



Native expression and purification of hormone-sensitive lipase from *Psychrobacter* sp. TA144 enhances protein stability and activity

Giuseppina Ascione^a, Donatella de Pascale^b, Concetta De Santi^b, Carlo Pedone^a, Nina Alayne Dathan^{a,*}, Simona Maria Monti^a

^a Institute of Biostructures and Bioimaging, National Research Council, Via Mezzocannone 16, I-80134 Naples, Italy

^b Institute of Protein Biochemistry, National Research Council, Via Pietro Castellino 111, I-80131 Naples, Italy

ARTICLE INFO

Article history:

Received 21 February 2012

Available online 13 March 2012

Keywords:

Lipase
Psychrophile
Trehalose
Benzyl alcohol
Molecular chaperones
Protein stability

ABSTRACT

Psychrobacter, a micro-organism originally isolated from Antarctic sea water, expresses an extremely active hormone-sensitive lipase (HSL) which catalyzes the hydrolysis of fatty acid esters at very low temperature and is therefore of great potential industrial and pharmaceutical interest. An insoluble form of the entire enzyme has previously been cloned and expressed in *Escherichia coli*, subsequently refolded and shown to be active, whilst a shorter but completely inactive version, lacking the N-terminal 98 amino acids has been expressed in soluble form. In this study the entire enzyme has been expressed as a fully soluble protein in *E. coli* in the presence of either the osmolyte trehalose, plus high salt concentration, or the membrane fluidizer benzyl alcohol. Trehalose promotes protein mono-dispersion by increasing the viscosity of the growth medium for bacterial cells, thereby helping circumvent protein aggregation, whilst the heat-shock inducer benzyl alcohol stimulates the production of a network of endogenous chaperones which actively prevent protein misfolding, whilst also converting recombinant aggregates to native, correctly folded proteins. The resultant recombinant protein proved to be more stable than its previously expressed counterpart, as shown by CD and enzymatic activity data which proved the enzyme to be more active at a higher temperature than its refolded counterpart. By light scattering analysis it was shown that the newly expressed protein was monomeric. The stability of the full length native protein will help in understanding the structure of PsyHSL and the role of its regulatory N-terminal for eventual application in a myriad of biotechnological processes.

© 2012 Elsevier Inc. All rights reserved.

1. Introduction

Lipases play a crucial role in lipid metabolism, catalyzing the hydrolysis of acylglycerides and a range of other fatty acid esters [1]. They are produced by organisms in a wide range of environments and are fully functional, often in an extreme range of conditions [2,3]. Due to their catalytic activities and adaptable nature they are potentially extremely useful enzymes for an ever growing number of industrial and pharmaceutical applications [4–6]. Cold-active enzymes produced by micro-organisms often show high catalytic efficiency at low temperatures, frequently associated with low stability at moderate and high temperatures. The specific activity of psychrophilic enzymes is higher than that of their mesophilic homologues at 0–30 °C. Over the past decade, the attention of many researchers has been focused on the biotechnological applications of these enzymes [5], thanks to the numerous eco-

nomical and ecological advantages of enzymes that operate at lower temperatures.

The bacterial species TA144 of *Psychrobacter* is a micro-organism which has been isolated from Antarctic sea water, where it efficiently catalyzes the hydrolysis of acylglycerides and other fatty acid esters in the process of degradation of organic matter via its highly active hormone-sensitive lipase [7]. Cold-adapted micro-organisms normally grow very slowly, but with the aid of genetic engineering it has become possible to clone their highly catalytic genes in host strains such as *Escherichia coli* as has been the case for the *lip2* gene, normally expressed in *Psychrobacter* sp. TA144, hereafter referred to as recombinant PsyHSL [8]. As with other members of the HSL family PsyHSL contains a highly conserved sequence of His-Gly-Gly-Gly upstream of the catalytic site, shown to be homologous to the corresponding region of human HSL, Gly-Asp-Ser-Ala-Gly [9]. In fact PsyHSL shows a surprising degree of homology around the catalytic domain, displaying 42% identity and 60% similarity over a span of 87 amino acids, this region being proposed to be a common feature in triacylglycerol lipases and esterases [10].

