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A novel helper phage for HaloTag-mediated co-display of enzyme and substrate on phage

Wouter Delespaul [a], Yves Peeters [a], Piet Herdewijn [b], Johan Robben [a, *]

[a] Department of Chemistry, KU Leuven, Celestijnenlaan 200G, B-3001 Heverlee, Belgium
[b] Rega Institute for Medicinal Research, KU Leuven, Minderbroedersstraat 10, B-3000 Leuven, Belgium

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ABSTRACT

Phage display is an established technique for the molecular evolution of peptides and proteins. For the selection of enzymes based on catalytic activity however, simultaneous coupling of an enzyme and its substrate to the phage surface is required. To facilitate this process of co-display, we developed a new helper phage displaying HaloTag, a modified haloalkane dehalogenase that binds specifically and covalently to functionalized haloalkane ligands. The display of functional HaloTag was demonstrated by capture on streptavidin-coated magnetic beads, after coupling a biotinylated haloalkane ligand, or after on-phage extension of a DNA oligonucleotide primer with a biotinylated nucleotide by phi29 DNA polymerase. We also achieved co-display of HaloTag and phi29 DNA polymerase, thereby opening perspectives for the molecular evolution of this enzyme (and others) towards new substrate specificities.

1. Introduction

Molecular evolution is a powerful technique to improve the properties of enzymes by screening or selecting from a library of variants. Phage display, although originally developed for the selection of short peptide binders [1] and antibodies [2,3], has also shown its potential in enzyme evolution by the development of, e.g., catalytic antibodies [4] and DNA polymerases with altered specificities [5]. However, selection for catalytic activity is not so straightforward. Researchers have achieved varying success by using transition state analogs or suicide inhibitors as a ligand [6,7], but this is always an approximation to the enzymatic reaction one is selecting for. Ideally, to select for catalytic turnover, phage displaying active enzymes should be directly selected on product formation. This can be realized if displayed enzymes tag the phage itself by converting a co-displayed substrate into product. Affinity purification based on product binding can then be used to recover active variants.

Several approaches have been described to link a substrate to a phage particle, either before or after conversion to product. If the substrate is a peptide, it can simply be produced as a translational fusion to one of the phage coat proteins [8,9]. A more general solution is proximity coupling, proposed by Winter and co-workers [10]. They used a maleimide-linked DNA primer that would cross-link to the phage main coat protein while being extended with a biotinylated nucleotide by a displayed polymerase. The primer, however, randomly cross-linked with the long filamentous phage particle, may be beyond reach of the co-displayed polymerase, hence favoring cross-reactivity and loss of the genotype–phenotype association. It would therefore be preferable to have both enzyme and substrate located in close proximity at the same end of the phage particle.

Schultz and co-workers achieved co-display of staphylococcal nuclease next to a DNA oligonucleotide by coupling it to a synthetic peptide capable of forming an artificially designed coiled coil [11]. One half of this coil is an acidic peptide encoded as an N-terminal fusion with phage coat protein g3p in the helper phage genome; the other half is a basic peptide, chemically synthesized and conjugated to the substrate. Both peptides associate in a leucine-zipper-like fashion and form a disulfide bond tying them together covalently. However, the synthesis of the basic peptide is challenging, considering its length (46 amino acids) and the requirement of a non-standard chemical modification. In addition, the conjugation to the substrate and coupling to phage is a complicated procedure. A similar approach is the display of enzyme-calmodulin fusion proteins to which substrate-conjugated calmodulin-binding peptide can be attached [12]. The main difference here is the

* Corresponding author.
E-mail addresses: wouter.delespaul@chem.kuleuven.be (W. Delespaul), yves.peeters@chem.kuleuven.be (Y. Peeters), piet.herdewijn@rega.kuleuven.be (P. Herdewijn), johan.robben@chem.kuleuven.be (J. Robben).

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display of enzyme and substrate-coupling protein as a double fusion to a phagemid-encoded g3p, instead of encoding the substrate-coupling domain in the helper phage. Walker and co-workers developed yet another method for the evolution of glycosyltransferases, in which a selenocysteine residue is incorporated at the g3p N-terminus, exploiting its higher nucleophilicity and reactivity at lower pH in comparison with cysteine. Phage could be selectively derivatized with substrates functionalized with an α-iodoacetamide group [13]. A last example of enzyme and substrate co-display was described by Sunbul et al. [14]. They fused helper phage g3p to an 11-residue peptide, ybbR, which can be modified with small molecules conjugated to coenzyme A by Sfp, a phosphopantetheinyl transferase.

In an attempt to simplify the process of displaying both an enzyme and its substrate on phage and make it more generically applicable, we present an alternative approach using a new helper phage displaying HaloTag, an engineered haloalkane dehalogenase (Fig. 1). Natural dehalogenases convert halogenated hydrocarbons into their corresponding alcohols by a nucleophilic displacement mechanism. In HaloTag, an essential histidine residue is mutated preventing the base-catalyzed hydrolysis of the alkyl-enzyme intermediate, resulting in a stable covalent adduct [15]. In this way, HaloTag can be linked to a plethora of useful molecules like fluorescent dyes, solid surfaces or small molecules. The protein tag was originally developed for cell imaging and isolation of protein complexes, but can in principle be used in any application, in vivo or in vitro, where a certain molecule needs to be linked to a protein. The reaction of HaloTag with its ligands is highly specific, fast and essentially irreversible under physiological conditions. Additionally, a number of commercially available ligand 'building blocks' facilitate the coupling of a large variety of molecules.

