様　式　Ｆ－１９－２

科学研究費助成事業　　研究成果報告書

令和　　元 年　　９ 月 ２４ 日現在

機関番号：１０１０１
研究種目：国際共同研究加速基金（国際共同研究強化）
研究期間：2016～2018
課題番号：１５ＫＫ０２６９

研究課題名（和文）Ecological function of wood insect associated microbes（国際共同研究強化）

研究課題名（英文）Ecological function of wood insect associated microbes(Fostering Joint International Research)

研究代表者
高須賀　太一（Takasuka, Taichi）

北海道大学・農学研究院・准教授

研究者番号：７０７４８４０９

交付決定額（研究期間全体）：（直接経費）　　9,800,000円
渡航期間：　　５ヶ月

研究成果の概要（和文）：将来枯渇する事が予想される化石資源の代替資源として，陸海域のバイオマス資源を利用した持続可能なバイオ燃料やバイオ化成品生産技術の確立が求められている．本研究では，酵素学的知見から，陸域のバイオマス作物や海域の褐藻の効率的な分解方法の開発を目指した．具体的には植物細胞壁の主要成分であるセルロースやヘミセルロース等を効率的に糖化する多機能性セルラーゼ（GH5_4ファミリー）についての研究を行う事で，セルラーゼの多機能性について本ファミリーに属する243種の酵素の機能・系統樹解析を行った．褐藻をターゲットとした研究では，2種類の酵素を組み合わせた褐藻の効率的な分解を実現した．

研究成果の学術的意義や社会的意義
本研究で明らかにした129種の多機能性セルラーゼは，一つの触媒ドメインがセルロースやキシランおよびマンナン（これらは植物細胞壁の主要成分）を分解する事ができる事から，従来の粗酵素抽出液中のセルラーゼ，キシラナーゼやマンナナーゼ酵素を一つの多機能性セルラーゼに置き換える事が可能となる．さらに，129種の酵素は異なる至適反応条件を示した事から，様々な植物バイオマスの糖化条件に適した酵素の利用が可能となり，効率的な植物バイオマス分解技術につながる．褐藻分解については，2種類の酵素を組み合わせる事により褐藻主要成分であるラミナリンおよびアルギン酸の効率的な分解を達成できた．

研究成果の概要（英文）：The establishment of a sustainable biofuels and biochemicals production technology using biomass resources in land and sea areas is required as an alternative resource for finite fossil resources that are expected to be diminished in the future. So that we aimed at the development of efficient decomposition method of biomass crops in land and kelp in the sea area.
Efficient plant biomass decomposition requires novel biomass-degrading enzymes, which might possess multi-substrate functions and high specific activities. To discover the new enzyme function and also to understand multifunctionality of the cellulase, we focused on 243 enzymes from the previously reported multifunctional enzyme family (GH5_4s) for functional characterization together with the Bayesian inference phylogenetic analyses.
Additionally, an efficient kelp degradation was achieved by a minimum two enzyme combination between Glycoside hydrolase 55 family (GH55) and alginate lyase (PL18).

研究分野：生化学

キーワード：plant biomass　kelp biomass　GH5　GH55

様　式　Ｆ－１９－２

１．研究開始当初の背景

Efficient plant biomass decomposition requires novel biomass-degrading enzymes, which might possess multi-substrate functions and high specific activities. To develop new enzyme cocktails for efficient plant biomass, we have researched on multifunctional endoglucanase family (glycoside hydrolyase 5 subfamily 4: GH5_4). Additionally, kelp, which is widely available in the ocean, was also targeted as a renewable resource for biofuels and bio-chemicals production, and an efficient kelp biomass decomposition method is needed. We have focused on defining a minimum enzyme combination for efficient enzyme hydrolysis of kelp by using glycoside hydrolase 55 family (GH55) and alginate lyases (PL18s), which target two major polymers present in kelp, laminarin and alginate, respectively.

