

Stabilization of Lipase from *Thermomyces lanuginosus* with *p*-Chloromercuribenzoic Acid

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Addition of *p*-chloromercuribenzoic acid (PCMB) to partially purified lipase from *Thermomyces lanuginosus* QM 225 increased its half-life at 50°C from 1 d to over 100 d. Addition of PCMB to the fermentor before harvest increased the yield of lipase by at least 50%. It was concluded that stabilization by PCMB resulted from inhibition of a sulfhydryl protease.

Industrial interest in applications of lipases for large-scale processing of oleochemicals has increased in recent years (1, 2). The cost of lipase, a critical factor (3, 4), is largely determined by the yield of the fermentation process. Our laboratory has been interested in enzymatic hydrolysis of tallow (fat splitting) (5), for which we require a non-specific, thermostable lipase. Because a suitable enzyme preparation was not commercially available, we studied the thermophilic fungus *Thermomyces* (formerly *Humicola*) *lanuginosus* as a potential source. Production and characterization of a non-specific, thermostable lipase from *H. lanuginosa* S-38 was described by Liu, Beppu and Arima (6).

It is well known that many crude enzyme preparations containing protease activity can be stabilized with protease inhibitors. For example, it was concluded that "decreased stability of lipase from *Geotrichum candidum* was correlated with increased proteolytic activity" (7). Also, it was reported that different cultures of *H. lanuginosa* produced proteases that were inhibited by *p*-chloromercuribenzoic acid (PCMB) (8, 9). Such proteases are called sulfhydryl proteases because PCMB reacts specifically with free sulfhydryl groups (10). We noticed some variability in the stability of our crude lipase preparations, so it seemed likely that a sulfhydryl protease was present. The objective of the work reported here was to discover fermentation conditions and develop purification methods for increased thermostability and yield of lipase from *T. lanuginosus*.

A culture of *T. lanuginosus*, QM 225 was obtained from the Northern Regional Research Center, ARS, USDA, Peoria, IL. Liquid cultures were grown at 45°C in a medium containing (per liter) 50 g corn steepwater (Corn Products, Englewood Cliffs, NJ), 20 g soluble potato starch, 5 g soybean oil, 2 g K₂HPO₄, 1 g MgSO₄·7H₂O, and 5 g CaCO₃ adjusted to pH 7 with NaOH (6). A 100-ml portion of sterile medium in a 500-ml flask was inoculated with 1 ml of thawed, deep-frozen spore suspension and incubated with shaking for 24 h. This entire culture was used to inoculate 10 l of the same medium in a 16-l fermentor. Agitation and aeration were maintained at 212 rpm and 10 l/min, respectively. Vessel pressure was less than 20 kPa, and foaming was controlled by automatic addition of antifoam. The fermentation was continued for 7 d, then

filtered. The crude culture filtrate containing about 5 g/l protein (Lowry) (11) and 150 IU/ml lipase was adjusted to pH 5.5 with acetic acid and centrifuged to remove the precipitate.

Lipase in the pH 5.5 supernatant was purified by adsorption to acrylic microporous membranes contained in a pleated capsule filter (Product no. 12117, Gelman, Ann Arbor, MI) (5). (Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.) Approximately 100 IU lipase per cm² of membrane was adsorbed irreversibly at pH 5.5, but could be completely recovered by desorption at pH 9 (0.2 M Na₂CO₃). The desorbed lipase was concentrated by ultrafiltration (H1P10 Hollow Fibers, Amicon, Danvers, MA), adjusted to pH 6.0, centrifuged to remove the precipitate, and freeze-dried. About 1/4 the activity of the original lipase remained in the freeze-dried powder, which had an activity of about 300 IU/mg and a specific activity of about 800 IU per mg protein. In subsequent fermentation experiments, 20 mg/l PCMB (Sigma, St. Louis, MO) was added in alkaline solution to the fermentor before harvesting. The partially purified freeze-dried lipase without PCMB was used for all other experiments.

