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Construction and characterization of chimeric enzymes of kojibiose phosphorylase and trehalose phosphorylase from *Thermoanaerobacter brockii*

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Abstract—Chimeric phosphorylases were constructed of the kojibiose phosphorylase (KP) gene and the trehalose phosphorylase (TP) gene from *Thermoanaerobacter brockii*. Four chimeric enzymes had KP activity, and another had TP activity. Chimera V–III showed not TP, but KP activity, although only 125 amino acid residues in 785 residues of chimera V–III were from that of KP. Chimera V–III had 1% of the specific activity of the wild-type KP. Furthermore, the temperature profile and kinetic parameters of chimera V–III were remarkably changed as compared to those of the wild-type KP. The results of the molecular mass of chimera V–III using GPC (76,000 Da) strongly suggested that the chimera V–III protein exists as a monomer in solution, whereas wild-type KP and TP are hexamer and dimer structures, respectively. The result of the substrate specificity for phosphorolysis was that the chimera acted on nigerose, sophorose and laminaribiose, in addition to kojibiose. Furthermore, chimera V–III was also able to act on sophorose and laminaribiose in the absence of inorganic phosphate, and produced two trisaccharides, β-D-glucosyl-(1→6)-laminaribiose and laminaritriose, from laminaribiose.

Keywords: Kojibiose phosphorylase; Trehalose phosphorylase; Chimeric enzyme; *Thermoanaerobacter brockii*; Glycoside hydrolase family 65

1. Introduction

Phosphorylases that have been reported to date are classified into 14 types based on the phosphorolyzed substrates.[1] All of them catalyze exo phosphorolysis at the nonreducing end of the glycosidic linkage, and most phosphorolyze glucosidic linkages. Recently, enzymes that phosphorolyze galactosyl linkages[2,3] and *N*-acetylglucosaminyl linkages[4,5a] have been reported.

Kojibiose phosphorylase (KP; EC 2.4.1.230) catalyzes the reversible phosphorolysis of the α-(1→2)-glucosylic bond of kojibiose (α-D-glucopyranosyl-(1→2)-D-glucopyranose), and trehalose phosphorylase (TP; EC 2.4.1.64) reversibly phosphorolyzes the α,α-(1↔1)-glucosylic bond of trehalose (α-D-glucopyranosyl α-D-glucopyranoside). Both phosphorylases produce β-D-glucose 1-phosphate (β-G1P) and D-glucose during the phosphorolysis as follows: kojibiose + inorganic phosphate (Pi) ⇄ β-G1P + D-glucose, trehalose + inorganic phosphate (Pi) ⇄ β-G1P + D-glucose. Functionally, KP and TP catalyze the phosphorolysis of the α-glucosylic bond with an inversion of the anomeric configuration. Structurally, they are classified into the glycoside hydrolase family 65 (GH-65) based on the amino acid sequence (http://afmb.cnrs-mrs.fr/CAZY/GH.html). *Thermonanaerobacter brockii* ATCC35047 produces both KP and TP.[5b] We have already purified both enzymes[6,7] and cloned their genes.[8,9]

Protein engineering techniques have often been employed to improve the characteristics of the targeted enzymes. For industrial purposes, the enhancement of the thermostability of the targeted enzymes could lead

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to an improved durability of the enzymes[10] or reduced contamination in a reaction. The change in specificities of the enzymes could increase the yield of the main products,[11,12] thereby reducing any unfavorable by-products, or facilitate the construction of novel enzyme activities.[13,14] Chimerization is used to improve the enzymatic properties. Hagihara et al.[15] constructed the thermostable α-amylase by chimerization, and Ohdan et al.[16] introduced raw starch-binding domains into *Bacillus subtilis* α-amylase. Furthermore, this technique has often been used to identify, which regions of the enzymes could be important for their activities or functions.[17–20] Therefore, constructing chimeric enzymes of KP and TP would provide an opportunity for the construction of novel enzyme activity.

In this paper, we report the construction and characterization of chimeric enzymes produced from two phosphorylases, KP and TP.

2. Results

2.1. Screening for active chimeras from the library

As shown in Figure 1, we selected four regions, IEGD, MYRYN, FETARFW, and VDNNAYT, to construct chimeric gene library. After screening about 480 clones, five active chimeric enzymes, II, III, I–III, V–III, and IV–I were obtained (Fig. 2). Four chimeric enzymes, II, III, I–III, V–III, had KP activity, and another, IV–I, had TP activity. Their enzymatic activities are summarized in Table 2. Each chimeric enzyme showed a lower activity than the wild-type enzyme. Interestingly, although approximately 84% of the amino acid sequence of chimera V–III is from that of TP, chimera V–III had KP activity, but no TP activity. Therefore, we examined the enzymatic properties of this chimera in detail.

2.2. Properties of the chimera enzyme V–III

We obtained 75.3 mg of the purified chimera V–III enzyme with 283 U (synthetic activity of KP). The specific activity was 3.76 U/mg-protein, 1% of the activity of the wild-type KP (389 U/mg-protein). The molecular mass of the enzyme was estimated to be 90,000 (by SDS-PAGE) and 76,000 (by GPC). Both of them agreed with the value of 91,000 deduced from the amino acid sequence of chimera V–III. This strongly suggested that chimera V–III was a monomeric protein. As for the phosphorolytic activity, the optimal temperature of the chimera enzyme was 35 °C, 30 °C lower than the wild-type KP. This enzyme was thermally stable at 50 °C or

