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Note

Inactivation of α-Amylases from *Thermoactinomyces vulgaris* R-47, TVA I and TVA II, by ω-Epoxyalkyl α-D-Glucopyranoside[*1]

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Abstract: We found here that ω-epoxyalkyl α-D-glucopyranosides consisting of three, four and five alkyl carbons (α-E3G, α-E4G and α-E5G, respectively), which are known to be affinity-labeling reagents of β-amylase, had the effect of inactivating two pullulan-hydrolyzing α-amylases from *Thermoactinomyces vulgaris* R-47, TVA I and TVA II, at high concentration (*ca*. 0.1 – 1.5 M). The inactivation exhibited saturation kinetics of a two-step mechanism, and an inactivation rate constant, k, and equilibrium dissociation constant, K_R, of α-E5G were calculated. The k/K_R values of α-E5G for TVA I and TVA II were 13.1 × 10^{-4} and 6.41 × 10^{-4} $M^{-1} \cdot s^{-1}$ respectively. In terms of the power of inactivation, the orders for TVA I and TVA II were α-E5G＞α-E3G≈α-E4G, and α-E5G＞α-E3G＞α-E4G, respectively. The findings indicated that the relation between the lengths of the alkyl carbons and the inactivation of TVA I and TVA II differs from that for β-amylase and isomalto-dextranase.

Key words: α-amylase, ω-epoxyalkyl α-D-glucopyranoside, affinity labeling, *Thermoactinomyces vulgaris*

Affinity labeling is one of the most effective ways to investigate the active site of enzymes.[1–6] In the case of glycosidases, acidic amino acid residues, Asp and Glu, are commonly reported as catalytic residues,[6–10] and ω-epoxyalkyl α-D-glucopyranosides (α-EAGs), which react with a functional carboxyl group in the active site, have been synthesized to investigate the catalytic mechanism of glycosidases.[2–6] The epoxides reportedly affected β-amylase[2–6] and isomalto-dextranase,[5] but had no significant influence on the activity of α-amylases.[3,5]

α-Amylase (1,4-α-D-glucanhydrolase; EC 3.2.1.1), belonging to glycoside hydrolase family 13, hydrolyzes α-1,4-glucosidic linkages of starch and related oligosaccharides to release α-anomer products. Most of the α-amylases show only limited hydrolysis of pullulan and cyclodextrins. *Thermoactinomyces vulgaris* R-47 produces two pullulan-hydrolyzing enzymes, TVA I[11] and TVA II,[12] as extracellular and intracellular enzymes, respectively. In addition to the efficient hydrolysis of pullulan, TVA I strongly hydrolyzes starch but less efficiently hydrolyzes α- and β-cyclodextrins, while TVA II shows outstanding kinetic values for small oligosaccharides and α-, β-, and γ-cyclodextrins.[11] To investigate the catalytic mechanism of TVAs further, α-EAGs, which consist of three to five alkyl carbons, were prepared. We found here that high concentrations of α-EAGs had the effect of the inactivating of both TVA I and TVA II, although the effect of the inactivation was much weaker than for β-amylase and isomalto-dextranase.

α-EAGs consisting of three, four and five alkyl carbons were prepared according to the method of Isoda *et al*.[2,5] 2′,3′-Epoxypropyl α-D-glucopyranoside (α-E3G), 3′,4′-epoxybutyl α-D-glucopyranoside (α-E4G) and 4′,5′-epoxypentyl α-D-glucopyranoside (α-E5G) were synthesized using allyl alcohol, 3-buten-1-ol and 4-penten-1-ol, respectively, and glucose as the starting materials. The structures of the synthesized α-EAGs were confirmed by NMR analysis (JEOL JNM-AL400). The purities of α-EAGs were also checked by thin layer chromatography. TVA I and TVA II were prepared and purified as described previously.[11,12]

The inactivation of the enzymes by α-EAGs was measured in 0.1 M sodium acetate buffer, pH 5.0 (for TVA I) or 0.1 M sodium phosphate buffer, pH 6.0 (for TVA II). Reaction mixtures consisting of TVA I (1.28 μM) or TVA II (1.56 μM), 0.05% bovine serum albumin and various concentrations of α-EAGs were incubated at 40°C. Samples (6 μL) of the reaction mixtures were taken, and added to 114 μL of *p*-nitrophenyl α-D-maltoside (0.63 mM, Sigma) dissolved in 0.1 M sodium acetate buffer, pH 5.0 (for TVA I) or 0.1 M sodium phosphate buffer, pH 6.0 (for TVA II) and incubated for 10 min at 40°C. The reaction was terminated by the addition of an equal vol-

