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Factors Affecting the Heat Stability of Lipase Produced by a Strain of *Pseudomonas fluorescens*

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ABSTRACT

Maximum lipase activity produced during growth of Pseudomonas fluorescens *strain 38 at 10°C was approximately fifteen times greater in half strength peptone water than in whole milk. Lipase production in the latter medium at 4°C was approximately 25% greater than at 10°C.*

Isoelectric focusing of lipase produced in both UHT whole milk and half strength peptone water gave one band of activity at pH 5·0. Also, both temperature and pH optima for lipase activity were similar for the enzyme produced in either of the two culturing media. The thermostability of lipase at 71·5°C was greater in whole milk cultures than in peptone water cultures. A simulated HTST pasteurization heat treatment (71·5°C for 15 s) caused complete deactivation of lipase in partially purified preparations obtained from both culturing media. However, after the same heat treatment in the presence of either Triton X-100 (0·1% v/v) or casein (3·5% w/v) approximately 65–70% and more than 90%, respectively, of the enzymic activity of partially purified lipases was retained.

INTRODUCTION

Extracellular heat stable lipases produced by psychrotrophic *Pseudomonas* spp. in raw milk during refrigerated storage may cause off-flavours to develop by the production of rancid free fatty acids in dairy products like cheese and UHT milk (Cogan, 1977; Law, 1979; Andersson *et al.*, 1981).

The thermostability of lipases produced by *Pseudomonas* spp. has been

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studied by other workers (Driessen & Stadhouders, 1974; Andersson *et al.*, 1979; Adams & Brawley, 1981; Fox & Stepaniak, 1983; Dring & Fox, 1983), using skimmed milk cultures to generate the enzyme. It has seemed to us probable that the constituents of culture media in which such lipases are produced may affect not only production of the enzymes, as reported by Bucky *et al.* (1986), but also their thermostabilities. For this reason we have determined the activity and heat stability of lipase produced in UHT whole milk and, for comparative purposes, half strength peptone water. In addition, pH and temperature optima, isoelectric point and approximate molecular weights of lipases were compared for enzyme produced in the two media.

MATERIALS AND METHODS

Organism identification and maintenance

The bacterium studied was originally isolated from refrigerated raw milk. It was identified as *Pseudomonas fluorescens* on the basis of Gram stain, catalase and oxidase production, motility, fluorescein production on King's B medium, acid production from glucose and gelatin liquefaction according to the scheme recommended by Cowan & Steel (1974). The organism was shown to be a particularly active lipase producer on solid growth media (tributyrin agar, Tween 80 agar and modified Crossley's victoria blue butterfat medium) according to methods recommended by Jonsson & Snygg (1974).

Cultures (24 h at 27°C) were prepared in Nutrient Broth (Oxoid) and stored under liquid nitrogen, a fresh culture being used for each growth experiment.

Culturing conditions and media

Samples (750 ml) of half strength peptone water (Oxoid) and UHT whole milk (Leeds Industrial Cooperative Society Dairy) were dispensed into 2·5 litre conical flasks. Inocula (1·0 ml) were obtained from 24 h subcultures, incubated at 27°C, of the same medium as in the subsequent test run. After inoculation cultures were incubated at either 4°C or 10°C in a refrigerated orbital incubator (Gallenkamp) under shaken (100 rpm) conditions. Samples (5 ml) were withdrawn at regular intervals for estimation of viable counts, using the spread plate technique, and measurement of lipase activity.

Enzyme and protein assays

Lipase activity
Lipase activity was assayed using triolein as substrate. The reaction mixture contained 2 ml of triolein emulsion [10% w/v triolein (practical grade, Sigma) emulsified in 10% w/v gum arabic (Sigma) using an Ultra-Turrax homogenizer (Sartorius, Gottingen), at maximum speed for 10 min], 0·5 ml of a suitable dilution of enzyme solution and 2 ml of 0·2M Tris-HCl [buffer] at pH 8·5 (the pH value optimum for lipase activity).

A 1 ml portion of the reaction mixture was transferred to 5 ml of Dole's reagent [Propan-2-ol, *n*-heptane and 2M H_2SO_4 (all Analar, BDH), 40:10:1 by volume] initially and after 1 hour's incubation at 40°C (the optimum temperature for activity). The amount of oleic acid released was determined by the method of Dole & Meinertz (1960) using oleic acid as standard. One unit of lipase activity is defined as the amount of enzyme releasing 1 μmol of oleic acid a minute.