Lipase was assayed by a flow-through pH-stat method (12) after dilution in a buffer (pH 8.0) containing 20 mM Tris-(hydroxymethyl) aminomethane, 40 mM KCl, 0.01% Triton X-100 (Rohm and Haas, Philadelphia, PA), and 0.02% sodium azide. In the activity vs. temperature experiments, the temperature of the continuous olive-oil emulsion was varied from its normal value of 37°C to between 31 and 69°C. In activity vs. pH experiments, the partially purified lipase was dissolved in one of two buffers. A 20 mM Tris-20 mM sodium phosphate buffer was used for pH's between 6.5 and 8.5, and a 20 mM sodium acetate-20 mM sodium ammonium phosphate buffer was used for pH's below 6.5 or above 8.5. In stability vs. temperature experiments, the partially purified lipase was dissolved in the normal dilution buffer, with pH adjusted and/or PCMB added as indicated. Solutions were incubated at the required temperature and sampled and assayed periodically.

Protease assays were conducted by a modification of the

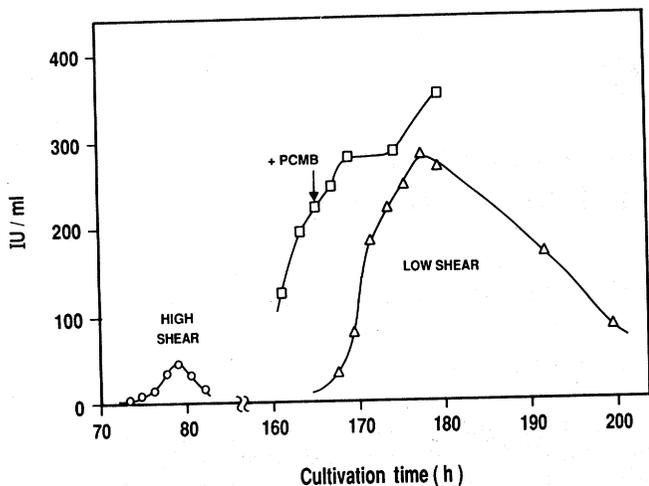


FIG. 1. Lipase activity in 10-l fermentations of *T. lanuginosus*. Air flowrate: 10 l/min. Temperature: 45°C. Symbols: ○, impellor diameter 7.6 cm, 700 rpm; □△, impellor diameter 12 cm, 250 rpm.

method of Ong and Gaucher (9) as follows. To 1.75 ml of 0.5% casein in 50 mM phosphate, pH 8.0 buffer at 37°C was added 0.25 ml enzyme solution in the normal dilution buffer with or without PCMB. After 60 min incubation, the reaction was stopped by the addition of 3.00 ml of 5% trichloroacetic acid (TCA), and allowed to remain for 30 min. The precipitate was removed by filtration. To 1.00 ml of the filtrate was added 5.00 ml of 0.2 N NaOH with 1% Na₂CO₃ and 1.00 ml of Folin phenol reagent diluted with 2 parts water to one part reagent. After 30 min the color was read with a spectrophotometer at 660 nm. The absorbance of a control, identical except that TCA was added before the enzyme, was subtracted to give A_{660}^{cor} .

Figure 1 shows the lipase activity in 10-l fermentations at high shear (700 rpm) and low shear (250 rpm), all other conditions being the same. Low shear resulted in a fermentation time about twice as long and a maximum lipase concentration of about 5 times as much. Microscopic examina-

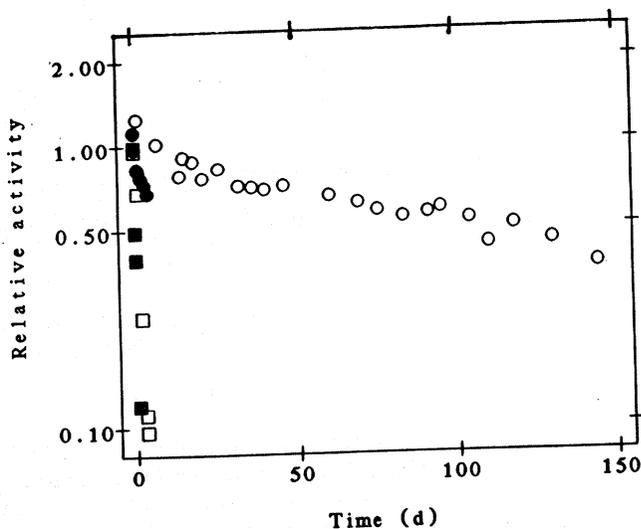


FIG. 2. Stability of partially purified lipase at 50°C in 20 mM Tris pH 7.5 or at 57°C in 20 mM Tris pH 8.0, with 40 mM KCl, 0.01% Triton X-100 and 0.02% sodium azide, and with or without 20 mg/l PCMB. Symbols: ○, 50°C with PCMB; □, 50°C without PCMB; ●, 57°C with PCMB; ■, 57°C without PCMB.