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Abbreviations: TVA, *Thermoactinomyces vulgaris* R-47 α-amylase; α-EAG, ω-epoxyalkyl α-D-glucopyranoside; α-E3G, 2′,3′-epoxypropyl α-D-glucopyranoside; α-E4G, 3′,4′-epoxy-butyl α-D-glucopyranoside; α-E5G, 4′,5′-epoxypentyl α-D-glucopyranoside; α-E6G, 5′,6′-epoxyhexyl α-D-glucopyranoside.

ume of 0.1 M Na_2CO_3, and the absorbance at 400 nm was measured to calculate the amount of *p*-nitrophenol released.

We first tested the reaction of α-E3G with TVAs. The residual activity of TVA I incubated with 1.9 M of α-E3G for 6 h was 45%. Moreover, the residual activity of TVA II with 1.2 M of α-E3G for 5 h was 10%. When TVAs were incubated with α-E3G under the conditions described above (*i.e.*, TVA I, 1.9 M for 6 h; TVA II, 1.2 M for 5 h), a competitive inhibitor, maltose, at a concentration equal to that of α-E3G, was added and the residual activity was measured. TVA I and TVA II were competitively protected for being inactivated, and the residual activity under these conditions was 83 and 78%, respectively. The results indicated that α-E3G is bound to the active site of TVAs. To assess whether these inactivations are irreversible, the excess of α-E3G was removed using a centrifugal microconcentrator (Suprec-02, Takara Bio, Japan) and the residual activity was measured. After the removal of α-E3G, the activity of neither TVA I nor TVA II was restored at all, indicating that the inactivations by α-E3G are irreversible.

To determine the residual enzyme activity precisely, samples of the reaction mixtures were taken at appropriate time intervals, and the time course of inactivation was monitored. The logarithm of the residual activity *versus* time was plotted, and time-dependent inactivations by α-EAGs of both TVA I and TVA II were revealed by the pseudo-first-order kinetics (Figs. 1, 2). The residual activities of TVA I incubated with 1 M α-E3G and α-E4G for 4 h were 75.8 and 74.7%, respectively (Figs. 1A, C). Those values of TVA II with 1 M α-E3G and α-E4G were 29.9 and 57.4%, respectively (Figs. 1 B, D). In contrast, the effects of the inactivation of α-E5G were markedly more effective than those of α-E3G and α-E4G, and the most effective inactivators were α-E5G for both TVA I and TVA II (Fig. 2). Incubation with even 0.2 M α-E5G for 4 h efficiently decreased the residual activities of TVA I and TVA II to 19.3 and 22.9%, respectively (Figs. 2A, B).

The error values of the plots of α-E3G and α-E4G for TVAs were large, because the power of inactivations of α-E3G and α-E4G was low, making precise measurements difficult. Thus, the values of pseudo-first-order rate constant, k_{app}, at various concentrations of α-E5G were only calculated in this study. The plots of k_{app} values for both TVA I and TVA II revealed a saturation curve (Fig. 2C). In the case of TVA I, the values with 1 M or higher of α-E5G did not fit the saturation curve, and these values were not included in the calculation described below. Similar results were observed in α-E4G for both TVA I and TVA II (Figs. 1C, D), probably because the extremely high concentration of α-EAGs had some different effects on the enzymes. Together with the observation described above, the reaction between α-EAG and the enzyme is consistent with a two-step mechanism.[1,2,5] A dissociable complex is formed between α-EAG and the enzyme, and the inactivation by α-EAG is as follows:

$$E + A \overset{K_R}{\rightleftharpoons} EA \xrightarrow{k} E_{inact}$$

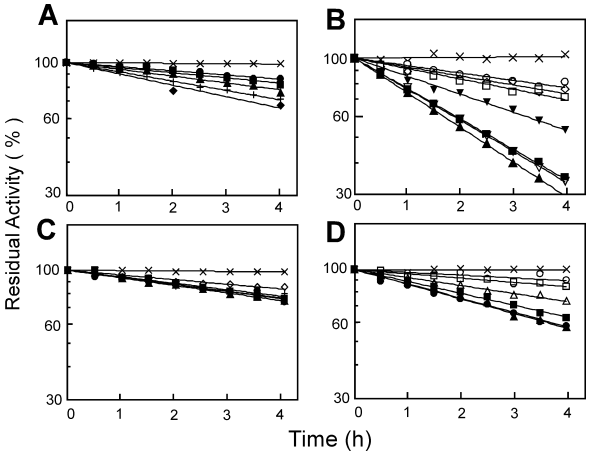

Fig. 1. Inactivation of TVA I and TVA II by α-E3G and α-E4G.

