Biochemical Engineering Journal 48 (2009) 118–123

Contents lists available at ScienceDirect

Biochemical Engineering Journal

journal homepage: www.elsevier.com/locate/bej

Increasing the substrate specificity of *Bacillus stearothermophillus* lactate dehydrogenase by DNA shuffling

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ARTICLE INFO

Article history:
Received 22 January 2009
Received in revised form 29 July 2009
Accepted 29 August 2009

Keywords:
Molecular modelling
NAD^+-dependent lactate dehydrogenase enzyme
Protein engineering
Recombinant protein
Steady-state kinetic
Substrate specificity

ABSTRACT

Previously it is suggested that a single mutation (Q102R) in the lactate dehydrogenase (LDH) gene from *Bacillus stearothermophillus* (*bs*) could switch the substrate specificity by 3 orders of magnitude from lactate to malate and produce a highly efficient malate dehydrogenase (MDH). In order to examine if random mutation and screening could improve this, a DNA-shuffling method would be used to generate a mutant LDH and recombinant LDH genes which will be later used for transforming *Escherichia coli*. The recombinant colonies are blotted and screened for their ability to catalyse the oxidation of malate. The most active MDH produced by this method is only slightly more efficient than the rationally designed Q102R variant. In addition to this mutation, the shuffled version incorporates further seven residue changes which are chemically conservative. These experiments demonstrate that the blind shuffling can achieve a huge shift in specificity which was a known, highly effective single-site mutation designed using structural knowledge.

1. Introduction

The L-lactate dehydrogenase (LDH) from *Bacillus stearothermophillus* (*bs*) is commercially significant owing to its use in the production of chiral building blocks for the synthesis of key pharmaceuticals and agrochemicals. Although *bs*LDH allows the synthesis of chiral hydroxy acids from their corresponding oxo-acids with high stereochemical fidelity, it has the disadvantage of having narrow substrate specificity. Hence, like many LDHs from thermophilic bacteria, it is extremely stable to heat, but limited in its substrate range; it is therefore a viable target for engineering specificity.

Previous studies of this enzyme have yielded useful information on both the switching of specificity and the broadening of substrate range using rational and randomized mutagenic methods. For instance it has been shown [1] that a complete switch of substrate specificity from the lactate–pyruvate pair to the oxaloacetate–malate pair could be achieved simply by a Q102R replacement which is found in the naturally occurring malate dehydrogenases. More recently [2] the substrate specificity of *bs*LDH has been successfully altered by a semi-random approach.

Despite these successes, large and desirable alterations of the properties of a given enzyme framework usually depend on an extensive knowledge of the mechanisms underlying molecular recognition, a multitude of crystal structures for a given enzyme framework and computer modelling analysis. Therefore, the rational design of enzymes with new properties is time consuming and only a limited number of variants can ever be evaluated for function and sometimes the results are not predictable.

More recently, Allen and Holbrook [3] used DNA shuffling to produce a novel *bs*LDH that no longer requires the activator fructose-1,6-bisphosphate (FBP), thereby simplifying the enzymatic system by locking it in the activated state, a desirable property for an enzyme being used in industrial processes. An essential prerequisite of the above screening assay is that the *bs*LDH activity detected with a specific hydroxyl acid substrate must not be affected by the proteins, intracellular substrates and reduced coenzymes of the *Escherichia coli* host. In the screen initially designed by El Hawrani et al. [2] host proteins were inactivated on the nitrocellulose filters using a heat step. We wish to combine this property of thermostability with either broad substrate specificity or selectivity towards substrates of commercial relevance. In order to alter specificity we have applied the DNA-shuffling approach.

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doi:10.1016/j.bej.2009.08.015

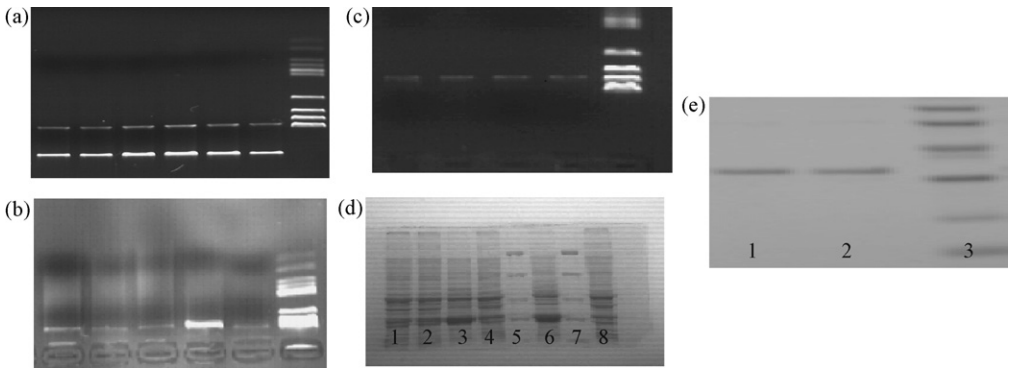

Fig. 1. DNA and protein gels in the production of shuffled-*bs*LDH. Gel (a): the central 6 lanes show the *bs*LDH gene and cut plasmid, the outer lanes in all agarose gels are size markers (fragment sizes: 72, 118, 194, 234, 271, 281, 310, 603, 872, 1078 and 1358 bp). Gel (b): the products of random cleavage of the *bs*LDH gene with a non-specific endo-DNAse I, the right most lane contains markers. Gel (c): the combinatorial library of shuffled-*bs*LDH gene fragments after reassembly, the rightmost lane contains markers. Gel (d): an example protein expression SDS-PAGE gel of the shuffled-*bs*LDH protein in JM105 *E. coli* (1–4, 6 and 8), markers (5 and 7), (fragment sizes: 16, 35, 50, 71 and 105 kDa) Gel (e): the protein purity after chromatography (1, shuffled-*bs*LDH; 2, native *bs*LDH and 3, markers (fragment size: 2, 16, 35, 50, 71 and 105 kDa, from bottom to top)).

