A motif rich in charged residues determines product specificity in isomaltulose synthase

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Abstract Isomaltulose synthase (PalI) catalyzes hydrolysis of sucrose and formation of α-1,6 and α-1,1 bonds to produce isomaltulose (α-D-glucosylpyranosyl-1,6-D-fructofranose) and small amount of trehalulose (α-D-glucosylpyranosyl-1,1-D-fructofranose). A potential isomaltulose synthase-specific motif (^{325}RLDRD^{329}), that contains a 'DxD' motif conserved in many glycosyltransferases, was identified based on sequence comparison with reference to the secondary structural features of PalI and homologs. Site-directed mutagenesis analysis of the motif showed that the four charged amino acid residues (Arg^{325}, Arg^{328}, Asp^{327} and Asp^{329}) influence the enzyme kinetics and determine the product specificity. Mutation of these four residues increased trehalulose formation by 17–61% and decreased isomaltulose by 26–67%. We conclude that the 'RLDRD' motif controls the product specificity of PalI.

Key words: Isomaltulose synthase; Motif; Sucrose isomerization; Isomaltulose; Trehalulose

1. Introduction

Isomaltulose (α-D-glucosylpyranosyl-1,6-D-fructofranose), a functional isomer of sucrose commonly referred to as palatinose, has generated significant interest in the last two decades. It has potential as a functional sugar for human consumption [1–3]. So far only a small range of bacterial species are known to be able to produce this sucrose isomer. These bacterial species include *Serratia plymuthica* [4], *Erwinia rhapontici* [1], *Klebsiella planticola* [5], *Pseudomonas mesoacidophila* [6], *Protaminobacter rubrum* and *Enterobacter* sp. [7]. Isomaltulose synthase has been purified and characterized biochemically [8,9], but not at the molecular level.

Isomaltulose synthase, besides catalyzing isomerization of sucrose to produce isomaltulose, also produces another sucrose isomer, trehalulose (α-D-glucosylpyranosyl-1,1-D-fructofranose), as well as small amounts of glucose and fructose as byproducts [5,8]. The ratio of the enzyme products varies depending on the bacterial strains. *E. rhapontici* NCPPB 1579 [1], *K. planticola* CCRC 19112 [5], *P. rubrum* [7], and *S. plymuthica* NCIB 8285 [10] produce mainly isomaltulose (75–85%) whereas *P. mesoacidophila* MX-45 [6] and *Agrobacterium radiobacter* MX-232 [11] produce significantly more trehalulose (about 90%) than isomaltulose. It is not yet clear what structural features of these enzymes determine their product specificity.

To understand the mechanism of sucrose isomerization at the molecular level and identify the key amino acid residues involved in the enzyme reaction, we recently cloned and sequenced the *palI* gene (GenBank accession number AY040843) encoding an isomaltulose synthase (PalI) from a bacterial isolate *Klebsiella* sp. LX3 [12]. The refinement of crystal structure of PalI (PDB code: 1M53) indicates that PalI possesses a $(\beta/\alpha)_8$-barrel, a characteristic feature of the glucoside hydrolase (GH) family 13 [13]. Moreover, the three-dimensional (3-D) structure of PalI is identical to the structures of oligo-1,6-glucosidase (OGL) from *Bacillus cereus* [14] and amylosucrase (AS) from *N. polysaccharea* [15] at the N-terminal catalytic domain (manuscript in preparation). Significantly, these structural homologs are functionally different. The enzyme OGL catalyzes a one-step enzyme reaction, i.e. hydrolysis of glycosidic bond, and AS is a hexosyltransferase that catalyzes the synthesis of an amylose-like polymer from sucrose [16]. PalI, however, is responsible for a two-step enzyme reaction, i.e. hydrolysis of the α-1,2 glycosidic bond of sucrose and then formation of α-1,6 and α-1,1 bonds simultaneously to produce isomaltulose and trehalulose. The functional difference suggests that PalI may contain unique structural features required for isomerization.

