

Protein engineering of amyloamylase from *Thermus aquaticus* with random and saturation mutageneses

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Abstract: Amyloamylase catalyses an intramolecular transglycosylation of α -1,4 glucan and produces cyclic α -1,4 glucan with a degree of polymerization (DP) of 17 and larger. These large cyclodextrins, referred to as cycloamylose in this article, can form inclusion complexes with various guest molecules, but differ from conventional cyclodextrins with a DP from 6 to 8, in their structures and properties. The amyloamylase from *Thermus aquaticus* is highly thermostable and thus potentially useful for the manufacture of cycloamylose. However, together with its major transglycosylation activity, the enzyme possesses weak but significant hydrolytic activity, which consequently reduces the yield of cycloamylose. In order to obtain an amyloamylase with lower hydrolytic activity, the amyloamylase gene from *T. aquaticus* was subjected to random and saturation mutageneses. Based on these studies we obtained a mutated amyloamylase with enhanced cyclization activity and dramatically decreased hydrolytic activity, which is suitable for manufacturing cycloamylose on an industrial scale. A study of various mutated enzymes also provided with considerable information that helps to understand the activity and specificity of this enzyme.

Key words: amyloamylase, *Thermus aquaticus*, protein engineering, cycloamylose, cyclic glucan.

Abbreviations: DP, degree of polymerization; CGTase, cyclodextrin glycosyltransferase; 4 α GT, 4- α -glucanotransferase; Taq, *Thermus aquaticus*.

Cyclic α -1,4 glucan

Cyclic α -1,4 glucans with a degree of polymerization (DP) of 6, 7 and 8 are generally called cyclodextrins and produced by the cyclization reaction of cyclodextrin glycosyltransferase (CGTase, EC 2.4.1.19) on starch. Cyclodextrins can incorporate various hydrophobic guest molecules into their central cavities and alter their properties, such as solubility, stability and other chemical properties. Because of their ability to form an inclusion complex, cyclodextrins are widely used in various industries, including pharmaceutical, cosmetics, food, toiletries, agriculture, and chemical. Larger cyclodextrins with a DP from 9 to 13 have been reported (FRENCH, 1957), but they are present only in trace amounts and believed to be produced as by-products of cyclodextrins by CGTase.

It had been believed that CGTase was the only en-

zyme that can produce cyclic α -1,4 glucan. However, TAKAHA et al. (1996) found that the disproportionating enzyme (D-enzyme, EC 2.4.1.25) from potato also catalyzed the cyclization reaction of linear α -1,4 glucan and produced cyclic α -1,4 glucan with a DP from 17 to several hundreds (referred to as cycloamylose in this article). Cycloamylose thus produced has been subjected to various studies to investigate its structures in solution (KITAMURA et al., 1997) and in a crystal form (JACOB et al., 1998; 1999; SAENGER et al., 1998; GESSLER et al., 1999), its physicochemical properties (ENDO et al., 2002; UEDA et al., 2002) and its inclusion complex formation (KITAMURA et al., 1999; ENDO et al., 2002). These results showed that the structure and properties of cycloamylose differed from the conventional cyclodextrins. While the structures of the conventional cyclodextrins are of a doughnut shape, the structure of cycloamylose with a DP of 26 determined (GESSLER et

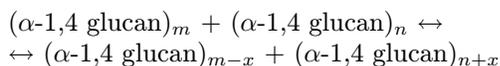
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al., 1999) is no longer taking a doughnut-like conformation, where two single-helical amylose moieties in anti-parallel arrangement are joined by the band-flip structure. Cycloamylose showed much higher solubility than the conventional cyclodextrins and it also has the ability to form an inclusion complex with guest molecules. It was recently reported that cycloamylose functions as an artificial chaperone to refold denatured protein into an active form (MACHIDA et al., 2000).