These results are also validated with Q102, single-site mutation designed using structural knowledge [1]. After screening for activity, a novel synthetic shuffled mutant was created (shuffled-*bs*LDH) that showed 10^3-fold improvement in catalytic efficiency with the malate–oxaloacetate system. The protein has eight amino acid changes. The steady-state kinetic properties of shuffled-*bs*LDH are compared with native *bs*LDH and with the rationally defined and highly efficient Q102R-*bs*LDH.

2. Materials and methods

2.1. DNA shuffling to construct shuffled-bsLDH

Bacterial strain *E. coli* JM105 {F′ traD36 proA$^+$ proB$^+$ laclq lacZΔM15/Δ (lac-pro) X111 thi rpsL (Strr) endA sbcB supE hsdR9} was used as a host to prepare all double-stranded DNA for mutagenesis and sequencing in the plasmid pKK223-3 (Pharmacia Biotech, Uppsala, Sweden). The plasmid pKK223-3 is a transcription expression vector and contains a TAC promoter (Isopropyl β-D-1-thiogalactopyranoside, (IPTG) inducible) which drives expression of recombinant genes and ribosomal termination sequences as well as ampicilin resistance [4]. The same *E. coli* strain was also used as a host for transformation and expression of *bs*LDH proteins in pKK223-3.

The shuffled-*bs*LDH gene was constructed by the method used by Stemmer [5] with some modifications as explained below. When the method was applied to LDH it first involved isolating the coding DNA (an about 1 kb fragment) with a 5′ region containing an EcoRI site and a 3′ region with a PstI site. The product is of the correct size as shown in Fig. 1(a). About 10 μg plasmid containing wild type LDH gene was digested at 37 °C for 1 h with EcoRI and PstI in 20 μl 10× digestion buffers. After purification from 0.8% agarose gel, 2 μg of the plasmid DNA of *bs*LDH was then randomly cleaved with a non-specific endo-DNAse I incubating the reaction mixture including 10 mM Tris–HCl (pH 7.4), 10 mM of $MnCl_2$ and 0.0015 U DNase I for 15 min at 20 °C. This should give a smear of randomly sized oligonucleotides smaller than 1 kb. These are visible in Fig. 1(b) together with a small amount of undigested gene.

Fragments with sizes 100–300 bp were purified from the corresponding region of a 2.5% agarose gel. These fragments were then amplified by PCR (94 °C for 30 s, 50 °C for 30 s and 72 °C for 30 s) but without adding primers. This critical step generates sequence variation in the fragments generated in step 1. As expected the products run on a gel as a characterless smear. The full-length gene is finally isolated by the second PCR (initial denaturation 96 °C for 2 min, 94 °C for 30 s, 55 °C for 30 s, 72 °C for 45 s, for 10 cycles and followed by another 14 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 45 s and final extension 72 °C for 12 min) step using the correct primers for the 5′ EcoRI (N-terminus) and 3′ PstI (C-terminus) sites and 4 μl of the first PCR product. The gel shown in Fig. 1(c) shows a high yield of a randomly, shuffled library of about 1 kb genes. All PCRs were carried out in the presence of 0.2 mM of each dNTPs, 1× *Taq* buffer, and 2.5 U *Taq/Pfu* (1:1) enzyme mixtures [6]. The about 1 kb product was digested with EcoRI and PstI prior to cloning into similarly cut pKK223-3 DNA and the ligation products were used to transform *E. coli* JM105 competent cells.

2.2. Screening for variants with substrate-specificity changes

The screen was initially designed by A.S. El Hawrani for use with a thermophilic protein. Having a thermophilic enzyme expressed in a mesophilic bacterial strain (JM105 *E. coli*) allows inactivation of host proteins on the nitrocellulose filters using a heat step [2]. The *bs*LDH library is blotted onto nitrocellulose filters and subjected to a number of steps. Lysis of *E. coli* cells on nitrocellulose filters was carried out and nitrocellulose filters were incubated at 65 °C [2].

The filters were incubated in the dark and any *E. coli* colonies expressing *bs*LDH protein with activity towards the hydroxy acid target substrate were detected by the appearance of a purple coloration around the edges of the bacterial colonies. Double-stranded DNA of shuffled LDH having activity with oxaloacetate was sequenced to confirm the changes on the gene. Protein over-expression is conveniently established by the appearance of an intense band on an SDS polyacrylamide gel stained with Coomassie Blue dye.

2.3. Construction of Q102R-bsLDH

Bacterial strains: Two different bacterial strains are used in this work namely, DH5α^{TM}-T1^R competent cells ([F$^-$ ϕ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 *ton*A]) which is supplied with Invitrogen^{TM} GeneTailor ^{TM} Site-Directed Mutagenesis System and JM105 as a host for transformation and expression of Q102R mutant of *bs*LDH protein in pKK223-3.

The mutation was introduced into the *bs*LDH gene at required positions by site-directed mutagenesis using Invitrogen Gene Tailor^{TM} Site-Directed Mutagenesis System (Invitrogen) according to the manufacturer's protocol. The following oligonucleotides (forward 5′GGTTGTCATTTGCGCCGGCGCC**CGC**CAAAAACCGG3′ and