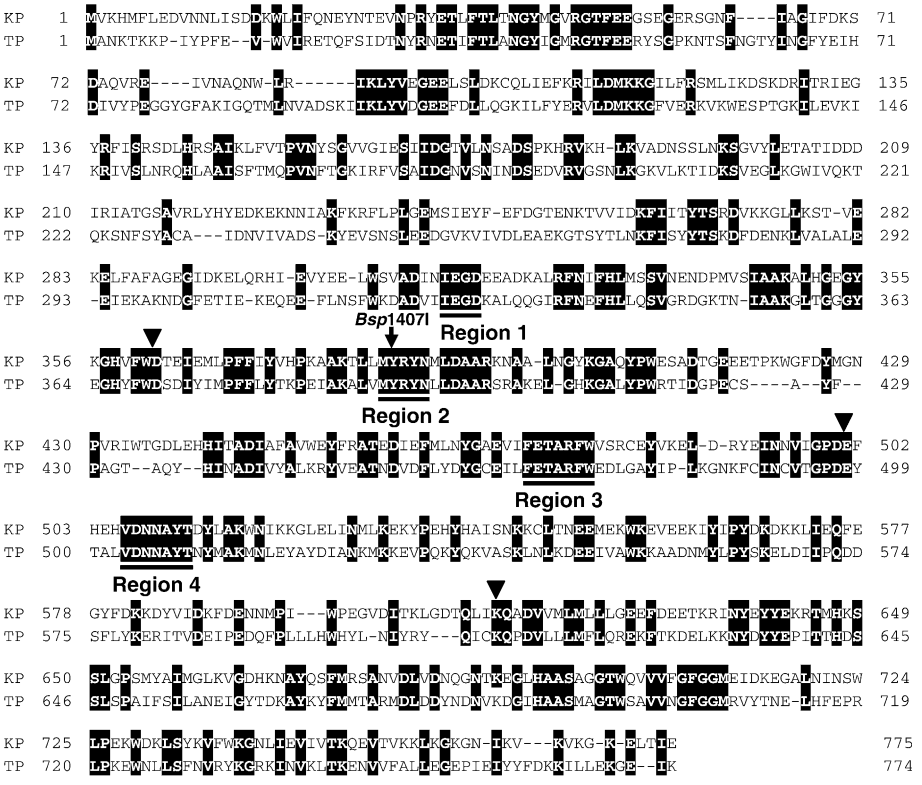

Figure 1. Alignment of amino acid sequences between kojibiose phosphorylase (KP) and trehalose phosphorylase (TP) from *T. brockii*. Residues in black boxes are conserved. The arrow indicates the restriction site of *Bsp*1407I. Regions 1–4 (underlined sequences) indicate the sites of recombination. Closed triangles indicate the putative catalytic residues of KP and TP based on the tertiary structure of maltose phosphorylase (MP) from *Lactobacillus brevis*.[21]

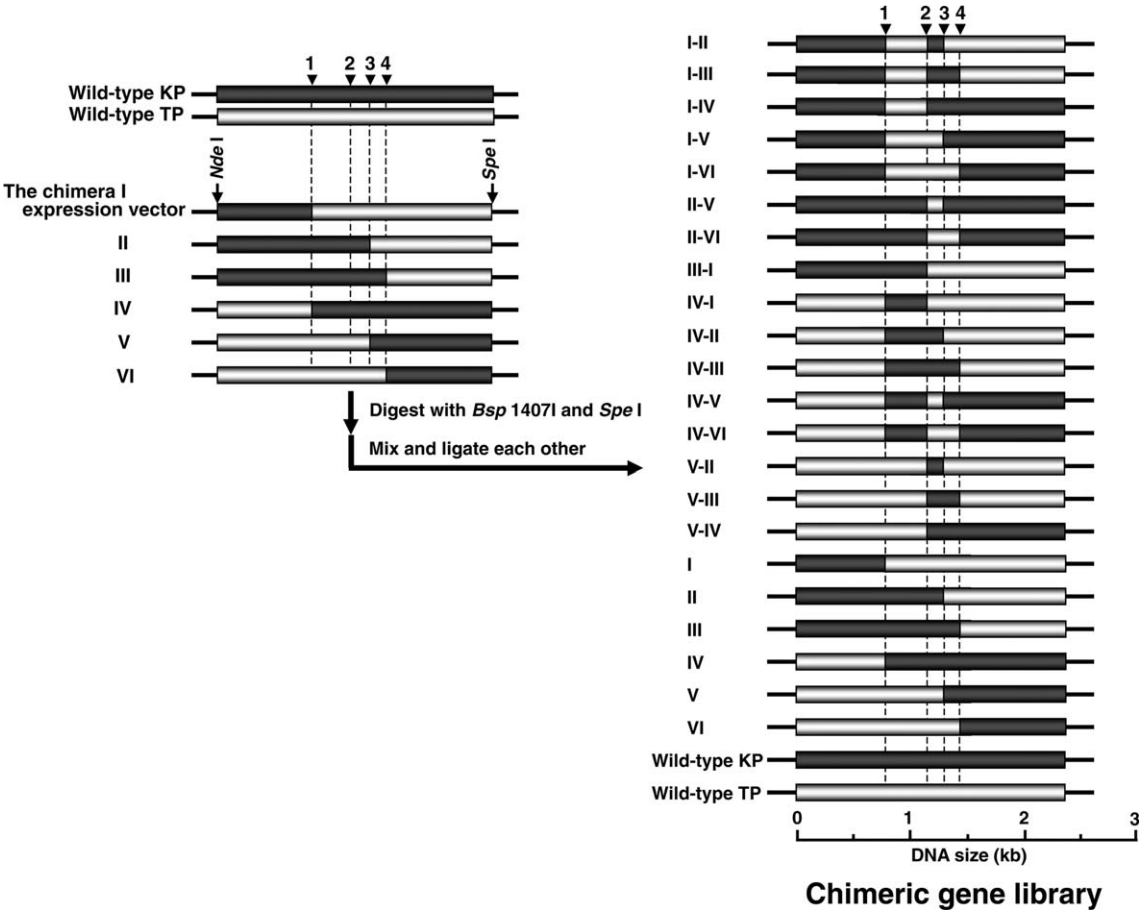

Figure 2. Construction schemes of the chimeric gene library. The methods of construction are described in Sections 4.4 and 4.5. Black and shaded bars indicate KP and TP genes, respectively. Closed triangles indicate the regions of recombination between KP and TP genes (see Fig. 1). There is a unique restriction site of *Bsp*1407I in region 2.

