, 40 mM Tris-HCl (pH 7.4), and 0.2 mg/mL BSA was incubated at 30°C. The enzyme concentrations were 4.0-10 nM, 746 nM, and 40 nM for D-mannose, D-talose, and D-lyxose, respectively. The reactions with D-mannose, D-talose, and D-lyxose were carried out for 15 min, 6 h, and 30 min, respectively. Fifty micro-liters of the reaction mixture were taken every 3 min, 1 h, and 5 min in the reaction with D-mannose, D-talose, and D-lyxose, respectively. The reaction was stopped by heating the sample at 100°C for 3 min. D-Fructose produced from D-mannose was measured as described above. D-Xylulose and D-tagatose, produced from D-lyxose and D-talose, respectively, were quantified according to the colorimetric quantification method of ketose⁵⁰. To the reaction mixture, 75 μ L of solution A [0.05% (w/v) resorcinol in absolute ethanol] and 75 μ L of solution B [0.216 g/L $\text{FeNH}_4(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$ in HCl] were added and incubated immediately at 80°C for 40 min. The sample was cooled down on ice, and absorbance at 480 and 640 nm of the resulting solutions were measured to determine D-xylulose and D-tagatose, respectively. As standard, 0-2.5 mM D-tagatose and 0-0.5 mM D-xylulose were used. The kinetic parameters were calculated by fitting the initial reaction velocity to the Michaelis-Menten equation.

IV-2-7. Purification of isomerization product from disaccharide substrates, Man₂, Glc-Man, and epilactose

A reaction mixture (1 mL) containing 4.20-7.91 μ M Marme_2490, 100 mM disaccharide substrates (Man₂, Glc-Man, and epilactose), and 40 mM Tris-HCl buffer (pH 7.4) was incubated at 30°C for 24 h. The products were purified by TLC. The sample was spotted on silica gel plate (sample 100 μ L per sheet), and developed as described above. Strips (1 cm) were cut off from the left and the right sides of the TLC plate, and carbohydrates were detected as described above. From the position of the reaction product, silica gel containing the product was scraped from remained TLC plate, suspended in 1 mL of distilled water, and centrifuged to remove the silica gel at 13,000 $\times$ g at 4°C for 5 min. The supernatant was dried *in vacuo* at 40°C. The dried product was dissolved in 1 mL of distilled water, and stored at -20°C. Chemical structures of the obtained products were analyzed by nuclear magnetic resonance (NMR) and Electrospray Ionization Mass

Spectrometry (ESI MS).

IV-2-8. Time course of conversion of D-mannose to D-fructose

A reaction mixture (375µL) containing 1.28 µM Marme_2490, 10 mM D-mannose, and 40 mM Tris-HCl (pH 7.4) was incubated at 30°C for 80 min. Twenty-five micro liters of the sample was taken at the indicated time, and the reaction was terminated at 100°C for 3 min. The concentration of D-fructose produced was measured as described above.

IV-3. Results and discussions

IV-3-1. Preparation of the recombinant Marme_2490 and EcAKI

The Marme_2490 and EcAKI genes were amplified by PCR in which genomic DNA from *M. meditteranea* and *E. coli* DH5α were used as template, respectively, and the stop codon of antisense primer was removed to attach the C-terminal His-tag to the protein (protein sequence consisting *Marme_2490/EcAKI* without stop codon, nucleotides encode six histidine residues, and stop codon, respectively). The PCR products of the amplified *Marme_2490* and *EcAKI* were 1,185 and 1,242 bp, respectively. These PCR fragments were cloned into pET23a vector and introduced into *E. coli* BL21(DE3) for the production of the recombinant proteins (Fig. IV.1).

IV-3-2. Protein purification

IV-3-2-1. Marme_2490

The *E. coli* BL21(DE3) transformant harboring the expression plasmid for Marme_2490 was grown in 1 L of LB medium supplemented with 100 µg/mL ampicillin at 37°C, and the production of the recombinant protein was induced by 0.1 mM IPTG at 16°C for 20 h. The recombinant Marme_2490 was successfully produced in the soluble fraction (Fig. IV.2). The cell-free extract was prepared by sonication and applied onto a Ni-chelating Sepharose Fast Flow column. Adsorbed protein was eluted by a linear gradient of imidazole from 10 mM to 0.5 M (total elution volume, 200 mL) (Fig. IV.3). The fractions 48-70 (150 mL) were all exhibited a single protein band (Fig. IV.4), pooled, and dialyzed against 10 mM sodium phosphate buffer (pH 7.0). The obtained protein (19.4 mg) showed

a single band on SDS-PAGE (Fig. IV.5).

IV-3-2-2. EcAKI

Recombinant EcAKI was expressed in *E. coli* BL21(DE3), and purified by Ni-chelating Sepharose Fast Flow column chromatography as Marme_2490. The cell-free extract from sonicated recombinant EcAKI cells (Fig. IV.6) (43 mL) was applied to a column, and adsorbed protein was eluted as recombinant Marme_2490 (Fig. IV.7). The fractions 85-135 with high purity of EcAKI were collected (250 mL) (Fig. IV.8), and dialyzed against 10 mM sodium phosphate buffer (pH 7.0). The yield of purified protein, showing a single band on SDS-PAGE (Fig. IV.9), was 24.0 mg.

IV-3-3. Screening of substrate of Marme_2490 and comparison of substrate specificities between Marme_2490 and EcAKI

Activity of Marme_2490 was investigated with 30 kinds of sugars. In 10-min reaction, 0.4 μ M Marme_2490 reversibly converted D-mannose and D-lyxose to D-fructose and D-xylulose, respectively (Fig. IV.10). Reaction products from D-talose, Man₂, Glc-Man, and epilactose were detected in the long-term reaction (16 h) using high concentration of enzyme (2.9-4.0 μ M) (Fig. IV.10). D-Talose was reversibly converted to D-tagatose, which was judged from the TLC experiment. The reaction products from the disaccharide substrates, Man₂, Glc-Man, and epilactose, were determined to be Man β 1-4Fru (Fig. IV.11.a), Glc β 1-4Fru (Fig. IV.11.b&Table IV.1), and Gal β 1-4Fru (Fig. IV.11.c&Table IV.1) by NMR and MS analyses, respectively. No product was detected by TLC from the reaction in the presence of Gal β -1,4Fru as a substrate indicating that the velocity of Marme_2490 to Gal β -1,4Fru was lower than Gal β -1,4Man (data not shown) corresponding to the result on TLC of D-fructose reaction which showed lower amount of converted product, D-mannose than the conversion of D-mannose to D-fructose (Fig. IV.10). Marme_2490 is registered as a putative AGE in database, but the recombinant Marme_2490 did not show any activity to *N*-acetyl-D-glucosamine. No activity was detected in the reaction with D-glucose and D-xylose which are the C-2 epimers of D-mannose and D-lyxose, respectively. Furthermore, no product was detected from the reaction in the presence of D-allose, D-galactose, methyl β -D-glucoside, methyl α -D-glucoside, 2-deoxy-D-glucose, 6-deoxy-D-glucose, 1,5-anhydro-D-glucitol, L-rhamnose, D-altrose (C-3 epimer of D-mannose), D-arabinose (C-3 epimer of D-lyxose), sugar alcohols

(D-glucitol, D-mannitol), sugar phosphate (D-mannose 6-phosphate), and disaccharides (cellobiose, maltose, lactose, sucrose, Man-Glc) by TLC under the same reaction conditions (long-term reaction).

The substrate specificities of EcAKI for various substrates were investigated. EcAKI acted on D-mannose, D-fructose, D-glucose, D-lyxose, and D-xylulose (Fig. IV.12) corresponding to reference results²⁸. As the substrate specificity of Marme_2490 was similar to that of EcAKI, the substrate specificity of EcAKI was compared with Marme_2490. In contrast to EcAKI, Marme_2490 did not show activity to D-glucose at all, and thus axial 2-OH group in aldose substrates is essential for the Marme_2490. Marme_2490 had weak isomerization activity towards D-talose and the disaccharide substrates, while EcAKI did not act on those substrate (Fig. IV.12), indicating that Marme_2490 has higher selectivity for OH-group at the C4 position of monosaccharide substrates, and disaccharide substrates than EcAKI. Although any β -(1 $\rightarrow$ 4)-linked disaccharides tested are acceptable for Marme_2490, but an α -(1 $\rightarrow$ 4)-linked disaccharide substrate, maltose, did not serve as substrate.

IV-3-4. Effects of temperature and pH on activity and stability

The maximal activity of Marme_2490 to 10 mM D-mannose was obtained at 30°C in 10 min reaction, and the enzyme was stable at 35°C and lower (at pH 7.0 for 30 min). The activity was highest at pH 7.3, and the enzyme was stable in a range of pH 5.5-9.4 (4°C for 24 h) (Fig. IV.13).

IV-3-5. Kinetic parameters for monosaccharide substrates

The kinetic parameters of Marme_2490 for the monosaccharide substrates, D-mannose, D-lyxose, and D-talose, were examined (Table IV.2). The k_{cat} value for D-mannose, 329 s⁻¹, was higher than those for D-lyxose and D-talose by 5- and 1,000-fold, respectively. The K_m value for D-mannose, 16.7 mM, was lower than those for D-lyxose and D-talose by 2.5- and 1.6-fold, respectively. The k_{cat}/K_m value of Marme_2490 for D-mannose, 19.7 s⁻¹mM⁻¹, was 13- and 1,700-fold higher than D-lyxose and D-talose, respectively. D-Mannose was the best substrate for this enzyme among the sugars tested, and thus, Marme_2490 is a kind of D-mannose isomerase.

The K_m value of Marme_2490 for D-mannose was approximately 6.5-, 8.0-, and 6.9-fold lower than those of EcAKI and *S. enterica* AKI (SeAKI) and MI from

Thermobifida fusca (TfMI), respectively. The k_{cat} value of Marme_2490 for D-mannose was 2.4-fold lower than TfMI, but 13- and 14-fold higher than those of EcAKI and SeAKI, respectively. The $k_{\text{cat}}/K_{\text{m}}$ value of Marme_2490 for D-mannose was 2.9-113-fold higher than TfMI, EcAKI, and SeAKI. The k_{cat} and K_{m} values of Marme_2490 for D-lyxose was 4.7-fold higher and 9.6-fold lower than those of EcAKI, respectively. The k_{cat} values of Marme_2490 and TfMI for D-lyxose were not significantly different, while K_{m} value of Marme_2490 for D-lyxose was 13-fold lower than TfMI. The $k_{\text{cat}}/K_{\text{m}}$ value of Marme_2490 for D-lyxose was 13- and 46-fold higher than those of EcAKI and TfMI, respectively. These results indicated that Marme_2490 has higher affinity toward D-mannose and D-lyxose than the known enzymes catalyzing isomerization of D-mannose and D-lyxose. MIs from *P. saccharophila* and *T. fusca* act on D-rhamnose, D-galactose, D-arabinose, D-xylose, D-glucose, and *N*-acetyl glucosamine^{28, 51}, but epimerization activity to these substrates was not detected in Marme_2490 in the assay (IV-3-3).

The amino acid sequence of several MIs²⁸ from *Enterobacter cloacae*, *Salinispora arenicola*, *Pseudomonas syringae*, and *T. fusca* are 40-88% identical to EcAKI¹. However, the amino acid sequence of Marme_2490 is approximately 20% identical with those of MIs and AKIs. Thus, the low sequence identity of Marme_2490 with the known MIs and AKIs might lead the difference of substrate specificity.

IV-3-6. Time course of conversion of D-mannose to D-fructose

The isomerization of D-mannose catalyzed by of Marme_2490 was monitored at 30°C (Fig. IV.14). Approximately 70% of D-mannose was converted to D-fructose at the late stage of the reaction. This result is consistent with the previously reported equilibrium ratio D-fructose:D-mannose = 35:65 – 25:75, the equilibration of MIs from *Xanthromonas rubrilineans*⁵², *X. pruni*⁵², *P. cepacia*⁵³, *T. fusca*²⁸, and *Mycobacterium smegmatis*⁵⁴.

IV-3-7. The comparison of amino acid residues forming the active site between known AGE superfamily enzymes and Marme_2490

Marme_2490 has two conserved catalytic His residues acting as general acid/base catalysts, His251 and His372, corresponding to His248 and His383 of EcAKI²⁸, His248 and His382 in AGE from *Anabaena* sp.²⁷, and His259 and His390 in RmCE²⁵, respectively. Glu254 and Arg57 of Marme_2490 correspond to the Glu and Arg forming hydrogen bonds with substrate in the other AGE superfamily members. W312 of Marme_2490

corresponds to Trp304, Trp316, and Trp322 of AGE, EcAKI and RmCE^{25, 27, 28}, respectively, which have stacking interaction with the substrate (reducing end part of substrate in RmCE) and are completely conserved in AGE superfamily. Asn174, Tyr113, and His178 of Marme_2490 correspond to Asn196, Tyr124, and His200 of RmCE²⁵, forming hydrogen bonds with 2-OH of reducing-end sugar residue. In EcAKI, 4-OH group of D-mannose is predicted to form hydrogen bond with main-chain carbonyl of Arg238²⁸. EcAKI does not act on 4-epimer of D-mannose, suggesting a possibly that this hydrogen bonding interaction is essential for enzyme activity of EcAKI. As Marme_2490 has low selectivity for 4-epimer of D-mannose, recognition of 4-OH group of substrate in Marme_2490 is probably less strict than in EcAKI. Consistent with this prediction, Marme_2490 has Leu241 at the corresponding position of Arg238 of EcAKI (Fig. IV.15). Marme_2490 does not have any amino acid residues corresponding to those of CEs important for binding to non-reducing end part of disaccharides substrates as discussed in Chapter III, thus Marme_2490 might have weak isomerization activity to disaccharides substrates.

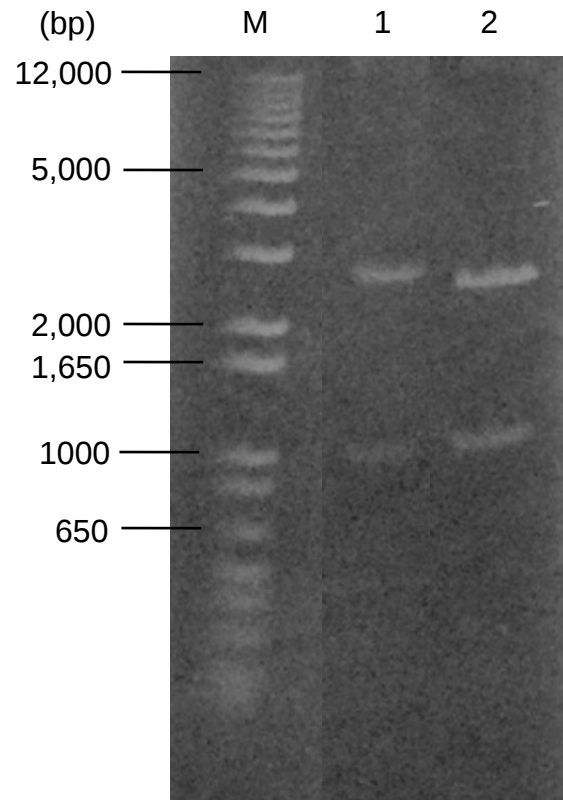


Fig. IV.1. Electrophoretic analysis of expression plasmid of *Marme_2490* and *EcAKI*.

M, 1 kb Plus DNA ladder; 1, expression plasmid of *Marme_2490* digested by *EcoRI* and *XhoI*; 2, expression plasmid of *EcAKI* digested by *NdeI* and *XhoI*.

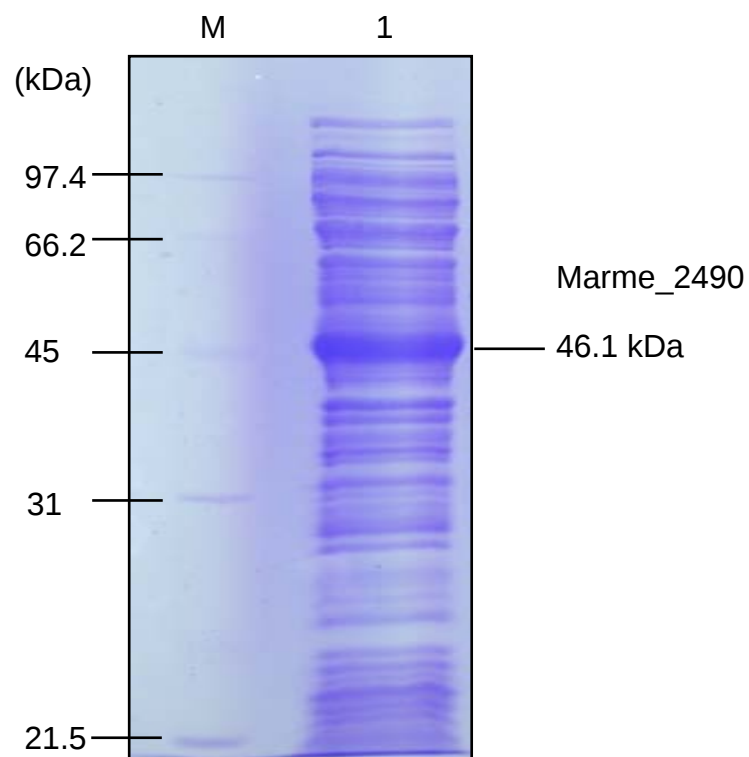


Fig. IV.2. SDS-PAGE analysis of cell-free extract of *E. coli* transformant producing recombinant Marme_2490.

SDS-PAGE was performed with 10% polyacrylamide gel, and protein was stained with the Coomessie Brilliant Blue. M, size marker; 1, cell-free extract.

