J. Appl. Glycosci., **52**, 277–279 (2005)

Note

Site-Directed Mutagenesis of Tryptophan 622 of *Thermoactinomyces vulgaris* R-47 Glucoamylase: pH Optima and Activities of Five Mutants*

(Received February 3, 2005 ; Accepted May 2, 2005)

Kazuhiro Ichikawa, Takashi Tonozuka,** Masahiro Mizuno, Atsushi Nishikawa and Yoshiyuki Sakano

Department of Applied Biological Science, Tokyo University of Agriculture and Technology
(3–5–8, Saiwai-cho, Fuchu 183–8509, Japan)

Abstract: In *Aspergillus awamori* glucoamylase, the optimal pH has been reported to increase to maintain activity by a mutation of Ser411 which forms a hydrogen-bond with a catalytic base (Fang and Ford, *Protein Eng*., 11, 383–388 (1998)). Most glucoamylases have either Ser or Gly at this position, whereas only *Thermoactinomyces vulgaris* R-47 glucoamylase (TGA) and two putative glucoamylases have Trp. We focused on Trp622 in TGA and examined the pH optima of five mutants, W622C, W622D, W622G, W622H and W622S. The pH optima of these mutants were 6.2–6.8, which was identical to or slightly lower than that of the wild-type enzyme. However, the activities of these mutants at pH optima decreased to 4.3–52% of that of wild-type enzyme. From these results and information on the crystal structures of glucoamylases, Trp622 in TGA is suggested to be an important residue for substrate binding rather than for determination of optimal pH.

Key words: glucoamylase, optimal pH, catalytic base

Glucoamylase (1,4-α-D-glucan glucohydrolase, E.C. 3.2.1.3, GA) is an exo-hydrolase that releases β-D-glucose from the non-reducing ends of starch and related oligo- and polysaccharides. GA has been extensively used in starch-processing industries, and because of its commercial importance, numerous GAs have been studied.[1] Fungal GAs prefer starch to maltooligosaccharides and many of them have a starch-binding domain in addition to a catalytic domain. Most fungal GAs have maximal activity in an acidic pH range from 4.0 to 6.0.[2–4] In contrast, bacterial and archaeal GAs hydrolyze maltooligosaccharides more efficiently than fungal GAs. Our previous report indicated that a thermophilic actinomycete, *Thermoactinomyces vulgaris* R-47 GA (TGA),[5] and a methanogenic archaeon, *Methanococcus jannaschii* GA,[6] degrade maltooligosaccharides more preferably than starch, and their pH optima are 6.8 and 6.5, which are almost neutral. *Thermoanaerobacterium thermosaccharolyticum* GA has been reported to show almost equal levels of activity for maltotetraose, maltoheptaose and starch, and is optimally active in a broad pH range between 4.0 and 5.5.[7]

The optimal pH of an enzyme is affected by ionization of its catalytic groups, which depends on the interactions involved in their microenvironments.[8,9] In the case of GA, the catalytic residues are two glutamic acids. Based on the crystal structure information,[10] Fang and Ford modified Ser411 in *Aspergillus awamori* GA by site-directed mutagenesis and successfully elevated the optimal pH.[11] In GA, five conserved regions were proposed by Coutinho and Reilly,[12] and Ser411 in *A. awamori* GA is located in region V. The corresponding residues of most of GAs were identified as either Ser or Gly, while those of only three proteins, TGA, *Methanosarcina acetivorans* C2A putative GA (MA4050) and *Methanosarcina mazei* Goe1 putative GA (MM0864), were found to be Trp. Trp has been reported to be an important residue for the binding and recognition of substrates in glycoside hydrolases.[13–15] We focused on Trp622 in TGA and constructed five TGA mutants, W622C, W622D, W622G, W622H and W622S, to examine whether Trp622 is a residue which affects the optimal pH of this enzyme.