below at pH 5.5. The pH profile of the chimera was the same as the wild type. Temperature and pH profiles for the synthetic reaction of chimera V–III had the same values as the phosphorolytic reaction (Table 3). Table 4 shows the kinetic parameters of chimera V–III. For each examined substrate, the K_m value was higher than the wild-type KP. The K_m value for kojibiose was estimated to be 280 mM or higher, since the v–[s] plot for kojibiose was linear up to 280 mM (data not shown). The k_{cat}/K_m value for kojibiose was estimated to be $0.0025\ mM^{-1}\ s^{-1}$ from the slope of the v–[s] plot. This is lower than the wild-type KP by five orders of magnitude, whereas the values for β-G1P and glucose were lower than the wild-type enzyme by two to three orders of magnitude.

2.3. Specificity of the chimera enzyme V–III

As shown in Table 5, the acceptor specificity of chimera V–III for synthesis was investigated. The acceptor specificity was almost the same between chimera V–III and the wild-type KP. This is reasonable since the chimera has KP activity. From the substrate specificity of chimera V–III for phosphorolysis, it was found that the chimeric enzyme acted on nigerose (α-D-glucopyranosyl-(1→3)-D-glucopyranose), sophorose (β-D-glucopyranosyl-(1→2)-D-glucopyranose), and laminaribiose (β-D-glucopyranosyl-(1→3)-D-glucopyranose), in addition to kojibiose, although the wild-type KP strictly recognized and phosphorolyzed kojibiose. The relative activities toward kojibiose, nigerose, sophorose, and laminaribiose were 100%, 0.039%, 0.21%, and 0.35%, respectively. Furthermore, the chimeric enzyme was able to act on sophorose and laminaribiose in the absence of inorganic phosphate (relative activities were 0.097% and 0.26%), and produced tri-, tetra-, and pentasaccharides.

2.4. Isolation of a trisaccharide from laminaribiose

We collected the trisaccharide fraction produced from laminaribiose by HPLC using an MCI GEL CK04SS column, and 70 mg of the trisaccharide was obtained. NMR spectroscopy suggested that the trisaccharide would be a mixture of two components (data not shown). They were designated saccharides A and B. The trisaccharide solution was then put through a re-

Table 1. Oligonucleotide primers used for the construction of the six chimeric enzyme genes from the KP and TP genes[a]

Primer	Nucleotide sequence (Amino acid sequence)	Sequence of the recombination	Constructed chimeras
P1	5′-**GCAGAGCTTT**ATCCCCTTCAATATTTATATC-3′ L A K D G E I N I D	IEGD (region 1: position 315–318 of KP)	Chimera I
P2	5′-TGAAGGGGAT**AAAGCTCTGCAGCAAGGCATAC**-3′ E G D K A L Q Q G I	IEGD (region 1: position 315–318 of KP)	Chimera I
P3	5′-**CCCAAAATCT**TGCAGTCTCAAAAATGACTTC-3′ W F R A T E F I V E	FETARFW (region 3: position 472–478 of KP)	Chimera II
P4	5′-TGAGACTGCA**AGATTTTGGGAAGATTTAGGAG**-3′ E T A R F W E D L G	FETARFW (region 3: position 472–478 of KP)	Chimera II
P5	5′-**TGGTATAAGC**ATTATTATCAACATGCTCATG-3′ T Y A N N D V H E H	VDNNAYT (region 4: position 506–512 of KP)	Chimera III
P6	5′-TGATAATAAT**GCTTATACCAATTATATGGCG**-3′ D N N A Y T N Y M A	VDNNAYT (region 4: position 506–512 of KP)	Chimera III
P7	5′-CGTCTTCTTC**ATCTCCTTCTATGATTACATC**-3′ A E E D G E I I V D	IEGD (region 1: position 324–327 of TP)	Chimera IV
P8	5′-**AGAAGGAGAT**GAAGAAGCAGATAAAGCTTTG-3′ E G D E E A D K A L	IEGD (region 1: position 324–327 of TP)	Chimera IV
P9	5′-CCCAAAACCT**TGCAGTTTCAAATAATATTTC**-3′ W F R A T E F L I E	FETARFW (region 3: position 469–475 of TP)	Chimera V
P10	5′-**TGAAACTGCA**AGGTTTTGGGTATCTAGATG-3′ E T A R F W V S R	FETARFW (region 3: position 469–475 of TP)	Chimera V
P11	5′-CAGTATAAGC**GTTATTGTCAACTAATGCCG**-3′ T Y A N N D V L A	VDNNAYT (region 4: position 503–509 of TP)	Chimera VI
P12	5′-**TGACAATAAC**GCTTATACTGATTACCTTGC-3′ D N N A Y T D Y L	VDNNAYT (region 4: position 503–509 of TP)	Chimera VI
KPF	5′-GATATA*CATATG*GTAAAGCACATGTTTTTAGAG-3′	5′ of the coding region of the KP gene	
TPF	5′-GATATA*CATATG*GCCAACAAAACGAAGAAACC-3′	5′ of the coding region of the TP gene	
KPR	5′-CTGCAG*ACTAGT*TCATTATTCTATTGTCAGCTC-3′	3′ of the coding region of the KP gene	
TPR	5′-CTGCAG*ACTAGT*TCATTACTTTATTTCTCC-3′	3′ of the coding region of the TP gene	

[a] The sequences of single underline and bold letters indicate the sequences from the KP and TP gene, respectively. Overlapped sequences with the KP and TP genes are shaded. The *Nde* I site in the KPF and TPF primers and the *Spe*I site in the KPR and TPR primers are indicated by italic letters.