Proteinase activity
Proteinase activity was measured by the method of Millet (1970) according to the modifications of Ingham *et al.* (1981) with azocasein (Sigma) as substrate. Assays were performed at 40°C and pH 6·0 which were optima for enzymic activity.

Protein assay
The concentration of protein in lipase extracts was determined either by absorbance at 280 nm, or by the method of Lowry *et al.* (1951) according to the modifications of Herbert *et al.* (1971) with bovine serum albumin as standard.

Crude lipase production

Peptone water and whole milk cultures were centrifuged (10 000 *g* for 20 min at 4°C) after approximately 100 hours' incubation at 10°C when lipase activity was maximal. The peptone water supernatant so produced was defined as crude lipase from this source. For whole milk, the cream layer which separated on centrifugation contained approximately 90% of the total lipase activity. In order to remove lipid material from the collected cream layer acetone was added (5 ml per gram of cream), mixed for 30 min at 0°C and subsequently centrifuged (20 000 *g* for 20 min at 0°C). The precipitate produced was washed (× 3) with acetone (5 ml per gram) and then (× 2) with diethyl ether (5 ml per gram) under vacuum at 4°C on filter paper (Whatman No. 1).

Resolubilization of this crude milk lipase was achieved by suspension of the precipitate in 0·05M Tris-HCl pH 8·5 + 0·05% v/v Triton X-100 (0·02 g per millilitre) for 30 min at room temperature, followed by centrifugation (20 000 *g* for 20 min at 4°C) to remove undissolved material.

Effect of storage on lipase activity and heat stability

In order to determine the effect of storage temperature on lipase stability, crude resolubilized lipase prepared from whole milk cultures was stored at either 4°C or 20°C for 72 h. Lipase activity and susceptibility to thermal deactivation (71·5°C for 15 s) were determined at 24-h intervals during this period.

Enzyme purification

Gel filtration on Sephadex G-100

Sephadex G-100 was used to remove low molecular weight material from a crude preparation of lipase produced in half strength peptone water. Peptone water culture supernatant (1000 ml) was concentrated (× 100) by ammonium sulphate precipitation, between 20% and 60% of saturation. After redissolving in 0·05M Tris-HCl, pH 8·5, gel filtration was carried out with Sephadex G-100 (column: 1·6 cm × 70 cm) using the same buffer. Eluted fractions (5 ml) collected at 4°C, which contained lipase activity, were combined and concentrated (× 10) by ultrafiltration using an Amicon apparatus fitted with a PM-10 Diaflo membrane (10 000 MW cut-off) prior to affinity chromatography.

The increased specific activity which resulted was not achieved for crude lipase prepared from whole milk cultures so this fractionation step was omitted for this preparation.

Affinity chromatography

After gel filtration peptone water lipase concentrate (3·0 ml) was passed down a column of Phenyl Sepharose CL-4B (1·0 cm × 16 cm) using 0·05M Tris-HCl, pH 8·5, as eluting buffer (20 ml per hour at 4°C), also used to pack the column. When protein could no longer be detected in the eluent by absorbance at 280 nm, three bed volumes of 0·05M Tris-HCl pH 8·5 + 2·0M KCl was passed through the gel bed. Lipase was then eluted with an increasing concentration gradient of Triton X-100 (0·5–1·5% v/v) in the same buffer over ten bed volumes.

Lipase in crude preparations from milk cultures did not bind to the affinity gel by the method described above. Binding was, however, achieved by mixing Phenyl Sepharose CL-4B (5 ml) pre-equilibrated with 0·05M Tris-HCl pH 8·5 + 1·0M KCl with concentrated (× 10) crude lipase (prepared by

ammonium sulphate precipitation between 20% and 60% of saturation and redissolving in the same buffer). After gentle mixing for 1 h at 4°C the gel slurry was packed into the column used above (10 ml per hour at 4°C). Elution was continued with the equilibrating buffer until protein could no longer be detected by absorbance (280 nm); lipase was then eluted from the column as for the peptone water enzyme.

