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Identification of Catalytic Amino Acid Residues by Chemical Modification in Dextranase

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A novel endodextranase isolated from *Paenibacillus* sp. was found to produce isomaltotetraose and small amounts of cycloisomaltooligosaccharides with a degree of polymerization of 7–14 from dextran. To determine the active site, the enzyme was modified with 1-ethyl-3-[3-(dimethylamino)-propyl]-carbodiimide (EDC) and α-epoxyalkyl α-glucosides (EAGs), an affinity labeling reagent. The inactivation followed pseudo first-order kinetics. Kinetic analysis and chemical modification using EDC and EAGs indicated that carboxyl groups are essential for the enzymatic activity. Three Asp and one Glu residues were identified as candidate catalytic amino acids, since these residues are completely conserved across the GH family of 66 enzymes. Replacement of Asp189, Asp340, or Glu412 completely abolished the enzyme activity, indicating that these residues are essential for catalytic activity.

Keywords: Dextranase, catalytic amino acids, chemical modification, carboxyl group

Introduction

Dextran is a polysaccharide consisting mainly of α-1,6-glucosidic linkages of D-glucose that is produced by some lactic acid bacteria [16]. Endodextranases, which hydrolyze the α-1,6-linkages of dextran at random, belong to glycoside hydrolase (GH) families 49 and 66, based on the similarity of their amino acid sequence [11]. Based on the CAZY database (http://www.cazy.org), GH family 66 enzymes consist of endodextranases (E.C. 3.2.1.11) and cycloisomaltooligosaccharide glucanotransferases (CITase; E.C. 2.4.1.248), which are found mainly in bacteria [10, 17, 18]. Crystal structures are available for *Streptococcus mutans* dextranase and *Bacillus circulans* T-3040 CITase [30], and these structures indicate that the GH family 66 enzymes have three conserved domains: the N domain with an immunoglobulin fold, the catalytic A domain with a (β/α)8-barrel structure, and the C domain with tandemly repeated Greek key motifs [29, 30].

Kinetics analysis, affinity labeling, and site-directed mutagenesis are powerful tools for the identification of catalytic amino acid groups or residues. Kinetics analyses generally assess the pK_e values, the heats of ionization, and changes in the pK_e caused by addition of an organic solvent [5]. Affinity labeling is one of the most effective ways to determine enzyme active sites [15, 19, 20, 25]. The catalytic amino acid residues of some glycosidases have been confirmed by chemical modification with conduritol B epoxide (1,2-anhydro-*myo*-inositol; CBE). CBE is known to be a mechanism-based irreversible inactivator for α-

glucosidases [19]; it binds specifically to the essential α-carboxyl group of the Asp residue at the active site, which is a catalytic nucleophile (-COO^-). α-Epoxyalkyl α-glucosides (EAGs) such as 2′,3′-epoxypropyl α-D-glucopyranoside (E3G) [15] or 4′,5′-epoxybutyl-α-D-glucopyranoside (E5G) [20] have been used for determination of the catalytic amino acid residues of α-amylase or isomaltodextranase.

The gene encoding extracellular dextranase or CITase has been studied in dextranases from *S. rattus* [14], *S. mutans* [13], *S. sobrinus* [2], *S. downei* [12], *S. salivarius* [21], *Paenibacillus* sp. [6–8], and CITases from *B. circulans* [24]. Recently, we isolated a novel enzyme, PsDex, from *Paenibacillus* sp. collected on the Hokkaido University campus. PsDex produced isomaltotetraose (IG4), and small amounts of CIs (degree of polymerization = 7–14; CI-7 to CI-14) having endodextranase and CITase activities [17]. The unique primary structure of PsDex contains four regions (the N domain region, the A domain region, the insertion region, and the C domain region), and the catalytic domain of PsDex shares highly conserved regions with dextranases from *S. mutans* UA159 [29] and CITases from *B. circulans* [30]. Truncated rPsDex bearing only the A domain region was produced with an activity of 433 U/l by culturing in the RIL strain of *Escherichia coli* BL21 (DE3) using the pET-23d vector [17]. The specific activity towards dextran T2000 was 6.5 U/mg. SDS-PAGE analysis showed that purified truncated rPsDex has a molecular mass of 143 kDa [17].

In this study, we used kinetic analysis and affinity labeling with 1-ethyl-3-[3-(dimethylamino)-propyl]-carbodiimide (EDC) and EAG to determine the essential groups of truncated rPsDex. We further used site-directed mutagenesis to determine the catalytic amino acids among those that are invariant in the GH family 66. Our findings strongly suggest that the carboxyl group in the catalytic amino acids was essential in the reaction.

Materials and Methods

Chemicals

EDC and glycine ethyl ester hydrochloride (GEE) were purchased from Nacalai Tesque Inc. (Kyoto, Japan). PCR products were sequenced in both orientations with an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). EAGs were kindly provided by Professor Kimura at Hokkaido University (Sapporo, Japan).

