Review

The conclusive proof that supports the concept of the α-amylase family: structural similarity and common catalytic mechanism

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Abstract: We previously found a new enzyme, neopullulanase (EC 3.2.1.135) from *Bacillus stearothermophilus*, and showed that it catalyzes the hydrolysis of α-1,4- and α-1,6-glucosidic linkages, as well as transglycosylation to form α-1,4- and α-1,6-glucosidic linkages. The replacement of several amino acid residues that constitute the active center of the neopullulanase showed that one active center of the enzyme participated in all four reactions described above. Pointing out the structural similarity and the common catalytic mechanism of the enzymes that catalyze these four reactions, we proposed and defined a general idea for the α-amylase family as a family of functionally related enzymes. We recently determined the crystal structure of the neopullulanase and its complexes with panose, maltotetraose, and isopanose. The electron densities in the complexes with panose, maltotetraose, and isopanose were clearly identified at the same active center, indicating that the cleavages of α-1,4 linkage of maltotetraose and α-1,6 linkages of isopanose are carried out by the same active center. Possible acceptor binding for transglycosylation was also observed. These mutational and structural analyses are the conclusive proof that supports the concept of the α-amylase family as enzymes with structural similarity and a common catalytic mechanism.

Key words: α-amylase family, neopullulanase, catalytic mechanism, structural similarity, mutational analysis, structural analysis.

Introduction

The concept of α-amylase family (Takata et al., 1992) was proposed independently from the classification of carbohydrate-active enzymes based on amino acid sequence similarities (Henrissat, 1991; for updated form see http://afmb.cnrs-mrs.fr/CAZY/). In 1989, we had already pointed out, in cyclomaltodextrin glucanotransferase, pullulanase, isoamylase, and neopullulanase, the existence of the four highly conserved regions that are well-known in α-amylases (Nakajima et al., 1986) and contain all the catalytic residues and the residues that bind glucosyl residues adjacent to the scissile linkage in the substrate, even though each enzyme has different function. We had also discussed the possibility of common catalytic machinery in these enzymes, based on the experimental results for neopullulanase (Kuriki & Imanaka, 1989) and the structure of Taka-amylase A (Matsuura et al., 1984).

Based on the structural similarity and common catalytic mechanisms among the enzymes described above, we first proposed and defined the concept of α-amylase family (Takata et al., 1992). We defined the α-amylase family as an enzyme family that fulfils the following requirements: the α-amylase family enzymes (i) act on α-glucosidic linkages; (ii) hydrolyze α-glucosidic linkages to produce α-anomeric mono- and oligo-saccharides or form α-glucosidic linkages by transglycosylations; (iii) have four highly conserved regions in their primary structures which contain all the catalytic and most of the important substrate-binding sites; and (iv) have Asp, Glu, and Asp residues as catalytic sites corresponding to Asp206, Glu230, and Asp297 of Taka-amylase A (Takata et al., 1992).

In order to explain the concept of the α-amylase family, we schematically represented the relationship of specificities for the target linkage and reaction of the enzymes typically belonging to the α-amylase family (Takata et al., 1992; Fig. 1). Hydrolysis of α-1,4-glucosidic linkages is typically catalyzed by α-amylase. Pullulanase and isoamylase hydrolyze α-1,6 linkages. Amylomaltase catalyzes transglycosylation to form α-1,4-glucosidic linkages. Branching enzyme catalyzes transglycosylation to form α-1,6 linkages. These four reactions and classification of these enzymes can be clearly distinguished. Each of the four reactions is cat-

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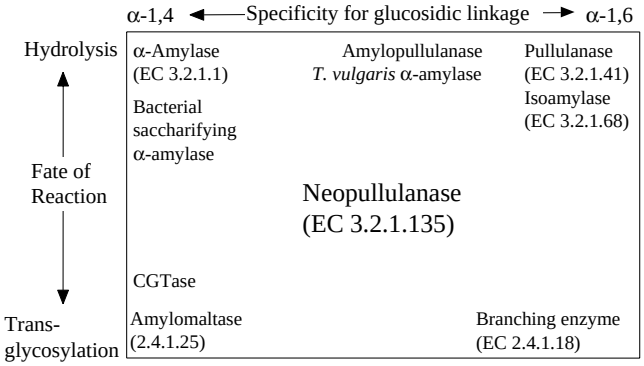

