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Article

Effects of amino acid substitutions at position 315 of GH10 xylanase XynR on its alkaliphily

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XynR is a thermostable and alkaline glycoside hydrolase (GH) 10 xylanase, produced by thermophilic and alkaliphilic *Bacillus* sp. strain TAR-1. We previously selected T315N as an alkaliphilic variant. In this study, we examined the effects of amino acid residue at position 315 of XynR on its alkaliphily. In the hydrolysis of beechwood xylan, all 19 variants at position 315 exhibited bell-shaped pH-activity profiles. T315H, T315N, T315Q, and T315S exhibited a broader bell-shaped pH-dependence of activity than WT. At pH 10.0, the activities were in the order of T315N > T315Q > T315S > T315H = WT.

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Introduction

Xylanase [EC 3.2.1.8] is a glycoside hydrolase (GH) that catalyzes the hydrolysis of internal β-1,4-linkage of xylan. Xylanase is industrially used in various fields such as paper and pulp, food, and biofuel industries [1–3]. In such industries, highly thermostable and alkaline-active xylanase is anticipated because the reaction is carried out at high temperature and pH conditions [4]. Most xylanases belong to the family GH10 or GH11. GH10 and GH11 xylanases have two conserved catalytic Glu residues in the active site. However, sequence or structural similarity is not observed between these two xylanases. GH10 xylanase has a $(\beta/\alpha)_8$ triose-phosphate isomerase (TIM) barrel fold while GH11 xylanase has a β-jellyroll fold composed of eight β strands [5]. The active-site cleft is shallower in GH10 xylanase and is deeper in GH11 xylanase. Generally, GH10 xylanases are less substrate specific and more thermostable than GH11 xylanases [6].

Generation of thermostable and alkaline-

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active xylanase has been an important protein engineering research target. To improve thermostability, mutation of the active-site aromatic residues [7,8], engineering of the N- or C-terminal flexible loop [9–11], replacement of amino acid residues in the external α-helix with Pro or Glu [12], replacement of Pro and Glu [13], and random mutation [14] have been applied. To improve alkaliphily, introduction of multiple Arg on protein surface [14], random mutagenesis [15], and amino acid substitutions adjacent to the active site and on protein surface [16] have been applied.

A GH10 xylanase XynR was identified in the culture broth of thermophilic and alkaliphilic *Bacillus* sp. strain TAR-1 isolated from a soil sample from Kanagawa, Japan [17]. We previously selected T315N as an alkaliphilic XynR variant from a site saturation mutagenesis library [18]. In this study, we examined the effects of amino acid residue at position 315 on alkaliphily of XynR.

Materials and Methods

Materials　3,5-Dinitrosalicylic acid (DNS) was purchased from Nacalai Tesque (Kyoto, Japan). Beechwood xylan was from Megazyme (Bray, Ireland). The concentrations of wild type (WT) and variants expressed in *Escherichia coli* were determined using Protein Assay CBB Solution (Nacalai Tesque) with bovine serum albumin (Nacalai Tesque) as a standard.

Expression and purification of WT and variants　For the construction of plasmids, site-directed mutagenesis was carried out using the Quikchange method with pET-21b(+)-XynR [18] as a template. Transformed *E. coli* BL21(DE3) was cultured in 100 mL of LB broth containing 50 μg/mL ampicillin at 37ºC with shaking. When OD_{660} reached 0.6–0.8, 25 μL of 500 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added, and growth was continued at 30ºC for 24 h. The cells were collected by centrifugation and suspended in 50 mL of 20 mM phosphate-NaOH buffer (pH 8.0) (buffer A) and disrupted by sonication. After centrifugation, the supernatant was collected, to which solid ammonium sulfate was added to be 50% saturation. After centrifugation, the pellet was collected, dissolved in buffer A containing 0.5 M NaCl (buffer B), and dialyzed against buffer A. The crude enzyme solution obtained above was applied to a HisTrap™ HP column (GE Healthcare, Buckinghamshire, UK) equilibrated with buffer B. The column was washed with 50 mL of buffer B and eluted with buffer B containing 0.5 M imidazole. Each fraction (5 mL) was assessed to contain WT or variants by SDS-PAGE. Active fractions were concentrated after desalting. Purified enzyme solution was stored at 4ºC before use.

