Influence of Milk Proteins on the Thermostability of the Lipase from *Pseudomonas fluorescens* 33

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ABSTRACT

The effects of some milk proteins on the thermostability of the lipase from *Pseudomonas fluorescens* 33 were investigated. All purified milk protein fractions except κ-casein that dissolved in phosphate buffer were effective for thermostabilization of the lipase. Thermal behavior of the lipase containing β-lactoglobulin was so specific that, after heating at 80 to 90°C, activity remained high and was comparable with that of unheated treatment. The thermostability of the lipase containing whey proteins in synthetic salts solution was extensively lowered, but that containing casein micelles retained 50% of original activity after heat treatment at 80°C for 10 min. Low temperature inactivation of the lipase was influenced by concomitant milk proteins.

(**Key words**: lipase, milk proteins, thermostability, psychrotrophs)

Abbreviation key: LTI = low temperature inactivation.

INTRODUCTION

Extracellular lipases and proteases produced by psychrotrophic bacteria are well-known causes of spoilage in dairy products. Most of these lipases and proteases are heat stable and survive under UHT conditions (1, 4, 7). Some studies (2) report a relatively heat-labile temperature zone (low temperature inactivation; LTI) that is lower than the stable UHT region for the enzymes. Maximum LTI effect occurs at around 55°C (9) and at 50 to 100°C (5, 10, 11, 13, 18) for proteases and lipases, respectively. The cause of LTI of protease is attributed to autodigestion (8, 16, 17), but the cause of LTI of lipase has not yet been fully elucidated. Our previous paper (14) reported the purification and characterization of the lipase from *Pseudomonas fluorescens* 33 cultivated in reconstituted skim milk. Maximum LTI effect occurred at 40°C for purified lipase (14) and at 60 to 70°C for the lipase in the culture supernatant (13). The results suggest that environmental conditions affect LTI temperature of the lipase because the culture supernatant contained milk constituents, metabolites of the bacterium, and hydrolystates of constituents by the action of the bacterial enzymes. The effect of the composition of the heating medium on temperature of LTI was also recognized for the lipase from *P. fluorescens* AFT 36 (11). For practical purposes, however, information should be obtained on the effect of milk constituents on the activity, LTI temperature, and thermostability of bacterial lipases.

This study investigated the influence of milk components, primarily milk proteins, on the thermostability of the purified lipase from *P. fluorescens* 33.

MATERIALS AND METHODS

Enzyme

Purification of the lipase from *P. fluorescens* 33 has been described elsewhere (14).

Measurement of Lipase Activity

Lipase activity was determined by the method previously described (13). Butter oil (3.6%), emulsified with 8.9% gum arabic in .1 M sodium phosphate at pH 7.5, was used as the substrate.

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Preparation of Milk Proteins

Fresh raw milk was obtained from the Holstein herd at the Hokkaido University Experimental Farm immediately after milking. The milk was skimmed at 1200 × g for 30 min at 20°C. Casein micelles were prepared by centrifugation at 70,000 × g, 20°C, for 70 min. The resulting pellet was resuspended in double-strength synthetic milk salts solution (12). The protein concentration was adjusted to 5.0%.

Whey proteins were obtained from isoelectric precipitation of skim milk at pH 4.6 with 1 M HCl, followed by concentration of the supernatant with ultrfiltration (molecular weight cutoff: 10,000 Da). The concentrated whey proteins were dialyzed against distilled water and dissolved in double-strength synthetic milk salts solution or .02 M sodium phosphate buffer, pH 6.6, at a protein concentration of 2 or .2%.

Casein fractions were isolated chromatographically by the method of Christensen and Munksgaard (6) and were dissolved in .02 M sodium phosphate buffer at pH 6.6 and a protein concentration of .2%.

α-Lactalbumin was purified by the method of Lindahl and Vogel (15) and adjusted to .2% with .02 M sodium phosphate buffer, pH 6.6. Purified β-lactoglobulin was purchased from Sigma Chemical Co. (St. Louis, MO) and dissolved at a concentration of .2% in .02 M sodium phosphate buffer at pH 6.6.

Effect of Milk Components on Thermostability

The purified lipase was diluted with deionized water to give a protein concentration of 20 µg/ml; then, equal volumes of .02 M sodium phosphate buffer, pH 6.6; double-strength synthetic milk salts solution, pH 6.6; or the milk protein solutions described were added. Otherwise, the purified lipase was diluted only with fresh raw skim milk.