(A, B) Semilogarithmic plots of the residual activity of TVA I (A) or TVA II (B) *versus* time at various α-E3G concentrations. (C, D) Semilogarithmic plots of the residual activity of TVA I (C) or TVA II (D) *versus* time at various α-E4G concentrations. Concentrations of α-EAGs (M) are as follows: 0, ×; 0.1, ○; 0.16, ◇; 0.2, □; 0.4, △; 0.5, ▼; 0.6, ●; 0.8, ■; 0.9, ▽; 1.0, ▲; 1.2, +; 1.4, ✻; 1.5, ◆.

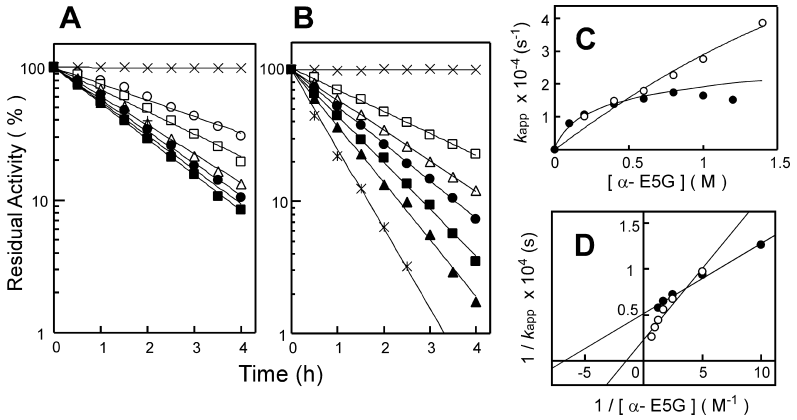

Fig. 2. Inactivation of TVA I and TVA II by α-E5G.

(A, B) Semilogarithmic plots of the residual activity of TVA I (A) or TVA II (B) *versus* time at various α-E5G concentrations. Concentrations of α-E5G (M) are as in Fig. 1. (C) Dependence of k_{app} on α-E5G concentration, and (D) double-reciprocal plots of (C): TVA I, ●; TVA II, ○.

Table 1. Parameters of inactivation of TVAs by α-E5G.

Enzyme	k $\times 10^{-4}(s^{-1})$	K_R (M)	k/K_R $\times 10^{-4}(M^{-1} \cdot s^{-1})$
TVA I	1.94±0.08	0.148±0.014	13.1±0.7
TVA II	4.35±0.87	0.67±0.22	6.41±0.76

where E and A are the enzyme and α-EAG, respectively, and E_{inact} is the inactivated enzyme. For this scheme, the following equation is derived:

$$k_{app} = \frac{k[I]}{K_R + [I]}$$

where k, K_R, and $[I]$ are values for the inactivation rate constant, the equilibrium dissociation constant and the inactivator concentration, respectively. By replotting the reciprocal of the k_{app} values *versus* the reciprocal of the inhibitor concentrations, k and K_R were calculated from the invert of the equation:[1,2]

$$\frac{1}{k_{app}} = \frac{K_R}{k}\frac{1}{[I]} + \frac{1}{k}$$

The values, k, K_R and k/K_R, were calculated by using the double-reciprocal plots of the k_{app} values *versus* α-E5G concentrations (Fig. 2D), and k/K_R values for TVA I and TVA II were 13.1×10^{-4} and 6.41×10^{-4} $M^{-1} \cdot s^{-1}$, respectively (Table 1).