Table 2. Enzymatic activities of the active chimeras from KP and TP[a]

Chimera	Type of activity	Activity (U/mL)		Synthesis/phosphorolysis
		Synthesis	Phosphorolysis	
II	KP	1.6×10^{-2}	N.D.	—
III	KP	3.8	6.4×10^{-1}	5.9
I–III	KP	1.6×10^{-1}	5.4×10^{-3}	2.9×10^{1}
V–III	KP	1.1×10^{-1}	2.0×10^{-3}	5.5×10^{1}
Wild-type KP	KP	2.6×10^{2}	4.8×10^{1}	5.5
IV–I	TP	9.6	1.1×10^{1}	9.1×10^{-1}
Wild-type TP	TP	9.4×10^{1}	4.1×10^{1}	2.3

N.D., not determined.

[a] Synthetic activities were determined using 1% β-G1P and 1% D-glucose as substrates, so that the amount of Pi released was measured by the method of Fiske and Subbarow.[29] Phosphorolytic activities of chimera II, III, I–III, V–III, and the wild-type KP were assayed by using 1% kojibiose as a substrate. The substrate for phosphorolytic activities of chimera IV–I and the wild-type TP was trehalose. The glucose released by phosphorolysis was measured by the glucose oxidase–peroxidase method.[28]

Table 3. Enzymatic properties of chimera V–III, the wild-type KP and TP

		Chimera V–III	Wild-type KP[a]	Wild-type TP[b]
Optimum pH	Synthesis	5.5	—	6.0–7.0
	Phosphorolysis	5.5	5.5	7.0-7.5
Optimum temperature (°C)	Synthesis	35	—	—
	Phosphorolysis	35	65	70
pH stability	Synthesis	4.6–9.5	—	—
	Phosphorolysis	4.6–9.5	5.5–9.7	6.0–9.0
Thermal stability (°C)	Synthesis	50	—	—
	Phosphorolysis	50	65	60
Molecular mass (Da)	SDS-PAGE	90,000	83,000	88,000
	GPC	76,000	500,000	190,000

[a,b] Data taken from Chaen et al.[6,7]

Table 4. The kinetic parameters of chimera V–III and the wild-type KP

		Substrate			
		β-G1P	Glucose	Kojibiose	Pi
k_{cat} (s^{-1})	V–III	28	13	N.D.[a]	N.D.[a]
	KP	650	650	170	170
K_m (mM)	V–III	9.3	120	>280	58
	KP	0.77	3.5	0.77	0.85
k_{cat}/K_m ($mM^{-1}\ s^{-1}$)	V–III	3.0	0.11	0.0025	N.D.[a]
	KP	840	180	220	200

[a] N.D., not determined.

peated preparative HPLC using an ODS-AQ AQ-303 column. The fractions containing saccharides A and B were separately collected and then evaporated. The amounts of purified saccharides A and B were 7.4 mg (purity: 91.2%) and 9.5 mg (92.5%), respectively.

2.5. Characterizations of saccharides A and B

The molecular mass of saccharide A was found to be 504 Da by measuring the $[M+Na]^+$ ion (*m*/*z* 527) by ESIMS. This value was identical to that of an acyclic trisaccharide composed of glucose. The methylation analysis of saccharide A showed the following ratios: 2,3,4,6-tetra-*O*-methyl product:2,4,6-tri-*O*-methyl product:2,3,4-tri-*O*-methyl product = 0.89:1.00:0.98. To confirm this structure, NMR spectroscopy measurements were done. In the ^1H NMR spectrum of saccharide A, three anomeric protons were observed, and the coupling constants of the anomeric protons indicated the β-configuration ($J_{1,2}$ 7.7–7.9 Hz). The ^{13}C NMR spectrum of saccharide A contained 18 signals indicating that it should be a trimer of glucose (data not shown). From these results, it was suggested that saccharide A had one β-(1→3)- and one β-(1→6)-glucosidic linkage. Furthermore, the digestive analysis of saccharide A was done to determine the location of the β-(1→6)-glucosidic linkage. Since saccharide A was negligibly hydrolyzed by β-glucosidase from almond under the conditions described in Section 4.13, it was suggested that this saccharide had a β-(1→6)-linkage at the nonreducing end of the glucose. Based on these results, saccharide A was deduced to be β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl-(1→3)-D-glucopyranose (β-D-glucosyl-(1→6)-laminaribiose).

