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Esterase 2 from *Alicyclobacillus acidocaldarius* as a reporter and affinity tag for expression and single step purification of polypeptides

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Abstract

A novel dual function (reporter and affinity) tag system has been developed. Expression vectors have been constructed to express polypeptides in *Escherichia coli* cells as C-terminal fusions with esterase 2, a 34-kDa protein from *Alicyclobacillus acidocaldarius*. Presence of esterase allows to monitor the expression of fusion proteins spectrophotometrically or by activity staining in the polyacrylamide gels. The fusion proteins can be purified from crude bacterial extracts under non-denaturing conditions by one step affinity chromatography on Sepharose CL-6B immobilized trifluoromethyl-alkyl-ketone. The esterase carrier can be cleaved from fusion proteins by digestion with amino acid sequence-specific proteases blood coagulation factor Xa. The system has been used successfully for the expression and purification of polypeptides from different prokaryotic and eukaryotic organisms.

Keywords: Esterase; Reporter enzyme; Affinity tag; Trifluoromethyl-alkyl-ketone

The simple, rapid purification and isolation of target proteins from complex biological mixtures remains one of the challenging tasks in biochemistry and biotechnology. Genetic engineering techniques allow the *in vitro* construction of gene fusions with proteinous tags for the purpose of affinity purification of recombinant proteins. To date, a large number of different gene fusion system, ranging in molecular size from only a few amino acid residues to large binding proteins have been described to serve as affinity tags for purification of *in vivo* expressed proteins [1]. These systems use different types of interaction, such as enzyme-substrate, bacterial receptor-serum protein, polyhistidines-metal ion and antibody–antigen. However, in most of commonly used affinity fusion systems such as polyhistidines, glutathione *S*-transferase, streptavidin and cellulose binding domain [2–5], the fusion proteins can only be detected by immunological methods. Therefore, the expression of fusion proteins is difficult to be followed in real time.

Here, we describe an affinity fusion system based on the esterase from thermophilic bacterium *Alicyclobacillus acidocaldarius* [6], which serves at the same time as an affinity tag and as a reporter to monitor the extent of expression. The 34 kDa esterase 2 is a thermostable, single chain polypeptide that folds into a one domain structure with one active center that possess a lipase-like Ser-His-Asp catalytic triad [7]. The overall fold is typical for α/β hydrolases [7] with N- and C-terminal ends of the protein exposed on the esterase surface and not involved in catalysis or structure stabilization. This provides the possibility to link the esterase with other polypeptides without affecting the native fold and the enzymatic activity.

Trifluoromethyl-alkyl-ketone (TFK)[1] derivatives have been shown to inhibit esterases. They are thought to mimic

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[1] *Abbreviations used:* TFK, trifluoromethyl-alkyl-ketone; TFK-Sepharose, trifluoromethyl-alkyl-ketone Sepharose CL-6B; Est2, esterase 2; Nox, NADH oxidase; peGFP-Est2, pIVEX2.3d-eGFP-Est2; eGFP, enhanced green fluorescence protein.

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the transition state of ester hydrolysis by forming a hemiketal linkage with the putative serine at the enzyme's active site. Accordingly, the trifluoromethyl-alkyl-ketone Sepharose CL-6B (TFK-Sepharose) is a suitable matrix to purify esterase in single step affinity chromatography [8].

Materials and methods

Materials

Pfu DNA polymerase was from Promega (Mannheim, Germany), *Taq* DNA polymerase was from Peqlab (Erlangen, Germany), T4-DNA-Ligase, 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). 3-Bromo-1,1,1-trifluoro-propan-2-one and CelLytic™ B bacterial cell lysis extraction reagent were from Sigma (Taufkirchen, Germany). NADH and FAD were from Serva (Heidelberg, Germany). Other analytical grade chemicals were obtained from Merck (Darmstadt, Germany).

Plasmid construction

Plasmid pT7SCII, containing the gene of the esterase 2 (Est2) was kindly provided by G. Manco, Napples, Italy. The gene was amplified by PCR with the primers Est2CT_for (5′-GAGCTCGGTACC**ATTGAGGGTCGC**GGTTCCGGCGGTGGTATGGCGCTCGATCCC-3′) and Est2CT_rev (5′-GGATCC**TCA**GGCCAGCGC-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 pIVEX2.3d vector through *Sac*I and *Bam*HI cleavage sites. The resulting plasmid pEst was used as a parental expression vector for further cloning.

