Suicide Substrate-based Inactivation of Endodextranase by ω -Epoxyalkyl α -D-Glucopyranosides

(Received June 15, 2010; Accepted July 17, 2010)

Hee-Kwon Kang,¹ Young-Min Kim,¹ Hiroyuki Nakai,¹ Min-Sun Kang,¹ Wataru Hakamada,²
 Masayuki Okuyama,¹ Haruhide Mori,¹ Toshiyuki Nishio² and Atsuo Kimura^{1,*}

¹Research Faculty of Agriculture, Hokkaido University
 (Kita-9 Nishi-9, Kita-ku, Sapporo 060-8589, Japan)

²College of Bioresource Sciences, Nihon University
 (1866 Kameino, Fujisawa 252-0880, Japan)

Abstract: Three kinds of ω -epoxyalkyl α -glucopyranosides (3',4'-epoxybutyl α -D-glucopyranoside (E4G), 4',5'-epoxypentyl α -D-glucopyranoside (E5G) and 5',6'-epoxyhexyl α -D-glucopyranoside (E6G)), having alkyl chains of different lengths at their aglycone moieties, inactivated the endodextranase from *Streptococcus mutans* ATCC 25175 (SmDex) irreversibly with the pseudo-first order kinetics. Alkyl chain length-dependent inactivation was observed and the degree of activity loss was E5G, E6G and E4G, in that order, implying that the distance between epoxide group and glucosyl residue of ω -epoxyalkyl α -glucopyranoside was important in the modification of endodextranase. Inactivation by E5G followed the model of reversible intermediate-complex formation mechanism (suicide inhibitor-based mechanism). The rate constant of irreversible inactivation (k) and the dissociation constant of intermediate-complex (K_R) of SmDex and E5G were 0.44 min⁻¹ and 1.45 mM, respectively. Hydrolytic reaction product (isomaltose) protected SmDex from E5G-inactivation, suggesting that E5G bound to the catalytic site of SmDex. This is the first report that ω -epoxyalkyl α -glucopyranoside becomes a suicide substrate for endodextranase.

Key words: ω -epoxyalkyl α -glucopyranosides, endodextranase, suicide substrate, affinity labeling

Dextranases are enzymes that hydrolyze the α -1,6-glucosyl backbone chain of dextran and isomaltooligosaccharides. Dextranases are classified as endolytic enzymes (endodextranase) or exolytic enzymes (exodextranase) by their reaction modes. Furthermore, dextranases are divided into several families on the basis of the similarity in their amino acid sequences.^{1,2)} Most endodextranases belong to glycoside hydrolase family (GH) 49 and 66, and exodextranases belong to GH13, 15, 27, 49 and 87.³⁾ The dextranase from *Streptococcus mutans* ATCC 25175 (SmDex) is an endodextranase (EC 3.2.1.11; 6- α -D-glucan 6-glucanohydrolase) that is classified under GH66. GH66 enzymes are composed of endodextranase and cycloisomaltooligosaccharide glucanotransferase⁴⁾ (EC 2.4.1.248; (1 $\rightarrow$ 6)- α -D-glucan:(1 $\rightarrow$ 6)- α -D-glucan 6- α -D-(1 $\rightarrow$ 6 α -D-glucano)-transferase (cyclizing)). In GH66 members, twenty-nine enzymes have been reported and sixteen enzymes have been characterized at present.

There are two types of chemical modification reagents based on their inactivation mechanisms,⁵⁾ i) two molecules reaction type (Equation (1)) and ii) intermediate-formation type (Equation (2)) as follows:

where E, R, E^{*}-R and E $\cdots$ R are intact enzyme, chemical modification reagent, inactivated enzyme bound by reagent covalently, and reversible intermediate-complex, respectively. In Equation (2), k and K_R are the rate constant of irreversible inactivation and dissociation constant of intermediate-complex, respectively. Inactivation of the suicide substrate (mechanism-based inactivator) follows the intermediate-formation type mechanism.⁶⁾ Suicide substrate itself is an inert compound that does not attack any free amino acid. When this compound is incorporated into the catalytic site of an enzyme, its inert active species is activated by enzyme reaction to form the covalent linkage with amino acid residue, resulting in the inactivation of the enzyme.⁶⁾

Epoxyalkyl-based suicide substrate is an effective material to label the catalytic residues and has been applied in a number of glycosidases.⁷⁻¹⁷⁾ ω -Epoxyalkyl α -glucopyranosides have already been reported to show irreversible inhibition to the isomalto-dextranase (IMDex) (EC 3.2.1.94; 6- α -D-glucan isomaltolase) from *Arthrobacter globiformis* T6 with the suicide substrate mechanism.¹⁸⁾ IMDex is an exodextranase which produces isomaltose from the non-reducing terminal of dextran, suggesting a possibility that ω -epoxyalkyl α -glucopyranoside also becomes a suicide substrate for endodextranase. Therefore, 3',4'-epoxybutyl α -D-glucopyranoside (E4G), 4',5'-epoxypentyl α -D-glucopyranoside (E5G), and 5',6'-epoxyhexyl α -D-

* Corresponding author (Tel. +81-11-706-2816, Fax. +81-11-706-2808, E-mail: kimura@abs.agr.hokudai.ac.jp).