The mass spectrum of saccharide B also showed an $[M+Na]^+$ ion peak with a *m*/*z* ratio of 527 for the true

Table 5. Acceptor specificities of chimera V–III, the wild-type KP and TP[a]

Acceptor	Product		
	Chimera V–III	KP	TP[b]
D-Glucose	+++	+++	+++
D-Arabinose	—	+	—
D-Fructose	—	—	—
D-Galactose	—	+	++
D-Mannose	—	—	+
L-Rhamnose	—	—	N.D.[c]
L-Xylose	—	+	—
D-Glucosamine	—	—	++
N-Acetyl-D-glucosamine	—	—	+
2-Deoxy-D-glucose	—	—	++
Sorbitol	—	—	—
Mannitol	—	—	N.D.[c]
Trehalose	+++	+++	—
Maltose	+++	+++	—
Isomaltose	+++	+++	—
Cellobiose	—	++	—
Sucrose	++	++	—
Melibiose	—	—	N.D.[c]
Lactose	—	—	—
Lactulose	—	—	N.D.[c]

[a] The reaction mixture (0.5 mL) containing 5.2% β-G1P, 2% acceptor, and the enzyme (3.85 U/g β-G1P) in 50 mM acetate buffer, pH 5.5, was incubated at 35 °C (chimera V–III) or 60 °C (the wild-type KP) for 72 h. The reaction products were analyzed by TLC and GLC under the condition described in Sections 4.14 and 4.15. The transfer ratio to acceptor was detected by GLC. +++, transfer ratio is more than 40%; ++, 40–20%; +, 20–0%; —, no detected transfer product to acceptor.

[b] Data taken from Chaen et al.[7]

[c] N.D., not done.

mass of 504 Da. This value was identical to that of an acyclic trisaccharide composed of glucose. The methylation analysis of saccharide B showed the following ratio: 2,3,4,6-tetra-*O*-methyl product:2,4,6-tri-*O*-methyl product = 0.89:2.00. The ^{1}H NMR spectrum of saccharide B was completely identical to that of laminaritriose (data not shown). From these results, saccharide B was deduced to be β-D-glucopyranosyl-(1→3)-β-D-glucopyranosyl-(1→3)-D-glucopyranose (laminaritriose).

3. Discussion

We constructed the chimeric proteins of KP and TP from *T. brockii* ATCC35047, and five active chimeric enzymes, II, III, I–III, V–III, and IV–I were isolated. Four chimeras, II, III, I–III, and V–III, had KP activity. Especially, although only 125 amino acid residues (from regions 2–4, Fig. 1) in 785 residues of chimera V–III were from those of the wild-type KP, chimera V–III showed not TP but KP activity. Since both KP and TP catalyze the transglucosylation using β-G1P as the glucosyl donor, it is strongly suggested that the 125 amino acid region (regions 2–4) plays an important role in determining the specificity of KP. Other chimeric enzymes, III and I–III, having this region also show a KP activity. Another chimeric phosphorylase, IV–I, had TP activity. In 775 amino acid residues of chimera IV–I, the 69 amino acid region (from regions 1–2, Fig. 1) was from that of the wild-type KP. Since chimera IV–I shows TP activity, it is strongly suggested that regions 1–2, exchanged from KP are not important for the enzymatic specificity. This would also be supported by the fact that regions 1–2 of chimera V–III are from TP. An alignment analysis of KP and TP showed that there was a low homology region at position 419–440 of KP between regions 2 and 4 (Fig. 1). This region includes the residue Asp459 that may be involved in binding to the saccharides.[8] Both KP and TP belong to GH-65, which includes phosphorylases that produce β-G1P by phosphorolysis of disaccharides having an α-glucosidic linkage. Maltose phosphorylase (MP; EC 2.4.1.8) is also a member of GH-65. Based on the tertiary structure of MP from *Lactobacillus brevis*,[21] regions 2–4 of KP and TP are the component of the active site of KP and TP, respectively (Fig. 3A and C). Furthermore, although the region at position 428–440 of KP forms a loop adjacent to the active site, the region at position 429–436 of TP corresponding to that of KP does not (Fig. 3A and C). The structural difference in this region between KP and TP would cause the relative specificities of the enzymes. In fact, chimera V–III that showed KP activity has this region (Fig. 3B).

We compared the enzymatic properties of chimera V–III with those of the wild-type KP. The temperature profile, specific activity, kinetic parameters and substrate specificity of the chimera were remarkably changed as compared to those of KP. The molecular mass of chimera V–III obtained by SDS-PAGE and GPC strongly suggests that the chimerization caused a structural change in the enzyme from the oligomeric to monomeric structure. Furthermore, on the basis of the molecular modeling, the loop adjacent to the active site of chimera V–III that corresponds to the region at position 428–440 of KP was remarkably changed by chimerization (Fig. 3A and B). These structural distinctions would be responsible for the differences in the temperature profile, specific activity, kinetic parameters, and substrate specificity between chimera V–III and the wild-type KP. Similar observations have been reported on various enzymes.[22–24]

Chimera V–III acted on disaccharides having a β-glucosidic linkage (sophorose and laminaribiose), although

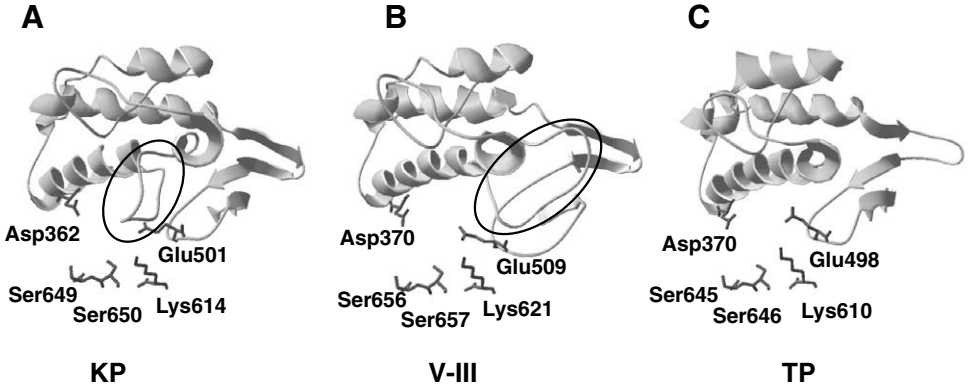

Figure 3. Putative tertiary structures of the regions 2–4 of KP (A), chimera V–III (B), and TP (C) based on the tertiary structure of MP.[21] The deduced amino acid sequence of MP was homologous to those of KP, chimera V–III and TP with 28.8%, 34.1%, and 34.3%, respectively. The main chains of proteins are shown as ribbons. The putative catalytic residues of KP are Asp362, Glu501, and Lys614 (chimera V–III, Asp370, Glu509, and Lys621; TP, Asp370, Glu498, and Lys610). The two serine residues in each model are phosphate binding sites. The circles indicate the loop (A and B). This figure is prepared using CPHmodels 2.0 X3M computer program to extract the 3D models (http://www.cbs.dtu.dk/services/CPHmodels/).

