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Thermodynamic analysis of *Bacillus cereus* oligo-1,6-glucosidase and its cumulatively proline-introduced mutant proteins by differential scanning calorimetry

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Abstract

Differential scanning calorimetry (DSC) was used to characterize thermodynamically the stability of *Bacillus cereus* ATCC7064 oligo-1,6-glucosidase (dextrin 6-α-D-glucanohydrolase, EC 3.2.1.10) and its proline-introduced mutants. DSC analysis revealed that the free energy change of unfolding for the mutants cumulatively increased as the number of introduced proline residues increased. The resulting increase in the free energy change was ascribed to the concomitant decrease in the entropy change of polypeptide backbone unfolding in the mutants. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: *Bacillus cereus* oligo-1,6-glucosidase; Proline-introduced; Differential scanning calorimetry

1. Introduction

In the field of protein engineering, general and steady strategies to enhance protein thermostability have been desired for years. We succeed in cumulative thermostabilization of *Bacillus cereus* ATCC7064 oligo-1,6-glucosidase (dextrin 6-α-D-glucanohydrolase, EC 3.2.1.10) by replacing with proline residues [1]. This strategy follows the proline rule (formerly proline theory) that is proposed by Suzuki et al. [2–4]. The principle of the rule is that the increase in the frequency of proline occurrence at β-turns and/or in the total number of hydrophobic residues can enhance protein thermostability. There is a rigid constraint around the $N-C^{\alpha}$ rotation of a proline residue in a polypeptide chain so that proline residue has less backbone configuration entropy than other amino acids in the chain. Therefore, proline substitutions at appropriate sites of a protein could decrease the backbone entropy of unfolding and increase the free energy of unfolding. As a result, the engineered protein is expected to be thermostabilized. Thermodynamic analysis on proline substitutions is demonstrated by Matthews et al. [5] with T4 lysozyme. However, the cumulative thermostabilization of *B. cereus* oligo-1,6-glucosidase by proline substitutions is not examined thermodynamically yet. Through

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our previous studies, protein stability is defined by using the temperature at which the enzyme is 50% inactivated in 10 min [1–4,6–8]. Such a method could not show whether the thermostabilization by proline substitutions is attributed to the entropic effect. So we examined the unfolding of *B. cereus* oligo-1,6-glucosidase and its proline-introduced mutants with differential scanning calorimetry (DSC). We chose five mutants (Mut-1, Mut-4, Mut-7, Mut-9, Mut-12; the number means the number of proline residues introduced) which differ in thermostability [1,4]. Thermodynamic analysis of *B. thermoglucosidasius* KP1006 oligo-1,6-glucosidase was also made as the enzyme has 72% sequence identity with the *B. cereus* enzyme and the former has 13 more proline residues than the latter [7].

2. Experimental

2.1. Enzyme purification of oligo-1,6-glucosidases

B. cereus ATCC7064 oligo-1,6-glucosidase was expressed in *Escherichia coli* MV1184 and purified as described before [6]. The enzyme mutants, Mut-1, Mut-4, Mut-7, Mut-9, and Mut-12, were prepared as reported previously [1]. These mutants contained the following proline substitutions: Mut-1, Lys121 → Pro; Mut-4, Lys121 → Pro/Glu175 → Pro/Glu290 → Pro/Glu208 → Pro; Mut-7, Lys121 → Pro/Glu175 → Pro/Glu290 → Pro/Glu208 → Pro/Glu270 → Pro/Glu378 → Pro/Thr261 → Pro; Mut-9, Lys121 → Pro/Glu175 → Pro/Glu290 → Pro/Glu208 → Pro/Glu270 → Pro/Glu378 → Pro/Thr261 → Pro/Glu216 → Pro/Asn109 → Pro; and Mut-12, Lys121 → Pro/Glu175 → Pro/Glu290 → Pro/Glu208 → Pro/Glu270 → Pro/Glu378 → Pro/Thr261 → Pro/Glu216 → Pro/Asn109 → Pro/Lys457 → Pro/Thr440 → Pro/Ile403 → Pro. The secondary structures of 12 positions of proline substitutions are classified into three groups: (1) the second positions of β-turns (Lys121, Glu208, Glu290, Lys457); (2) the N1 positions of α-helices (Asn109, Glu175, Thr261, Ile403); and (3) the positions on flexible loops (Glu270, Glu378, Glu216, Thr440) [7,9]. The 12 positions were corresponded to those at which proline residues occur in the extremely thermostable oligo-1,6-glucosidase from *B. thermoglucosidasius* KP1006 [7]. The *B. thermoglucosidasius* enzyme was purified as described previously [7]. The purity of all enzymes used was judged by SDS-PAGE. Before DSC, enzyme samples were dialyzed extensively at 4°C for 15 h against buffer I (50 mM potassium phosphate; pH 7.0). Buffer I was used as the reference for DSC. The samples were ultracentrifuged at $100{,}000 \times g$ for 30 min at 4°C to remove minute contaminants.

