Biologia, Bratislava, **57/Suppl. 11:** 71—76, 2002 **Review**

Crystal structures and substrate specificities of two α-amylases hydrolyzing cyclodextrins and pullulan from *Thermoactinomyces vulgaris* R-47**

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Thermoactinomyces vulgaris R-47 produces two α-amylases, TVA I and TVA II, both of which hydrolyze pullulan to produce panose and also hydrolyze cyclodextrins. Here, we report the crystal structures of a TVA II-substrate complex and of unliganded TVA I. Using a mutated TVA II with modified catalytic residues, we determined the structure of TVA II bound to β-cyclodextrin, methyl β-cyclodextrin, and maltohexaose. TVA II has four domains, A, B, C, and N, and is observed as a dimer in the crystal. The substrates bound to the active-site cleft, which is formed by domains A and B. The overall structures of TVA II and other α-amylase family enzymes are quite different because the similarity of domains other than domains A, B, and C of these enzymes is not found. However, the positions and structures of the sugars bound to TVA II were similar to those observed in the other α-amylase-family enzymes. Crystals of TVA I were obtained using polyethylene glycol 10,000, and the crystal structure of TVA I was determined. Like TVA II, the structure of TVA I was composed of domains A, B, C, and N. However, unlike TVA II, TVA I was observed as a monomeric form in the crystal, and the interaction between domain N and other domains is quite different. When domain N of TVA I and TVA II was deleted, to investigate the roles of domain N for both enzymes, the activities of these mutated enzymes were greatly reduced.

Key words: TVA, *Thermoactinomyces vulgaris,* pullulan, cyclodextrin, α-amylase, neopullulanase, maltogenic amylase.

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**Recent studies show that TVAI and TVAII are not typical "α-amylases". However, because both enzymes have properties of neopullulanase, cyclomaltodextrinase, and maltogenic amylase, it is difficult to give only one of these specific names for describing TVAI and TVAII. Thus we use the term "α-amylase" in this paper.

TVA I, TVA II, and related enzymes

Thermoactinomyces vulgaris R-47 α-amylases, TVA I and TVA II, hydrolyze specific ($\alpha 1 \rightarrow 4$)-glucosidic linkages of pullulan to produce panose and also hydrolyze cyclodextrins (CDs). TVAs also catalyze transglycosylation to the C-4 and C-6 positions of acceptors like glucose. Their primary structures are homologous (30–50 % identity) (reviewed by PARK et al., 2000) to those of neopullulanases (EC 3.2.1.135) (KURIKI et al., 1988), cyclomaltodextrinases (EC 3.2.1.54) (SAHA & ZEIKUS, 1992) and maltogenic amylases (EC 3.2.1.133) (PARK et al., 2000). It is noteworthy that the identity between TVA II and neopullulanases and cyclomaltodextrinases (ca. 50%) is higher than that between TVA II and TVA I (ca. 30%).

Neopullulanases are enzymes that hydrolyze pullulan to produce panose, and they also hydrolyze starch, but less efficiently. Cyclomaltodextrinases are enzymes which efficiently hydrolyze CDs, and, interestingly, OGUMA et al. (1993) reported that pullulan is a poor substrate for the cyclomaltodextrinase from *Bacillus sphaericus*. Although both TVA I and TVA II hydrolyze pullulan and cyclodextrins, TVA I strongly hydrolyzes starch but less efficiently hydrolyzes α- and β-CDs, while TVA II shows outstanding kinetic values for small oligosaccharides and CDs. Therefore, the substrate specificties of these pullulan/CD-hydrolyzing enzymes are clearly different while their primary structures are homologous, and the molecular evolution and the relationships between their structures and functions are intriguing. In this study we have investigated and compared the structures and functions of TVA I and TVA II by means of X-ray crystallography and site-directed mutagenesis.

Three-dimensional structure of TVA II

We have reported the unliganded crystal structure of TVA II (KAMITORI et al., 1999). TVA II is composed of four domains, A, B, C, and N (Fig. 1A). Domain A has a $(\beta/\alpha)_8$ barrel structure with a

