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The esterase from *Alicyclobacillus acidocaldarius* as a reporter enzyme and affinity tag for protein biosynthesis

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Abstract Esterase from thermophilic bacteria *Alicyclobacillus acidocaldarius* can be produced up to 200 μg/ml by coupled in vitro transcription/translation system derived from *Escherichia coli*. The synthesized thermostable enzyme can be determined by photometrical and fluorescent assays at least up to 10^{-8} M concentration or by activity staining in the polyacrylamide gels. Enhanced green fluorescence protein-esterase fusion protein was bound to a matrix with immobilized esterase inhibitor and purified by affinity chromatography. Thus, the esterase is suited as a reporter enzyme to monitor the expression of polypeptides coupled to its N-terminus and simultaneously, as a cleavable tag for polypeptide purification.

Keywords: In vitro translation; Esterase; Reporter enzyme; Affinity tag; Protein engineering

1. Introduction

Cell free translation systems for protein production are used for synthesis of functionally active proteins and study of protein biosynthesis in vitro [1]. To monitor the gene expression by coupled transcription/translation, reporter proteins were introduced. The most widely used reporters are the green fluorescent protein (GFP) [2], firefly luciferase [3], dihydrofolate reductase [4], chloramphenicol acetyl transferase [5] and β-galactosidase [6]. Here, we report about the esterase from thermophilic bacterium *Alicyclobacillus acidocaldarius* [7] that can be used as a convenient reporter group to monitor protein biosynthesis. The 34 kD esterase is a thermostable, single chain protein that folds into a one domain structure with one active center that possess a lipase-like Ser-His-Asp catalytic triad [8]. The overall fold, typical for α/β hydrolases, shows a central eight-stranded mixed β-sheet surrounded by five helices with a helical cap on a top of the C-terminal end of the central β-sheet. The N- and C-terminal ends of the protein are not involved in catalytic center of the enzyme and are exposed on the esterase surface [8] providing a possibility for the protein to be fused with other polypeptides without altering the esterase native fold.

2. Materials and methods

2.1. Materials

Taq polymerase was from Qiagen (Hilden, Germany), T4-DNA-Ligase from Promega (Mannheim, Germany), Factor Xa protease and restriction enzymes were from NewEngland Biolabs (Frankfurt, Germany). Fast Blue BB Salt, *p*-Nitrophenyl acetate and β-Naphthyl-acetate were from Fluka (Steinheim, Germany). 5-(and 6-)Carboxy-2′,7′-dichlorofluorescein diacetate was from Molecular probes (Eugene, USA). Other analytical grade chemicals were obtained from Roth (Karlsruhe, Germany). Radioactive [^{14}C]leucine (54 mCi/mmol) was purchased from Amersham, Life Sciences (Freiburg, Germany).

2.2. Plasmid construction and purification

Plasmid pT7SCII, containing the gene of the esterase (Est2) [7] was kindly provided by G. Manco, Naples, Italy. The gene was amplified by PCR with the primers Est2_for (5′-CCATGGCGCTCGATCCC GTCATTCAGC-3′) and Est2_rev (5′-GAGCTC**CTA**GGCCAGCGC GTCTCG-3′). The primers create the *Nco*I and the *Sac*I cleavage sites (underlined letters) upstream and downstream of the Est2 gene, respectively. The primer Est2_rev contains a UAG stop codon (bold letters). The PCR product was sequenced and cloned into the vector pIVEX2.3d (Roche Diagnostics, Mannheim, Germany). The resulting plasmid, pIVEX2.3d-Est2_RF1 (pEst2), was used for in vitro translation.

The pIVEX2.3d-eGFP-Est2_RF1 (peGFP-Est2) plasmid was constructed as follows. The gene of enhanced green fluorescence protein (eGFP) was amplified from the plasmid pSL1180-eGFP (provided by G. Krauss, Bayreuth) by PCR with the primers eGFP_for (5′-CCAT GGTGAGCAAGGGCG-3′) and eGFP_rev (5′-GCGG CCGCCTTT GTACAGCTCGTCCAT-3′). The primers introduce the *Nco*I and the *Not*I cleavage sites (underlined letters) upstream and downstream of the eGFP, respectively. The PCR product was sequenced and cloned into the pIVEX2.3d vector resulting in the pIVEX2.3d-eGFP (peGFP) plasmid. The Est2 was amplified with primers Est2CT_for (5′-GAG-CTCGGTACC**ATTGAGGGTCGC**GGTTCCGGCGGTGGTATGG CGCTCGATCCC-3′) and Est2CT_rev (5′-GGATCC**TCA**GGCCAG CGC-3′). The primers create the *Sac*I and the *Bam*HI cleavage sites (underlined letters) upstream and downstream of the Est2, respectively. The primer Est2CT_for contains the cleavage site of protease Factor Xa coding sequence (bold letters) and the primer Est2CT_rev contains the UAG stop codon (bold letters). The PCR product was sequenced and cloned into the peGFP plasmid. The resulting plasmid, peGFP-Est2, was used for in vitro translation.

