Scientific Research

Characterization of a Novel *Pseudomonas stutzeri* Lipase/Esterase with Potential Application in the Production of Chiral Secondary Alcohols

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Abstract

The search for new enzymes is facilitated by the rapidly growing number of genome sequences from different organisms. However, the discovery of functional proteins is still time intensive and complex alignments have to be performed. Herein, a genome database search identified a new, until now undescribed, putative lipase from *Pseudomonas stutzeri* strain A1501. The gene was cloned and expressed as functional protein in *E. coli*. A biochemical characterization provided an indication that the enzyme could be classified as an esterase with an alkaline pH optimum and a temperature optimum at 50°C. The enzyme was able to perform the kinetic resolution of racemic esters and could therefore be an interesting candidate for chiral synthesis.

Keywords

1. Introduction

Lipolytic enzymes are of great interest for biotechnological processes. Their stereo- and regioselectivity, no co-factor dependency as well as their high stability and performance in diverse organic media make them highly interesting for chemical production.

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In principal, lipolytic enzymes can be divided into two categories, namely esterases (E.C. 3.1.1.1) and lipases (E.C. 3.1.1.3) [1]. Both possess an α/β hydrolase fold including a catalytic triad, which is typically formed by the amino acids Ser-His-Asp/Glu [2] [3]. The esterases are restricted to small ester containing compounds, whereas lipases are able to convert water-insoluble long-chain triacylglycerides. A second difference is attributed to a flexible amphiphilic loop which is only present in lipases [4] [5]. This lid covers the active site in aqueous environments (close conformation) but undergoes a conformational change in contact to a lipid water interface (open conformation) [4] [6] [7]. This process, called interfacial activation, is exclusively found in lipases and was demonstrated to play a role in their activation [4].

Nowadays, these enzymes are used in the pharmaceutical and cosmetic industry, are involved in biodiesel processing and are applied in food industry [8]-[10]. Furthermore, their high regio- and stereoselectivity makes them interesting for the synthesis of chiral compounds [11]. Especially, bacterial enzymes play an important role in these processes and are preferred over plant or animal derived enzymes. They are easily accessible and can be produced in necessary quantities in cheap media and often downstream processing is simple. Among all, *Pseudomonas* sp. are the origin of a large amount of known esterases and lipases and some representatives are already commercially available [12] [13]. Most of them show a high thermal stability and are active in alkaline media [14]. Furthermore, the large substrate scope displays the versatility of esterases and lipases derived from *Pseudomonas* species. *Pseudomonas stutzeri* is an outstanding representative with a large genotypic diversity [15] and several genomic sequences from different strains are available in public databases.

Here we describe the identification of a new, until now undescribed, lipase/esterase through a mere database screening. Within the genome from *Pseudomonas stutzeri* strain A1501 a putative lipase of unknown function was found. The codon optimized gene of this candidate was cloned in different pET vectors and the resulting proteins were recombinantly expressed in *E. coli*. All constructs were tested for soluble expression and the best candidate was characterized in terms of buffer stability as well as pH and temperature optimum. Finally, the enzyme was investigated for ester hydrolysis to generate chiral alcohols.

2. Material and Methods

2.1. Preparation of Expression Plasmids

In order to avoid deficient translation, the lipase gene was ordered from Geneart (Regensburg, Germany) as synthetic gene with optimized codon usage for expression in *E. coli*. Different vectors were chosen (pET32b+ (Trx-tag), pET39b+ (DsbA-tag), pET40b+ (DsbC-tag) and pET44b+ (NusA-tag)) to achieve tag-mediated soluble expression of the corresponding proteins. Appropriate primer pairs were used to amplify the lipase gene by standard PCR methodology using *Pfu* DNA polymerase (Promega, Fitchburg, USA) and to prolong the sequence by the restriction sites for *NcoI* or *SacI* (forward) and *XhoI* (reverse). After purification of the PCR products, double digestion of the PCR fragments as well as all vectors was performed in accordance to the manufacturer's protocol. Finally, lipasegene and vectors were ligated by T4 ligase (Thermo Fisher Scientific, Waltham, USA) at 22˚C overnight. The resulting constructs were transformed into *E. coli* DH5α and correctness of all constructs was confirmed by sequencing of the entire coding sequence (Eurofins MWG Operon, Ebersberg, Germany).