tion revealed many bent and broken cells under high-shear conditions but not under low shear. Therefore, cell damage could be responsible for the low yield under high-shear conditions. The effect of added PCMB, also shown in Fig. 1, will be discussed below.

Addition of PCMB had no effect on activity at any temperature and data with and without PCMB were averaged. Maximum activity occurred at about 55–60°C and activity was at least 50% of the maximum between 30°C and 65°C. The results show the competing effects of increased reaction rate (Arrhenius law) and increased enzyme inactivation as the temperature is increased. The mean residence time in the continuous emulsion reactor, equivalent to the reaction time in a batch reaction, was only about 10 min (12). The half-life of the partially purified

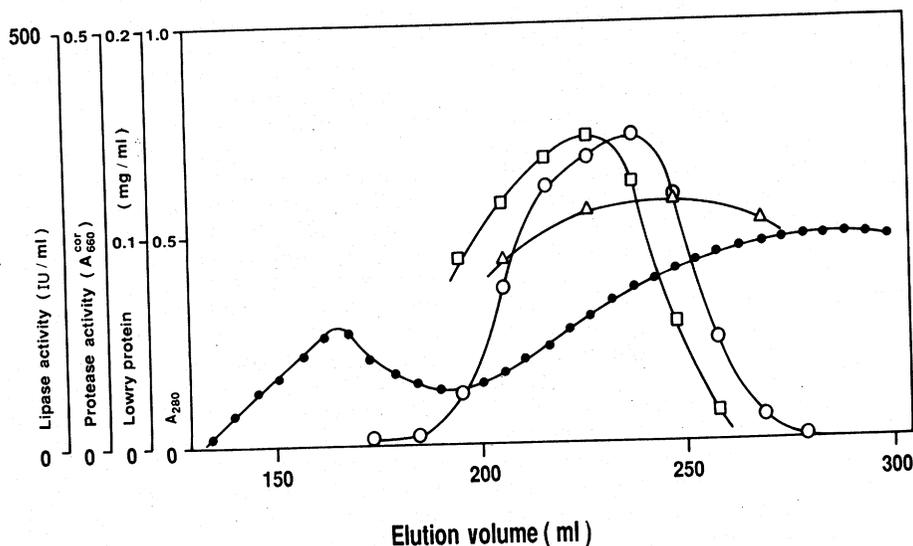


FIG. 3. Sephadex G-75 chromatography of partially purified lipase. Bed dimensions: 2.2 × 107.5 cm. Void volume: 167 ml. Total volume: 472 ml. Sample: 24 mg Lowry protein dissolved in 7.0 ml lipase assay buffer and eluted with the same. Symbols: ○, lipase activity; □, protease activity; △, lowry protein; ●, A₂₈₀.

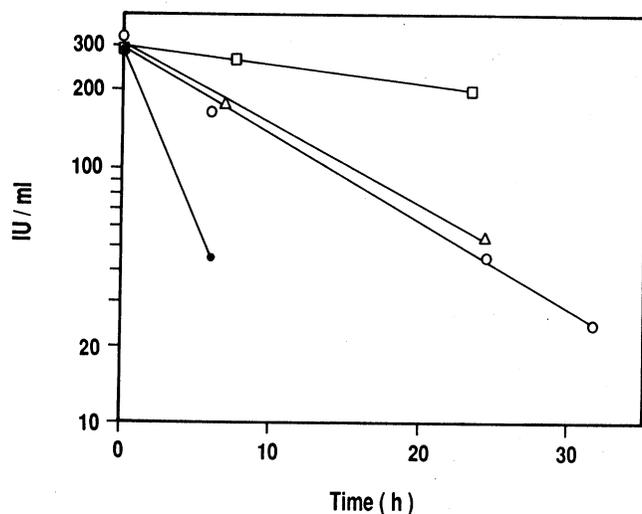


FIG. 4. Stability at 57°C of lipase in high-protease and low-protease fractions from G-75 chromatography with or without addition of 20 mg/l PCMB. Symbols: ●, high protease, without PCMB; ○, low protease, without PCMB; △, high protease, with PCMB; □, low protease, with PCMB.