* Corresponding author. Fax: +39 0812534574.

E-mail address: nina.dathan@unina.it (N.A. Dathan).

PsyHSL has previously been expressed in the bacterial vector pET22b, but proved to be highly insoluble. It has however been successfully refolded and shown to be catalytically active, although this activity does not confirm the true nature of the enzyme; all attempts to crystallize the refolded protein have proved unsuccessful [8]. However a deleted version of PsyHSL, lacking the initial 98 residues has been expressed in fully soluble form but not extensively characterized since this deleted version was completely inactive [8]; suggesting that the N-terminal region of HSL, like its human counterpart, could be considered as a regulative domain of the protein, rendering the attainment of the soluble full length protein to be of considerable potential interest [9].

The aim of this study was to find suitable expression conditions in order to provide us with a high level of native, soluble and fully active protein for extensive biochemical characterization and, eventually, crystallographic studies. For this purpose PsyHSL was initially re-cloned in two simple 6×His-tag (N-terminal and C-terminal) modified pET vectors pETM11 and pETM13 (EMBL, Heidelberg) via PCR using specific oligonucleotide primers and their soluble expression levels were confronted with that obtained from pET22b. Further to altering growth conditions two additives were also used to encourage correct folding of the recombinant protein expressed in *E. coli*. These were benzyl alcohol, a membrane fluidizer [11] known to artificially cause a heat-shock response in the modified membrane of bacterial cells, causing severe stress and inducing the expression of the cells own chaperone network, as well as the osmolyte trehalose which exerts an anti-aggregation effect on proteins in the presence of high salt concentration thereby encouraging uptake of the osmolyte into individual bacterial cells [12]. The addition of these compounds proved to be decisive in procuring a high expression level of stable, soluble, full length recombinant protein.

2. Materials and methods

2.1. Materials

Oligonucleotide primers for PCR were purchased from PRIMM srl (Milan), DNA extraction and purification kits were from Qiagen (Germany), Phusion polymerase (Finnzymes, Milan), expression vectors pETM11 and pETM13 were kindly provided by the Protein Expression and Purification Core Facility, EMBL (Heidelberg), restriction enzymes were from New England Biolabs (Milan), anti-His-HRP conjugated mouse monoclonal IgG from Santa Cruz (USA), HisTrapHP and Superdex 200 columns from GE Healthcare (Milan), Bio-Sep SEC3000 from Phenomenex (Germany). Bacterial expression strains were from Novagen, whilst the cloning strain TOP10F' was from Invitrogen (Milan). Benzyl alcohol, trehalose and other chemicals were from Sigma-Aldrich (Milan).

2.1.1. Construction of pETM11-HSL and pETM13-HSL

HSL ([8] GenBank X53868.1) cDNA was PCR-amplified from pET22b-HSL with Phusion polymerase using the site-specific oligonucleotide primers: HSL-F CGCGCGCCATGGCATGCTATTCTAC-CAGTACCGGC with HSL-11R CGCGCGCTCGAGTTACTACGCTCTGA GATTGGCTTATCAC and again HSL-F with HSL-13R CGCGCGCTC-GAGCGCTCTGAGATTGGCTTATCAC and inserted in the appropriately cut expression vectors pETM11 and pETM13 before being transformed in TOP10F'. The resulting plasmids were named 11-HSL and 13-HSL, whilst the original was known as 22-HSL.

2.1.2. Expression screening of recombinant clones

Expression of recombinant HSL was screened in strains: BL21(DE3), BL21(DE3)pLysS, Rosetta(DE3), Rosetta(DE3)pLysS and RosettaGami2(DE3), using various bacterial growth media. Cultures

were induced in exponential phase for 16 h at 20 °C with 0.2 mM IPTG. All pellets were lysed in B-PER (Pierce, Milan) to obtain soluble and insoluble fractions and loaded on 12% SDS/PAGE. Expression was confirmed by Western Blot with anti-His-HRP conjugated antibody before scaling-up optimized conditions.