2.2. Expression and Purification of Functional Lipase/Esterase

Functional plasmids were transformed into *E. coli* BL21 (DE3) and cells were grown overnight at 37˚C in 5 mL LB medium with the appropriate antibiotic. Antibiotic concentration was 100 µg/mL for ampicillin and 50 µg/mL for kanamycin, respectively. The preparatory culture was transferred into the main culture (500 mL TB media in a 2 L Erlenmeyer flask), grown for several hours at 37˚C and protein expression was induced with IPTG (0.1 mM final concentration) at an OD_{600} of 1.5. Low temperature expression was chosen at 17˚C for 24 h and 150 rpm. The resulting proteins were named Trx-lipase, DsbA-lipase, DsbC-lipase and NusA-lipase, respectively.

The cells were harvested by centrifugation (7500 × g for 5 min at 4˚C), washed in 50 mM Tris-HCl buffer (pH 8.0) and finally resuspended in 5 mL of the same buffer. Cell lysis was done by sonication and the soluble protein fraction was separated by centrifugation (15,000 × g for 20 min at 4˚C).

Purification was achieved by IMAC using an ÄKTA FPLC system (GE Healthcare, Chalfont St Giles, UK)

equipped with a 5 mL Ni-NTA superflow cartridge (Qiagen, Venlo, Netherlands). For all steps a flow rate of 5 mL/min was applied. Prior to the purification, the column was equilibrated with 5 - 15 mL equilibration buffer (50 mM Tris-HCl, 0.3 M NaCl, 20 mM imidazole, pH 8.0). The protein containing sample was supplemented with 20 mM imidazole, filtered through a sterile filter (0.22 µm) and injected into the chromatography system. After sample loading, the column was washed with 15 mL equilibration buffer. Elution of the target protein was obtained by an elution gradient from 20 - 500 mM imidazole over 10 mL and a final step using 5 mL elution buffer (50 mM Tris-HCl, 0.3 M NaCl, 500 mM imidazole, pH 8.0). Protein containing fractions were pooled and residual imidazole was removed by a desalting step using a HiPrep 26/10 Desalting column (GE Healthcare, Chalfont St Giles, UK). The column was equilibrated with desalting buffer (1 mM Tris-HCl, pH 8.0) and desalting occurred over two column volumes at a flow rate of 5 mL/min. Afterwards, the protein preparation was lyophilized and stored at $-80°C$.

2.3. Characterization of the New Lipase/Esterase

Lipase lyophilisates were resolved in the appropriate buffer. Protein concentrations were determined by Bradford assay and a stock solution (100 µg/mL) was further diluted according to the specific assay. In general, enzyme activity assays contained 1780 µL of the corresponding buffer, 20 µL enzyme solution and 200 µL substrate solution.

2.4. Specific Activity and Substrate Specificity

Substrate specificity was determined using a set of p-nitrophenyl esters with saturated carboxylates ranging from C_4 to C_{16} (p-nitrophenyl butyrate (pNPB), p-nitrophenylcaprylate (pNPC), p-nitrophenyllaurate (pNPL), p-nitrophenylpalmitate (pNPP)). Esters were dissolved in 2-propanol to form the substrate solution (1 mM). In these experiments 50 mM Tris-HCl pH 8.0, supplemented with 1% triton X-100 as surfactant, served as standard buffer. The absorption at 405 nm (absorption maximum of p-nitrophenol (pNP)) was investigated over 2 min and the slope was calculated to determine specific activities. The molar extinction coefficient of pNP for the different pH values and buffers was determined separately (data not shown).

2.5. Stability, Temperature Optimum and pH Optimum

Determination of the stability as well as temperature and pH optimum was performed with the activity assay described above. Noteworthy, no surfactant was supplemented in the buffers and the buffer strength was 50 mM each. Hydrolysis of pNPC served as standard for all measurements. The stability was tested by incubation of the enzyme in buffer at room temperature over several days. The temperature optimum was determined in potassium phosphate buffer pH 8.0. The pH optimum was investigated over a pH range of pH 4 - 11. Therefore, the following buffers were used: citrate buffer (pH 4 - 6), potassium phosphate buffer (pH 6 - 8), Tris-HCl buffer (pH 8 - 9) and CAPS buffer (pH 10 - 11). If buffers with pH < 7 were used, the absorption maximum was measured at 320 nm instead of 405 nm.