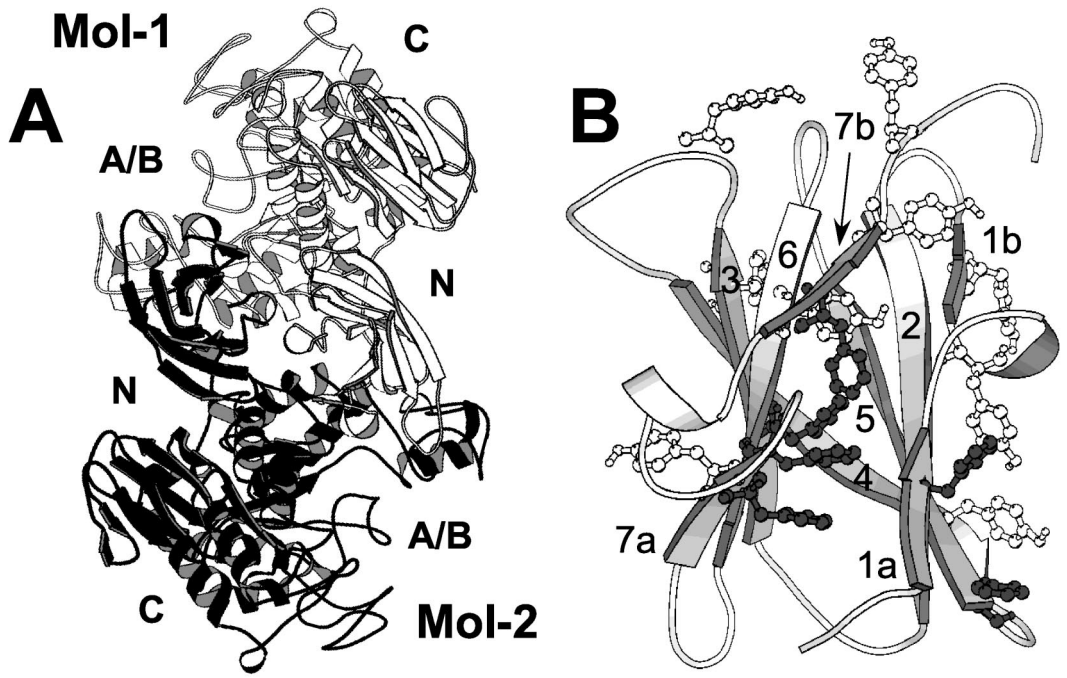

Fig. 1. Three-dimensional structure of TVA II. (**A**) A view from the non-crystallographic 2-fold axis of TVA II as illustrated by the program MOLSCRIPT. Domains N, A/B, and C are indicated. Mol-1 and Mol-2 are in the light and dark colors, respectively. (**B**) Domain N of TVA II. Phenylalanine residues (black), tyrosine residues (white), and the seven β-strands (1a, 1b, 2–6, 7a, and 7b) are indicated.

small domain protruding from it , domain B. Domain C contains the C-terminus, and it consists of eight β-strands. Domains A/B and C are commonly found in α-amylase family enzymes. Domain N contains the N-terminus, and it consists of seven β-strands. Only a few enzymes in the α-amylase family have domains located prior to the N-terminus of domains A, B, and C, thus, domain N is one of the distinguishing structural features of TVA II.

Domain N consists of seven β-strands, and the first and seventh β-strands are divided into strands 1a and 1b, and strands 7a and 7b, respectively (Fig. 1B). Domain N forms a distorted incomplete β-barrel structure, and if one more β-strand were located in front of the open barrel, the barrel structure would be a completely closed form, but it is an incomplete and open form. Thus many hydrophobic residues in the barrel are located on the exterior to form the solvent accessible surface. In particular, many Phe and Tyr residues are located on and in the barrel. There are many reports that aromatic residues such as Phe, Tyr, and Trp residues of carbohydrate-hydrolyzing enzymes function in substrate recognition and binding (MATSUI et al., 1994, PENNINGA et al., 1995) Thus these residues in TVAII and also the hydrophobicity associated with them may play an important role in substrate recognition and binding.

Another important role of domain N is to drive the formation of the dimeric structure of TVA II. Typical α-amylases do not form a dimer and function as a monomer. In the monomeric structure, domain N seemed to be isolated from other domains, and interacts only with the sixth α-helix of domain A. However, in the crystal, the distance between domain N of one molecule and that of another molecule was much less and interactions were tighter than those between other domains; thus TVA II formed a dimeric structure due to the interaction of domains N (Fig. 1A). We also confirmed that TVA II formed the dimer in solution (YOKOTA et al., 2001a).

