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In vitro biosynthesis of 3-mercaptolactate by lactate dehydrogenases

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ABSTRACT

3-Mercaptolactate (3ML) is an interesting mercapto compound with special regard to the biosynthesis of new polythioesters (PTEs). Unfortunately, this thioester analog of lactic acid is currently not commercially available. For this reason, we developed an *in vitro* biosynthesis pathway to convert cysteine to 3-mercaptopyruvate (3MPy), which is then rapidly and efficiently converted to 3ML by suitable lactate dehydrogenases (LDHs). As liver LDH from *Rattus norvegicus* (LDH_{Rn}) was previously described to Exhibit 3MPy reduction activity, *in silico* studies based on homology to LDH_{Rn} were performed and led to the identification of four potentially suitable bacterial LDH candidates from *Escherichia coli* (LDH_{Ec}), *Corynebacterium glutamicum* (LDH_{Cg}), *Bacillus cereus* (LDH_{Bc}) and *Gloeobacter violaceus* (LDH_{Gv}). After heterologous expression in *E. coli* followed by purification, the enzymes were assessed for their potential to reduce 3MPy to 3ML in comparison to LDH_{Rn}. With 3MPy, LDHs from *E. coli, C. glutamicum* and *B. cereus* showed no or only very low specific activities of 0.23 ± 0.1 U/mg (LDH_{Cg}) and 0.08 ± 0.2 U/mg (LDH_{Bc}), respectively. In contrast, LDH_{Gv} exhibited a remarkable specific activity of 63.6 ± 8.1 U/mg, being even twice as active as the *R. norvegicus* LDH. To verify LDH-catalyzed biosynthesis of 3ML we developed and optimized a detection method allowing qualitative analysis and quantification of 3MPy and 3ML by derivatization with Ellman's reagent and liquid chromatography-mass spectrometry. This study shows once more the impressive versatility of LDHs and presents a rapid and efficient biosynthesis process for 3ML, a biotechnologically interesting, yet hard-to-obtain, compound.

1. Introduction

3-Mercaptolactate (3ML; IUPAC: (2*R*)-2-hydroxy-3-sulfanylpropionate) is an organic sulfur compound and the sulfur-containing structural analog of lactate. In eukaryotes, 3ML naturally occurs when cysteine is degraded via the transamination pathway by cysteine aminotransferase forming 3-mercaptopyruvate (3MPy), which is then further metabolized by 3MPy sulfurtransferase (3MST) or reduced to 3ML by lactate dehydrogenases (LDH) [1–3]. As a result of the rare inheritable disorder mercaptolactate-cysteine disulfiduria caused by MPST deficiency, high concentrations of mercaptolactate-cysteine disulfide are found in the urine [4–6]. In prokaryotes, the cysteine transamination pathway has never been investigated in detail, but it was recently shown that bacterial aminotransferases from several organisms also catalyze transamination of cysteine [7]. While a biotechnical synthesis of 3ML has never been described, there are several reports on the chemical synthesis of 3ML with rather unsatisfactory results [8,9]. Therefore, 3ML is not commercially available and the chemical synthesis on request is paralleled by high costs.

LDHs are ubiquitous in nature and catalyze the interconversion of pyruvate and lactate. There are two distinct enzyme classes within the LDHs each comprising two subclasses: NAD dependent (nLDH) and flavin-containing, NAD-independent (iLDH), each class including members being specific for either L- or D-lactate, respectively (L-nLDH: EC 1.1.1.27, D-nLDH: EC 1.1.1.28 and L-iLDH: EC 1.1.2.3; D-iLDH: EC 1.1.2.4) [10]. All known nLDHs are cytosolic enzymes, while iLDHs are frequently associated with membranes and use cytochrome as an electron acceptor [11]. The LDHs specific for L-isomers share highly homologous sequences, which can also be observed among the subclass of D-specific LDHs. However, there are barely homologies between L- and D-LDHs [12].