Heat treatment of the enzyme was carried out as previously described (14). Remaining activity was measured and expressed as the percentage of the activity before heating.

Protein Determination

The protein concentration was determined by the method of Bensadoun and Weinstein (3), using bovine serum albumin as a standard.

Statistical Analysis

Data were analyzed by t test.

RESULTS

Remaining activity of the lipase in phosphate buffer heated at 50°C was significantly higher than lipase heated at 40°C ($P < .05$), but not in synthetic milk salts system (Figure 1). Figure 1 shows that the lipase in synthetic milk salts solution was significantly less stable than in phosphate buffer above 70°C ($P < .01$).

The lipase in skim milk was significantly more stable ($P < .01$) than in synthetic milk salts solution except in the temperature range of 60 to 80°C (Figure 2). The thermostability of lipase in 2.5% casein micelle suspension was higher ($P < .01$) than that in skim milk. In contrast, the lipase in synthetic milk salts solution containing 1 or .1% of whey proteins was completely inactivated at 60 to 80°C; after heating at 90°C, however, a trace of activity was detectable (Figures 2 and 3). When sodium phosphate buffer was substituted for synthetic milk salts solution as the buffer system, the lipase retained activity above 60°C. Moreover, activity was higher after heat treatment at 80 to 90°C than after heating at 50 to 70°C ($P < .01$) (Figure 3).

Further observations after addition of purified milk protein fractions to the lipase revealed that, among casein fractions, α_s-casein was the most effective for thermostabilization
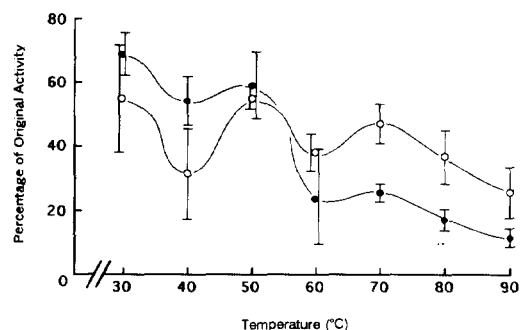

Figure 1. Thermostability at different temperatures of *Pseudomonas fluorescens* 33 lipase in .01 M sodium phosphate buffer, pH 6.6 (○; n = 4), or in synthetic milk salts solution, pH 6.6 (●; n = 6); incubation was for 10 min. The enzyme protein was at 10 µg/ml. Vertical bars indicate standard deviations of the means.
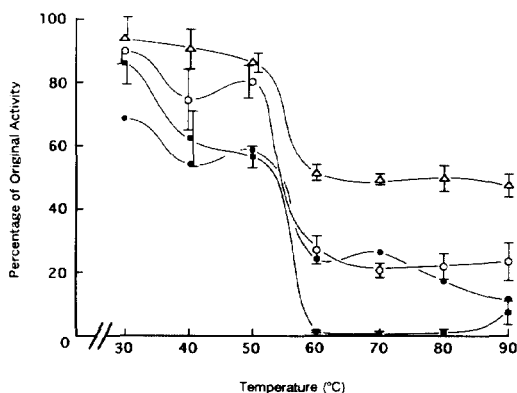
Figure 2. Thermostability at different temperatures of *Pseudomonas fluorescens* 33 lipase in synthetic milk salts solution, pH 6.6, containing 2.5% casein micelles (△; n = 6) or 1.0% whey proteins (■; n = 6), and in skim milk (O; n = 6); incubation was for 10 min. The enzyme protein was at 10 μg/ml. The lipase in synthetic milk salts solution, pH 6.6, containing no milk proteins (●), is included for comparison.
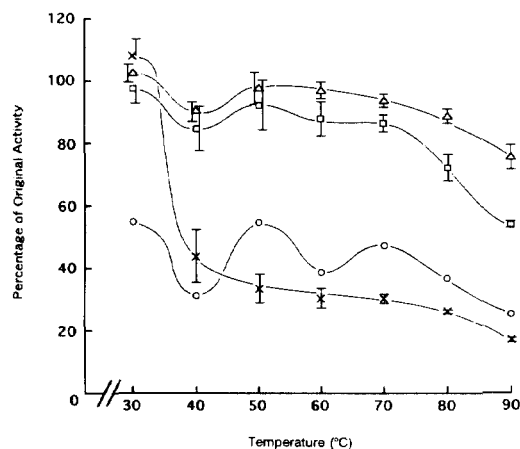

Figure 4. Thermostability at different temperatures of *Pseudomonas fluorescens* 33 lipase in .01 *M* sodium phosphate buffer, pH 6.6, containing α_s-casein (△; n = 4), β-casein (□; n = 4), or κ-casein (×; n = 4); incubation was for 10 min. The enzyme protein was adjusted to 10 μg/ml. Phosphate buffer containing no caseins (O) is included for comparison.

