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研究課題名（和文）Development of single molecule display platform for directed evolution of enzymes

研究課題名（英文）Development of single molecule display platform for directed evolution of enzymes

研究代表者

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研究成果の概要（和文）：産業、診断、研究用改変酵素や酵素基質の開発を目指し、分子ライブラリを酵素活性で迅速に選抜するためのcDNAディスプレイシステムを開発した。従来のタンパク質工学の手法と比べて、本システムは、短時間かつ少ない労力と許容範囲の費用で、所望の特性を持つ改変タンパク質分子を得ることができる。まずトランスグルタミナーゼ(TG)基質の選択システムを最適化した。次にランダム化した基質ライブラリーを用い、TG1およびTG2の反応性の高いペプチド基質をすることに成功するだけでなく、それら酵素の基質選択性を網羅的に明らかにした。さらに酵素(オキシダーゼ)を選択するためのcDNAディスプレイ法の諸条件を検討した。

研究成果の学術的意義や社会的意義
選択された生体分子配列の詳細な解析は、酵素の基質特異性や構造-機能相関に関する包括的な知識を提供し、酵素の機能理解や将来の応用設計に重要であることから、科学に貢献する。酵素とその基質の開発は、付加価値の高い製品の効率的かつ持続的な生産、高感度かつ迅速な診断ツール、効率的な治療薬を可能にするため、社会にとって有益である。

研究成果の概要（英文）：We developed a platform for rapid activity-based screening of combinatorial biomolecular libraries during protein engineering, aiming to enable rapid development of modified enzymes and enzymatic substrates for use in industry, diagnostics, and research. Accuracy and in-depth analysis of the selected sequences was enabled by next-generation sequencing and bioinformatics. Unlike conventional methods for protein engineering, this platform takes short time and less labor under acceptable expenses to yield a modified biomolecule with desired properties. Screening and selection system was first optimized using simple model libraries followed by screening of the combinatorial libraries of interest. We have selected very reactive peptide substrates of transglutaminases (TG), TG1 and TG2, and optimized the screening system for the selection of enzymes, e.g. oxidase. Oxidase and transglutaminase are important for industry and diagnostics and for this reason we focused on these two enzymes.

研究分野：Applied biochemistry (応用生物化学)

キーワード：biomolecular engineering　in vitro display　cDNA display　transglutaminase　oxidase　NGS　bio informatics

１．研究開始当初の背景 (Background at the beginning of the study)

Biocatalysis is gaining importance in all aspects of the industry, medicine, diagnostics, environmental remediation, bio-refinery, and research, as seen by the constant increase of the global enzyme market which now stands at 10 billion USD. Under SDGs 6, 7, 12, and 13, we need to achieve a sustainable society in terms of energy utilization, and environmental issues. Biocatalysis is desirable since enzymes operate under mild reaction conditions and do not require the usage of excess energy or hazardous chemicals. The range of available reactions catalyzed by enzymes is in rapid increase, largely due to the increased use of metagenomic approaches for enzyme discovery, and improvements in enzyme engineering by directed evolution through the development of computational and high-throughput screening technologies.

Directed evolution mimics the natural selection in a test tube, however at high speed and guided by the selection pressure we set. Its importance in enzyme engineering has been recognized by the Nobel prize in Chemistry awarded to Prof. Frances Arnold in 2018. Due to the large generated diversity (e.g. randomization of 5 amino acid positions produces a library of 10^6), selection is the most critical step and has long been a bottleneck in terms of time, labor and cost.

In vitro screening and selection methods are advantageous to *in vivo* methods where library size is limited by the efficiency of DNA transformation into *E. coli* or yeast. *In vitro* methods utilize cell-free protein synthesis and various display technologies to physically link genotype and phenotype on a single-molecule level. mRNA/cDNA display pioneered by Roberts and Szostak group (Roberts and Szostak, PNAS 1997, p.94) and Nemoto and Yanagawa group (Nemoto et al., FEBS Lett 1997, p.414) relies on the formation of a covalent link between the genotype and phenotype via puromycin linker. Complete control of expression/selection conditions, stable genotype-phenotype linkage, available library size (up to 10^{12}), robustness, and short operation time make mRNA/cDNA display a superior selection technology over the other *in vitro* methods.