Site-directed mutagenesis was performed using a QuikChange Site-Directed Mutagenesis Kit (Stratagene) to modify an expression plasmid, pTGA6060. To construct W622C, W622D, W622G, W622H and W622S, oligonucleotides 5′-GGG AAA GCG GCT **TGC** GT*G* GTA CCG CTT ACT TGG-3′, 5′-CG GGG AAA GCG GCT **GAC** GT*G* GTA CCG CTT ACT TGG TCC-3′ 5′-GGG AAA GCG GCT **GGG** GT*G* GTA CCG CTT ACT TG-3′ 5′- CG GGG AAA GCG GCT **CAC** GT*G* GTA CCG CTT ACT TGG TCC-3′ 5′-GGG AAA GCG GCT **TCG** GT*G* GTA CCG CTT ACT TG-3′ and their complementary primers were used. The designed mutations are shown in bold, and the silent mutations designed to introduce the *Kpn*I site (underline) to facilitate the selection of the positive clones are shown in italics. All desired mutations were confirmed by DNA sequencing.

All TGA mutants were prepared from *E. coli* MV1184 and purified with the same procedure as for the wild-type enzyme.[5] Their purities were confirmed by SDS-PAGE.[16] Protein concentrations were determined by the measurement of absorbance at 280 nm using the formula of Gill and von Hippel.[17]

TGA activity was assayed as described previously,[5] and

---

* This paper is dedicated to our mentors, the late Professors Dr. Michinori Nakamura, Dr. Susumu Hizukuri and Dr. Toshiaki Komaki, in memory of their numerous pioneering works and leadership in the field of starch and its related science.

** Corresponding author (Tel. +81–42–367–5702, Fax. +81–42–367–5705, E-mail: tonozuka@cc.tuat.ac.jp).

Abbreviations: GA, glucoamylase; TGA, *Thermoactinomyces vulgaris* R-47 glucoamylase.

optimal pH was investigated at 40°C for 30 min using 6.0 mM maltotetraose (corresponds to *ca.* 6-fold of K_m value of wild-type TGA at pH 6.5) in 80 mM sodium citrate buffer (pH 4.5–6.0), sodium phosphate buffer (pH 6.0–8.0) and bicine-NaOH buffer (pH 8.0–9.0).

The pH optima of W622C, W622D, W622G, W622H and W622S were 6.6, 6.2, 6.4, 6.8 and 6.6, which were identical or slightly lower than that of the wild-type enzyme (6.8).

However, the activities of these mutants at pH optima were lower than that of the wild-type enzyme. Although W622H retained 52% of the wild-type enzyme activity, W622G, in which a residue with the smallest side-chain was introduced, drastically decreased to 4.3% of the wild-type enzyme activity. Also, W622D had only 5.0% of the wild-type enzyme activity. In W622C and W622S, the activities at pH optima exhibited 13 and 17% of that of the wild-type enzyme. The decrease in the activities of these mutants at pH optima is not due to a decrease in their thermal stability because these mutants in 10 mM Tris-HCl buffer (pH 7.5) are stable at 50°C for 30 min (data not shown).

Next, the effect of pH on the activities of mutants was compared with that of wild-type TGA. As shown in Fig. 1, all TGA mutants showed narrower bell-shaped curves than the wild-type enzyme. The bell-shape curves of W622C, W622G and W622S, in which a neutral amino acid residue was introduced, were similar and loss of activity in both acidic and basic pH ranges was observed. W622D, in which an acidic amino acid residue was introduced, showed the narrowest bell-shaped curve among these mutants and was drastically reduced under basic conditions compared to that of the wild-type enzyme. In W622H, in which a basic amino acid residue was introduced, a significant decrease in activity occurred in the acidic pH range, but the bell-shaped curve of this mutant in the basic pH range was almost identical to that of wild-type TGA.

