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Lipase of *Penicillium caseicolum*

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The lipase activity of *Penicillium caseicolum* was tested in a cell-free broth of the organism and in a partially purified preparation of the enzyme. The optimum reaction pH of the enzyme was 9.0 and the optimum temperature 35 °C. A mixture of $CaCl_2$ and sodium taurocholate enhanced the activity of the enzyme toward butter oil. The heat inactivation of this lipase was exponentially related to temperature. A z value of 16 °C and an inactivation energy of 32 200 cal/mol were calculated for this enzyme. Triglycerides of lower molecular weight fatty acids were better substrates than those of higher molecular weight fatty acids for this lipase.

Lipases are widely distributed in nature. By hydrolyzing lipids, lipases contribute significantly to the development of flavor, pleasant or unpleasant, in many foods, especially dairy products (Schwimmer, 1981). Of particular interest to the cheese industry are the lipases produced by microorganisms (Chandan et al., 1969; Shahani et al., 1976). Two fungi commonly used in the manufacture of Camembert and Brie cheeses are *Penicillium camemberti* and *Penicillium caseicolum* (or *Penicillium candidum*). While some work on the lipases of these molds has already been published (Lamberet and Lenoir, 1976), much remains to be learned about them. This study deals with certain aspects of the catalysis by *P. caseicolum* lipase.

MATERIALS AND METHODS

A culture of *P. caseicolum*, obtained from G. Roger Laboratories, Paris, France, was grown in mycological broth (Difco Labs, Detroit, MI) containing 1% corn oil at room temperature with rotary shaking (120 rpm) for 3 days. The growth medium was filtered, and the cell-free broth was assayed for lipase activity. For the determination of optimum pH for the enzyme, the reaction was carried out a seven pH levels in the range 5.0–11.0. The substrate solution contained 10% gum arabic, 2 mM $CaCl_2$, and 10% tributyrin or 10% butter oil, both emulsified by homogenization. The pH was adjusted by adding 0.1 N NaOH. Substrate solution (5 mL) was transferred into the reaction vessel of a pH-Stat (Metrohm, Herisan, Switzerland), the temperature was adjusted to 35 °C, 0.2 mL of cell-free broth was added, and the reaction was allowed to proceed for 5–10 min. The volume of 0.02 N NaOH consumed, to keep the pH constant, was recorded automatically on a strip chart, and the slope of the recorded line (which was straight for the first few minutes) was taken as a measure of the reaction rate. The lipase activity was expressed in micromoles of fatty acid(s) liberated per minute.

For the determination of optimum temperature, the reaction system just described was set to pH 9.0 and the reaction was run at temperatures varying from 25 to 55 °C.

The effect of sodium taurocholate, sodium deoxycholate, and $CaCl_2$ on the lipase hydrolysis was also studied. The salts were added by themselves or in combination at concentrations vary-

Table I. Relative Activity of *P. caseicolum* Lipase toward Natural and Synthetic Lipids[a]

lipid	rel rate of hydrolysis
tributyrin	100
tricaproin	47
tricaprylin	40
tristearin	23
triolein	26
trilaurin	34
trimyristin	16
tripalmitin	9
corn oil	34
sunflower oil	33
grape seed oil	30
almond oil	29
safflower	29
soybean oil	28
peanut oil	28
butter oil	24
sesame oil	24
lard	23
hazelnut oil	22
walnut oil	21
olive oil	18

[a] Reaction mix: 5 mL containing 0.2 mg of purified enzyme, 10% lipid, 10% gum arabic, 8 mM in $CaCl_2$, 8 μM in sodium taurocholate. pH 9.0, 35 °C. Actual lipase activity against tributyrin: 6.2 μmol of FFA/mg of protein per min.

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Table II. Free Fatty Acid Distribution (%) in Butter Oil before and after the Action of *P. caseicolum* Lipase[a]

fatty acid	FFA in lipolyzed butter oil	FFA in butter oil	fatty acid compn of butter oil
C_4	19.0	0	3.3
C_6	1.8	1.2	2.4
C_8	1.6	0.4	1.4
C_{10}	2.3	3.7	2.8
C_{12}	4.0	4.1	4.0
C_{14}	12.7	13.6	12.1
$C_{14:1}$	6.6	7.4	2.9
C_{16}	22.1	18.8	29.0
$C_{16:1}$	7.3	12.8	4.5
C_{18}	8.0	14.1	10.7
$C_{18:1}$	13.0	17.5	22.2
$C_{18:2}$	1.6	6.4	3.3

[a] Reaction conditions similar to those cited in Table I.