GH5 hydrolyzes various types of polysaccharides. Recent our studies and others suggested that some of the subfamilies of this enzyme class are capable of hydrolyzing more than three different polysaccharides such as cellulose, xylan, mannnan by one catalytic domain. These "multi functional" enzymes were mainly distributed in the GH5 subfamily 4 class (GH5_4). Such enzyme was determined in the *Clostridium thermocellum* secretome (Takasuka et al., Methods Mol. Biol., 2014, Deng et al., ACS Chem. Biol., 2014). Although we utilized this particular enzyme to enhance plant biomass decomposition (Walker et al., Biotechnol. Biofuels., 2015), we did not know a basic question of “how this type of enzyme can accommodate multiple substrates at the binding cleft?”. Thus, we attempted to find out the answer to this question by conducting phylogenetic-based protein functional analysis described in sections 2 to 4 below.

GH55 is another class of enzymes that can hydrolyze laminarin polymers largely found in brown kelp. Brown kelp is consists of laminarin, alginate, mannitol, and fucoidan, and we found previously the bacterial GH55 (SactELam55A) that can completely decompose laminarin into glucose, gentiobiose, and laminaribiose (Bianchetti and Takasuka et al., 2015). We took a phylogenetic-based approach to envision more than 40 different bacterial GH55s reported at NCBI database, and found that the SactELam55A was one of the best enzymes in terms of a wide range of pH and temperature optima besides very high laminarinase activity. Furthermore, this enzyme was easily produced by a recombinant method. To search for the potential use of this enzyme for a kelp conversion, it was necessary to test this enzyme toward actual kelp substrate, yet we did not perform.

２．研究の目的

Determining functional diversity in glycoside hydrolase subfamily 4 enzymes (GH5_4s).

To determine their biochemical functions, we decided to extract all of the currently available GH5_4 sequences (NCBI), synthesize genes, and produce enzymes followed by assays. Furthermore, we attempted to determine more than ten crystal structures of those multi-functional enzymes in order to understand structurally how they can recognize various polysaccharides as substrate.

Kelp hydrolysis by minimum enzyme combination between glycoside hydrolase 55 (GH55) and alginate lyase (PL18).

We decided to utilize the SactELam55A with different enzyme, polysaccharide lyases (PL18s), that can hydrolyze alginate polymers, also abundant in kelp.

３．研究の方法

Methods of determining functional diversity of GH5_4 enzymes.

Over 250 catalytic domains of GH5_4 were chosen for gene synthesis in collaboration with the U.S. Department of Energy, Joint Genome Institute (DNA synthesis section: lead by Dr. Yoshikuni). 243 were successfully synthesized and delivered at the University of Wisconsin - Madison, then we utilized a wheat cell-free protein synthesis to produce all of the synthesized genes and quantitated productivity by the Gel-Doc system (Takasuka et al., Methods Mol. Biol., 2014). Various substrates including crystalline cellulose (Sigmacell-20), amorphous cellulose (PASC), lichenan, xylan, xyloglucan, mannan, galactoglucomannan, chitin were used, and each enzyme was tested reactivity on listed substrates for a range of reaction temperature and pH for 20 hrs followed by DNS assay to monitor reduced end products of polysaccharide substrates. A Bayesian inference phylogenetic tree was constructed from the 638 reported GH5_4 sequences, and the 243 synthesized and tested GH5_4s were mapped on the tree. More than 10 GH5_4 that showed multifunctionality were crystallized and X-ray diffraction patterns were collected at the U.S. Department of Energy, Argonne National Laboratory, Illinois, U.S.A. for structure analysis.

Combinatorial enzyme function screening toward efficient kelp hydrolysis.

Six polysaccharide lyase 18 enzymes (PL18s) including the previously reported aly-Sj02 from *Pseudoalteromonas* sp. SM0524 (Dong et al., J. Biol. Chem., 2014) were gene synthesized in collaboration with the U.S. Department of Energy, Joint Genome Institute. Enzymes were recombinantly expressed in *Escherichia coli*, followed by protein purification by an affinity chromatography. Alginate lyase function was tested together with SactELam55A enzyme on a pure commercial alginate or kelp (*Laminaria digitata*) at 40 to 60˚C at around pH 6.0 for 20 hrs, followed by DNS assay. Nano-structure Initiator Mass Spectrometry (NIMS) was performed to detect soluble end products after hydrolysis and numerical simulation was performed to determine kinetics. End product determination was also performed by 2D HSQC NMR analysis.