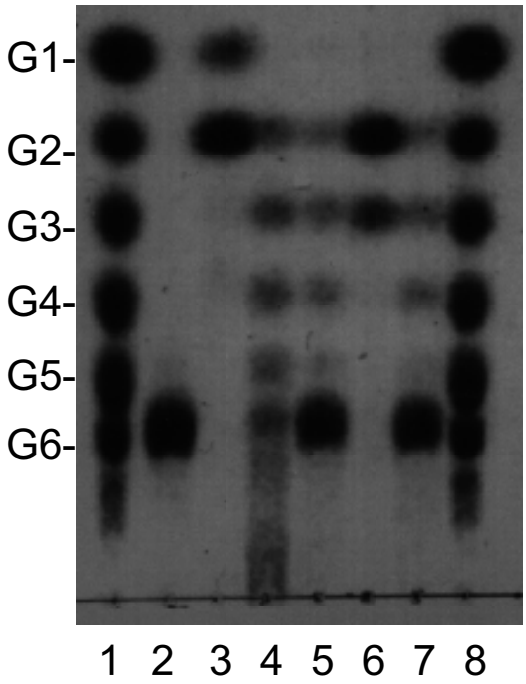

Fig. 2. Thin-layer chromatograms showing maltohexaose hydrolysis of wild-type and mutated TVA II. Abbreviations are as follows: G1, glucose; G2, maltose; G3, maltotriose; G4, maltotetraose; G5, maltopentaose; G6, maltohexaose. Lanes 1 and 8, maltooligosaccharides marker (G1-G6); 2, maltohexaose (no enzyme added); 3, wild-type TVA II; 4, D421N; 5, D325N; 6, E354D; 7, E354Q. The reaction mixture containing 80 μL of 0.5% maltohexaose and 20 μL of each enzyme (wild-type = 0.22 mg/mL, mutants = 1.8 mg/mL) was incubated at 40 ℃ for 1 day.

Crystal structure of TVA II complexed with substrates

To determine the crystal structure of TVA II-substrate complexes, the three catalytic residues, Asp325, Glu354, and Asp421, were modified. The activities of these mutated enzymes were markedly reduced (less than 0.05% of the activity of wild-type enzyme) but traces of activity still remained (ICHIKAWA et al., 2000). The hydrolyzing patterns of the mutated enzymes were further investigated. The action pattern of D421N was markedly different from that of the wild-type and the other three mutated enzymes, and oligosaccharides larger than maltohexaose were observed in the products in addition to maltose, maltotriose, maltotetraose and maltopentaose (Fig. 2). This result indicated that the D421N enzyme catalyzes transglycosylation rather than hydrolysis. In the three catalytic acidic amino-acid residues, Asp, Glu, and Asp, of α-amylase-family enzymes, the third Asp residue has been proposed to capture water molecules during hydrolysis (reviewed by KURIKI & IMANAKA, 1999), and the report supports our results in this study.

The structure of E354A TVA II complexed with β-CD (KONDO et al., 2001), and D325N TVA II complexed with methyl β-cyclodextrin (mβ-

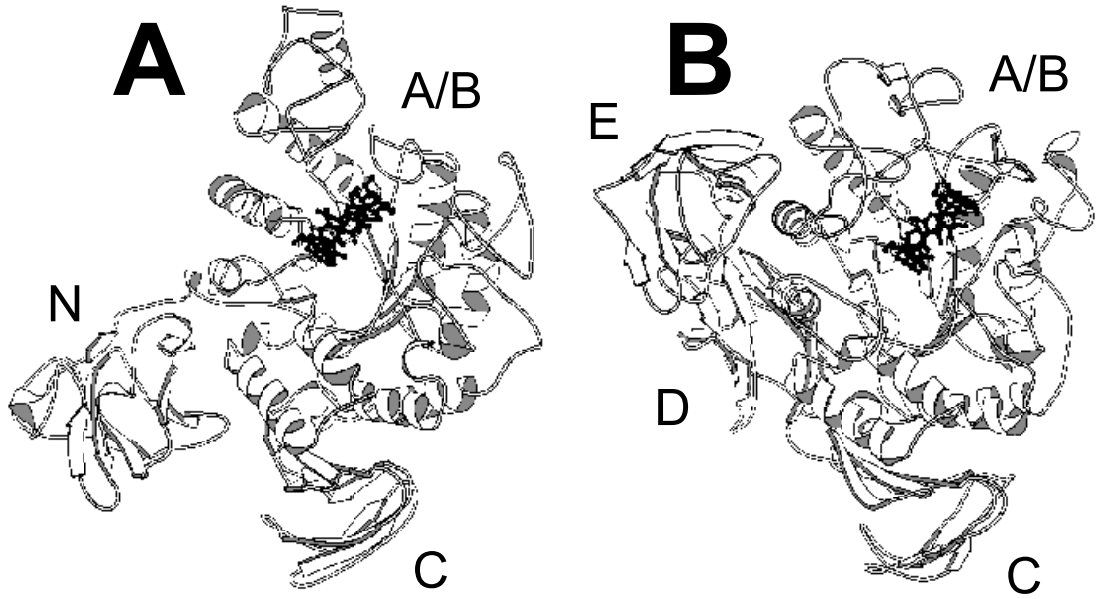

Fig. 3. Comparison of three-dimensional structures of TVA II (**A**) and CGTase (**B**), both of which bind to β-CD. Domains N, A/B, C, D and E, and β-CD are indicated.

