Abstract of Doctoral Dissertation

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Characterization and ligand-binding manner of EHEP and BGL for producing biofuel from brown algae

褐藻からバイオ燃料を生産するタンパク質 EHEP と BGL の特性およびリガンド結合様式の研究

Dramatic increases in fuel demands globally have prompted a search for renewable energy sources. Biofuel is a promising alternative to fossil fuels because of its lower cost, renewable supply, and reduced greenhouse gas emissions. Brown algae are considered ideal feedstocks for producing biofuel because their many advantages. The cultivation does not need arable land, only use fresh water. The brown algae do not contain lignin, making the enzymatic hydrolysis of brown algae relatively easy.

The sea hare *Aplysia kurodai* consumes brown algae as a staple food, using *β*-glucosidases (*aku*BGLs) as catalysts for hydrolyzing the glycosidic bonds of laminarin abundant in brown algae to produce glucose, making it an excellent model for investigating the biofuel production process. However, phlorotannins which are also abundant in brown algae, inhibit the hydrolysis reaction of *aku*BGLs, bringing the serious problem in production of biofuel source glucose from brown algae. Interestingly, Eisenia hydrolysis enhancing protein (EHEP) identified in the digestive fluid of *A. kurodai*, was found recently to protect *aku*BGL from this inhibition by binding and then precipitating with phlorotannins, despite not possessing catalytic function. Hence, EHEP and *aku*BGL have high potential for the application in the biofuel industry. An understanding of phlorotannin-binding manner with EHEP and *aku*BGL will lead us to elucidate protective mechanisms of EHEP and the phlorotannin-inhibitory mechanism of *aku*BGL. Consequently, these knowledge could provide information on how increase the activity of *aku*BGL and how EHEP can be used recycled, and knowledge of the three-dimensional structures of *aku*BGL and EHEP is indispensable for this.

Because phlorotannin is very difficult to isolate from brown algae and it has been reported that tannic acid (TNA) is an analog of phlorotannins, we explore firstly the inhibitory activity of TNA for *aku*BGL and whether EHEP binds TNA to protect *aku*BGL from TNA-inhibition by analyzing the *aku*BGL

activity using ortho-Nitrophenyl-β-galactoside (ONPG) as the substrate. The results showed that the activity of *aku*BGL decreased with increasing the concentration of tannic acid. About 70% activity of *aku*BGL was inhibited with a concentration of 40 μM TNA. When adding EHEP, the more activity of *aku*BGL was recovered with increasing the concentration of EHEP. About 80% activity of *aku*BGL was recovered with a concentration of 3.96 μM EHEP. Therefore, instead of phlorotannins, we used TNA to investigate the protective mechanism of EHEP and the phlorotannin-inhibitory mechanism.

To understand the TNA-binding manner of EHEP, we tried to analyze structures of EHEP in apo and TNA-binding form. Because EHEP is a novel and unique protein, with no structures of homologous proteins were reported, we determined the structure of EHEP by single-wavelength anomalous dispersion using sulfur atoms in the native protein as scattering factors (native-SAD) using a long wavelength. To obtain the structure of EHEP complexed with TNA (EHEP-TNA), we attempted to soak apo crystal into TNA solution for one month. The structure of EHEP consists of three chitin-binding domains (ChBD1, ChBD2, and ChBD3) linked by two long loops. In the EHEP-TNA structure, TNA is located on the surface of EHEP, and outmost five gallic acids of TNA cannot be visualized due to structural disorder. The TNA-binding pocket was formed by C-terminal α-helix of ChBD1, N-terminal α-helix of ChBD2, and middle part (loop) of ChBD3 with the help of two linker loops. TNA bound to EHEP by hydrogen bond and hydrophobic interactions, and the binding did not induce large conformation changes. Based on structural information, we consider that precipitation of EHEP with TNA should be recovered and successfully dissolve the precipitate of EHEP-TNA using buffer with alkaline pH.

Furthermore, we determined the structure of *aku*BGL. The structure consists of two GH1 domains (GH1 domain1, 29-492 GH1-DI and GH1 domain2, 513-974: GH1-DII) which adopt almost the same structure with a rmsd of 0.59 Å for 371 Cα atoms (40.47 % sequence identity). This is the first time to visualize the structure that contains two GH1 domains of BGLs. As other structures of GH1 family enzymes, each GH1 domain exhibits a classical $(\beta/\alpha)_8$-barrel fold. D2 domain processes an active site containing two conserved carboxylic acid residues (E675, E885), however, the first catalytic residue in D1 domain was mutated to D192. The two-domain architecture might represent a specific adaption towards brown algae. Moreover, docking was performed to analysis the inhibition mechanism of tannic acid with *aku*BGL.