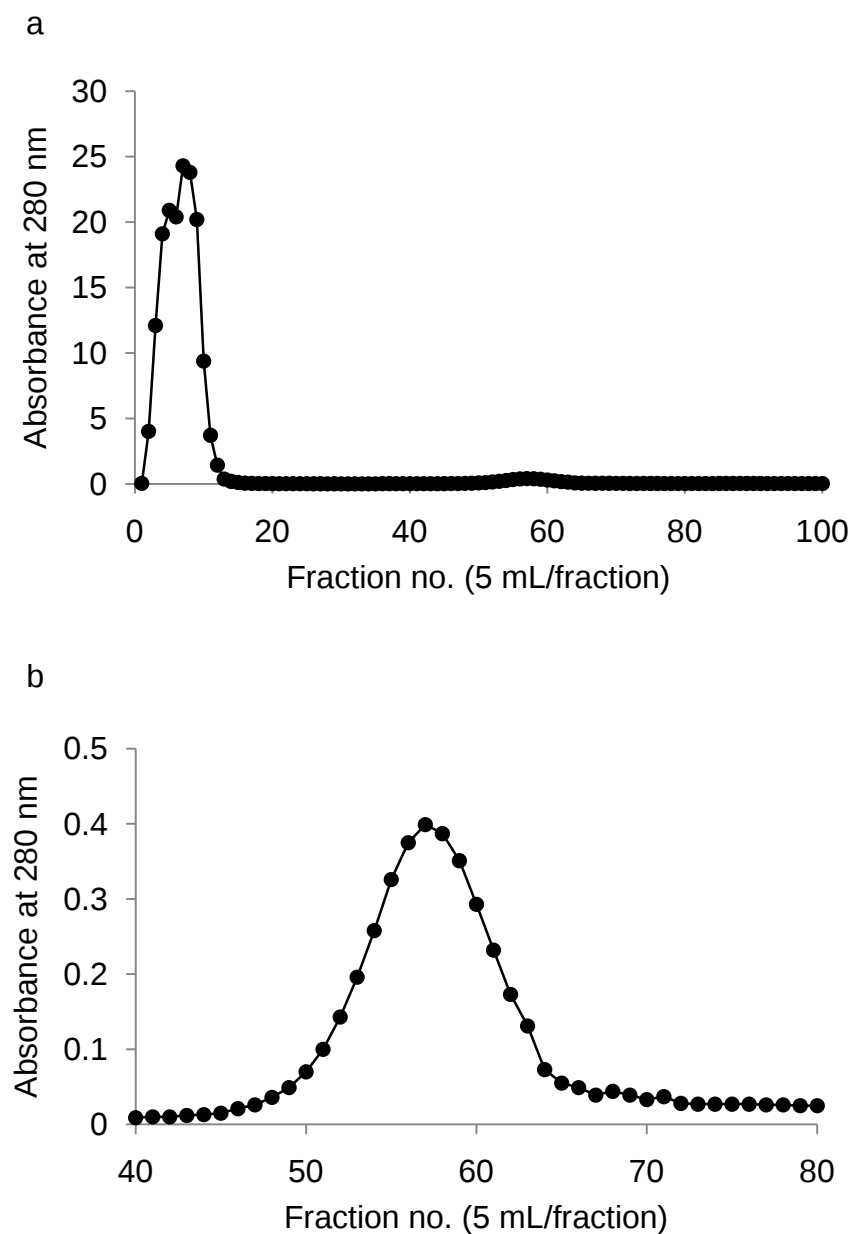


Fig. IV.3. Ni-chelating Sepharose Fast Flow column chromatography of Marme_2490.

Absorbance at 280 nm (-●-). Ni-chelating Sepharose Fast Flow column (i.d. 3.0×10.5 cm) was equilibrated with 20 mM sodium phosphate buffer (pH 7.0) containing 10 mM imidazole. The elution was done with a linear gradient of imidazole from 10 mM to 0.5 M, and the fractions 48-70 were pooled (150 mL). a, absorbance at 280 nm from fraction 1-100; b, close up view of elution fractions of Marme_2490.

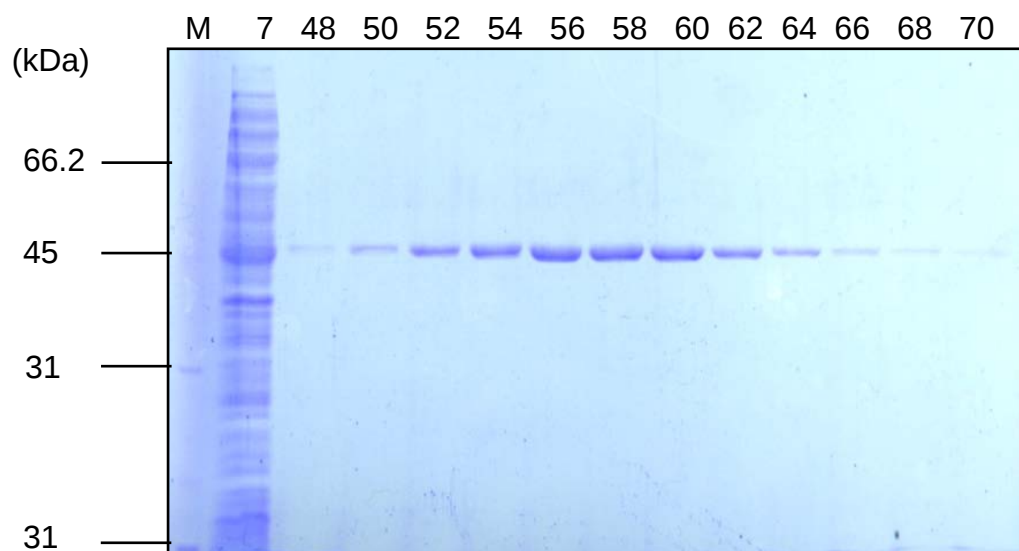


Fig. IV.4. SDS-PAGE analysis of adsorbed proteins containing Marme_2490 in Ni-chelating Sepharose Fast Flow column chromatography.

Adsorbed protein was eluted with a linear gradient of 10 mM-0.5 M imidazole. Fractions containing Marme_2490 were investigated by SDS-PAGE; the fractions 48-70 were pooled (150 mL), and dialyzed against 10 mM sodium phosphate buffer (pH 7.0). SDS-PAGE was performed with 10% polyacrylamide gel, and protein was stained with the Coomessie Brilliant Blue. M, size marker; 7-70, fraction number (10 μ L was loaded) (fraction 7 is non-adsorbed protein; 48-70 are adsorbed protein).

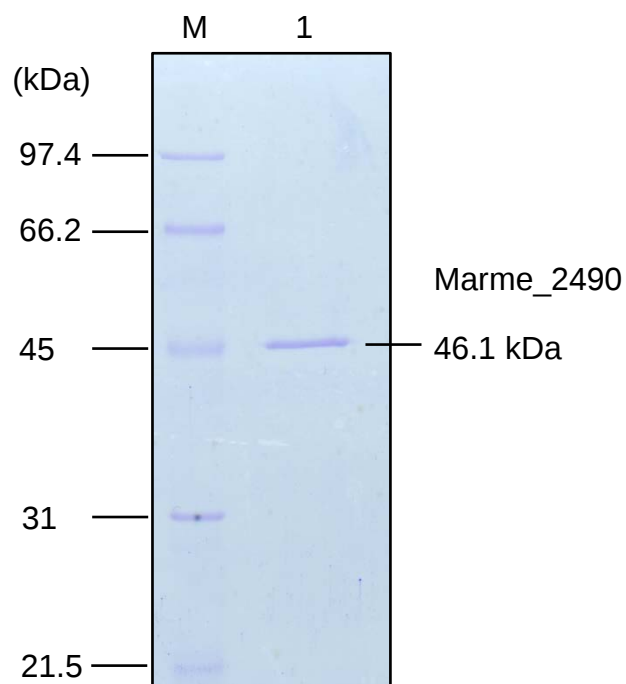


Fig. IV.5. SDS-PAGE analysis of purified Marme_2490.

M, size marker; 1, purified Marme_2490 (0.5 μ g). Molecular masses of standard proteins are indicated on the left lane. The protein was stained with Coomassie Brilliant Blue.

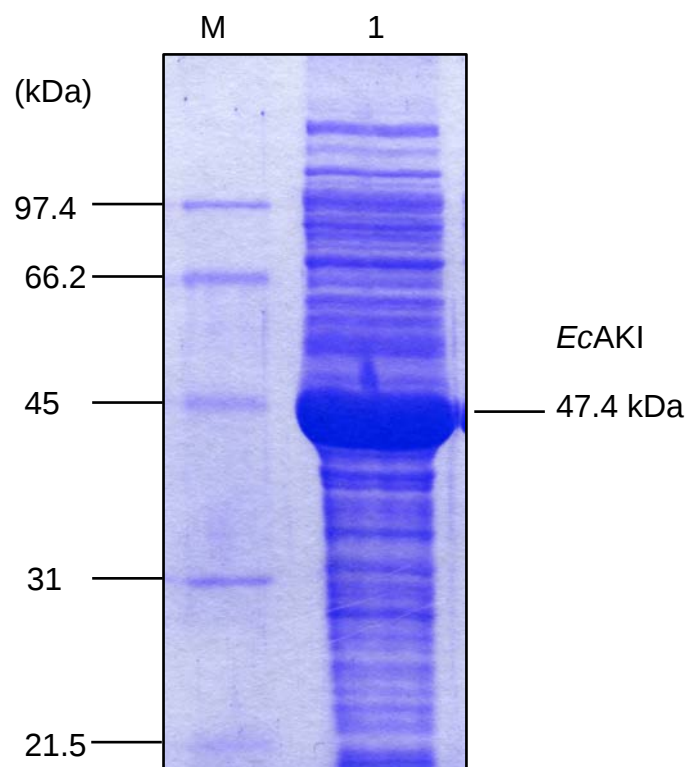


Fig. IV.6. SDS-PAGE analysis of cell-free extract of *E. coli* transformant producing recombinant EcAKI.

SDS-PAGE was performed with 10% polyacrylamide gel, and protein was stained with the Coomessie Brilliant Blue. M, size marker; 1, cell-free extract.

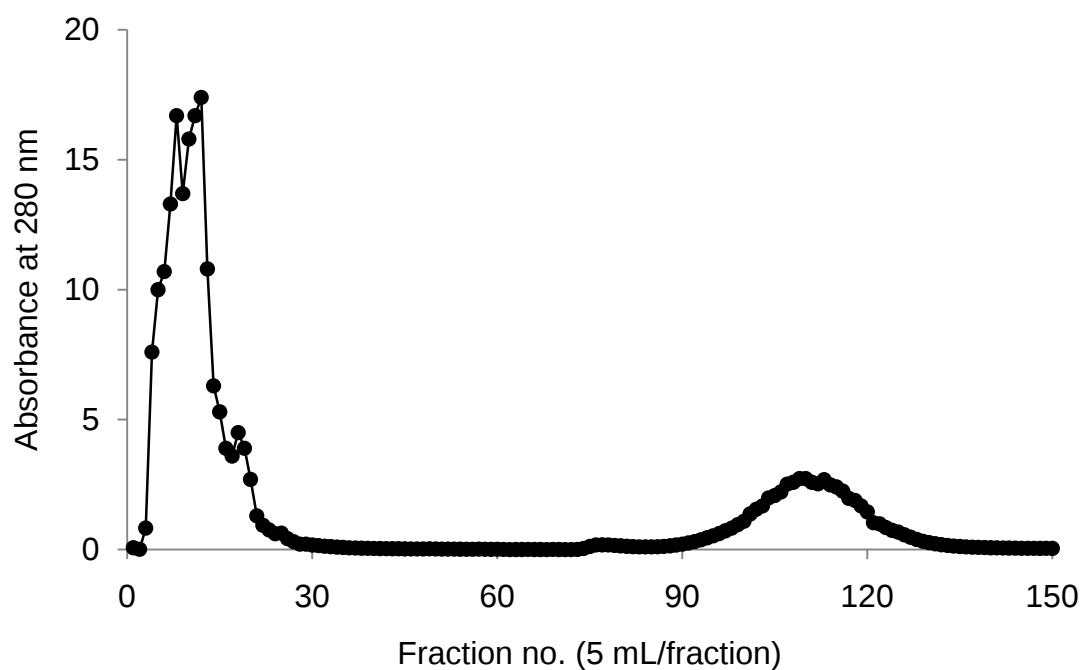


Fig. IV.7. Ni-chelating Sepharose Fast Flow column chromatography of EcAKI.

Absorbance at 280 nm (-●-). Ni-chelating Sepharose Fast Flow column (i.d. 3.0×10.5 cm) was equilibrated with 10 mM sodium phosphate buffer (pH 7.0) containing 0.5 M NaCl and 10 mM imidazole. The elution was done with a linear gradient of imidazole from 10 mM to 0.5 M, the fractions 85-135 were pooled (250 mL).

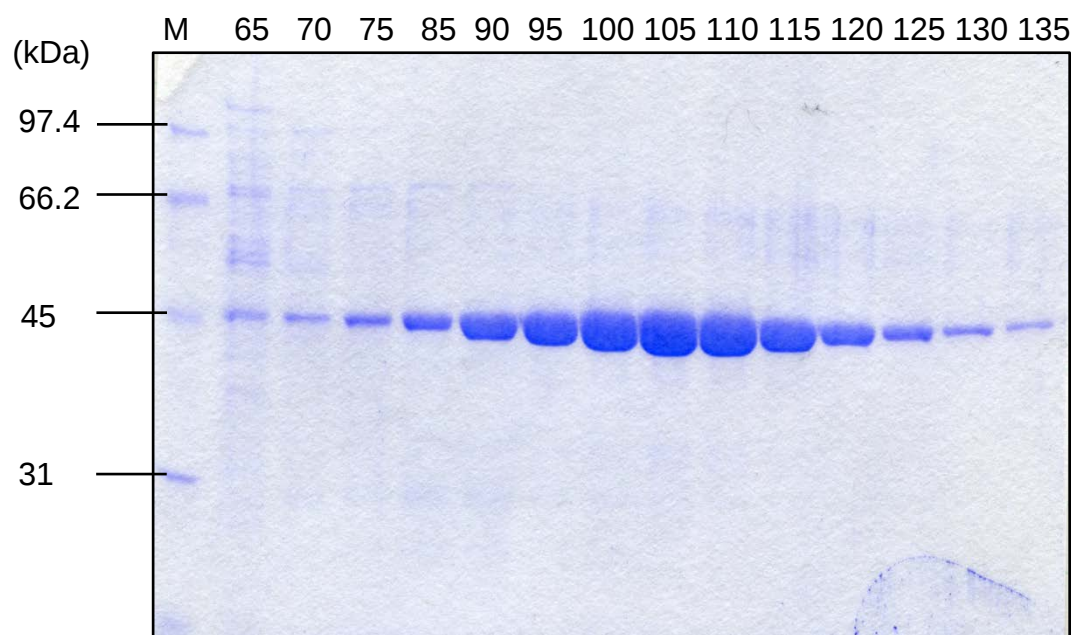


Fig. IV.8. SDS-PAGE analysis of adsorbed protein containing EcAKI in Ni-chelating Sepharose Fast Flow column chromatography.

Adsorbed protein was eluted by a linear gradient of 10 mM-0.5 M imidazole. Fractions containing Marme_2490 were investigated by SDS-PAGE, the fractions 85-135 were pooled (250 mL), and dialyzed against 10 mM sodium phosphate buffer (pH 7.0). SDS-PAGE was performed with 10% polyacrylamide gel, and protein was stained with the Coomessie Brilliant Blue. M, size marker; 65-135, fraction number (10 μ L was loaded).

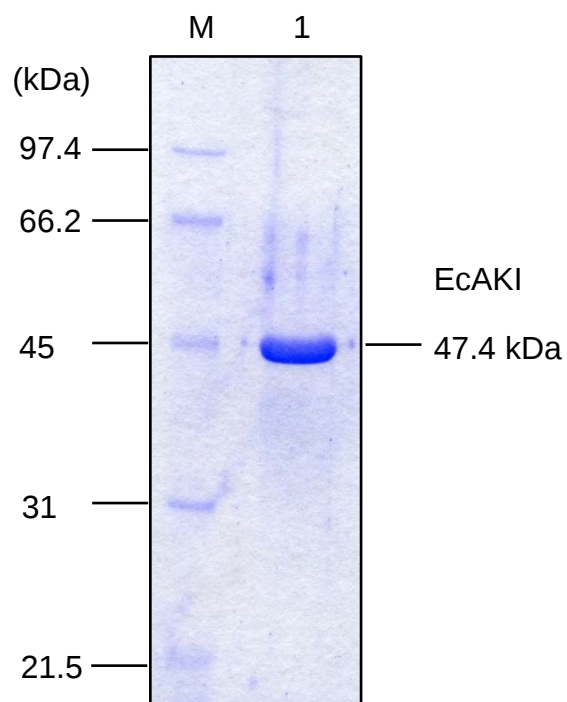


Fig. IV.9. SDS-PAGE analysis of purified EcAKI.

M, size marker; 1, purified EcAKI (1 μ g). Molecular masses of standard proteins are indicated on the left lane. The proteins were stained with Coomassie Brilliant Blue.

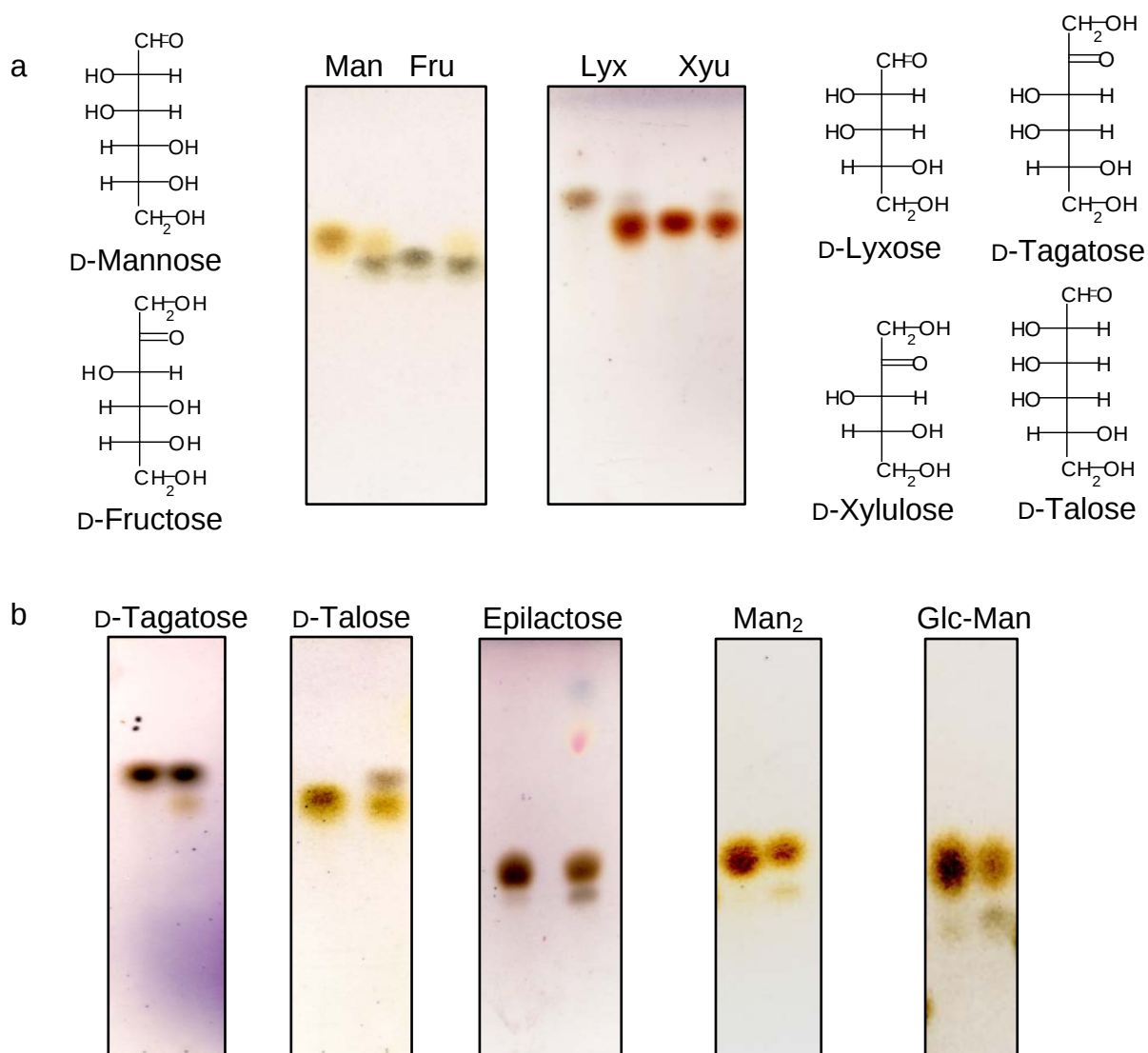


Fig. IV.10. TLC analysis of the reactions of Marme_2490 with various sugars.