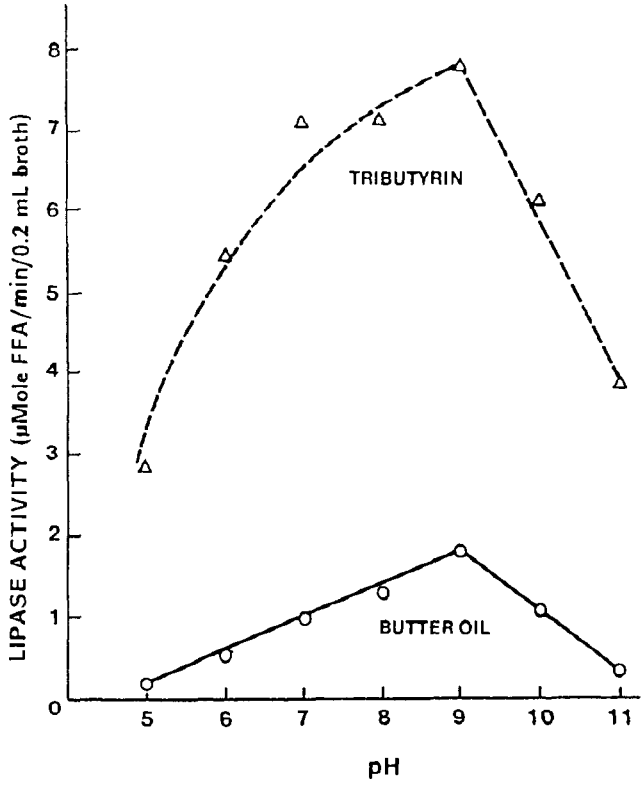

Figure 1. Effect of pH on the lipase activity of *P. caseicolum* with tributyrin and butter oil as substrates. Reaction mix: 5 mL containing 0.2 mL of broth, 10% lipid, 10% gum arabic, 2 mM $CaCl_2$; 35 °C.

ing from 0 to 10 μM for the cholate and 0–10 mM for $CaCl_2$. The reaction was run at 35 °C and pH 9.0.

The thermal stability of the lipase in the cell-free broth was studied at temperatures ranging from −27 °C to +100 °C. The storage time at each temperature varied from 1 month at the subfreezing temperatures to a few minutes at 100 °C. At the end of each storage period an appropriate aliquot of the broth was tested for lipase activity with use of the substrate mixture described previously. The pH of the reaction mixture was 9.0 and the reaction temperature 35 °C.

For the substrate specificity tests the enzyme was partly purified as follows: Ammonium sulfate was added to 100 mL of cell-free broth to reach 70% saturation, and the mixture was held at 4 °C for 2 h and subsequently centrifuged at 18000*g* for 20 min in a Sorvall RC23 centrifuge (DuPont, Newtown, CT) refrigerated at 4 °C. A pellet was formed and was dissolved in 20 mL of distilled water and dialyzed for 24 h, at 4 °C, against distilled water in cellulose acetate tubing possessing a cutoff molecular weight of 16 000–18 000. The protein content of the enzyme preparation was estimated by the Bradford (1976) dye-binding procedure. The substrate specificity of the enzyme toward 21 synthetic and natural lipids (Table I) was tested at pH 9.0