Abbreviations: E4G, 3',4'-epoxybutyl α -D-glucopyranoside; E5G, 4',5'-epoxypentyl α -D-glucopyranoside; E6G, 5',6'-epoxyhexyl α -D-glucopyranoside; GH, glycoside hydrolase family; IMDex, isomalto-dextranase; SmDex, dextranase from *Streptococcus mutans* ATCC 25175.

were predicted to share the common catalytic domain of $(\beta/\alpha)_8$ -barrel structure.²⁰⁾ SmDex and IMDex displayed the identical alkyl chain length-dependent inactivation (E5G > E6G > E4G), supporting this prediction. For further studies, E5G was used for an analysis of inactivation.

By varying the concentration of E5G, the inactivation was investigated (Fig. 3(A)). From plots of Fig. 3(A), showing the pseudo-first order process, the k_{app} values were calculated and their dependence on the concentration of E5G was studied (Fig. 3(B)). Plots of the k_{app} values versus E5G concentration displayed a hyperbolic shape (Fig. 3(B)), which means that an intermediate of dissociable complex of the enzyme and E5G, like the enzyme-substrate complex of the Michaelis-Menten type in the enzyme reaction, is formed before the covalent binding of SmDex and E5G. The inactivation model follows the above-described intermediate-formation type mechanism (Equation (2)). In the case of two molecule reaction type inactivation (Equation (1)), the k_{app} value is proportional to the concentration of chemical modification reagent and the plots of the k_{app} values against [reagent] exhibit a linear curve. In intermediate-formation type inactivation, when the inhibitor concentration is a large excess over the

enzyme concentration and the inhibitor is not depleted by spontaneous or enzyme-catalyzed side-reactions, the k_{app} value is given by the following equation^{5,6)}:

$$k_{app} = k [E5G]/(K_R + [E5G]), \text{ giving } 1/k_{app} = K_R/k/[E5G] + 1/k \quad (3)$$

From the latter equation, both parameters of k and K_R are obtained by the double reciprocal plots of k_{app} versus $[E5G]$ (Fig. 3(C)). The k and K_R values were estimated to be 0.44 min^{-1} and 1.45 mM , respectively. Since isomaltose, a product of SmDex-catalyzed hydrolytic reaction, protected SmDex from E5G-inactivation, E5G was considered to modify the catalytic site of SmDex. Our above-described results suggest that E5G of ω -epoxyalkyl α -glucopyranoside is a suicide substrate of endodextranase.

The k/K_R value ($0.303 \text{ min}^{-1}\text{mM}^{-1}$) corresponds to the second-order rate constant of the reaction between SmDex and E5G. The value k/K_R is about 10^5 times larger than the second-order rate constant of spontaneous addition of anion to epoxide,²¹⁾ also supporting the suicide substrate-based inactivation of E5G. The k/K_R value for E5G-inactivation of IMDex was reported to be $1.16 \text{ min}^{-1}\text{mM}^{-1}$,¹⁸⁾ which is 3.8 times higher than that of SmDex. This rapid inactivation of IMDex by E5G is mainly due to the high affinity of IMDex to E5G (K_R value of 0.120 mM ; A. Kimura, unpublished datum). When accepting E5G at the catalytic site, IMDex is considered to have higher affinity at subsite -1 and -2 than SmDex (Fig. 1(B)).

Yamamoto *et al.* reported that Asp145 and Asp270 were the catalytic amino acids of cycloisomaltotransferase from *Bacillus circulans* T-3040.²²⁾ As this enzyme and SmDex are members of GH66, the catalytic amino acids of SmDex should be Asp residues. Very recently, Asp243 of GH66 endodextranase from *Thermotoga lettingae* TMO²³⁾ was suggested as a catalytic amino acid residue. According to the general mechanism of inactivation of glycosidases by an epoxide,²⁴⁾ a carbon on the epoxy ring generates the covalent linkage with a catalytic carboxylate, suggesting that E5G binds one of two carboxylate groups of Asp residues (corresponding to Asp145 and Asp270 of cycloisomaltotransferase). Figure 1(C) depicts the possible scheme of the E5G-labeling reaction. In this scheme, E5G reacts to SmDex through two processes²⁵⁾: i) the epoxide ring opening by addition of a proton to its oxygen atom and ii) the attack of the catalytic carboxylate group of SmDex on a carbon atom of epoxide. It is of interest that there are two routes (route-a and route-b in Fig. 1(C)) for the process of ii). Route-a is that the carboxylate forms an ester linkage with 4'-C of 4',5'-epoxypentyl chain of E5G with the formation of a new chiral carbon at 4'-C. In route-b, the carboxylate reacts to the terminal carbon of 5'-C. In order to learn the inactivation mechanism of SmDex with ω -epoxyalkyl α -glucopyranoside, the further studies including structural analysis of modified SmDex with a biochemical approach are currently in progress.