Several detailed kinetic studies using α-EAGs have been reported, and the effects of α-EAGs on each enzyme differ.[2,4,5] Isoda *et al*. reported that soybean β-amylase was inactivated by α-E3G, and the k, K_R and k/K_R values were 1.14×10^{-3} s^{-1}, 0.12 M and 9.5×10^{-3} $M^{-1} \cdot s^{-1}$ respectively.[2] Kimura *et al*. reported that the inactivation by α-E4G of the soybean β-amylase was much more effective than that by α-E3G or α-E5G, and in terms of the power of inactivation, the order was α-E4G > α-E5G > α-E3G > α-E6G.[5] Kimura *et al*. also reported that α-E4G, α-E5G and α-E6G were effective in inactivating of isomalto-dextranase (the k/K_R values were 1.65, 19.4 and 3.25 $M^{-1} \cdot s^{-1}$, respectively) but no significant inactivation was observed with α-E3G, and the order in terms of the k/K_R values for isomalto-dextranase was α-E5G > α-E6G > α-E4G.[5] Compared to β-amylase and isomalto-dextranase, TVAs had much lower k/K_R values (*ca*. $10 - 10^4$ -fold less), and in terms of the power of inactivation, the orders for TVA I and TVA II were α-E5G > α-E3G ≈ α-E4G, and α-E5G > α-E3G > α-E4G, respectively. Thus, the relation between the lengths of the alkyl carbon chains of α-EAGs and the degree of inactivation differ for each of the enzymes.

We have already reported the three-dimensional structures of TVA I and TVA II. TVA I and TVA II have a relatively wide and shallow cleft compared to α-amylases which do not hydrolyze pullulan and cyclodextrins, and this cleft has been proposed to favor the binding of various substrates and versatility in substrate specificity.[13,14] This wide and shallow cleft may allow α-EAGs to bind the catalytic center of TVAs. Compared to TVA I, TVA II hydrolyzes a wider variety of small oligosaccharides, for example α- and β-cyclodextrins, much more efficiently.[11] When the same alkyl carbon chain of 1 M α-EAGs was used, the inactivations were more effective for TVA II than TVA I, and it is likely that the difference in substrate specificity between TVA I and TVA II results in the different inactivations.

In soybean β-amylase, α-E3G formed a covalent bond with Glu186, which functions as a general acid catalyst.[4] The crystal structures of the *Bacillus cereus* var. *mycoides* β-amylase complexed with α-EAGs also showed that a glucose unit of α-E3G and α-E4G was bound at subsite −2.[6] In TVAs, Glu396 (TVA I)/354 (TVA II) protonates the glycoside oxygen at a hydrolyzing site, and subsequently Asp356 (TVA I)/325 (TVA II) attacks the C-1 atoms of the glucose unit at subsite −1. Asp472 (TVA I)/421 (TVA II) has been suggested to be involved in fixing the substrate and in stabilizing the transition state.[7,15] It is likely that the glucose unit of α-EAGs is bound at subsite −2 of TVAs, and the epoxide could react with a functional carboxyl group of either Glu396 (TVA I)/354 (TVA II) or Asp356 (TVA I)/325 (TVA II). Based on these assumptions, the models of α-EAGs were placed in the catalytic cleft of TVAs using the Swiss-Pdb Viewer software.[16] The alkyl carbon chains of α-E3G and α-E4G were short and the structures appear insufficient to form a covalent bond with either of these Glu and Asp residues. The alkyl carbon chain of α-E5G may be a more suitable length for forming a covalent bond with the functional carboxyl group.

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REFERENCES

1) F. Wold: Affinity labeling—An overview. *Methods. Enzymol*., **46**, 3–14 (1977).
2) Y. Isoda and Y. Nitta: Affinity labeling of soybean β-amylase with 2′,3′-epoxypropyl α-D-glucopyranoside. *J. Biochem*., **99**, 1631–1637 (1986).
3) Y. Isoda, S. Asanami, K. Takeo and Y. Nitta: Attempt at affinity labeling of α- and β-amylases by α- and β-D-glucopyranosides and α- and β-maltooligosaccharides with 2′,3′-epoxypropyl residue as aglycone: specific inactivation of β-amylases. *Agric. Biol. Chem*., **51**, 3223–3229 (1987).
4) Y. Nitta, Y. Isoda, H. Toda and F. Sakiyama: Identification of glutamic acid 186 affinity-labeled by 2,3-epoxypropyl α-D-glucopyranoside in soybean β-amylase. *J. Biochem*., **105**, 573–576 (1989).
5) A. Kimura, T. Nishio, W. Hakamada, T. Oku, S.S. Mar, G. Okada and S. Chiba: Affinity labeling of glycosidase by ω-epoxyalkyl α-glucoside. *J. Appl. Glycosci*., **47**, 235–241 (2000).
6) T. Oyama, H. Miyake, M. Kusunoki and Y. Nitta: Crystal structures of β-amylase from *Bacillus cereus* var *mycoides* in complexes with substrate analogs and affinity-labeling reagents. *J. Biochem*., **133**, 467–474 (2003).
7) K. Ichikawa, T. Tonozuka, T. Yokota, Y. Shimura and Y. Sakano: Analysis of catalytic residues of *Thermoactinomyces vulgaris* R-47 α-amylase II (TVA II) by site-directed mutagenesis. *Biosci. Biotechnol. Biochem*., **64**, 2692–2695 (2000).
8) T. Kuriki and T. Imanaka: The concept of the α-amylase family: Structural similarity and common catalytic mechanism. *J. Biosci. Bioeng*., **87**, 557–565 (1999).
9) R. Kuroki, L.H. Weaver and B.W. Matthews: A covalent enzyme-substrate intermediate with saccharide distortion in a mutant T4 lysozyme. *Science*, **262**, 2030–2033 (1993).
10) H. Akeboshi, T. Tonozuka, T. Furukawa, K. Ichikawa, H. Aoki, A. Shimonishi, A. Nishikawa and Y. Sakano: Insights into the reaction mechanism of glycosyl hydrolase family 49:

