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Production and characterization of two N-terminal truncated esterases from *Thermus thermophilus* HB27 in a mesophilic yeast: Effect of N-terminus in thermal activity and stability

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ABSTRACT

Two N-terminally truncated variants of the esterase E34Tt from *Thermus thermophilus* HB27 (YP_004875.1) were expressed in *Kluyveromyces lactis*. Production and biochemical properties of both recombinant proteins were investigated. The esterase activity was greatly increased compared to the wild-type strain. In particular, the extracellular production of the ΔN16 variant (KLEST-3S) was 50-fold higher than that obtained with *T. thermophilus* HB27. Response surface methodology was applied to describe the pH and temperature dependence of both activity and stability. When compared with the wild type esterase, the optimal temperature of reaction decreased 35 and 15 °C for ΔN16 and ΔN26, respectively. KLEST-3S showed a maximum of activity at pH 7.5 and 47.5 °C, and maximal stability at pH 8.1 and 65 °C. KLEST-5A (ΔN26) did not show an absolute maximum of activity. However, best results were obtained at 40 °C and pH 8.5. KLEST-5A showed also a lower stability. In the presence of a surfactant, both proteins showed lower stability at 85 °C ($t_{1/2}$ < 5 min) than the wild-type enzyme ($t_{1/2}$ = 135 min). However, in the absence of detergent, the stability of KLEST-3S was higher ($t_{1/2}$ = 230 min, at 85 °C) than that of the mutant KLEST-5A (12 min) or the wild type enzyme (19 min). Minor differences were observed in the substrate specificity. Our results suggest that the N-terminal segment is critical for maintaining the hyperthermophilic function and stability.

Introduction

Bacterial esterases, which catalyze the hydrolysis of ester bonds, are interesting in biocatalysis due to their broad substrate specificity, high regio- and enantioselectivity, and ability to synthesize active chiral compounds [1,2].

The demand for novel enzymes with better activity and stability properties increases exponentially [3], and thermal stability is one of the most valuable characteristics in new enzymes. Extreme thermophiles (microorganisms living at temperatures above 70 °C) are an interesting source of stable enzymes as, besides thermoresistance, thermozymes frequently present an unusual resistance towards a number of chemical and physical denaturing agents [4], which make them suitable for harsh industrial conditions where conventional enzymes lose their function.

The thermophilic bacterium *Thermus thermophilus* HB27 produces two enzymes with lipase/esterase activity and molecular weights of 34 and 62 kDa, which have been isolated from several cellular compartments, including the cytoplasm, periplasmic space and membranes [5–7]. In a previous work, the 34-kDa protein (E34Tt) was purified and characterized. By using mass fingerprinting, peptides were found to share identity with the YP_004875.1 protein, which was annotated as putative esterase in the genome analysis of *T. thermophilus* HB27, although experimental evidence was lacking at that moment. No sequence homology was detected with any known lipase or esterase. However, the purified enzyme exhibited esterase activity with preference for medium chain esters (C10) [8]. Moreover, YP_004875.1 was further expressed in *Saccharomyces cerevisiae* and the esterasic character was confirmed [9].

The wild type enzyme resulted a very attractive biocatalyst for biotechnological purposes. E34Tt was noticeable for its high thermophilicity; the optimal reaction temperature was higher than 80 °C and the half-life of thermal inactivation at 85 °C was 135 min, which makes it even more thermostable than some esterases from hyperthermophilic microorganisms.

Hydropathy analysis of the sequence of 329 amino acid residues that comprise the esterase E34Tt isolated from wild-type *T.*

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thermophilus HB27 shows a highly hydrophobic sequence between residues 4 and 26. E34Tt is a secretory protein, with a cleavage site between residues 16 (alanine) and 17 (glutamine) [10]. However, in cultures of wild-type *T. thermophilus*, E34Tt was recovered mainly in the membrane fraction. Detergent was required during its solubilization and purification from bacterial lysates [8]. Moreover, after solubilization in the presence of 1% w/v CHAPS, the detergent was still needed for maintaining activity and stability at high temperatures, characteristics of an integral membrane protein [11].

The sequence of E34Tt was cloned in the mesophilic yeast *S. cerevisiae*, replacing its secretion signal by the yeast α-factor secretion signal contained in the YEpFLAG-1 plasmid [9]. With this construction, a high level of expression was reached. Total lipolytic activity produced by the recombinant strain ScEST-O3 was more than 30-fold higher than the obtained with the wild type microorganism. However, the expressed protein was not properly secreted. It remained mainly cell-associated (more than 80% was retained within the periplasmic space), due to aggregation problems that hindered its secretion. Moreover, the optimal temperature of activity dropped from over 80 to 40 °C, indicating that some protein domain may be destabilized.

These behaviors may be due to hydrophobic interactions involving some highly hydrophobic residues at the new N-terminus of the processed protein. Moreover, a number of reports describe dimerization or oligomerization processes through hydrophobic areas of the N-terminal region [12–14].