To identify the specific features involved in isomerization of sucrose, we located a putative sucrose isomerization motif by sequence comparison with reference to the secondary structural features of PalI and its homologs. The influences of the charged residues in this motif on sucrose isomerization were investigated by site-directed mutagenesis and enzymatic analysis.

2. Materials and methods

2.1. Bacterial strains, plasmids and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* DH5α was used as the host strain for both cloning and expression of *palI* and its derivatives. The strain was grown aerobically at 37°C in Luria–Bertani (LB) medium supplemented with 50 g sucrose per l. Ampicillin (100 mg/l) was added at 100 mg per l when required.

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2.2. Site-directed mutagenesis and DNA manipulation

Site-directed mutagenesis of *pal*I was performed using the QuikChange site-directed mutagenesis kit (Stratagene). Plasmid clone pGEK, in which *pal*I was fused to the gene encoding glutathione S-transferase (GST) in the same open reading frame [12], was used as a template. Each desired amino acid replacement was generated by using two synthetic oligonucleotide primers. After 12 amplification cycles (95°C, 30 s; 55°C, 1 min; 68°C, 12 min) with *pfu* Turbo DNA polymerase (Stratagene), the polymerase chain reaction (PCR) products were treated with *Dpn*I and the nicked plasmid DNA incorporating the desired mutation was transformed into the competent cells of *E. coli* DH5α. The mutations were confirmed by DNA sequencing. Seven derivatives of pGEK were created (Table 1). DNA manipulation was carried out according to standard procedure [17].

2.3. Enzyme purification

Overexpression and purification of PalI and its mutant versions were carried out following the procedure described previously [18]. In brief, PalI and derivatives were purified by affinity chromatography using glutathione-Sepharose 4B (Amersham Pharmacia Biotechnology), and separated from GST after digestion with thrombin. The purified proteins were analyzed by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) [19] and stained with Coomassie blue R-250 (Bio-Rad). Protein concentration was determined by Bradford method using bovine serum albumin as the standard [20].

2.4. Isomaltulose synthase assay

Unless otherwise stated, 2 μg of enzyme was mixed with 400 μl of 0.1 M citrate-phosphate buffer (pH 6.0) containing 4% sucrose in a final solution of 500 μl and incubated at 35°C for appropriate time with gentle agitation. Reducing sugar was determined by dinitrosalicylic acid (DNS) methods [21], using isomaltulose (Sigma) as the standard. One unit of PalI activity is defined as the amount of enzyme required to catalyze formation of 1 μmol of isomaltulose in 1 min under assay conditions. Data presented are the means of three individual experiments.

2.5. In vitro conversion of sucrose

In vitro conversion of sucrose was carried out in a test tube containing 2 ml of 4% sucrose solution and 20 μg of enzyme at 35°C in a shaking water bath for 4–6 h. Individual sugar formed from sucrose was analyzed by high performance liquid chromatography (HPLC).

2.6. Sugar analysis by HPLC

Quantitative analysis of individual sugars was undertaken by HPLC (Waters 2690) using a Symmetry® C18 column (4.6×250 mm) and a Refractive Index Detector (Waters 2410). Samples were passed through 0.2 μm membrane filters, injected to HPLC by autosampler, and eluted isocratically with water at a flow rate of 1 ml/min. Individual sugars were identified by comparison of retention times with those of standards. The relative percentage of each sugar was calculated based on its peak area. Standard sugar samples were obtained from Sigma.