The plasmids were purified as described [9].

2.3. Cell-free transcription/translation experiments

Transcription/translation Kits were the gift from RiNA GmbH (Berlin, Germany) and the reaction was performed at 37 °C according to the manual provided by the supplier with 0.5 mM [^{14}C]leucine (17.3 mCi/mmol). The templates were added up to 5 nM concentration.

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Aliquots, 3 μl, were withdrawn at different time intervals and the newly synthesized protein was determined by radioactivity measurement in 10% hot trichloroacetic acid precipitate. Protein composition was analysed by SDS–PAGE [10]. The gels were fixed with 15% formaldehyde in 60% methanol and stained with Coomassie Blue G-250. The dried gels were exposed to an imaging plate for radioactivity analysis with the PhosphorImager SI (Molecular Dynamics, Sunnyvale, USA).

2.4. Esterase activity assays

Esterase activity assay was performed as described [7]. Aliquots of 1 μl transcription/translation mixture were added to 1 ml of 50 mM phosphate buffer, pH 7.5, containing 0.025 mM *p*-nitrophenyl acetate. The production of *p*-nitrophenoxide was monitored at 405 nm in 1 cm path-length cells with UV-Spectral photometer DU 640 (Beckman, Fullerton, USA) at 25 °C. Initial rates were calculated by linear least-square analysis of time courses comprising less than 10% of the total substrate turnover.

Esterase activity was also determined by fluorescence assay. At each time interval 1 μl was withdrawn from transcription/translation mixture and added to 1 ml of 50 mM phosphate buffer, pH 7.5, with 0.025 mM 5-(and 6-)carboxy-2′,7′-dichlorofluorescein diacetate. Production of 5-(and 6-)carboxy-2′,7′-dichlorofluorescein was measured at 25 °C by Luminescence spectrometer LS50B (Perkin Elmer, Boston, USA) with λ_{ex} at 500 nm and λ_{em} at 525 nm. Since hydrolysis of the 5-(and 6-)carboxy-2′,7′-dichlorofluorescein leads to fluorescence appearance at 525 nm intensity of fluorescence was recorded in arbitrary units as time function. Initial rates of esterase activity were calculated in arbitrary units per minute by linear least-square analysis of time courses comprising less than 10% of the total substrate turnover.

Activity staining of the esterase in polyacrylamide gel after electrophoretic separation was performed according to [11] with Fast Blue BB Salt and β-naphthyl-acetate.

2.5. Affinity purification of eGFP-esterase fusion

The pIVEX2.3d-eGFP-Est2_RF1 plasmid was expressed in vitro as described above. The fluorescence at 507 nm of eGFP-esterase fusion protein was monitored at $\lambda_{ex} = 488$ nm and 25 °C directly in the translation mixture without dilution using a 150 μl quartz cell. The esterase activity was monitored by photometric assay in parallel with eGFP fluorescence assay. Then 200 μl of the translation mixture was incubated with 25 μl of TFK-matrix (trifluoromethyl ketone Sepharose CL-6B, prepared as described [12]) equilibrated with 100 mM Na-phosphate, pH 7.5, at 37 °C for 4 h. The TFK-matrix was spun down and the supernatant was analyzed for the eGFP fluorescence and the esterase activity. The remaining pellet of TFK-matrix was washed with 3 ml of 100 mM Na-phosphate, pH 7.5, with 100 mM NaCl. Then the TFK-matrix was resuspended in 175 μl of 40 mM Tris, 200 mM NaCl, 4 mM $CaCl_2$, pH 8.0, and treated with 20 μg Factor Xa protease for 15 h at 23 °C. The TFK-matrix was spun down and the supernatant was analyzed for the eGFP fluorescence and the esterase activity. The remaining material was removed from TFK-matrix by boiling it for 5 min at 95 °C in 10% SDS. The aliquots from each step of purification were also analysed by SDS–PAGE.

