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Enhanced Activity and Enantioselectivity of a Hyperthermophilic Esterase from Archaeon *Aeropyrum pernix* K1 by Acetone Treatment

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Abstract To improve the activity and enantioselectivity of hyperthermophilic archaeon *Aeropyrum pernix* K1 esterase (APE1547) and its mutants, they were purified by acetone-treated method. It was found that the acetone treatment not only caused APE1547 and its mutants to display higher activity and enantioselectivity but also saved more than 90% of time spent in purifying them by Ni-chelating column. In hydrolysis of *p*-nitrophenyl caprylate, the acetone-treated APE1547 and mutant A containing the following substitutions R11G, L36P, V225A, I551L, and A564T showed 5.7- and 6.9-fold active increase, respectively. In the resolution of 2-octanol acetate, the acetone-treated mutant A had a 9-fold enantioselective increase relative to that purified by Ni-chelating column. In addition, the impact of pH, temperature, and chemical reagents on activity of APE1547 and mutant A was discussed in this paper.

Keywords Catalytic activity · Enantioselectivity · Hyperthermophilic esterase · 2-Octanol acetate · Acetone treatment

Introduction

Hyperthermophilic esterases are a class of recently developed biocatalysts with high catalytic efficiency, substrate specialty, and thermostability, etc., which means that they have more potential in industry than mesophilic and psychrophilic enzymes [1–3]. So many efforts have been made to further optimize their property. Among them, genetic engineering was an available strategy to modify hyperthermophilic enzymes and endow them with super ability against high temperatures and organic reagents, e.g., decontaminants and denaturants, so as to be free of denaturation and to be feasibly employed in food, medicine,

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leather treatment, oil exploitation, waste disposal, etc. [4]. Although only a few successful attempts have been reported in the exploration of thermostability and industrial application of hyperthermophilic esterases, we have successfully developed a method to select mutants from a pool of mutants obtained by the site-directed mutation with focus on improving activity and enantioselectivity of esterase APE1547 [5]. Its mutants showed obviously active improvement than the wild-type protein even if they had less structural change judged by the available model of APE1547 [6, 7]. In order to further improve activity and enantioselectivity of APE1547 and its mutants, here they were treated with acetone.

Improvement of enzymatic activity and enantioselectivity by chemical reagents has been reported by many [8–15], e.g., *Candida rugosa* lipase treated with 2-propyl alcohol [16] or with arylpropionic acid [17]. This was because the enzymatic conformation could be altered and stabilized by some organic reagents to immunize from contamination, which inhibit enzymatic activity and/or reduce enantioselectivity [16]. However, those procedures usually required expensive preparation and exhaustive dialysis to eliminate organic reagents in the end [8–13]. Contrastively, a facile and effective way for improvement of activity and enantioselectivity should be ascribed to the acetone treatment due to its less preparation time [18–21]. So we employed acetone to purify APE1547, mutant A (with sequence analysis), and the other mutants (without sequence analysis) to improve their activity and enantioselectivity and discussed the corresponding mechanism.

Materials and Methods

Materials

The wild-type APE1547, mutant A, and the other mutants were expressed by the method as described in literature [5]; *p*-nitrophenyl caprylate (pNPC) and sodium dodecyl sulfate (SDS) were purchased from Sigma. 2-Octanol acetate was synthesized in our lab. All other reagents were commercially available.

Purification of Esterases

The frozen cells were thawed in 50 mM Tris–HCl buffer (pH 8.0) at room temperature and lysed by sonication for 15 min. The cell suspension was centrifuged at 8,000 rpm for 20 min, and then, the supernatant was subjected to heat shock at 85 °C for 30 min. The supernatant containing crude esterase was centrifuged at 14,000 rpm and 4 °C for 20 min. The obtained crude esterase was dialyzed and then lyophilized to obtain pre-purified esterase, which could be further purified by Ni-chelating column and acetone treatment, respectively.

The lyophilized protein (500 mg) was eluted with six bed volumes of buffer (0.5 M NaCl; 20 mM Tris–HCl, pH 7.9; 100 mM imidazole) in a Ni-chelating column (3 mL), which was pre-eluted orderly with deionized water, five bed volumes of buffer solution (0.8 M $NiSO_4$; 20 mM Tris–HCl, pH 7.9), and three bed volumes of buffer solution (0.5 M NaCl; 20 mM Tris–HCl, pH 7.9). The eluted protein was dialyzed against water and phosphate buffer (50 mM, pH 8.0) successively, lyophilized, and stored at −20 °C. Then the esterase purified by Ni-chelating column was gotten.