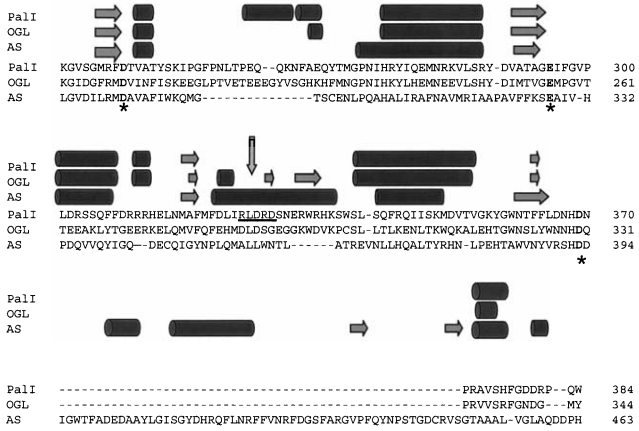

Fig. 1. Comparison of partial secondary structure in the catalytic domain between PalI (1M53), *B. cereus* OGL (1U0K) and *N. polysaccharea* AS (1G5A). The DALI server was used for secondary structure alignment. The secondary structure of PalI was resolved at 2.2 Å (manuscript in preparation) and the genuine α-helix (horizontal column) and β-sheet (horizontal arrow) of PalI, OGL and AS were shown at the top of the sequence. Three catalytically active residues (D^{241}, E^{295}, and D^{369} in PalI) were indicated by stars. The vertical arrow shows the isomaltulose synthase motif 'RLDRD'.

3. Results

3.1. Identification of potential motif in PalI that affects isomerization

Based on the preliminary X-ray data of PalI [13], we worked out the secondary structure of PalI, which was aligned with the OGL of *B. cereus* and the AS of *N. polysaccharea*. We also searched for potential motifs by using bioinformatic tools. As shown in Fig. 1, an interesting short sequence ^{325}RLDRD^{329} was identified. The motif is situated at a loop region that shares little similarity in the secondary structural architecture with those of OGL and AS (Fig. 1). Besides, the short sequence contains the 'DxD' motif that has been identified in 13 families of glycosyltransferases and shown to be critical for enzyme activity [22,23]. Moreover, this short sequence contains four charged amino acid residues, i.e. two Asp and two Arg residues. It was proposed that the charged amino acid residues at the active center could play an important role [9]. Identical sequence is also found in the predicted peptide sequences of the isomaltulose synthase genes from *E. rhapontici* (^{325}RLDRD^{329}) [24], *P. rubrum* (^{325}RLDRD^{329}) and *Enterobacter* sp. SZ62 (^{325}RLDRD^{329}) [7]. Interestingly, we noted that the sucrose isomerase from *P. mesoacidophila* MX-45, which is known to produce more than 90% of trehalulose and small amount of isomaltulose [6], contains a differ-

Table 1
Bacterial strains and plasmids used in this study

Strain/plasmids	Genotype or phenotype	References
E. coli DH5α	*recA1endA1hsdR17supE4gyrA96 relA1 Δ(lacZYAargF)U169(80DLACzΔM15)*	[17]
pGEK	*pal*I ORF without signal peptide coding sequence fused with GST in PGEX-2T	[12]
pGEK/R^{325}D	Arg residue at 325 in PalI was replaced by Asp in pGEK	this study
pGEK/R^{325}L	Arg residue at 325 in PalI was replaced by Leu in pGEK	this study
pGEK/D^{327}R	Asp residue at 327 in PalI was replaced by Arg in pGEK	this study
pGEK/D^{327}N	Asp residue at 327 in PalI was replaced by Asn in pGEK	this study
pGEK/R^{328}D	Arg residue at 328 in PalI was replaced by Asp in pGEK	this study
pGEK/R^{328}L	Arg residue at 328 in PalI was replaced by Leu in pGEK	this study
pGEK/D^{329}N	Asp residue at 329 in PalI was replaced by Asn in pGEK	this study