SDS-PAGE　Samples were mixed with five volumes of the SDS-PAGE sample buffer (0.25 M tris-hydroxymethyl aminomethane (Tris)-HCl buffer (pH 6.8), 50% v/v glycerol, 10% w/v SDS, 5% v/v 2-mercaptoethanol, 0.05% w/v bromophenol blue) and were incubated at 100°C for 5 min. The solution (10 μL) was applied to a 10% polyacrylamide gel with a constant current of 40 mA for 40 min. After electrophoresis, proteins in the gel were stained with 0.25% Coomassie Brilliant Blue R-250, 50% methanol, and 7% acetic acid.

Hydrolysis of beechwood xylan　The activity was measured as described previously [16,17]. Briefly, the reaction was initiated by mixing 10 μL of enzyme solution and 90 μL of substrate solution (10 mg/mL beechwood xylan in 100 mM acetate-sodium acetate buffer at pH 3.5–5.5, 100 mM phosphate-NaOH buffer at pH 6.0–8.5, or 100 mM carbonate-bicarbonate buffer at pH 9.0–11.0) both pre-incubated at 37°C. The reaction solution was incubated at 37°C for

predetermined time, and 100 µL of DNS solution (0.5% w/v DNS, 1.6% w/v NaOH, 30% w/v potassium sodium tartrate) was added to stop the reaction. After incubating at 100°C for 15 min and at 0°C for 3 min, 80 µL of the solution was added to 120 µL of water, and A_{540} was measured with a multimodal plate reader EnSight (PerkinElmer, Waltham, MA). To estimate the concentrations of reducing sugars, xylose was used to make a standard curve. The initial reaction rate was estimated from the time-course for production of reducing sugars.

Circular dichroism (CD) measurement The CD spectra of WT, variants, and control solutions were measured using a J-820 spectropolarimeter (Jasco, Tokyo, Japan) with a Peltier system of cell temperature control under the following conditions: spectral range 200–250 nm; 100 mdeg sensitivity; 0.2 nm resolutions; 1 s response time; 10 nm min^{-1} scan rate; and 5 accumulations. CD spectra were recorded at 37°C using a 2-mm cell. The concentration of each enzyme was 1.0 µM. CD spectra were processed with a Jasco software, and finally expressed in mean-residue molar ellipticity units, $[\theta]$ (deg cm^2 $dmol^{-1}$).

Results and Discussion

Preparation of WT and variants

Figure 1A shows the whole and active-site structures of WT [19]. Glu150 and Glu256 are the catalytic residues. Thr315 lies in the active site, and is close to Glu256 and the Ca^{2+} ion. As shown in Fig. 1B, the Ca^{2+} ion is surrounded by five amino acid residues including Thr315.

We expressed the C-terminally $(His)_6$-tagged WT and 19 site-saturation variants at position 315 in *E. coli* and purified them from the cells by nickel affinity chromatography. Starting with 100 mL of *E. coli* cultures, 0.1–2.0 mg of purified enzymes were recovered. Upon SDS-PAGE under reducing conditions, each yielded a single band with a molecular mass of 40 kDa (Fig. 2A).