Construction, Expression, and Purification of Truncated rPsDex and Four Mutants

Construction and production of truncated rPsDex and the D189N, D340N, E412Q, and D1252N mutant enzymes were performed as described by Kim *et al.* [17]. Site-directed mutagenesis for D189N, D340N, E412Q, and D1252N was performed by megaprimer PCR with specific primers for the mutant [4]. The RIL strain of *E. coli* BL21 (DE3) harboring PsDex-pET23d, D189N-pET23d, D340N-pET23d, E412Q-pET23d, and D1252N-pET23d was grown in 1 L of LB containing 50 μg/ml of ampicillin and 35 μg/ml of chloramphenicol at 37°C to an optical density of 0.5, and then transferred at 18°C. The recombinant protein was induced by addition of 0.2 mM isopropyl-1-thio-β-D-galactopyranoside at 18°C over the course of 20 h. The resultant expressed proteins were purified to homogeneity by nickel-chelating chromatography using Chelating Sepharose Fast Flow (Amersham Pharmacia Biotech., Piscataway, NJ, USA).

Dextranase Activity

The dextranse activity was assessed by the modified Somogyi assay documented by Nelson [23, 27, 28] for determination of the released reducing sugar in the reaction mixture. The dextranase assay involved incubation in 20 mM sodium acetate (pH 5.5) with 0.4% (w/v) dextran T2000 at 35°C. The reactions were terminated by addition of Somogyi reagent and boiling for 10 min, followed by spectrophotometric assay. One unit (U) of activity was defined as the amount of enzyme that caused the release of 1 μmol of reducing power per minute, under the conditions of the assay.

The reaction mixture (0.5 ml) containing 32 mM McIlvaine buffer (pH 3.14–9.45), 27.84 nM of the enzyme, and 0.008% dextran T2000 (1/5 of K_m) was incubated at 35°C for 10 min. The reaction mixture (0.5 ml) containing 27.84 nM of dextranase and 0.008% (w/v) dextran T2000 was incubated in McIlvaine buffer (pH 3.2–9) at 35°C for 10–25 min in the presence of 20% (v/v) methanol or in its absence. The reaction mixture (0.5 ml) containing 27.84 nM of dextranase and 0.008% (w/v) dextran T2000 was also incubated in McIlvaine buffer (pH 5.5) at 15°C, 20°C, 25°C, 30°C, and 35°C for 10–30 min in the presence of 20% (v/v) methanol or in its absence. The determination of the liberated reducing sugar was performed according to the copper bicinchoninate procedure [22] using glucose as a standard.

Chemical Modification by EDC

The modification mixture contained 10–40 mM of EDC, 100 mM GEE, and truncated rPsDex (3.18 μM) in 20 mM sodium-acetate buffer (pH 5.5) and was incubated at 35°C. At various times, 10 μl of enzyme solution treated with EDC was collected and diluted 10-fold with 20 mM sodium-acetate buffer (pH 5.5); the residual enzyme activity was then measured under the following conditions: the reaction mixture (0.1 ml) containing 20 mM sodium-acetate buffer (pH 5.5), modified enzyme (63.7–118 nM), and 0.4% (w/v) dextran T2000 was incubated at 35°C for 10 min. Determination of the quantity of reducing sugar liberated was performed according to the copper bicinchoninate procedure [22] using glucose as a standard.

Modification of EAGs

For mechanism-based inactivation, the reaction mixture

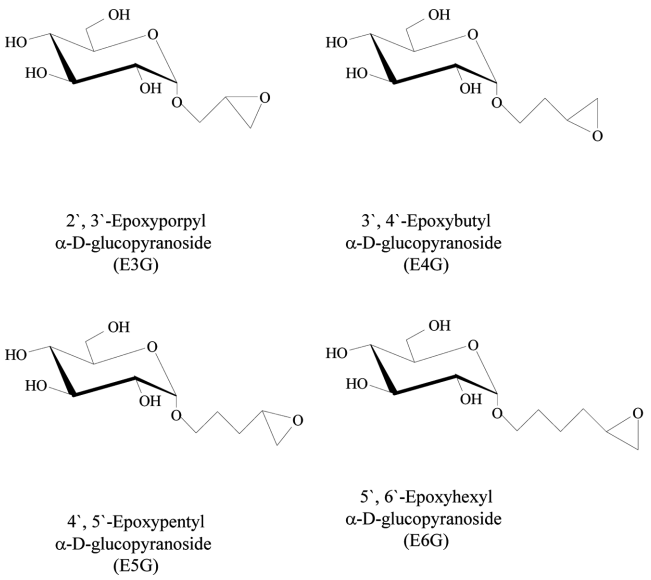

Fig. 1. Structures of EAGs.

containing truncated rPsDex (4.49 μM) and 50 mM sodium-acetate buffer (pH 5.0) was incubated at 35°C with 20 mM E3G, E4G, E5G, or E6G (Fig. 1). For the experiment involving protection by IG4, a reaction mixture containing truncated rPsDex (4.49 μM), 50 mM sodium acetate buffer (pH 5.0), and 20 mM E5G and 5 mM IG4 was incubated at 35°C. The residual activity was then examined and compared with that of a reaction mixture to which 5 mM IG4 had not been added.