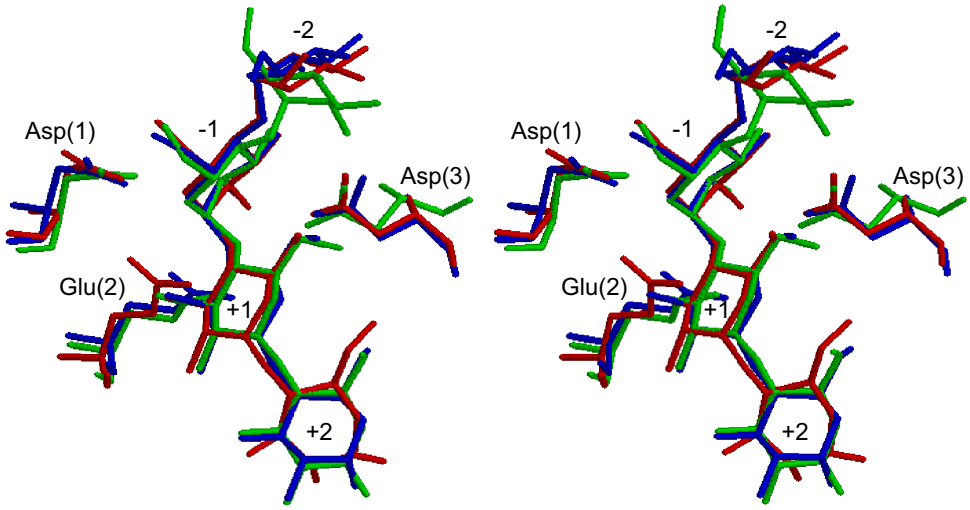

Fig. 4. Superimposition of active centers of TVA II (red), *Bacillus subtilis* α-amylase (green), and CGTase (blue) complexed with a maltooligosaccharide. The PDB codes are 1JIB, 1BAG, and 1CXF, respectively. The three conserved catalytic amino acid residues of α-amylase-family enzymes are shown as Asp(1), Glu(2), and Asp(3), and they correspond to residues D325, E354, and D421 of TVA II respectively. Numbers -2 to $+2$ indicate glucose units, and the enzymatic cleaving point is located between glucose -1 and $+1$.

CD) (Yokota et al., 2001b) were determined. β-CD and mβ-CD are bound to the active-site cleft, which is formed by domains A and B (Fig, 3A). In the α-amylase family enzymes, the structure of cyclodextrin glycosyltransferase (CGTase) complexed with β-CD was reported (Knegtel et al., 1995). The overall structures of TVA II and CGTase are quite different because similarity between