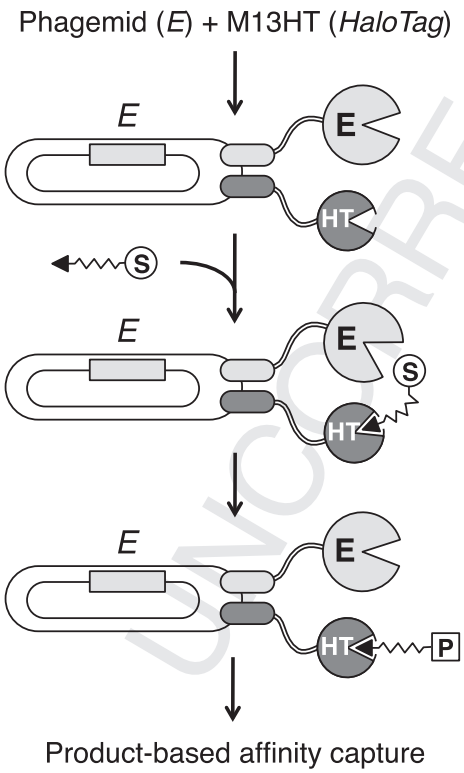

Fig. 1. Enzyme selection by HaloTag-mediated substrate co-display. Phagemid particles co-displaying HaloTag (HT) and enzyme (E) are produced by infecting phagemid-containing cells (encoding enzyme *E*) with M13HT helper phage (encoding HaloTag). The substrate (S) is covalently attached to HaloTag by means of the conjugated haloalkane ligand (represented by a black arrow). After conversion into product (P) by the enzyme, product-based affinity capture is used to capture phage displaying active enzymes.

As an example, we demonstrate the covalent coupling of a DNA oligonucleotide to phage particles through the HaloTag fusion. We show that the attached oligonucleotide, annealed with a template molecule, can be extended by phi29 DNA polymerase [16], a mesophilic enzyme that is used in numerous biotechnological applications [17–19]. Also, the simultaneous display of functional HaloTag and phi29 DNA polymerase is achieved, indicating the potential of this phage display system for the molecular evolution of this polymerase towards, e.g., enhanced incorporation of non-natural nucleotides.

2. Materials and methods

2.1. Construction of M13HT helper phage

The helper phage displaying HaloTag protein was derived from M13KO7 (New England Biolabs). First, we engineered an EagI restriction site just after the secretion signal in the g3p coding sequence using QuickChange mutagenesis (Agilent Technologies) with primers KO7-EagI-F (CAACAGTTTCGGCCGAGTGAGAATAGAAAGG) and KO7-EagI-R (CCTTTCTATTCTCACTCGGCCGAAACTGTTG), and M13KO7 replicative form (RF) DNA as a template. The product was transformed into *Escherichia coli* DH5α and positive clones were identified by PCR screening and digestion of the PCR product with EagI (Thermo Scientific). Plasmid DNA was prepared and sequence-verified to yield M13KO7-EagI.

The HaloTag-coding sequence was isolated from plasmid pH6HTN (Promega) by PCR with primers HT-F (TTTCTATTCTCACTCGGCGGCCGCAGAAATCGGTACTGGCTTTCC) and HT-R (AACAGTTTCTGCGGCCGCGTTATCGCTCTGAAAGTACAGATCC), thereby adding flanking NotI restriction sites to generate EagI-compatible overhangs. The PCR product was digested with NotI (Thermo Scientific), purified and ligated with M13KO7-EagI RF DNA digested and dephosphorylated with EagI and FastAP (Thermo Scientific). After transformation in DH5α and PCR screening, RF DNA was isolated and fully sequenced. RF DNA was then transformed into XL1-Blue MRF' (Agilent Technologies) yielding plaques from which phage, termed M13HT, was prepared using standard protocols. The M13HT genome sequence was deposited into Genbank under accession number KM505149.

2.2. Determination of functional HaloTag display

To 400 μl of TBS buffer (25 mM Tris–HCl pH 7.4, 140 mM NaCl, 2.5 mM KCl), 100 μl of M13HT phage suspension (10^{10}–10^{11} cfu/ml) and 1 μl of a 100-μM solution of HTBL (HaloTag® PEG-Biotin Ligand, Promega) were added, and the mixture was incubated for 1 h at room temperature. The phage were precipitated with polyethylene glycol (PEG) to remove unbound HTBL, and resuspended in 400 μl of TBS. Next, 50 μl of streptavidin-coated paramagnetic beads (Dynabeads® MyOne Streptavidin T1, Life Technologies) were washed three times in two volumes of TBST (TBS + 0.5% Tween-20), and added to the phage suspension. The mixture was incubated at room temperature for 15 min with gentle rotation to keep the beads suspended, followed by 5 washes with 200 μl of TBST. Bound helper phages were eluted by resuspending the beads in 500 μl of TEV protease reaction mix (AcTEV Protease, Life Technologies) and incubating at room temperature for 1 h with gentle rotation to cleave off bound phage. Helper phage concentrations were determined by spot titration.

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