

Quaternary structure and enzymatic properties of cyclomaltodextrinase from alkalophilic *Bacillus* sp. I-5

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Abstract: Cyclomaltodextrin (CD)-degrading enzymes, such as cyclodextrinase (CDase), maltogenic amylase (MAase), neopullulanase (NPase), and *Thermoactinomyces vulgaris* α -amylase II (TVA II), are mainly multi-subunit enzymes that hydrolyze various carbohydrate substrates, such as maltooligosaccharides, starch, and pullulan. The analysis of the catalytic properties of this subgroup of enzymes suggests that they may play an important role in carbohydrate metabolism in the bacterial cell. We overexpressed CDase from alkalophilic *Bacillus* sp. I-5 (CDase I-5) and characterized the quaternary structure of the enzyme using analytical ultracentrifugation, size-exclusion chromatography, and differential scanning calorimetry (DSC). The purified recombinant CDase I-5 was found to be a dodecameric structure composed of six homodimers, which are the basic structural unit of CD-degrading enzymes. A plot of apparent molecular mass as a function of pH suggested that CDase I-5 exists as a dimeric form at pH values lower than 6.0, whereas the dimers associate to form a dodecamer at pH values greater than 6.5. The kinetic parameters of CDase I-5 for the hydrolysis of maltooligosaccharides and CDs demonstrated that the dodecameric form of the enzyme at pH 7.0 exhibited much higher activity than did the dimeric form at pH 6.0. We also investigated dimer/dodecamer stability using DSC. The results reveal that the supramolecular assembly contributes to an increase in the thermostability of CDase I-5 across a wide range of pH values. Factors affecting the dissociation/association of the oligomeric enzyme, including pH, salt, and pressure, are also discussed.

Key words: cyclomaltodextrinase, maltogenic amylase, dissociation/association, oligomerization, dodecamer, quaternary structure.

Abbreviations: CD, cyclomaltodextrin; CDase, cyclodextrinase; CDase I-5; cyclodextrinase from alkalophilic *Bacillus* sp. I-5; DSC, differential scanning calorimetry; MAase, maltogenic amylase; NPase, neopullulanase; ThMA, maltogenic amylase from *Thermus*.

Introduction: dimerization/oligomerization of cyclomaltodextrin (CD)-degrading enzymes

Dimerization or oligomerization is an important property of many natural enzymes. It not only provides various structural and functional advantages, including increased catalytic efficiency, thermostability, and regulation of biological systems, but also helps to minimize the size of a cell's genome (MARIANAYAGAM et al., 2004). Quaternary structure is a very common property of human enzymes, as exemplified by hemoglobin. Only a few enzymes in the α -amylase family GH13, however, are known to form dimers or oligomers. These

include cyclodextrinase (CDase) and maltogenic amylase (MAase) (LEE et al., 2002).

CDase (EC 3.2.1.54) is known to hydrolyze CD much faster than starch and hence can be easily distinguished from α -amylases by its substrate preference (BENDER, 1986). Following the first report of CDase from *Bacillus macerans* (DEPINTO et al., 1968), many CDases have been isolated from various microbial sources including *Bacillus coagulans* (KITAHATA et al., 1983), *Clostridium thermohydrosulfuricum* 39E (recently reclassified as *Thermoanaerobacter ethanolicus* 39E) (PODKOVYROV et al., 1992), alkalophilic *Bacillus* sp. (YOSHIDA et al., 1991), *Bacillus sphaericus* E-

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Table 1. Physicochemical data for the association/dissociation of oligomeric enzymes.

Factor	Effective range	Driving force	Enzymes ^a	Relative rate
pH	5~7	Electrostatic repulsion of positively charged residues (histidine at C-/N-terminus)	CDase D-Amino acid transaminase	slow slow
Salt	0~2 M	Charge shielding/ hydrophobic interaction	F1-ATPase MAase β -Galactosidase	slow rapid rapid
Pressure	0~3 kbar	Pressure induced-hydration	Alcohol dehydrogenase Alkaline phosphatase	slow slow

^aReferences: CDase – this work; D-amino acid transaminase (KISHIMOTO et al., 2000); F1-ATPase (CABEZON et al., 2000); MAase (this work); β -galactosidase (GALLAGHER et al., 1997); alcohol dehydrogenase and alkaline phosphatase (CIONI & STRAMBINI, 1996).