The reaction products were detected on TLC from the reaction mixture of Marme_2490 with various sugars. a, D-Mannose (Man), D-fructose (Fru), D-lyxose (Lyx), and D-xylulose (Xyu) were converted to D-fructose, D-mannose, D-xylulose, and D-lyxose, respectively, in 10-min reaction. b, D-tagatose, D-talose, epilactose, Man₂, and Glc-Man served as substrates in 16-h reaction. Standard substrates were spotted on the left side, and samples after the reactions were spotted on the right side. The substrate in each reaction was written above the figure.

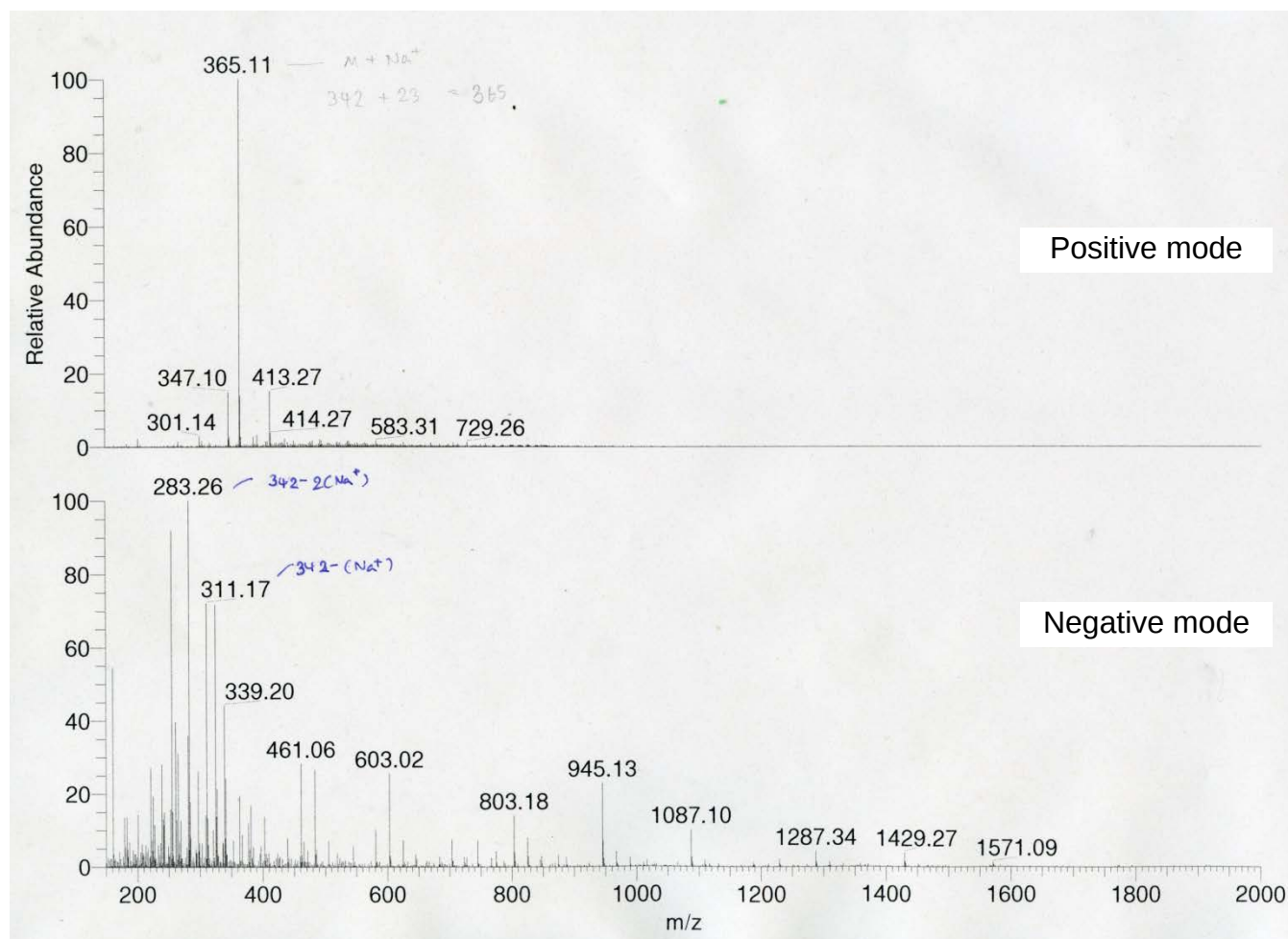


Fig. IV.11.a.1. ESI MS of Manβ1-4Fru.

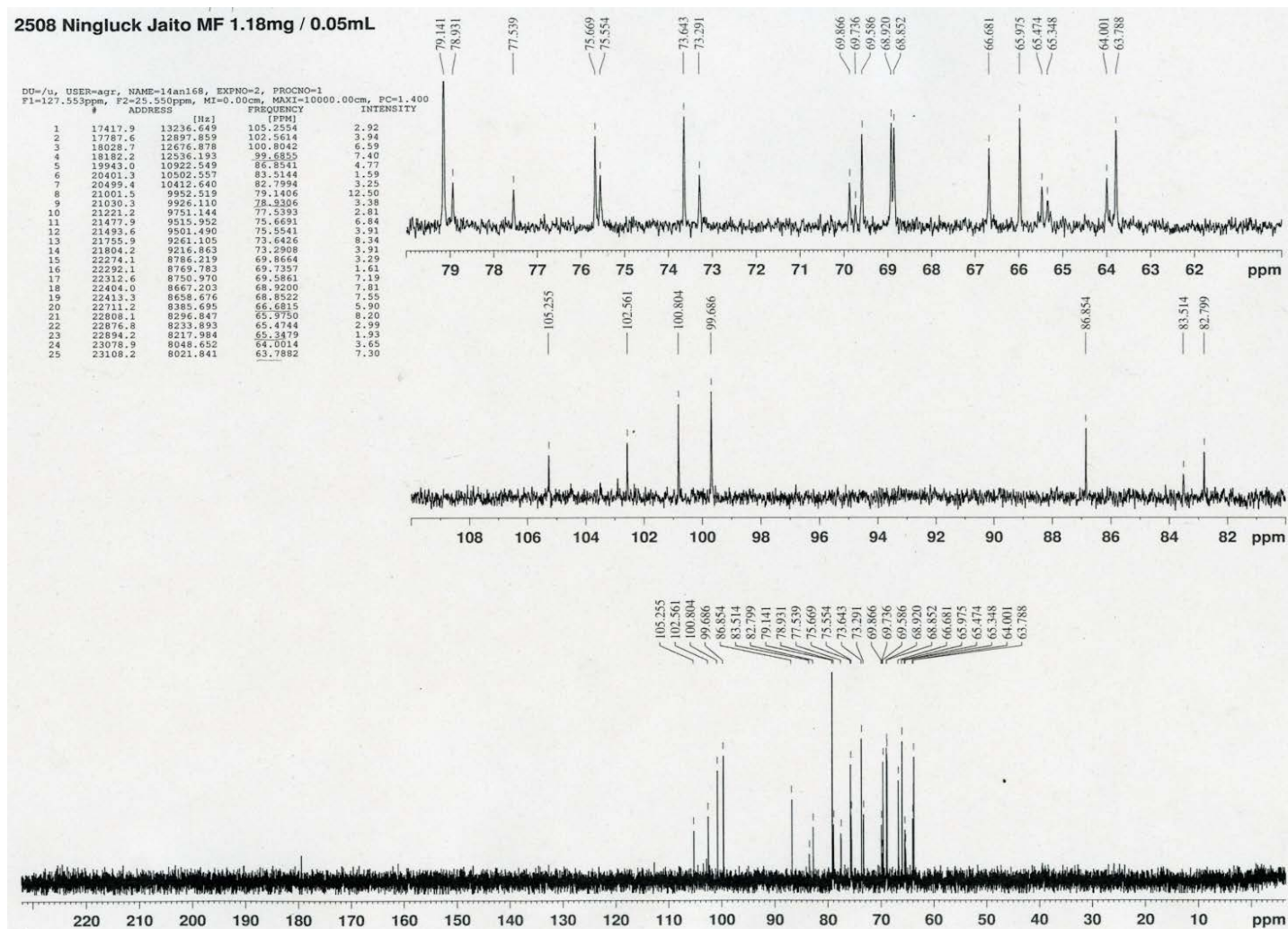


Fig. IV.11.a.2. NMR of Man β 1-4Fru.

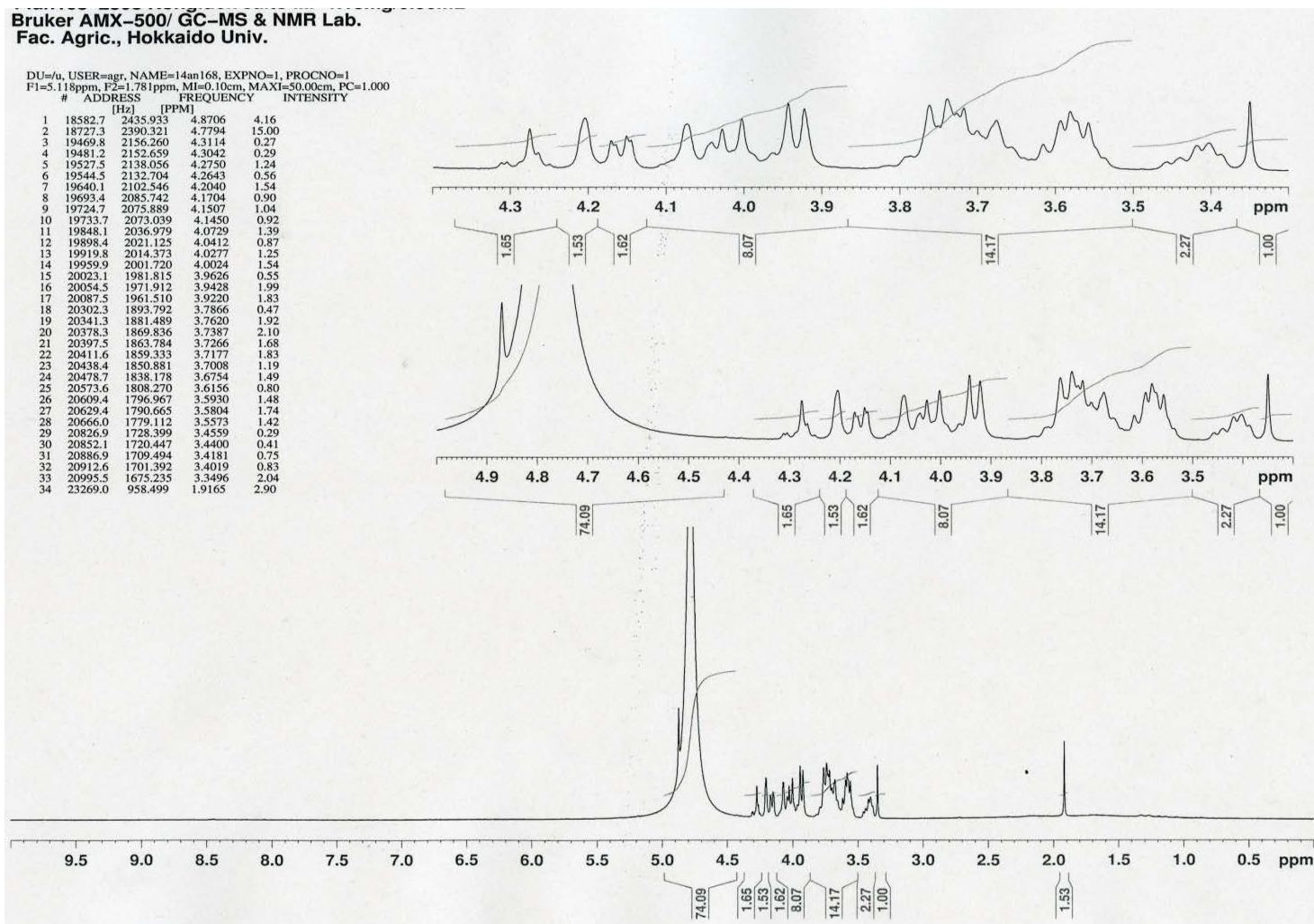


Fig. IV.11.a.3. NMR of Manβ1-4Fru.

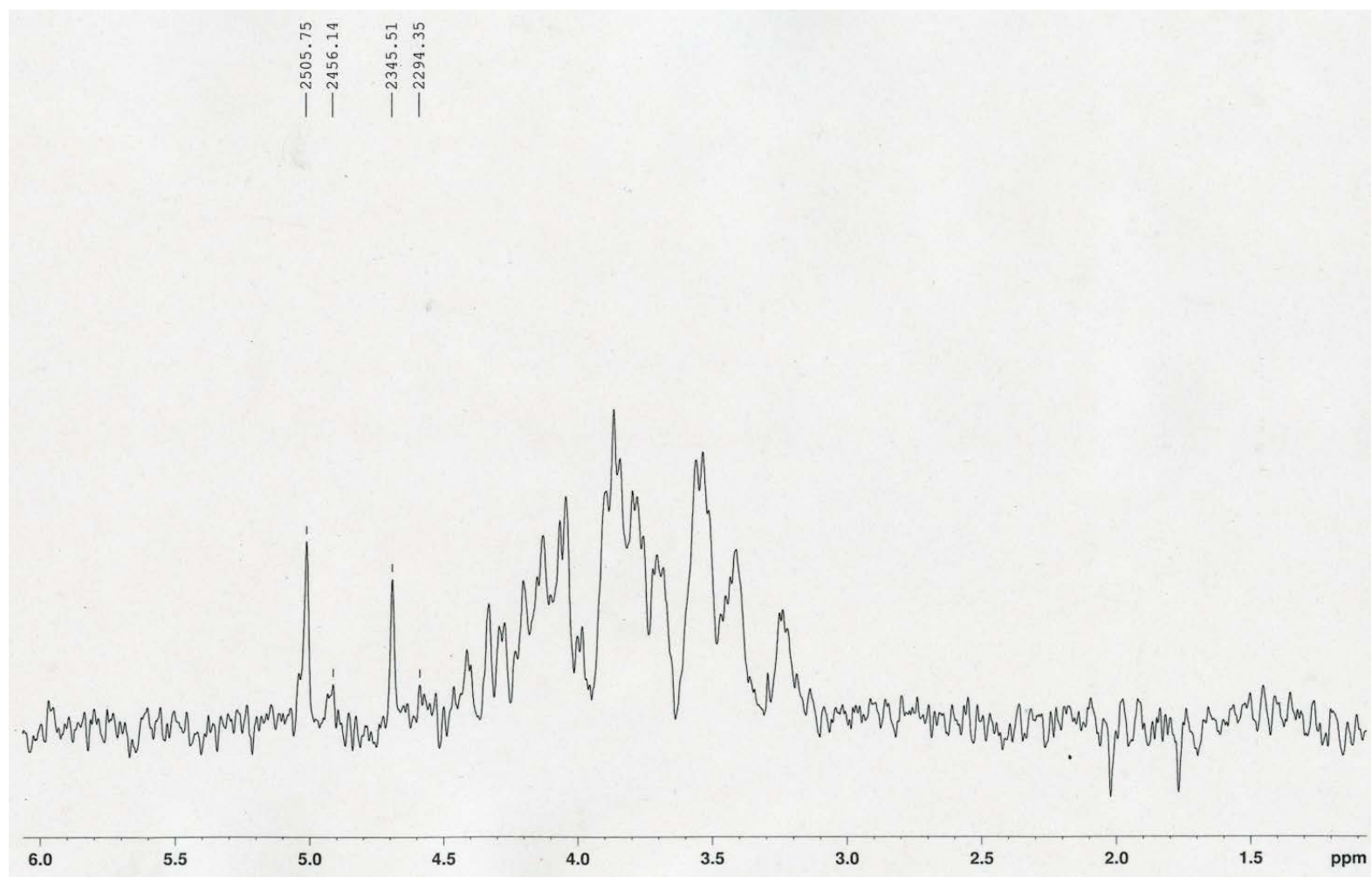


Fig. IV.11.a.4. NMR of Manβ1-4Fru.

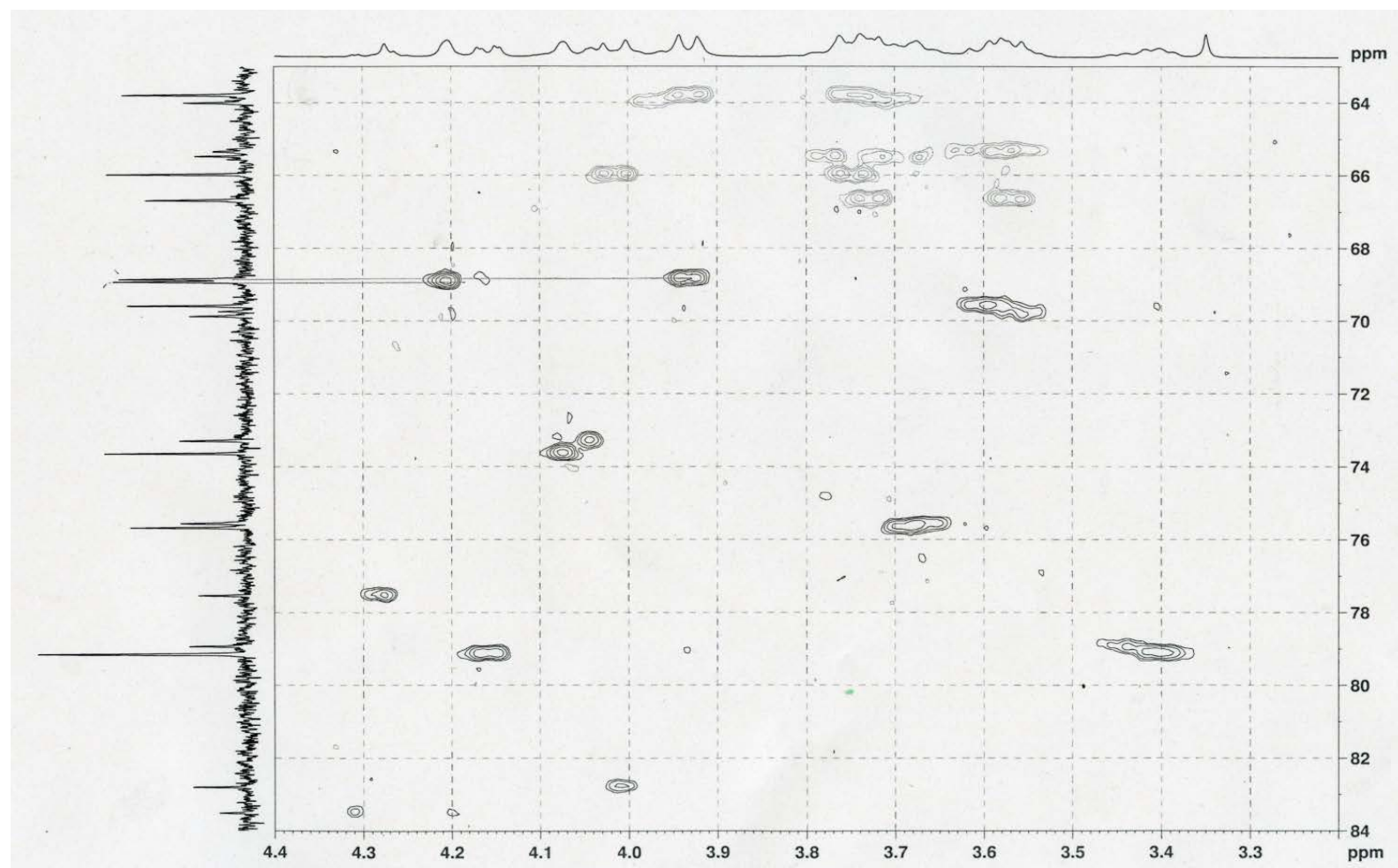


Fig. IV.11.a.5. Two dimensional NMR (HSQC) of Man β 1-4Fru.