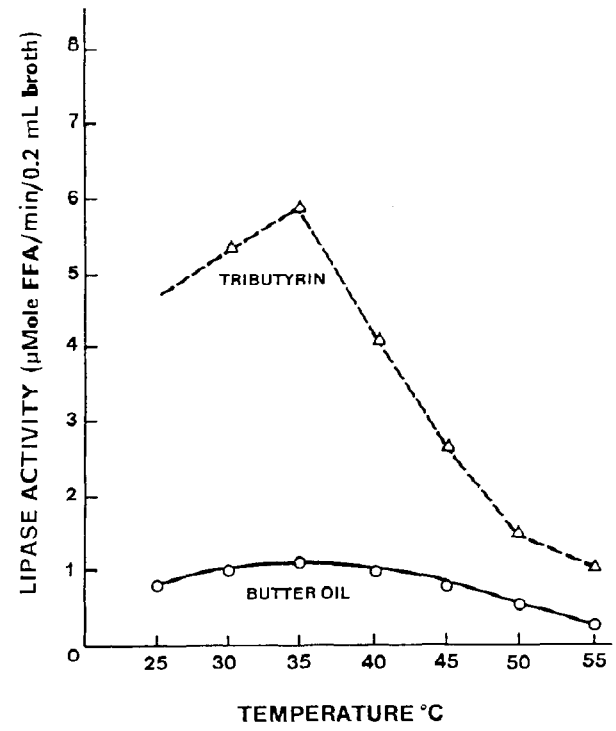

Figure 2. Effect of temperature on the lipase activity of *P. caseicolum* with tributyrin and butter oil as substrates. Reaction mix: 5 mL containing 0.2 mL of broth, 10% lipid, 10% gum arabic, 2 mM $CaCl_2$; pH 9.0.

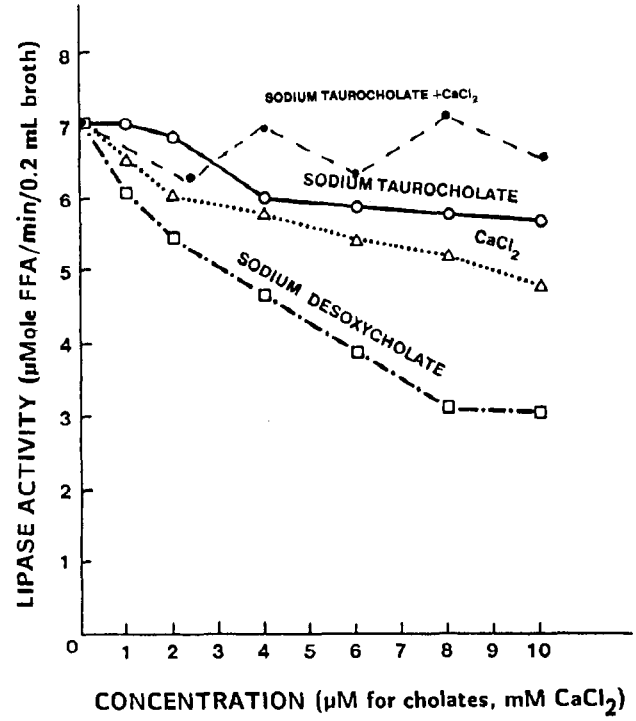

Figure 3. Effect of sodium taurocholate, sodium desoxycholate, and $CaCl_2$ on the lipase activity of *P. caseicolum* with tributyrin as substrate.

and 35 °C. Ten-gram portions of each lipid were emulsified in 100 mL of 10% gum arabic solution, which also contained $CaCl_2$ (8 mM) and sodium taurocholate (8 μM).

The free fatty acids of butter oil, before and after the action of *P. caseicolum* lipase on butter oil, were determined by column chromatography after their butyl esters were formed and the esters injected in a Hewlett-Packard 5840A gas chromatograph equipped with a 2 m × 2 mm (i.d.) DEGS on Chromosorb column and a flame ionization detector (Supelco, 1979).

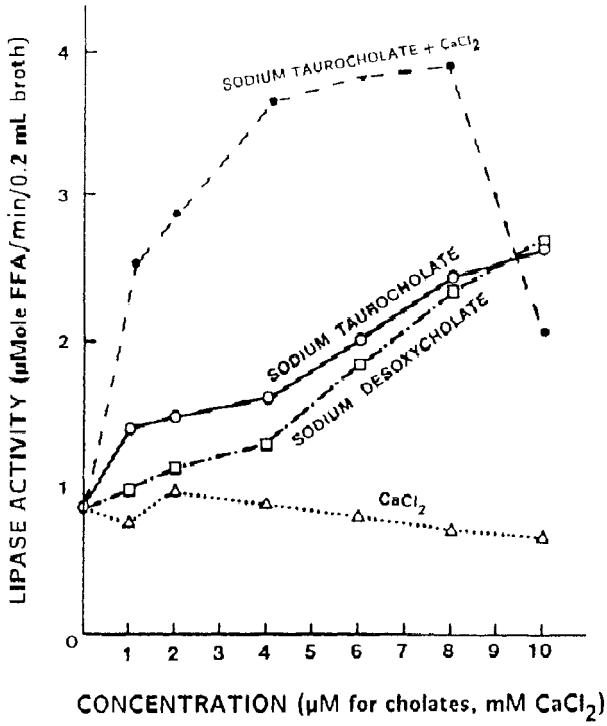

Figure 4. Effect of sodium taurocholate, sodium desoxycholate, and $CaCl_2$ on the lipase activity of *P. caseicolum* with butter oil as substrate.