At appropriate intervals, 10 μl of enzyme solution treated with EAGs was collected and diluted 10-fold with 20 mM sodium-acetate buffer (pH 5.5), and the residual enzyme activity was then measured. A reaction mixture containing 20 mM sodium-acetate buffer (pH 5.5), modified enzyme (89–179 nM), and 0.4% (w/v) dextran T2000 was incubated at 35°C and comparatively assessed against a reaction mixture to which the inhibitor had not been added. Determination of the quantity of reducing sugar liberated was performed according to the copper bicinchoninate procedure [22] using glucose as a standard.

Thin-Layer Chromatography (TLC)

The reaction mixtures were placed on silica gel 60 plates (Merck, Darmstadt, Germany) and developed twice using a solvent system of acetonitrile:H_2O:EtOAc:1-propanol = 85:70:20:50 (v/v/v/v) with isomaltooligosaccharides (from glucose to isomaltoheptaose) as the standards. The carbohydrates were visualized on the TLC plate by dipping into 0.03% (w/v) *N*-(1-naphthyl)ethylenediamine and 5% (v/v) H_2SO_4 in methanol, followed by heating at 120°C for 5 min [26].

Protein Concentration and Electrophoresis

The protein concentration of the cell-free extract was determined by the method described by Bradford [3] using bovine serum albumin as a standard. The concentration of purified protein was calculated from the amino acid content of the protein hydrolysate (6 M HCl, 24 h, 110°C), determined using a JEOL JLC/500V instrument equipped with a ninhydrin detection system. The protein concentrations for truncated rPsDex and the D340N, D189N, E412Q, and D1252N mutants were calculated based on values of $A^{1\%}_{1cm}$ = 5.54, 5.96, 4.29, 4.87, and 3.67, respectively, as determined by amino acid analysis.

Results

Kinetics Analysis

To determine the catalytic groups, the effects of pH, heat, and the dielectric constant on the ionization constant of the catalytic groups were investigated using truncated rPsDex. An initial velocity of hydrolysis of 0.008% (w/v) (20% of K_m for dextran T2000) was measured in the pH range of 3.2–9 in 32 mM McIlvaine buffer. The pH activity profile of dextranase was found to be bell-shaped, with an optimum pH of 5.5 (Fig. 2A), and the ionization constants toward dextran T2000 were found to be 4.5 and 7.3 (Table 1). The measured pK_{e1} value of 4.5 and pK_{e2} value of 7.3 suggested that carboxylate and carboxyl groups are the essential ionizable groups for the enzyme.

The heat of ionization (H) is also important for identifying the ionizable group. The heat of ionization value was measured according to the effects of temperature on the pK_e using van't Hoff's equation [5]. As shown in Fig. 2B, the ΔH for the acid and alkaline ionizable groups changed by −1.88 and 0.87 kcal/mol (Table 1) depending on the temperature. Generally, a ΔH value of less than 2 kcal/mol suggests that a carboxyl is the essential ionizable group [5].

Evaluation of the effect of the dielectric constant of the solvent on the pK_e provides important results on the essential ionizable groups for catalysis [5]. As shown in the right side of Fig. 2C, the dielectric constant decreased in 20% (v/v) methanol and both ionization constants shifted to a higher pH (by 0.2 and 0.3 pH units for pK_{e1} and pK_{e2}, respectively). We can conclude that carboxyl groups are the essential ionizable groups on truncated rPsDex from the effect of the dielectric constant of the solvent. The p*K*

Table 1. Characteristics of essential groups of truncated rPsDex.

Substrate	Ionizable group 1			Ionizable group 2		
	pK_{e1}	ΔH_{e1}	ΔpK_{e1}	pK_{e2}	ΔH_{e2}	ΔpK_{e2}
Dextran T2000	4.54	-1.88	+ 0.2	7.33	0.87	+ 0.3
Ionizable group	Carboxylate (-COO-)			Carboxyl (-COOH)		

