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Purification and characterization of a new inducible thermostable extracellular lipolytic enzyme from the thermoacidophilic archaeon *Sulfolobus solfataricus* P1

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ABSTRACT

A new thermostable extracellular lipolytic enzyme, induced from the thermoacidophilic archaeon *Sulfolobus solfataricus* P1 using corn oil as an inducer, was purified to apparent homogeneity by butanol extraction and two column chromatographies using DEAE-Sepharose followed by Butyl-Sepharose. The purified enzyme assessed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and gel filtration was approximately 45 kDa and monomeric. The maximal activity examined using *p*-nitrophenyl palmitate as a substrate was observed at 98 °C and pH 6.0. The enzyme showed remarkable thermostability: It retained 51% of its activity after 120 h at 80 °C. In addition, the enzyme displayed extremely high stability against water-miscible alcohols, SDS, and urea, even at high concentrations. This high stability of the enzyme against protein-denaturing agents indicates a high potential in industrial applications. The enzyme has broad substrate specificity, exhibiting not only carboxylesterase activity toward short-chain acyl esters but also lipase activity toward long-chain acyl esters including triacylglycerols regardless of saturated and unsaturated fatty acids. The k_{cat}/K_m ratios of the enzyme for *p*-nitrophenyl palmitate (C_{16}), the most preferable substrate among all tested, was 93.4 s^{-1} μM^{-1}. Together, it was identified by thin-layer chromatography (TLC) that the enzyme can hydrolyze all positions of the three ester bonds in triolein. The enzyme is a serine esterase belonging to the α/β hydrolase family containing a typical catalytic triad composed of serine, histidine, and aspartic acid in the active site of the enzyme. The enzyme is the first purified inducible extracellular lipolytic enzyme from archaea.

1. Introduction

Lipolytic enzymes are ubiquitous enzymes existing widely in animals, plants, and microorganisms [1]. They include two major groups, carboxylesterases (EC 3.1.1.1) and lipases (EC 3.1.1.3), which show a preference toward short-chain acyl esters (≤C10) and long-chain acyl esters (≥C10) containing triacylglycerols, respectively. Most of them belong to the α/β hydrolase family containing a characteristic Ser-Asp-His catalytic triad and the most conserved pentapeptide sequence, GXSXG (where X is any residue) around the active-site serine [2].

Lipolytic enzymes play an important role as biocatalysts for biotechnological applications. Bacterial lipolytic enzymes, particularly thermophilic and extracellular lipolytic enzymes, are attracting enormous attention because of their applications in industry, such as medical biotechnology, detergent production, synthetic chemistry, flavor and aroma synthesis, and other food-related processes [3,4]. Moreover, the enzymes from hyperthermophilic archaea, which inhabit extremely high-temperature environments, have recently attracted increasing attention due to their several possible applications. In fact, their exceptional stability to denaturing agents is specifically useful for industrial biotransformation applications. In addition, they could provide new study models for both evolutionary clarification of the function and basic elucidation of the structure–stability relationship of this class of protein [5,6]. *Sulfolobus solfataricus* (*S. solfataricus*) P1 used in this study belongs to the thermoacidophilic archaea, which is isolated from sulfur-rich volcanic hot springs and grows at around 80 °C and pH 4 [7].

So far, only few lipolytic enzymes have been characterized from archaea in contrast to the large numbers from bacteria. Most of the archaeal lipolytic enzymes are intracellular carboxylesterases, such

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as *S. acidocaldarius* [8–10], *S. solfataricus* P2 [11,12], *S. solfataricus* P1 (DSM 5354) [13], *S. solfataricus* P1 (DSM 1616) [14,15], *S. solfataricus* MT4 [16], *Pyrobaculum calidifontis* [17], *Archaeoglobus fulgidus* [18], and *Pyrococcus furiosus* [19]. On the other hand, a few extracellular enzymes showing lipolytic activity have been reported from halophiles such as *Haloarcula marismortui* [20] without any addition of inducer and from thermoacidophilic *Sulfolobus shibatae* [21] with the addition of Tween compounds as an inducer in culture medium. However, there have been no reports on the purification of their enzymes.

In this study, we describe the purification and characterization of a new inducible extracellular thermostable lipolytic enzyme from *S. solfataricus* P1 (DSM 1616). The enzyme was induced using 0.5% corn oil as an inducer and purified from culture medium, and then its various biochemical properties were examined. This is the first report on the purification of the inducible extracellular lipolytic enzyme from archaea.