both KP and TP phosphorolyze only the α-glucosidic linkage. The transglucosylation products from laminaribiose were determined to be β-D-glucosyl-(1→6)-laminaribiose and laminaritriose, respectively. When the cell extract prepared from the host *Escherichia coli* was used as the enzyme solution under the same conditions, the transglucosylation products were not synthesized. Furthermore, when the protein concentration of the wild-type KP was equalized with chimera V–III, no transglucosylation product was detected. These facts would rule out the possibility that this β-transglucosylation is due to either the contamination of enzymes from the host cells or a side reaction of the native enzyme. We have already reported the substrate recognition of KP.[25] KP strictly recognizes the configuration of the hydroxyl groups of the acceptor glucose molecule at positions 2, 3, and 4, and catalyzes the transfer reaction to the hydroxyl at position 2 of the glucosyl residue. This substrate recognition to laminaribiose proposed three transglucosylation patterns as follows: first, chimera V–III may recognize the configuration of the hydroxyl groups of the glucose molecule at positions 3, 2, and 1, and catalyze the transfer reaction to the hydroxyl at position 3 of the glucosyl residue. Second, the chimera may recognize the configuration of the hydroxyl groups at positions 6, 4, and 3, and transfer the glucose residue to the hydroxyl at position 6. Finally, chimera V–III might catalyze the transfer reaction to the hydroxyl group at position 2 of the glucose recognized at positions 2, 3, and 4. In fact, laminaritriose and β-D-glucosyl-(1→6)-laminaribiose were synthesized in the first and second cases mentioned above, respectively. This suggests that the substrate recognition of KP was applicable to that of chimera V–III. In this study, the transfer product to laminaribiose by the β-(1→2)-linkage was not detected. The conformation of the reducing-end glucose molecule in laminaribiose might interfere with its binding to the enzyme or inhibit the transglucosylation.

In this study, we found the region that determined the specificity of KP by chimerization of two enzymes, KP and TP, belonging to the same family. Furthermore, chimera V–III acquired the unique activity that enabled it to act on disaccharides having a β-D-glucosidic linkage, although both KP and TP phosphorolyze only the α-D-glucosidic linkage. Chimerization bodes well for future prospects for making various enzymes for a variety of purposes.

4. Experimental

4.1. Materials

Trehalose was purchased from Hayashibara Shoji (Okayama, Japan). Kojibiose and β-G1P were prepared in our laboratory.[26,27] All other chemicals and reagents were of analytical or commercial grade.

4.2. Microorganisms and cultivation

E. coli JM109 (*recA*1, *endA*1, *gyrA*96, *thi*-1, *hadR*17, *sup*E44, *rel*A1, Δ(*lac-proAB*), [F′, *traD*36, *proAB*, *lacI*^{q}Z ΔM15]) was used as a host for gene cloning. *E. coli* BL21 [F-,*ompT*, $hsdS_B(r_B^- m_B^-)$, *gal*, *dcm*] was used for gene expression. *E. coli* cells were grown at 37 °C on LB agar plates containing 1% Bacto-Tryptone (Difco Laboratories, Detroit, MI, USA), 0.5% Bacto-Yeast Extract (Difco), 1% NaCl, and 1.5% Bacto-Agar (Difco). *E. coli* cells were then cultured at 37 °C in 2 × YT medium containing 1.6% Bacto-Tryptone (Difco), 1% Bacto-Yeast Extract (Difco), and 0.5% NaCl. When necessary, kanamycin (100 μg/mL) was added to the medium.

4.3. Plasmids

The plasmid pET38b(+) (Novagen, Darmstadt, Germany) was used as a vector for the gene cloning and expression. The plasmids pKBK14 containing the KP gene[8] and pKTP1 containing the TP gene[9] were used as templates for the PCR amplification.

4.4. Construction of six chimeric genes

To construct six chimeric genes of the KP and TP genes, we selected three regions, IEGD (region 1, position 315–318 of KP, or 324–327 of TP), FETARFW (region 3, position 472–478 of KP, or 469–475 of TP), and VDNNAYT (region 4, position 506–512 of KP, or 503–509 of TP), for recombination, since their sequences are completely identical between KP and TP (Fig. 1). The six genes of the chimeric enzymes, I–VI, were constructed using the synthetic oligonucleotide primers (Sigma Genosys Japan, Hokkaido, Japan) listed in Table 1. To construct the gene of chimera I, the 5′-side part of the gene was amplified by PCR using two primers, KPF and P1. Plasmid pKBK14 was used as the template. The PCR mixture (50 μL) consisted of 100 pmol of each primer, 0.2 mM of each dNTP, and 2.5 U of Takara *Pyrobest* DNA polymerase (Takara Shuzo, Kyoto, Japan). The template DNA (10 ng) was initially denatured for 1 min at 95 °C. The amplification profile (20 s at 98 °C, 30 s at 60 °C, and 2.5 min at 72 °C) was repeated for 25 cycles. In the same way, we also amplified the 3′-side part of the gene using two primers, P2, and TPR. Plasmid pKTP1 was used as the template. Finally, the chimera I gene was constructed by overlap PCR using two primers, KPF and TPR. The 5′- and 3′-parts of the genes were used as a set of templates, coding the entire chimera I. PCR amplification was done as described above except for the amount of the template (100 ng of each amplified DNA). Genes of the chimeras