Engineering of enzymes and enzymatic substrates such as peptides has long been done by laborious and slow traditional colony screening methods or expensive robotic screening systems.

In vitro methods have a great advantage over both methods since they require substantially less labor and time compared to colony screening methods and for much less cost compared to robotic systems. However, the application of *in vitro* display methods to enzyme engineering, substrate profiling, and substrate engineering is still very limited (Seelig and Szostak, Nature 2007, p.448; Fleming et al., JACS 2020, p.142; Kozlov et al., PLOS ONE 2012, 7).

２．研究の目的 (Research objective / Purpose of the study)

To enable the efficient development of enzymes and enzymatic substrates for the growing demand in industry, medicine and pharmaceutical science, our objective is to harness the benefits of *in vitro* display methods and develop a system for efficient engineering of enzymes and enzymatic substrates. To achieve this objective, we decided to use cDNA display technology, as

one of the most efficient and stable *in vitro* display systems, in collaboration with its founder Professor Nemoto from Saitama University. Our first enzyme targets for engineering were horseradish peroxidase (HRP) and manganese peroxidase due to their wide substrate specificity and high potential in the synthesis of chemicals, pharmaceuticals and polymers, and bioremediation. However, due to numerous problems in recombinant expression of these enzymes (with highly similar complex structures) in active form, we focused on other important biocatalysts, namely transglutaminase (TG) and D-amino acid oxidase (DAO). We aimed at studying the substrate profile of TG1 and TG2, two very important representatives of mammalian TGs, and microbial TG (mTG), a representative of TGs with industrial importance. Based on the substrate profile, we planned to design highly specific glutamine peptide probes for measurement of TG (TG1 and TG2) activity in tissue samples and site-specific protein cross-linking in biotechnology (mTG). DAO has great potential for specific detection of D-amino acids in human samples, however, its specificity is too broad for detection and quantification of one specific kind of D-amino acid. To make DAO applicable in diagnostic kits, we aimed at engineering its substrate specificity towards D-Asp, an indicator of endocrine function.

３．研究の方法 (Research method)

The selection using the cDNA display system was done as illustrated in Figure 1. DNA libraries were order-synthesized as ssDNA fragments with randomized positions of interest by the NNK codon. ssDNA library was converted to a dsDNA library containing all necessary parts for cDNA display. After *in vitro* transcription of the random DNA library, the mRNA library is bound to the puromycin linker and subjected to *in vitro* translation to obtain the mRNA display library. Here, the protein/peptide variants encoded by the mRNA are synthesized and chemically bound to their corresponding mRNA-linker complex via puromycin of the linker which incorporates

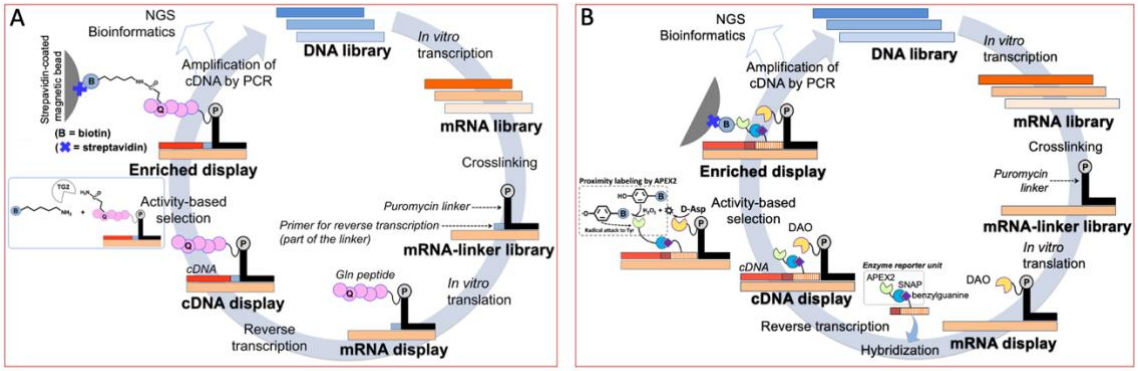

Figure 1. A) System for selection of TG substrate peptides; B) System for selection of DAO.