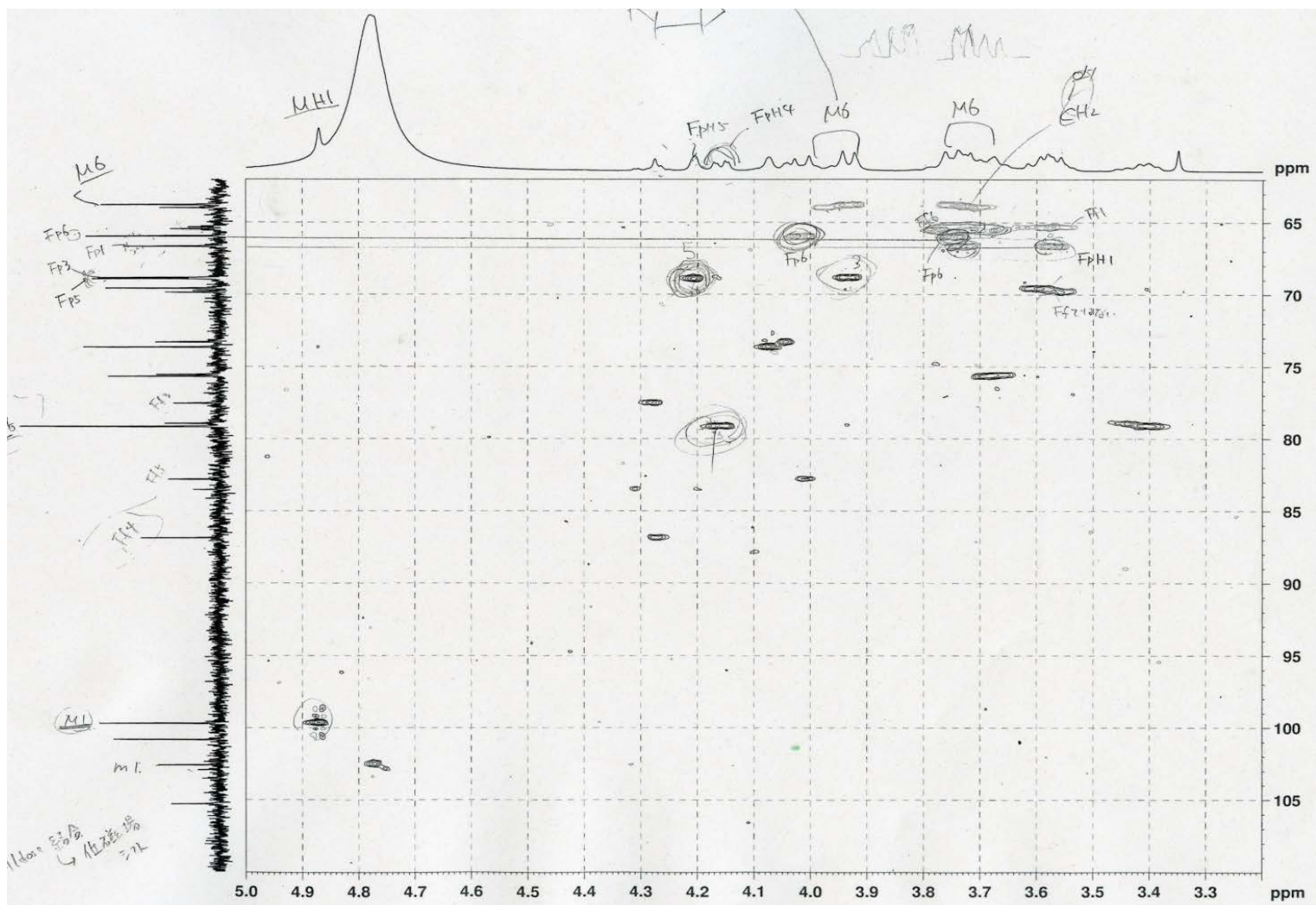


Fig. IV.11.a.6. Two dimensional NMR (HSQC) of Man β 1-4Fru.

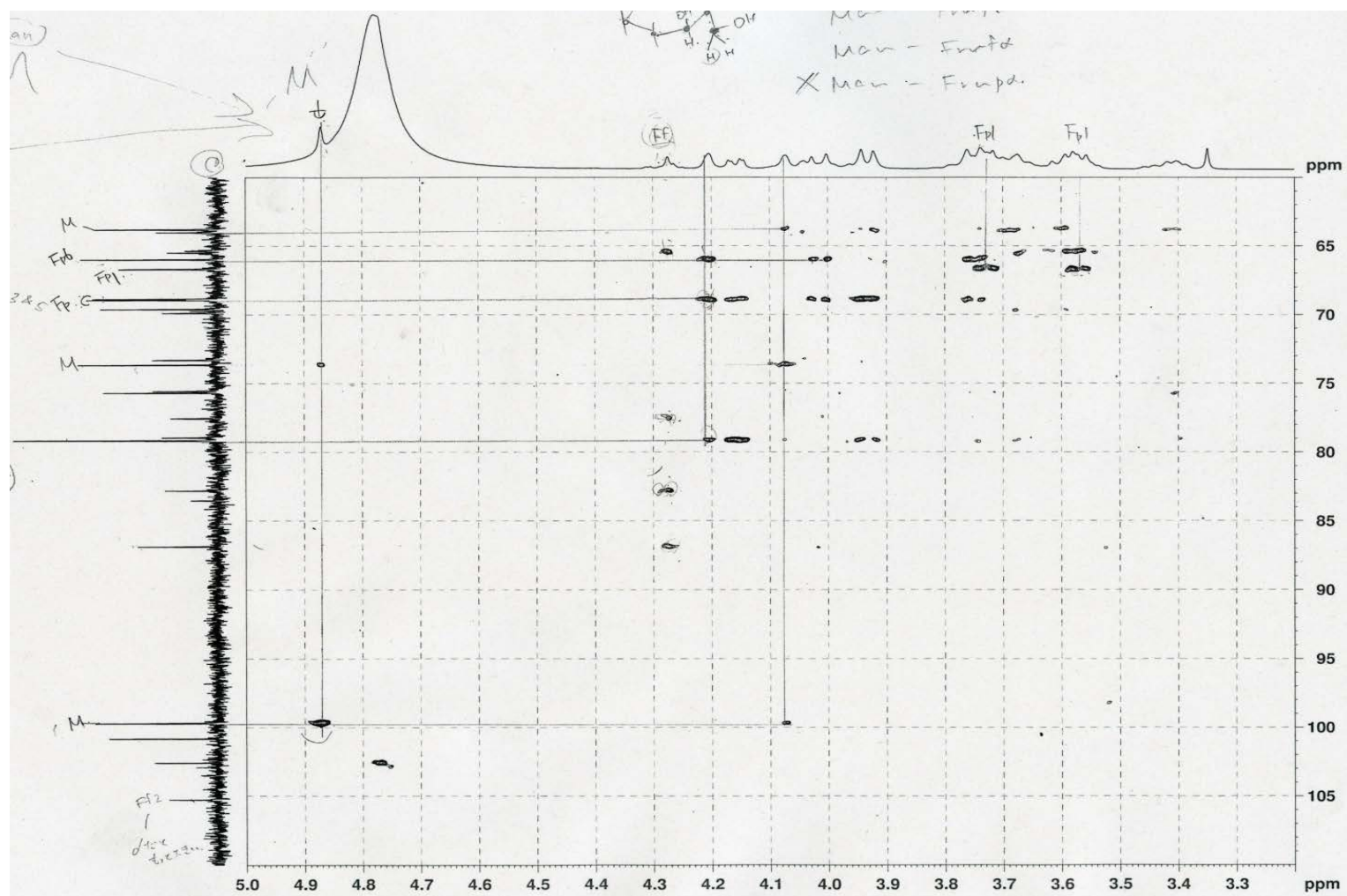


Fig. IV.11.a.7. Two dimensional NMR (HSQC) of Man β 1-4Fru.

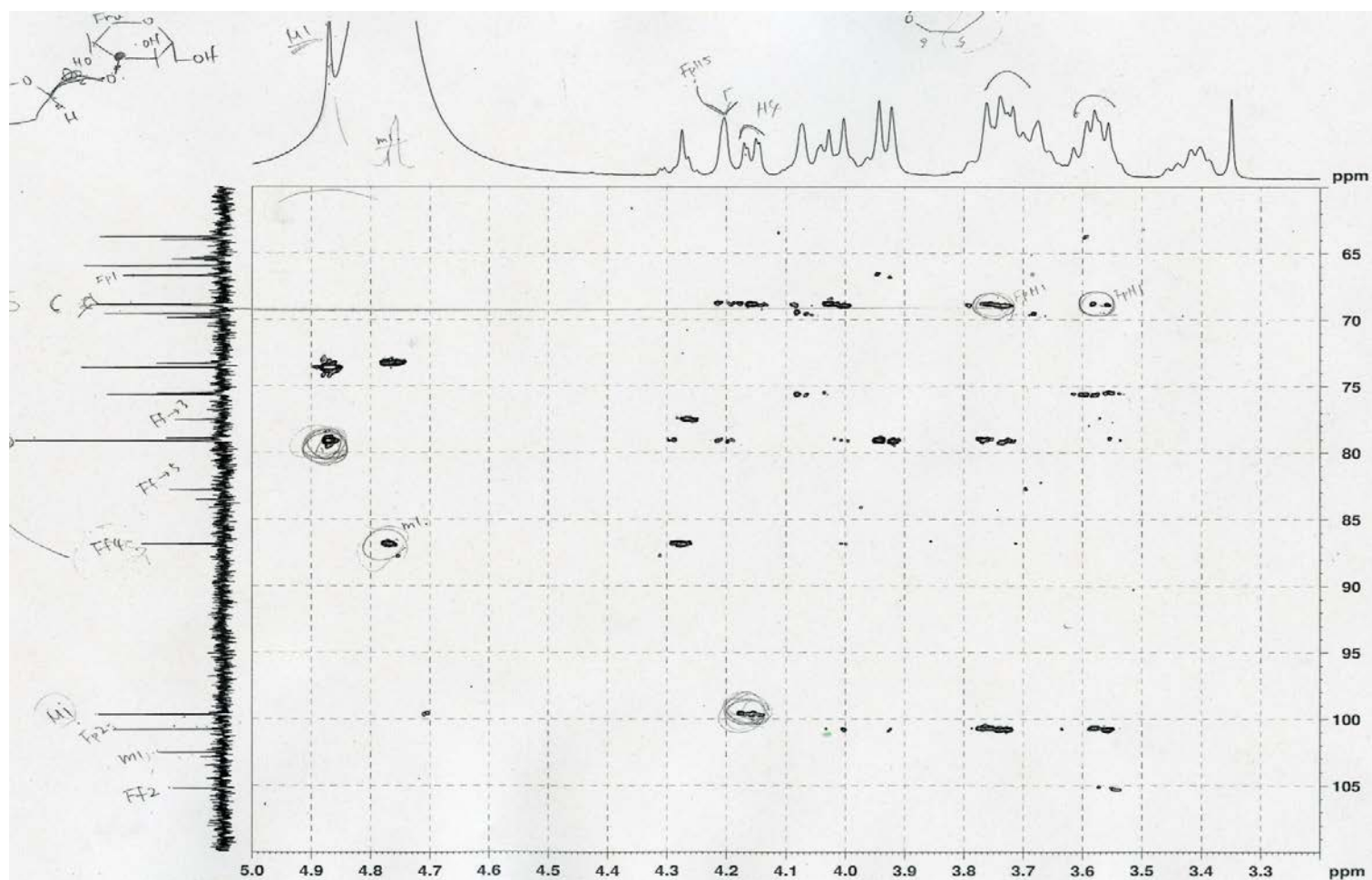


Fig. IV.11.a.8. Two dimensional NMR (HMBC) of Man β 1-4Fru.

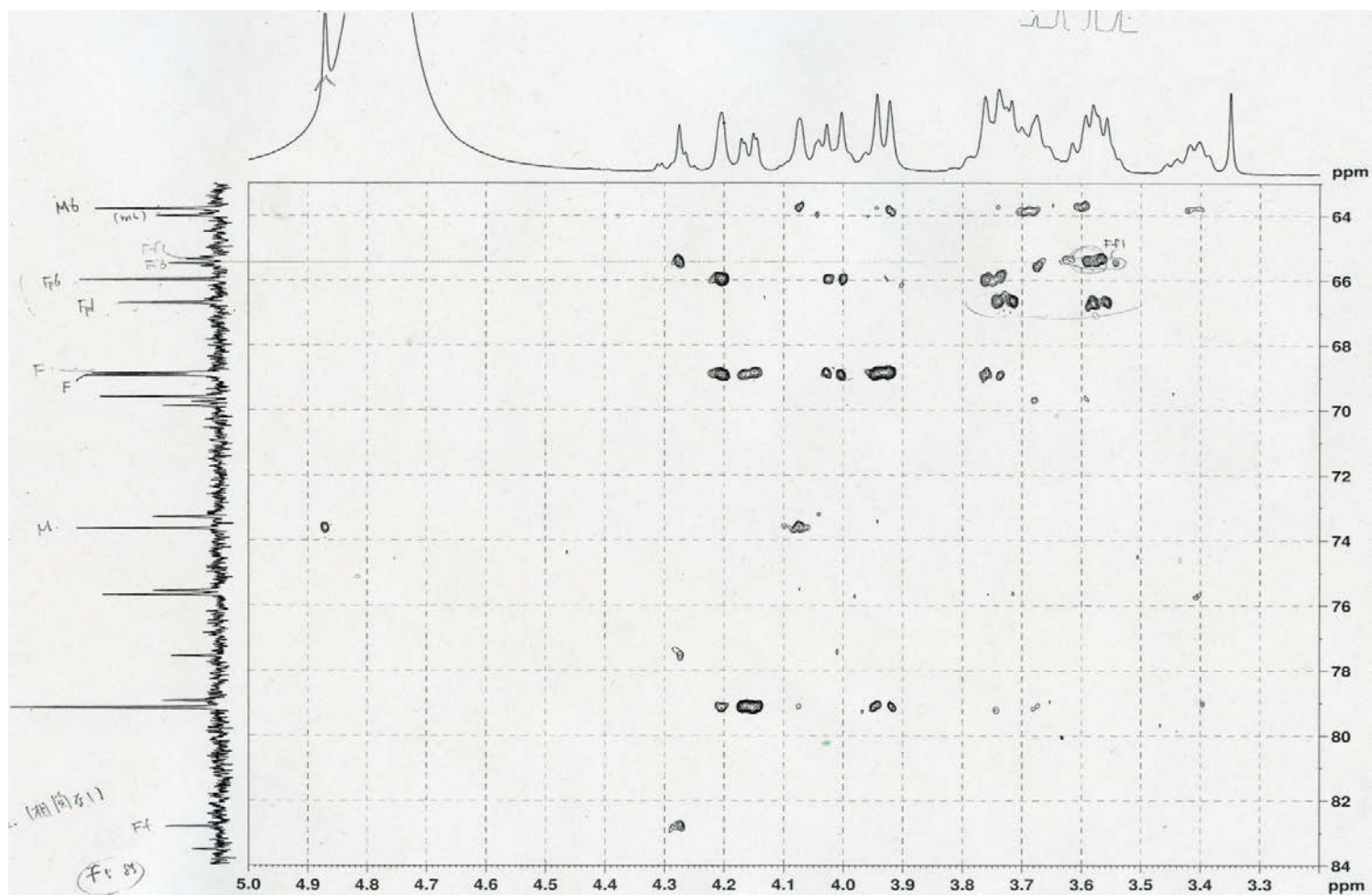


Fig. IV.11.a.9. Two dimensional NMR (HSQC-TOCSY) of Man β 1-4Fru.