nLDHs catalyze the reduction of pyruvate to lactate using the cofactor NADH or *vice versa* the oxidation of lactate to pyruvate with the concomitant reduction of NAD^+. Together with alcohol dehydrogenase

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and NAD(P)H-linked L-malate dehydrogenase, nLDHs are members of the large family of dehydrogenases [13]. As alternative substrates to pyruvate and lactate, LDHs can also convert α-hydroxybutyrate, α-ketobutyrate and also 3MPy [14]. In mammals, LDH is a cytoplasmic protein and present in almost all tissues. A high amount of the enzyme is found in heart, liver, muscle and kidney tissue. Functional LDH enzymes consist of four subunits encoded by two genes (LDH-A and LDH-B), which are assembled in homo- or heterotetramers. While humans and other higher animals as well as plants possess only L-LDH, invertebrates, lower fungi and prokaryotes additionally possess D-LDH [11,12].

Bacterial LDHs are active as dimers or tetramers, and consequently, their molecular weight ranges between 70–140 kDa [10]. In bacteria, LDH is the key enzyme during lactic acid fermentation, the anaerobic version of converting sugars into energy. By reducing pyruvate to lactate, the organism can recycle oxidized NAD^+ for glycolysis. Bacteria that form large amounts of lactate by fermentation are referred to as lactic acid bacteria [15]. Due to the increasing knowledge about metabolic flux, LDH kinetics and optimal cultivation conditions, the biotechnical production of lactic acid has reached high yields of up to 98 % [16].

A simple and cost-effective method for the synthesis of 3ML would not only be of academic interest. The close structural analog 3MP is currently used as one of the few well-established precursor substrates for biosynthesis of polythioesters (PTEs) [17,18] and 3ML as a potential alternative PTE precursor could lead to the synthesis of the new and promising biologically persistent biopolymer poly(3ML) (PML). Furthermore, PML represents the sulfur-containing structural analog to polylactic acid (PLA), which is already widely used to substitute petroleum-derived plastics [19]. Non-biodegradable biopolymers like PTEs combine two important benefits: a sustainable bio-based production as well as potential applications requiring persistent materials [20].

This study focused on finding suitable LDHs to develop a simple and affordable method for the biosynthesis of 3ML from 3MPy. In combination with the previously established synthesis of 3MPy from cysteine [7], this enables biosynthesis of the PML precursor 3ML from the cheap substrate cysteine (Fig. 1). Thus, we present a rapid and efficient biosynthesis process for 3ML, a biotechnologically interesting, yet hard-to-obtain, compound.

2. Material & methods

2.1. Materials

Chemicals were purchased from Merck (Merck KGaA, Darmstadt, Germany), Roth (Carl Roth GmbH & Co. KG, Karlsruhe, Germany), or Sigma-Aldrich (Sigma-Aldrich Co., St. Louis, MO, USA) and enzymes or kits from Thermo Fisher Scientific (Waltham, MA, USA) or VWR (International GmbH, Darmstadt, Germany). Oligonucleotide synthesis and sequencing was performed by Eurofins Genomics (GmbH, Ebersberg, Germany).

2.2. Identification of bacterial LDHs with activity on 3MPy

In order to identify interesting LDH candidates, the amino acid sequence of L-LDH from *Rattus norvegicus* (UniProt No.: P04642) was used as query searching the Swissprot database of the National Center for Biotechnology Information (NCBI; http://blast.ncbi.nlm. nih.gov/Blast.cgi). Selected amino acid sequences (Table 1) were then aligned employing ClustalX algorithm [21] embedded in the Bioedit program [22]. The phylogenetic tree was then constructed with the neighbor joining method using NJplot [23].

2.3. Bacterial strains and cultivation conditions

All bacterial strains used in the present study are described in Table 2. The donor strains for *ldh* were cultivated at 30 °C using lysogeny broth (LB) medium [24]. *E. coli* Top10 strains were cultivated in LB medium at 30 °C under aerobic conditions on a rotary shaker at 120 rpm. Media for plasmid-harboring strains were supplemented with 100 μg/ml ampicillin. Cultures were harvested by centrifugation (10 min, 3500 × *g*, 4 °C), and cells from ampicillin-containing cultures were additionally washed with 0.9% (w/v) sodium chloride before the pellets were stored at −20 °C.