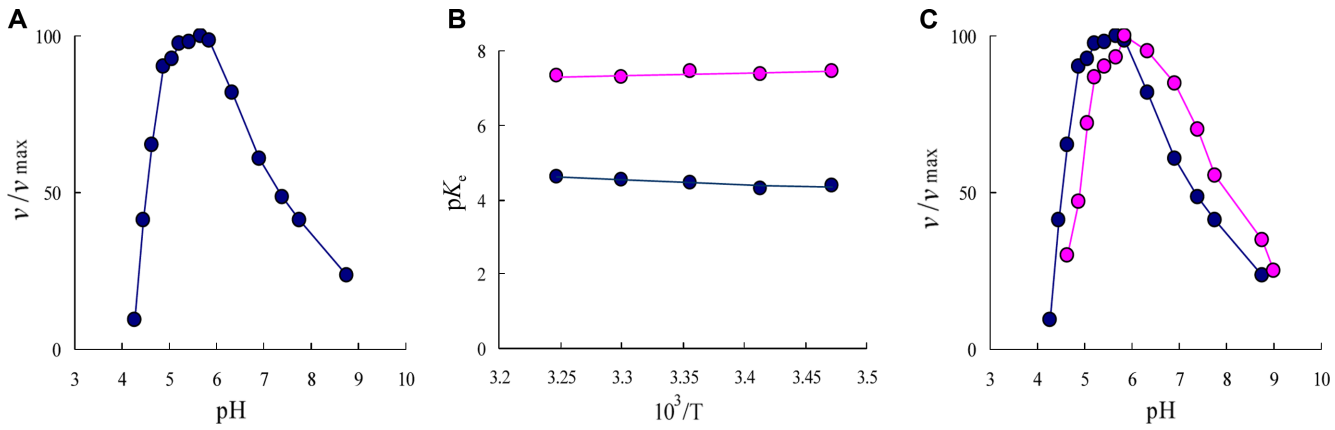

Fig. 2. Characterization of the activity of truncated rPsDex.
(**A**) pH activity curve for truncated rPsDex. A reaction mixture (0.5 ml) containing 32 mM McIlvaine buffer (pH 3–10), 27.8 nM of the enzyme, and 0.008% dextran T2000 (1/5 of K_m) was incubated at 35°C for 10 min. (**B**) Temperature dependence of the pK_{e1} and pK_{e2} values of truncated rPsDex. The ΔH_e values used for calculations using the van't Hoff equation were –1.88 kcal/mol for the acid (ΔH_{e1}) and 0.87 kcal/mol for the alkaline (ΔH_{e2}) ionizable group. Pink circle: acid ionizable group; blue circle: alkaline ionizable group. (**C**) Effects of methanol on hydrolysis of dextran T2000 with variation of the pH. A reaction mixture (0.5 ml) containing 27.8 nM of dextranase and 0.008% dextran T2000 was incubated in 20 Mcllvaine buffer (pH 2.75–9) at 35°C for 10–20 min in the presence or absence of 20% (v/v) methanol. Pink circle: in the absence of methanol; blue circle: in the presence of 20% (v/v) methanol.

values of ionizable group 1 and ionizable group 2 for the acidic side and the alkaline side were estimated, as summarized in Table 1.

Chemical Modification by EDC

Truncated rPsDex was progressively inactivated by incubation with EDC, a specific modifier for the carboxyl group, in the presence of GEE at pH 5.5 and 35°C. The inactivation followed pseudo first-order kinetics in relation to the concentration of EDC (Fig. 3). A parallel loss of activity on dextran T2000 was observed; 96% of the initial dextranase activity was lost after exposure to 40 mM EDC for 40 min. This strongly implies that the carbodiimide reacted with the information of amino acid residues at the active site.

Inactivation of PsDex by EAG

Incubation of dextranase with a molar excess of EAGs resulted in complete inactivation of the enzyme (Fig. 4). With equivalent concentrations of inhibitors (20 mM), the relative values of the inactivation rates were 16:69:96:100 for propyl (E3G), butyl (E4G), pentyl (E6G), and hexyl (E5G) derivatives at 20 min, respectively. Truncated rPsDex modified by treatment with E5G and E6G (20 mM) for 1 h did not hydrolyze the dextran T2000 or isomaltopentaose (IG5), as determined by TLC analysis (Fig. 5). Truncated rPsDex was allowed to react with various concentrations of E5G, and the residual enzyme activity was measured at 35°C, pH 5.5. The final concentration of E5G was 0 (Control sample), 0.25, 0.5, 1.0, 1.5, 2.5, 5, 10, and 20 mM (Fig. 6A). As shown in Fig. 6, the inactivation of truncated rPsDex by

