Microbial Lipolysis at Low Temperatures

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It was found that lipase production during the growth of Pseudomonas fluorescens was not a function of the total number of bacteria. The optimal temperatures for bacterial growth and lipase production were determined as 20 and 8°C, respectively. The lipolytic activity was studied in emulsions of olive oil at temperatures ranging from +8 to −30°C. After an initially rapid lipolysis, the reactions retarded at different levels depending on storage temperature. Transfer to a higher temperature resulted in a resumed lipolysis. Also, at low temperatures, lipolysis was studied as a function of water activity and was found to occur in dehydrated substrates.

Refrigerated storage of foods is a universally accepted method for prolonging their shelf life. One limiting factor of such storage is the presence of psychrotrophic bacteria (11), of which the Pseudomonas bacteria are regarded as one of the most common genera which spoil foodstuffs at low temperatures (A. Björklund, Ph.D. thesis, University of Otaniemi, Helsinki, Finland, 1970). The ability to produce fat-splitting enzymes, lipases, is a property common to most bacteria (14). In chilled stored foods the lipolytic activity of psychrotrophic microorganisms can give rise to quality changes (3).

The optimum conditions of enzyme production generally differ from those of bacterial growth. The optimum temperature of lipase production is often lower than that of microbial growth and thus is not a function of total cell growth (2, 11, 17).

Deterioration of frozen foods is often attributed to enzymatic reactions caused by enzymes like invertase, proteases, and lipases, which can be secreted by microorganisms before freezing. These enzymes can still be active in frozen foods although the organisms are dormant (6, 7). Microorganisms like Pseudomonas fragi, Staphylococcus aureus, Geotrichum candidum, Candida lipolytica, Penicillium roqueforti, and Penicillium sp. have been reported to produce lipases active at −7, −18, and −29°C (3). Pancreatic lipases have also been studied and found to be active in frozen systems (16, 18).

The restricted water activity of frozen foods may be considered as analogous to the condition in dehydrated foods. Enzymatic reactions can, however, occur in foods which have been protected against microbial spoilage by lowering the water content, and the enzyme activity is related to the remaining water content of the food (1, 19). Apparently it is the availability of the water present, rather than the absolute water content, that is decisive for the activity. The water activity (aw) is a better measure of the availability of water in food than its water content. Water activity is often defined by the equation aw = p/p0, where p is the vapor pressure of water in a food system at a certain temperature and p0 is the vapor pressure of pure water at the same temperature (12). The relative humidity (in percent) = aw × 100 (12). The interrelationship of water content and water activity, determined as equilibrium relative humidity, is often complex and represented by adsorption and desorption isotherms (12). For dehydrated foods these isotherms are generally sigmoidal (1).

MATERIALS AND METHODS

Organism. Stock cultures of P. fluorescens SIK W1 were maintained as slant cultures on tryptone glucose extract agar (Difco Laboratories, Detroit, Mich.). Before being used as inoculum, the organism was subcultured once in nutrient broth (Difco) for 20 h at 20°C.

Culture conditions. The bacterium was cultured in 2-liter Erlenmeyer flasks containing 1 liter of nutrient broth, autoclaved at 121°C for 17 min before inoculation. The cultures were incubated in thermostated incubation rooms at 4, 8, 15, 20, and 30°C (±1°C) under reciprocal shaking at 75 strokes per min. Samples were withdrawn at regular intervals for determination of total bacterial count and lipolytic activity.

Assay of total count. From appropriate dilutions of the culture samples in 0.85% NaCl solution, 1 ml was withdrawn and mixed with molten and tempered tryptone glucose extract agar (45 to 48°C) in a petri dish. The colonies were counted after 2 days of incubation at 30°C. The viable count was expressed as the average of two samples.

Assay of lipase activity. The lipase activity in the culture media was determined by a pH-stat method described earlier (4). Olive oil was used as a substrate, and the activity was expressed as micromoles of fatty acids released per minute per milliliter of medium.
Determination of titratable acidity. The amount of fatty acids released during lipolysis was determined by titration with alcoholic NaOH, as previously described (8). The concentration of free fatty acid was expressed as titratable acidity in micromoles of acid per milliliter of medium.

Enzyme preparations. A cell-free lipase solution was obtained by centrifuging a 2-day culture of P. fluorescens at 24,000 x g for 20 min at 4°C. The solution was concentrated approximately 20 times by ultrafiltration, as previously described (4). In the water activity experiments, this enzyme solution was lyophilized at 0.1 mm of Hg for 2 days. This enzyme powder was stored at −18°C.

Crystallized subtilisin type Carlsberg (Novo Industri A/S, Bagsvaerd, Denmark) was used to study the lipase stability in the presence of a proteolytic enzyme.

Emulsified substrate preparation. Olive oil emulsions were prepared by first emulsifying 5 ml of olive oil in 45 ml of a 10% water solution of gum arabic by sonication at 1.5 A for 2 min (Ultra Sonic; Measuring & Scientific Equipment, Ltd., London, England). A 100 ml amount of a solution consisting of 30 ml of water, 30 ml of 0.075 M CaCl2, 20 ml of 3 M NaCl, and 20 ml of sodium taurocholate (15 mg/ml) was then added to the emulsion.

