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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 of their many advantages.

The sea hare *Aplysia kurodai* consumes brown algae as a staple food, using β-glucosidases (akuBGLs) 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. However, phlorotannin which is also abundant in brown algae, inhibits the hydrolysis reaction of akuBGLs, bringing the serious problem in the production of biofuel source glucose from brown algae. Interestingly, Eisenia hydrolysis enhancing protein (EHEP) in the digestive fluid of *A. kurodai*, was found recently to protect akuBGL from this inhibition by binding and then precipitating with phlorotannin. An understanding of phlorotannin-binding manner with EHEP and akuBGL will lead us to elucidate protective mechanisms of EHEP and the phlorotannin-inhibitory mechanism of akuBGL. The knowledge could provide information on
how to increase the activity of akuBGL and how EHEP can be used recycled and the
three-dimensional structures of akuBGL and EHEP are indispensable.

To understand the TNA-binding manner of EHEP, we tried to analyze the structure of EHEP
firstly. 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 protein as scattering factors (native-SAD) using a long wavelength. We
also attempted to get the complex structure of EHEP with tannic acid (TNA), an analog of
phlorotannins by soaking apo crystal into TNA solution for one month. The structure of EHEP
consists of three chitin-binding domains (ChBD1, 2, and 3) linked by two long loops. In the
EHEP-TNA structure, TNA is located on the surface of EHEP, and outmost five gallic ac
cannot be visualized due to structural disorder. TNA bound to EHEP by hydrogen bond and
hydrophobic interactions, and the TNA-binding did not induce large conformation changes of
EHEP. Based on structural information, we consider that precipitation of EHEP with TNA should
be recovered and successfully dissolve the precipitate of EHEP-TNA in alkaline pH.

Furthermore, we determined the structure of akuBGL which consists of two GH1 domains (D1
and D2) and they adopt almost the same structure with a rmsd of 0.59 Å for 371 Cα atoms (40.5%
sequence identity). This is the first time to visualize the structure that contains two GH1 domains
of BGLs. Like other structures of GH1 family enzymes, each domain exhibits a classical
($\beta/\alpha$)$_{8}$-barrel fold. D2 processes an active site containing two conserved carboxylic acid residues
(E675, E885), while the first catalytic residue in D1 was mutated to D192. The two-domain
architecture might represent a specific adaption towards brown algae. Moreover, docking was
performed to analyze the inhibition mechanism of tannic acid with akuBGL. Taken all results
together, we proposed the inhabitation and protection mechanism.

In conclusion, in this study, the applicant has new findings on phlorotannins binding manner of
EHEP and $anaBGL$, which does not only contribute important information to life science and
provide also molecular bases for designing new enzymes to produce biofuel from brown algae. In
addition, through this study, the applicant has acquired a scientific intellectual and acting power.

Therefore, we acknowledge that the applicant is qualified to be granted the Doctorate of Life
Science from Hokkaido University.