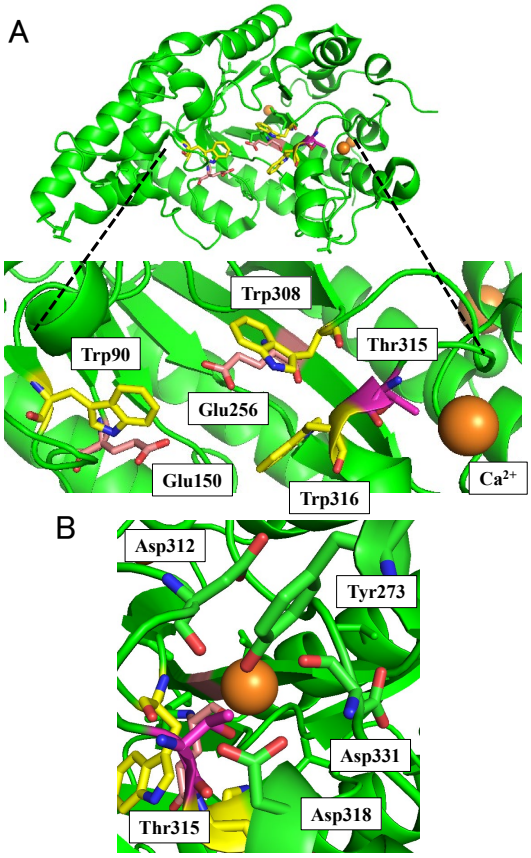

Fig. 1. Structure of XynR. The structure is based on PDB code 7CPK [19]. (A) Whole structure and close-up view of the active site. The overall protein structure (ribbon model), Ca^{2+} (orange sphere), and Trp90, Glu150, Glu256, Trp308, and Trp316 (ball and stick model) are shown. (B) Close-up view of the Ca^{2+}-binding site. Ca^{2+} (orange sphere), Tyr273, Asp312, Thr315, Asp318, and Asp331 (cylinder) are shown.

We assessed the activities of WT and 19 variants in the hydrolysis of beechwood xylan at pH 8.0 and 37°C (Fig. 2B). Compared with WT, four variants (T315S, T315H, T315N, and T315Q) exhibited 90–110% activity, four variants (T315G, T315A, T315C, and T315K) 50–80% activity, and other 11 variants less than 40% activity, indicating that Thr315 is not indispensable for, but is involved in catalysis.

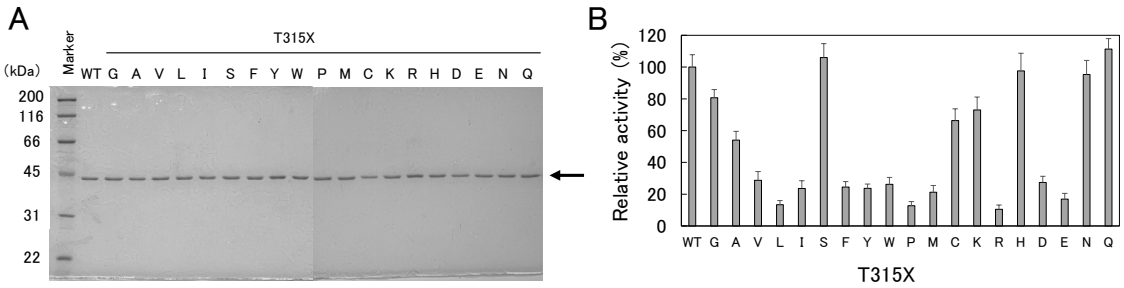

Fig. 2. SDS-PAGE and activities of purified WT and variants. (A) Coomassie Brilliant Blue-stained 10% SDS-polyacrylamide gels is shown. (B) Hydrolysis reaction of beechwood xylan was carried out in 100 mM phosphate-NaOH buffer (pH 8.0) at 37ºC with the enzyme and initial substrate concentrations of 0.5 μM and 9 mg/mL, respectively. Relative activity indicates the value compared to WT. Error bars indicate SD values of triplicate determinations.

