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

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Received 7 December 2004; revised 24 February 2005; accepted 24 February 2005  
Available online 10 March 2005

Edited by Christian Griesinger

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 enzyme to monitor the expression of polypeptides coupled to its N-terminus and simultaneously, as a cleavable tag for polypeptide purification. 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

2. Materials and methods

2.1. Materials

Tag 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-Nitrophenol acetate and β-Naphthyl-acetate were from Fluka (Steinheim, Germany), 5-(and 6-)Carboxy-2′,7′-dichlorodihydrofluorescein diacetate was from Molecular probes (Eugene, USA). Other analytical grade chemicals were obtained from Roth (Karlsruhe, Germany). Radioactive [¹⁴C]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'-CCATGGGCGCTCGATCCC GTCATTACGC-3') and Est2_rev (5'-GAGCTCCTAGGGCCAGCC GTCTCG-3'). The primers create the Neol and the SauI 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'-CCGG CCGCC TTT GTCAGGCTGTCGAC-3'). The primers create the Neol and the NorI 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). The plasmid was transformed with primers Est2CT_for (5'-GAGCTCGGTACCATGAGGGTCCGGTGTCGCGGCGGTGGTATGG CGTTCCGGC-3') and Est2CT_rev (5'-GGATCTCAGGCCAG CGC-3'). The primers create the SauI and the BamH1 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 [¹⁴C]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 \( \lambda_{\text{ex}} \) at 500 nm and \( \lambda_{\text{em}} \) 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 \( \beta \)-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 \( \lambda_{\text{ex}} = 488 \) 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 CaCl₂, 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 [14C]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](image-url)
The in vitro produced esterase possesses high enzymatic activity (Fig. 1B). Thus, even thousand fold dilution of the in vitro synthesized esterase in the assay mixture, which results in $10^{-8}$ M final esterase concentration, provides well-detectable initial rates of the enzymatic activity. In contrast, the level of esterase activity in the absence of esterase gene is very close to the background (Fig. 1B) providing evidence for the absence of endogenous E. coli esterase activity in the translation system. Besides the standard photometric detection the fluorescence measurement of the esterase activity was carried out. Enzymatic hydrolysis of the 5-(and 6-carboxy-2,7'-dichlorofluorescein diacetate by the esterase leads to appearance of the fluorescent product (Fig. 1C). Kinetics of esterase production detected by fluorescence coincide with ones determined photometrically or by polypeptide-incorporated radioactivity. The SDS–PAGE of the total protein from the reaction mixture with subsequent detection of radioactivity distribution and staining of the gel for esterase activity were also performed for in vitro synthesized esterase in comparison with EF-Ts. The protein samples analyzed by SDS–PAGE contain many endogenous E. coli proteins (Fig. 2A), which are, however, not labeled with [14C]leucine (Fig. 2B). The autoradiogram of the gel (Fig. 2B) reveals distinct bands at the position of the esterase ($M_w \sim 34.4$ kD) and of the control protein EF-Ts ($M_w \sim 31.6$ kD) with more than 90% of incorporated radioactivity belonged to the full-length products in both cases. The faster migrating bands are probably incomplete polypeptides translated from truncated mRNAs. The in situ activity staining of the esterase in polyacrylamide gel detects only one band that corresponds to the full-length esterase. This is in contrast with the lack of esterase activity in the lines related to EF-Ts and in the control without template (Fig. 2C).