lipase without PCMB at 57°C in the absence of substrate was about 8 h, so the data indicate a more rapid inactivation occurring in the presence of substrate by mechanisms other than simple thermal denaturation or proteolysis.

Maximum activity occurred at about pH 8.0 and activity was at least 50% of the maximum between pH 5.1 and 9.3. Titration of fatty acid is incomplete below pH 8. Because titration is more nearly complete as the pH is increased, most pH-stat methods for lipase activity will tend to show an optimum pH above the true optimum for the enzyme. The flow-through pH-stat method is not subject to this error because the pH of titration is separately controlled at pH 10.4 (12).

Addition of PCMB to the lipase assay dilution buffer had no effect on lipase activity. Figure 2 shows the effect of 20 mg/l PCMB on the stability of aqueous lipase solutions held at 50°C and 57°C. Half-lives were increased from 0.9 to 111 d (two orders of magnitude) at 50°C and from 7.8 to 250 h (between one and two orders of magnitude) at 57°C. The effect of PCMB was less at higher temperatures (data not shown). Half-lives at 50°C with 20 mg/l PCMB were also obtained at pH 6.5 (105 d) and pH 8.5 (101 d). To show that the stabilization by PCMB was by inactivation of a sulfhydryl protease in the lipase preparation, which in the absence of PCMB could digest the lipase, the protease activity was measured with and without addition of PCMB. Assay of the freeze-dried preparation dissolved at 1 mg/ml in dilution buffer without PCMB gave $A_{580}^{580} = 0.473$. When dissolved at 2.5 mg/ml in the same buffer with 50, 100 or 150 mg/l PCMB the results were $A_{580}^{580} = 0.079, 0.041$ and 0.030 respectively.

To examine the possibility that PCMB might also be reacting directly with the lipase, stabilizing it without inactivating it, the preparation was further purified by chromatography on Sephadex G-75 (Pharmacia, Piscataway, NJ). Although considerable purification was achieved (maximum specific activity of lipase was over 3000 IU per mg Lowry protein), the lipase and protease were only partially separated (Fig. 3). A fraction, however, was obtained from the leading edge of the lipase

peak which was enriched in protease relative to lipase, and a fraction from the trailing edge of the lipase peak was enriched in lipase relative to protease. These two fractions were tested for stability with and without PCMB at 57°C. Results (Fig. 4) indicate that the low-protease fraction without PCMB was almost as stable as the high-protease fraction with PCMB. The difference in stability between the two fractions with PCMB was probably due to residual protease activity, since the data presented above showed that the protease activity was not completely abolished even at 150 mg/l. These results support a conclusion that the stabilization by PCMB results from inhibition of a sulfhydryl protease present in the impure preparation.

The steady decline of activity in Fig. 1 following the maximum in fermentations without added PCMB may have resulted from activity of the same sulfhydryl protease in the crude culture. To test this premise, 20 mg/l PCMB was added to an identical 10-l fermentation at the time that it normally would have been harvested. Results showed that PCMB was not as effective in the fermentor at stabilizing lipase. In further experiments (not shown), the fermentation time was extended beyond those of Fig. 1; the activity eventually declined even with 50 mg/l PCMB. The protease activity assay indicated considerable proteolytic activity that was not inhibited by PCMB was present in the crude culture filtrate. However, lipase activity continued to increase after addition of PCMB (Fig. 1). The maximum lipase activity was maintained for a much longer time so that timing of the harvest was less critical and the yield was at least 50% higher than without PCMB. Unfortunately, PCMB is too toxic for industrial-scale fermentations. Experiments are currently underway to try to find a cheap, non-toxic reagent that will perform the same function.

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