Gel filtration on Sephadex G-200

Gel filtration on Sephadex G-200 was carried out using 0·05M Tris-HCl, pH 8·5, buffer at 5 ml per hour, and 4°C. The column (1·6 cm × 70 cm) was calibrated using blue dextran, ox liver catalase, bovine serum albumin and cytochrome c according to the method of Andrews (1965). Concentrated lipase (2·0 ml) prepared by ultrafiltration (× 10 concentration) of fractions eluted from Phenyl Sepharose was passed down the Sephadex G-200 column. Fractions (5 ml) containing lipase activity were again combined and concentrated as above.

Enzyme properties

Isoelectric focusing

Half-strength peptone water culture supernatants were dialysed against distilled water and concentrated (× 10) by PM-10 ultrafiltration prior to isoelectric focusing. Crude resolubilized lipase prepared from the fat pellet of whole milk was subjected to ultrafiltration using an XM-300 Diaflo membrane (300 000 MW cut-off) to produce a clear filtrate containing lipase activity. This additional step was necessary to remove high molecular weight material which interfered with enzyme mobility during isoelectric focusing. The filtrate so produced was dialyzed against distilled water and concentrated (× 10) by PM-10 ultrafiltration. Isoelectric focusing was carried out for 2 h on thin-layer polyacrylamide gels (PAGPLATE, pH 3·5–9·5, LKB) using the LKB 2117 Multiphor system. Limiting power was 25 W, maximum potential difference was 1500 V and maximum current was 25 mA. In order to determine the pH gradient in the gel, isoelectric marker proteins (BDH) were focused concurrently. Lipase activity was detected by zones of hydrolysis in overlaid tributyrin agar [1% w/v tributyrin (practical grade, Sigma) homogenized in 1·5% w/v purified agar (Oxoid)].

Heat treatments

Samples (1·5 ml; see below) were injected into sterile polythene bags (Seward Surgical Ltd) compressed between hinged copper plates immersed in a water bath at 71·5°C. After holding at this temperature for between 15 s and 30 min the whole apparatus was plunged into a water bath at 10°C to facilitate rapid cooling. A double heat treatment at 71·5°C was also used,

with a total holding period of 30 s (2 × 15 s) interrupted by cooling to 10°C for approximately 2 min.

Single heat treatments (15 s to 30 min) were carried out on whole milk and peptone water culture samples collected towards the end of logarithmic growth when maximum lipase activities were approached. Also, additional milk culture samples were subjected to the double heat treatment. All samples were adjusted to pH 6·6 using 1M NaOH prior to heat treatments.

Crude preparations of lipase from peptone water and whole milk cultures previously concentrated (× 10) by PM-10 ultrafiltration were diluted (× 10) in sterile UHT whole milk before single heat treatment for 15 s.

The effect of pH value on lipase thermostability was determined by dilution of these concentrates (× 10) in 0·05M Tris-HCl buffer (pH 7·0–9·0) before the 15-s heat treatment.

The effect of Triton X-100 on the thermostability of lipase was determined, since this detergent was used during lipase fractionation. The crude lipase concentrates used above were diluted (× 10) with 0·05M Tris-HCl, pH 8·5, or the same buffer containing Triton X-100 (0·025–0·2% v/v) before heat treatment for 15 s. In addition, partially purified lipase concentrates (see section below headed 'Enzyme purification') were diluted (× 50) with Tris-HCl buffer (see above) or the same buffer containing either Triton X-100 (0·1% v/v) or casein (Technical grade, Sigma, 3·5% w/v) before 15 s heat treatment. Lipase solutions were buffered at pH 8·5 since heat stability was greatest at this initial pH.

All of the data shown represent means of triplicate determinations for lipase deactivation studies.

RESULTS

Effect of culturing conditions on lipase production

During culturing at 10°C lipase activity was first detected in peptone water and whole milk cultures towards the end of logarithmic growth. However, maximum levels of activity were approximately × 12 to × 15 greater in peptone water (Figs 1 and 2). Also, maximum lipase activity in whole milk during culturing at 4°C was approximately 25% greater than at 10°C (Fig. 2).