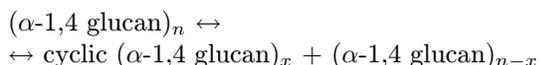
Enzymes for the synthesis of cycloamylose

4- α -Glucanotransferases (4 α GTs) are well known as the member of the α -amylase family and mostly catalyze the transglycosylation reaction (KURIKI et al., 2005). 4 α GT activities are widely distributed in bacteria, yeasts, plants and animals, and categorized by at least five structurally distinct enzyme groups. Both CGTase, and D-enzyme (amylomaltase) are members of this enzyme group, together with three other enzymes, including glycogen debranching enzyme, 4 α GT from *Thermotoga maritima*, and other 4 α GTs. This enzyme group is mainly found in Archaea (for a review, see TAKAHA & SMITH, 1999). Various 4 α GTs have been tested for their ability to produce cycloamylose. As summarized in Table 1, various enzymes belonging to 4 α GTs also catalyze the cyclization reaction and produce cyclic α -1,4 glucans (TAKAHA & SMITH, 1999; FUJII et al., 2003). These results suggest that the cyclization reaction is not a special activity present in a particular enzyme, but it is a common feature of 4 α GTs. However, the smallest cyclic glucan produced depended on the type of 4 α GT employed, as shown in Table 1.

The action of 4 α GTs can be summarized as follows:



This action is the inter-molecular glucan transfer reaction, and is often called the disproportionation reaction. The enzyme catalyzes the transfer of the α -1,4 glucan chain from a donor molecule to an acceptor molecule. As described above, most 4 α GTs also catalyze an intramolecular glucan transfer reaction (cyclization reaction), within a single linear glucan molecule, creating a cyclic glucan product, as follows:



This reaction is reversible, and the reverse reaction is often referred to as a coupling reaction when the donor molecule is a cyclic glucan. In addition to these transglycosylation reactions, most 4 α GTs also catalyze the hydrolysis of α -1,4-glucosidic linkage. The ratio of hydrolytic to transglycosylation activities differs among enzymes.

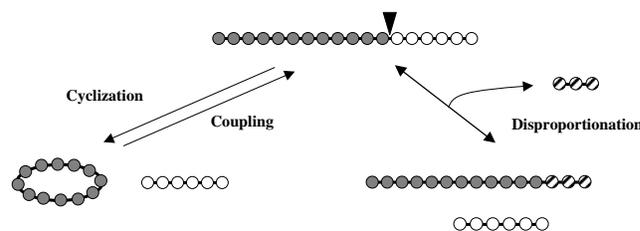


Fig. 1. Reactions of 4 α GTs on α -1,4 glucans. Each circle represents a glucose molecule.

Conversion of linear amylose into cycloamylose with 4 α GTs proceeds by the combination of these transglycosylation activities (Fig. 1). Linear amylose is cleaved by 4 α GTs, then the cleaved fragment is transferred either to the nonreducing end of a separate linear acceptor molecule (disproportionation reaction) or to its own nonreducing end (cyclization reaction). The reversibility of these reactions allows high molecular weight cyclic molecules to be linearized again by the coupling reaction. If the enzyme has no hydrolytic activity, the composition of the final products should be determined by the equilibrium of these activities. If the enzyme has hydrolytic activity, a whole glucan should be consequently converted into a smaller one, causing a reduction in the yield of cycloamylose.

In addition to the production of cycloamylose, amylomaltase can be applied to starch processing. The amylomaltase from *Thermus thermophilus* can produce a strong thermo-reversible gel from starch, which is very similar to gelatin (KAPER et al., 2004).

Amylomaltase from *Thermus aquaticus*

Among the enzymes that can produce cycloamylose, amylomaltase from *Thermus aquaticus* (Taq) is particularly interesting because it produces cycloamylose with a DP of 22 and larger. The smallest cycloamylose produced by this enzyme is larger than that produced by the potato D-enzyme by 5-glucose units; it is the largest cycloamylose produced by the enzymes shown in Table 1. Taq amylomaltase exhibits maximum activity at 75 °C and is stable at temperatures up to 85 °C. In spite of these differences, Taq amylomaltase exhibits a high level of homology with the potato D-enzyme (41% at the amino acid sequence level).