2.4. Expression of recombinant lactate dehydrogenases

The selected genes encoding bacterial LDHs were PCR-amplified from genomic DNA of *B. cereus*, *C. glutamicum*, *E. coli* and *G. violaceus* using Phusion High-Fidelity polymerase (Thermo Fisher Scientific) and oligonucleotides listed in Table 2. The approximately 1000 kb PCR products were subcloned into pJET1.2/blunt (Thermo Fisher Scientific), verified by sequencing and excised with *Nde*I and *Xho*I to ligate the purified insert fragments with *Nde*I-*Xho*I-digested pET19b expression vectors. The resulting pET19b:ldh_x plasmids encode the respective LDH with N-terminal His_{10}-tag to facilitate purification. In contrast, *ldh* of *G. violaceus* was directly introduced into the pET19b vector via the Gibson Assembly method [25]. For this purpose, the pET19b vector was restricted with *Nde*I and *Xho*I and incubated with the amplified *ldh* of *G. violaceus* (containing overlapping sequences to pET19b) at 50 °C for 20 min. The mixture containing the recombinant vector was directly used for transformation of *E. coli* Top10.

The eukaryotic LDH encoding gene from *R. norvegicus, ldha,* contains exons. Therefore, the amino acid sequence of LDH_{Rn} (UniProt No. P04642) was used as the template for *in silico* reverse translation. The sequence was codon optimized for expression in *E. coli* and a stop codon as well as restriction sites (*Nde*I & *Xho*I) were added. This artificial and optimized gene sequence was then synthesized by Gene Art (Thermo Fisher Scientific). The supplied pMA-T vector was treated with *Nde*I and *Xho*I, and the purified insert fragment was ligated with the *Nde*I-*Xho*I-digested pET19b expression vector.

For expression of LDHs from *B. cereus*, *C. glutamicum*, and *E. coli*, the expression host *E. coli* C43 (DE3) harboring the respective expression vector (Table 2) was cultivated in LB medium supplemented with 100 μg/ml ampicillin. The main cultures, 1 l baffled flasks containing 250 ml LB, were inoculated with 1 % (v/v) of the final volume of an overnight grown pre-culture and cultivated at 30 °C until reaching an optical density at 600 nm (OD_{600}) of 0.6. They were then induced with 1 mM IPTG and further cultivated for 20 h at 20 °C. For heterologous expression of LDH from *G. violaceus* and *R. norvegicus* the expression host *E. coli* BL21 (DE3) was chosen, and cultivation was performed for 20 h at 20 °C in ZYP-5052 autoinduction medium [26].

2.5. Purification of recombinant LDHs

To purify recombinant LDHs, *E. coli* cells were resuspended in 4 ml

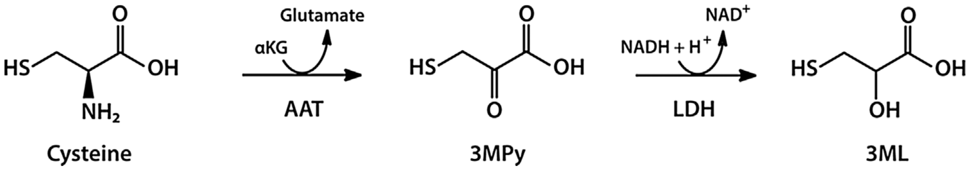

Fig. 1. Schematic reaction of cysteine transamination by AAT to 3MPy, which then serves as substrate for LDH. Abbreviations: αKG, α-ketoglutarate, AAT, aspartate aminotransferase; LDH, lactate dehydrogenase; 3ML, 3-mercaptolactate; 3MPy, 3-mercaptopyuruvate; NADH, nicotinamide adenine dinucleotide.