2.6. Enantioselectivity of Lipase from *P. stutzeri*

Enantioselectivity was determined by the enzymatic hydrolysis of α-methylbenzyl butyrate (MBB) to 1-phenylethanol. The assay was performed in Tris-HCl pH 8.0, the starting concentration of MBB was 400 mM. Reaction vessels were shaken at 25°C and samples of 200 µL were taken periodically. 1 mL ethyl acetate was added to the samples and intensive mixing transferred the products to the water-free phase. Samples were dried with ammonium sulfate to remove remaining water and analyzed by gas chromatography. R- and S-enantiomer of 1-phenylethanol were separated in a GC 2010 gas chromatograph (Shimadzu, Kyoto, Japan) equipped with an AOC-20i auto injector. For separation of all chiral compounds a FS-Cyclodex β-I/P column (CS—Chromatographie Service GmbH, Langerwehe, Germany) was used. The carrier gas (N_2) was applied with a pressure of 50 kPa and 1 µL of the extracted samples were injected on the column with split flow. The optimized method was 60°C for 2 min, 60°C - 90°C for 5 min (slope: 6°C/min), 90°C - 150°C for 20 min (slope: 3°C/min) and 150°C for 2 min. Retention times were 5.4 min for vinyl butyrate, 22.1 min for (*R*)-1-phenylethanol and 22.6 min for (*S*)-1-phenylethanol.

2.7. Software Tools and Databases

Nucleotide and amino acid sequences of the different proteins were found at NCBI and homology arrangements were performed by the BLAST tool of NCBI. The visualized model of the *P. stutzeri* lipase was obtained from Swiss-Model [16]-[18] and processed and displayed with Yasara v.14.2.12.

3. Results and Discussion

The *P. stutzeri* A1501 genome is completely sequenced and available on public databases (NCBI reference sequence number: NC_009434.1, GenBank: CP000304.1). Within the sequence between base 4,349,096 and 4,349,944 a probable reading frame for an unknown lipase/esterase was spotted. This gene was formerly annotated to encode for a lipase and also the protein sequence can be found in the NCBI database (NCBI reference sequence: YP_001174476.1). However, the functional protein was neither described or expressed nor characterized. After a protein BLAST analysis was performed, it turned out that the enzyme was conserved over different *P. stutzeri* strains with sequence identities over 90%. Furthermore, it is closely related to a lipase from *P. mendocina* [19] (PDB ID: 2fx5). Apart from the fact that the new lipase/esterase possesses an N-terminal overhang of 23 amino acids, both enzymes have a high grade of similarity (identities: 83%, positives: 92%, gaps: 0%). The alignment of both sequences is depicted in **Figure 1(a)**. The missing N-terminal peptide (first 23 amino acids) was identified to be a signal peptide using SignalP [20]. Based on the solved structure of *P. mendocina* lipase, a putative model of *P. stutzeri* lipase/esterase was created *via* Swiss-Model web server [16]-[18] giving a first impression of the possible enzyme structure and illustrating the typical α/β hydrolase fold (**Figure 1(b)**). The centre forms a parallel β-sheet probably containing the catalytic groups. This β-sheet is surrounded by loops and helices, whereby one of the last may be the lid which covers the catalytic centre. As mentioned above, lipases and esterases are hardly distinguishable when comparing their primary sequences [2]. Therefore, it is not possible to determine the correct classification of the enzyme from the alignment and further biochemical studies have to be performed.