Enzyme purification

Lipase was eluted from Sephadex G-100, Phenyl Sepharose CL-4B and Sephadex G-200 as a single band. On the basis of specific activity

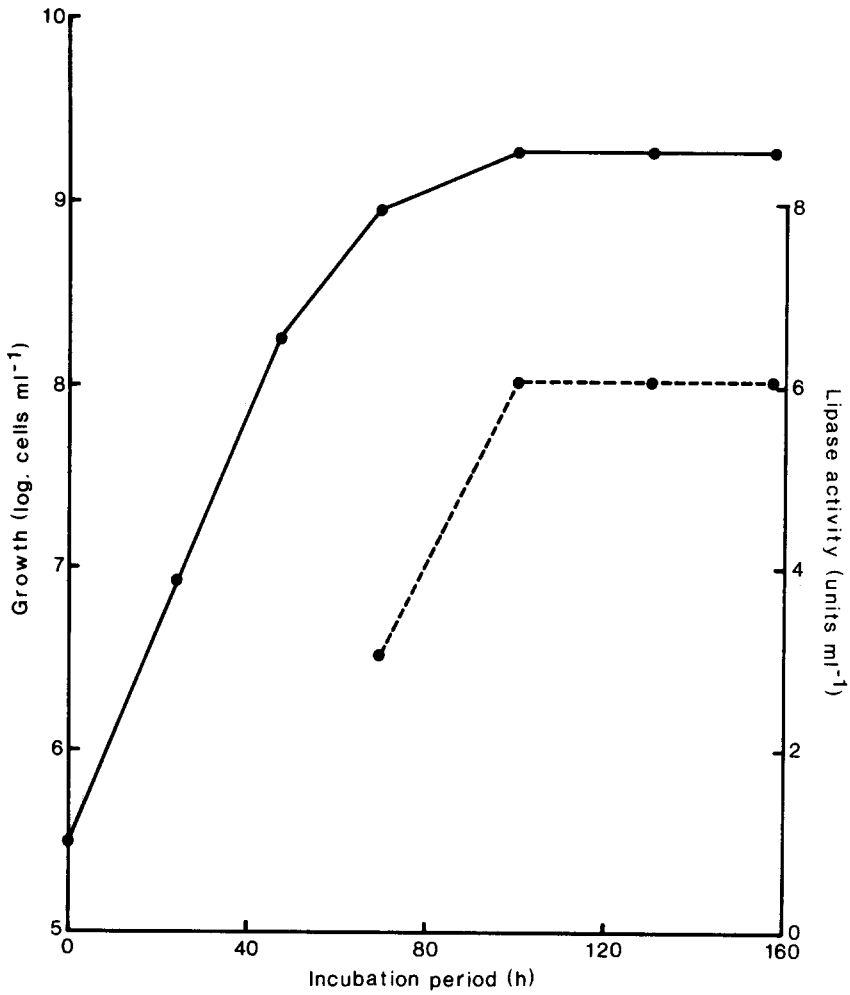

Fig. 1. Growth (●——●) and lipase production (●- - -●) by *Pseudomonas fluorescens* strain 38 in shaken half strength peptone water at 10°C.

measurements the final purifications achieved for the enzyme generated in whole milk and half strength peptone water were 48-fold and 68-fold, respectively. Proteinase activity present in the crude lipase preparations was substantially removed by the purification methods employed and no proteolytic activity was detected in the lipase fractions eluted from Sephadex G-200.

On the basis of elution from Sephadex G-200 using the calibration curve of K_{av} versus log MW for the standard proteins described above, the molecular weights of milk and peptone water lipase were estimated to be 155 000 and 175 000, respectively. After isoelectric focusing a single lipase