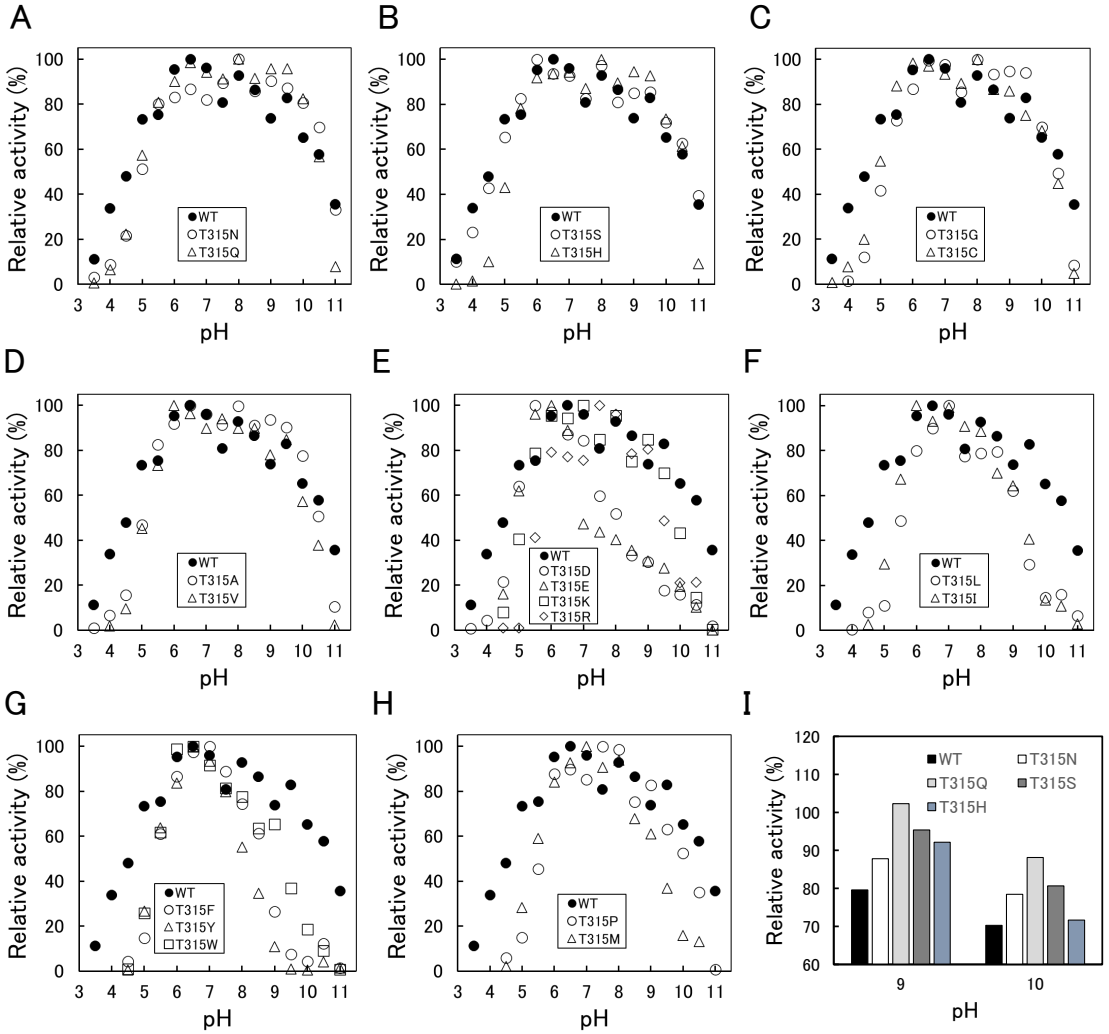

Fig. 3. Effect of pH on activities of WT and variants. Hydrolysis reaction of beechwood xylan was carried out at 37ºC with the enzyme and initial substrate concentrations of 0.5 μM and 9 mg/mL, respectively. The reaction buffers were 100 mM acetate-sodium acetate buffer (pH 3.5–5.5), 100 mM phosphate-NaOH buffer (pH 6.0–8.5), and 100 mM carbonate-bicarbonate buffer (pH 9.0–11.0). Relative activity indicates the value compared to that at the optimal pH of WT or each variant (A–H) or that of WT at pH 8.0 (I). One of the results of triplicate determinations is shown.