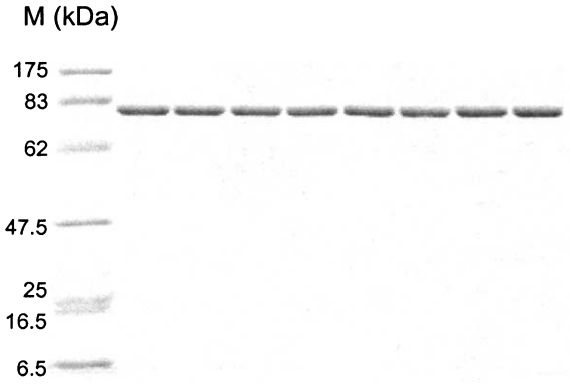

Fig. 2. SDS–PAGE analysis of the purified PalI and its variants. Symbol: M, protein marker. Protein samples from left to right: PalI, PalI:R^{325}D, PalI:R^{325}L, PalI:D^{327}R, PalI:D^{327}N, PalI:R^{328}D, PalI:R^{328}L, PalI:D^{329}N.

ent corresponding short sequence (^{311}RYDRA^{315}) [7]. These data suggest that the short sequence '^{325}RLDRD^{329}' could play an important role in specifying isomaltulose production.

3.2. Effect of mutations on PalI activity

To test the role of the '^{325}RLDRD^{329}' motif, we replaced separately the two positively charged Arg residues in the motif with negatively charged Asp (PalI:R^{325}D, PalI:R^{328}D) and hydrophobic Leu (PalI:R^{325}L, PalI:R^{328}L); and the two negatively charged Asp residues of the motif with positively charged Arg (PalI:D^{327}R) and hydrophilic Asn (PalI:D^{327}N, PalI:D^{329}N) by site-directed mutagenesis (Table 1). To characterize these PalI derivatives, the *palI* and its mutant versions were overexpressed and the enzymes were purified (see Section 2). The mutations did not appear to affect the protein expression level as visualized by SDS–PAGE (Fig. 2), but resulted in changes in enzyme kinetics. The kinetic analysis of these mutants showed that the K_{cat} was decreased by 1.5–13.5-fold and the K_m increased by about 2–4-fold, except PalI:D^{327}N and PalI:D^{329}N with close K_{cat} values, and PalI:R^{325}D(L) showing similar K_m parameters to the native PalI, respectively (Table 2).

3.3. Effect of mutation on PalI product specificity

To investigate the role of the '^{325}RLDRD^{329}' short sequence in sucrose isomerization, we analyzed the product profiles of PalI and its derivatives. After the reaction, samples were analyzed by HPLC and individual sugars were identified by

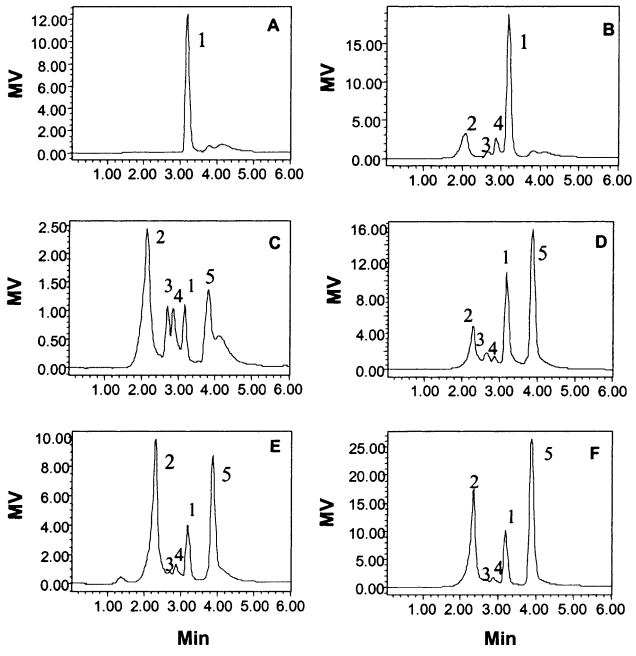

Fig. 3. HPLC analysis of the sugars converted by PalI and its four mutants from sucrose. A: Isomaltulose. B–F: The reaction products of PalI, PalI:R^{325}D, PalI:D^{327}R, PalI:R^{328}D, and PalI:D^{329}N. Standards were used to determine the retention times of isomaltulose (3.18 min), trehalulose (2.2 min), glucose (2.67 min), fructose (2.87 min) and sucrose (3.88 min), and assigned the HPLC peaks accordingly (1, isomaltulose; 2, trehalulose; 3, glucose; 4, fructose; 5, sucrose). MV, recorder output (in mV). $MV = 2000 \times SF \times S \times \Delta n$, where SF is the scale factor, S the sensitivity setting, and Δn the refractive index.