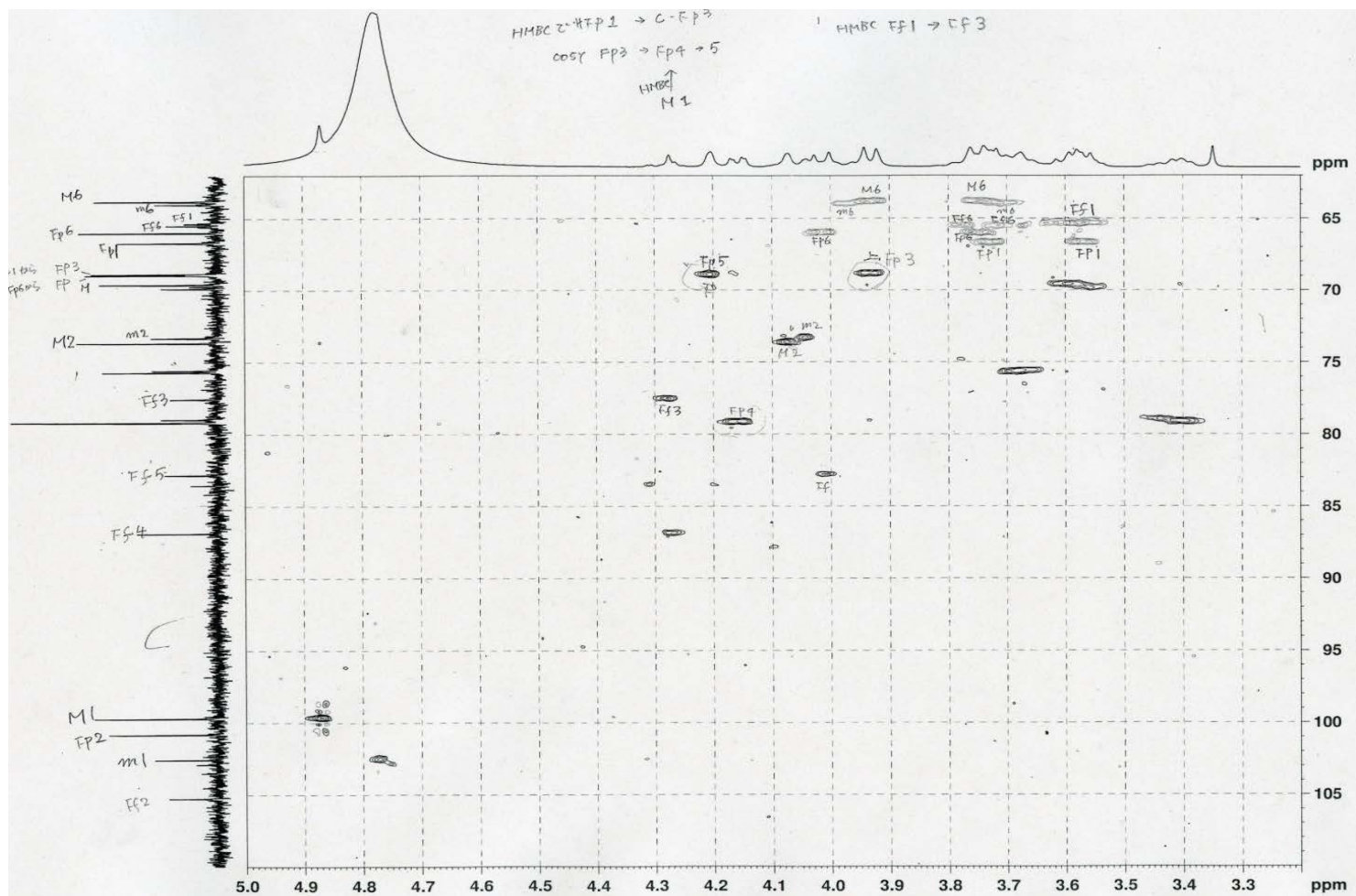


Fig. IV.11.a.10. Two dimensional NMR (HSQC) of Man β 1-4Fru.

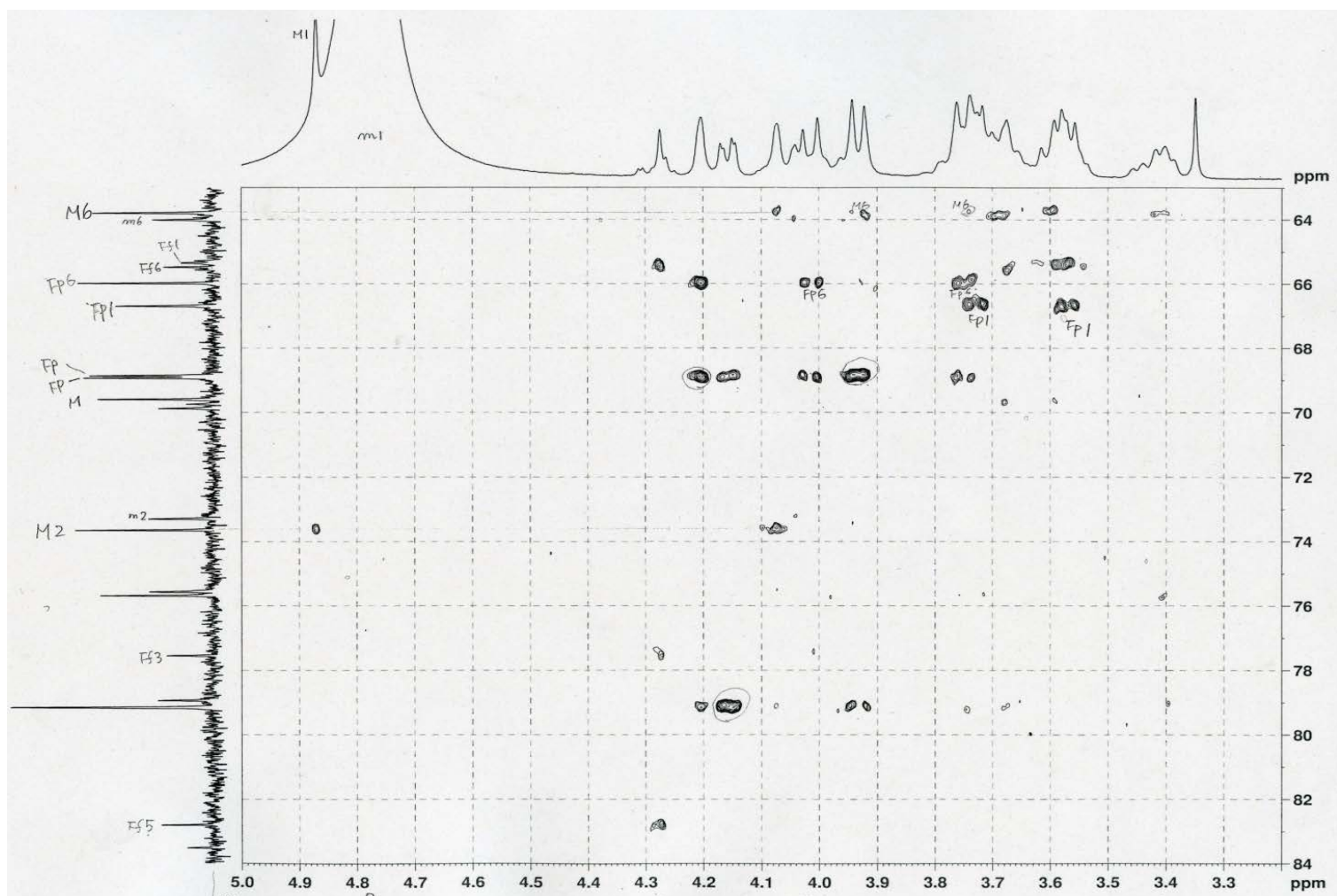


Fig. IV.11.a.11. Two dimensional NMR (HSQC-TOCSY) of Man β 1-4Fru.

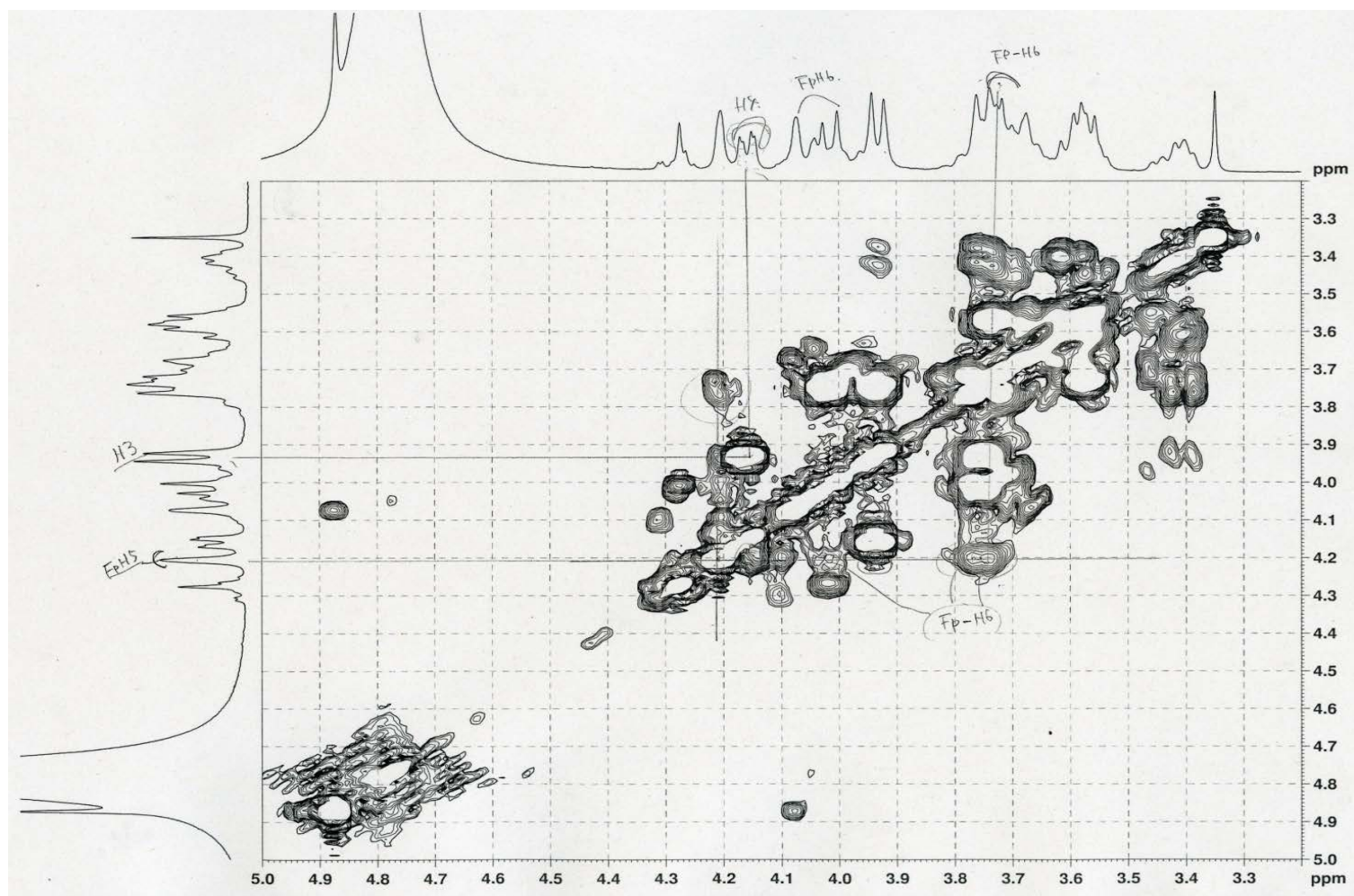


Fig. IV.11.a.12. Two dimensional NMR (COSY) of Man β 1-4Fru.

Table IV.1. Comparison of chemical-shift assignment and D.I.S. value of keto disaccharides of Gal β 1-4Fru, Glc β 1-4Fru, and those of putative samples.

Shifts and d.i.s. in p.p.m.				
Carbon atom	Gal β 1-4Fru ^a (β -pyranose)	Putative Gal β 1-4Fru sample	Glc β 1-4Fru ^a (β -pyranose)	Putative Glc β 1-4Fru sample
C1	101.5	101.6	101.1	101.1
C2	71.73	71.58	74.00	73.88
C3	73.65	73.43	76.68	76.74
C4	69.66	69.52	70.63	70.67
C5	76.03	76.09	76.87	76.88
C6	62.06	61.97	61.76	61.73
C'1	65.08	64.74	65.00	64.74
C'2	98.76	64.72	99.10	98.96
C'3	67.16	66.93	67.08	67.87
C'4	78.34	78.23	78.41	78.29
C'5	67.70	67.59	67.71	67.52
C'6	63.88	63.83	63.88	63.84

a, Ref.-55

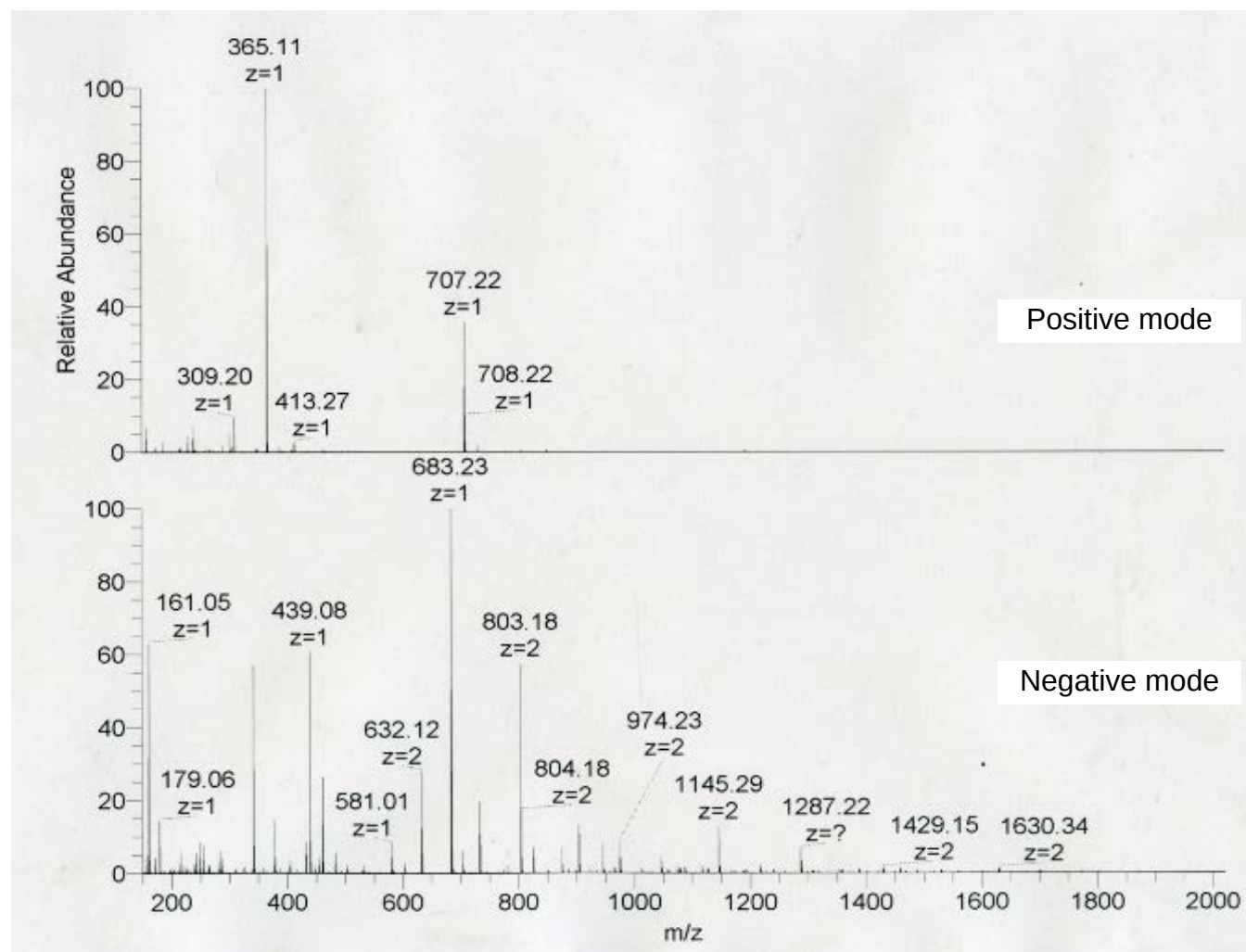


Fig. IV.11.b. ESI MS of Glc β 1-4Fru

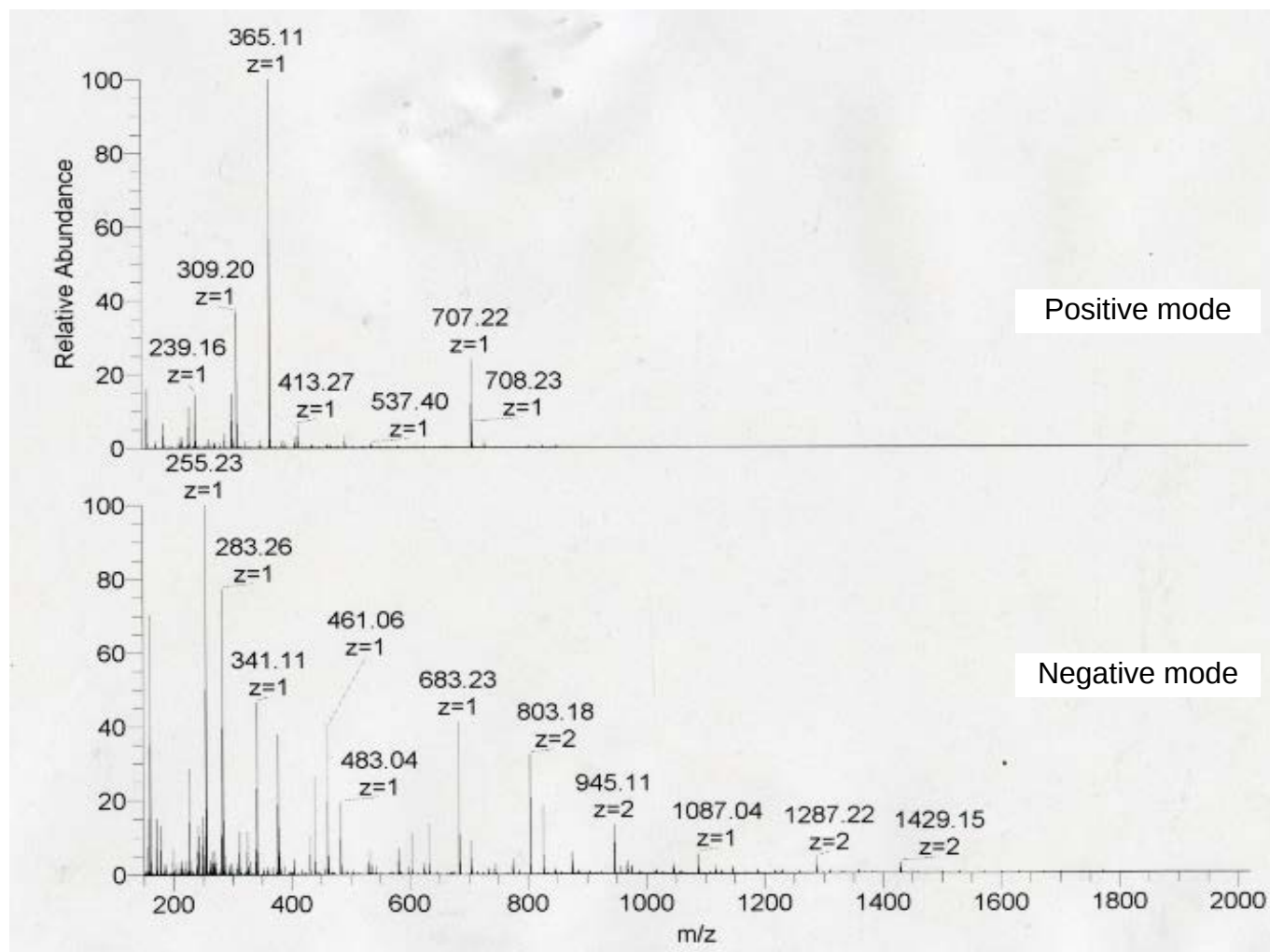


Fig. IV.11.c. ESI MS of Gal β 1-4Fru.

