Heat Inactivation of the Extracellular Lipase from *Pseudomonas fluorescens* MC50

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Characteristics of the heat inactivation of a partially purified form of lipase produced by *Pseudomonas fluorescens* MC50 were examined in a simulated milk salt system. At relatively low enzyme concentrations, first-order inactivation was observed in both the low-temperature range (50–90 °C) and the high-temperature range (100–150 °C). A discontinuity is observed in an Arrhenius plot of the data, yielding two lines with corresponding activation energies of 10.7 kcal/mol in the low-temperature range and 21.8 kcal/mol in the high-temperature range. Also in the high-temperature range biphasic inactivation curves were obtained at high enzyme concentrations. The presence of calcium appeared to stabilize the enzyme in the low-temperature range but had no effect at 150 °C. These results suggest that irreversible inactivation in the two temperature ranges is characterized by different denatured states reached from different intermediates having distinctly different degrees of molecular unfolding.

Lipases (triacylglycerol acylhydrolase, EC 3.1.1.3) produced by psychrotrophic bacteria have been found to be stable to extreme heat treatments (Adams and Brawley, 1981a,b; Andersson et al., 1979; Griffiths et al., 1981; Mottar, 1981; Law, 1979; Driessen and Stadhouders, 1974; Lawrence, 1967a,b). A survey of 12 psychrotrophs commonly found in raw milk indicated that all strains produced lipases that survived 63 °C for 30 min to varying extents (Law et al., 1976). Furthermore, *Pseudomonas fluorescens* and *Pseudomonas fragi* produced lipases that partially survived 100 °C for 10 min, and cheese made from milks containing high counts of these ogranisms developed rancidity. In fact, lipases produced by *P. fluorescens* have been shown to partially survive treatment in the "ultrahigh temperature" (UHT) range (130–150 °C for 2–10 s) (Driessen and Stadhouders, 1974; Andersson et al., 1979). Consequently, although products may be commercially sterile, deterioration due to surviving enzyme activity may still occur (Andersson et al., 1981).

The question of thermostability of proteins is of fundamental interest to the understanding of protein structure. Some information and understanding have been obtained by comparison of the structures of analogous proteins produced by mesophiles and by thermophiles (Perutz, 1978; Argos et al., 1979; Zuber, 1976). However, very little is known of the reasons for heat stability of enzymes produced by psychrotrophic organisms. Furthermore, very few studies have addressed the characteristics of lipase stability in the UHT range (Driessen and Stadhouders, 1974; Andersson et al., 1979; Adams and Brawley, 1981a,b).

The present study was undertaken to characterize the heat stability of a substantially purified lipase produced by *P. fluorescens* MC50 in both the low- and high-temperature ranges. Some characteristics of a crude preparation of this enzyme have been previously reported (Adams and Brawley, 1981a,b).

EXPERIMENTAL SECTION

Enzyme Isolation. *P. fluorescens* MC50 was originally isolated from raw milk (Adams and Brawley, 1981a). The crude enzyme was prepared as described in the preceding paper (Bozoğlu et al., 1984).

Enzyme for this study was further purified by gel chromatography on Sephacryl S-500 (Pharmacia, Piscataway, NJ). Crude lyophilized enzyme was dissolved in 10 mM sodium citrate, pH 8.5, and chromatographed on a 0.9 cm × 30 cm column of Sephacryl S-500. The column was equilibrated and eluted with the same buffer at a flow rate of 31.4 mL h^{-1} cm^{-2} (Bozoğlu et al., 1984). Fractions having high lipase activity were combined, filter sterilized, and lyophilized.

Enzyme Activity Assay. Lipolytic activity was assayed by using a pH-stat method (Parry et al., 1966) and an automatic titration system as described in the preceding paper (Bozoğlu et al., 1984). The standard substrate was a corn oil emulsion prepared by mixing 38 mL of water, 2.5 mL of corn oil, 9 mL of 10% gum arabic, 1 mL of 2% sodium deoxycholate, and 0.1 mL of 20% $CaCl_2$ (all from Fisher Scientific, Raleigh, NC) and homogenizing the mixture with a hand homogenizer. One unit of activity is defined as 1 mequiv of alkali added/mL at a temperature of 41 °C and a pH of 8.5.

Heat Treatment. Enzyme solution was prepared in simulated milk ultrafiltrate buffer, pH 6.5 (Jenness and Koops, 1962), to give a concentration of approximately 0.5 unit/mL unless otherwise specified. One milliliter of enzyme solution was sealed in 2-mL freeze-drying tubes (0.7-mm o.d., Bellco Glass Inc., Vineland, NJ) and heated in a circulating oil bath (Haake, Berlin, West Germany) thermostated at temperatures between 50 and 150 °C. A correction for lag in heat penetration was made for all temperatures. Following being heated, the tubes were cooled quickly by immersion in an ice bath and kept at 4 °C until assayed.