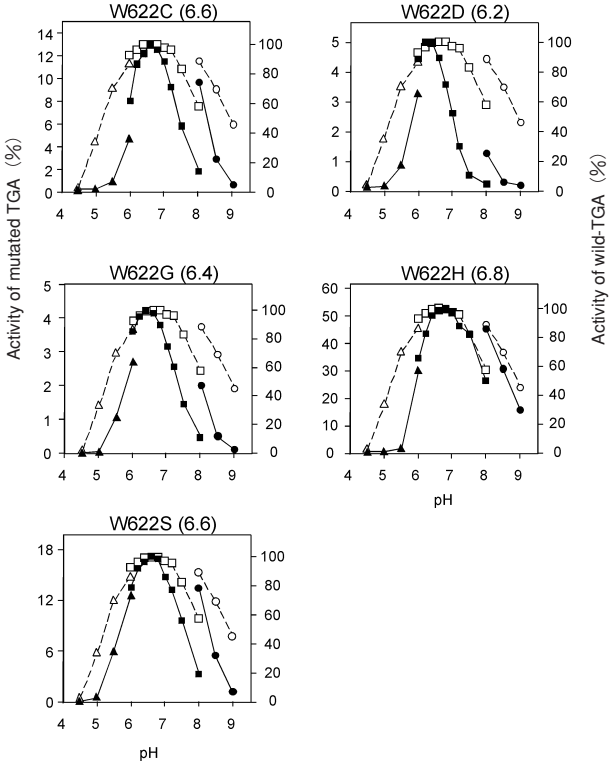

Fig. 1. Effect of pH on the activities of wild-type TGA (△, □, ○) and Trp622-mutants (▲, ■, ●).

In the pH range 4.5–6.0, 6.0–8.0, 8.0–9.0, sodium citrate buffer (▲, △), sodium phosphate buffer (■, □), and bicine-NaOH buffer (●, ○) were used. Activities of TGA mutants with respect to the activity of the wild-type enzyme at optimal pH are shown. The pH optima of these mutants are given in parentheses.

We analyzed the effect of size and charge of an amino acid residue at position 622. Introduction of a neutral amino acid residue with a smaller side-chain such as Gly, Cys or Ser caused a decrease in activity, and particularly, the substitution of the smallest Gly residue resulted in the biggest decrease among all mutants. Mutation with the acidic amino acid residue Asp also caused a significant decrease in activity. On the other hand, in the case of the bulky residues Trp and His at this position, TGA activities were maintained at a high level. Thus, bulky residues such as Trp and His at position 622 have been shown to be important for maintaining TGA activity. However, W622H drastically reduced the activity below pH 6.0, unlike the wild-type enzyme. Since this pH is consistent with the expected pK value of the His imidazole side-chain, the significant decrease in activity for W622H was probably due to the protonation of the His imidazole side-chain. Thus, TGA activity is also influenced by the electronic state of the amino acid residue at position 622 in TGA.

With respect to optimal pH, although W622D and W622G most changed (decreased by 0.6 and 0.4 pH units compared to the wild-type enzyme), a marked decrease in activity and narrower bell-shaped curves were also observed for these mutants. In *Aspergillus awamori* GA, five mutants of the corresponding residue, S411A, S411C, S411D, S411G and S411H, have been reported.[11] In particular, S411A most increased the optimal pH for maltose hydrolysis by 0.84 U while maintaining a similar level of activity to the wild-type enzyme. The mutant N304T of soybean β-amylase, an exo-type inverting enzyme like GA, also increased optimal pH by 1.2 U with 32% the activity of the wild-type enzyme.[9] Both *A. awamori* GA and soybean β-amylase successfully increased the optimal pH while maintaining activity by introducing a mutation to remove the hydrogen bond from the catalytic base. Thus, the role of Trp622 in TGA may be different from those of Ser411 in *A. awamori* GA and Asn340 in soybean β-amylase. The acarbose-complexed structures of *A. awamori* var. *X100* GA[10] and *Thermoanerobacterium thermosaccharolyticum* GA[18] indicate that the positions of C_α in their corresponding residues are virtually identical at the bottom of the active cleft. Based on structural information, Trp622 in TGA was expected to locate near subsite 1 and/or 2. Our previous paper[5] indicated the sum of subsite affinities 1 and 2 of TGA is higher than those of other GAs whose subsite structures have been reported[13,19,20]; for example, *A. awamori* GA. Generally, tryptophan plays an important role in the binding and recognition of substrates in glycoside hydrolases and mutation of this residue has been reported to result in a significant decrease in activity.[13,14] Therefore, Trp622 in TGA is suggested to be an important residue for substrate binding