2.1.3. Purification of recombinant HSL

Expression was scaled up under sequentially optimized conditions to 400 ml cultures grown in 1.8 L baffled shake flasks, with vigorous shaking, inducing HSL expression 16 h at 20 °C with 0.2 mM IPTG, before harvesting 15 min at 3000g, 4 °C. One gram pellets were resuspended in 10 ml of: 50 mM Tris-HCl, 40 mM imidazole, 0.1% Tween 20, 30 mM MgCl₂, 5 mM DTT, 1 mM PMSF, 10 µg/ml lysozyme, 5 µg/ml DNaseI, pH 8.0. The lysate was adjusted to 500 mM NaCl and sonicated gently on ice. The soluble fraction was recovered by centrifugation and loaded on a 1 ml HisTrapHP column, connected to an FPLC ÄKTA system, in the presence of 50 mM Tris-HCl, 500 mM NaCl, 40 mM imidazole, 2 mM DTT, 100 µM PMSF, pH 8.0. Peak fractions eluted at 100 mM and 250 mM imidazole, concentrated on Amicon 10 kDa MWCO and loaded on Superdex 200, Superose 6 or Bio-Sep SEC 3000 columns in 50 mM Tris-HCl, 150 mM NaCl, 2 mM DTT, pH 8.0.

2.1.4. Enzymatic activity of 13-HSL

13-HSL was tested for esterase activity as previously described [8].

2.1.5. Optimization of expression for correct protein folding

Recombinant HSL underwent growth and expression in the presence of additives to optimize the level of correct protein folding in bacteria. Either trehalose or benzyl alcohol were added at the initiation of the exponential stage of bacterial growth at various concentrations. Growth was continued and 13-HSL expression induced with IPTG. Lysates from 2 ml cultures were prepared in B-PER and 100 µg from each soluble fraction was subjected to 2 h digestion at 25 °C in the presence of 5 ng trypsin to determine optimal additive concentration.

Once optimized, growth was scaled up to 400 ml cultures as above and followed either to OD₆₀₀ = 0.2 at 37 °C, before adding 50 mM trehalose and 400 mM NaCl, switching to 20 °C for 45 min before adding 0.2 mM IPTG and expressing 24 h at 16 °C; or to OD₆₀₀ = 0.4 at 37 °C, before adding 20 mM benzyl alcohol, switching to 20 °C for 20 min before adding 0.2 mM IPTG and expressing 24 h at 16 °C. Pelleted cells were resuspended and treated as before, although following HisTrap purification, concentrated samples were further purified directly on Bio-Sep SEC 3000. Using this new purification procedure the protein yield was 1.6 mg/g cells, while refolding from inclusion bodies yielded 20 mg/g cells.

2.1.6. Circular dichroism

All CD spectra were recorded with a Jasco J-715 spectropolarimeter equipped with a Peltier temperature control system [Model PTC-423-S]. Molar ellipticity per mean residue, $[\theta]$ in deg cm² × dmol⁻¹, was calculated from the equation: $[\theta] = [\theta]_{\text{obs}} \times \text{mrw} / 10 \times l \times C$, where $[\theta]_{\text{obs}}$ is the ellipticity measured in degrees, mrw is the mean residue molecular mass, C is the protein concentration in mg × mL⁻¹, and l is the optical path length of the cell in cm. Far-UV measurements (183–250 nm) were carried out at 20 °C, at time constant of 4 s, 2 nm band width, scan rate of 10 nm min⁻¹, using a 0.1 cm optical path length cell and a protein concentration of 0.2 mg × mL⁻¹ in 6.6 mM buffer phosphate pH 8.0. CD spectra were signal averaged over at least three scans, and the baseline was corrected by subtracting a buffer spectrum. Thermal unfolding curves were determined by recording the molar ellipticity at 222 nm, using a scanning rate of 1 °C/min.

Download English Version:

<https://daneshyari.com/en/article/1929803>

Download Persian Version:

<https://daneshyari.com/article/1929803>

[Daneshyari.com](https://daneshyari.com)