itself into the growing polypeptide and halts its further growth. From this step onward, the workflow is different for the selection of glutamine peptide substrates of TG (Fig. 1A) and the selection of DAO (Fig. 1B).

For the selection of peptide substrates of TG, mRNA display is first converted to cDNA display and then subjected to activity-based selection. The selection step relies on TG-mediated crosslinking of displayed Gln peptides with biotin-pentylamine resulting in biotinylation of the

reactive peptide sequences which can be captured by streptavidin-coated magnetic microbeads and pooled down. After the selection and subsequent processing, the cDNA of the original (display pool before selection) and enriched (display pool enriched on the surface of the microbeads) display pools were PCR-amplified and analyzed by next-generation sequencing and bioinformatics.

For the selection of DAO, after mRNA display generation 18-20 bs-long ssDNA carrying enzyme reporter unit (ascorbate peroxidase 2, APEX2) is hybridized to the mRNA part and reverse transcription is done to stabilize the genetic information. During the activity-based selection, complexes carrying active DAO are bound to the surface of the streptavidin-coated magnetic beads and pulled down for NGS analysis. Selection is achieved by the biotin-phenol mediated proximity labeling catalyzed by the enzyme reporter unit.

４．研究成果 (Research results)

(1) Selection of reactive TG peptide substrates and TG substrate profiling

① Random library design

The DNA sequences of the peptides named K5, T26 and M48 previously selected as artificial substrates for TG1, TG2, and MTG, respectively (Sugimura *et al.*, FEBS J 2008, 275, p.5667; Sugimura *et al.*, J Biol Chem 2006, 281, p.17699; Sugimura *et al.*, Arch Biochem Biophys 2008, 477, p.379) by phage display were used as templates for mutagenic library design (Figure 2).

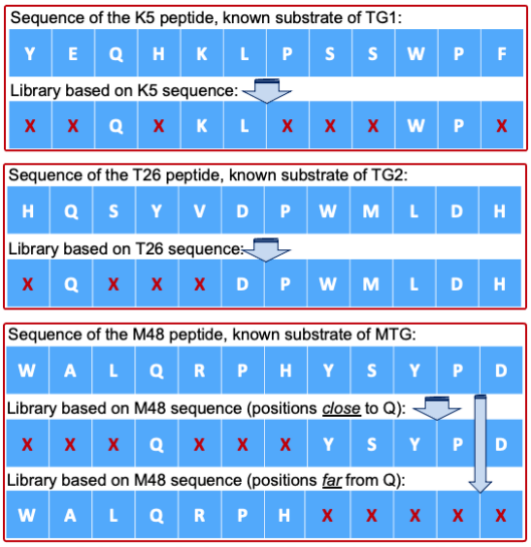

Figure 2. Library design for TG substrate peptides.

② Analysis of the selected peptide sequences

Libraries based on K5 and T26 sequences have been subjected to selection and data analysis. Two

