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Short communication

Conversion of a *Serratia* sp. phospholipase into a true esterase by new genetic diversification method

Jae Kwang Song [a], Bora Chung [b,1], Joon Shick Rhee [b,*]

[a] *Applied and Engineering Chemistry Division, Korea Research Institute of Chemical Technology, P.O. Box 107, Yuseong-gu, Daejeon 305-600, South Korea*
[b] *Department of Biological Sciences, Korea Advanced Institute of Science and Technology, 373-1 Guseong-dong, Yuseong-gu, Daejeon 305-701, South Korea*

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Random point mutation and recombination of DNA represent the current approach to the generation of genetic diversity for directed protein evolution. Most notably, various methodologies that generate homologous and/or non-homologous crossovers, such as family DNA shuffling and sequence homology-independent protein recombination (SHIPREC), are generally used as strategies to obtain enzymes with new or improved properties [1–4]. Compared with in vitro evolution process, natural mutations have arisen, however, from more various recombination including deletions, insertions, duplications or fusions [5]. So far, such a typical process employed by natural evolutionary mechanisms had not been practically used to alter enzymatic properties in the field of directed evolution. Recently, some methods based on insertion and/or deletion were developed to add more diverse searchable sequence space to the current repertoire of in vitro evolution process [6–8]. For example, the thermostability of catalase I and the fluorescence property of green fluorescent protein were diversified by random elongation mutagenesis [7] and random insertion/deletion mutagenesis [8], respectively.

As described in our previous report [9], we also developed the mutagenic and unidirectional reassembly (MURA) method for the creation of DNA shuffled and truncated enzyme libraries. This method involves reassembling small-length fragments of template genes with a unidirectional primer containing an appropriate restriction site, and then cloning the genetic variants both truncated randomly in a desired direction and recombined in vitro. The MURA method enabled us to increase the dimension of the sequence space, thus changing from a substrate specificity of phospholipase (GenBank accession no. U37262) to that of esterase including lipase. The NPL variants with newly obtained esterase activity were isolated by screening the MURA library-harboring *E. coli* cells on tributyrin-emulsified agar plates, and they were one of the phospholipase A_1 (PlaA from *Serratia* sp. MK1 KCTC 2865) variants from which the N-terminal region had been deleted (Fig. 1). For example, NPL variants of PlaA, into which both random point mutations and unidirectional random truncation have been introduced simultaneously as shown in sequences of NPL-0602, can rarely be generated by error-prone PCR and/or various DNA shuffling methods (Fig. 2).

Previously, we examined the catalytic activities of the wild-type PlaA and the NPL variants with substrate solution of tributyrin (C4:0). It had been found that the NPL variants showed a significant level of

* Corresponding author. Tel.: +82-42-869-2613; fax: +82-42-869-2610.
E-mail address: jsrhee@mail.kaist.ac.kr (J.S. Rhee).
[1] Present address: AmorePacific Corporation R&D Center, Yongin-si, Gyeonggi-do 449-729, South Korea.

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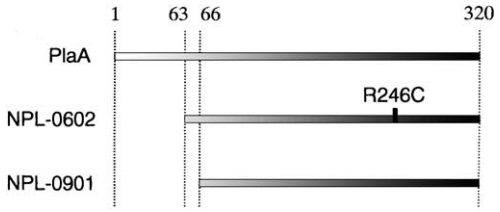

Fig. 1. The wild-type PlaA and its NPL variants described in this report. The PlaA of *Serratia* sp. strain MK1 encodes 320-amino acid monomer. NPL-0602 and NPL-0901 are N-terminal-truncated and shuffled variants of PlaA. R246C on the bars representing NPL-0602 indicates the amino acid substitution that had been introduced during shuffled and unidirectional reassembly PCR.

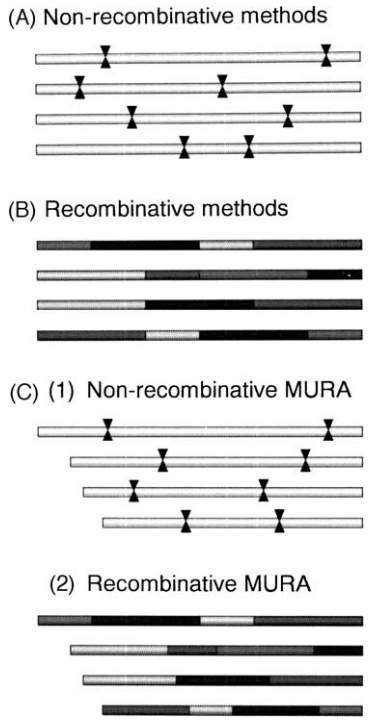

Fig. 2. Schematic comparison between MURA methods and other non-recombinative or recombinative methods. (A) Random point mutations are introduced into a single polypeptide backbone of interest by error-prone PCR, site saturation mutagenesis, etc. (B) In vitro exchanges of DNA sequences between two or more genes are achieved by various recombinative methods including family DNA shuffling. (C) Random truncated variants of an enzyme are obtained by unidirectional reassembly of its own small-length fragments, at the same time possessing new point mutations (1). It is possible that, if two or more closely related genes are employed, chimeric variants truncated incrementally from any direction are obtained by using pools of each appropriate MURA primer or by applying degenerate MURA primers (2).

esterase activity towards tributyrin (C4:0), whereas the wild-type PlaA exhibited no activity under the same assay conditions. To evaluate the catalytic properties of NPL enzymes in detail, we examined the chain length specificity and the interfacial catalytic property of NPL enzymes.