4．研究成果

Results of determining functional diversity of GH5_4 enzymes (Glasgow et al., J. Mol. Biol., 2019).

243 selected GH5_4 enzymes were produced in soluble forms, of which 229 enzymes showed at least one activity toward a panel of substrates mentioned in **3.METHODS**. Out of 229 enzymes, 126 showed reactivity on lichenan (beta-1,4 linked glucan), xylan and mannan (trifunctional), 77 showed reactivity on lichenan and xylan (bifunctional), and 11 showed reactivity on lichenan and mannan (bifunctional) (Fig. 1). By specific activity measurements of these enzymes, we found that lichenan- and xylan-degrading activities are highly correlated, while mannan-degrading activity was not.

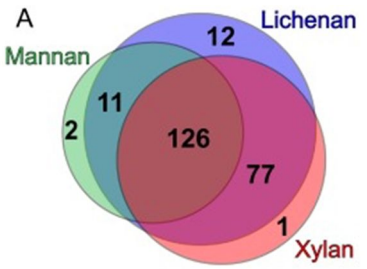

Fig. 1. Venn diagram showing assay-defined mono-, bi- and trifunctional characteristics of 229 enzymes.

We then asked whether evolution of this class of enzymes correlates with observed multifunctionality (Fig. 2). Based on the Bayesian inference phylogenetic tree and observed reactivities, we made arbitrary clades 1, 2, and 3 in GH5_4.

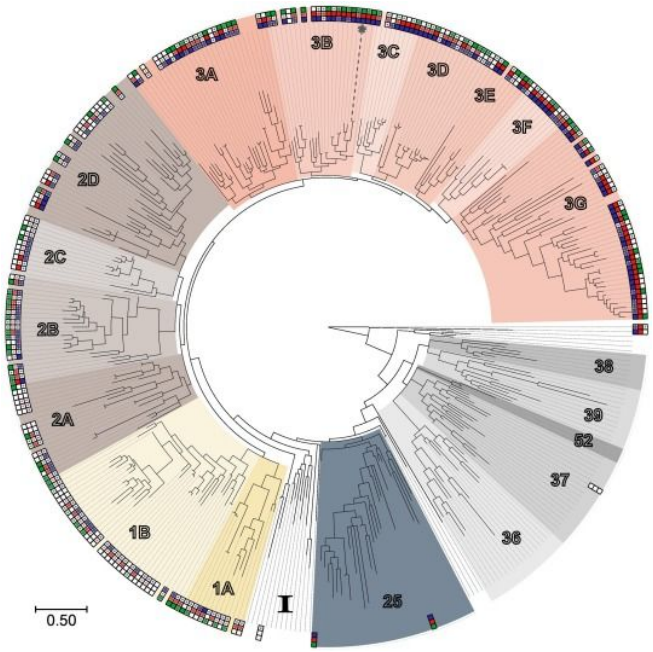

Fig. 2. Major clades within GH5 subfamily 4 are denoted by yellow, brown, and orange highlighting, with varying saturation and each color corresponded to subclade partitioning; other subfamilies are indicated by varying shades of gray. Enzyme sequences that were tested for activities are shown by a three-cell table at the outer shell of the tree. The presence of blue, red, or green circles within each cell indicates lichen-, xylan-, or mannan-degrading activity, respectively, and circle size indicates measured strength of specific activity. The largest, most intense symbols reflect enzymes with specific activities ranking in the top third. *indicated the location of previously reported enzyme (Walker et al., Biotechnol. Biofuels., 2015).