comparison with standards (see Section 2). Fig. 3 shows the HPLC profiles of the reaction products from PalI and its four mutants. Only four products (isomaltulose, trehalulose, glucose, and fructose) were identified by the method used. We calculated the relative percentage of individual sugar based on its peak area. As shown in Table 2, replacements of any of the four residues Arg^{325}, Arg^{328}, Asp^{327} and Asp^{329} reduced the percentage of isomaltulose but increased the percentage of trehalulose in the reaction products. In particular, mutation of Asp^{327} and Asp^{329} respectively to Asn decreased the isomaltulose percentage by 43–52%, and increased the trehalulose percentage by 41–54%. Mutation of Arg^{325} and Arg^{328} separately to Asp or Leu decreased the isomaltulose percentage by 52–67%, and increased the trehalulose percentage by

Table 2
Influence of residue substitutions in isomaltulose synthase motif on enzyme kinetics[a] and conversion of sucrose to isomaltulose and trehalulose[b]

Mutants	K_m (mM)	k_{cat} (min^{-1})	Isomaltulose		Trehalulose	
			Content	Decrease (%)	Content	Increase (%)
PalI	54.3 ± 1.5	173.9 ± 3.5	82.8 ± 3.6	–	12.1 ± 1.3	–
PalI:R^{325}D	44.0 ± 1.6	12.0 ± 1.2	15.8 ± 2.0	67.0	55.7 ± 4.1	43.6
PalI:R^{325}L	49.1 ± 1.9	12.0 ± 1.2	28.5 ± 5.3	54.3	43.4 ± 5.0	31.3
PalI:D^{327}R	121.7 ± 4.6	71.0 ± 2.4	56.3 ± 4.4	26.5	29.8 ± 3.8	17.7
PalI:D^{327}N	89.9 ± 2.3	122.8 ± 2.8	39.1 ± 4.6	43.7	53.1 ± 4.3	41.0
PalI:R^{328}D	108.9 ± 2.8	14.6 ± 1.4	19.2 ± 1.6	63.6	73.3 ± 4.0	61.2
PalI:R^{328}L	195.5 ± 3.9	34.5 ± 1.9	30.9 ± 2.4	51.9	62.4 ± 5.4	50.3
PalI:D^{329}N	157.7 ± 4.7	148.3 ± 1.5	30.4 ± 1.5	52.4	66.8 ± 2.8	54.7

[a]Enzyme (2 μg) was mixed with different concentrations of sucrose (10–150 mM) dissolved in 0.1 M citrate-phosphate buffer (pH 6.0) at a final volume of 500 μl. The K_m and k_{cat} values were calculated based on the *Michaelis–Menten* equation.
[b]Enzyme (20 μg) was mixed with 2 ml of 5% sucrose solution and incubated at 35°C for about 4–6 h. The converted products were analyzed by HPLC and the percentage of individual sugar was calculated based on peak area.

31–61% (Table 2). Mutation of Arg^{325} to Asp or Leu significantly enhanced the percentage of glucose and fructose in the reaction product by about 16% and 21%, respectively, while replacement of the other three residues has minor impact on glucose and fructose formation.