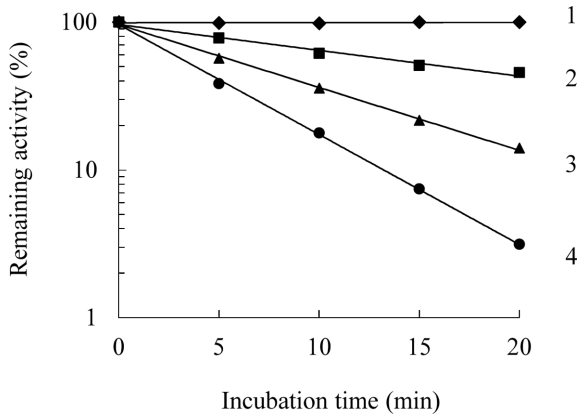

Fig. 3. Chemical modification of truncated rPsDex in EDC.
The modification mixture contained 10–40 mM EDC, 100 mM GEE, and truncated rPsDex (3.18 μM) in 20 mM sodium-acetate buffer (pH 5.5) and was incubated at 35°C. At various times, 10 ml of enzyme solution treated with EDC was collected and diluted 10-fold with 20 mM sodium-acetate buffer (pH 5.5) to stop the reaction; the residual enzyme activity was then measured. A reaction mixture containing 20 mM sodium-acetate buffer (pH 5.5), modified enzyme, and 0.4% dextran T2000 was incubated at 35°C for 10 min. Curves 1: buffer; 2: 10 mM EDC; 3: 20 mM EDC; 4: 40 mM EDC.

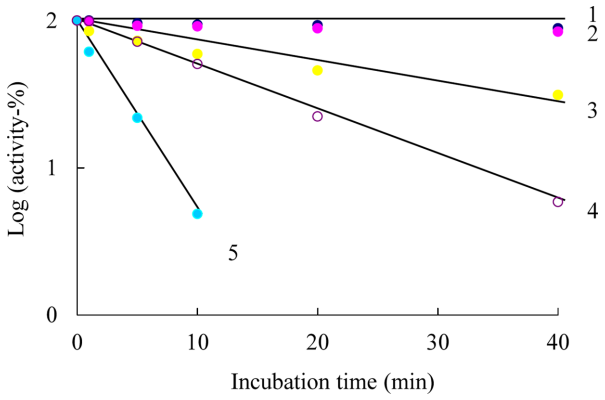

Fig. 4. Inactivation of truncated rPsDex by EAGs (3, 4, 5, and 6).
A reaction mixture containing truncated rPsDex (4.49 μM) and 50 mM sodium-acetate buffer (pH 5.5) was incubated at 35°C with 20 mM E3G, E4G, E5G, or E6G. At appropriate intervals, 10 ml of enzyme solution treated with an EAG was collected and diluted 10-fold with 20 mM sodium-acetate buffer (pH 5.5) to stop the reaction, and the residual enzyme activity was then measured. A reaction mixture containing 20 mM sodium-acetate buffer (pH 5.5), modified enzyme, and 0.4% dextran T2000 was incubated at 35°C for 10 min and compared with enzyme controls incubated in the absence of the inhibitor. Curves 1: buffer; 2: 20 mM E4G; 3: 20 mM E3G; 4: 20 mM E6G; 5: 20 mM E5G.

E5G followed pseudo first-order kinetics. Furthermore, all enzyme activity was not observed after dialysis. This implies that E5G binds irreversibly to the enzyme. The plot of the observed first-order rate against the E5G concentrations was hyperbolic (Fig. 6B), and the double-reciprocal plot of k_{obs} versus E5G concentration, [E5G], was indeed linear (Fig. 6C). These observations suggest that the strong inactivation arises by the formation of a dissociation complex between the enzyme and E5G, similar to the Michaelis–Menten-type enzyme-substrate complex, during the enzymatic reaction, as described in reaction scheme (1):

$$E + E5G \leftrightarrow E^*E5G \rightarrow E - E5G \quad (1)$$

where E*E5G is a reversible complex formed between the enzyme and E5G; E-E5G is the inactivated enzyme with covalently bound E5G; K_R is the dissociation constant of E*E5G; and k is the rate constant of irreversible inactivation as like Kimura *et al.* [20]. When the E5G concentration is sufficiently in excess of the enzyme concentration, the relationship among the parameters can be described as follows (2):

$$1/k_{obs} = (K_R/[E5G])/k + 1/k \quad (2)$$

The double reciprocal plots of k_{obs} versus the E5G concentration yielded K_R and k values of 1.78 mM and 2.64×10^{-3} s^{-1}, respectively (Fig. 6C). As shown in Kimura's previous report [20], the stoichiometry for binding of E5G to truncated rPsDex was determined from replots of log (k_{obs}) versus log [E5G] under the condition of $[E5G] << K_R$, where the slope was estimated to be 0.62, implying 1:1 stoichiometry between E5G and PsDex. These results indicated that truncated rPsDex was inactivated by modification of the active site at a 1:1 mol/mol ratio with E5G. In the presence of 5 mM IG4, >48% of the residual activity was retained, in comparison with the approximately 98% reduction in activity in the presence of 5 mM E5G (Fig. 7).