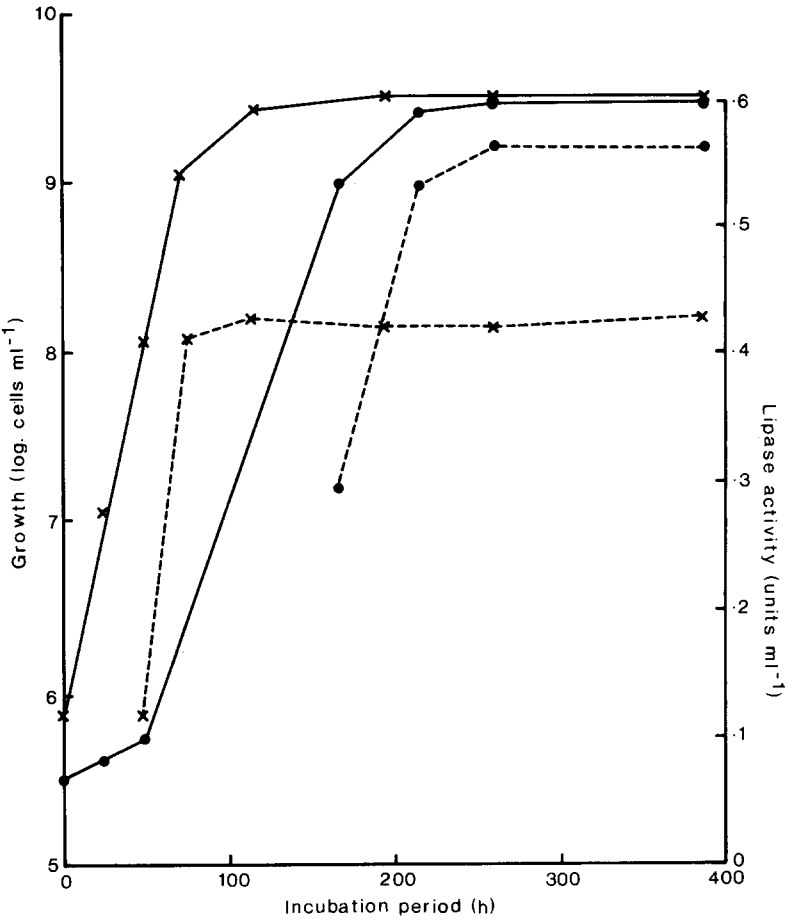

Fig. 2. Growth at 10°C (×——×) and 4°C (●——●) and lipase production at 10°C (×---×) and 4°C (●---●) by *Pseudomonas fluorescens* strain 38 in shaken whole milk.

band at pH 5·0 was detected for the enzyme generated in both culture media.

Heat stability

Lipase activity in whole milk and peptone water cultures declined non-linearly with time at 71·5°C. The initial rapid loss of activity was followed by a more gradual but progressive decline (Fig. 3). Lipase was far more thermostable in the former system. However, after a double heat treatment (2 × 15 s) interrupted by cooling to 10°C, compared to a continuous heat treatment for 30 s, there was a considerable enhancement of the loss of lipase activity in the milk cultures (Table 1).

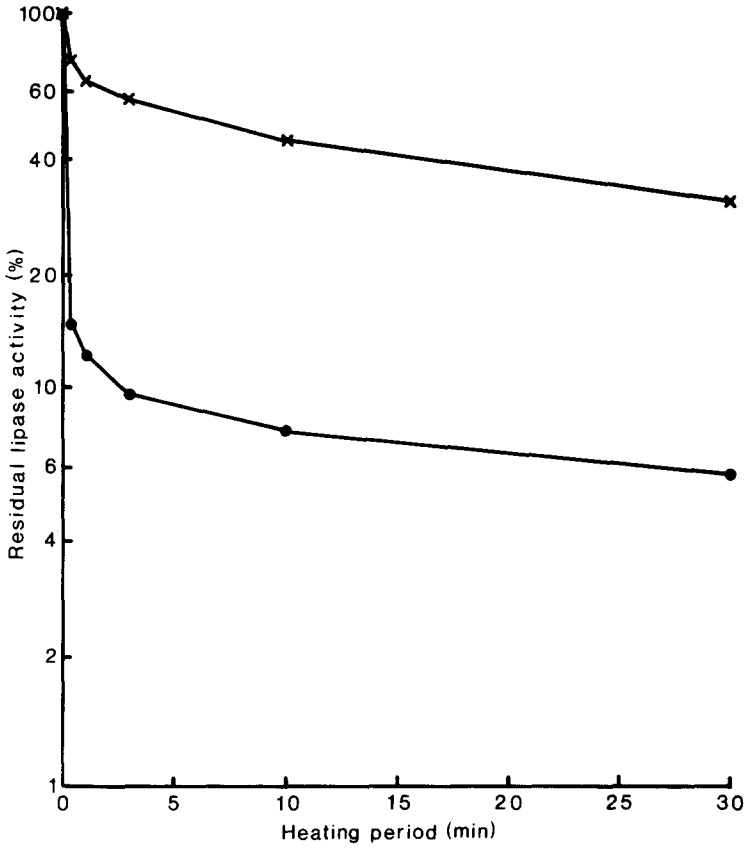

Fig. 3. Thermal deactivation curves for *Pseudomonas fluorescens* strain 38 at 71·5°C in whole milk culture samples (×——×) and half strength peptone water culture samples (●——●).