3.1. Cloning, Expression and Purification of the New Lipase/Esterase (*P. stutzeri*)
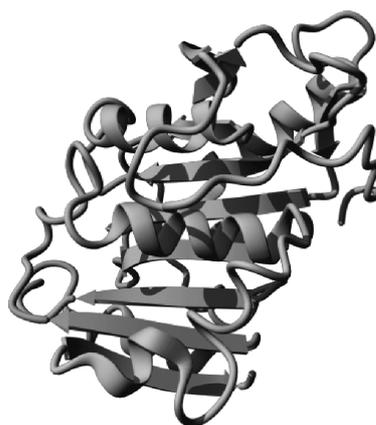
The identified gene was synthesized and cloned into different expression vectors. Noteworthy, codon usage of the wild type gene was adapted for expression in *E. coli* to avoid incorrect translation. Unfortunately, expression without solubilizing tags resulted in insoluble protein located in inclusion bodies (data not shown). Therefore, the expression of fusion proteins with enhanced solubility was chosen [21], which was formerly described to be transferable to lipases. For example, Narayanan and co-workers reported the soluble expression of a lipase from *Burholderia* when fused to DsbA or DsbC [22]. Also the soluble expression of lipase B from *Candida antarctica* was improved by N-terminal addition of a thioredoxin tag [23]. We selected four different tags, namely thioredoxin (Trx, 12 kDa), two thiol: disulfide interchange proteins (DsbA, 23 kDa and DsbC, 25.5 kDa) and the transcription termination/antitermination protein NusA (NusA, 55 kDa). Expression plasmids were constructed resulting in the expression of the lipase/esterase with the respective N-terminal tag. The proteins were named Trx-lipase, DsbA-lipase, DsbC-lipase and NusA-lipase. Overexpression in *E. coli* BL21 (DE3) resulted in soluble expression of all four fusion proteins (**Figure 2(a)**). According to their molecular mass, all the constructs were clearly assignable. Since smaller tags would have lower influence on the overall structure of the fusion protein, its enzyme activity was investigated. All the fusion proteins were active with the highest activity for Trx-lipase (Trx-Lip, **Figure 2(b)**). Because of the smallest tag size and the highest activity in the cell raw extracts, Trx-Lip was selected for further investigation. Purification by IMAC was followed by desalting and lyophilisation and produced the target enzyme in adequate purity.

3.2. Biochemical Characterization

First of all, substrate range and specific activities of purified Trx-Lip were determined. Therefore, a set of different p-nitrophenyl esters, ranging from C_4 to C_{16} was used (**Figure 3**). Trx-Lip showed maximum activity towards middle chain substrates (C_4 to C_8), whereas esters with unsaturated long chain fatty acids (C_{12} to C_{16}) were converted very slowly or not at all. The K_M-value for the best substrate (pNPB) was determined to be 0.43 mM calculated by Lineweaver-Burk plot. This indicates a high affinity to short and medium chain fatty acid substrates.

(a)

(b)

Figure 1. Alignment and putative model of the identified lipase/esterase from *Pseudomonas stutzeri* A1501. (a) Alignment of the putative functional gene and a closely related lipase gene from *P. mendocina.* Bright grey: identical amino acids, dark grey: similar amino acids, white: no consensus; (b) Homology model of *Pseudomonas stutzeri* A1501 lipase/esterase. The crystal structure of *Pseudomonas mendocina* lipase (identities: 82%, PDB 2fx5A) is the best available structural approximation and was used to model the possible structure. The centre forms a parallel β-sheet, which is surrounded by helices, whereby one of them may be the lid covering the catalytic centre. The model was obtained from Swiss-Model [16]-[18], processed and displayed with Yasara v.14.2.12.
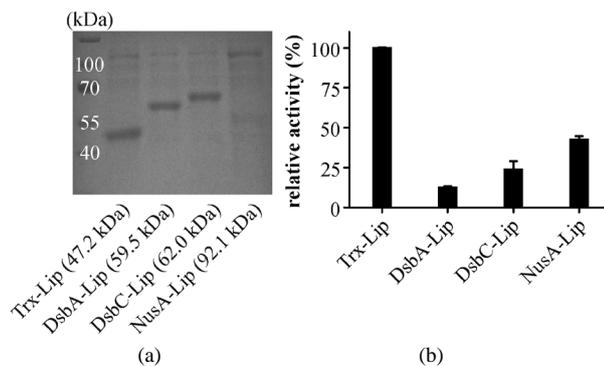

(a)

(b)