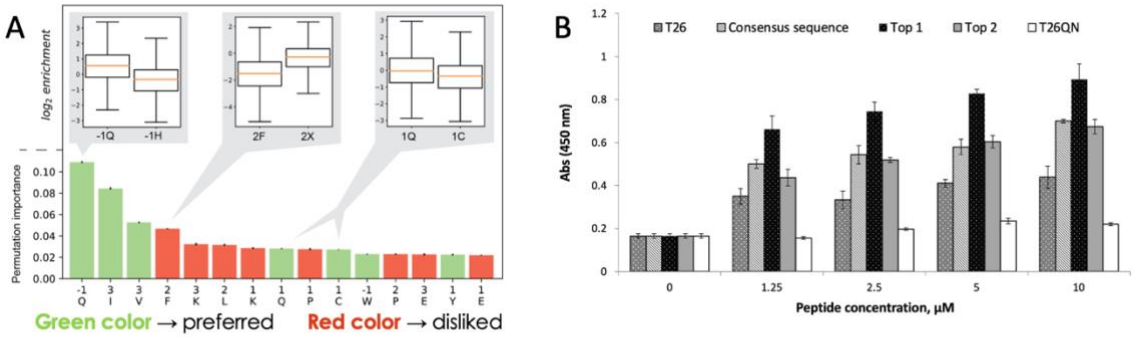

Figure 3. A) Importance of each amino acid at each mutated position of the peptide. B) Reactivity of the top enriched peptides, Top 1 and Top 2, and heatmap-derived sequence (Q-Q-C-Y-I-) in comparison to T26 and non-substrate peptide, T26QN, determined by the TG2 enzymatic assay.

M48-based libraries are still being analyzed; thus, the data is not yet available. Bioinformatics analysis of TG2-preferred peptides (selected peptide sequences) is given in Figure 3A. We found

that Gln at position -1, followed by Ile/Val at position 3, and Gln/Cys at position 1, are the most preferred substrate sequences of TG2, while Phe at position 2 followed by Lys at position 3, Leu at position +2 and Lys/Pro at position +1 represent the sequences disliked by TG2. We also ranked the selected peptide sequences according to their enrichment factor and identified peptides with the highest enrichment, which we tested in TG2 enzymatic assay alongside previously found T26 peptide and non-substrate peptide (T26QN). The results (Fig. 3B) indicate that our most enriched newly selected peptide sequence has a reactivity higher than that of the T26 peptide.

Analysis of TG1-preferred peptides (per-residue amino acid enrichment) is given in Figure 4. We found that TG1 prefers His/Tyr at position -1, His/Lys at position +1, and Pro at position +9, while it dislikes Pro at position +1.

Figure 4. The heatmap showing the color-coded enrichment factor of each amino acid at randomized positions of the K5 peptide library.

(2) Establishment of the DAO selection system

Up to date, there is only one report on the application of *in vitro* display to engineering of a (bond-forming) enzyme, RNA ligase (Seelig and Szostak, Nature 2007, p.448), while our system represents the first *in vitro* display system targeting any other kind of enzyme. We first tested *in vitro* translation of DAO and demonstrated the formation of the DAO display (Fig. 5). For this analysis, we separated soluble from insoluble fractions after *in vitro* translation and purified display molecules from both fractions via streptavidin-coated magnetic beads (linker contains biotin). We digested mRNA part of the display to reduce its size and released the display complexes from the beads by RNase T1 digestion (linker contains the recognition site). After this, crosslinking product and digested display product were analyzed by SDS-PAGE and detected by fluorescein attached to the linker. Our results indicate that DAO can be displayed in soluble form. Single-point mutations were also inserted into the DAO gene to prevent non-essential dimerization which would otherwise negatively affect the selection. The next step was the optimization of the enzyme reporter unit with APEX2 fused to a carrier protein, SNAP-tag. The reporter unit showed high activity and good expression yield. Optimization of the selection step is ongoing.