On the other hand, the pre-purified esterase (500 mg) was dissolved in phosphate buffer (2.5 mL, 50 mM, pH 8.0) under gentle stirring and at 4 °C, then the acetone (1.25 mL) was slowly dropped into the enzyme solution over 1 h. The precipitate appearing in solution could

be collected by centrifugation at 8,000 rpm for 15 min and further collected by adding more acetone to the supernatant and then centrifuging. The combined protein precipitate was distilled under vacuum and stored at −20 °C, to obtain the esterase purified by acetone treatment.

Assay of Enzymatic Activity

The activity unit was defined as the amount of enzyme needed to hydrolyze pNPC to produce 1 μmol *p*-nitrophenol (pNP). The esterase-catalyzed hydrolysis of pNPC was tracked via monitoring pNP at 405 nm on a HTACHI 557 ultraviolet–visible spectrophotometer equipped with a temperature controller. In the standard assay, the solution of pNPC in acetonitrile (20 μL, 10 mM) was added to the reaction mixture to obtain a final concentration of 0.2 mM in a phosphate buffer (50 mM, pH 8.0), and then, the reaction mixture was incubated at 50 °C or 70 °C.

Enantioselective Analysis

Enzymatic kinetics in hydrolysis of 2-octanol acetate (2%, *v*/*v*) in phosphate buffer (25 mM, pH 8.0, 500 IU) was detected by GC set at 200 °C for injector and 290 °C for detector, from 110 °C to 210 °C as temperature procedure, and at 60 mL/min as flow rate of nitrogen carrier [22]. The two derivatives of (*S*)-2-octanol and (*R*)-2-octanol, got by reacting with (*R*)-1-phenylethyl isocyanate, were analyzed at the temperature procedure from 110 °C to 222 °C [22]. The kinetic conversion (*c*) was obtained based on the decreased peak area of 2-octanol acetate. The enantiomeric excess of products (ee_p) was determined by calculating the peak areas of two derivatives. The enantioselectivity of enzyme in resolution of 2-octanol acetate (*E*) was calculated by the formula: $E=[\ln[1-c(1+ee_p)]/[\ln[1-c](1-ee_p)]$.

Effect of Temperature, pH, and Chemical Reagents on Activity

To determine the effect of temperature on activity, the enzymes were pre-incubated in Tris–HCl buffer (50 mM, pH 8.0) at each different temperature for 30 min. Then each sample was mixed with substrate solution. The enzymatic activity was assayed under the standard condition as described in the section Assay of enzymatic activity. The effect of pH on activity was measured at 70 °C in CH_3COOH–CH_3COONa buffer (50 mM, pH 3.5∼5.5), $Na_2B_3O_7$–HCl buffer (pH 8.0∼9.0), and $Na_2B_3O_7$–NaOH buffer (pH 9.0∼11.0), respectively. All buffers contained 1 mM pNPC as substrate. The assayed enzymes were pre-incubated in the same buffer at 70 °C for 30 min, and then, the remaining activities were measured by the standard assay procedure. The effect of chemical reagents on activity was performed as follows: the tested enzymes were pre-incubated in Tris–HCl buffer (50 mM, pH 8.0) with various chemical reagents at 70 °C for 60 min and then mixed with substrate solution, and the enzymatic activities were assayed under the standard condition.

Results and Discussion

Purification of Recombinant Esterases

During the course of pre-purification, 30% impure proteins in supernatant were denatured by heat treatment at 85 °C for 30 min. The pre-purified enzyme could be further purified by Ni-chelating column or acetone treatment. SDS-polyacrylamide gel electrophoresis (SDS-

PAGE) showed that the purities of target proteins obtained by the two purified methods were almost same (Fig. 1). Moreover, the acetone treatment took less time than the common purification by Ni-chelating column. This was because the acetone treatment took no more than 5 h composed of 2.5 h for precipitation, 0.5 h for centrifugation, and 2 h for vacuum distillation, and the purification by Ni-chelating column took almost 88 h composed of 4 h for column chromatography, 72 h for dialysis, and 12 h for lyophilization. Therefore, the acetone treatment saved more than 90% of time taken in the common purification.

Change of Activity

The enzyme activity was determined by hydrolytic rate of pNPC. Figure 2 showed that almost all of the proteins (wild-type APE1547, mutant A, and other mutants) showed increased activity after acetone treatment even if the activities were different among them. The wild-type APE1547 and mutant A treated twice by acetone had 5.7- and 6.9-fold active increase relative to those purified by Ni-chelating column, respectively. The reason may be that acetone, with a low dielectric constant (21.4 at 20 °C) compared with water (80 at 20 °C), availably increases the interaction in-between electrified protein particles, which promotes protein coagulation and deposition by peeling hydration sheath off the protein and reducing dielectric constant of the solvent. As a result, much of the hydrophobic region of the protein is exposed along with the durational addition of acetone [23], which allows the substrate easy