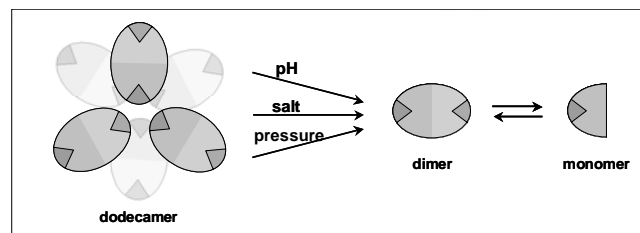


Fig. 1. Scheme of the dissociation/association of the oligomeric enzyme.

244 (OGUMA et al., 1990; OGUMA et al., 1993), alkalophilic *Bacillus* sp. I-5 (KIM et al., 1998), *Flavobacterium* sp. (BENDER, 1993), *B. sphaericus* ATCC7055 (GALVIN et al., 1994), *Klebsiella oxytoca* M5a1 (FEEDERLE et al., 1996; FIEDLER et al., 1996), *Thermococcus* sp. B1001 (HASHIMOTO et al., 2001), *Anoxybacillus flavithermus* (TURNER et al., 2005), and *Laceyella sacchari* (TURNER et al., 2005). The molecular weights of these enzymes, ranging from 62 to 90 kDa, are higher than those of α -amylases. Many of the CDases described above show multi-substrate specificities toward CDs, soluble starch, amylose, and pullulan. The relative rates of hydrolysis of the various substrates differ among the enzymes, but are generally in the order of CD = maltooligosaccharides \gg pullulan $>$ starch.

MAase (EC 3.2.1.133) and neopullulanase (NPase) (EC 3.2.1.135) have recently been reported to be capable of hydrolyzing CDs, pullulan, and starch, similarly to CDase. We previously reported that these three groups of enzymes are similar enough to all be classified as CD-degrading enzymes on the basis of their three-dimensional structures and multi-substrate specificities (LEE et al., 2002; OH, 2003).

The elucidation of the structure of CDase from *Bacillus* sp. I-5 reveals a dodecameric assembly composed of six sets of homodimer units, which are the basic structural units of the MAase of *Thermus* (ThMA; KIM et al., 1999) and the NPase of *Bacillus stearothermophilus* (HONDOH et al., 2003) and *Thermoactinomyces vulgaris* α -amylase II (KAMITORI et al., 1999).

Factors affecting the dissociation/association of the oligomeric protein are known to include pH, salt, and pressure (MARIANAYAGAM et al., 2004), as illus-

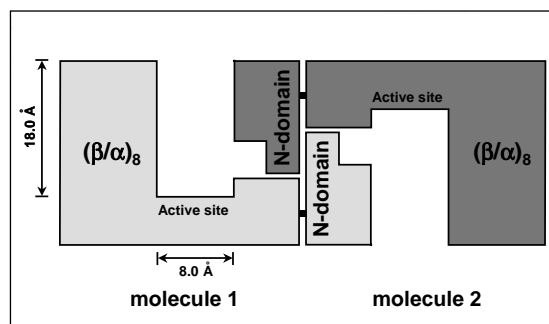


Fig. 2. Dimeric unit of CDase: Domain-swapping dimer.

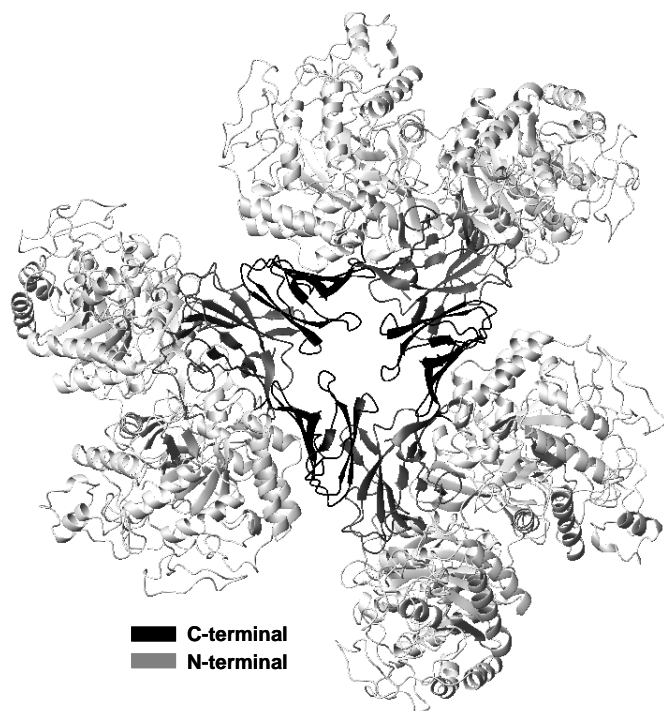
trated in Figure 1 and documented in Table 1. However, there is a relative paucity of biophysical knowledge compared with the information available in a number of biochemical databases.