RESULTS AND DISCUSSION

pH and Temperature Optima. Figure 1 illustrates the results of the pH optimization experiment. At pH 9.0 the hydrolysis of both tributyrin and butter oil proceeds at its fastest rate. The results of the temperature optimization test are shown in Figure 2. With either of the two substrates the optimum temperature appears to be 35 °C, although this optimum is much clearer with tributyrin as a substrate. It is noteworthy that in both the pH and the temperature optimization tests tributyrin was shown to be a more rapidly processed substrate than butter oil.

Effect of Salts. The effects of the bile salts and $CaCl_2$ on the activity of *P. caseicolum* lipase are depicted in Figure 3 and 4. When tributyrin was used as substrate, all three additives had an inhibitory effect on lipase, desoxycholate more so than the other two salts (Figure 3). With butter oil as substrate (Figure 4), the bile salts enhanced the activity of lipase, while $CaCl_2$ acted as a very weak inhibitor. $CaCl_2$ in combination with sodium taurocholate, however, greatly accelerated the lipolysis of butter oil up to certain concentrations of these salts. The effect of the same salt combination on the lipolysis of tributyrin was not as clear as on butter oil.

Stability of *P. caseicolum* Lipase. Storage of the cell-free mold broth at −27 or −15 °C for 1 month did not result in loss of lipase activity. Also, no decrease in lipase activity was observed when the broth was stored at 0 or 4 °C for up to 72 h. At 25 °C, 1% of the lipase activity was lost after 24 h and 1.7% after 72 h. For higher temperatures, a *D* value (hours for a 90% reduction of the original activity) was calculated and plotted against the corresponding storage temperature (Figure 5). It can be seen that the thermal inactivation of *P. caseicolum* lipase is in semilogarithmic relationship with temperature, and a *z* value (temperature increase associated with a 10-fold decrease in *D* value) equal to about 16 °C can be derived. An activation energy of 32 200 cal/mol was calculated from these data for the inactivation reaction of lipase.

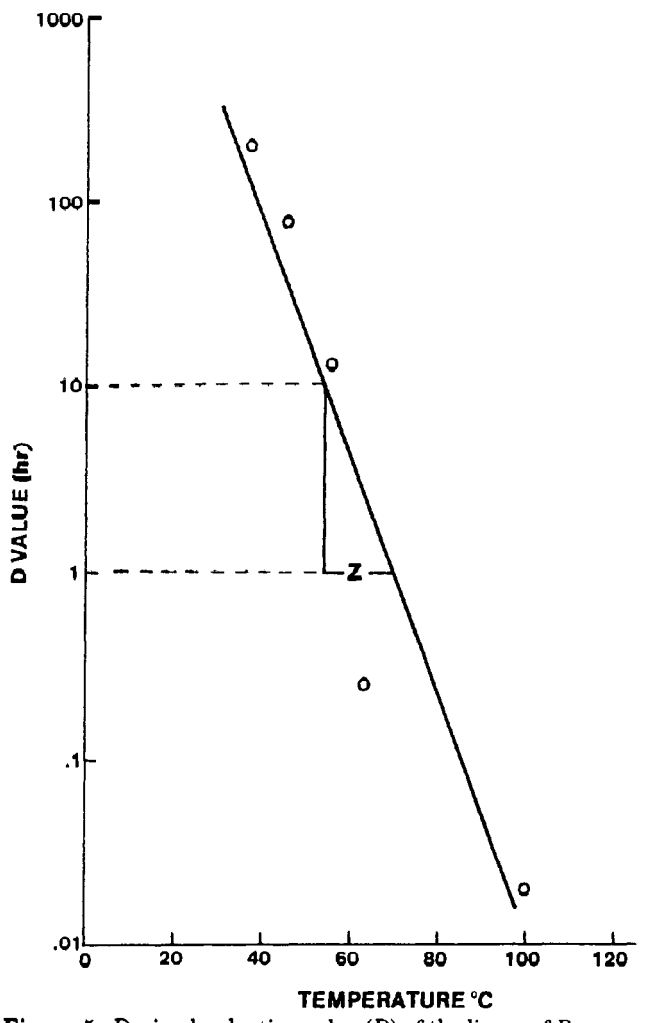

Figure 5. Decimal reduction value (*D*) of the lipase of *P. caseicolum* at various temperatures with tributyrin as substrate.