The crystal structure of Taq amylomaltase has already been determined in its native form (PRZYLAS et al., 2000b) and in a complex with acarbose molecules (PRZYLAS et al., 2000a). In the analysis of the enzyme-acarbose complex, two acarbose molecules were bound to the enzyme, one in the active site groove at subsite -3 to +1 and the other one around Tyr54, which is 14 Å away from the catalytic site (Fig. 2). It is considered that Tyr54 interacts with substrate and helps to form a curved conformation of the glucan chain in this region. The catalytic center of Taq amylomaltase is

Table 1. 4 α GTs which can produce cyclic glucan.

Enzyme (source)	EC number	4 α GT Family ^a	CAZy ^b	DP of smallest cyclic glucan (Ref.)
CGTase (<i>Bacillus macerans</i>)	EC 2.4.1.19	Type I	GH13	6 (FRENCH, 1957)
D-enzyme (<i>Solanum tuberosum</i>)	EC 2.4.1.25	Type II	GH77	17 (TAKAHA et al., 1996)
Amylomaltase (<i>Thermus aquaticus</i>)	EC 2.4.1.25	Type II	GH77	22 (TERADA et al., 1999)
GDE (<i>Saccharomyces cerevisiae</i>)	EC 3.2.1.33	Type III	GH13	11 (YANASE et al., 2002)
4 α GT	EC 2.4.1.25	Type IV	GH13	nt ^c (LIEBL et al., 1992)
(<i>Thermotoga maritima</i>)				
4 α GT	EC 2.4.1.25	Type V	GH57	16 (IMAMURA et al., 2001)
(<i>Thermococcus litoralis</i>)				
4 α GT	EC 2.4.1.25	Type V	GH57	16 (TACHIBANA et al., 1997)
(<i>Pyrococcus kodakaraensis</i>)				

^a 4 α GT Family types were defined by TAKAHA & SMITH (1999).

^b CAZy family name was obtained from Carbohydrate-Active Enzymes server at URL: <http://afmb.cnrs-mrs.fr/CAZY/>.

^c nt = not tested.

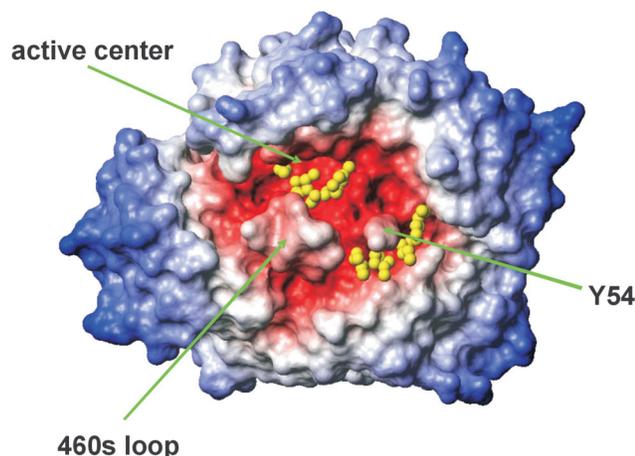


Fig. 2. Molecular surface of Taq amylomaltase in complex with acarbose. The surface is colored according to the distance to the center of the mass, going from red via white to blue. The two acarbose molecules are shown in yellow. The active center, Tyr54 and 460s loop are also indicated.

partially covered with two loops (the 250s loop and the 460s loop), which are proposed to prevent the formation of small cyclic glucans with a DP of 21 and smaller by steric hindrance. A hypothetical pathway for the formation of the smallest cycloamylose starting from the active center, via Tyr54 and the 460s loop, is equivalent to the length for α -1,4 glucan with about 20-glucose units, which corresponds well to the actual minimum size; a DP of 22 (STRÄTER et al., 2002). Therefore, it has been anticipated that Tyr54 and the 460s loop might affect the composition and the smallest size of cycloamylose produced by the Taq amylomaltase.