pH dependence of activity

Figure 3 shows the pH dependences of activity of WT and 19 variants in the hydrolysis of beechwood xylan at 37°C. They all exhibited bell-shaped pH-activity profiles. T315N, T315Q, T315S, and T315H exhibited higher relative activities at pH 9.0–10.0 than WT (Fig. 3A, B), suggesting that the amino acid residue whose side chain has an amido or hydroxyl group at position 315 makes XynR alkaliphilic. T315G, T315C, T315A, and T315V exhibited similar profiles to WT (Fig. 3C, D). Other 11 variants exhibited markedly less activities at pH 10.0–11.0 than WT (Fig. 3E–H). The activities at pH 10.0 were in the order of T315Q > T315S = T315N > T315H = WT (Fig. 3I).

Activity at pH 10.0

We selected T315H, T315N, T315Q, and T315S that exhibited 90–110% of that of WT (Fig. 2B) and a broader bell-shaped pH-dependence of activity at alkaline side than WT (Fig. 3) for subsequent analyses.

Figure 4 shows the CD spectra of WT and four variants (T315H, T315N, T315Q, and T315S) at pH 8.0 and 25°C. Each exhibited negative ellipticities at around 200–250 nm with minimum values around 222 nm, indicating the mutation at position 315 did not elicit drastic structural changes.

Figure 5 shows the activities of WT, T315H, T315N, T315Q, and T315S in the hydrolysis of beechwood xylan at pH 10.0, at 37°C. The reducing sugars increased with increasing the reaction time. The activities were in the order of T315N > T315Q > T315S > T315H = WT. In XynR, Glu150 and Glu256 are thought to be catalytic residues by comparison with the amino acid and tertiary structures of a GH10 xylanase from an alkalophilic *Bacillus* sp. NG-27 [20]. Thr315 is closer to the catalytic residue Glu256 than Glu150. We presume that the mutation of Thr315 to His, Asn, Gln, or Ser stabilizes the protonation of the catalytic residue in the alkaline side, presumably Glu256, resulting in an increase in pK_a value. Elucidation of these mechanisms by crystallographic analyses is the next subject.

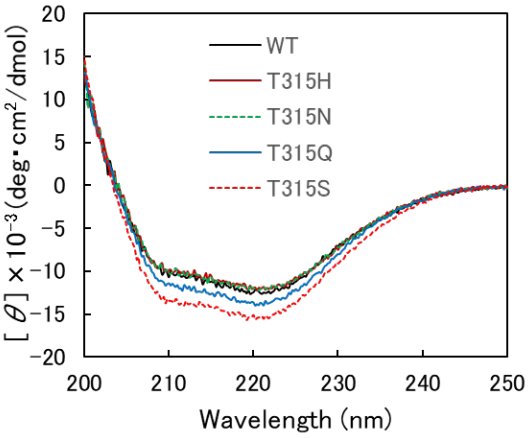

Fig. 4. CD spectroscopy at 200–250 nm. $[\theta]_{200–250}$ of the enzymes were measured in 20 mM phosphate-NaOH buffer (pH 8.0) without $CaCl_2$ at 25ºC.

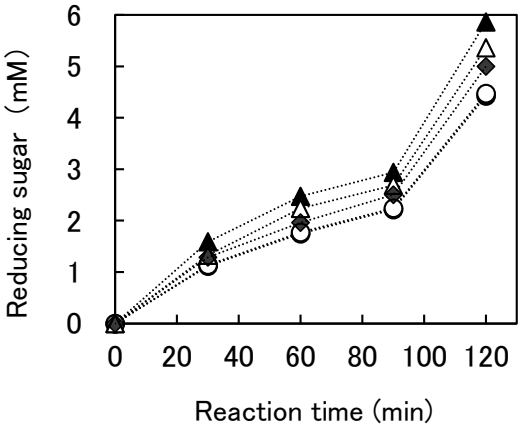

Fig. 5. Activities of WT and variants at pH 10.0. Hydrolysis reaction of beechwood xylan was carried out at 37ºC with the enzyme and initial substrate concentrations of 0.5 μM and 9 mg/mL, respectively. The reaction buffer was 100 mM carbonate-bicarbonate buffer (pH 10.0). Symbols: WT, closed circle; T315H, open circle; T315N, closed triangle; T315Q, open triangle; T315S, closed diamond. One of the results of triplicate determinations is shown.