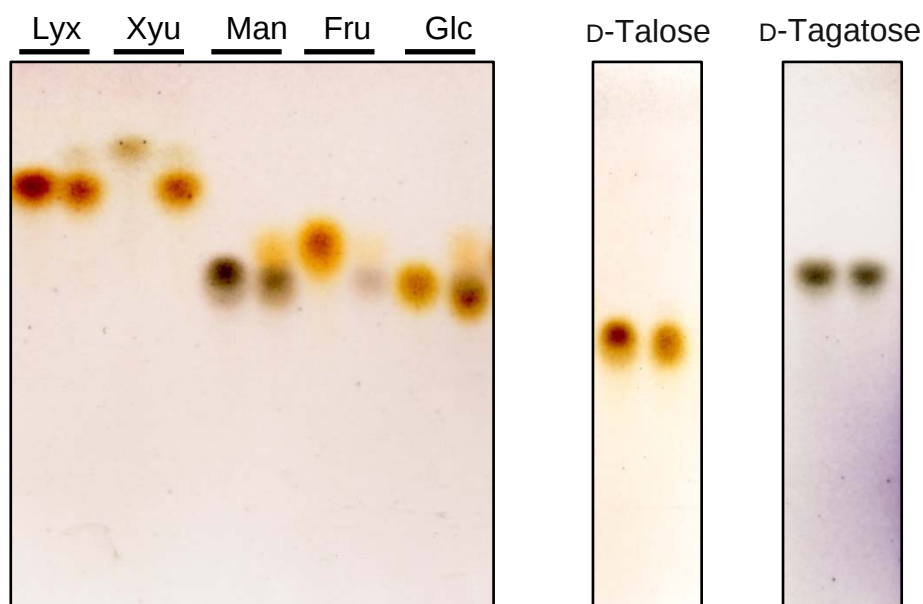


Fig. IV.12. TLC analysis of the reaction of EcAKI with various sugars.

Products from various sugars were analyzed by TLC. D-Lyxose (Lyx), D-xylulose (Xyu), D-mannose (Man), D-fructose (Fru), and D-glucose (Glc) served as substrate within 16 h reaction. No product was detected in TLC analysis from D-talose and D-tagatose with the same reaction condition (time and enzyme concentration) for the others. Substrates were spotted on the left side, and reacted samples were spotted on the right side. Carbohydrates used for substrate are shown above.

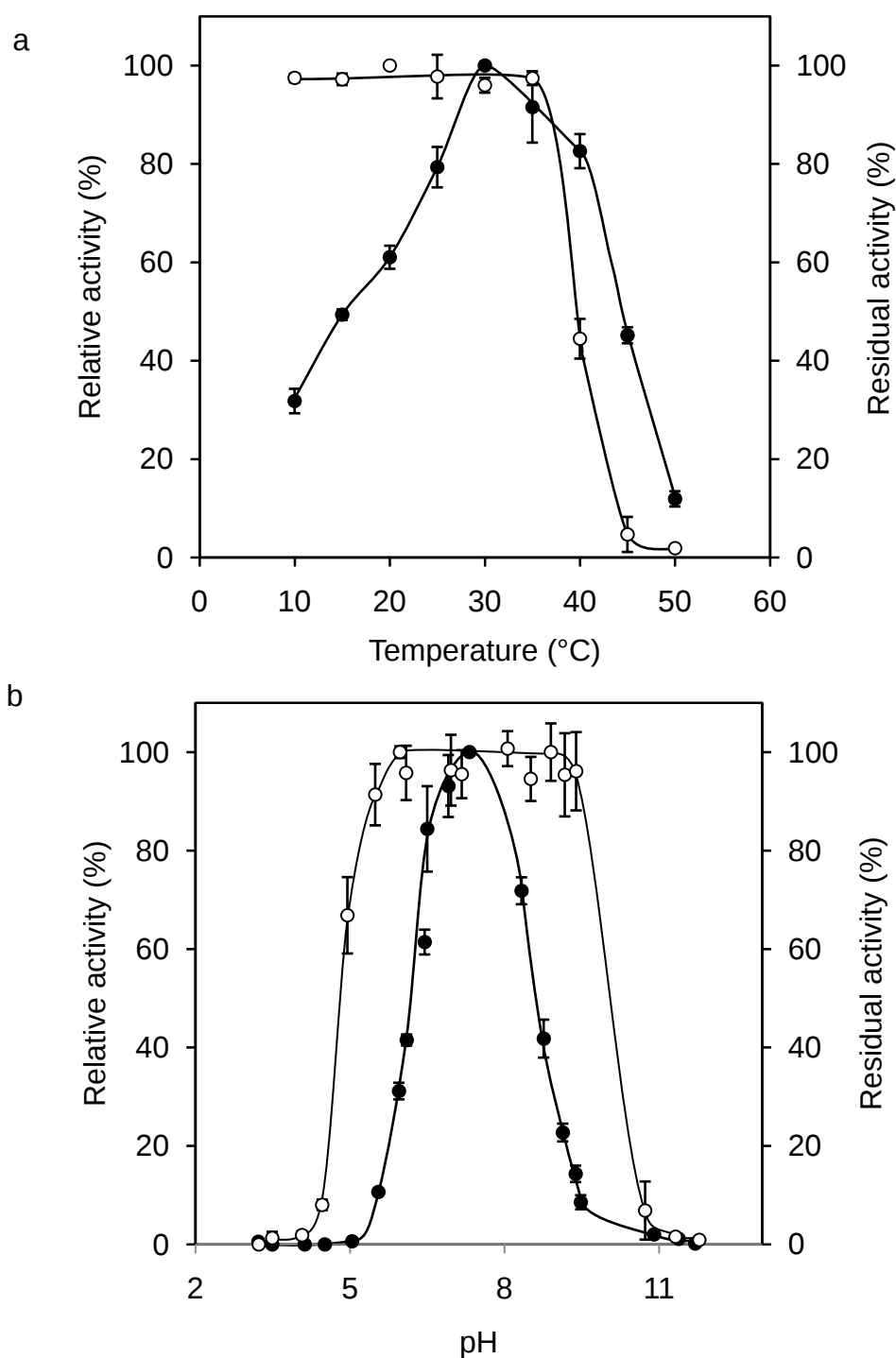


Fig. IV.13. Effects of temperature and pH on activity and stability of Marme_2490.

Relative isomerization activity from 10 mM D-mannose was measured at various pH values in a 10-min reaction at 30°C (a, closed circles) and temperature in 1 reaction at pH 7.4 (b, closed circles). Stability was evaluated with residual activity after the incubation at various temperature at pH 7.4 for 30 min (a, open circles), and various pH at 4°C for 24 h (b, open circles).

Table IV.2. The kinetic parameters of Marme_2490 and related enzymes.

Enzyme	D-Mannose			D-Lyxose			D-Talose		
	k_{cat}	K_{m}	$k_{\text{cat}}/K_{\text{m}}$	k_{cat}	K_{m}	$k_{\text{cat}}/K_{\text{m}}$	k_{cat}	K_{m}	$k_{\text{cat}}/K_{\text{m}}$
	(s ⁻¹)	(mM)	(s ⁻¹ mM ⁻¹)	(s ⁻¹)	(mM)	(s ⁻¹ mM ⁻¹)	(s ⁻¹)	(mM)	(s ⁻¹ mM ⁻¹)
Marme_2490	329 ± 2.2	16.7 ± 1.8	19.7	64.4 ± 3.6	42.3 ± 3.4	1.52	0.319 ± 0.05	27.8 ± 7.69	0.0115
EcAK ^a	25.3 ± 1.0	108 ± 11	0.234	13.5 ± 0.6	405 ± 41.7	0.033	N.D.	N.D.	N.D.
SeAKI ^a	23.3 ± 0.9	134 ± 15	0.174	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
TfMI ^b	788 ± 40	115 ± 15	6.85	63.3 ± 6.5	537 ± 12	0.118	N.D.	N.D.	N.D.

Data are mean ± S.D. for three independent experiments. N.D., no data available.

a, Ref-28; b, Ref-29.

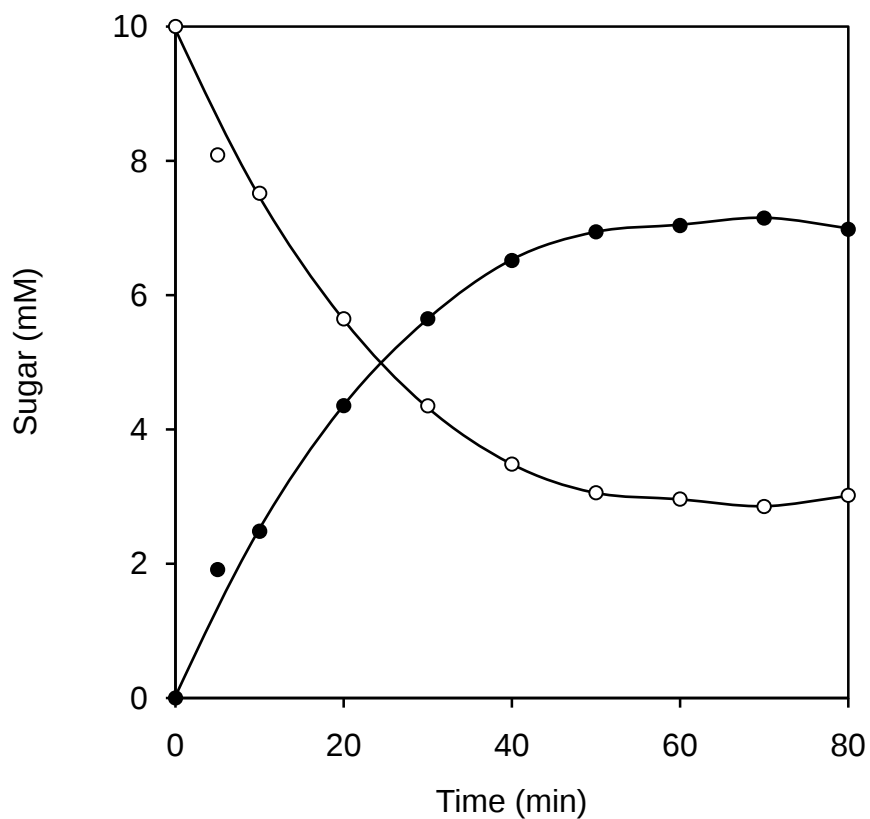


Fig. IV.14. Time course of the conversion of D-mannose to D-fructose.

D-Fructose produced from D-mannose was quantified, and D-mannose concentration (mM) was calculated using the following equation $[\text{Man}] = 10 - [\text{Fru}]$. The reaction mixture containing 10 mM D-mannose as a substrate, 40 mM Tris-HCl (pH 7.4), and 1.28 μM Marme_2490 was incubated at 30°C for 0-80 min. ●, D-Fructose; ○, D-mannose.

CHAPTER V. Colorimetric quantification of β -(1 $\rightarrow$ 4)-mannobiose and 4-O- β -D-mannosyl-D-glucose

V-1. Introduction

Man₂ is a potential prebiotic disaccharide, which is highly resistant to digestive enzymes and fermented to produce short chain fatty acids by human fecal bacteria⁵⁶. This disaccharide possesses innate immune modulating activity^{57, 58}. It enhances antibacterial defenses in chicken macrophages, and oral administration of Man₂ prevents *Salmonella enteritidis* infection, which is a food-borne pathogen often found in broilers⁵⁹.

Compared with chromatographic techniques such as HPLC, spectrophotometric methods can process a larger number of samples more easily. As D-glucose can be easily quantified by spectrophotometric methods^{60, 61}, colorimetric quantification methods of maltose and cellobiose were established by coupling the colorimetric quantification of D-glucose and phosphorylation of these oligosaccharides with maltose phosphorylase (EC 2.4.1.8) and cellobiose phosphorylase (EC 2.4.1.20), respectively⁶²⁻⁶⁴. In this chapter, MGP and CE are candidate enzymes for the colorimetric quantification of Man₂ and Man-Glc. MGP and CE for the colorimetric quantification of Man-Glc and Man₂ were established by combination of the enzymatic quantification of D-glucose and reactions of MGP and CE.

V-2. Material and methods

V-2-1. RaCE, RmCE, and RaMGP

Recombinant *R. albus* CE (RaCE) and RaMGP were prepared as described previously^{19, 65}. *E. coli* transformants harboring expression plasmids for RaCE and RaMGP derived from pET23a^{5, 65}. Recombinant RmCE was obtained from Chapter III. Protein purification of RaCE and RmCE were done as described in Chapter III, while recombinant RaMGP was produced and purified as described previously⁵. The purification steps of these three enzymes were described shortly as follows:

For the recombinant RaCE, the *E. coli* transformant was cultured in 3 L of LB containing 50 μ g/mL ampicillin at 37°C until OD₆₀₀ reached 0.6. Then, 0.1 mM IPTG was added into the culture for enzyme expression and incubated at 16°C for 20 h. The cultured cells were centrifuged, re-suspended in 10 mM sodium phosphate buffer pH 7.0, and

disrupted using an Ultrasonic Disruptor UD-201 (Tomy, Tokyo). Supernatant was obtained by centrifugation, and applied onto a Toyopearl DEAE-650M column and a Toyopearl Butyl-650M column as described in chapter III. The purified protein was investigated by SDS-PAGE, and stored in 50% glycerol at -20°C.

For the recombinant RmCE, the recombinant RmCE was produced in *E. coli* BL21(DE3) in 3 L of LB containing 50 µg/mL ampicillin. The expression of protein was induced by adding 0.1 mM IPTG when absorbance at 600 nm reached 0.6, and the culture broth was further incubated at 16°C for 20 h. Bacteria cells were harvested, re-suspended in buffer, and disrupted. The cell-free extract obtained by centrifugation was incubated at 70°C for 30 min and centrifuged at 13,000 × g at 4°C for 10 min to remove *E. coli* derived proteins. The supernatant was applied onto a Toyopearl DEAE-650M column and a Toyopearl Butyl-650M column¹⁹. The purity of purified protein was investigated using SDS-PAGE, and the sample was stored at -20°C in the presence of 50% glycerol.

For the recombinant RaMGP, the recombinant RaMGP prepared according to Kawahara *et al.*⁵ was used. *E. coli* BL21(DE3) transformed with RaMGP expression plasmid was cultured in 1 L of LB containing 50 µg/mL ampicillin at 37°C. The protein was induced with 0.1 mM IPTG when absorbance at 600 nm reached 0.6, and culture both was incubated at 18°C for 24 h. Harvested bacteria cells were disrupted by sonication. The obtained cell-free extract was applied onto Toyopearl DEAE-650M column, Toyopearl Butyl-650M column, and Sephacryl S-300 column chromatography. The purified protein was investigated on SDS-PAGE, and freeze with liquid nitrogen for storing at -80°C.

V-2-2. Protein assays

The protein assays were carried out according to the chapter II-2-5. The protein concentration of the cell-free extract and the protein concentrations of column chromatography fractions were measured using the Bradford method and the UV method, respectively. The concentration of the purified enzyme was calculated from the amino acid concentrations of an acid hydrolysate.

V-2-3. Colorimetric quantification of Man-Glc and Man₂

V-2-3-1. Man-Glc

Fifty micro-liters of 0–0.5 mM Man-Glc was mixed with 100 μ L of the enzyme solution, containing 1.3 μ g/mL RaMGP, and 70 mM sodium phosphate buffer (pH 6.3), and 20 μ L of the D-glucose quantification reagent, and incubated at 37°C for 30 min. The D-glucose quantification reagent was prepared as follows: a bottle of the coloring reagent Glucose CII-Test (Wako Pure Chemical Industries) was dissolved in 175 mL of 100 mM sodium phosphate buffer (pH 6.0) containing 2.8 mM 4-aminoantipyrine and 40 mM phenol as substitute for 350 mL of the original buffer in the kit.

V-2-3-2. Man₂

Fifty micro-liters of 0–0.5 mM Man₂ was mixed with 100 μ L of the enzyme solution, containing 1.3 μ g/mL RaMGP, 12 μ g/mL RaCE, and 70 mM sodium phosphate buffer (pH 6.3), and 20 μ L of the D-glucose quantification reagent, and incubated at 37°C for 30 min.

V-2-4. Kinetic analysis of RaCE and RmCE

The CE reaction mixture (50 μ L), containing 2.5–25 mM Man₂, 40 mM sodium phosphate buffer (pH 7.0), and the enzyme, was incubated at 37°C (RaCE) or 60°C (RmCE) for 10 min. The concentrations of RaCE and RmCE in the reaction mixture were 7.48 nM and 13.7 nM, respectively. The reactions were terminated by addition of 20 μ L of 0.1 M HCl and heating the sample at 100°C for 3 min. To neutralize the sample, 20 μ L of 0.1 M NaOH was added. As HCl and NaOH were added to the sample, following procedures for determination of Man-Glc were modified (concentrations of phosphate and RaMGP were not changed significantly from the experiments described above). The sample was mixed with 60 μ L of the enzyme solution, containing 2.2 μ g/mL RaMGP and 83 mM sodium phosphate buffer (pH 6.3), and 20 μ L of the D-glucose quantification reagent, and incubated at 37°C for 30 min. The concentration of Man-Glc produced was determined based on the absorbance at 505 nm using D-glucose as standard. The kinetic parameters were calculated by fitting the velocities at various substrate concentrations to the Michaelis-Menten equation. Non-linear regression was carried out using Grafit version 7.0.2.

V-3. Results and discussions

V-3-1. The quantification of Man-Glc and Man₂

In the CE-MGP pathway, CE converts Man₂ to Man-Glc which is phosphorylated to Man1P and D-glucose by MGP. D-Glucose is quantified using glucose oxidase-peroxidase method by measuring absorbance at 505 nm. In the reactions for both Man-Glc and Man₂, a good linear relationship between the concentrations of the sample and absorbance at 505 nm was obtained (Fig. V.1). The concentrations of D-glucose were consistent with the results of various concentrations of Man-Glc and Man₂, indicating that Man-Glc and Man₂ were completely consumed in this reaction system. No difference of the absorbance obtained in the experiments of D-glucose, Man-Glc, and Man₂ indicates that Man1P at ≤ 0.5 mM does not prevent the quantification reactions with the indicated concentration of RaMGP, although Man1P at ≥ 1 mM causes product inhibition in the phosphorylation of Man-Glc⁵. The colorimetric quantification methods established here for Man-Glc and Man₂ are rapid and simple for quantification of many samples.

As Man₂ and Man-Glc are the reaction products of mannan 1,4-mannobiosidase (EC 3.2.1.100)⁶⁶ and CE, the quantification methods of these oligosaccharides can be utilized for activity assays. CE is a useful utilized enzyme for the production of a prebiotic oligosaccharide, epilactose^{65, 67}. The colorimetric Man-Glc quantification makes the enzyme assay for CE easier.