This work was partially supported by the Program for Promotion of Basic and Applied Researches for Innovations in Bio-oriented Industry (BRAINI).

Fig. 3. Inactivation of SmDex by E5G and its kinetic analysis.

(A) Inactivation of SmDex by E5G. E5G-treatment of SmDex was done by varying the concentration of E5G (0.5 mM (a), 0.75 mM (b), 1 mM (c), 2 mM (d)) at 35°C in 50 mM sodium acetate buffer (pH 5.0). (B) Plots of $[E5G]$ versus k_{app} value. The k_{app} values were estimated from Fig. 3(A). (C) Plots of $1/[E5G]$ versus $1/k_{app}$ value.

REFERENCES

- 1) B. Henrissat: A classification of glycosyl hydrolases based on amino-acid sequence similarities. *Biochem. J.*, **280**, 309–316 (1991).
- 2) B. Henrissat and A. Bairoch: New families in the classification of glycosyl hydrolases based on amino-acid sequence similarities. *Biochem. J.*, **293**, 781–788 (1993).
- 3) E. Khalikova, P. Susi and T. Korpela: Microbial dextran-hydrolyzing enzymes: fundamentals and applications. *Microbiol. Mol. Biol. Rev.*, **69**, 306–325 (2005).
- 4) T. Oguma, K. Tobe and M. Kobayashi: Purification and properties of a novel enzyme from *Bacillus* spp. T-3040, which catalyzes the conversion of dextran to cyclic isomaltooligosaccharides. *FEBS Lett.*, **345**, 135–138 (1994).
- 5) M. Dixon and E.C. Webb: *Enzymes*, 3rd Ed., Longman Group, London, pp. 369–372 (1979).
- 6) G. Legler: Glycoside hydrolases: mechanistic information from studies with reversible and irreversible inhibitors. *Adv. Carbohydr. Chem. Biochem.*, **48**, 319–384 (1990).
- 7) G. Legler and E. Bause: Epoxyalkyl oligo-(1,4)- β -D-glucosides as active site directed inhibitors of cellulases. *Carbohydr. Res.*, **28**, 45–52 (1973).
- 8) M.L. Shulman, S.D. Shiyan and A.Y. Khorlin: Specific irreversible inhibition of sweet-almond β -glucosidase by some β -glycopyranosylepoxyalkanes and β -D-glucopyranosyl isothiocyanate. *Biochim. Biophys. Acta*, **445**, 169–181 (1976).
- 9) A.J. Clarke and H. Strating: Affinity labelling of *Schizophyllum commune* cellulase with [^3H]-4,5-epoxypentyl β -cellobioside: synthesis of inhibitor and stoichiometry of interaction. *Carbohydr. Res.*, **188**, 245–250 (1989).
- 10) P.B. Hoj, R. Condrón, J.C. Traeger, J.C. McAuliffe and B.A. Stone: Identification of glutamic acid 105 at the active site of *Bacillus amyloliquefaciens* 1,3-1,4- β -D-glucan 4-glucanohydrolases using epoxide-based inhibitors. *J. Biol. Chem.*, **267**, 25059–25066 (1992).
- 11) P.B. Hoj, E.B. Rodrigues, J.R. Iser, R.V. Stick and B.A. Stone: Active site-directed inhibition by optically pure epoxyalkyl cellobiosides reveals differences in active site geometry of two 1,3-1,4- β -D-glucanohydrolases. *J. Biol. Chem.*, **266**, 11628–11631 (1991).
- 12) T. Keitel, O. Simon, R. Borris and U. Heinemann: Molecular and active-site structure of a *Bacillus* 1,3-1,4- β -glucanase. *Proc. Natl. Acad. Sci. USA*, **90**, 5287–5291 (1993).
- 13) R. Macarron, J. Vanbeeumen, B. Henrissat, I. Delamata and M. Claeysens: Identification of an essential glutamate residue in the active site of endoglucanase III from *Trichoderma reesei*. *FEBS Lett.*, **316**, 137–140 (1993).
- 14) R. Havukainen, A. Torronen, T. Laitinen and J. Rouvinen: Covalent binding of three epoxyalkyl xylosides to the active site of endo-1,4-xylanase II from *Trichoderma reesei*. *Biochemistry*, **35**, 9617–9624 (1996).
- 15) G. Sulzenbacher, M. Schulein and G.J. Davies: Structure of the endoglucanase I from *Fusarium oxysporum*: Native, cellobiose, and 3,4-epoxybutyl β -D-cellobioside-inhibited forms at 2.3 Å resolution. *Biochemistry*, **36**, 5902–5911 (1997).
- 16) P. Ntarima, W. Nerinckx, K. Klarskov, B. Devreese, K.M. Bhat, J. Van Beeumen and M. Claeysens: Epoxyalkyl glycosides of D-xylose and xylooligosaccharides are active-site markers of xylanases from glycoside hydrolase family 11, not from family 10. *Biochem. J.*, **347**, 865–873 (2000).
- 17) T. Laitinen, J. Rouvinen and M. Perakylä: Inversion of the roles of the nucleophile and acid/base catalysts in the covalent binding of epoxyalkyl xyloside inhibitor to the catalytic glutamates of endo-1,4- β -xylanase (XYNII): a molecular dynamics study. *Protein Eng.*, **13**, 247–252 (2000).
- 18) 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).
- 19) R.F. McFeeter: A manual method for reducing sugar determinations with 2,2'-bicinchoninate reagent. *Anal. Biochem.*, **103**, 302–306 (1980).
- 20) D.J. Rigden: Iterative database searches demonstrate that glycoside hydrolase families 27, 31, 36 and 66 share a common evolutionary origin with family 13. *FEBS Lett.*, **523**, 17–22 (2002).
- 21) W.C.J. Ross: The reactions of certain epoxides in aqueous solutions. *J. Chem. Soc.*, 2257–2272 (1950).
- 22) T. Yamamoto, K. Terasawa, Y.M. Kim, A. Kimura, Y. Kitamura, M. Kobayashi and K. Funane: Identification of catalytic amino acids of cyclodextran glucanotransferase from *Bacillus circulans* T-3040. *Biosci. Biotechnol. Biochem.*, **70**, 1947–1953 (2006).
- 23) Y.M. Kim and D. Kim: Characterization of novel thermostable dextranase from *Thermotoga lettingae* TMO. *Appl. Microbiol. Biotechnol.*, **85**, 581–587 (2010).
- 24) B.P. Rempel and S.G. Withers: Covalent inhibitors of glycosidases and their applications in biochemistry and biology. *Glycobiology*, **18**, 570–586 (2008).
- 25) A. Kimura, M. Takata, Y. Fukushi, H. Mori, H. Matsui and S. Chiba: A catalytic amino acid and primary structure of active site in *Aspergillus niger* α -glucosidase. *Biosci. Biotechnol. Biochem.*, **61**, 1091–1098 (1997).