Fig. 1 SDS-PAGE of APE1547: *1* purified with Ni column, *2* treated twice with acetone, *marker* cell lysate

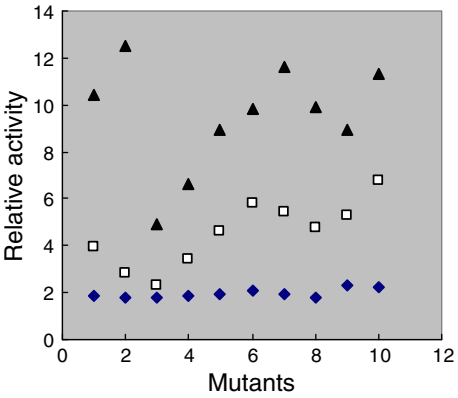

Fig. 2 Activity of enzymes: purified by Ni-chelating column (*violet diamonds*) and by acetone treatment once (*white squares*) and twice (*black triangles*); numbers *1* APE1547, *2* mutant A, and *3–10* mutants without sequence analysis

access to the active center in the hydrophobic region and then causes enzymatic activity increase [10].

Change of Enantioselectivity

Table 1 showed that the mutant A treated twice with acetone had a 9-fold increase for enantioselectivity in resolution of 2-octanol acetate relative to that purified by Ni-chelating column, but the wild-type APE1547 was less sensitive to acetone in terms of enantioselectivity. Based on the earlier reports on improvement of enzymatic enantioselectivity with organic reagents [24, 25], it is presumed that acetone could conveniently cause the hydrophobic core of mutant A to re-pack for the site-directed mutations (R11G, L36P, V225A, I551L, and A564T) [5], which accordingly changed the conformation of active site as described in literature [26, 27]. Another possible reason is that the acetone–carbonyl carbon bonds with the amino nitrogen of one critical residue, which might optimize the protein configuration [21].

Effect of pH, Temperature, and Chemical Reagents

Optimum pH and Temperature

The acetone treatment also shifted the optimum pH of APE1547 and mutant A. The optimum pH of APE1547 purified by acetone treatment and Ni-chelating column was 9.0

Table 1 Enantioselectivity of APE1547 and mutant A in hydrolysis of 2-octanol acetate

Esterase	Method	Specific activity (IU/mg)	*c* (%)	ee_P (%)	*E*
APE1547	Ni-chelating column	15.7	11.3	25.6	1.74
	Acetone treatment twice	5.7	58.0	38.0	3.53
Mutant A	Ni-chelating column	10.9	16.0	52.0	3.47
	Acetone treatment twice (TBC26-AC)	6.9	61.5	62.0	31.2

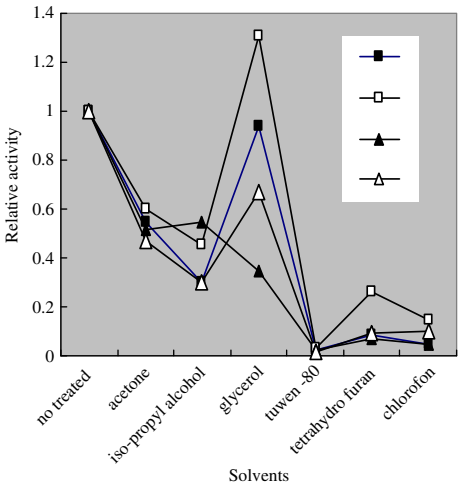

Fig. 3 Effect of solvents on activity of APE1547 (*black squares*), acetone-treated APE1547 (*white squares*), mutant A (*black triangles*), and acetone-treated mutant A (*white triangles*)

and 8.0, respectively. And the optimum pH of mutant A purified by acetone treatment and Ni-chelating column was 9.0 and 8.3, respectively. It was obvious that the acetone treatment brought some increase for their optimum pH. However, their optimum temperatures were not changed by acetone treatment and were still at 98 °C.

Effect of Chemical Reagents

The measurement on activity of all proteins in mixed reaction solutions containing different chemical reagents at 70 °C showed that most of the chemical reagents gave obvious inhibition on both APE1547 and mutant A no matter that they are or are not treated by acetone (Fig. 3). Among them, Tween-80 was the most harmful to enzymatic activity. The just exception was glycerol that gave some increase for the activity of APE1547 treated with acetone. As to the inhibitive mechanism, it needs to be further investigated in the future.

Conclusions

In this paper, it is comprehended that a desired purification method, acetone treatment, was very critical to improve the activity and enantioselectivity of enzymes. The acetone treatment has effectively improved the activity and enantioselectivity of APE1547 and its mutants. This will inspire us to further explore the application of this acetone-treated method in industry.

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