The effect of Ca^{2+} on heat stability was examined by replacing the 10 mM $CaCl_2$ in simulated milk ultrafiltrate buffer with 10 mM $MgCl_2$.

RESULTS

Enzyme solutions in simulated milk ultrafiltrate (pH 6.5) were heated between temperatures of 50 and 150 °C. The survival curves plotted in semilogarithmic form are shown in Figures 1 and 2. Linear relationships were observed, indicating first-order inactivation kinetics for all temperatures. Linear correlation coefficients ranged from 0.930 to 0.997 (Table I). Calculation of $t_{1/2}$ and first-order rate constants (Table I) indicated that the half-life decreased with a temperature increase between 60 and 90 °C, but an abrupt increase was observed when the temperature reached 100 °C. For example, the half-life at 90 °C was 16.5 s but increased to 6.9 min at 100 °C, followed by a

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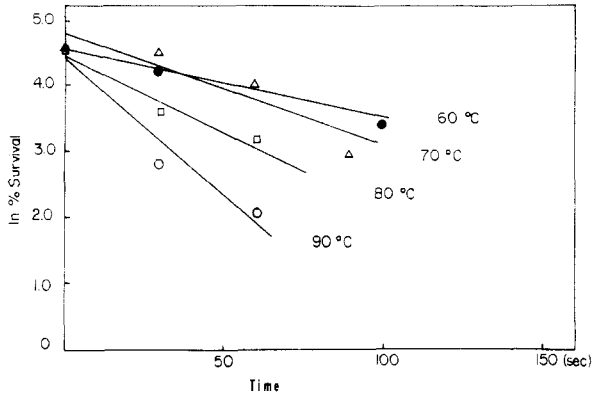


Figure 1. Semilogarithmic plot of the data for heat inactivation of *P. fluorescens* MC50 lipase in the temperature range of 60–90 °C. Data are shown for 60 (●), 70 (△), 80 (□), and 90 °C (○). A point is not included for 210 s at 60 °C because of the scale, but it was used for calculations summarized in Table I. Data were obtained in the milk salt buffer.

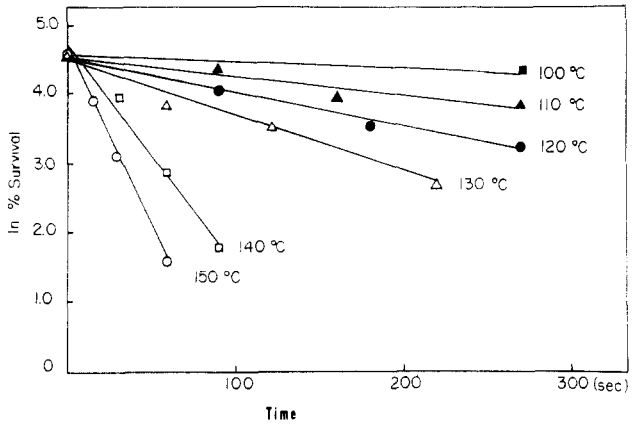


Figure 2. Semilogarithmic plot of the data for heat inactivation of *P. fluorescens* MC50 lipase in the temperature range of 100–150 °C. Data are shown for 100 (■), 110 (▲), 120 (●), 130 (△), 140 (□), and 150 °C (○). Points are not included for 480 or 660 s at 100 °C because of the scale, but they were used for calculations summarized in Table I. Data were obtained in the milk salt buffer.

Table I. First-Order Thermal Inactivation Rates and Half-Life Values Obtained for *P. fluorescens* MC50 Lipase in the Temperature Range of 50–150 °C

temperature, °C	linear correlation coefficient	$k \times 10^2$, s^{-1}	$t_{1/2}$, s
50	0.987	0.135	513.3
60	0.997	1.020	67.9
70	0.930	1.842	37.7
80	0.932	2.321	29.9
90	0.969	4.207	16.5
100	0.990	0.168	412.5
110	0.976	0.297	248.4
120	0.986	0.504	137.5
130	0.956	0.825	84.0
140	0.988	3.166	21.8
150	0.997	4.902	14.1

steady decrease to 14.1 s at 150 °C. This discontinuity in the values for $t_{1/2}$ was characterized by a 25-fold increase at 100 °C. Consequently, the half-life for irreversible loss of enzymic activity was similar at temperatures of 90 and 150 °C.