Figure 2. SDS-PAGE and activity of all fusion proteins. Trx-Lip—lipase N-terminally tagged with thioredoxin, DsbA-Lip —lipase N-terminally tagged with DsbA, DsbC-Lip—lipase N-terminally tagged with DsbC, NusA-Lip—lipase N-terminally tagged with NusA. (a) SDS-PAGE of cell raw extracts from the different investigated lipase variants after expression in *E. coli*. A soluble expression could be realized with all four fusion proteins; (b) Enzymatic hydrolysis of p-nitrophenolbutyrate (pNPB) by cell raw extracts. All four variants were active after recombinant expression in *E. coli*, with Trx-Lip showing the highest activity for pNPB.
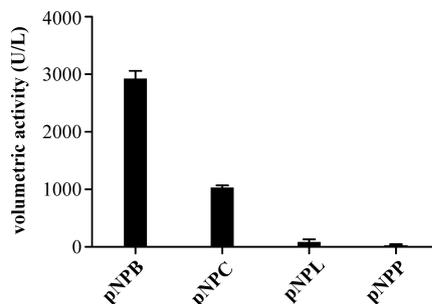
Figure 3. Volumetric activities of Trx-Lip for substrates with different chain length. pNPB: p-nitrophenylbutyrate, pNPC: p-nitrophenylcaprylate, pNPL: p-nitrophenyllaurate, pNPP: p-nitrophenylpalmitate; The highest activity was observed for pNPB hydrolysis.

It was suggested that a preliminary classification of esterases and lipases can be done from their substrate spectrum using p-nitrophenyl esters [24]. Esterases hydrolyze small alkyl esters like pNPB, while lipases are able to hydrolyze long chain fatty acid esters like pNPP. Nowadays, this classification is controversially discussed [25] [26], but still the hydrolytic behaviour can give a first hint. Our findings suggested that Trx-Lip corresponded more to an esterase than to a lipase. Furthermore, interfacial activation of the enzyme could not be observed, which is in accordance to the behaviour of the structurally related lipase from *P. mendocina* [19].

Especially for industrial applications, it is necessary to know which conditions can be exposed to a certain enzyme. First, the stability of the recombinant enzyme was tested in buffer. Lyophilized Trx-Lip was resuspended in Tris-HCl buffer pH 8.0 and incubated at room temperature. After 24 h a relative activity of 96% was detected and even after 8 days the lipase still displayed a residual activity of 86% (**Figure 4(a)**). Additionally, temperature and pH spectra were investigated (**Figure 4(b)** and **Figure 4(c)**). The temperature spectrum of Trx-Lip showed a linear slope with increasing temperature and a maximum activity at 50°C. At higher temperatures the lipase became unstable and lost its activity very fast. The pH range of Trx-Lip was very broad with the highest activity between pH 7.5 - 10 and a maximum at pH 9. The high activity at alkaline pH and the temperature optimum above 40°C referred this enzyme to typical representatives of lipases and esterases [14]. Additionally, these facts indicate the potential application of the new lipase in industrial processes, for example in the detergent industry.

3.3. Enantioselectivity of Lipase from *P. stutzeri*

Enantioselective synthesis of chiral compounds is a major concern in modern pharmaceutical production. Lipases display attractive catalysts because they can either hydrolyse prochiral substrates or convert one enantiomer from a racemic mixture [27]. This increases reactant flexibility and enables different synthetic routes. The possible application of the new enzyme was investigated by the hydrolysis of α-methylbenzyl butyrate to 1-phenylethanol. A commercial lipase from *P. stutzeri* (Lipase TL®), which has been shown to process bulky secondary alcohols [28] [29], was used as positive control. Resulting products were analysed by GC and the amount was calculated from appropriate standard curves (**Figure 5**).