2.2. Protein concentration

An aliquot of protein samples was exhaustively dialyzed against ultrapure water, lyophilized, and then weighted. After dissolving the dried material in buffer I, protein was determined by the method of Lowry et al. [10]. Protein concentration for DSC was adjusted to 0.5–0.7 mg ml^{-1} in buffer I.

2.3. DSC

DSC was done with a MicroCal MC-2D differential scanning microcalorimeter (MicroCal). The scanning rate was 1°C min^{-1}, and the data were sent every 0.015°C change. Before the measurements, protein samples or the reference (buffer I) was degassed with stirring in an evacuated chamber for 15 min at room temperature and then immediately applied into the calorimeter cell. DSC curves of protein unfolding were recorded between 15°C and 65°C. Instrumental baselines were determined with both cells filled with buffer I. These backgrounds were subtracted from each measured DSC datum. The temperature of unfolding (T_d, the temperature at the midpoint of unfolding transition) and the enthalpy changes of unfolding (ΔH_{cal} or the experimental transition enthalpies, $\Delta H(T_d)$)

were obtained from DSC curves. The heat capacity change of unfolding (ΔC_p, the difference in heat capacity between the folded and unfolded states) was evaluated at T_d from the observed pretransition and posttransition base lines in DSC curves [11].

2.4. Calculation of thermodynamic stability

Protein thermostability was referred from the definitions of Becktel and Schellman [12] as a function of the free energy change (ΔG_d) of unfolding vs. temperature. ΔG_d was calculated from $\Delta H(T_d)$ and ΔC_p using the modified Gibbs–Helmholtz Eqs. 1–3 as shown below [12]. ΔC_p was assumed to be independent of solutions used and temperatures at which the present study was made [13,14]:

$$\Delta H_d(T) = \Delta H(T_d) + \Delta C_p(T - T_d), \quad (1)$$

$$\Delta S_d(T) = \Delta S(T_d) + \Delta C_p \ln(T/T_d), \quad (2)$$

$$\Delta G_d(T) = \Delta H(T_d)(1 - T/T_d) - \Delta C_p \times (T_d - T) - \Delta C_p T \ln(T/T_d), \quad (3)$$

where T and T_d refer to absolute temperature (K). Thermodynamic data were analyzed by using the computer software, DSC Data Analysis in Origin^{TD} version 2.9 provided by MicroCal.

3. Results and discussion

3.1. DSC curve and reversibility of unfolding of oligo-1,6-glucosidase

A typical DSC curve for *B. cereus* oligo-1,6-glucosidase at pH 7.0 is given in Fig. 1. The reversibility of the thermal unfolding was examined by repeating DSC scanning of the sample, and by evaluating the DSC curve recovered. The extent of reversibility was found to be dependent on the temperature at which the first scanning was terminated and to be independent of the scanning rate. This finding suggested that

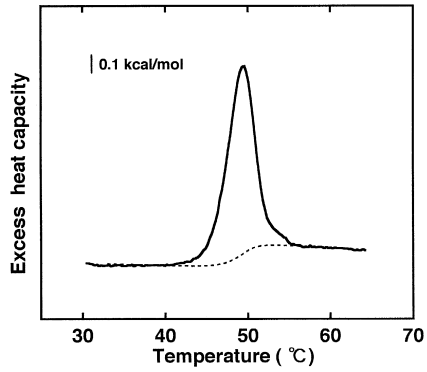

Fig. 1. Typical DSC curve for *B. cereus* oligo-1,6-glucosidase at 1°C min^{-1} and pH 7.0. Protein concentration was 0.46 mg ml^{-1}. The vertical bar is 0.1 kcal mol^{-1}. The broken line represents a baseline used for ΔH_{cal} calculation.

DSC data could be analyzed using thermodynamic models [15].