The gene of NADH oxidase (Nox) from *Thermus thermophilus* was amplified from the plasmid pTthnadox310 [9] by PCR with the primers Nox_for (5′-CATATGGAGGCGACCCTTCCCGTTTTG-3′) and Nox_rev (5′-GAGCTCGCGCCAGAGGACCACCCGCTCCAGGG-3′). The primers introduce the *Nde*I and the *Sac*I cleavage sites (underlined letters) upstream and downstream of the Nox, respectively. The PCR product was sequenced and cloned into the pEst2 vector resulting in pNox-Est plasmid.

The pIVEX2.3d-eGFP-Est2 (peGFP-Est2) was constructed as follows. The gene of enhanced green fluorescence protein (eGFP) from the bioluminescent jellyfish *Aequoria victoria* was amplified from the plasmid pSL1180-eGFP (provided by G. Krauss, Bayreuth) by PCR with the primers eGFP_for (5′-CCATGGTGAGCAAGGGCG-3′) and eGFP_rev (5′-GCGGCCGCCTTTGTACAGCTCGTCCAT-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 pEst2 vector resulting in the peGFP-Est plasmid.

The vectors pNox-Est and peGFP-Est were transformed into *Escherichia coli* BL21 (DE3) (Novagen Merck Bioscience, Schwabach, Germany) for fusion protein expression.

*Preparation of soluble TFK inhibitor 3-butylsulfanyl-1,1,1-trifluoro-propan-2-one (**3**) and TFK-modified Sepharose (**4**)*

3-Bromo-1,1,1-trifluoroacetone (**2**) (0.5 ml, 0.92 g, 4.8 mmol) was dissolved in 15 ml CH_2Cl_2 under nitrogen atmosphere. 10 ml mixture of butanethiol (**1**) (0.46 ml, 0.39 g, 4.3 mmol) and pyridine (0.34 ml, 0.34 g, 4.3 mmol) in CH_2Cl_2 was added dropwise into ice-cooled solution of 3-brom-1,1,1-trifluoroacetone. The resulting reaction mixture was stirred for further 1.5 h at 0 °C, then diluted with 20 ml CH_2Cl_2, washed with 50 ml 1 M HCl, following by 50 ml saturated NaCl. Organic layer was separately dried over Na_2SO_4, filtered and concentrated. Flash column chromatography (hexane/ethylacetate = 5:1) afforded pure product 3 as colorless oil in 74% yield (0.64 g).

^{1}H NMR (270 MHz, $CDCl_3$): 0.90 (t, 3H), 1.38 (m, 2H), 1.55 (m, 2H), 2.49 (t, 1H), 2.69 (t, 1H), 2.88 (s, 1H), 3.46 (s, 1H); ^{13}C NMR (90 MHz, $CDCl_3$): 13.59, 21.82, 30.65, 33.37, 37.83, 92.72, 124.98; EI-MS, *m*/*z* (%): 200 $[M]^+$ (65), 157 (8), 127 (12), 129 (14), 103 (53), 88 (35), 61 (100).

Expression of fusion proteins in E. coli

An overnight culture of *E. coli* BL21 (DE3) transformed with corresponding plasmids was diluted 1:200 in 1 L of fresh LB medium and grown at 37 °C. One milliliter culture was collected each hour for determination of the expression rate. The cells were harvested by centrifugation at 10,000*g* for 1 min and the pellets were resuspended in 100 μl CelLytic™ B bacterial cell lysis extraction reagent. After 2 min vigorous stirring (Vortex), the cell debris were pelleted by centrifugation at 14,000*g* for 5 min. The supernatant was carefully transferred to an Eppendorf tube. The expression of fusion protein was monitored by esterase activity assay [6].

Affinity purification of NADH oxidase-esterase 2 fusion protein (NADOX-EST2)

Cells with NADOX-EST2 expression were harvested and resuspended in 10 ml of buffer A (50 mM Tris–HCl pH 7.5, 300 mM NaCl, 1 mM 2-mercaptoethanol, 1 mM EDTA, 5% glycerol). The cells were extracted by ultrasonication and subjected to centrifugation at 20,000*g* for 15 min at 4 °C. The supernatant was loaded on the 5 ml TFK-Sepharose column (1 × 1 cm) [10] which was equilibrated with buffer A. The column was then washed to the baseline by buffer A. The fusion protein was eluted by 5 ml buffer A containing 20 mM of soluble inhibitor **3**.