of maltose or glucose at subsite 1 and/or 2, rather than for determination of optimal pH, unlike Ser411 in *A. awamori* GA.

This study was supported in part by the Novozymes Japan Research Fund.

REFERENCES

1) P.J. Reilly: Protein engineering of glucoamylase to improve industrial performance. *Starch/Stärke*, **51**, 269–274 (1999).
2) Y. Tsujisaka: Fungal glucoamylase (*Asp. niger, Asp. awamori, Rhi. delemar, Rhi. niveus et al.*), in *Handbook of Amylases and Related Enzymes: Their Sources, Isolation Methods, Properties and Applications,* The Amylase Research Society of Japan, ed., Pergamon Press, Oxford, pp. 117–120 (1988).
3) P.M. Coutinho and P.J. Reilly: Glucoamylase structural, functional, and evolutionary relationships. *Proteins*, **29**, 334–347 (1997).
4) J. Sauer, B.W. Sigurskjold, U. Christensen, T.P., Frandsen, E. Mirgorodskaya, M. Harrison, P. Roepstorff and B. Svensson: Glucoamylase: structure/function relationships, and protein engineering. *Biochim. Biophys. Acta*, **1543**, 275–293 (2000).
5) K. Ichikawa, T. Tonozuka, R. Uotsu-Tomita, H. Akeboshi, A. Nishikawa and Y. Sakano: Purification, characterization, and subsite affinities of *Thermoactinomyces vulgaris* R-47 maltooligosaccharide-metabolizing enzyme homologous to glucoamylases. *Biosci. Biotechnol. Biochem*., **68**, 413–420 (2004).
6) R. Uotsu-Tomita, T. Tonozuka, H. Sakai and Y. Sakano: Novel glucoamylase-type enzymes from *Thermoactinomyces vulgaris* and *Methanococcus jannaschii* whose genes are found in the flanking region of the α-amylase genes. *Appl. Microbiol. Biotechnol*., **56**, 465–473 (2001).
7) D. Ganghofner, J. Kellermann, W.L. Staudenbauer and K. Bronnenmeier: Purification and properties of an amylopullulanase, a glucoamylase, and an α-glucosidase in the amylolytic enzyme system of *Thermoanaerobacterium thermosaccharolyticum*. *Biosci. Biotechnol. Biochem*., **62**, 302–308 (1998).
8) M.D. Joshi, G. Sidhu, I. Pot, G.D. Brayer, S.G. Withers and L. P. McIntosh: Hydrogen bonding and catalysis: a novel explanation for how a single amino acid substitution can change the pH optimum of a glycosidase. *J. Mol. Biol*., **299**, 255–279 (2000).
9) A. Hirata, M. Adachi, A. Sekine, Y.N. Kang, S. Utsumi and B. Mikami: Structural and enzymatic analysis of soybean β-amylase mutants with increased pH optimum. *J. Biol. Chem*., **279**, 7287–7295 (2004).
10) A.E. Aleshin, L.M. Firsov and R.B. Honzatko: Refined structure for the complex of acarbose with glucoamylase from *Aspergillus awamori* var. *X100* to 2.4-A resolution. *J. Biol. Chem*., **269**, 15631–15639 (1994).
11) T.Y. Fang and C. Ford: Protein engineering of *Aspergillus awamori* glucoamylase to increase its pH optimum. *Protein Eng*., **11**, 383–388 (1998).
12) P.M. Coutinho and P.J. Reilly: Structure-function relationships in the catalytic and starch binding domains of glucoamylase. *Protein Eng.,* **7**, 393–400 (1994).
13) M.R. Sierks, C. Ford, P.J. Reilly and B. Svensson: Site-directed mutagenesis at the active site Trp120 of *Aspergillus awamori* glucoamylase. *Protein Eng*., **2**, 621–625 (1989).
14) T. Christensen, B.B. Stoffer, B. Svensson and U. Christensen: Some details of reaction mechanism of glucoamylase from *Aspergillus niger*: Kinetic and structural studies on Trp52→Phe and Trp317→Phe mutants. *Eur. J. Biochem*., **250**, 638–645 (1997).
15) M. Mizuno, T. Tonozuka, A. Uechi, A. Ohtaki, K. Ichikawa, S. Kamitori, A. Nishikawa and Y. Sakano: The crystal structure of *Thermoactinomyces vugaris* R-47 α-amylase II (TVA II) complexed with transglycosylated product. *Eur. J. Biochem*., **271**, 2530–2538 (2004).
16) U.K. Laemmli: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680–685 (1970).
17) S.C. Gill and P.H. von Hippel: Calculation of protein extinction coefficients from amino acid sequence data. *Anal. Biochem*., **182**, 319–326 (1989).
18) A.E. Aleshin, P.H. Feng, R.B. Honzatko and P.J. Reilly: Crystal structure and evolution of a prokaryotic glucoamylase. *J. Mol. Biol*., **327**, 61–73 (2003).
19) A. Tanaka, Y. Fukuchi, M. Ohnishi, K. Hiromi, S. Aibara and Y. Morita: Fractionation of isozymes and determination of the subsite structure of glucoamylase from *Rhizopus niveus. Agric. Biol. Chem*., **47**, 573–580 (1983).
20) H. Ohnishi, H. Kitamura, T. Minowa, H. Sakai and T. Ohta: Molecular cloning of a glucoamylase gene from a thermophilic *Clostridium* and kinetics of the cloned enzyme. *Eur. J. Biochem*., **207**, 413–418 (1992).