In this review we discuss the physicochemical properties of CDase I-5 in relation to the association/dissociation of the supramolecular assembly.

Dodecameric structure of CDase I-5: a supramolecular assembly of dimeric units

The monomeric structure of CDase I-5 contains a distinct N-domain in addition to a central $(\beta/\alpha)_8$ -barrel domain and a C-domain. Unlike the central $(\beta/\alpha)_8$ -barrel domain, the N-domain (residues 1-123) and C-domain (residues 505-583) are composed exclusively of β -strands. Two molecules of CDase monomer form a dimer in which the N-domain of one molecule is involved in extensive interactions with the $(\beta/\alpha)_8$ -barrel domain of the adjacent molecule. Figure 2 is a schematic of the dimeric unit of CDase I-5 as observed in the typical three-dimensional domain-swapped dimer. In the dimeric structure, the C-domain is distinctively separated from the active-site groove and is not involved in main-chain-to-main-chain hydrogen bonding with either the N- or the $(\beta/\alpha)_8$ -barrel domain. Instead, the interface between the C-domain and the $(\beta/\alpha)_8$ -barrel domain consists predominantly of hydrophobic residues. Interestingly, the C-domain is critically involved in the supramolecular assembly of CDase as described below.

(a)



(b)

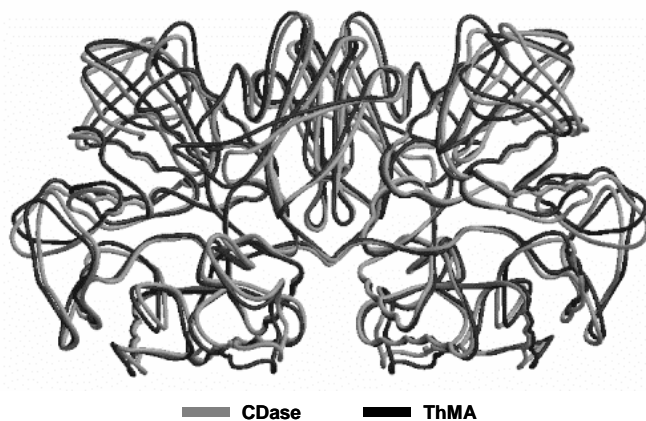


Fig. 3. Three-dimensional structure of CDase I-5. (a) Dodecameric structure. (b) Superposition of the $C\alpha$ traces of the dimeric structures of CDase and ThMA. The rmsd of the $C\alpha$ atoms after the superposition is 2.28 Å.

The crystal packing of CDase I-5 reveals an assembly composed of six dimeric units corresponding to the ThMA dimer. The dimeric units are related by the crystallographic two- and three-fold symmetry axes of the cubic cell, resulting in a tightly packed hexameric assembly of the dimer (Fig. 3a). The predominant intermolecular interactions between the dimers are mediated by the C-domain of one molecule and the N-domain of an adjacent molecule. The superposition of the $C\alpha$ atoms of the dimeric or monomeric unit of CDase I-5 and of ThMA shows that the relative orientations of the C- and N-domains with respect to the central domain are very similar in the two structures (Fig. 3b).