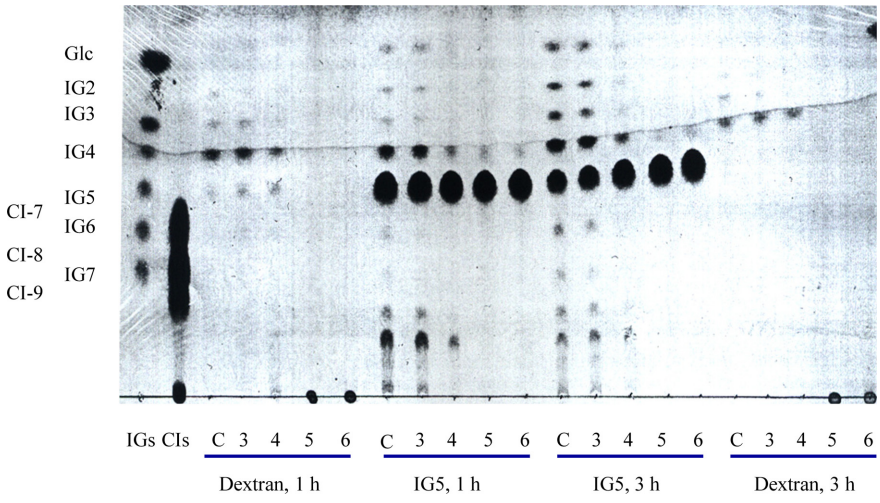

Fig. 5. TLC analysis of hydrolysis of dextran T2000 or IG5 by rPsDex that was inactivated and truncated by 20 mM EAGs.
IGs: isomaltooligosaccharides (glucose to isomaltoheptaose); CIs: cyclodextran DP 7, 8, 9; C: control experiment; 3: E3G; 4: E4G; 5: E5G; 6: E6G.

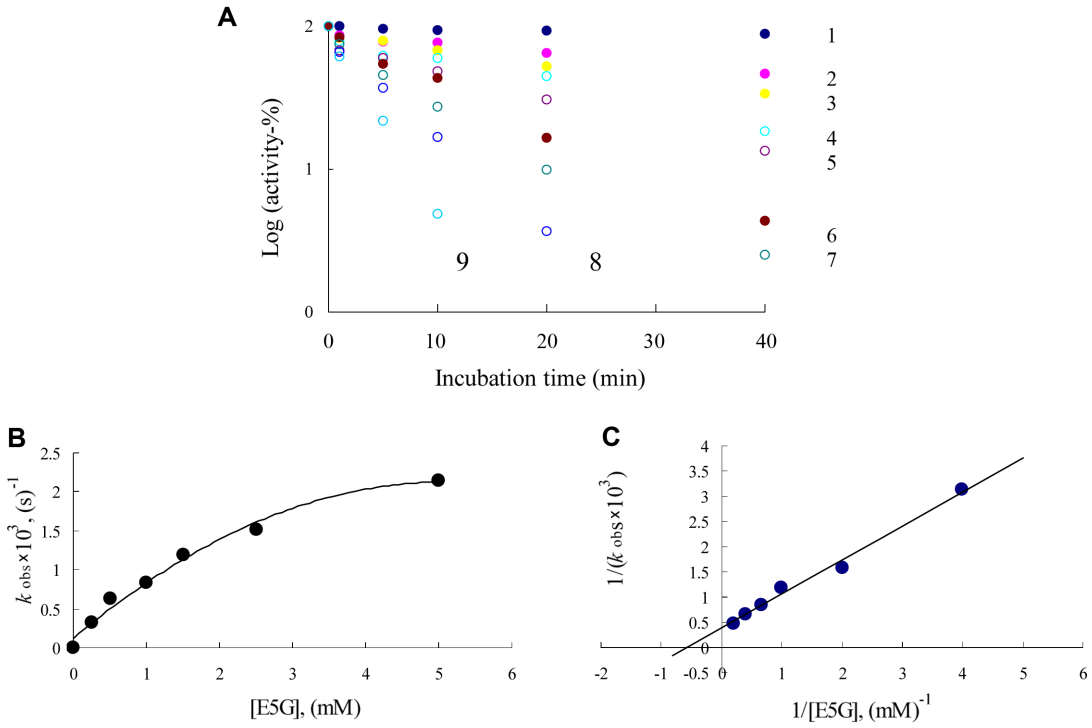

Fig. 6. Kinetics analysis of truncated rPsDex inactivation by E5G.

(**A**) Inactivation of truncated rPsDex by E5G. A reaction mixture containing truncated rPsDex (4.49 μM) and 50 mM sodium acetate buffer (pH 5.5) was incubated at 35°C with various concentrations of E5G. At appropriate intervals, 10 ml of enzyme solution treated with EAGs was collected and diluted 10-fold with 20 mM sodium-acetate buffer (pH 5.5) to stop the reaction, and the residual enzyme activity was then measured. A reaction mixture containing 20 mM sodium-acetate buffer (pH 5.5), modified enzyme, and 0.4% dextran T2000 was incubated at 35°C for 10 min and compared with enzyme controls incubated in the absence of the inhibitor. Curve 1: 0 mM E5G; 2: 0.25 mM E5G; 3: 0.5 mM E5G; 4: 1 mM E5G; 5: 1.5 mM E5G; 6: 2.5 mM E5G; 7: 5 mM E5G; 8: 10 mM E5G; 9: 20 mM E5G. The k_{obs} values (**B** and **C**) were calculated from (**A**) and replotted for this figure.