Lipolysis at 1, 4, and 8°C. To 375 ml of olive oil emulsion, 25 ml of enzyme solution was added. Formaldehyde was added to a final concentration of 0.04% to prevent microbial growth. Olive oil emulsions without added enzyme solution were used as controls. The emulsions were incubated at 1, 4, and 8°C (±1°C), and samples were periodically withdrawn for determination of titratable acidity. After about 2 weeks, 100 ml of the emulsions incubated at 1 and 4°C were transferred to new flasks, which were then incubated at 8°C. The rest of the emulsions were retained at 1 and 4°C.

Lipolysis at subzero temperatures. Test tubes, each containing a mixture of 1 ml of enzyme solution and 9 ml of olive oil emulsion, were frozen in an alcohol bath immediately after the addition of enzyme. The contents had reached a temperature below −30°C within 2 min. Replicate samples were stored at −10, −20, and −30°C (±3°C). Test tubes containing olive oil emulsion but no enzyme solution were prepared and used as controls. Samples were removed periodically and thawed at +4°C before determination of titratable acidity. After about 1 year, some tubes were transferred from −30 and −20°C to −10°C, where the incubation continued.

Enzyme stability during storage. Lipase solutions from a 2-day culture of lipase solution, 0.5 ml of a water solution of subtilisin (0.5 mg/ml) was added. The solutions were sterilized by membrane filtration (0.22 µm; Millipore Corp., Bedford, Mass.), and the mixture was incubated at 20°C. The lipase activity in the stored samples was determined periodically by the pH-stat technique and expressed as percent initial activity.

Lipolysis at different water activities. Conditions and incubations were performed in milieus with different αw values, prepared by adding saturated salt solutions (see Table 1) to sealed jars at 20°C. The αw values were taken from the literature (13). One gram of lyophilized lipase, previously conditioned to the selected αw at 20°C for 48 h, was added to 20.0 g of preconditioned, spray-dried, full-fat (2.8%) milk powder. The water content of the milk powder was about 4%, corresponding to an αw of about 0.20 (15). Thus, the milk powder was conditioned to different αw values according to the adsorption isotherm (12), except in the case of P2O5 and LiCl conditions. To prevent microbial growth, methyl-p-hydroxybenzoate (AB Bforfs Nobelkrut, Bofors, Sweden) was added to a final concentration of 0.1% and the air was replaced by nitrogen. The degree of lipolysis was determined after 30 days by determining the amount of titratable acid in the following way. To 10.0 g of milk powder 75 ml of distilled water was added. After mixing, 10.0 ml of the mixture was transferred to an Erlenmeyer flask and 20.0 ml of distilled water was added. The sample was then titrated to pH 8.30 by 0.1 M NaOH in an automatic titration device (Mettler DK 10, DK 11, DV 11, and DV 101; Greifensee, Switzerland), and the acidity was expressed as millimoles of NaOH per gram of milk powder. Samples of milk powder, without the addition of lipase, were used as controls.

RESULTS

Growth and lipase production as a function of temperature. Growth curves of P. fluorescens cultivated at five different temperatures are shown in Fig. 1 and 2. The bacteria multiplied most rapidly at 20 and 30°C but produced the largest number of cells at 4 to 15°C. The generation times at 4, 8, 15, 20, and 30°C were calculated to be 316, 145, 64, 47, and 48 min, respectively. The amount of lipase in the culture media varied with the cultivation temperature and reached a maximal value at 8°C (Fig. 3). Only low lipase activity was observed in cultures incubated at 30°C, although the bacterial counts were high. The lipase activity was always higher at temperatures below the optimal temperature for bacterial growth (20°C), indicating that the lipase production of P. fluorescens is not directly correlated to the bacterial growth, measured as total numbers.

| Table 1. Water activity values (αw) for saturated salt solutions at 20°C* |
|---------------------------------|---|
| Salt                            | αw |
| P2O5                            | 0  |
| LiCl                            | 0.14 |
| MgCl2                           | 0.33 |
| Mg(NO3)2                        | 0.54 |
| NaCl                            | 0.76 |
| (NH4)2SO4                       | 0.81 |
| Li2SO4                          | 0.85 |
| Na2HPO4                         | 0.96 |

* Results of Labuza et al. (13).
Lipolysis at 1, 4, and 8°C. The optimal temperature for P. fluorescens lipase has been reported as 34°C (4), and in the present investigation the lipase action was found to proceed at temperatures used for the cold storage of foods (Fig. 4). The reaction rates were initially rapid, but after 3 days a slower rate of hydrolysis was noticed. When samples from the 1 and 4°C experiment after 12 days were transferred to 8°C, the lipolysis was again accelerated and the final levels of hydrolysis reached those observed in the 8°C experiment. The autohydrolysis was low at all temperatures used and did not exceed 5% of the enzyme-containing samples.