Oligomeric states and substrate hydrolysis of CDase I-5 in solution at various pHs

Sedimentation equilibrium analysis was performed at pH values between 5.0 and 8.5 to determine the effect of pH on dodecamer dissociation and to investigate changes in the association/dissociation equilibrium at various pHs. The apparent molecular mass was determined by analytical ultracentrifugation analysis. The estimated molecular mass of CDase I-5 was plotted as a function of pH (PARK et al., 2002). The results showed that CDase I-5 exists in dimeric form at pHs between 5.75 and 6.0. These dimers associate to form a dodecamer with a transition midpoint at pH 6.2, resulting in the dodecameric CDase I-5 being the predominant form of the enzyme at pHs greater than 6.5.

To compare the catalytic efficiency of the dodecameric and dimeric forms of the CDase for maltotooligosaccharides and CDs, we measured the enzyme

Table 2. Specific activity and thermostability of the dimeric and dodecameric forms of CDase I-5.

	Specific Activity (U/mg)		Thermostability by DSC (°C)		
	β -CD	Amylose	T_o	T_p	T_c
Dodecamer	219	72	63.9	67.0	68.7
Dimer	73	38	55.1	60.9	64.0

activity of the dodecameric form at pH 7.0 and of the dimeric form at pH 6.0. We generated the dodecameric form of the enzyme by equilibrating CDase I-5 with buffer solution at pH 7.0 and the dimeric form by equilibration with buffer at pH 6.0 for 3 days. Aliquots of the enzyme solution were reacted with substrates at pH 7.0 or pH 6.0, respectively. The enzyme activity of the dodecameric form was observed to be nearly the same at pH 7.0 as at pH 6.0, showing that pH was not a major factor in determining enzymatic activity. This result indicates that the 2- to 3-fold higher catalytic efficiency of the dodecameric form at pH 7, compared with the activity of the dimeric form at pH 6, arises from the physical differences between the oligomeric states (Table 2). As LEE et al. (2002) previously reported, all twelve active sites of the monomeric unit of the enzyme are located externally on the dodecameric assembly. Hexamer formation does not shield any of the active sites from access by bulk molecules, so the entrance of a substrate molecule to the active site cannot be prohibited by dodecamerization. While two of the active sites on the dimer are at a 180° angle to one another, the clustered active sites face each other. This spatial arrangement of the active sites implies that the supramolecular assembly of ThMA or CDase could confer an enzyme activity superior to that of the dimeric form because a hydrolyzed product, which is released from one active site on the assembly, could readily be accepted into another active site in the cluster. This represents a novel example of how the oligomerization of an enzyme can increase the catalytic efficiency of hydrolysis of a polymeric substrate requiring multiple entries into the active site for complete degradation. In a dimeric enzyme, the active sites are frequently formed at the interfaces between subunits, resulting in an increase in the local concentration of catalytic sites (MARIANAYAGAM et al., 2004).

Intrinsic capability for supramolecular assembly: role of histidine in N-/C-domains

The three-dimensional structure of CDase I-5 has shown (LEE et al., 2002) that His539 in the C-terminal region and His49 and His89 in the N-terminal region are located at the interface between the dimeric units. Each of these three histidine residues was replaced with a valine residue by site-directed mutagenesis to investigate the role of histidine residues in supramolecular assembly.

To compare the role of each histidine residue, the

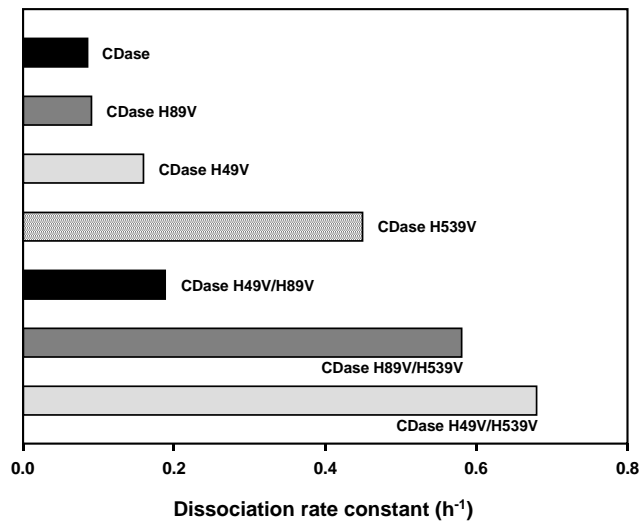


Fig. 4. Dissociation rate constants of CDase I-5 variants compared with wild-type CDase I-5.