(Figure 4); heating above 70°C was required to inactivate the lipase gradually. In contrast, κ-casein was ineffective for thermostabilization of the lipase. In the presence of α-lactalbumin, LTI temperature was centered at 40 to 50°C

(Figure 5). The lipase containing β-lactoglobulin retained higher activity as the heating temperature increased above 50°C; remaining activity after heating at 80 to 90°C was comparable with that of unheated lipase
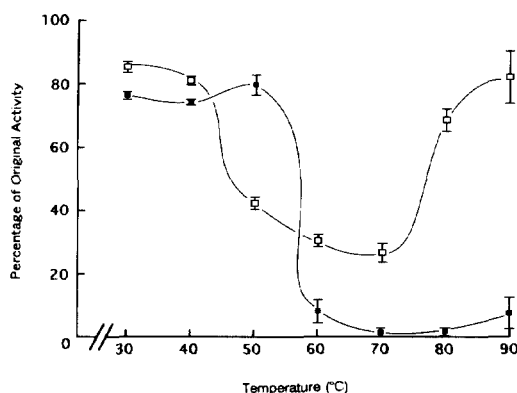

Figure 3. Thermostability at different temperatures of *Pseudomonas fluorescens* 33 lipase in .1% whey proteins buffered with .01 *M* sodium phosphate, pH 6.6 (□; n = 4), or synthetic milk salts solution, pH 6.6 (■; n = 4); incubation was for 10 min. The enzyme protein was at 10 μg/ml.
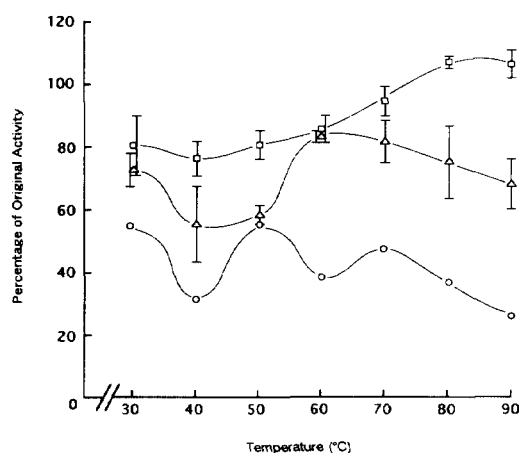

Figure 5. Thermostability at different temperatures of *Pseudomonas fluorescens* 33 lipase in .01 *M* sodium phosphate buffer, pH 6.6, containing α-lactalbumin (△; n = 4) or β-lactoglobulin (□; n = 4); incubation was for 10 min. The enzyme protein was adjusted to 10 μg/ml. Phosphate buffer containing no whey protein (O) is included for comparison.

(Figure 5). Among milk proteins used in this study, only α-lactalbumin and whey proteins in phosphate buffer offered LTI.

DISCUSSION

Some workers (11, 18) described the thermostabilizing effect of Ca^{2+} on bacterial lipases. However, some constituents of synthetic milk salts solution seemed to be effective in making the *P. fluorescens* 33 lipase susceptible to thermal inactivation above 60°C (Figure 1).

We hypothesized that the thermostability of the lipase in skim milk consisted of a balance of the thermal stabilization of casein micelle suspension and the thermal destabilization of whey proteins (Figure 2). We did not investigate the effects of some low molecular weight materials that appeared in skim milk but not in synthetic milk salts solution (e.g., lactose, glucose, vitamins, or trace minerals) for their effect on the thermostability of the lipase.

The effect of casein in thermal stabilization of the lipase is mainly derived from α_s-casein and β-casein (Figure 4).

The LTI temperature of the lipase in phosphate buffer shifted to 60 to 70°C in the presence of whey proteins (Figure 3). The shift was ascribed to interaction between the lipase and β-lactoglobulin at elevated temperatures (Figure 5). However, whey proteins provide extensive thermal inactivation in the presence of synthetic milk salts, which suggests that a multiple system might be involved in the inactivation of the lipase.

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