TABLE 1

Thermostability of Lipase Produced by *Pseudomonas fluorescens* Strain 38 in Half Strength Peptone Water and Whole Milk

Heating medium	*Heat treatment at 71·5°C*	*Per cent residual lipase activity (means of triplicate determinations)*
Peptone water	1 × 15 s	15+/−3
Whole milk	1 × 15 s	75+/−5
	1 × 30 s	70+/−5
	2 × 15 s	33+/−3

Crude lipase concentrates, prepared from either whole milk or peptone water cultures and diluted in sterile UHT whole milk, retained similar percentage levels of enzyme activity (approximately 75%) after heat treatment for 15 s. For these concentrates, diluted in Tris-HCl buffer (pH 7·0 to 9·0), lipase thermostability was found to be greatest at pH 8·5 (Fig. 4).

The presence of Triton X-100 greatly increased the thermostability of

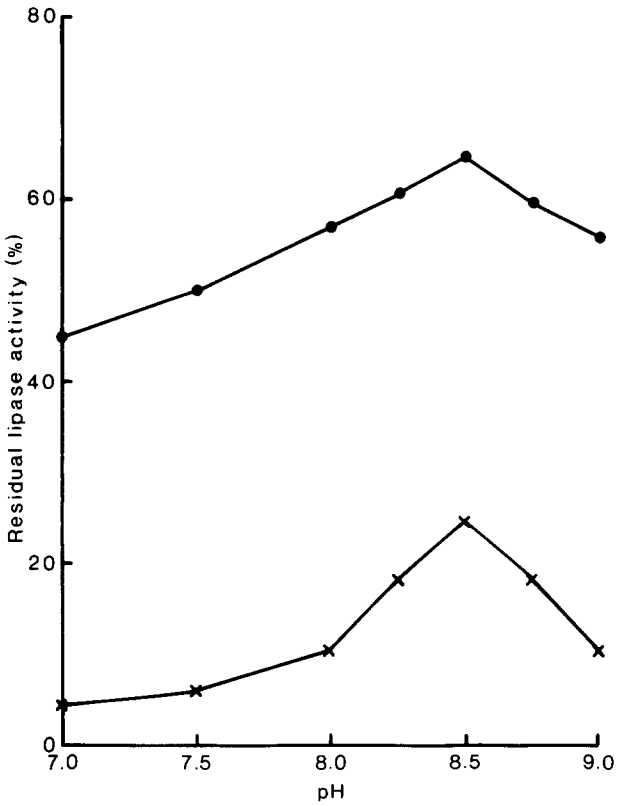

Fig. 4. Effect of pH on the thermal stability (71·5°C for 15 s) of *Pseudomonas fluorescens* strain 38 lipase in whole milk culture samples (●——●) and half strength peptone water samples (×——×).

crude peptone water-generated lipase (Fig. 5), the effect being concentration dependent. In the presence of 0·05% (v/v) of Triton X-100, approximately 90% of the original enzyme activity was retained after heat treatment at 71·5°C for 15 s. However, for crude lipase from milk cultures in the presence or absence of the detergent, approximately 70% of the original activity remained after the same heat treatment.