References

[1] Kumar, D., Kumar, S.S., Kumar, J., Kumar, O., Mishra, S.V., Kumar, R., Malyan, S.K. (2017) *Biochem. Cell. Arch.* **17**, 353–360.

[2] Beg, Q.K., Kapoor, M., Mahajan, L., Hoondal, G.S. (2001) *Appl. Microbiol. Biotechnol.* **56**, 326–338.

[3] Kalim, B., Böhringer, N., Ali, N., Schäberle, T.F. (2015) *Brit. Biotechnol.* **7**, 1–20.

[4] Horikoshi, K. (1999) *Alkaliphiles* Tokyo (Japan), Kodansha.

[5] Linares-Pasten, J.A., Aronsson, A., Karlsson, E.N. (2018) *Curr. Protein Pept. Sci*. **19**, 48–67.

[6] Liu, L., Sun, X., Yan, P., Chen, H. (2012) *PLoS One* **7**, e45762.

[7] Moreau, A., Shareck, F.D., Kluepfel, Z., Morosoli, R. (1994) *Enzyme Microb. Technol*. **16**, 420–424.

[8] Liu, L., Zhang, G., Zhang, Z., Wang, S., Chen, H. (2011) *J. Biol. Chem*. **286**, 44710–44715.

[9] Roberge, M., Shareck, F., Morosoli, R., Kluepfel, D., Dupont, C. (1998) *Protein Eng*. **11**, 399–404.

[10] Bhardwaj, A., Leelavathi, S., Mazumdar-Leighton, S., Ghosh, A., Ramakumar, S., Reddy, V.S. (2008) *PLoS One* **3**, e3063.

[11] Irfan, M., Gonzalez, C.F., Raza, S., Rafiq, M., Hasan, F., Khan, S., Shah, A.A. (2018) *Enzyme Microbial. Technol.* **111**, 38–47.

[12] Wang, K., Luo, H., Tian, J., Turunen, O., Huang, H., Shi, P., Hua, H., Wang, C., Wang, S., Yao, B. (2014) *Appl. Environ. Microbiol.* **80**, 2158–2165.

[13] Watanabe, M., Fukada, H., Ishikawa, K. (2016) *Biochemistry* **55**, 4399–4409.

[14] Turunen, O., Vuorio, M., Fenel, F., Leisola, M. (2002) *Protein Eng*. **15**, 141–14.

[15] Shibuya, H., Kaneko, S., Hayashi, K. (2005) *Biosci. Biotechnol Biochem.* **69,** 1492–1497.

[16] Umemoto, H., Ihsanawati, Inami, M., Yatsunami, R., Fukui, T., Kumasaka, T., Tanaka, N., Nakamura, S. (2009) *Biosci. Biotechnol. Biochem.* **73**, 965–967.

[17] Nakamura, S., Nakai, R., Wakabayashi, K., Ishiguro, Y., Aono, R., Horikoshi, K. (1994) *Biosci. Biotechnol. Biochem*. **58**, 78–81.

[18] Kuwata, K., Suzuki, M., Takita, T., Yatsunami, R., Nakamura, S., Yasukawa, K. (2021) *Biosci. Biotechnol. Biochem.* **85**, 1853–1860.

[19] Suzuki, M., Takita, T., Kuwata, K., Nakatani, K., Li. T., Katano, Y., Kojima, K., Mizutani, K., Mikami, B., Yatsunami, R., Nakamura, S., Yasukawa, K. (2021) *Biosci. Biotechnol. Biochem.* **85**, 386–390.

[20] Manikandan, K., Bhardwaj, A., Gupta, N., Lokanath, N.K., Ghosh, A., Reddy, V.S., Ramakumar, S. (2006) *Protein Sci.* **15**, 1951–1960.

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