When an Arrhenius plot, ln k vs. $1/T$, was constructed, a discontinuity yielding two different slopes was observed (Figure 3). The activation energy for inactivation was calculated to be 10.7 kcal/mol for temperatures between 60 and 90 °C, whereas the activation energy observed

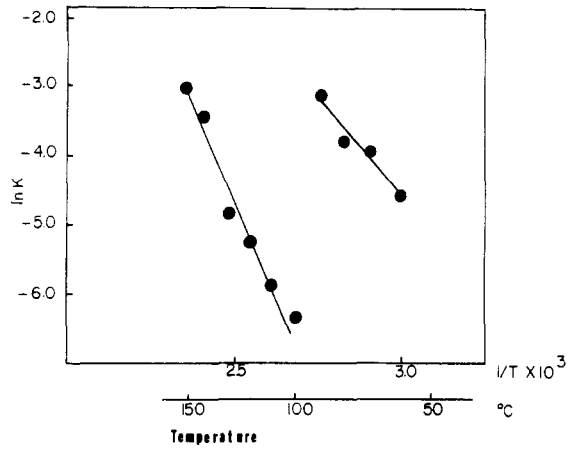


Figure 3. Arrhenius plot of thermal inactivation data for *P. fluorescens* MC50 lipase between 60 and 150 °C.

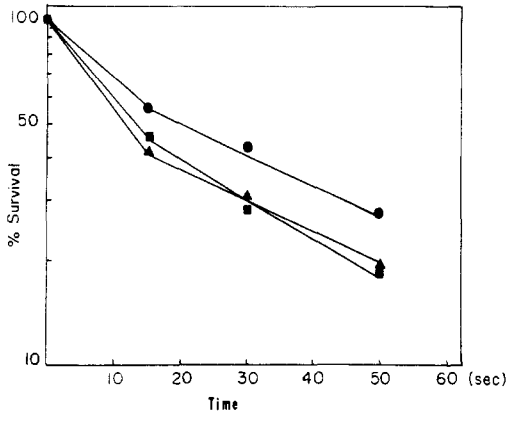


Figure 4. Effect of calcium on the thermal inactivation of *P. fluorescens* MC50 lipase at 150 °C. (●) Milk, correlation coefficient 0.965; (■) milk salt buffer, correlation coefficient 0.950; (▲) milk salt buffer without calcium, correlation coefficient 0.943. The correlation coefficients were calculated for a linear regression including the zero-time point. The enzyme concentrations were approximately 0.7 unit/mL.

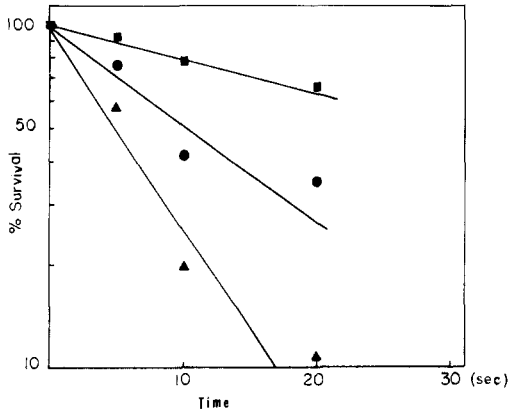


Figure 5. Effect of calcium on the thermal inactivation of *P. fluorescens* MC50 lipase at 60 °C. (●) Milk, correlation coefficient 0.909; (■) milk salt buffer, correlation coefficient 0.988; (▲) milk salt buffer without calcium, correlation coefficient 0.965. The correlation coefficients were calculated for a linear regression including the zero-time point. The enzyme concentrations were approximtely 1.6 units/mL.

betwen 100 and 150 °C was 21.8 kcal/mol.

The effect of Ca^{2+} on the heat stability of MC50 lipase was examined in both temperature ranges. Solutions of enzyme were heated in buffer, milk, and buffer containing Ca^{2+} at 60 and 150 °C. Data shown in Figure 4 indicate that Ca^{2+} did not influence the stability of MC50 lipase

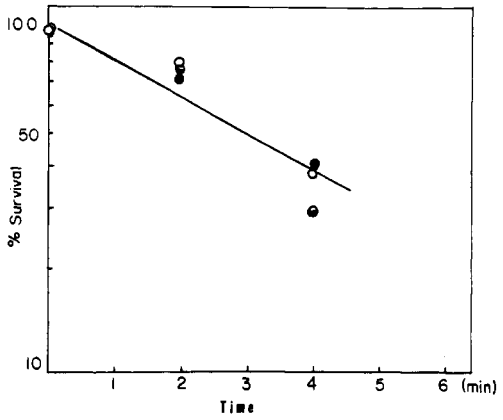


Figure 6. Effect of enzyme concentration on the thermal inactivation of *P. fluorescens* MC50 lipase at 60 °C. Concentrations of enzyme were (○) 0.742 unit/mL, correlation coefficient 0.960, (◒) 0.530 unit/mL, correlation coefficient 0.957, and (●) 0.305 unit/mL, correlation coefficient 0.964. The correlation coefficients were calculated for a linear regression including the zero-time point.