Fig. 1. Schematic representation of the relationship of specificities for the target linkage and reaction of the enzymes typically belonging to the α-amylase family.

alyzed by one of four individual types of enzyme. However, some exceptional examples have been reported. Several α-amylases have been shown to catalyze α-1,4 transglycosylation in addition to the main reaction, α-1,4 hydrolysis. Cyclomaltodextrin glucanotransferases feebly but significantly catalyze α-1,4 hydrolysis. We may, therefore, reasonably conclude that the boundary between glucanohydrolases and glucanotransferases is not clear. Some enzymes other than neopullulanase have been reported to hydrolyze not only α-1,6- but also α-1,4-glucosidic linkages. Now, therefore, we are aware of the existence of enzymes at the boundary between α-amylase and pullulanase or isoamylase. Neopullulanase catalyzes all four reactions: hence, the enzyme should be located in the center as shown in Figure. 1 (TAKATA et al., 1992; KURIKI, 1999; for a review see KURIKI & IMANAKA, 1999).

We describe here the conclusive proof that supports the concept of the α-amylase family, focusing attention on our work. Other important contributions to establish the concept of the α-amylase family have been described in excellent reviews (SVENSSON, 1994; MACGREGOR et al., 2001; JANECEK, 2002).

Why is the concept of the α-amylase family important for us?

α-Amylases have been used industrially for many years. Pullulanases and isoamylases have also been used for various industrial purposes. Cyclomaltodextrin glucanotransferases are useful for the production of various cyclodextrins and glycosides. We have first industrialized branching enzyme and developed a new material: Cluster Dextrin^{TM} (FUJII et al., 2003). We have also first industrialized amylomaltase and developed cyclic α-1,4 glucans with degrees of polymerization of 22 and higher (FUJII et al., 2003). Thus, we have been demonstrating industrial uses for various α-amylase family enzymes. We have also been trying to interconvert glucanohydrolases/glucanotransferases, and their specificity and create tailor-made industrially useful enzymes based on the concept of the α-amylase family. We have controlled the specificity toward α-1,4- and α-1,6-glucosidic linkages and the transglycosylation activity of neopullulanase (KURIKI et al., 1996), and have changed the substrate and product specificities of branching enzymes (KURIKI et al., 1997). We have also introduced new function to an α-amylase by domain fusion with cyclomaltodextrin glucanotransferase (OHDAN et al., 2000) and have engineered amylomaltase to essentially erase hydrolytic activity (FUJII et al., 2005). We are going further to improve enzymes based on the concept of the α-amylase family.

Mutational analyses to provide evidence that one active center of neopullulanase participates in all four reactions; hydrolysis of α-1,4- and α-1,6-glucosidic linkages and transglycosylation to form α-1,4- and α-1,6-glucosidic linkages

Neopullulanase was the key enzyme to open the door for formulation of the concept of α-amylase family (Fig. 1). Neopullulanase catalyzes α-1,4 and α-1,6 hydrolysis and α-1,4 and α-1,6 transglycosylations. If one active center of neopullulanase definitely catalyzes all four reactions, we can conclude that the catalytic mechanisms of the enzymes belonging to α-amylase family are basically the same, thus providing conclusive proof that supports the concept of the α-amylase family of enzymes with structural similarity and a common catalytic mechanism. If the same active center of neopullulanase participates in all four reactions, we should obtain the following results: (i) neopullulanase should simultaneously lose all activities following the replacement of one of the catalytic residues; and (ii) the enzyme specificity toward each type of glucosidic linkage should be altered by the replacement of the residue involved in substrate recognition. When one of the catalytic residues, Asp328, Glu357, or Asp424, was replaced by their amide form, Asn, Gln, or Asn, respectively, neopullulanase lost all four activities (KURIKI et al., 1991; Fig. 2). When one or two of the residues involved substrate recognition were changed, the mutated neopullulanases exhibited different specificities toward α-1,4- and α-1,6-glucosidic linkages (KURIKI et al., 1991; Fig. 2 and Table 1). This was the first clear evidence, by mutational analysis, showing that one active center of an enzyme participates in hydrolysis of α-1,4 and α-1,6-glucosidic linkages and transglycosylation to form α-1,4- and α-1,6-glucosidic linkages. These observations were the key evidence used to formulate the concept of the α-amylase family as described previously.