Dimers and oligomers are often the functional form of proteins and may have been evolutionarily selected to confer thermostability on the proteins because subunit associations can result in extra stabilization of the proteins [15,16]. Indeed, many enzymes from thermophilic organisms are capable of forming higher order oligomers. The hyperthermostability of the esterase EstE1 seems to be achieved mainly by its dimerization through hydrophobic interactions [17]. In the same line, wild type esterase from *T. thermophilus* HB27 (E34Tt) undergoes a reversible temperature-dependent trimerization process likely promoted by hydrophobic interactions [6,8].

In order to gain insights into the role of the hydrophobic N-terminal region, two molecular variants (KLEST-3S and KLEST-5A) of the esterase E34Tt (Accession No. YP_004875.1) previously purified from *T. thermophilus* HB27 were produced. KLEST-3S (ΔN16) was obtained by removing the putative secretion signal (amino acids 1–16) of the native protein and KLEST-5A (ΔN26) by the deletion of the first 26 residues of the N-terminal sequence. *Kluyveromyces lactis* (NRRL-Y1140) was used as host for the heterologous expression using the vector pKLAC1. In addition, production and biochemical properties of the two truncated forms were investigated. Using second-order rotatable designs, empirical models were obtained to describe the pH and temperature dependence of activity and stability. We found that the N-terminal segment is critical for maintaining the hyperthermophilic function and stability. However, only minor differences were observed in the substrate specificity.

Materials and methods

Materials

p-Nitrophenyl laurate, sodium cholate, Triton X-100, Coomassie Brilliant Blue R-250, Fast Red and α-naphthyl acetate were from Sigma (St. Louis, MO, USA). Molecular weight markers for electrophoresis were obtained from Bio-Rad (Richmond, CA, USA). All other chemicals were of the purest grade available.

Gene isolation, cloning and expression

Conventional DNA recombinant techniques were used except otherwise stated. The pKLAC1 vector (New England Biolabs) and the *K. lactis* NRRL-Y1140 (CBS-2359) strain, were used to express two N-terminal truncated variants (ΔN16 and ΔN26) of the esterase E34Tt (YP_004875.1) from *T. thermophilus* HB27. Protein secretion using pKLAC1 was achieved by generating a fusion between the protein and the α-MF secretion domain present in the vector. The recombinant strains obtained were named KLEST-3S (ΔN16) and KLEST-5A (ΔN26).

Genomic DNA from *T. thermophilus* HB27 was used as template for PCR amplification of the putative esterase gene. For the construction of the KLEST-3S strain the primers EST1KF2 (TTTCTCGAGAAAAGAcagggcctcgaggccttctgg) and EST1KR (TTTGGTACCtcaaggccgcacccgggggggcgt) were used, which contain restriction sites for *XhoI* and *KpnI*, respectively, and amplify the full coding sequence except the putative secretion signal. For the construction of the KLEST-5A strain, the primers EST1KF (TTTCTCGAGAAAAGAgaggtgcccggtggggtctgc) and EST1KR (TTTGGTACCtcaaggccgcacccgggggggcgt) were used, which contain restriction sites for *XhoI* and *KpnI*, respectively, and amplify the coding sequence except the putative secretion signal and N-terminal residues from 16 to 26.

Due to the high GC-content of the template DNA, PCR cycling conditions were optimized to improve specificity and product yield. Best results were obtained with the following conditions: initial denaturation (95 °C, 3 min); followed by 30 cycles of denaturation (95 °C, 30 s), annealing (55 °C, 30 s), extension (72 °C, 1 min); and a final cycle of 72 °C, 8 min; using Taq DNA polymerase (Roche) and 4% DMSO in the buffer. The resulting PCR product was purified by precipitation with ethanol and sodium acetate, and TA-cloned into a T-vector [18]. The correctness of the constructions was verified by PCR, restriction analysis and sequencing.

From these intermediary clones in T-vector, the sequences of interest were obtained by digestion with *XhoI* and *KpnI* and ligated to pKLAC1 digested with the same enzymes. *Escherichia coli* DH10B cells (Invitrogen) were transformed with the ligation reaction, and the transformants were selected by growing in LB medium supplemented with ampicillin (70 μg/mL). The recombinant plasmids were extracted from the transformants using the Spin Clean™ Plasmid Miniprep Kit (Metabion) and the correctness of the construction was confirmed by PCR and restriction analysis.

K. lactis NRRL-Y1140 cells were transformed with the recombinant plasmids, previously linearized by digestion with *SacII*. The 5′ PLAC4 and 3′ PLAC4 sequences direct insertion of the expression cassette into the promoter region of the LAC4 locus in the *K. lactis* genome. Selection of transformants was performed by culture in a nitrogen-free minimal medium containing acetamide.

For detection of the secreted enzyme, the transformants were grown in 2 mL of YPGal medium (2% bactopeptone, 1% yeast extract and 2% galactose, w/v) in sterile culture tubes. The cultures were incubated with shaking (250 rpm) at 30 °C for 48 h and extracellular lipolytic activity was measured.

The correct integration of the expression cassette in the transformants was identified by PCR using the procedure and primers supplied with the pKLAC1 vector by New England Biolabs. Multicopy integration was detected for both KLEST-3S and KLEST-5A strains.

Culture conditions

Cultures were performed at 30 °C and 250 rpm without pH control in a 10-L stirred-tank bioreactor filled up with 8 L of YPL medium, containing 2% (w/v) peptone; 1% (w/v) yeast extract and 2%