Not surprisingly, the specific activity of Lipase TL® (starting activity: 5660 U/mg protein) is higher than that of Trx-Lip (starting activity: 523 U/mg protein). Both enzymes are able to hydrolyse the R-enantiomer with good enantiomeric excess (ee). The ee for Lipase TL® was calculated to be >99%. In the first 2 h, also Trx-Lip showed ee-values > 93%, but within the next 15 h the ee decreased to 83%. Nevertheless, the exclusive hydrolysis of the R-enantiomer at the beginning indicates not only a stereoselective preference, but also illustrates the ability to perform kinetic resolution of chiral esters. Furthermore, Lipase TL® is able to perform the (dynamic) kinetic resolution of α-hydroxyketones [29] [30]. In our experiments, Trx-Lip was not able to catalyse kinetic resolution of benzoin, and the sterically less complex α-hydroxypropiophenone could also not be converted (data not shown). Despite the same origin (both enzyme preparations are from *Pseudomonas stutzeri*), Trx-Lip behaves different from Lipase TL® and was thereby not a component of the commercial product. This illustrates that Trx-Lip indeed presents a new until now unknown lipase/esterase. It is known that the enantioselectivity of lipases can be influenced by many parameters like the choice of solvent [31] or by immobilization [32]. Optimized conditions could thereby improve the stereoselectivity of the new lipase/esterase. Herein also just one
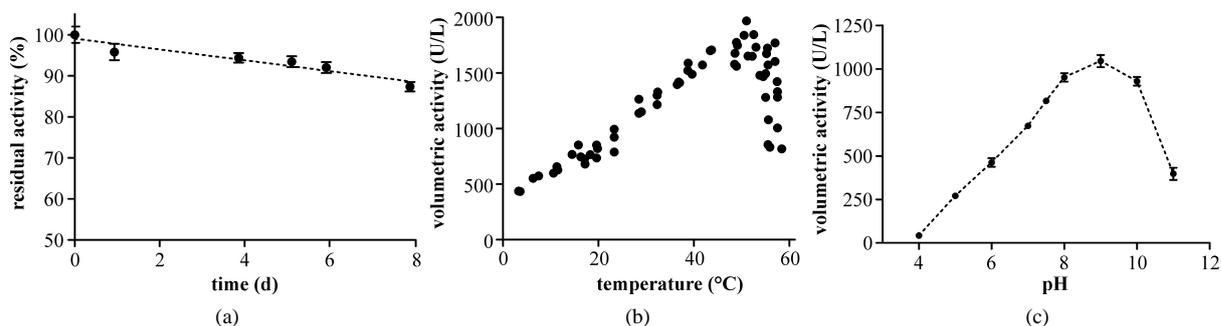
Figure 4. Biochemical characterization of Trx-Lip. (a) Stability of Trx-Lip in buffer, even after 8 days > 85% residual activity was observable; (b) Volumetric activity of Trx-Lip at varying temperatures, the temperature optimum is ~50˚C; (c) Volumetric activity of Trx-Lip at varying pH values, the pH optimum was determined to be at pH 9.0.
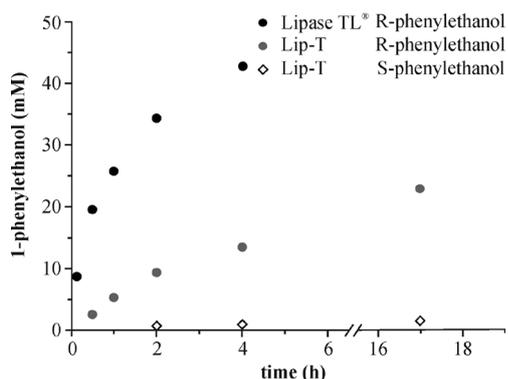

Figure 5. Hydrolysis of *α*-methylbenzyl butyrate by Trx-Lip and Lipase TL®. Lipase TL® hydrolyzes exclusively the R-enantiomer (black). In contrast Trx-Lip hydrolyzes the R-enantiomer (gray) faster than the S-enantiomer (white), but not exclusively.

substrate was tested for enzymatic performance and enantiomeric selectivity. Additional substrate screenings could identify enzyme requirements and will lead to a better understanding of this new enzyme.

4. Conclusion

In summary, a database search identified a new lipase/esterase that was related to lipase from *P. mendocina*. This enzyme could be expressed in an active form in *E. coli* when fused to different solubilizing tags. The Trx-tag turned out to be the most effective tag due to its small size and outstanding solubilizing performance. The resulting fusion protein (Trx-Lip) was purified and biochemically characterized. It showed the highest activity to mid-chain (C_4-C_8) esters, and had a pH optimum of 9.0 and a temperature optimum at 50˚C. Furthermore, stereospecificity was tested by the hydrolysis of *α*-methylbenzyl butyrate. The enzyme was R-specific and was able to perform the kinetic resolution of chiral esters. Although a substrate screening would be necessary to gain further information on enzyme properties, the new lipase/esterase represents a promising candidate for the synthesis of chiral compounds also in industrial processes. Additionally, the authors would propose to classify the enzyme as an esterase instead of a lipase.

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