Structural analyses to provide evidence that one active center of neopullulanase participates in all four reactions; hydrolysis of α-1,4- and α-1,6-glucosidic linkages and transglycosylation to form α-1,4- and α-1,6-glucosidic linkages

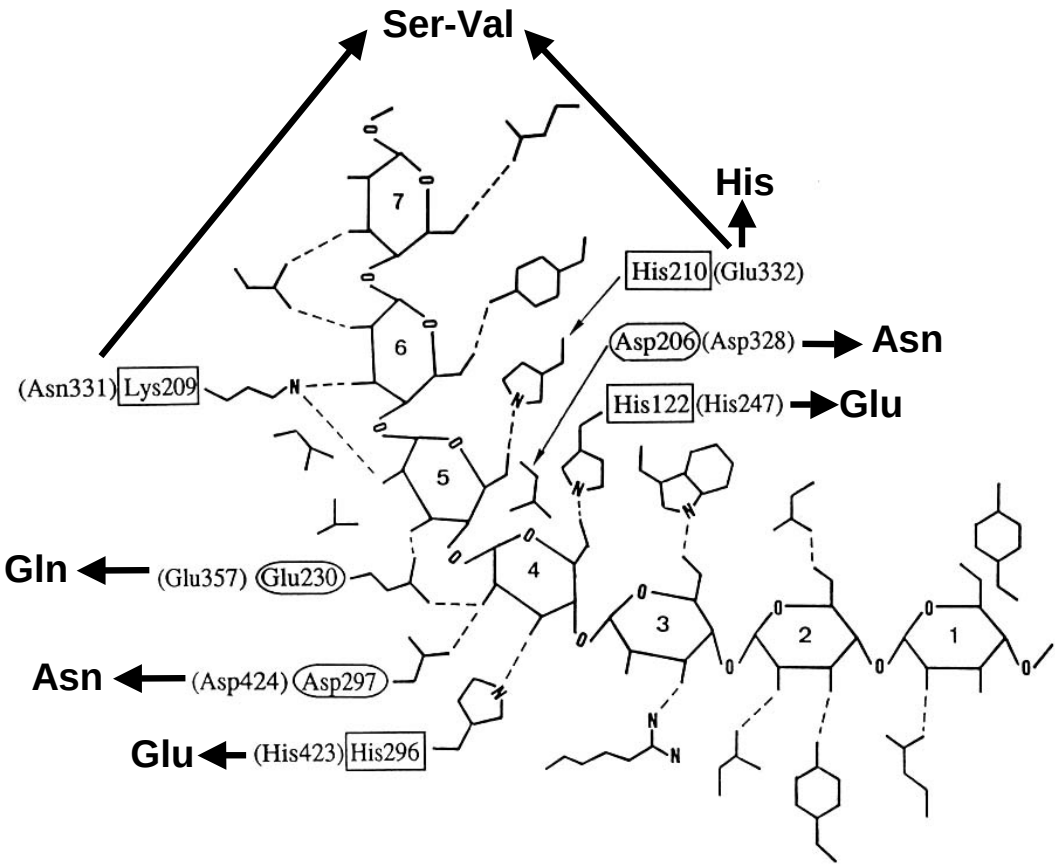

Fig. 2. Preliminary substrate-binding model of neopullulanase based on that of Taka-amylase A, showing mutations at catalytic and substrate-binding sites. Catalytic and substrate-binding sites for Taka-amylase A are indicated as oblongs and rectangles, respectively (Matsuura et al., 1984). Corresponding amino acid residues of neopullulanase are shown in parentheses (Kuriki et al., 1991). The cleavage point lies between residues 4 and 5.