3.2. Validity of two-state model

When an equilibrium holds between the folded and unfolded states of a protein, the van't Hoff enthalpy change (ΔH_{vH}) of unfolding is obtained from the equation:

$$d \ln K/dT = \Delta H_{vH}/RT^2,$$

where K is an equilibrium constant and R is gas constant. ΔH_{vH} should be equal to ΔH_{cal} (see Section 2.4). Thus, $\Delta H_{cal}/\Delta H_{vH}$ values are close to unity [13]. ΔH_{cal} values for *B. cereus* oligo-1,6-glucosidase and its mutant Mut-1 at pH 6.0–7.5 were shown in Table 1 with their corresponding $\Delta H_{cal}/\Delta H_{vH}$ values. It is observed with several globular proteins that $\Delta H_{cal}/\Delta H_{vH}$ values are between 1.00 ± 0.05, indicating two-state behaviors of unfolding [11,13]. This was the case for $\Delta H_{cal}/\Delta H_{vH}$ values for the *B. cereus* enzyme and its mutant Mut-1 except for the *B. cereus* enzyme at pH 7.3 and 7.5. The same was observed for mutants Mut-4, Mut-7, Mut-9 and Mut-12 (data not shown). So the unfolding of the *B. cereus* enzyme and its mutants followed the two-state transition of unfolding in DSC.

Table 1
DSC data on *B. cereus* oligo-1,6-glucosidase and its mutant Mut-1 at different pH values

	pH	Protein concentration (mg ml^{-1})	T_d (°C)	ΔH_{cal} (kcal mol^{-1} K^{-1})	$\Delta H_{cal}/\Delta H_{vH}$
Wild-type	6.0	0.487	48.5	233	1.03
	6.3	0.511	49.4	267	0.996
	6.5	0.526	50.0	281	0.971
	6.8	0.527	49.6	277	0.979
	7.0	0.534	49.3	260	1.00
	7.3	0.498	47.8	245	1.09
	7.5	0.561	47.0	240	1.11
Mut-1	6.0	0.645	49.6	235	0.985
	6.3	0.654	50.5	264	0.952
	6.5	0.648	51.1	290	0.954
	6.8	0.650	51.1	295	0.953
	7.0	0.704	50.6	265	0.955
	7.3	0.678	49.3	256	1.00
	7.5	0.668	48.3	245	1.05

3.3. Dependence of T_d on pH

The DSC curves of *B. cereus* oligo-1,6-glucosidase at pH 6.0–7.5 are given in Fig. 2. DSC curves for some protein unfolding are deconvoluted into a few individual contours under the assumption of a two-state model of unfolding [16,17]. However, the oligo-1,6-glucosidase made a single main peak in DSC without any other peak or shoulder, although it had different T_d values dependent on the pH change.

3.4. Comparison of thermodynamic parameters for B. cereus oligo-1,6-glucosidase and its mutants

Table 2 shows T_d, ΔC_p, and $\Delta H(T_d)$ measured at pH 7.0 for *B. cereus* oligo-1,6-glucosidase, its mutants, and *B. thermoglucosidasius* oligo-1,6-glucosidase. To evaluate the stability of these enzymes under the same condition, ΔH_d, ΔS_d, and $\Delta^2 G_d$ (the difference in ΔG_d between the *B. cereus* enzyme and its each mutant) calculated from the modified Gibbs–Helmholtz equations were compared in Table 2 at 49.3°C, the T_d of the *B. cereus* enzyme. T_d values of mutants were progressively elevated as the number of proline substitutions increased. $\Delta^2 G_d$ in ΔG_d for the mutants except for mutant Mut-7 increased with increasing number of proline residues introduced. These findings showed strongly that the *B. cereus* enzyme could be cumulatively thermostabilized by proline substitutions. In Table 2, the changes in $\Delta^2 G_d$ reflected those in T_d well. However, Mut-7 seemed insignificant because it showed only marginal decrease (0.3 kcal mol^{-1}) in $\Delta^2 G_d$ and a small increase (0.2°C) in T_d between Mut-7 and Mut-4. The increases in $\Delta^2 G_d$ and in T_d for each mutant against the *B. cereus* enzyme were between 1.1 and 3.7 kcal mol^{-1} and between 1.3°C and 5.4°C, respectively. The increases in $\Delta^2 G_d$ and in T_d per each proline residue introduced were comparable to those obtained for T4

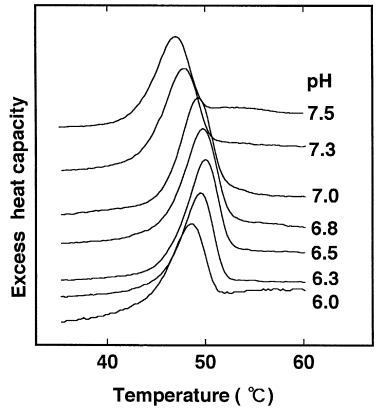

Fig. 2. pH dependence of the excess molar heat capacity of *B. cereus* oligo-1,6-glucosidase. Each line shows the excess heat capacity change at different pH indicated in the figure.