V-3-2. The kinetic parameter of RaCE and RmCE for the epimerization of Man₂

The kinetic parameters of RaCE and RmCE for the epimerization of Man₂ have not been determined thus far, although the involvement of these enzymes in the metabolism of β -mannan was predicted. Using the assay method for the quantification of Man-Glc, the kinetic parameters for the epimerization of Man₂ were determined. The colorimetric method made it possible to quantify many samples, which is required for the determination of kinetic parameters. As summarized in Table V.1, RaCE and RmCE showed 5–15-fold higher k_{cat}/K_m values for Man₂ than those for cellobiose and lactose. The reaction conditions of RaCE for Man₂ (pH 7.0, 37°C, and 2.5–25 mM Man₂) were different from those for cellobiose and lactose (pH 7.5, 30°C, and 10–100 mM substrate). As epimerization activity of RaCE at pH 7.0 at 37°C is slightly lower than that obtained at pH 7.5 at 30°C⁶⁵, k_{cat}/K_m value for Man₂ at pH 7.5 at 30°C is estimated to be higher than the value shown in Table V.1. High preference of RaCE and RmCE for Man₂ supports the

prediction that these enzymes epimerize Man₂ to Man-Glc for further phosphorolysis by MGP in the β -mannan metabolic pathway^{5, 68}.

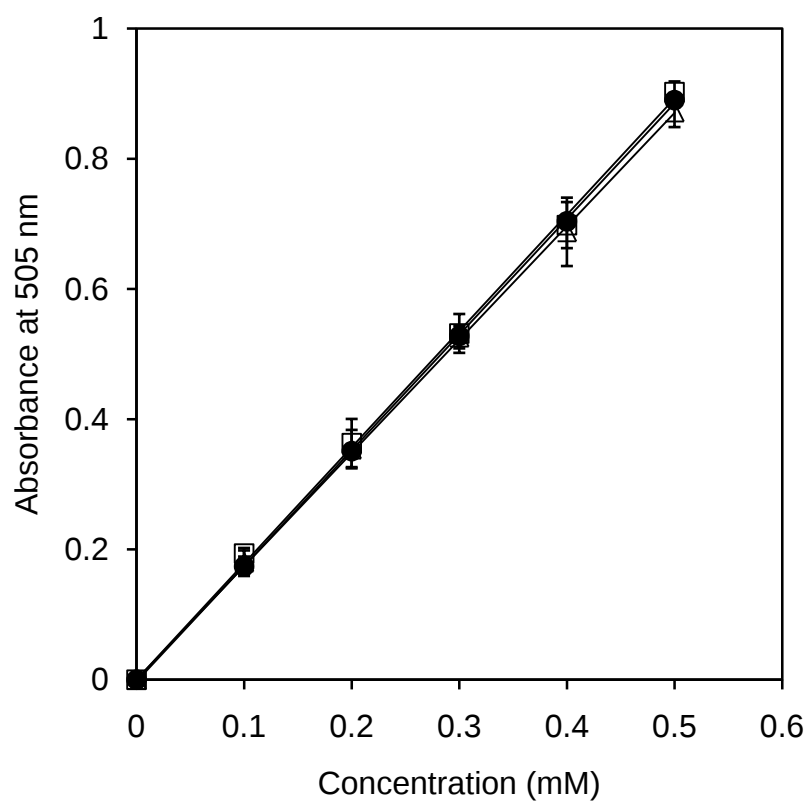


Fig. V.1. Colorimetric quantification of Man-Glc and Man₂.

Man-Glc, open triangles; Man₂, open squares; D-glucose (control), filled circles. Data represent the mean $\pm$ standard deviation (error bars) for three independent experiments.

Table V.1. Comparison of kinetic parameters of RaCE and RmCE.

Enzyme	Man ₂			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)	k_{cat} (s ⁻¹)	K_{m} (mM)	$k_{\text{cat}}/K_{\text{m}}$ (s ⁻¹ mM ⁻¹)
RaCE	201 ± 1	8.74 ± 0.13	23.0	63.8 ^a	13.8 ^a	4.62 ^a	52.1 ^a	33.0 ^a	1.58 ^a
RmCE	94.7 ± 4	3.53 ± 0.41	26.8	80.8 ^b	27.2 ^b	2.97 ^b	111 ^b	28.8 ^b	3.85 ^b

Data are mean ± S.D. for three independent experiments.

a, Ref-65. Reaction at pH 7.5, 30°C. b, Ref-19. Reaction at pH 7.0, 60°C. To quantification of Man₂, the reaction mixture of Man₂ (50 µL) containing 0.25-25 mM Man₂, 40 mM sodium phosphate buffer (pH 7.0), and 7.48 nM RaCE/13.7 nM (RmCE) was incubated at 37°C (RaCE)/60°C (RmCE). The reaction was stopped by 20 µL of 0.1 M HCl and heat at 100°C. D-Glucose was quantified as follows: added 20 µL of 0.1 M NaOH, 20 µL of D-glucose quantification reagent, 10 µL of 37 µM RaMGP, 50 µL of 100 mM sodium phosphate buffer (pH 7.0), and 20 µL of water. The sample was incubated at room temperature for 30 min, and D-glucose was quantified at absorbance 505 nm.

CHAPTER VI. General discussions

β -Mannan is one of the major component of hemicellulose in plant cell wall. To degrade this polysaccharide, several β -mannan-hydrolyzing enzymes including β -mannanase and β -mannosidase are required. Mannooligosaccharides and D-mannose are the products from β -mannan hydrolysis. They can be used in several application fields such as medical, pharmaceutical, and food-biochemical fields⁶⁹⁻⁷⁴. The mannooligosaccharides have prebiotic activity of enhancing growth of bifidobacteria and lactobacilli in human intestine⁶⁹, prevent adherence of toxic bacteria to the intestinal wall^{70, 71}, and exhibit fibrinolytic activity⁷² which prevents blood clots formation. D-Mannose helps maintain a healthy bladder and urinary tract⁷³. Furthermore, the addition of locust bean gum and guar gum (containing high degree of mannose) improve the texture and reduce melting of food product⁷⁴.

MGP of the aerobic bacterium

There are many bacteria which produce enzymes degrading β -mannan. Recently, the CE-MGP pathway, discovered only in anaerobic bacteria, *R. albus*⁵ and *B. fragilis*⁴, is a new metabolic pathway for β -mannan degradation. The *Rmar_2440* from *R. marinus*, is encoding a putative MGP which located in the upstream of *CE*. After investigation, *Rmar_2440* exhibited MGP activity toward Man-Glc to Man1P and D-glucose. *Rmar_2440* activity was highest at pH 6.5 and 70°C, while BfMGP showed the highest activity at pH 7.0 and 50°C⁴. RaMGP has the same optimum pH as RmMGP, but the activity was highest at 50°C⁵. In the reverse phosphorolysis reactions, D-glucose, 6-deoxy-D-glucose, and D-xylose served as acceptor substrates for RmMGP. Methyl β -D-glucoside and 1,5-anhydro-D-glucitol were also used as acceptors for RmMGP, but not for RaMGP⁴. Furthermore, the molecular mass of *Rmar_2440* was 222 kDa, while RaMGP was 80 kDa under non-denaturing condition⁴. This is the first enzymatic characterization of an MGP of the aerobic bacterium. The presence of MGP suggests that the degradation of β -mannan mediated by CE and MGP is not limited to anaerobe, but also distributed to aerobe.

Two amino acid residues involved in the binding to disaccharide substrates

CE is also a key enzyme in CE-MGP metabolic pathway, and belongs to AGE superfamily with AKI, AGE, and MI. CE is sole enzyme acting on disaccharides, while

other enzymes in this superfamily act on monosaccharide as a substrate. The comparison of the known AGE superfamily enzymes structures found that at non-reducing end part of substrate, Asp and Trp residues are conserved only in CE. The site-directed mutagenesis of these two residues supported that Asp and Trp, localized suitable for the disaccharide binding, are significantly involved in the binding to disaccharide substrates. Ser is also predicted on the structural basis to form hydrogen bond with 4-OH of the non-reducing end part of substrate²⁵. This residue should be analyzed in the future.

CE has been found in various bacteria. Almost all CEs catalyze only epimerization activity. But, three CEs from *Dityoglomus turgidum*, *Spirochaeta thermophila*, and *Caldicellulosiruptor saccharolyticus* catalyze isomerization in addition of epimerization⁷⁻⁹. The substrate specificity of CEs is diverse for cellobiose and lactose as substrates. The substrate specificities of RmCE and EcCE for lactose were better than cellobiose^{19, 20}. In contrast, cellobiose, served as a substrate for BfCE, RaCE and DtCE, was better than lactose^{4, 65, 32}. Furthermore, the substrate specificity for trisaccharides substrates; maltotriose, cellotriose, and mannotriose were determined from StCE, and DtCE^{31, 32}. Therefore, the difference substrate specificity among CEs should be analyzed in the future.

Conserved region prediction of function-unknown enzymes belonging to AGE superfamily

The phylogenetic analysis of AGE superfamily enzymes is shown in Chapter I, Fig. I.5. AGE was first found in porcine kidney²⁶, and *Anabeana* sp. AGE was the second found²⁷. AKI and MI were found in *E. coli*²⁸, *S. enterica*²⁸, and *T. fusca*²⁹, respectively. CE has been found in various bacteria^{3, 4, 19- 21, 30-32}. The cluster of CE and putative CE enzymes were functionally separated from AGE, AKI, and MI, supporting that amino acid sequences in each cluster have low similarity. To predict the unknown enzymes in AGE superfamily, the conserved region of the amino acid sequence should be considered. The conserved amino acid residues of several CEs have three regions corresponding to reference results⁷⁵, while AGE has only one conserved region (Fig. VI.1). MI maintains loop which has high homology to AKIs as shown in Fig. VI.2.

In the phylogenetic tree constructed in Chapter I, Fig. I.5 is made by known enzymes and several unknown enzymes. A function unknown enzyme, Marme_2490 from *M. medditernea*, belongs to AKI cluster, but its amino acid sequence exhibits low identity

to AKIs. The characterization of Marme_2490 suggests that Marme_2490 is a novel type of MI as follows: Marme_2490 has only isomerization activity toward D-mannose which is the best substrate, and the $k_{\text{cat}}/K_{\text{m}}$ values of Marme_2490 for D-mannose is higher than any known enzymes, TfMI, EcAKI, and SeAKI^{28, 29}. Marme_2490 has low isomerization activity to D-talose, but enzyme in the same cluster, EcAKI, is not act on D-talose at all. Marme_2490 slightly showed isomerization activity to disaccharide substrates, Man₂, Glc-Man, and epilactose. AGE superfamily still has many function-unknown enzymes which should be studied in the future based on conserved region as described above, and these unknown proteins are possible to be new enzymes which produce interesting new sugars useful for human life.

Application from enzymes involved in the CE-MGP pathway

Man₂ has beneficial potential for use as a prebiotic digosaccharide in food material. Man₂ inhibit the localization of *Salmonella enteritidis* which is a food-borne pathogen often found in broilers⁵⁹. The quantification of Man₂ is usually determined by HPLC^{4, 20, 21, 30, 31, 65} which is an accurate technique and usually analyzes the saccharide components in chemistry, but this technique spends long time to analyze the samples. This is the first alternative method for quantification of Man₂ and Man-Glc using spectrophotometer easily and accurately. The colorimetric quantification of Man-Glc and Man₂ using the CE-MGP pathway enzymes was developed. CE acts on only Man₂ as a substrate, while MGP acts on only Man-Glc as a substrate. For quantification of Man-Glc, MGP was coupled with quantification of D-glucose by the glucose oxidase-peroxidase method. In addition of CE in the reaction of MGP, Man₂ was also quantified accurately. Therefore, to know the concentration of Man₂, this spectrophotometric quantification method is easy method and save time for multiple samples determination.

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RmCE      1  ---TETIPDVRRLRALQAEVHEELTENILKFWATRTHDPVHGFFVGRVGPDRGRHPPEAPRGAILNARILWTFAAAYRO 75
CpCE      1  -MNTSDEKQQLPVNTLKQELLOELKR-ILVYNTTETVDERNGGFIGRISAHNVKDPYAVKGSVLNTRILWTFSAAYNL 77
BfCE      1  -----MDEILKQEMOKELTTRILPYMERMDQENGFFYGRITGOEELPRADKGAILNARILWTFSAAYRL 67
PhCE      1  -----MSEILIOEFKELAG-MLNYNATYTVQVHTGFYGOVDNDNNAIEKADKGAVLNLRILWTFSAAYNY 66
FjCE      1  -----MPANLKQKLSLTAELDS-ILNYSKHTLDNNGEFGVGOIDFNHIVANAEGSVLNSRILWTFSSVOI 69
DfCE      1  -----MAAFTPEAFKKEFIA-ILDYNEKYGLDSEKGGFYGRVNYENOPVKDAKSVLTGRILWTFSLAHLR 66
SlCE      1  -----MDLKQRAELRQEHKD-ILAYNTRYAPDRVHGFFYGRVNYONOPPIAEKGIVLNARILWTFSAALRE 67
HaCE      1  MANLVDGGFDHGLREMWQAFRDEVLNLPFMANHTLDHEGGFYGGLTNDLTIHNEFPFSAVLCGRILWTFACAYRM 79
RaCE      1  -----MMISEIROELTDHIIPFWNK-LRDDENGFFYGYLSYGLLELDDKADKGVILSRILWTFSNAYMT 63
EcCE      1  -----MKNEVVYKQTEKILPFWNA-MRDDENGFFYGYMSDLHDDHADKGCILNSRILWTFSTAYMY 63
DtCE      1  -----MDLKVLKSEIFEHLNNKIIPFWE-LKDENGFFYISYVGFDLKDDPYAPKGLVLSRILWTFSSRLWNO 67
CsCE      1  -----MDITRFKEDLKAHLEEKIIPFWS-LKDDFEGGYGYMDFNLNDRKAOKGCILNSRILWTFSSACVNV 67
SdCE      1  -MGVPLKKNLNYSDLNNAFSESLHA-IADWVDNTVDEIHGGFVGYMSVDNKNVATADKGILNTRILWTFSEALF 76
TtCE      1  -METETDTRQILILLONNCAELAH-LVSNWASNVVG--DGNFSGEINSQGLPOHAGKGGIYSRLLWTFSEVALG 74
StCE      1  -MPSSRSLRSTLROIRSELASOLFHDHILPFWLA-QDDPHGGFFYGSIT--TTPDGPAGKGLVMTARHLWTFSAFLS 73
SeAKI     1  -----MKWFNTLSHNRWLEQETD-RIFNFGK-NAVV--PTGFGW-LGNKGQIKEMGTHLITARMLHVSVVAAS-- 65
EcAKI     1  -----MKWFNTLSHNRWLEQETD-RIFDFGK-NSWV--PTGFGW-LGNKGQIKEMGTHLITARMLHVSVVAAA-- 65
TfMI      1  -MTLWTARAARHLDAEAF-RLVDFAA--AADHPHGFAW-LDGGGAPLPGQGVHTVITCRVTHVAALAHL-- 67
Marme_2490 1  -MSYPADFSTKTFLEAHTE-KTMAFYFPTCIDP-EGGFFQFCKDDGSVYDPNTRHLVSSTRFIFNFAQAYLH- 68
AnabeanaAGE 1  -KNLOALAQLYKNALLNDVLPFENHSLDS-EGGYFTCLDRQKGVYD-TDKFILLQNRQVWTFSLMNCQ- 66
PorcineAGE 1  -MEKERETLQA-KERVQGLD-RVMAFWLEHSHDREHGGFFTCLEGRDGRVYD-DLYVYLQGRQVWTFYCRLYRK- 71

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RmCE      141 EALALARSIDYLIETHCADRVHGGYVEACDRAWRPLEDARLSAKDAPEPRSIINTHLHYLEAVANLYR-VMPET----- 213
CpCE      143 ETAKQTAIAMWHAIROYAYDKIYGGYIEACTODWOPLSDLRLSLKDANERKSINTHLHYLEAMTTLYE-IWPE----- 215
BfCE      133 PEADYAVRLFNDIESHSDGLKNGYCEALTREWNIEADMRSLSEKDNANERKTINTHLHILEPYTNLYR-WMKDA----- 205
PhCE      132 EEALALAKILYADIEKHSFDPINNGYFAFSQWSELTDORLSKDNANEKKTINTHLHYLEAYTSLYT-WMPDE----- 204
FjCE      135 OKALETAVNLKYSIOKYSYDPVNGYLEAFTRWIOPIEDRLSDKDNANEKKTINTHLHYLEAVANLFK-WMKDE----- 207
DfCE      132 KPALKQAQSLFEIEKHAFDPVNGGYREAFADWSATDDVILSKSPWN--KSHINTHLHYLEAYTNLYS-WMPDA----- 202
SlCE      133 EPALALAKEVFQNMVKHAYDPKKGGFVEALARDWSPATDVIISKYDNOEIKTINTHLHILESFTCLYH-WMPDK----- 205
HaCE      145 OSALALEAQTFLHLENHAFDAVYGGYIEGCDRVWQPLGDSRLSKLEPEARKTMMTILHMEAYANLRL-WMDVA----- 217
RaCE      129 KEALALAYRLFEDIEKNTLYE--YGYREAFDROWRLVDNEALSENGLKADKTMNAILHLIEAYTELYK-ADGNE----- 199
EcCE      129 IEATIAEIIYEKIEEDTMRDT--KGYLEAFTRDFRADNOKLSENGVMAERTMNTLLHIEAYSALVH-ALRKKVADPAK 205
DtCE      133 KESLDLALEIYKIEEVCKNDV--GYKEEFDEKNPKENIIVSEYGIICERSMNTLLHIEAYTNLFT-ATYDQ----- 203
CsCE      133 EEALHMAKRLFEILETKCKRE--NGYTEOFERNWQEKENRFLSENGVIASKTINTHLHYLESYTNLYR-LLKLD----- 203
SdCE      142 ADVLAQAHAFELTEANADENHOGYLEAFSNTWGRDDMRSLSEKDLNSPKSINTHLHILEAYTALHI-AAPSP----- 214
TtCE      151 EHALLAALTIYELVEEFALDSAHGGYFAFSREHRELDRISEKDLASPKTINTHLHILEAYTGILLR-ALROA----- 223
StCE      139 QDALRIARETDLVETRGDRTHSGGYEAFTDWTPLPEPLGEGETPAKKTINTHLHYLEAYSAFSR-IDREP----- 211
SeAKI     129 PEARKLLDYTIETIEKYFSEEEQKLESMDAFASTQED-----YRGGANMHAVEAFLIYVD-VTHDK----- 191
EcAKI     129 PEARKLLDYTIETIEKYFSEEEQKLESMDAFASTQEE-----YRGGANMHAVEAFLIYVD-VTHDK----- 191
TfMI      132 PGARELLDAAAVIEQRFEETETGRRESMDAAHHADEP-----YRGANSMHLVEAFLAADF-ATGDR----- 194
Marme_2490 131 SEAEVIEVTVOLLTEHFHFNKHLGLYDEISSDKTVSP-----YRGONANMHMEALMSAADF-ATQNP----- 193
AnabeanaAGE 132 EWAKDVAQVANNVLRRT-----RPMKALAVPMILANLLEME-WLLPO----- 174
PorcineAGE 141 ARYQSEAVDMMDOIVHWREDPSG-----LGRP-----QLPGVASESMVPMMLCLVEQLGEEDEELA----- 200