During storage of crude lipase preparations obtained from milk cultures

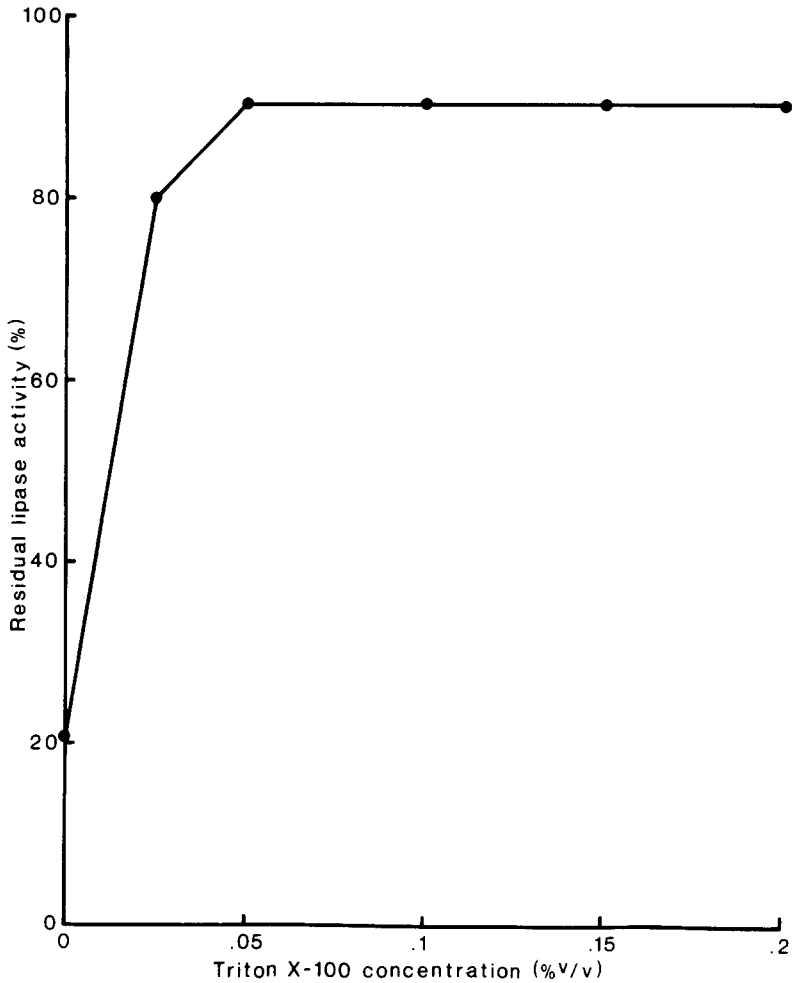

Fig. 5. Effect of Triton X-100 on the thermal stability (71·5°C for 15 s) of *Pseudomonas fluorescens* strain 38 lipase in crude preparations of the enzyme produced in shaken half strength peptone water at 10°C.

at 4°C and 20°C, lipase activity remained constant, whereas thermostability, as determined by a 15 s treatment at 71·5°C, declined linearly and more rapidly at the higher storage temperature (Fig. 6).

For partially purified lipase from whole milk and peptone water, complete deactivation occurred after a 15 s heat treatment in Tris-HCl buffer. However, in the presence of Triton X-100 (0·1% v/v) approximately 70% of the enzyme activities before heat treatment remained. Casein (3·5% (w/v)) further increased the thermostabilities of lipase produced in either culture medium so that more than 90% of the original activities were retained after the same 15 s heat treatment.