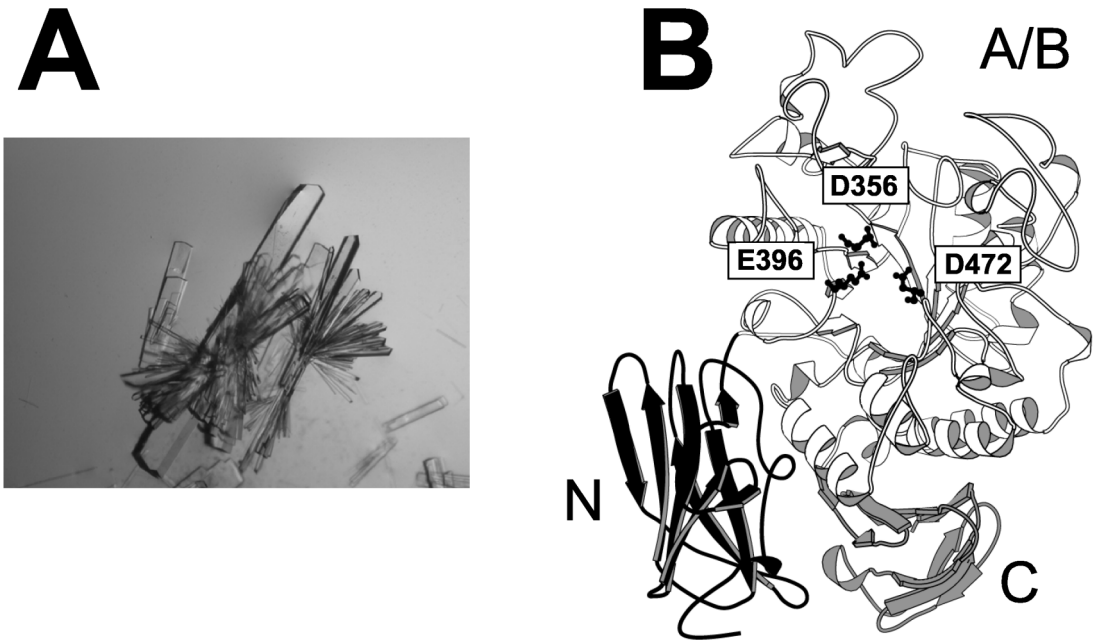

Fig. 5. X-ray crystallography of TVA I. (**A**) A photograph of crystals of TVA I. (**B**) The three-dimensional structure of TVA I. Domains N, A/B, and C, and the three catalytic amino acid residues, D356, E396, and D472 are indicated.

domains of these enzymes is not found, except in domains A, B, and C. However, the position of β-CD in the crystal structure of CGTase resembled that in the monomeric form of TVA II (Fig. 3B).

The structure of D325N TVA II complexed with maltohexaose (G6) was also investigated (YOKOTA et al., 2001b). Only four glucose units can be identified in the annealed omit map, although the electron density map of these four glucose units was clearly obtained. The torsion angles of glucosidic bonds at the site of cleavage of G6 deviate considerably from those in a regular helical structure of amylose. Structures of *Bacillus* α-amylase and CGTase complexed with a maltooligosaccharide have been already reported, thus these structures were superimposed (Fig. 4). Although the positions of glucose units located relatively far from the hydrolyzing site (-2 and $+2$) were different, the positions and torsion angles of glucosidic bonds at the cleaving point (between -1 and $+1$) were very similar.

Crystal structure of TVA I and analyses of roles of domain N for both enzymes

Crystals of TVA I were obtained using the hanging drop vapor diffusion method at 20 °C, with a well solution of 0.5–2% (w/v) polyethylene glycol 10,000 in 50 mM of MES buffer (pH 6.5) and a protein solution of 30 mg/mL (KONDO et al., 2000) (Fig. 5A). The heavy atom derivatives were also prepared by soaking with the salts of platinum and lead, and diffraction data were measured using a Rigaku R-AXIS IIc imaging plate system and also from synchrotron at Photon Factory, Tsukuba, Japan. Phasing and refinement were performed in XtalView and CNS, and the crystal structure of TVA I was determined by multiple isomorphous replacement at 1.6 Å (KAMITORI et al., 2002) (Fig. 5B).

Like TVA II, the structure of TVA I is composed of domains A, B, C, and N, and domains N of both enzymes mainly consist of β-strands. However, unlike TVA II, TVA I is observed as a monomeric form in the crystal, and the interaction between domain N and other domains is quite different. Domain N of TVA II in the monomer is isolated from other domains with a few interactions with domain A, while domain N of TVA I forms many interactions with domains A and C to stabilize the whole enzyme structure.

The substrate-accessible cleft at the active site of TVA I is wide and shallow, thus large polysaccharides such as starch and pullulan seem

to have easy access, while small oligosaccharides such as CDs and maltooligosaccharides seem less suitable to stay in the catalytic cleft. In contrast, TVA II forms a dimer and the substrate-accessible surface of TVA II is partially covered by the partner molecule of the dimeric structure. Therefore the catalytic cleft of TVA II could be more effective in holding small maltooligosaccharides and CDs.

To investigate further the roles of domain N for both enzymes, domain N of TVA I and TVA II was removed (Yokota et al., 2001b). We first deleted regions encoding domain N from the expression plasmids, but soluble enzymes were not obtained, suggesting that domain N is also essential for the folding process of TVAs. Therefore, a sequence composed of six amino acids, Leu-Val-Pro-Arg-Gly-Ser, the cleaving site of thrombin protease, was genetically inserted between domain N and domain A of TVA I and TVA II, and domain N was planned to be removed proteolytically. The results of SDS-PAGE showed that domain N of the enzymes was lost in *E. coli* during cultivation, and we obtained the soluble TVA I and TVA II without domain N (designated as TVA I-ΔN and TVA II-ΔN, respectively). The proteolytic fragments of domain N were not observed on SDS-PAGE and could not be purified.

The activities of both TVA I-ΔN and TVA II-ΔN for polysaccharides, maltooligosaccharides, and CDs were greatly decreased (ca. 0.010–0.062% of those of wild-type enzymes). The findings suggest that, as well as participating in the formation of dimer in TVA II, domain N is essential for binding and recognition of substrates in both TVA I and TVA II.

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