The wild-type PlaA and the NPL variants, NPL-0602 and NPL-0901, were fused with six consecutive histidine affinity tag, and then purified from crude cell extract as described in our previous report [10]. Their activities were measured with the triglyceride-emulsified substrate solutions, such as tributyrin (C4:0), tricaproin (C6:0), tricaprylin (C8:0) and triolein (C18:1), and quantified according to the NaOH consumption recorded [10,11]. The wild-type PlaA showed only a negligible amount of catalytic activity towards triglycerides, whereas the NPL enzymes exhibited relatively high activity towards triglycerides, especially showing a preference for the short-chain length (Fig. 3). Meanwhile, lipases usually exhibit a phenomenon termed interfacial activation (i.e. a profound increase in the rate of hydrolysis of substrate when substrate concentration exceeds the critical micelle concentration). Esterases, however, prefer water-soluble substrates, thus showing no interfacial activation. Interfacial activation of the NPL enzyme (NPL-0901) was examined by measuring its specific activity as a function of the concentration

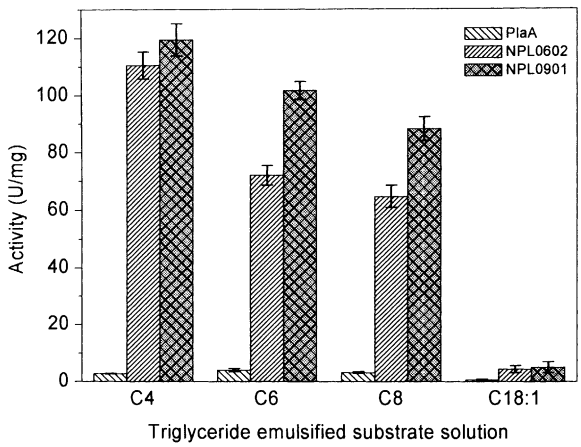

Fig. 3. Substrate specificity of PlaA, NPL-0602 and NPL-0901 towards triglycerides with different chain lengths. Activities were measured with the pH-titration method, and all values whose error ranges were within 5% were averaged from at least triplicate assays.

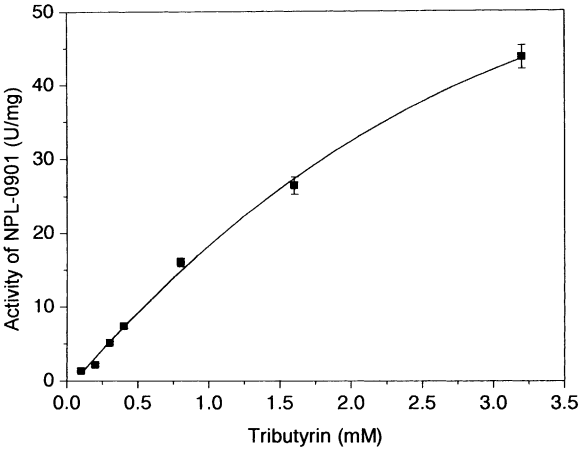

Fig. 4. Catalytic activity of NPL enzyme on various concentrations of tributyrin substrate. The concentration of tributyrin-emulsified substrate solution was above and below critical micelle concentration.

of tributyrin. Assays were carried out at the ionic strength of 0.15 M NaCl [12]: at this point the solubility limit of tributyrin was 0.41 mM. A significant activity was shown below the concentration at which isotropic micelle solutions of substrates are formed (Fig. 4). Moreover, the NPL enzyme as a whole exhibited Michaelis–Menten kinetics with no interfacial activation. Judging from the fact that the NPL variants with newly obtained triglyceride-hydrolyzing activity prefer short-chain fatty acid esters and obey the Michaelis–Menten kinetics, the NPL variants are, therefore, considered to have a typical esterase activity and not a lipase activity.

In this report, we addressed the enzymes with esterase activity that had evolved through new genetic diversity-generation method. First, we believe that new mutant enzymes are valuable to a research field concerned with the biotechnological applications and the structural relationships of lipolytic enzymes. More importantly, it is anticipated that a more efficient approach to access a more diverse sequence space for directed protein evolution will be developed by improving more closely nature-mimicked methods, including this MURA method developed in our laboratory.

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