Lipolysis at subzero temperatures. Figure 5 shows the formation rate and amount of acid released by the P. fluorescens lipase at −10, −20, and −30°C. After an initially rapid lipolysis, the reactions stopped at different levels, depending on the storage temperature. The reactions which had stopped at −20 and −30°C resumed when the samples were transferred to −10°C (Fig. 5). The new levels were similar to those obtained for samples which had been stored at −10°C all the time.

Enzyme stability. Figure 6 shows the lipase activity as a function of storage time at different temperatures. Frozen storage did not affect the enzyme activity to any greater extent, and at 20°C the lipase still possessed 50% of its original activity after about 3 months. However, subtili-
Increased with lipolysis constant level.

Solubility of different temperatures showing inactivated the proteolytic enzymes present in the medium can inactivate lipases.

**Lipolysis as a function of a\textsubscript{w}** Lipolysis depended on water activity (Fig. 7). At a\textsubscript{w} values of 0.54 and lower, lipolysis took place at a fairly constant level. At higher a\textsubscript{w} values, however, the lipolysis increased with increasing a\textsubscript{w}. At an a\textsubscript{w} value of 0.85, the lipolysis was about three times that obtained at an a\textsubscript{w} of 0.54.

**DISCUSSION**

The *P. fluorescens* cultures reached larger cell densities at temperatures below that at which the exponential growth was most rapid. The explanation for this might be the one given by Sinclair and Stokes (20), who found that the solubility of oxygen increased when the incubation temperature was lowered and thus more oxygen became available for growth, resulting in a larger number of viable cells.

The amount of lipase present in the media is dependent on several factors which regulate the production and destruction of lipase. The apparent productivity, determined as lipase activity in the culture media, is thus the difference between production and destruction of lipase. In this investigation there was no correlation between the total number of cells and the apparent lipase production at different temperatures. Furthermore, it was noticed that increasing temperature had a depressing effect on the apparent lipase production. This is in agreement with other findings for both lipases (2, 11, 17) and protease (10). Alford and Elliott (2) suggested that lipase production of *P. fluorescens* is higher at lower temperatures, whereas Nashif and Nelson (17) claimed that the difference is possibly due to a higher rate of enzyme inactivation at higher temperatures. In the present investigation a comparison between growth curves (Fig. 1 and 2) and lipase stability (Fig. 6) suggests that the differences are due to a real decrease in lipase production, rather than to an accelerated destruction of lipase at higher temperatures. An explanation might be that the enzyme systems involved in lipase formation are repressed at higher temperatures, thus resulting in a lower productivity. Subtilisin, however, inactivated the lipase, and thus proteolytic enzymes can be one destructive factor. Such an effect of proteolytic enzymes has been discussed previously for *P. fluorescens* (9), and this investigation indicates that for bacteria producing both proteases and lipases proteolytic enzymes can inactivate lipases in their native form.

Lipolysis was shown to occur at temperatures used for both cold storage and frozen storage of foods. The reaction rates were always higher at the beginning of the storage period, and the ultimate acid accumulation depended on the incubation temperature. Similar results have been observed previously (3, 16, 18). The level at which the retardation of lipolysis starts depended on the temperature and might be due to inactivation of the lipase with time. However,
the reactions resumed when the temperature was raised, and thus an irreversible inactivation of the enzyme had not occurred. Another explanation can be that reaction products, such as fatty acids, inhibit the lipolysis and that this inhibition is temperature dependent. Benzonana and Desnuelle (5) showed that lipase was inactivated by fatty acids which accumulate at the lipid-water interface, where the reaction takes place. In partly frozen systems, a crystalline structure of water can affect the enzymatic reactions by reducing the area of the lipid-water interface. Besides, the mobility of enzyme, substrate and product molecules is limited in such systems. The activity noted when the emulsions were removed from a low temperature to a high temperature probably does not depend on the existence of more than one lipase, since isoelectric focusing results in only one peak with lipolytic activity (4).

Since the lipase is active at subzero temperatures where ice formation reduces water activity (18), it is not surprising that the lipase is also active in other products with reduced \(a_w\) values, like dehydrated foods. Acker (1) noticed that different values of hydrolysis were attained at different stages of \(a_w\) and that the enzyme reactions started again after raising the \(a_w\) value. The results of this investigation of lipolysis at cold-storage temperatures are thus identical to the findings of Acker, and some of his explanations probably apply to this study. At low \(a_w\) values, the amount of water available is limited, thus changing the lipid-water interface whereby the number of reaction sites may be reduced. The fact that substrates for lipases are water insoluble and that the water phase alone is not the location for the enzyme reactions can explain why lipolysis occurs at subfreezing temperatures and at low \(a_w\) values.

In a previous report, lipase from \(P.\) fluorescens was shown to be extremely resistant against both heat and chemical denaturation (4). Pasteurization or even sterilization processes before storage of foods will kill the bacteria, whereas heat-resistant lipase previously produced by them will remain active. Since the enzyme is also stable and active during storage at low temperatures, as well as at low water activities, lipase produced by \(P.\) fluorescens can cause changes in quality in fatty foods during prolonged chilled or dehydrated storage.

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LITERATURE CITED