Although we were able to show an extensive, quantitative demonstration of a relationship between substrate specificities of 243 GH5_4s and evolutional link, more work is needed to entirely under how the protein sequence dictates substrate specificity. On the other words, we have not established the way to predict multifunctionality of enzyme based on the protein sequence. Currently, we have been analyzing the crystal structures of 10 selected trifunctional GH5_4s, which we successfully solved in the past several years, to fill our knowledge among protein sequence, protein structure and function.

Successful kelp hydrolysis by minimum two enzyme system (Takasuka et al., in preparation).
To develop a minimum two enzyme kelp hydrolysis method, six PL18s (Alginate lyases) were produced with purified form, and tested for purified alginate conversion as well as kelp conversion with SActELam55A. Results showed that the CaPL18A (E7 enzyme) resulted in the highest %kelp conversion when the enzyme was mixed with SActELam55A. Thus, we choose CaPL18A for following kelp hydrolysis experiments.

When SActELam55A was reacted with kelp, nearly 70% of laminarin fraction in kelp was released as glucose detected by NIMS analysis. In the presence of both enzymes in kelp hydrolysis, we were able to determine both laminarin and alginate end products (manuscript in preparation). Compared to kelp control, disappearance of original laminarin and alginate polymers can be seen, and this result indicated that SActELam55A and E7 enzymes are a minimum enzyme pair, which hydrolyze kelp efficiently.

Unfortunately, we were not able to assign the DP of alginate end products determined by 2D HSQC NMR, and thus we are currently trying to determine end product polymer lengths of alginate end products by the NIMS. Shortly, we will write up the manuscript.

５．主な発表論文等
（研究代表者は下線）

〔雑誌論文〕（計 2 件）

1. Glasgow, E.M., Vander Meulen, K.A., Takasuka, T.E., Bianchetti, C.M., Bergeman, L.F., Deutsch, S. and Fox, B.G. Extent and origins of functional diversity in a Subfamily of Glycoside Hydrolases. *J. Mol. Biol.*, **査読有**, Vol. 431, p1217-1233, (2019).

2. Takasuka, T.E.*, Bianchetti, C.M., Deng, K., Beebe, E.T., Bergeman, L.F., Vander-Meulen, K.A., Deutsch, S., Kim H., Ralph, J., Northen, T.R. and Fox, B.G. Laminarinase and alginate lyase hydrolysis of kelp to monooligosaccharides. (*In preparation*).

*Corresponding

〔学会発表〕（計 3 件）

1. 高須賀太一. 「**セルロース分解性放線菌における分泌酵素遺伝子の転写制御機構の解明**」 ***第 32 回セルラーゼ研究会***, **長野**, 7 **月** 2018.

2. Takasuka, T. Novel insect symbionts cellulolytic Streptomyces potentiate plant biomass refinery. ***International Symposium on Biomass Refinery: From Biomass Crops to Chemicals and Fuels***, **札幌**, 2 **月** 2018.

3. Takasuka, T. Omics and genome-enabled technology to understand phylogenetic-based enzyme functions. ***環境微生物系学会合同大会***, **仙台**, 8 **月** 2017.

〔図書〕（計 0 件）

〔産業財産権〕
○出願状況（計 0 件）

名称：
発明者：
権利者：
種類：
番号：
出願年：
国内外の別：

○取得状況（計 0 件）

名称：
発明者：
権利者：

種類：
番号：
取得年：
国内外の別：

〔その他〕
ホームページ等
Not applicable.
６．研究組織

研究協力者

〔主たる渡航先の主たる海外共同研究者〕
研究協力者氏名：ブライアン　フォックス
ローマ字氏名： Brian, Fox
所属研究機関名：University of Wisconsin -Madison
部局名：Department of Biochemistry
職名：Department Chair and Professor

研究協力者氏名：吉国　泰男
ローマ字氏名：Yoshikuni, Yasuo
所属研究機関名：The U.S. Department of Energy, Joint Genome Institute
部局名：Division of gene synthesis
職名：Project leader

〔その他の研究協力者〕
研究協力者氏名：
ローマ字氏名：