Engineering of Taq amylomaltase with decreased hydrolytic activity

As described above, Taq amylomaltase is highly ther-

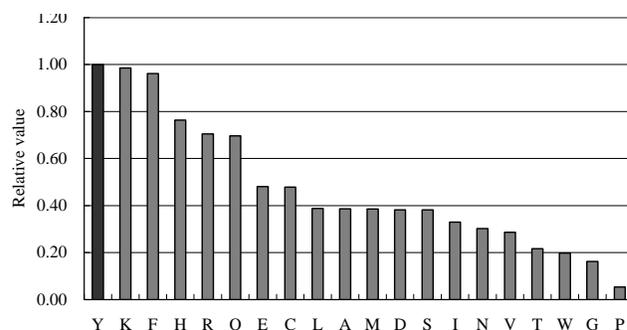


Fig. 3. Hydrolytic activity of Tyr54-mutated enzymes. The Tyr54-mutated enzymes and the wild-type enzyme were extracted from recombinant *E. coli*. Each enzyme solution was purified by heat treatment (at 70 °C for 30 min) and hydrophobic chromatography (Phenyl-TOYOPERAL, Tosoh). The enzyme thus prepared was incubated with 0.5% (w/v) cycloamylose at 70 °C, pH 5.5. The hydrolytic activities were determined by measuring the increase in the reducing power and are expressed relative to the wild-type enzyme. The wild-type enzyme is shown in dark gray, while the mutants in light gray. All of the mutated enzymes, including the wild-type enzyme, are expressed with a single letter of an amino acid residue at the 54th position.

mostable and potentially suitable for the production of cycloamylose on an industrial scale. However, the enzyme not only catalyzes the transglycosylation reaction, but also the hydrolysis of glucan and cycloamylose, which reduces the yield of cycloamylose. In order to obtain a suitable enzyme for the industrial production of cycloamylose, mutated Taq amylomaltases with lower hydrolytic activity were created (K. FUJII et al., submitted manuscript). Random mutations were introduced into the structural gene of the Taq amylomaltase by error-prone PCR and the plasmid library was constructed in *Escherichia coli* as a host. Each clone was grown in LB liquid medium, and the cell extract was prepared and then heated at 70 °C for 30 min to deactivate amylases in the host cell. The enzyme solution thus prepared was subjected to hydrolytic and cyclization activity assays; the methods are described in

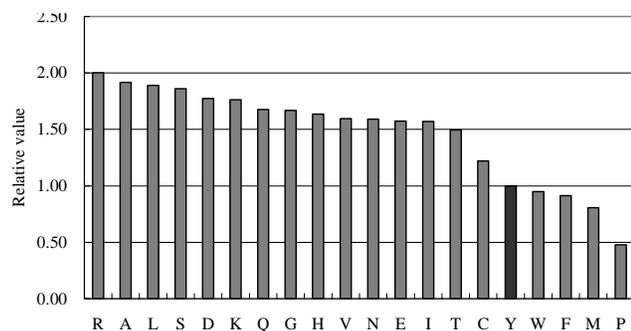


Fig. 4. Cyclization activity of Tyr54-mutated enzymes. The enzyme solution prepared (see the legend to Figure 3) was incubated with 0.1% (w/v) amylose at 70 °C, pH 5.5, and then mixed with iodine solution. The cyclization activities were determined by measuring the decrease in the A₆₆₀ of the amylose-iodine complex and are expressed relative to the wild-type enzyme. The wild-type enzyme is shown in dark gray, while the mutants in light gray. All of the mutated enzymes, including the wild-type enzyme, are expressed with a single letter of an amino acid residue at the 54th position.

Figures 3 and 4, respectively. From this screening program, three mutated enzymes with reduced hydrolytic activity but still retaining cyclization activity were obtained. Surprisingly, all three clones have a single mutation at Tyr54, indicating that an amino acid replacement at this position does affect the hydrolytic activity of this enzyme. Tyr54 was then replaced with all other amino acids by site-directed mutagenesis. The hydrolytic and cyclization activity of these mutants was measured, and the results are summarized in Figures 3 and 4. The hydrolytic activity of the wild-type enzyme was higher than that of any other mutated enzyme. When Tyr54 was substituted with an aromatic or basic amino acid residue, Y54F, Y54K, Y54H or Y54R, these mutated Taq amyloamylases retained higher hydrolytic activity than others, while the hydrolytic activity of the Y54G mutated enzyme was dramatically decreased to 10% of the wild-type enzyme (Fig. 3). In contrast to the hydrolytic activity, the replacement of Tyr54 resulted in the increase of cyclization activity in most cases (Fig. 4). The Y54R mutated enzyme exhibited the highest cyclization activity, reaching double of that of the wild-type enzyme, whereas the activity of the Y54W, Y54F or Y54M mutated enzymes was slightly decreased (Fig. 4).