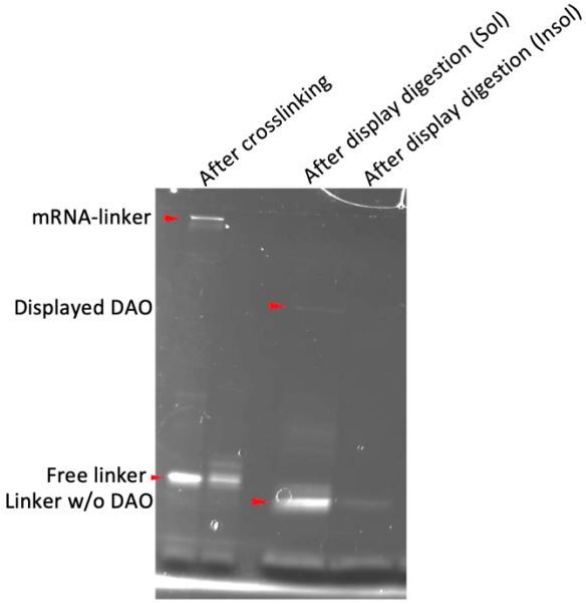

Figure 5. SDS-PAGE analysis of the DAO display formation.

５．主な発表論文等

〔雑誌論文〕　計1件（うち査読付論文　0件／うち国際共著　0件／うちオープンアクセス　1件）

１．著者名 Damnjanovic Jasmina, Odake Nana, Fan Jicheng, Jia Beixi, Kojima Takaaki, Nemoto Naoto, Hitomi Kiyotaka, Nakano Hideo	４．巻 -
２．論文標題 cDNA display coupled with next-generation sequencing for rapid activity-based screening: Comprehensive analysis of transglutaminase substrate preference	５．発行年 2021年
３．雑誌名 bioRxiv	６．最初と最後の頁 -
掲載論文のDOI（デジタルオブジェクト識別子） 10.1101/2021.09.08.459404	査読の有無 無
オープンアクセス オープンアクセスとしている（また、その予定である）	国際共著 －

〔学会発表〕　計5件（うち招待講演　0件／うち国際学会　1件）

１．発表者名 Damnjanovic Jasmina, 大嶽 七菜, 兒島 孝明, 根本 直人, 中野 秀雄
２．発表標題 Novel cDNA display platform for activity-based selection in protein engineering
３．学会等名 日本農芸化学会2021年度大会
４．発表年 2021年

１．発表者名 Damnjanovic Jasmina, Odake Nana, Kojima Takaaki, Nemoto Naoto, Hitomi Kiyotaka, Nakano Hideo
２．発表標題 cDNA display coupled with next-generation sequencing for activity-based selection: Comprehensive analysis of transglutaminase substrate preference
３．学会等名 2021 Sakura-Bio Meeting (international conference)
４．発表年 2021年

１．発表者名 Damnjanovic Jasmina, Nezu Moeri, Jia Beixi, Odake Nana, Hitomi Kiyotaka, Kojima Takaaki, Nemoto Naoto, Nakano Hideo
２．発表標題 Comprehensive analysis of transglutaminase substrate preference by cDNA display
３．学会等名 日本農芸化学会2022年度大会
４．発表年 2022年

１．発表者名 Damnjanovic Jasmina, Odake Nana, Kojima Takaaki, Nemoto Naoto, Hitomi Kiyotaka, Nakano Hideo
２．発表標題 cDNA display coupled with next-generation sequencing for rapid activity-based screening: Analysis of transglutaminase substrate preference
３．学会等名 The 3rd Aachen Protein Engineering Symposium (AcES)　European Federation of Biotechnology（国際学会）
４．発表年 2021年

１．発表者名 Munaweera T.I.K., Damnjanovic Jasmina, Nezu Moeri, Camagna Maurizio, Kojima Takaaki, Hitomi Kiyotaka, Nakano Hideo
２．発表標題 Comprehensive analysis of transglutaminase 1 substrate preferences using cDNA display
３．学会等名 第1回天野エンザイム酵素研究助成報告会
４．発表年 2022年

〔図書〕　計0件

〔産業財産権〕

〔その他〕

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６．研究組織

	氏名 （ローマ字氏名） （研究者番号）	所属研究機関・部局・職 （機関番号）	備考

７．科研費を使用して開催した国際研究集会

〔国際研究集会〕　計0件

８．本研究に関連して実施した国際共同研究の実施状況

共同研究相手国	相手方研究機関