Table 2
Thermodynamic data for *B. cereus* oligo-1,6-glucosidase and its mutants at pH 7.0

	T_d[a] (°C)	ΔC_p[b] (kcal mol^{-1} K^{-1})	$\Delta H(T_d)$[c] (kcal mol^{-1})	$\Delta H_d(49.3)$[d] (kcal mol^{-1})	$\Delta S_d(49.3)$[e] (cal mol^{-1} K^{-1})	$\Delta^2 G_d(49.3)$[f] (kcal mol^{-1})
B. cereus oligo-1,6-glucosidase						
Wild-type	49.3	5.03	260	260	800	0
Mut-1	50.6	6.15	265	257	794	1.1
Mut-4	52.9	6.10	262	240	736	2.8
Mut-7	53.1	3.65	231	217	666	2.5
Mut-9	54.3	6.20	248	217	662	3.6
Mut-12	54.6	4.51	222	198	603	3.7
B. thermoglucosidasius oligo-1,6-glucosidase	74.4	11.6	504	213	581	25.7

[a] T_d, the temperature at the midpoint of unfolding transition.
[b] ΔC_p, the difference in the heat capacity between the folded and unfolded states obtained from the pre- and posttransition base lines.
[c] $\Delta H(T_d)$, the calorimetric enthalpy change of unfolding at the temperature at the midpoint of unfolding transition of each protein.
[d] $\Delta H_d(49.3)$, the enthalpy change calculated at 49.3°C, the T_d of *B. cereus* oligo-1,6-glucosidases.
[e] $\Delta S_d(49.3)$, the entropy change calculated at 49.3°C.
[f] $\Delta^2 G_d(49.3) = \Delta G_d(49.3)$ (mutant) $- \Delta G_d(49.3)$ (*B. cereus* oligo-1,6-glucosidases) at 49.3°C.

and human lysozymes [5,18]. T4 lysozyme Ala82 → Pro mutant, and human lysozyme Ala47 → Pro and Val110 → Pro mutants show the increase in $\Delta^2 G_d$ by 0.3, 0.1 and 0.5 kcal mol^{-1}, and the increase in T_d by 0.7°C, 0.3°C and 1.2°C, respectively. ΔH_d and ΔS_d for the mutants of the *B. cereus* enzyme decreased with increasing the number of proline residues introduced (Table 2). However, their $\Delta^2 G_d$ was increased by large concomitant yields of a negative ΔS_d compensating those of a negative ΔH_d. The enhanced thermostability in the mutants could be attributed to the decrease in ΔS_d induced by proline residues introduced. The decrease in both ΔH_d and ΔS_d takes place on Ala19 → Pro substitution of bovine pancreatic RNase although the mutant protein makes less effect on thermostability as a net [19].

The thermodynamic data for *B. thermoglucosidasius* oligo-1,6-glucosidase showed the characters extrapolated from those for *B. cereus* oligo-1,6-glucosidase and its mutants. The decrease in ΔS_d was also observed for the *B. thermoglucosidasius* enzyme compared with the *B. cereus* enzyme. The *B. thermoglucosidasius* enzyme has no disulfide bridge within the molecule. So the large decrease in ΔS_d and the increase in ΔG_d could not be due to the S–S bridge formation as in human lysozyme [20,21]. The *B. cereus* enzyme has 13 positions at which proline residues occur in the *B. thermoglucosidasius* enzyme [7]. Twelve of the 13 positions in Mut-12 were already occupied by proline residues. Therefore, the entropic effect of proline substitutions might be nearly saturated in Mut-12.

The contribution of a single amino acid substitution to protein stability is generally, at most, 1–2 kcal mol^{-1} in ΔG_d of unfolding [5]. Each proline substitution makes a small but definite and additive contribution to protein thermostabilization if the sites satisfy the requirements for proline substitution [4]. This study afforded conclusive evidence that the strategy of proline substitutions is valuable for protein thermostabilization as one of the most promising and general strategies [22].

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