II and III were also constructed by a procedure similar to that of the chimera I construction (Fig. 2). Constructions of the chimeras, IV–VI, were done in a manner similar to chimeras I–III. For chimera IV, to amplify the 5′-part of the chimera IV gene, two primers, TPF and P7, were used. Plasmid pKTP1 that includes the TP gene was used as the template. The 3′-part of the chimera IV gene was amplified by using two primers: P8 and KPR. Plasmid pKBK14 was used as the template. Finally, chimera IV was constructed by overlap PCR using two primers (TPF and KPR) and the templates (5′- and 3′-part of the chimera IV gene). Chimeras V and VI were also constructed by a procedure similar to that of the chimera IV construction (Fig. 2). The six amplified chimeric genes were digested with *Nde*I and *Spe*I and then ligated into pET38b(+) cleaved with the same restriction enzymes.

4.5. Construction of chimeric gene library

There is a unique restriction site of *Bsp*1407I on the KP and TP genes. This site is located from Met384 to Asn388 on KP, or from Met392 to Asn396 on TP (region 2 in Fig. 1). Recombination between the KP and TP genes generated no frame shift at this restriction site. Using this site, we shuffled the six chimeric genes and constructed a chimeric gene library. This library was expected to contain 24 genes including 22 chimeras and the wild-type KP and TP genes (Fig. 2). The expression vectors of the chimeras I–VI were separately digested with *Bsp*1407I and *Spe*I. All the digested DNA fragments were mixed together and then ligated to give the chimeric gene library (Fig. 2).

4.6. Screening for active chimeras from the library

E. coli BL21 transformed by the chimeric gene library were cultured at 30 °C for 5 h in 5 mL of the 2 × YT medium. An amount of 0.1 mM isopropyl β-D-thiogalactopyranoside was then added, and the cells were cultivated for 19 h at 30 °C. After cultivation, each enzyme solution was prepared as follows: the cultured broth was mixed with an equal volume of lysozyme solution (400 μg/mL, in 50 mM acetate buffer, pH 6.0) and incubated at 37 °C for 3 h. The reaction mixture containing 200 mM β-G1P, 100 mM glucose and enzyme solution in 50 mM acetate buffer, pH 5.5, was incubated at 50 °C overnight. Each reaction mixture was then subjected to TLC product analysis. Clones producing oligosaccharides were selected. The plasmid DNAs were then prepared from the selected clones. Each plasmid was digested with several restriction enzymes, and agarose gel electrophoresis was completed to investigate the digestive pattern of the plasmids. Clones harboring the plasmids with digestive patterns different from that of both the wild-type KP and TP genes were selected.

4.7. Enzyme purification

E. coli harboring an expression vector of a chimeric enzyme was aerobically cultured with shaking at 37 °C in 100 mL of 2 × YT medium in a 500-mL Erlenmeyer flask. *E. coli* cells were harvested by centrifugation (10,000*g* for 20 min) and washed with 20 mM acetate buffer, pH 6.0. The washed cells were resuspended in lysozyme solution (400 μg/mL, in 20 mM acetate buffer, pH 6.0) and incubated at 37 °C for 2 h. The lysozyme-treated cell suspension was sonicated for 2 min (Ultrasonic Homogenizer UH-600; SMT Company, Tokyo, Japan). After sonication, the cell suspension was centrifuged at 4 °C for 20 min. The supernatant was heat treated by incubation at 50 °C for 3 h in a water bath. Solid $(NH_4)_2SO_4$ was added to 70% saturation. The mixture was left at 4 °C for 48 h and then centrifuged (10,000*g* for 20 min). The precipitate was recovered, dissolved in 20 mM acetate buffer, pH 6.0, and dialyzed against the same buffer. The dialyzed solution was then put on a DEAE-Toyopearl 650 S column (2.5 × 45 cm; Tosoh Corp., Tokyo, Japan) equilibrated with 20 mM acetate buffer, pH 6.0. The adsorbed enzyme was eluted with a linear gradient of 0–0.4 M NaCl in the same buffer. The active fractions were collected, and solid $(NH_4)_2SO_4$ was added to a final concentration of 1.5 M. This solution was put on a Butyl-Toyopearl 650 M column (1.6 × 49 cm; Tosoh) equilibrated with 20 mM acetate buffer, pH 6.0, containing 1.5 M $(NH_4)_2SO_4$. The adsorbed enzyme was eluted with a linear gradient of 1.5–0 M $(NH_4)_2SO_4$ in the same buffer. The active fractions were pooled and dialyzed against 20 mM acetate buffer, pH 6.0. The dialyzed solution was collected as the purified enzyme preparation.

4.8. Enzyme assay

Phosphorolytic activity of KP was assayed using kojibiose. The substrate solution contained 1% (w/v) kojibiose and McIlvaine buffer (pH 5.5; Pi concentration, 0.1 M) in a total volume of 2.0 mL. The enzyme solution (0.2 mL) was then added, and the reaction was initiated. After incubation at 50 °C for 30 min, the reaction was stopped by boiling for 10 min. The released glucose was measured by the glucose oxidase–peroxidase method.[28] One unit of enzyme activity was defined as the amount of enzyme liberating 1 μmol glucose per min under the above-mentioned conditions. Phosphorolytic activity of TP was assayed using the substrate solution containing 1% (w/v) trehalose and McIlvaine buffer (pH 7.0; Pi concentration, 0.1 M). The reaction condition was described in the 'phosphorolytic activity of KP'.