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RmCE      76  -----LG--TPLYREMAERAYRVFVRHFVD-AEHGGVYVMVAADGRPLDTRKHVYAQSFAIYALSENHRATGG 140
CpCE      78  -----LK--TDQVLTLANRWDYLYAHFAD-KVHGGLYNTVDYTGKAVDGKKQVYAIAGFYVACSEYFKASRN 142
BfCE      68  -----LG--REYKEMANRAKRYLIDHFYD-SEFGGVYHSLNRYGEPLDTKKOYIATGFAIYGLSEFHRATGD 132
PhCE      67  -----KS--MPESLHLARSYDYRIDHFVD-KOFGGVYNSVDFOGKPADTKKQYALAFALYGITFYFKASRE 131
FjCE      70  -----TK--KENHKEIAKRAFEYLSKYFYD-PEFNGLFWSINADKTPKDTKNQIYALAFAYIYGLSEYVYISQD 134
DfCE      67  -----LK--EAKYLTADRAYOOLARHFFD-PEHGGVYNSVADGSPLETKQIYGNAFAYMYGLAEYVRYTHF 131
SlCE      68  -----TO--HEEYRSIADQAFQYIKTYFVD-PEYGGIYNSVDATGKPKNALHLYGDAFTLYGLSEYVRATKS 132
HaCE      80  -----LG--DPRDLAVAAYAYVYLKQAFMD-QTYGGLYNSIDANGOPADHKQTYAQSFAIYGLAEYVRATGD 144
RaCE      64  -----LG--GDELLDNKHAHAYEFLIKSFID-YEYGGVYNMDFEGKPADTKHTYNTIAFAIYALSSYRASGD 128
EcCE      64  -----LQ--DEKLLDNKHAHAYEFLIKTYCFD-PMCGGIFNSVRYNGKPADTKHTYNTYDAFAIYALSAIYEATGS 128
DtCE      68  -----LR--KEEFINFADHSYKFLIKSFID-KENKGFYNMVDYKGEPIDKRKHLYGDAFVLYGLSEYKATOK 132
CsCE      68  -----LK--SEKCKEMAFHAFELKIKFMD-KEYEGLFNSVSHKGVDPVDTKHVYVQAFGIYGLSEYEAASGD 132
SdCE      77  -----TG--NKRVAQMAERAYYIVDHFID-TEHGGVFWALDYRGVWNNRKOYVAAQFAIYGLSAIYRLTKO 141
TtCE      75  RDSLAPASGPTSG--LASAKVSAQALYQVFSHFID-PEYGGVNHLEHANGELRDGRKOIYAAQFAIYCLSAIYRLTSD 150
StCE      74  -----RP--NPAYLEAAGNAYRFLTHAFYD-ATHRGFFNSVHPDGAPLSRVKLYGNAFAYIYALSLEYEATGD 138
SeAKI     66  -----MG--RPGAYDLVDHGKAMNGALRD-KKYGGWYACVN-DEGVVDASKOGYQHFALLGAASAVTT--GH 128
EcAKI     66  -----MG--RPGAYSLVDHGKAMNGALRD-KKYGGWYACVN-DEGVVDASKOGYQHFALLGAASAVTT--GH 128
TfMI      68  -----EG--TPGASALADHGLRALAGLRD-PEHDGWFALDSRGTVADSRKEAYQAFVLYLAAASATVA--GR 131
Marme_2490 69  -----TN--TAEYKHAAYHGIQVLRORHO--SQSGGVYVLLD-GGTLNDETNHCYGLAFVLYLAYSALQAT--GL 130
AnabeanaAGE 67  -----LE--KRENLIKIAIRNGAKFLAHDGRD--DEGNIYFALTTRGGEPLVQPYNFVSDCFAAAFSAFSAASGE 131
PorcineAGE 72  -----LERFHRPELLDAKAGGEFLLRHARVAPPEKKCAFVLTRDGRPVKVQVRSFSECYTMANNELKRVTA 140

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RmCE      214  -----ELAARLQALIELFLRATYH-PATGHLLIFFDERNRDR-----SRAVSFCHDIEASWLLLEAV 269
CpCE      216  -----OLKADILELLMLFDKQIHH-PTTHHLQLFRTDNMTVK-----STLISYCHDIEASWLLLEAA 271
BfCE      206  -----RLEROLYNLIGLFTEKILD-KDTHSLQLFFDNDWQSK-----YPVVSYCHDIEASWLLHEAA 261
PhCE      205  -----GLGRQIRNLLGVFTDKIID-RDTHHLMLFFDESWSHK-----SRAISFCHDIEASWLLLEAA 260
FjCE      208  -----TLKKDSIELLETIEKHFIN-TETGHLRLFFDENWIEK-----PDVISYCHDIEASWLLLOCA 263
DfCE      203  -----RLKKOTAGMLDTIITRIVN-PKTETMOLFFDEQWAK-----DNIVSYCHDIEASWLLFETA 258
SlCE      206  -----SVAEOMRGMHLMFOIHIID-PTTYRMNLFDMNMKVR-----RTAVSYCHDIEASWLLPEAA 261
HaCE      218  -----DVROOLASLIEACCEHIID-PVQGRFHLFFDDQNNHH-----EHGISYCHDIEASWLLMEAA 273
RaCE      200  -----DVADRLLKFGOLGMDRIVYT-PDINALKVFFDTAFNLV-----GDIHSYCHDIEATWLMDRAC 255
EcCE      206  GDVRDELFMNVNENKLAALIELMRDKFYN-SDRHRLDVFFDKYESL-----IDLSYCHDIEASWLLLEAA 271
DtCE      204  -----SIKKKIEDLILFLKEKIYD-SKTHHLVYFFDQKKNPI-----IDATSYCHDIEATWLMDEAL 259
CsCE      204  -----DYAEALWIVRLFVDKIYK-KGTGHFKVFCDDNNEL-----TKAVSYCHDIEASWLLDOAA 259
SdCE      215  -----KVSAAIKHCLFLDRYIIN-KANHLRMFMQMDWEDV-----SASYSYCHDIEASWLLMEAV 270
TtCE      224  -----ESPAVOOVAASVOQLLEYVSRVYN-SSSRHVHFMFNTHWED-----SLAFSYCHDIEASWLLWEAA 285
StCE      212  -----TVREAMEHLIRIFRTHI--ASSEHLGLYFTEDWSPM-----GGGISFCHDIEATWLMEEAV 265
SeAKI     192  -----KVLDRALRIASVITHVAR-NGDYRVNEHFDQWNPFIIDYKNKDNPAHRFAFGGTPGHIENGRLMLHLH 260
EcAKI     192  -----KVLDRALRIASVITHVAR-NNHYRVNEHFDQWNPFIIDYKNKDNPAHRFAFGGTPGHIENGRLMLHLH 260
TfMI      195  -----VLAERALRIAHFEVHVAAP-EDWRLEPHETPDQVQVADYNTDDRAHPEPYGVTVGHVLEARLLVHVE 263
Marme_2490 194  -----KVLDRALKLAKINICQKQASLSNSNEVHEVYTNWDQIDYKNKDPKHLFPMGPOPHQTEWAKLLMLD 263
AnabeanaAGE 175  -----LELENVLAATVQEVMGDFLD-DEQGLMYENVAPDGSIDC-----REGRLINPGHIEAMWIFINDIA 235
PorcineAGE 201  -----GRYALQGLHGCARRIL-QHVQ-RDQGVAVLENVSEDEGEELSG-----CLGRHONPGHAEAGHFLRHHS 260

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Fig. VI.1.a. Multiple alignment of amino acid sequences of AGE superfamily enzymes including CEs, AKIs, AGEs, and MI using MAFFTash. The red box sequences are the conserved regions. Blanket is a possible loop which is conserved in AKI and MI.

Multiple alignment of amino acid sequences of AGE superfamily enzymes including CEs, AKIs, AGEs, and MI using MAFFTash. The red box sequences are the conserved regions.

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RmCE      270 DVL-----GOATLRPRVQASLH-LARATLAEGRA-PDG-SLYYEIGE-----OGHLDTRHMMPOAEALVGFNLAYOES 336
CpCE      272 IAV-----GDEQLSRFRVRRAIN-LVRAA-LKGM-KDG-GLWYEYDTA-----EOHLIAEKHMMPQAEALVGLVNAWELS 338
BfCE      262 RVL-----GDAGLIAETEPVVKK-IAAAA-SEGLT-SDG-GMIYEKNLT-----TGHDIDGYHMMVQAEATVVGYYNLFYRF 328
PhCE      261 ESL-----GDENLIQFKDVAVK-MAMAS-IOGLD-ENG-GLNYEFE-----PSNMISREKHMVQAEAMVGFFNAFOLT 325
FjCE      264 EVL-----EEENLIANYKKHAVQ-IAEVT-KEGFD-TDG-GLNYEFEPK-----ENELIAEKHMVQAEALIGFYNAVOLT 330
DfCE      259 EIL-----HDEKLIGRMKQKSLA-MAATA-AKGLG-ADG-ALNYEYDPE-----TKHTOTDRSMVVAEOLVGFYNAYOLT 325
SlCE      262 DLLAANKPADKPKLOKAFQHIGVK-MARAA-STGYDPTDG-GMNYELEP-----DGHLNKERSMMVLAEAMVGFMNAVOLT 333
HaCE      274 HVL-----GDEHLIAKAETLAIG-MADAVYRNGRH-ADG-STIHERAP-----DGSINLERHMMVQAEAVVGFINAYOAT 340
RaCE      256 DVL-----GDEDLKKQFAEMDLK-ISHNIQDIALE--DG-ALNNERD-----KNEIDKTRVMVQAEAVVGFINAYOHS 320
EcCE      272 GIL-----DDEEITESLHPISSD-LVEKVYKEAFD--GHSIVNECE-----DGDVNTDRIMVQAEASVLGFLKAFERE 336
DtCE      260 RYI-----DNNKLIKEMSEINLK-IAEKVLEEAFE--SG-SLLNERV-----RGIVDKNRIWVQAEALVGFNLAYOKS 324
CsCE      260 KYI-----KDEKLKEEVEKLAL-VAQITLKEAFD--GQSILNEMI-----EDRIDRSKIWVQAEATVVGFINAYOKT 324
SdCE      271 ESL-----ESELHAHYKPAVIA-LAETCLEOGIG-RYN-EVLDAYNFE-----TQTLVDERVMVQAEALVGFNLAYHLA 338
TtCE      286 DVL-----GDDALKQSYRHDVIA-LAETCLAQARA-TDG-SLYDVFDKQ-----ROLNIPERVMVQAEAMVGFFNAWOLT 353
StCE      266 ELL-----YGHPLPDNFLSWIRP-MVEET-AQALEAHCG-SLPNEQRE-----DGSFDRARVMVQAEAFVGFNLAYQVF 332
SeAKI     261 AAL-----EARFETPPANLLLEDAKG-LFHATIRDAWAPDGDGFVYSVDMD--GKPIVRERVRNPIVEAMGTAYALMTLT 332
EcAKI     261 AAL-----EARCEQPPANLLLEDAKG-LFNATVRDAWAPDGDGFVYTVDNE--GKPVVRERVRNPIVEAMGTAYALMTVT 332
TfMI      264 AAL-----PDPPSWLLADAEA-MFAAAVARGNSVDGTEGFVYTLDDY--DTPVVRSRMHWVAAEISAAAVLGORT 331
Marne_2490 264 KRS-----PENNYLPKAKY-LFDLAYKKANDTKKG-GLHYGYAPD--GTVCDDPKYFVQAEASFAAALLKYAT 328
AnabeanaAGE 236 RRK-----ND-----SKTINQAVD-VVLNLIINFAND-NEYGGLYFMDAAGHP--POOLEWDQKLWVHLESVLALAMGYRLT 304
PorcineAGE 261 SRS-----GD-----AKLRAHVITDTFLLPFRSGND-ADHGGLFYFQDADGLCP--TQLEWAMKLMVPHSEAMIAFLMGVSES 330

RmCE      337 GEVLFYEAEDVMRYIRERORDTRG-GEWFAVRDDGAPY-P---DDKVDFWKGPYHNGRACLEATORLRLLEHVRSR-----
CpCE      339 GDPYFLDKAMASWOFIOOFLDKTD-GEWHWVGLDGKPI-E---EVKVGILKCPYHARACMEVVKRLK-----
BfCE      329 GDRGALOHSIDCNEFIKRLHLDVVH-GEWFWSLRADGSLNRD--DDKAGFWKCPYHGRMCIELLGE-----
PhCE      326 KEQTYDKFLKCNFEFTKAHIINTOK-GEWFWGVNEDLSLME--OYKVGILKCPYHGRACLEMIRRLGVNFDFFS-----
FjCE      331 GNEEYLKIVFENNFIKKHILDHKN-GEWFWGIHKDYSLLT-Q---KDKAGFWKCPYHGRACLELINRIKT-----
DfCE      326 KDAQFTKAEKSNDYIVNEFIDHER-GEWFGTVKEDGTPV-K---GDKINFWKCPYHARACAEEMRRTGKA-----
SlCE      334 HEKPFLDKSIKNEFTKKYLLDRON-GENYMGVTGDHTIV-G---KDKISFWKCPYHGRACLEMLERLDHLHG-----
HaCE      341 GKPEFAQAAYDSNFIORYFIDHDH-GDWFKILDAAONPLGA---IPKVGPFKCPYHARVCFEMIERLAEHNVSVQMRG-----
RaCE      321 GDEKFLSAKSVNENIKEYIIDKREGGENYSEVTFDHTPHDY--KETVGFWKCPYHGRMCMEVITRGVDI-----
EcCE      337 GKEEVRKAHEILAFILDKOVDKREGSEWFEMLKEDGTPC-H---KPMVREWKCPYHGRMCLEILKSGIEIG-----
DtCE      325 KLDKFLKAVFELNEFIKDFLDKRAQGEWFWKLDENYIPS-P---MPVEDLWKCPYHGRMCLEVIKRI-----
CsCE      325 KEEKYLDAAIKTWEIFIKHLVDRRKNSEWLKVNEDLEAV-N---MPIVEQWKCPYHGRMCLEIIRKVD-----
SdCE      339 GEORYLDAALNVNEFINSVQKDSVG-GEWHWLSRLDLPHT-G---DCKMGFWKAPYHNGRAMSISIKLVORIFGNNE-----
TtCE      354 GOKYLDVAVFSLNRYLEAEFI-TPG-SEWPWLARSDOGAGYR---AYLAGFWKGPYHNGRALMELISRINSVLNVDC-----
StCE      333 GEARYLEHALTVNRFIMHLDVDRG-GEWFWAVTPEGTLPLAG--YKGGFWKASVHNSRACLEGMRRIDAILLEERT-----
SeAKI     333 DDOYEEHYOKMNDYCIKYLMDYEN-GSNWQELDADINKVT-TKVNDGKQ---DIYHLHLCLVIPRLPLAPGLAPAVAAQLDINAHHHH
EcAKI     333 GDOVETWYQTMNEYCIKYLMDYEN-GSNWQELDADINKVT-T---GKQ---DIYHLHLCLVIPRIPLAPGLAPAVAAQLDINAK--
TfMI      332 GDERVEHYMYRTWIDHAATYFVDITVQ-GSNHHELDPTLAPPGGTWSGKP---DVYHAYQATRLPLPLAPSLAGALATVG-----
Marne_2490 329 KDETYYKQYLTLEWFSWNHMTDTE-GANVRI-DENNAAQY-D---NNKSPAGRTDVTHTMGACEVLTLTTL-----
AnabeanaAGE 305 GRDACWANYOKMHDYSWOFADPEY-GEWFGYLNRRGEVL-L---NLKGGKNKGFHVPRAMYLCWOQFEALS-----
PorcineAGE 331 GDPALLRLFLYQVAEYTFRQFRDPEY-GEWFGYLNRRGEKVA-L---TIKGGPFKGFHVPRCLAMCEMLLSALLSRLA-----

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Fig. VI.1.b. Multiple alignment of amino acid sequences of AGE superfamily enzymes including CEs, AKIs, AGEs, and MI using MAFFTash. The red box sequences are the conserved regions.

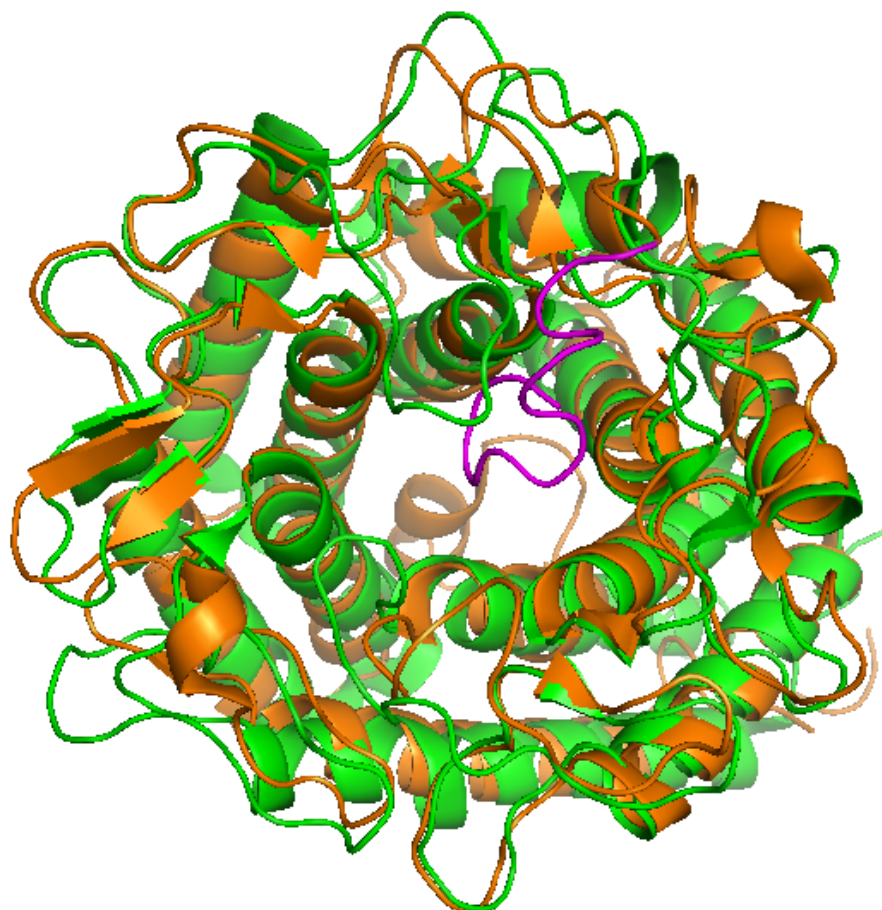


Fig. VI.2. Alignment structure of SeAKI and RmCE.

Comparison of overall structures of SeAKI (orange) and RmCE (green) is shown schematically in a ribbon model. Pink color is the conserved loop in AKIs.

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