As demonstrated in experiments shown in Fig. 3 the esterase from A. acidocaldarius can be used as an affinity tag for purification of protein esterase fusions. The synthesis of
eGFP-esterase fusion protein is shown by SDS-PAGE and autoradiography of the [14C]leucine labelled protein (Fig. 3A, lane 1). Trifluoromethyl-alkyl ketones are efficient competitive inhibitors of the esterases with the inhibition constant in nM range [13]. Immobilized trifluoromethyl-alkyl ketones can be, therefore, used for affinity purification of esterases [12]. After addition of TFK-Sepharose to translation mixture the eGFP-esterase is almost completely removed from the supernatant (Fig. 3A, lane 2). Cleavage of eGFP from affinity matrix was achieved via the build-in protease sensitive linker by Factor Xa protease. Therefore, after this step the eGFP polypeptide appears in the supernatant (Fig. 3A; lane 3). For release of esterase from affinity matrix harsh conditions (95 °C, 1% SDS) had to be used. In the Fig. 3B the esterase activity and florescence of eGFP are demonstrated in different fractions of the affinity-purification steps. The synthesized fusion protein possesses both activities (Fig. 3B; bar 1). After treatment with the TFK-Sepharose the supernatant has strongly diminished esterase activity and low eGFP fluorescence due to immobilization of the fusion protein (Fig. 3B; bar 2). After protease cleavage the fluorescent eGFP appears in the supernatant whereas the esterase, as expected, remains attached to the matrix. Correspondingly, no esterase activity can be detected in the supernatant after protease treatment (Fig. 3B; bar 3).

4. Discussion

Several reporter proteins such as GFP [2], firefly luciferase [3], dihydrofolate reductase [4], chloramphenicol acetyl transferase [5] and β-galactosidase [6] are used to monitor translation. However, these reporters have some disadvantages that confine their practical use. They originate from mesophilic organisms and therefore possess no thermostability [14, 15]. GFP requires time for maturation [2]. Detection of chloramphenicol acetyl transferase activity is complicated by the requirement of radioactive substrate or HPLC analysis [16]. On the other hand, the esterase from A. acidocaldarius as a reporter group for monitoring protein biosynthesis possesses all necessary features required for such a purpose. The active enzyme can be translated in vitro using E. coli system with relatively high yield (200 pg/ml), without any codon usage adjustment. The activity of the produced esterase can be monitored directly in the translation mixture. The photometric assay presented in Fig. 1B allows the detection of 10–12 mol of esterase in 100 µl assay volume. Application of micro plates allows to increase this detection limit by a factor of 10–100, reaching the sensitivity comparable with radioisotope labeling. The utilization of carboxyfluorescein diacetates as the esterase substrates allows fluorescent detection (Fig. 1C) that can be used for various applications in cell biology. For example, a fusion of the esterase with polypeptides allows the cellular localization by confocal microscopy. A remarkable feature of the esterase from A. acidocaldarius is its fast folding into a stable, active, single domain structure [8]. This allows a refolding and detection of the esterase activity in polyacrylamide gels after SDS electrophoresis and removal of the SDS. The sensitivity of this activity detection is well comparable with the sensitivity of the detection of 14C-labelled proteins by autoradiography (Fig. 2). Stability of the esterase from A. acidocaldarius at wide temperature range (10–75 °C) and activity over a broad pH (5–8) [7] may allow its wide applications as a reporter enzyme. The presence of the esterase on protein C-terminus allows using it for both the purification and the detection of recombinant proteins at the same time. As demonstrated in present communication, the esterase can be fused with a target polypeptide resulting in a product that possesses activities of both conjugated proteins (Fig. 3, bars 1). The presence of the esterase on the C-terminus of the target protein permits quantitative determination of expression level by esterase activity measurement since esterase translation is possible after completion of a target gene. This could be used for a rapid optimization of in vitro or in vivo expression conditions. Then the recombinant proteins can be one step isolated by esterase inhibitor based affinity chromatography. The esterase from A. acidocaldarius combines the properties of the robust and sensitive reporter protein along with the high affinity binding tag in one molecule.

Acknowledgments: This work was supported by Deutsche Forschungsgemeinschaft, DFG Sp 243/12-1, the Alexander von Humboldt Foundation (fellowship to D.E.A.), Fonds der Chemischen Industrie and the Bavarian HTO initiative. We thank Dr. G. Manco for providing the plasmid with the esterase gene and Dr. W. Stiege, RnNA GmbH, for supplying us with the in vitro transcription/translation kits. We thank Drs. A. Wolfrum and S. Voertler for discussion and comments and S. Kacmar for preparation of TFK matrix.

References