Mutational Analysis of Catalytic Amino Acids

The kinetics analysis and chemical mutation results indicated that acidic amino acid residues, Asp and Glu, were candidate catalytic residues. Analysis of the primary structure of 14 enzymes in the GH66 family showed that three Asp and one Glu residue were highly conserved, and these were considered to be candidate catalytic groups [17]. The Asp189, Asp340, Asp1252, and Glu412 units of rPsDex were selected as targets for site-directed mutagenesis, and each invariant Glu or Asp residue was substituted with Gln or Asn. The truncated rPsDex-pET23d plasmid was used as a template for megaprimer PCR [4]. Four mutant enzymes were purified to homogeneity by Ni^{2+}-Sepharose column chromatography. SDS-PAGE analysis indicated a molecular mass corresponding to 143 kDa with a yield of 35 mg/l of D189N, 54 mg/l of D340N, 25 mg/l of E412Q, and 38 mg/l of D1252N. The dextranase activities of the four mutant enzymes were compared with that of the truncated rPsDex on dextran T2000 (Table 2). The D1252N mutant retained 36% of the activity of truncated rPsDex, and the D189N, D340N, and E412Q mutants showed considerable decreases in dextranase activity (Table 2). The three amino acid

Table 2. Specific activity of purified truncated rPsDex mutants.

Position of mutation	Specific activity (U/mg)[a,b]	Relative activity (%)[c]
Truncated rPsDex	11.9	100
D194N	7.5×10^{-3}	6.3×10^{-4}
D340N	1.5×10^{-4}	1.2×10^{-5}
E412Q	2.2×10^{-3}	1.8×10^{-4}
D1252N	4.3	36

[a]Calculated from A_{280} using $E^{1\%}_{1cm}$ = 5.54, 4.29, 5.96, 4.87, and 3.67 for truncated rPsDex, D189N, D340N, and D1252N, respectively, as determined by amino acid analysis.

[b]0.4% Dextran, pH 5.5, at 35°C, according to the Somogyi–Nelson method [23, 27, 28].

[c]Specific activities as compared with that of truncated rPsDex.

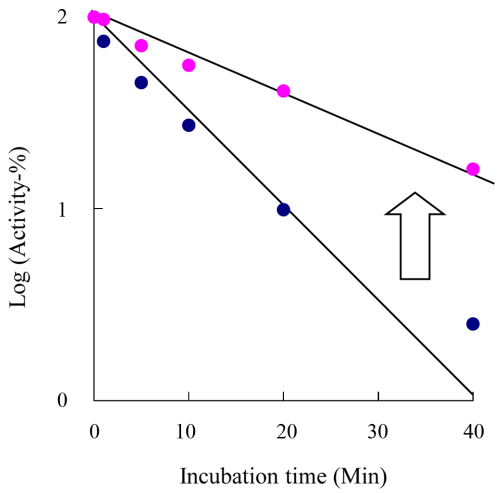

Fig. 7. Inactivation of truncated rPsDex with E5G in the presence of 5 mM isomaltotetraose (IG4).

A reaction mixture containing truncated rPsDex (4.49 μM), 50 mM sodium-acetate buffer (pH 5.5), 5 mM E5G, and 5 mM IG4 was incubated at 35°C. At appropriate intervals, 10 ml of enzyme solution treated with EAGs was collected and diluted 10-fold with 20 mM sodium-acetate buffer (pH 5.5) to stop the reaction, and the residual enzyme activity was then measured. The reaction mixture containing 20 mM sodium-acetate buffer (pH 5.5), modified enzyme, and 0.4% dextran T2000 was incubated at 35°C for 10 min.

residues are located in highly conserved regions 2, 4, and 5, among the nine conserved regions [17]. These data provide evidence of the essential catalytic role of Asp189, Asp340, and Glu412 in truncated rPsDex.