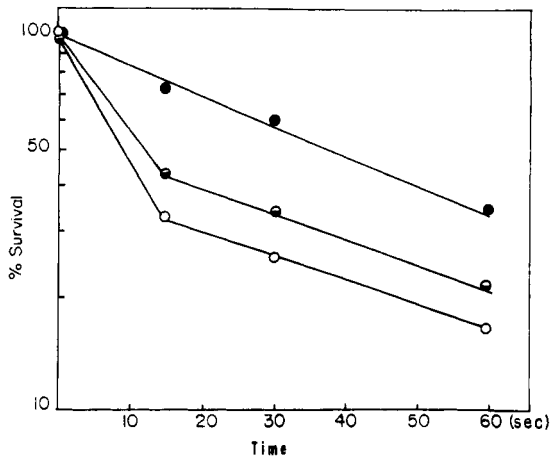


Figure 7. Effect of enzyme concentration on the thermal inactivation of *P. fluorescens* MC50 lipase at 150 °C. Concentrations of enzyme were (○) 0.982 unit/mL, correlation coefficient 0.908, (◒) 0.387 unit/mL, correlation coefficient 0.939, and (●) 0.186 unit/mL, correlation coefficient 0.998. The correlation coefficients were calculated for a linear regression including the zero-time point.

at 150 °C. Furthermore, the enzyme's stability was only slightly affected by the presence of milk proteins. However, at 60 °C the presence of Ca^{2+} stabilized the enzyme to heat inactivation (Figure 5). Also, it appeared that inactivation was slightly more rapid in milk than in buffer containing Ca^{2+}.

The effect of enzyme concentration on heat stability in the two temperature ranges was determined by heating solutions of varying enzyme concentrations at 60 and 150 °C. At 60 °C, concentrations ranging from 0.3 to 0.8 unit/mL were examined, all of which resulted in linear inactivation curves. Linearity was indicated by high linear correlation coefficients (0.949–0.965) for each concentration (Figure 6). The half-life calculated for all concentrations was 153 ± 20 s.

However, at 150 °C, biphasic inactivation curves were obtained for higher concentrations (Figure 7). Thus, when all the data points are included, the linear correlation coefficients become progressively poorer as the enzyme concentration increased. When the initial data points (0 s) are excluded from the regression analysis, the linear correlation coefficients obtained are 0.999, 0.999, and 0.996 for 0.98, 0.39, and 0.19 unit/mL, respectively. These results suggest an initial, rapid, concentration-dependent inactivation followed by a slower first-order process. The half-life for the slower phase of inactivation at the two higher enzyme concentrations and for the lowest enzyme concentration is 43.6 ± 2.3 s. It should be noted that similar biphasic inactivation kinetics were observed for the study on effect of Ca^{2+} at 150 °C as shown in Figure 4, where the enzyme concentration was higher than that yielding linear curves in Figure 7.

DISCUSSION

Lipases produced by other strains of *P. fluorescens* appear to be slighltly more thermostable, especially at the higher temperatures. For example, the time required for 90% inactivation was 2.0 min for strain SIK WI at 140 °C (Andersson et al., 1979) and 4.8 min for strain 22F at 150 °C (Driessen and Stadhouders, 1974) as compared to a value of 1.2 min at 140 °C found in this study. At temperatures of 100 and 120 °C, values for the pseudo-first-order inactivation rate constants and the half-life in skim milk (Andersson et al., 1979) were very similar to those obtained for MC50 lipase in simulated milk ultrafiltrate. However, it should be noted that the previous studies were conducted with enzyme preparations that had not been purified or with whole cultures. The half-life obtained for thermal inactivation of partially purified MC50 lipase in simulated milk ultrafiltrate at 150 °C is similar to that found for the crude enzyme in whole milk, ~18.9 s (Adams and Brawley, 1981a), but slightly less than that for the crude enzyme in water at pH 8.5, ~25.2 s (Adams and Brawley, 1981b). Similarity of the value for the crude enzyme in milk to that found here suggests that interactions with milk constituents other than the milk salt system do not affect the stability.