dissociation rate constants of the mutant and wild-type CDase I-5 were investigated. The dissociation process was analyzed in a universal buffer of pH 6.0 using a Superdex 200 HR 10/30 column. The peak area corresponding to the dodecamer diminished with incubation time. The progress curves of the dissociation of dodecamer to dimer fitted a single exponential decay. The dissociation rate constants of the single-mutation forms of CDase I-5 were increased. The ratios of the dissociation rate constants of mutant CDase to wild-type CDase were 5.24, 1.86, and 1.06 for the H539V mutant, H49V mutant, and H89V mutant, respectively (LEE, 2002). The H539V mutation showed the highest effect on the dissociation of the dodecamer to the dimer, while the H89V mutation had little effect on the dissociation. With regard to the double mutants, the dissociation rate constants were 7.92 for the H49V/H539V mutant, 6.76 for the H89V/H539V mutant, and 2.19 for the H49V/H89V mutant (Fig. 4). The results show that the histidine residues located at the N- and C-termini are involved in the interactions of the subunits of CDase I-5.

Supramolecular assembly increases the thermostability of CDase I-5

The dissociation of CDase I-5 was investigated by gel permeation chromatography in the presence of various concentrations of KCl (0–2.0 M). The results in-

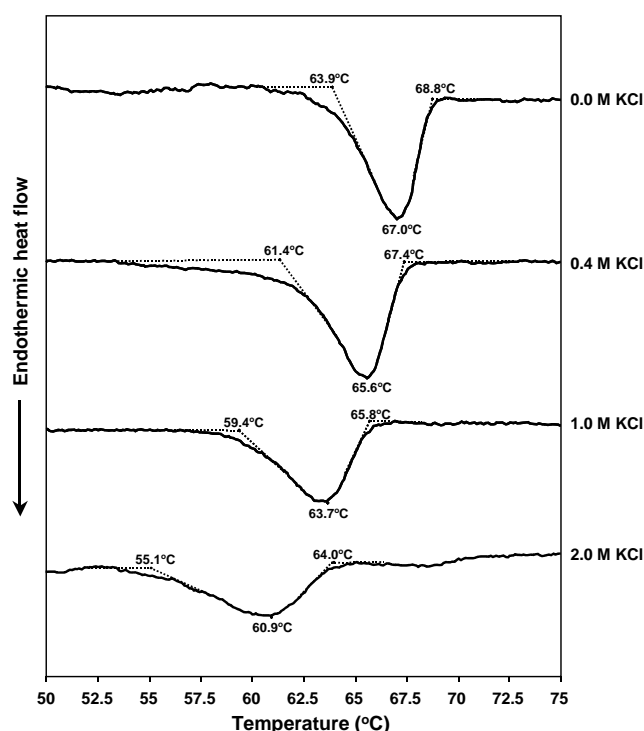


Fig. 5. Thermal denaturation of CDase I-5 analyzed by DSC.

indicate that the peaks corresponding to the dodecamer decreased, while the peaks corresponding to the dimer increased as the concentration of KCl increased (LEE, 2002). Sedimentation equilibrium analysis also reveals that the dodecamer dissociated into dimers in 1.0 M KCl, which decreased the degree of dodecamerization of CDase I-5 to 69% (PARK et al., 2002).

Differential scanning calorimetry (DSC) analysis was performed in the presence of KCl to investigate the thermal denaturation characteristics of CDase I-5 in various oligomeric states (Fig. 5). The temperature of denaturation, T_o , of CDase I-5 was 63.9°C in the absence of KCl, as compared with 55.1°C in the presence of 2.0 M KCl (LEE, 2002). This result implies that CDase is less stable in conditions under which it exists predominantly as a dimer than in conditions under which the dodecameric form predominates, such as low concentrations of salt.

In summary, the physicochemical factors affecting the dissociation/association of oligomeric enzymes, including environmental factors, driving forces, and relative rates, were identified (Table 1). The mechanism of dissociation/association seems to be specific to the protein involved. There have been many recent reports of the physical properties of protein dimerization or oligomerization, and these have provided insights into biological functions. Cumulative information obtained from various experimental data will enable us to understand the mechanism, function, and evolution of amylolytic enzymes in biology.

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