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Protein Expression and Purification 45 (2006) 368–373
Protein Expression & Purification
www.elsevier.com/locate/yprep

Identification of a thermostable and enantioselective amidase from the thermoacidophilic archaeon *Sulfolobus tokodaii* strain 7

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Received 17 May 2005, and in revised form 24 June 2005
Available online 2 August 2005

Abstract

We have characterized an amidase expressed from the putative amidase gene (*ST0478*) selected from the total genome analysis from the thermoacidophilic archaeon, *Sulfolobus tokodaii* strain 7. The ORF was cloned and expressed as an insoluble aggregated 6× His-tagged fusion protein in *Escherichia coli*. The protein was purified with denaturing, refolding on affinity column chromatography, size exclusion filtration, and heat treatment. The enzyme exhibited high thermostability and the optimum activity for amide cleavage against benzamide was observed at around 75 °C and pH 7.0–8.0. It also showed enantioselectivity for (*R,S*)-2-phenylpropionamide and preferentially hydrolyzed the *S*-enantiomer. This novel enzyme is the second characterized archaeal amidase.

Keywords: Thermostable amidase; Archaeon; Recombinant fusion protein; High-throughput refolding; Enantioselectivity

Amidases (EC 3.5.1.4) are widespread in various different organisms and they catalyze the hydrolysis of amides into the corresponding carboxylic acids and amines. The importance of hydrolases in biotechnology is growing rapidly due to their expanding applications in chemical and pharmaceutical industries for the production of optically pure compounds [1–4]. Moreover, from the industrial point of view, processes at elevated temperature bring many advantages, such as higher reaction rate, reduced contamination, and higher substrate solubility. Therefore, such enzymes that have relatively high stability at elevated temperatures both in aqueous medium and organic media are desirable for industrial use. Further, these enzymes are excellent subjects for investigation of the molecular basis of protein thermostability.

As for screening of thermostable enzymes, extreme thermophilic archaea are considered to be the most promising candidates [5–7]. Archaea represent third domain of life, in addition to well-known eukarya and bacteria [8]. Recently, several archaeal esterases have been isolated and characterized, and are shown to possess some interesting properties [9–13]. However, a variety of hydrolases from thermophiles still need to be investigated to analyze the characteristics under high temperature. Moreover, as far as amidases are concerned, only a few of them have been reported to show thermophilic properties, including bacterial cases [14–17].

Sulfolobus tokodaii strain 7 is an aerobic thermoacidophilic crenarchaeon with the optimum growth temperature at 80 °C, at low pH [18]. The sequence analysis of the whole genome was completed at the National Institute of Technology and Evaluation (Tokyo, Japan) [19]. Here, we report the characterization of an amidase expressed from the putative amidase gene (*ST0478*, encoding 396 amino acids) selected from the total genome analysis of the strain.

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doi:10.1016/j.pep.2005.06.017

Materials and methods

Strains, plasmids, and chemicals

Thermoacidophilic *S. tokodaii* strain 7 was obtained from the Japan Collection of Microorganisms (JCM 10545). *Escherichia coli* TOP10, BL21(DE3), BL21 Star(DE3), and the cloning vector pET101 (pET101 Directional TOPO Expression kit, for C-terminal 6× His tagging) were purchased from Invitrogen (Carlsbad, CA, USA). Furthermore, *E. coli* BL21-CodonPlus(DE3)-RIL (Stratagene, La Jolla, CA, USA), and six strains of (DE3) Competent Cell Set 2 (Novagen, Madison, WI, USA) were used as the additional host cells. Benzamide and other chemicals were commercially available (Aldrich Chemical, Milwaukee, WI, USA; Kanto Chemical, Tokyo, Japan; Tokyo Kasei Kogyo, Tokyo, Japan; Wako Pure Chemical industries, Osaka, Japan). Racemic 2-phenylpropionamide was synthesized chemically based on literature method [20].