The *z* values appearing in the literature for lipase vary greatly: from 8.9 °C for the *Pseudomonas fluorescens* 22 F lipase (Driessen and Stadhouders, 1974) to 36 °C for the *Pseudomonas* sp. MC_{50} lipase (Adams and Brawley, 1981) to 55 °C for the *Pseudomonas* sp. 21B lipase (Kishoti, 1975). The figures cited by Lamberet and Lenoir (1976) for the heat inactivation of *P. caseicolum* indicate an enzyme considerably more sensitive to heat than the enzyme encountered in this work.

Substrate Specificity. The enzyme used in the substrate specificity tests had an activity against tributyrin equal to 6.2 μmol of freed fatty acid/mg of protein per min. This activity was 49 times higher than that of the cell-broth. Table I lists the lipids tested as substrates for the *P. caseicolum* lipase and the corresponding rates of the enzymic hydrolysis. The rates are given as percent of the value for the hydrolysis of tributyrin. For the first three or four triglycerides of the list, the hydrolysis rate decreased as the molecular weight of the fatty acid increased. This trend, however, was not followed strictly for the higher molecular weight fatty acids. Most of the natural oils tested manifested a hydrolysis rate in the range of 20–30% of that for tributyrin. These results generally agree with those reported for other fungal lipases (Eitenmiller et al., 1970; Belloc et al., 1975; Chander et al., 1979). When butter oil was used as a substrate for the *P. caseicolum* lipase, the percentage composition of the free fatty acid mixture again indicated that the glycerides of lower molecular weight fatty acids are hydrolyzed more readily than those of higher molecular weight fatty acids (Table II).

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Registry No. Ca, 7440-70-2; lipase, 9001-62-1; sodium taurocholate, 145-42-6.

Effect of Dietary Iron Level on in Situ Turkey Muscle Lipid Peroxidation†

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The main object of this study was to evaluate the effect of removing the iron (Fe) supplement from the diet fed prior to slaughtering on the stability of turkey muscle tissue stored at 5 °C. The results demonstrate that a decrease in nutrititional Fe 3–7 weeks prior to slaughtering could result in a reduction of more than 50% in lipid peroxidation in in situ turkey dark muscle. Turkey light muscle, which is by its nature more stable to lipid peroxidation, was affected during the first experiment but not during the second. Removal of the Fe supplement from the diet fed during 3–7 weeks prior to slaughtering did not affect body weight or blood hematocrit, although the latter was slightly decreased.

It is becoming increasingly apparent that in vivo lipid peroxidation and several other tissue injuries derived from oxidative processes are accompanied by iron (Fe) toxicity (Halliwell and Gutteridge, 1984; Aust et al., 1985; Jacobs, 1977; Beanish et al., 1974).

Iron overload is a medical problem. The potential toxicity associated with excessive iron intake is exemplified by the tissue damage observed in human patients suffering from disorders commonly called hemochromatoses and those receiving blood transfusions for treatment of β-thalassemia (Jacobs, 1977; Beanish et al., 1974).

Goldberg's group (1962) performed some of the first studies linking iron-loading to lipid peroxidation. These authors demonstrated that iron–dextran injections caused accumulation of Fe in certain organelles and the changes noted closely resembled those observed in animals deficient in vitamin E. It has been shown similarly that a vitamin E deficiency increases lipid peroxidation induced by Fe–dextran (Dillard et al., 1984).

Lipid peroxidation is one of the primary reactions leading to quality deterioration in stored foods and especially in muscle tissues (Wilson et al., 1976). One of the main problems with regard to lipid peroxidation in muscle tissue was identification of the nature of the predominant form of Fe catalysts, e.g., myoglobin, non-heme iron or "free" iron ions involved in the initiation of this process. Most recently, it was found by us that free iron ions are the most important catalysts of in situ muscle lipid peroxidation (Kanner et al., 1988a–c). These results led us to develop the recent study.

The Fe requirement of turkeys at age 16–24 weeks is a 50 mg/kg diet (National Research Council, 1984). According to Scott et al. (1976) the Fe content of normal feedstuffs should be sufficient to meet the nutritional requirements of chickens without the use of iron supplements. Nevertheless, commercial diets are usu-

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