For the efficient production of cycloamylose, it is considered that the ratio of hydrolytic activity to cyclization activity in the enzyme is required to be as low as possible. The ratio of hydrolytic to cyclization activity is shown in Figure 5. The ratio of wild-type and Y54F enzymes was much higher than the ratios of the others; so these two enzymes are assumed not suitable for cycloamylose production. The Y54G mutated enzyme is expected to be the best enzyme for cycloamylose production, since its ratio is the lowest one (Fig. 5). The time course of cycloamylose syntheses by wild-type and Y54G mutated enzyme is shown in Figure 6. In the

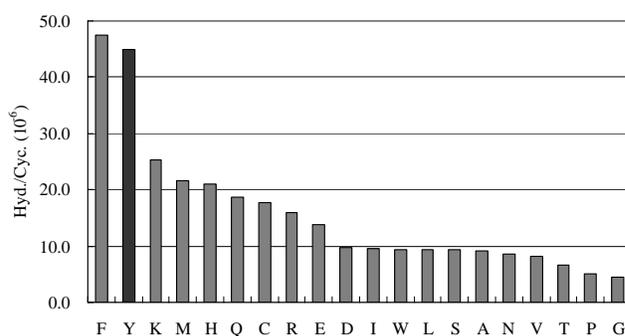


Fig. 5. The ratio of hydrolytic to cyclization activity in Tyr54-mutated enzymes. The ratio of hydrolytic to cyclization activity is expressed relative to the wild-type enzyme. The wild-type enzyme is shown in dark gray, while the mutants in light gray. All of the mutated enzymes, including the wild-type enzyme, are expressed with a single letter of an amino acid residue at the 54th position.

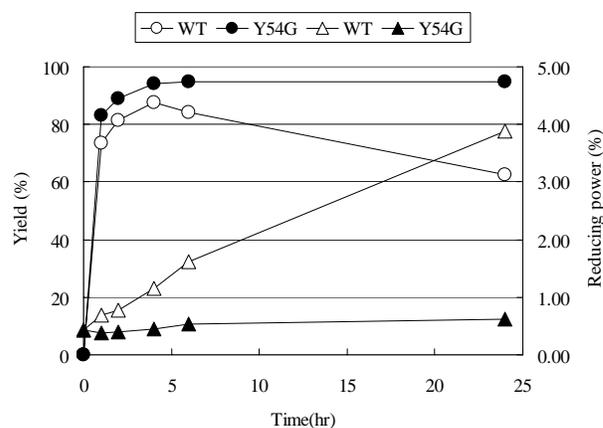


Fig. 6. Time course of cycloamylose production by the Y54G mutant enzyme. The action of the Y54G mutated enzyme (closed symbols) or the wild-type enzyme (open symbols) on amylose. The reducing power when all the amylose is broken down to glucose is defined as 100%. Circles and triangles represent the yield of cycloamylose and the reducing power in the reaction solution, respectively.

case of the wild-type enzyme, the yield of cycloamylose decreased gradually after four hours and dropped to 60% at the end of the reaction (24 hours). In the case of the Y54G mutated enzyme, the yield was not decreased during the reaction time, maintaining a level of more than 90% until the end of reaction (Fig. 6). The reducing power was significantly increased with the wild-type enzyme but not with the Y54G mutated enzyme. Therefore, it is strongly suggested that the presence of hydrolytic activity in the wild-type enzyme causes an increase in reducing power and a concomitant decrease in the yield of cycloamylose. Synthesis of cycloamylose by other mutated enzymes was also carried out, and significant correlations between the ratio of hydrolytic to cyclization activity and the yield of cycloamylose or the reducing power in the reaction solution after 24 hours were confirmed.