4. Discussion

Isomaltulose synthase (PalI) from *Klebsiella* sp. LX3 is a novel member of the α-amylase family [12]. Most members of the family catalyze either hydrolysis of carbohydrates to produce monomers or formation of polymers from mono sugars, whereas PalI has dual functions – hydrolysis of sucrose at the α-1,2 bond and then formation of α-1,6 and α-1,1 bonds separately to produce isomaltulose and trehalulose. By using site-directed mutagenesis and the subsequent enzyme product analysis, the current study established an isomaltulose synthase-specific motif '^{325}RLDRD^{329}' which contributes to the unique feature of PalI. The motif is rich in charged amino acid residues. Replacement of the charged residues of the motif with neutral or opposite charged residues may have significant effect on enzyme kinetics, and dramatically increases the percentage of trehalulose in the reaction product, demonstrating that the '^{325}RLDRD^{329}' motif not only plays an important role in the isomerization process but also controls the PalI product specificity.

The '^{325}RLDRD^{329}' motif is located in a region showing a distinct secondary structural feature different from the *B. cereus* OGL and *N. polysaccharea* AS, the close homologs of PalI (Fig. 1). Not surprisingly, the 'RLDRD' motif was not found in OGL and AS, as these two enzymes are functionally different from PalI. Analysis of the putative peptide sequences of several isomaltulose synthase genes confirmed that all contained the 'RLDRD' motif [7,24], although the homology of these peptide sequences to PalI varies from 67 to 99% [12]. The motif is rich in charged amino acid residues. To probe the role of the charged amino acid residues in the motif, we used either neutral or the opposite charged residues to replace the native charged residues in the motif by site-directed mutagenesis. Our results show that mutation of any of the four charged residues in the short sequence, ^{325}RLDRD^{329}, did not abolish enzyme activity. Rather, it resulted in an increased ratio of trehalulose to isomaltulose in the enzyme reaction products (Table 2). The most striking mutations were PalI:R^{328}D and PalI:D^{329}N, which did not significantly affect glucose and fructose formation but resulted in a 61 and 54% increase in trehalulose production, respectively. Especially, PalI:D^{329}N showed a similar K_{cat} value to its parental enzyme PalI (Table 2). Charge distributions in the isomaltulose synthase motif appear to influence α-1,1 and α-1,6 glucosidic bond formation and the stability of glucose and fructose binding to the enzyme. In particular, the Arg^{328} and Asp^{329} residues of PalI contribute significantly to the formation of isomaltulose.

Our data provide molecular evidence that the charged amino acids at the enzyme active center play important roles in sucrose isomerization and product specificity. The finding appears to be consistent with the finding that the major product of sucrose isomerase from *P. mesoacidophila* MX-45, in which the corresponding motif is ^{311}RYDRA^{315} [7], is trehalulose but not isomaltulose [6,25].

The DxD motif is conserved in many nucleoside diphosphate-binding glucosyltransferases [23]. The motif has been shown to be crucial for enzyme activity [22,23,26,27]. It has been suggested that the DxD motif participates in the coordination of the divalent cations (Mn^{2+}, Mg^{2+}) that are required for the binding of nucleotide sugar [22,23]. Crystal structure analysis on a bovine β4-galactosyltransferase and its complex with UDP-galactose showed that the ^{252}DxD^{254} motif was located at the bottom of the substrate binding and catalytic pocket, and Asp^{254} was involved in binding of the β-phosphate group of the substrate [28]. However, the study on the notch signaling regulator fringe, which was characterized recently as a glycosyltransferase, showed that the DxD motif is important for enzyme activity but not for nucleotide sugar binding [26]. These findings indicate that not all DxD motifs are functionally equivalent and our data support the findings. PalI converts sucrose into its isomers without participation of UDP-glucose, albeit that Mn^{2+} and Mg^{2+} enhance PalI activity [12]. We noted that although the DxD motif is highly conserved, the amino acid residues surrounding the motif are highly variable among the glucosyltransferase [23]. Apparently, different types of enzymes may use the conserved DxD motif as the core, together with different combinations of the adjacent amino acids, to serve for different catalytic functions.

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