ω -エポキシアルキル α -D-グルコピラノシドによる エンド型デキストラナーゼの自殺基質型失活

カン ヒゴン¹, キム ヨンミン¹, 中井博之¹, カン ミンソン¹

袴田 航², 奥山正幸¹, 森 春英¹, 西尾俊幸²

木村淳夫¹

¹ 北海道大学大学院農学研究院

(060-8589 札幌市北区北 9 条西 9 丁目)

² 日本大学生物資源科学部生命化学科

(252-0880 藤沢市亀井野 1866)

Streptococcus mutans ATCC 25175 由来のエンド型デキストラナーゼ (SmDex) に対し、3 種類の ω -エポキシアルキル α -D-グルコピラノシド (3',4'-エポキシブチル α -D-グルコピラノシド (E4G), 4',5'-エポキシペンチル α -D-グルコピラノシド (E5G) および 5',6'-エポキシヘキシル α -D-グルコピラノシド (E6G) : アグリコンのアルキル鎖長が異なる) を作用させると、SmDex は擬一次的な活性低下を示した。アルキル鎖長に依存した失活が認められ、失活の度合いは E5G > E6G > E4G であった。したがって ω -エポキシアルキル α -D-グルコピラノシドのグルコース残基とエポキシ基の距離が、SmDex の失活に対し重要であることが判明した。E5G は可逆的な中間体を形成する失活機構 (自殺基質型の失活機構) を与え、不活性化の一次定数 (k) と中間体の解離定数 (K_R) はそれぞれ 0.44 min^{-1} および 1.45 mM と算出された。SmDex の加水分解反応の生成物であるイソマルトースの存在により E5G の失活が防御されたため、E5G は SmDex の触媒部位に結合すると示唆された。本論文は、 ω -エポキシアルキル α -D-グルコピラノシドがエンド型デキストラナーゼの自殺基質にすることを示す初めての報告である。