Thermoactinomyces vulgaris R-47 由来グルコアミラーゼのトリプトファン 622 の部位特異的変異導入
—5 種類の変異型酵素の至適 pH と活性—

市川和洋，殿塚隆史，水野正浩，西河　淳
坂野好幸
東京農工大学大学院共生科学技術研究部生命農学部門
（183–8509 府中市幸町 3–5–8）

Aspergillus awamori 由来グルコアミラーゼでは，触媒残基と水素結合しているセリン 411 の変異によって，至適 pH が上昇したという報告がある。そのセリン 411 に相当する残基は，ほとんどのグルコアミラーゼでセリンあるいはグリシンであるのに対し，*Thermoactinomyces vulgaris* R-47 由来グルコアミラーゼ（TGA）ではトリプトファンと異なっていた。そこで，本酵素のトリプトファン 622 が至適 pH に影響を与える残基であるかどうかを調べるために，5 種類の変異型酵素 W622C，W622D，W622G，W622H，W622S を構築した。これらの変異型酵素の至適 pH は 6.2–6.8 となり，野生型酵素と同等あるいはわずかに低下したのみであったが，どの変異型酵素も活性の低下が認められた。至適 pH における W622H の活性は野生型酵素の 52% と比較的保持していたが，他の変異型酵素では 4.3–17% となり，大幅に減少した。これらの結果とグルコアミラーゼの立体構造をふまえると，TGA のトリプトファン 622 は *Aspergillus awamori* 由来グルコアミラーゼのセリン 411 とは異なり，至適 pH に影響を及ぼす残基というよりもむしろ基質結合に重要な残基であることが示唆された。