3. Results

Esterase from *A. acidocaldarius* was synthesized by coupled in vitro transcription/translation system derived form *E. coli*. Although, the codon usage of the esterase gene was not adjusted to the codon usage of *E. coli* the synthesis of the thermostable esterase proceeds with similar efficiency in this heterologous system as the synthesis of one most abundant *E. coli* proteins, the elongation factor Ts. The Fig. 1 demonstrates the in vitro [^{14}C]leucine incorporation into the esterase (Fig. 1A) with the simultaneous monitoring of the esterase activity (Fig. 1B). The system produces the target proteins linearly up to 60 min of incubation. The estimated yield for the EF-Ts and the esterase was approximately 350 and 200 μg of the protein per 1 ml of the reaction mixture, respectively

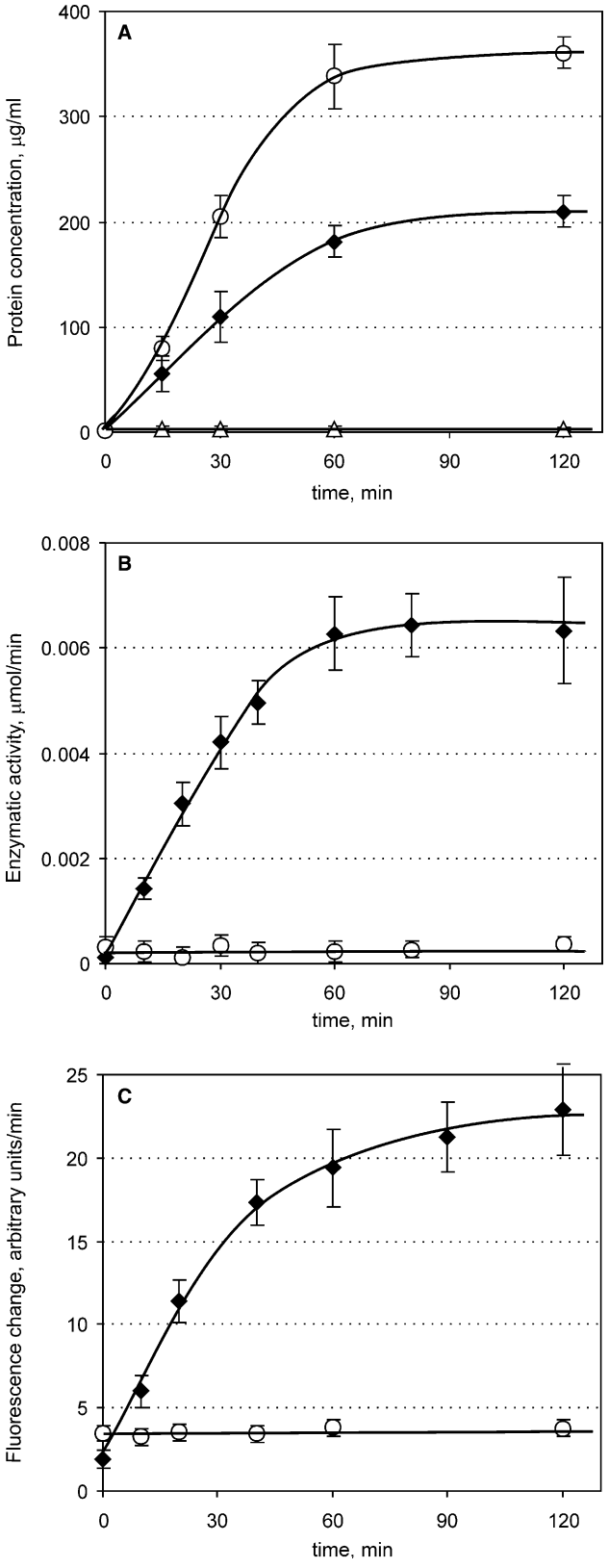

Fig. 1. In vitro synthesis of the esterase from *A. acidocaldarius* in *E. coli* translation system. The templates for the protein biosynthesis were: open circles, EF-Ts gene; filled squares, pEst2; open triangles, control without template. (A) Accumulation of newly synthesized protein measured by [^{14}C]leucine incorporation. (B) Activity of the esterase determined by hydrolysis of *p*-nitrophenyl acetate. (C) Activity of the esterase determined by hydrolysis of 5-(and 6-)carboxy-2′,7′-dichlorofluorescein diacetate.