2. Materials and methods

2.1. Strains, growth conditions, and chemicals

S. solfataricus P1 (DSM 1616) was purchased from American Type Culture Collection (ATCC). *S. solfataricus* P1 was aerobically grown at 75 °C, pH 4.0 in a 5-l fermentor with moderate stirring (120 rpm). The growth medium containing 0.2% (w/v) yeast extract and mineral bases was used according to the description of ATCC media formulations. Cells were harvested by centrifugation (4000 × *g*) for 45 min at 4 °C. For the induction of the extracellular lipolytic enzyme from the *S. solfataricus*, the harvested cells from 15-l cultivation in the yeast extract medium were suspended into the new medium (1 l, pH 4.0) containing 0.5% (w/v) glucose, using the mineral bases and 0.5% (v/v) corn oil as an inducer. The suspended cells were further cultured aerobically at 75 °C for 24 h. The induction by corn oil was designed by modifying the method of Huddleston et al. who used Tween compounds (*e.g.*, Tween 20, 40, 60, and 80) as inducers for the induction of the extracellular esterase from *S. shibatae* [21]. In this study, the inductions by 0.5% (v/v) olive oil and 0.2% Tween compounds (Tween 20, 60, and 80) were also examined to compare them with that by 0.5% corn oil using the same method as described above. In addition, the inductions of the extracellular lipolytic enzyme from *S. solfataricus* by three kinds of Tween compounds (Tween 20, 60, and 80) as inducers to compare with those of the extracellular esterase from *S. shibatae* were examined after cultivation for 24, 48, 72, and 96 h. The general yeast medium containing *S. solfataricus* without an inducer and 0.5% glucose medium containing an inducer without the *S. solfataricus* P1 strain were used as controls after cultivation under the same conditions. All chemicals used in this study were purchased from Sigma Chemical Co., Ltd. unless otherwise stated.

2.2. Purification of extracellular lipolytic enzyme

All purification steps were performed at room temperature unless otherwise stated. The existence of the induced extracellular lipolytic enzyme in each fraction during purification procedures was traced preferentially by the formation of a clear zone on a tributyrin-emulsified agar plate and further identified by the methods of activity staining and enzyme assay as described below. After cultivation in an inducible growth medium, cells were precipitated by centrifugation (6000 × *g*) for 30 min at 4 °C. When olive oil or corn oil was used as an inducer in a culture medium, the supernatant contained a lipid layer upon a medium layer that was transferred to the separating funnel and left to stand for a while until the upper lipid layer was clearly separated.

The lipid composition of the upper lipid layer separated from the lower medium layer using the funnel was later analyzed on a thin-layer chromatography (TLC) plate after the lipid extraction from the layer. The lower medium layer containing the induced extracellular lipolytic enzyme was collected from the separating funnel. The purification of the induced extracellular lipolytic enzyme from the collected medium layer was performed after butanol extraction to remove any possible hydrophilic (short-chain fatty acids) and/or micellar products produced by the enzyme reaction that could disturb the purification by the following column chromatography. The butanol extraction was done in another funnel by the addition of 10 mM sodium phosphate buffer (buffer A, pH 6.0)-saturated 1-butanol to the medium layer at the ratio of 1:1. The funnel containing two solutions was shaken vigorously to ensure it was mixed well and left to stand until the upper butanol layer was separated spontaneously.

The 1-butanol of the upper butanol layer was evaporated using a rotary vacuum evaporator (EYELA, USA) for the TLC analysis of the lipid products in it. The lower medium layer containing the enzyme was collected from the funnel for the purification of the enzyme. The collected lower solution containing the enzyme was dialyzed twice to remove the remaining butanol against the 5 l of buffer A for 16 h. The dialyzed enzyme solution was applied to a column of DEAE-Sepharose (5.5 by 30 cm) equilibrated with buffer A and eluted with 600 ml of buffer A to remove unbound proteins to an anion exchanger (DEAE). Subsequent elution was performed with continuous linear gradients using 720 ml of buffer A containing 0–0.15 M and 0.15–0.3 M NaCl, and finally with 240 ml of buffer A containing 0.3 M NaCl. The fractions containing the lipolytic enzyme activity were collected and combined. The final salt concentration of the combined protein solution was adjusted to 2 M by powdered NaCl, loaded onto a Butyl-Sepharose column (3.0 × 15 cm) equilibrated with buffer A containing 2 M NaCl, and then washed with 500 ml of buffer A containing 2 M NaCl to remove unbound proteins. After washing, the bound proteins were eluted with 500 ml of buffer A containing 0 M NaCl. Subsequent elutions were performed with a series of step gradients using 150 ml of buffer A containing 30%, 40%, 50%, 60%, and 70% (v/v) ethylene glycol. The fractions showing lipolytic activity on a tributyrin-emulsified agar plate, and having a single band on a sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) gel, were collected and dialyzed twice to remove ethylene glycol against 5 l of buffer A for 18 h. The dialyzed protein solution was used as the purified enzyme solution. The protein concentration was determined by the method of Bradford [22], using bovine serum albumin as the standard.

2.3. Identification and determination of molecular mass

The molecular mass of the purified extracellular lipolytic enzyme was determined on 12.5% SDS-PAGE gel with and without mercaptoethanol in the sample buffer using the low molecular mass standards [23]. Gels were stained by silver [24]. The molecular mass of the native protein was also determined by high performance liquid chromatography (HPLC) [GPC column (Protein-Pak^{TM} 300S, 7.8 × 300 mm, Waters)] using the molecular mass standards (Sigma MW-GF-200 kit).

2.4. Enzyme assays and activity staining

A standard enzyme assay was carried out using an artificial chromogenic substrate, *p*-nitrophenyl (PNP) palmitate (C_{16}) [14,25]. The enzyme reaction was started by the addition of 0.1 ml of the purified extracellular lipolytic enzyme solution (0.6 μg/ml) to 0.1 ml of freshly prepared and prewarmed PNP-palmitate solution (5 mM) as a substrate and 0.8 ml of prewarmed