1 Table 1. Activity ratio of wild-type and mutated neopullulanase toward α-1,4 and α-1,6 linkages (Kuriki et al., 1991).

Mutation	Activity ratio for α-1,4 /α-1,6 linkages
Wild type	4.6
H247E	6.8
H423E	6.9
E332H	2.1
N331S-E332V	2.2

We recently determined the crystal structure of *Bacillus stearothermophilus* neopullulanase and its complexes with panose, maltotetraose, and isopanose (Hondoh et al., 2003). The active enzyme forms a dimer in the crystalline state and in solution. The monomer enzyme is composed of four domains, N, A, B, and C, and has a $(\beta/\alpha)_8$-barrel in domain A. The active site lies between domain A of one monomer and domain N from the other monomer. Figure 3 shows the *Fo*-*Fc* electron density omit map with structure models of bound panose, maltotetraose, and isopanose. Panose is the main product of pullulan hydrolysis by neopullulanase (Kuriki et al., 1988). Neopullulanase mainly cleaves the middle position of the three α-1,4 linkages of maltotetraose and exclusively cleaves the α-1,6 linkage of isopanose (Imanaka & Kuriki, 1989). Since maltotetraose and isopanose are substrates of neopullulanase, a deactivated mutant, with the proton donor, Glu357, replaced by Gln, was used for complex crystallization with maltotetraose and isopanose. In the panose complex, the cleavage point is located at the reducing end, i.e. the reducing-end glucose lies in the position of glucose residue 4 of Figure 2. In the maltotetraose complex, the cleavage point is located at the central α-1,4 linkage. Thus the reducing-end glucose lies in the position of residue 6 of Figure 2. In the isopanose complex, the cleavage point is located at the α-1,6 linkage. In this case the reducing-end glucose lies in the position of residues 5 of Figure 2. Indeed, the electron densities of bound oligosaccharides were clearly identified at the same active center (Fig. 3). As mentioned previously, neopullulanase strongly catalyzes a transglycosylation reaction to form both α-1,4- and α-1,6-glucosidic linkages. A significant electron density was observed at the proximate position of bound maltotetraose and isopanose, but not at bound panose. Therefore, we conclude that this electron density is maltose, which works as an acceptor for transglycosylation.

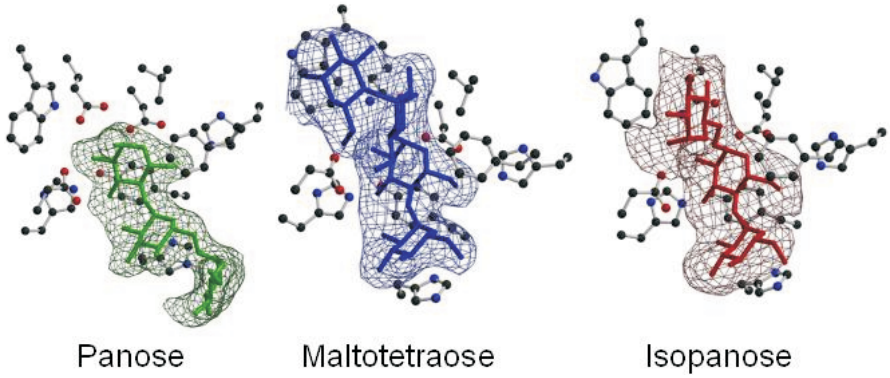

Fig. 3. *Fo–Fc* electron density omit map for the active site of *Bacillus stearothermophilus* neopullulanase with structure models of bound oligosaccharides. The reducing ends of the oligosaccharides lie toward the top of the picture.

This was also the first clear structural evidence showing that one active center of an enzyme participates in hydrolysis of α-1,4 and α-1,6-glucosidic linkages and transglycosylation to form α-1,4- and α-1,6-glucosidic linkages.

Concluding remarks

As new enzymes have been discovered, the main focus of biocatalysis has been on distinguishing and classifying enzymes. However, the study of neopullulanase was the key to open the door for the formulation of the concept of the α-amylase family: "Not only the structural similarity but also the common catalytic mechanism lie in a significant number of glucanohydrolases and glucanotransferases".

These structural and mutational analyses, described in this article, provide conclusive proof that supports the concept of an α-amylase family having structural similarity and a common catalytic mechanism.

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