The compositions of cycloamylose produced by the

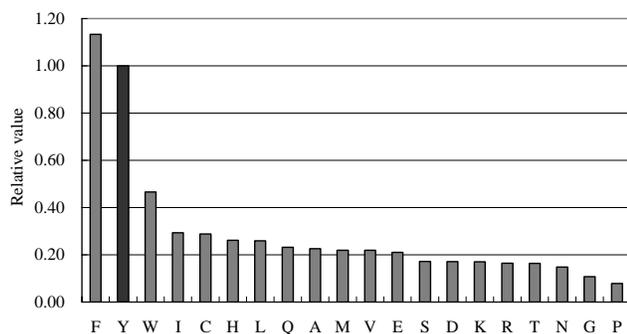


Fig. 7. Disproportionation activity of Tyr54-mutated enzymes. The enzyme solution prepared (see the legend to Figure 3) was incubated with 10% (w/v) maltotriose at 70 °C, pH 5.5. The disproportionation activities were determined by measuring the increase of glucose, and are expressed relative to the wild-type enzyme. The wild-type enzyme is shown in dark gray, while the mutants in light gray. All of the mutated enzymes, including the wild-type enzyme, are expressed with a single letter of an amino acid residue at the 54th position.

19 mutated enzymes were then compared with that produced by the wild-type enzyme. There was no difference in either the smallest DP of cycloamylose produced by the mutated enzymes or the distribution of DP. Although Tyr54 was anticipated to be involved in determining the smallest size of cycloamylose produced (PRZYLAS et al., 2000a), these results strongly suggest that Tyr54 is not involved in such a function.

Role of Tyr54 in the activity of Taq amylo-maltase

Since an amino acid replacement at Tyr54 produces a dramatic effect on both cyclization and hydrolytic activity, the role of Tyr54 in the activity of this enzyme is of great interest. To investigate the function of Tyr54 in a more detail, the disproportionation activity of 19 mutated enzymes was measured (Fig. 7). Surprisingly, the disproportionation activities of all the mutated enzymes except Y54F were remarkably decreased. An aromatic residue at the 54th position has enormous significance in the function of this enzyme *in vivo*.

As described above, a single amino acid replacement at Tyr54 causes various effects on the cyclization, hydrolytic and disproportionation reactions in the Taq amylo-maltase. Tyr54 has been suggested as the secondary substrate-binding site of this enzyme. However, since it is located about 14 Å away from the catalytic residues, it is unlikely that this residue is directly involved in determining the reaction specificity. These results raise a very interesting question on how an amino acid residue far from the catalytic site can affect the specificity of the enzyme so significantly. Although it is still too early to determine the function of Tyr54 in the activity of this enzyme, the tentative explanations from available information are as follows.

In the hydrolytic reaction, water molecules should

be present near the active site. A binding of substrate to Tyr54 might cause a conformational change in this enzyme. With the structural change, the hydrophilicity near the acceptor-binding site may be elevated and the hydrolytic activity is consequently increased.

In the cyclization reaction, the large glucan chain, such as amylose, binds to both the active site and Tyr54 in the wild-type enzyme. However, in Tyr54 mutants, the glucan chain does not bind to the 54th position of the enzyme and is expected to be more flexible in the enzyme. To complete a cyclization reaction, a non-reducing end of glucan bound to the enzyme should be turned back to the active site. In the Tyr54-mutated enzymes, the non-reducing end of glucan may have more opportunities to do this. Then, the cyclization activity of Tyr54-mutated enzyme is increased.

In the disproportionating reaction, an acceptor molecule is required to be present in the acceptor-binding site which should be close to the active site. Tyr54 might be the secondary substrate-binding site and help an acceptor molecule to be sent to the acceptor-binding site. The diffusion rate of substrate from the secondary binding site (Tyr54) to the acceptor-binding site is much faster than that from the solution. The Tyr54-mutated enzymes lacking an aromatic residue at the 54th position will lose their function as the secondary binding site and, as a result, the disproportionation activity is significantly decreased.

Conclusion and future prospects

An amylo-maltase suitable for the industrial production of cycloamylose has been successfully engineered by random and saturation mutageneses. Taq amylo-maltase with a Y54G mutation exhibited significantly higher cyclization activity but dramatically lower hydrolytic activity than the wild-type enzyme did. It is very interesting that the reaction specificity of the enzyme is significantly changed by the replacement of an amino acid residue positioned about 14 Å away from the active site. We believed that the findings presented in this study help to understand the reaction mechanism of this enzyme.

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