Cloning and expression of the S. tokodaii ST0478 gene in E. coli

Plasmid named pETstAmiC carrying *ST0478* gene was constructed by introduction of the 1.2 kbp region into cloning vector pET101. The DNA fragment was obtained by PCR amplification, using the extract of the boiled culture of *S. tokodaii* as a template. For PCR amplification, two synthetic oligonucleotides (5′-**CACC**ATGAAGATGTCATTAGAAGAATTAAATACAAAATAC-3′) and (5′-CTTAATAAAACCAACAATTCCCATTATTTCCTCTGC-3′) were used as the forward and reverse primers, respectively. The overhang sequence, boldfaced CACC, for directional TOPO vector was introduced at the 5′ end of the forward primer. PCR was carried out with the KOD plus DNA polymerase (Toyobo, Osaka, Japan). The obtained PCR product was introduced into pET101 by the topoisomerase reaction, resulting in the formation of plasmid pETstAmiC. A plasmid subcloning experiment was performed in *E. coli* TOP10, and the sequence was confirmed using a DNA sequencer (Applied Biosystems, Foster City, CA, USA).

Escherichia coli BL21-CodonPlus(DE3)-RIL was transformed with pETstAmiC and cultivated at 30 °C in Luria–Bertani medium containing 50 μg/ml ampicillin (up to OD = 0.6). Then, isopropyl-β-D-thiogalactopyranoside (0.1 mM at final concentration) was added for induction, and cultivation was kept for additional 5 h. The resulting broth (1 L from five flasks) was centrifuged at 4700*g* for 30 min at 4 °C.

Purification of the recombinant protein from E. coli

The recombinant protein corresponding to the *ST0478* expression product was renatured and purified as a 6× His-tagged fusion from its insoluble inclusion bodies. The harvested recombinant *E. coli* BL21-CodonPlus(DE3)-RIL cells from 1 L culture were suspended in 50 ml of 20 mM phosphate buffer (pH 7.4). The cells were disrupted by sonication for 15 min on ice, and the insoluble pellet was collected by centrifugation (15200*g*, 10 min, 4 °C). The pellet was solubilized in 50 ml of 20 mM phosphate buffer containing 0.5 M NaCl, 10 mM imidazole, and 6 M guanidine–HCl (pH 7.4). After centrifugation (15200*g*, 30 min, and 4 °C) and filtration of its supernatant, an aliquot of the solution corresponds to 50 ml culture was applied to a HiTrap Chelating HP (Amersham Biosciences, Uppsala, Sweden) Ni^{2+} chelating affinity column equilibrated with the phosphate buffer mentioned above. After washing the column with the same buffer, the stepwise reduction of guanidine–HCl concentration (from 6 to 0 M) was performed as the refolding strategy for the protein binding on the column. Finally, the 6× His-tagged recombinant protein was eluted with 20 mM phosphate buffer containing 0.5 M NaCl and 0.5 M imidazole (pH 7.4). The fractions containing amidase (estimated via SDS–PAGE band intensity) were collected and concentrated using an Amicon Ultra-15 centrifugal filter device (Millipore, Bedford, MA, USA). The enzyme solution was incubated at 60 °C for 25 min, followed by brief centrifugation to remove the denatured proteins. Protein concentration was determined by the method of Bradford [21] with the protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA), and bovine serum albumin (BSA) was used as the standard. Excluding heat treatment, the purification procedures were carried out at 4 °C as much as possible. The concentrated enzyme solution was used for the characterization experiments.

Gel electrophoresis, Western blot analysis, and gel filtration analysis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)[1] was performed according to the procedure of Laemmli [22]. Proteins were revealed by staining with Coomassie brilliant blue R-250. Purified enzyme (the recombinant 6× His-tagged fusion protein) was separated by SDS–PAGE and blotted onto a polyvinylidene difluoride (PVDF) membrane. Western blot was carried out with anti-His (C-term) antibody and was developed with alkaline phosphatase-conjugated anti-mouse IgG using a 5-bromo-4-chloro-3-indolyl-1-phosphate/nitro blue tetrazolium (BCIP/NBT) staining immunodetection kit (Invitrogen). The molecular mass of the native enzyme was estimated by analytical gel

[1] *Abbreviations used:* SDS–PAGE, Sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; BCIP/NBT, 5-bromo-4-chloro-3-indolyl-1-phosphate/nitro blue tetrazolium; TFA, trifluoroacetic acid.