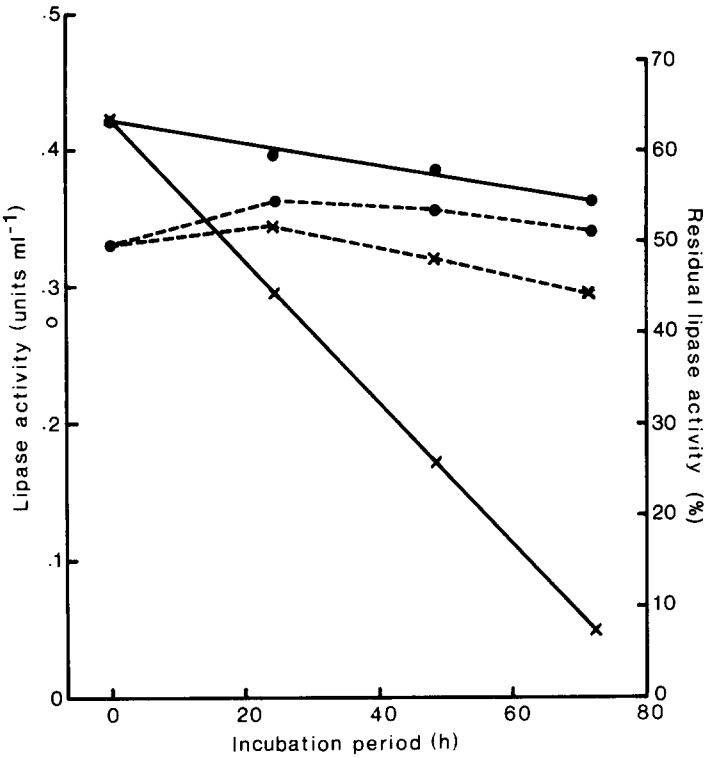

Fig. 6. Lipase activity during storage at 4°C (●---●) and 20°C (×---×), and lipase thermal stability (71·5°C for 15 s) during storage at 4°C (●——●) and 20°C (×——×) for crude preparations of the enzyme produced in shaken whole milk by *Pseudomonas fluorescens* strain 38 at 10°C.

DISCUSSION

Maximum lipase activity in whole milk was greater during culturing of the test strain of *Pseudomonas fluorescens* at 4°C than 10°C. A similar effect of growth temperature has also been reported for other strains of *Pseudomonas fluorescens* (e.g. Lawrence *et al.*, 1967; Andersson, 1980). However, during culturing at 10°C the maximum level of the microbial lipase activity recorded in whole milk was very much less than in peptone water. It is suggested that the same single lipase enzyme may perhaps have been produced in both culture media, since only one lipase band at pH 5·0 was detected on isoelectric focusing gels. Also, the pH and temperature optima for microbial lipase activities from both sources were similar. Thus, the principal distinction between the two media used for growth of the microorganism seems to be related to the rates of lipase synthesis and/or secretion, rather than the production of different microbial lipases.

However, the molecular weights determined for lipase after partial purification were related to the culture medium in which the enzyme had been produced. Although the production of two distinct lipases cannot be entirely discounted, self-association and aggregation with other components, has been reported for lipases (Lawrence *et al.*, 1967; Kimura *et al.*, 1972; Roy, 1981), and this may account for the difference in molecular weights.

Lipase in whole milk cultures was more resistant to thermal deactivation than in peptone water cultures. The loss of lipase activity was non-linear with time in either culture medium and a similar observation has also been reported for other pseudomonad lipases (e.g. Driessen & Stadhouders, 1974; Dring & Fox, 1983). In contrast, partially purified preparations of the enzyme prepared from either culture medium retained no lipase activity after the shortest testing period used (15 s) and therefore it is proposed that both media offer some protection against thermal deactivation. Also, since the thermostability of crude peptone water-generated lipase was enhanced by dilution in milk, it is concluded that milk components are able to stabilize the enzyme to a greater extent than those in peptone water. A similar protective effect by milk compared with phosphate buffer on the heat stability of extracellular lipase produced by two pseudomonads has also been reported by Law *et al.* (1976).

As Triton X-100 enhanced the thermostability of lipase in partially purified preparations of the enzyme from both cultures, it is possible that hydrophobic bonding is involved in the stabilization of the enzyme. Since Triton did not significantly affect lipase thermostability in crude preparations of the enzyme obtained from whole milk cultures it seems that the interaction between the detergent and lipase was hindered by association with other milk constituents. Such a proposal is further supported by the observation that whole casein also enhanced the thermostability of partially purified lipases.

Surprisingly, the lipase activity in whole milk cultures was substantially reduced by the short period double heat treatment. In this way it seems that the enzyme was sensitized to the second heat treatment. Additionally, as thermal stability of the lipase activity in crude preparations from milk cultures gradually declined during prolonged storage, while total lipase activity remained constant, it may be that prolonged storage, which is likely to involve proteolysis, also sensitizes the lipase to heat treatment.

Therefore, overall, it is suggested that lipase thermal stability may have been enhanced by interactions with milk proteins. The protective effect of Triton X-100 strongly suggests the involvement of either intramolecular or intermolecular hydrophobic bonding. During the double heat treatment procedure such hydrophobic bonding interactions may perhaps be readily

disrupted by rapid changes in temperature which might result in exposure and increased susceptibility of the enzyme to heat.

This study has also shown that in assessing the heat stability of bacterial lipases with a potential for causing the development of off-flavours, whole milk should be used as the experimental culture medium. Peptone water did not offer the same protection against thermal deactivation, even though the microbial lipase produced in both media was apparently the same.

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