In any case, the thermostability of MC50 lipase is more than 150-fold greater than that of *Bacillus stearothermophilus* or Putrefactive Anaerobe (PA) 3679 spores (Speck and Busta, 1968), which are commonly used to determine the processing conditions required for commercial sterilization. Thus, a UHT treatment of 150 °C for 3.42 s would reduce the PA 3679 viable spore population by 12 log cycles, but MC50 lipase would retain 85% of its original activity.

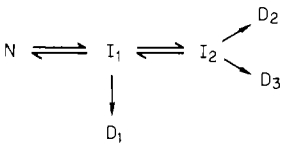
A most unusual feature, the discontinuity in the Arrhenius plots of inactivation data, was observed in this study. Consequently, a large discrepancy occurs between the observed half-lives in the low-temperature range and that predicted from the activation energy obtained in the high-temperature range. Similar large discrepancies were observed between measured and predicted times required for 90% inactivation of a proteinase produced by *P. fluorescens* (Barach et al., 1976). These authors attributed the low-temperature inactivation to an interaction between the enzyme and casein micelles (Barach et al., 1978). However, this mechanism cannot account for the abrupt change in activation energy for MC50 lipase since these experiments were performed in the absence of milk proteins. Furthermore, it now appears that both activation energies reflect thermal inactivation since there was no evidence of contaminating proteolytic activity at the lower temperatures. It also appears unlikely that the different activation energies reflect two lipase activities with different heat stabilities since further purification gave no evidence for two forms of activity (Bozoğlu et al., 1984). Moreover, most of the inactivation curves were monophasic except under particular conditions (see below).

The abrupt change in activation energy for irreversible inactivation suggests that different mechanisms are operative in the two temperature ranges. Several other ob-

servations support this argument. Thus, calcium did not affect the enzyme's stability at 150 °C but its presence stabilized the enzyme at 60 °C. Also, when examined in the same enzyme concentration range, biphasic inactivation curves were observed in the high-temperature range while these curves were monophasic in the low-temperature range.

Others (Driessen and Stadhouders, 1974; Andersson et al., 1979) have observed biphasic inactivation curves for lipase under specific conditions. Previously, these observations have been attributed to the presence of two lipases with differing heat stabilities (Driessen and Stadhouders, 1974; Cogan, 1977) or to association with other components in skim milk (Andersson et al., 1979). The latter authors gave evidence for homogeneity of their lipase, and likewise only one electrophoretic band of activity was observed in this study. Moreover, the biphasic quality was concentration dependent, suggesting the existence of two denatured states, one of which results from interaction between unfolded enzyme molecules. It appears from these results that the biphasic nature of the inactivation of other lipases and proteinsases should be reexamined for the effects of enzyme and possibly total protein concentration.

The characteristics of the molecular processes leading to irreversible thermal denaturation of lipase may be discussed in terms of a model such as



(1)

It seems reasonable to expect that different denatured states could be formed under different denaturing conditions. Thus, at low temperatures, denaturation may proceed to denatured state D_1 through intermediate I_1, which is not as extensively unfolded as intermediate I_2. Hence, this pathway would be characterized by a lower activation energy. In the high-temperature range intermediate I_2 is favored, which is more unfolded than I_1 and which leads irreversibly to denatured state D_2 through a first-order process or an associated denatured state D_3 through a higher order process. Intermediate I_2 is connected to the native state through a reversible pathway but it is not directly connected to denatured state D_1; thus, at temperatures favoring I_2, the enzyme has increased stability. Of course, other models could also account for the observations; for example I_2 could be in direct equilibrium with the native state rather than with I_1. These subtleties would depend on the folding pathway of the enzyme. This analysis also suggests that such observations may be a general phenomenon for proteins and enzymes since they result from general characteristics of protein structure.

Finally, one may question why a psychrotroph would produce a heat-stable enzyme. Seemingly, the structure was not evolutionally selected for this characteristic. A distinction that must be borne in mind is that studies such as those presented here do not require that the enzyme remain active at high temperatures, as would be the case for enzymes from a thermophile, but only that the structural change be reversible. Hence, thermostability as defined by these studies may be more concerned with the protein's folding pathway than with the structurally determined thermodynamic stability required for enzymes in thermophilic organisms. Consequently, one should not expect similarities with those specific structural characteristics (Perutz, 1978; Argos et al., 1979; Zuber, 1976) peculiar to thermophilic enzymes.

Registry No. Calcium, 7440-70-2; lipase, 9001-62-1.

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