Site-directed mutagenesis and substrate preference of isopullulanase. *Eur. J. Biochem*., **271**, 4420–4427 (2004).
11) T. Tonozuka, S. Mogi, Y. Shimura, A. Ibuka, H. Sakai, H. Matsuzawa, Y. Sakano and T. Ohta: Comparison of primary structures and substrate specificities of two pullulan-hydrolyzing α-amylase, TVA I and TVA II, from *Thermoactinomyces vulgaris* R-47. *Biochim. Biophys. Acta*, **1252**, 35–42 (1995).
12) T. Tonozuka, M. Ohtsuka, S. Mogi, H. Sakai, T. Ohta and Y. Sakano: A neopullulanase-type α-amylase gene from *Thermoactinomyces vulgaris* R-47. *Biosci. Biotechnol. Biochem*., **57**, 395–401 (1993).
13) S. Kamitori, S. Kondo, K. Okuyama, T. Yokota, Y. Shimura, T. Tonozuka and Y. Sakano: Crystal structure of *Thermoactinomyces vulgaris* R-47 α-amylase II (TVA II) hydrolyzing cyclodextrins and pullulan at 2.6 Å resolution. *J. Mol. Biol*., **287**, 907–921 (1999).
14) S. Kamitori, A. Abe, A. Ohtaki, A. Kaji, T. Tonozuka and Y. Sakano: Crystal structures and structural comparison of *Thermoactinomyces vulgaris* R-47 α-Amylase I (TVAI) at 1.6 Å resolution and α-amylase II (TVAII) at 2.3 Å resolution. *J. Mol. Biol*., **26**, 443–453 (2002).
15) A. Ohtaki, M. Mizuno, T. Tonozuka, Y. Sakano and S. Kamitori: Complex structures of *Thermoactinomyces vulgaris* R-47 α-amylase II with acarbose and cyclodextrins demonstrate the multiple substrate recognition mechanism. *J. Biol. Chem*., **279**, 31033–31040 (2004).
16) N. Guex and M.C. Peitsch: Swiss-Model and the Swiss-PdbViewer: An environment for comparative protein modeling. *Electrophoresis*, **18**, 2714–2723 (1997).

ω-Epoxyalkyl α-D-Glucopyranoside による *Thermoactinomyces vulgaris* R-47 由来 α-アミラーゼ，TVA I および TVA II の不活性化

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アルキル炭素鎖が3から5で構成されたω-epoxyalkyl α-D-glucopyranosides（それぞれα-E3G,α-E4G,α-E5Gと略す）は，β-アミラーゼのアフィニティーラベリング試薬として知られているが，高濃度（0.1–1.5 M程度）では *Thermoactinomyces vulgaris* R-47 由来のプルラン分解 α-アミラーゼ（TVA I および TVA II）を不活性化することが分かった（Fig. 1，2）．本不活性化は2段階から成る反応機構であると考えられ，α-E5G に対する不活性化定数 k および解離定数 K_R を計算した（Fig. 2）．α-E5G に対する TVA I と TVA II の k/K_R は 13.1×10^{-4} および 6.41×10^{-4} $M^{-1}\cdot s^{-1}$ であった（Table 1）．また，不活性化効果は TVA I および TVA II でそれぞれ α-E5G＞α-E3G≒α-E4G，および α-E5G＞α-E3G＞α-E4G の順番となった．この結果，アルキル炭素鎖の長さと不活性化の効果の関係は TVA I および TVA II とβ-アミラーゼやイソマルトデキストラナーゼで異なることがわかった．