Discussion

Kinetics Analysis of Truncated rPsDex

The kinetics analysis revealed carboxylate groups, -COO^-, and –COOH for essential ionizable groups, as summarized in Table 1. The k_{cat}/K_m value for cleavage of dextran T2000 at each pH was determined by the substrate depletion method, where k_{cat}/K_m is the pseudo first-order rate constant for the reaction at a substrate concentration <20% of the K_m value for dextran T2000. From the characteristic pK_e and ΔH values, ionizable group 1 for the acidic side was estimated to be a carboxylate group. For ionizable group 2 for the alkaline side, the pK_e value implies either an imidazole group with a low pK or a carboxyl group with a high pK, which is shifted by interaction with other vicinal amino acids in the active site. Since the ΔH value for a carboxyl group is approximately ±1.5 kcal/mol, and that for an imidazole group is approximately 7.0 kcal/mol as like Kimura *et al*. [20], the observed ΔH_2 value of approximately 0.87 kcal/mol suggested that ionizable group 2 is a carboxyl group (-COOH). This was supported by the shift of pK_{e2} to a higher value when the dielectric constant of the medium was lowered by addition of methanol. However, in the case of an imidazole group, the total change in the ionizable reaction would be little affected by a change in the dielectric constant [5]. The carboxyl group (-COOH) for the alkaline side is an active form of the essential ionizable group of the enzyme, and it may be involved in breakdown of the enzyme-substrate complex.

Chemical Modification of Truncated rPsDex by EDC or EAGs

Moreover, analysis of the results of chemical modification with EDC indicated that the carboxyl group is essential for enzyme activity. The catalytic ionizable groups of truncated rPsDex were investigated by chemical modification with EDC to specifically react with the carboxyl or carboxylate groups in the protein, with GEE as a general nucleophile for the EDC-modification experiment. Funane *et al*. [9] reported modification of CITase and mutants from *B. circulans* T-3040 using EDC. Whereas CITase was completely inactivated with 1 mM EDC after 30 min of incubation, truncated rPsDex was less susceptible.

Several detailed kinetics analyses using α-EAGs have been reported, including the effects of α-EAGs on enzymes [15, 19, 20, 25]. Isoda and Nitta [15] reported that soybean α-amylase was inactivated by α-E3G, and Kimura *et al*. [20] reported that inactivation of soybean α-amylase by α-E4G was much more effective than inactivation by α-E3G or α-E5G; in terms of inactivation power, the order was α-E4G > α-E5G > α-E3G > α-E6G. Kimura *et al*. [20] also reported that based on the k/K_R values, isomaltodextranase was inactivated by the enzymes in the following order: α-E5G > α-E6G > α-E4G (19.4, 3.25, and 1.65 $M^{-1}\cdot s^{-1}$, respectively) and lower inactivation was observed for α-E3G. Compared to α-amylase and isomaltodextranase, truncated rPsDex had a k/K_R value of 1.48 $M^{-1}\cdot s^{-1}$ for E5G; truncated rPsDex was inactivated by 20 mM α-EAGs in the following order of inactivation power: α-E5G > α-E6G > α-E4G, and no significant inactivation was observed with α-E3G, as shown by the behavior of isomaltodextranase. Thus, the relationship between the length of the alkyl carbon chains of the α-EAGs and the degree of inactivation differs for each enzyme. IG4 was not a substrate for truncated rPsDex. The presence of IG4 protected the enzyme from inactivation by E5G. This suggests that IG4 competitively protects the active site of the enzyme against inactivation by E5G. These

results indicate that E5G binds covalently to carboxyls near the active site.

Mutational Analysis

The catalytic domain of rPsDex shows significant similarity to that of 14 kinds of dextranases and CITases belonging to GH family 66. As suggested by Aoki and Sakano [1], these enzymes showed high homology in nine conserved regions [17]. Four acidic amino acid residues, three invariant Asp (Asp189, Asp340, and Asp1252) and one Glu (Glu 412), were identified as candidate catalytic residues. To investigate the role of each amino acid residue, each was replaced by different amino acids, such as Asn or Gln, via site-directed mutagenesis. Asp189, Asp340, and E412 of rPsDex were considered to be essential for the catalytic reaction (Table 2). In dextranase from *Streptococcus mutans* Ingbritt, the substitution of Asp385 (corresponding to Asp340 of rPsDex) by Glu, Asn, Thr, or Val completely erased the enzymatic activity [29]. The results are similar to the data presented herein, implying that the carboxyl group in this position may be generally required for the catalytic activity of dextranase.

It has been determined that the three-dimensional structure of the dextranases and CITases in the GH family 66 enzymes comprises an $(\alpha/\beta)_8$ TIM-barrel in the domain A structure, based on sequence similarity [29, 30]. Based on possession of a similar active center, the Asp residue (corresponding residue of PsDex, Asp340) was expected to act as a nucleophile, and three acidic amino acid residues (two Asp and one Glu) were determined to be candidate proton donors, as in our previous report [17]. In domain A, Asp189, Asp340, and Glu412 are located at the β2, β4, and β6 positions of the $(\alpha/\beta)_8$ TIM-barrel, respectively [29]. These results are similar to the data presented herein, implying that the carboxyl group of Asp340 may be generally required for expression of the catalytic activity of dextranase [29]. To elucidate the 3D structure of PsDex, the structure of the active site must be determined using various inhibitors, and the roles of Asp189, Asp340, and Glu412 in hydrolytic reactions should also be investigated.

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