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学位論文内容の要旨

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Reaction mechanism of glycosidases by studying 1,5-anhydro-D-fructose production

(1,5-アンヒドロ-D-フラクトース生成によるグリコシダーゼの反応機構の究明)

Even though two catalytic reaction models are proposed for glycoside hydrolases (the single or double nucleophilic displacement reaction mechanism and the oxocarbenium ion intermediate reaction mechanism), nowadays, the former model is believed. It is important to learn the reaction mechanism by obtaining obvious fact.

Several carbohydrate-active enzymes are classified into glycoside hydrolase family (GH) 31, where α -1,4-glucan lyase (GLase) and α -glucosidase (AGase) are members of this family. Full amino acid sequences of GLase and AGase display the similarity of 23-28%. Both GH31 enzymes share the identical catalytic residues of acidic amino acids. Conventional inhibitors of GH31 AGase also inhibits GLase, supporting that those enzymes share the mechanistic basis of substrate specificity and inhibitor specificity, even through the both of AGase and GLase catalyze the different reactions: AGase performs the hydrolytic reaction to produce glucose and GLase performs the elimination reaction to synthesize 1,5-anhydro-D-fructose (AF) as a product. These facts suggest that GH31 AGase also generates AF. The purpose of this study is the investigation of AF-production by GH31 AGase-catalyzed reaction.

1. Investigation AF-production by LC-MS-MS approach

For investigation of AF-production, an internal standard of [U - ^{13}C]AF was synthesized by GLase-catalyzed reaction with [U - ^{13}C]starch from algae (Chlorella Ind. Co. Ltd., Tokyo, Japan): GLase was incubated with [U - ^{13}C]starch at 37°C for 4 h in 0.1 M sodium acetate buffer (pH 4.0). The prepared [U - ^{13}C]AF was purified with Bio-Gel P2, followed by HPLC. The molecular mass of [^{13}C]AF was confirmed to be 168. Using *O*-ethylhydroxylamine, [^{13}C]AF was modified to its ethyloxime-derivative ([^{13}C]AF-OX; molecular mass, 211; molecular formula, [^{13}C]C₆[^{12}C]C₂H₁₅O₅N). Resultant [^{13}C]AF-OX was used as internal standard for LC-MS-MS approach to investigate AF-production by *Aspergillus niger* AGase (ANGase; GH31 enzyme).

2. Production of AF by ANGase (GH31 AG)

For analysis of AF-formation by ANGase, the reaction mixture was treated with *O*-ethylhydroxylamine/HCl to investigate the formation of 1,5-anhydro-D-fructose *O*-ethylloxime ($[^{12}\text{C}]$ AFOX; molecular mass, 205; molecular formula, $[^{12}\text{C}]\text{C}_8\text{H}_{15}\text{O}_5\text{N}$). It was found that a small amount of AF was produced by ANGase from maltooligosaccharides (maltose to maltohexose), suggesting that ANGase (GH31 AGase) catalyzes the elimination reaction to synthesize AF. Furthermore, bacteria AGase also generated AF during its hydrolytic reaction.

Formation of AF is considered to be directly related to the reaction mechanism, where two models are proposed: a nucleophilic displacement mechanism (Fig. A) and an oxocarbenium ion intermediate mechanism (Fig. B). If ANGase catalyzes the reaction via the oxocarbenium ion intermediate mechanism, AF is rationally produced (Fig. C and D), since the H-atom of the C2-H is easily extracted by two possible routes [carboxylate ion-associated mechanism (Fig. C) and water molecule-associated mechanism (Fig. D)], when ring oxygen atom is positively charged. However, this extraction is not likely to be observed via a nucleophilic displacement mechanism, and thus, AF would not be expected to be formed (Fig. A), suggesting that ANGase follows the oxocarbenium ion intermediate mechanism (Fig. B).