Synthetic activity of KP was assayed using β-G1P and glucose. The substrate solution contained 1% (w/v) glucose, 1% (w/v) β-G1P, and 50 mM acetate buffer,

pH 5.5, in a total volume of 2.0 mL. The enzyme solution (0.2 mL) was then added, and the reaction was started. After incubating this mixture at 50 °C for 30 min, the reaction was stopped by boiling for 10 min. The amount of released Pi was measured by the method of Fiske and Subbarow.[29] One unit of enzyme activity was defined as the amount of enzyme liberating 1 μmol Pi per min under the above-mentioned conditions. The synthetic activity of TP was assayed using the substrate solution containing 1% (w/v) glucose, 1% (w/v) β-G1P, and 0.1 M Tris–HCl buffer, pH 7.0. The reaction condition was described in the 'synthetic activity of KP'.

4.9. Electrophoresis of proteins

SDS-PAGE and Native-PAGE of the enzymes were done using the method of Laemmli with a gradient gel (5–20% polyacrylamide gel; Atto, Tokyo, Japan).[30] A broad-range size marker (Bio-Rad Laboratories, CA, USA) was used as the standard proteins for SDS-PAGE.

4.10. Gel-permeation chromatography (GPC)

A GPC was generated using a Superose™ 6 HR 10/30 column (1.0 × 30 cm; Amersham Biosciences, NJ, USA) equilibrated with 20 mM acetate buffer, pH 6.0, containing 0.2 M NaCl. A gel-filtration standard (Bio-Rad) was used as the standard proteins for GPC.

4.11. Preparation and isolation of trisaccharides from laminaribiose

To prepare the trisaccharides, a reaction mixture containing 58 mM of laminaribiose, chimera V–III (8.21×10^{-1} U/mmol for laminaribiose), and 10 mM $CaCl_2$ in 32.5 mL of 100 mM acetate buffer, pH 5.5, was incubated at 35 °C for 96 h. Proteins were removed from the resulting reaction mixture by filtration using an ultrafilter unit, USY-1 (Advantec, Tokyo, Japan), and then the mixture was desalted by passing it through ion-exchange resins (Diaion PK218:WA30 (Mitsubishi Chem., Tokyo, Japan): IRA411S (Japan Organo, Tokyo, Japan) = 1:2:1 (v:v:v)). The eluent was concentrated to 5 mL by evaporation at 40 °C. The saccharide solution was analyzed by HPLC equipped with an MCI GEL CK04SS column (1.0 × 20 cm × 2; Mitsubishi Chem.). The fractions containing the trisaccharides were then concentrated to 5 mL by evaporation at 40 °C. The trisaccharide solution was repeatedly put through the HPLC equipped with an ODS-AQ AQ-303 column (0.46 × 25 cm, YMC Co., Kyoto, Japan). Each trisaccharide solution was then concentrated to 1 mL by evaporation at 40 °C.

4.12. High-performance liquid chromatography (HPLC)

The compositions of the neutral saccharides in the samples were determined by HPLC. An HPLC analysis was performed using an ODS-AQ AQ-303 column (0.46 × 25 cm; YMC), or an MCI GEL CK04SS column (1.0 × 20 cm × 2; Mitsubishi Chem.).[27]

4.13. Hydrolysis with β-glucosidase

To confirm the structure of the trisaccharides, they were hydrolyzed with β-glucosidase (EC 3.2.1.21) from almond (Bioenzyme Laboratories, South Wales, UK). This enzyme catalyzes the exo hydrolysis at the non-reducing end of the glycosyl linkage and hydrolyzes the β-(1→3)-linkage, but does not easily digest the β-(1→6)-linkage. The reaction mixture containing 100 μL of 1% (w/v) sample and 100 μL of the enzyme solution (0.1 U/mL in 20 mM acetate buffer, pH 4.5) was incubated at 40 °C for 24 h. The reaction mixture was then analyzed by gas–liquid chromatography (GLC).

4.14. Thin-layer chromatography (TLC)

TLC was performed using Silica Gel 60 F254 plates (E. Merck, Darmstadt, Germany) with a solvent containing 6:4:1 *n*-butanol–pyridine–water. The sugars were detected by spraying with 20% sulfuric acid in MeOH solution and heating at 110 °C for 10 min.

4.15. Gas–liquid chromatography (GLC)

A sample of the saccharides was dried in the decompression chamber and then subjected to trimethylsilylation.[31] GLC analysis was performed using a GC-16 instrument (Shimadzu Corporation, Kyoto, Japan) with a stainless steel column packed with 2% silicone OV-17 on Chromosorb W (60–80 mesh, GL Sciences Inc., Tokyo, Japan) at 160–320 °C (7.5 °C/min). The carrier gas was helium at a flow rate of 1.0 mL/min. Detection was by a flame-ionization detector (FID).

4.16. Methylation analyses

Methylation analyses were performed according to the method of Hakomori.[32] The resulting partially methylated alditol acetates were analyzed by GLC (GC-14B, Shimadzu) using a DB-5 capillary column (J&W Scientific Co., Folsom, CA, USA) at 130–250 °C (5 °C/min).[27]

4.17. MS analysis

The molecular mass was determined by electrospray-ionization mass spectrometry (ESIMS).[27]

4.18. NMR spectroscopy

The NMR spectral data were recorded for 1% solutions in D_2O at 27 °C using a JNM-AL300 spectrometer (^1H 300.4 MHz, ^{13}C 75.45 MHz: JEOL, Tokyo, Japan).

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