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PRODUCTION AND PROPERTIES OF THE EXTRACELLULAR LIPASE OF *ACHROMOBACTER LIPOLYTICUM**

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SUMMARY

Achromobacter lipolyticum produced a maximum amount of extracellular lipase (glycerol ester hydrolase, EC 3.1.1.3) when grown at 21° for 36 h in a casitone broth medium at pH 7.0. The addition of 1% olive oil, corn, oil, or milk fat to the growth medium and slow stirring during incubation stimulated the lipase production 3-fold.

The enzyme possessed a pH optimum of 7.0 and a temperature optimum of 37°. The lipase appeared to be fairly heat stable. Heating at 71° for 180 min destroyed the activity by only 47%. However, the enzyme lost all its activity when heated at 99° for 40 min or when autoclaved at 121° for 15 min. Various salts stimulated the lipase activity, $MgCl_2$ being the most stimulatory and $CaCl_2$ the least.

The enzyme could lipolyze natural as well as synthetic glycerides. 10% substrate concn. seemed to be optimum for the enzyme action. Milk fat was lipolyzed faster than corn oil, olive oil, or any synthetic glyceride investigated. Oleyl glycerides were hydrolyzed more rapidly than butyryl glycerides. The lipase selectively liberated oleic, linoleic, and linolenic acids from milk fat.

INTRODUCTION

Several bacteria and fungi have been known to cause deterioration of a variety of foods, such as meat, fish, and dairy products, even when stored under refrigeration[1,2]. One of the most common defects encountered, particularly in the fatty foods, is the hydrolytic rancidity effected by the lipase(s) (glycerol ester hydrolases, EC 3.1.1.3) elaborated by the spoilage organisms. However, under certain conditions the action of such lipases is desirable. For example, in the ripening of Roquefort and Blue cheeses the lipases of *Penicillium roqueforti* aid in the development of the typical flavor. Also, during Cheddar cheese ripening, the lactobacilli upon autolysis release intracellular

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lipases which hydrolyze milk fat. The hydrolytic products thus formed contribute to the characteristic flavor development in Cheddar cheese[2].

Achromobacter lipolyticum is an active lipolytic organism which gains entry during production, processing, shipment, or storage of a variety of foods. HUSS[3] in 1908 identified *A. lipolyticum* as an organism contributing to hydrolytic rancidity in milk. Later, COLLINS AND HAMMER[4], LONG AND HAMMER[5], FOUTS[6], PETERS AND NELSON[7], and NASHIF AND NELSON[8] confirmed that the organism produces rancidity in dairy products.

Considerable work has been done on milk lipase and several fungal lipases with regard to their action on various glycerides and dairy products[9–14]. Relatively limited information is available on the characteristics of bacterial lipases, particularly the lipase of *A. lipolyticum*. This investigation was undertaken to study the factors affecting the production, the activity, and the specificity of the extracellular lipase of *A. lipolyticum*, with a view to controlling the deleterious effect of this organism upon the flavor quality of food products.

MATERIALS AND METHODS

Culture and preparation of extracellular lipase source

An active lipase producing culture of *A. lipolyticum* was obtained from the late Dr. W. O. NELSON, University of Illinois, Urbana. This culture had been originally obtained by him from Kraft Food Co., Glenview, Ill. A stock culture of the organism was maintained on tryptose–glucose agar slants, incubated at 21° for 24 h, stored at 4°, and subcultured monthly. For use in this study, a fresh inoculum of the organism was prepared by growing the organism in a basal growth medium containing 3% casitone (Difco), 0.1% Na_2HPO_4, and 0.01% KCl and incubating it at 21° for 24 h (ref. 11). One ml of the culture inoculum was inoculated into a 250-ml erlenmeyer flask containing 98 ml of sterile casitone medium *plus* 1 ml of sterile olive oil and incubated at 21° for 36 h. After incubation, the broth was centrifuged at 3000 × *g* for 15 min, and the cell-free broth was used as the extracellular enzyme source.

Lipase assay

The assay substrate was a freshly prepared emulsion of milk fat. Twenty ml of milk fat were dispersed in 80 ml of 10% gum arabic solution (in 0.025 M phosphate buffer, pH 7.0) at 40° and homogenized 5 times in a hand homogenizer. The substrate and the cell-free broth were tempered to 37°, 2.5 ml of the substrate was mixed with 2.5 ml of the cell-free broth, and the mixture was incubated at 37° for 15 min. The pH of the incubated samples was controlled at 7.0 by the addition of 0.1 M NaOH every 5 min. These samples were then assayed for the fatty acids liberated by the lipase action, using the silica-gel method of HARPER, SCHWARTZ AND EL-HAGARAWY[15]. The free fatty acids eluted from the silicic acid column with chloroform–butanol eluant (95:5, v/v) were then titrated with 0.01 M ethanolic KOH to the phenol red end point. The control consisted of 2.5 ml of the substrate and 2.5 ml of the autoclaved cell-free broth. The titration value obtained with the control sample was subtracted from the experimental value, and the net titration values, expressed as μmoles of free fatty acids, represented the lipase activity.

Concentration and purification of lipases

The lipase of *A. lipolyticum* was concentrated from 500 ml of cell-free broth by precipitation at 50% satn. of $(NH_4)_2SO_4$. The precipitate was dissolved in 10 ml of water and dialyzed against 2 l of deionized water. Milk lipase used in this study was purified by the method of CHANDAN AND SHAHANI[9].

Preparation and gas–liquid chromatography of methyl esters

The free fatty acids were separated from silica-gel eluates (described above) by the method of MCCARTHY AND DUTHIE[16]. In order to minimize loss of short-chain acids, the free fatty acid portions from the MCCARTHY AND DUTHIE columns were collected in a flask cooled with a salt-ice mixture and heat was avoided during concentration of the fractions.

Methyl esters of the free fatty acids were prepared by the technique of METCALF AND SCHMITZ[17], and the esters were extracted from the esterification mixture by a modified procedure of GANDER, JENSEN AND SAMPUGNA[18]. The modification involved the saturation of the mixture with NaCl followed by extraction of the esters with light petroleum. The modified technique increased significantly the recovery of the fatty acid esters, particularly those of the short-chain fatty acids. The recovery of butyric acid from the combined preparatory, esterification, and ester extraction steps ranged from 85–95%, and the recovery of caproic and longer-chain acids was virtually complete.

An Aerograph Model A-90-P chromatograph, consisting of a 5 ft × 1/4 inch stainless-steel column packed with 20% diethyleneglycol succinate on 60–80 mesh Chromosorb-P treated with hexamethyldisilazane, was used to fractionate the esters. A helium flow rate of 60 ml/min was maintained, and the temperature was manually programmed from 90° to 165°. Quantitative results with the National Heart Institute fatty acid standard mixtures A, D, E, and F agreed with the stated composition data, with the relative error being less than 1.0% of a given peak area for the components reported herein. Standard fatty acids used for percentage recovery calculations were obtained from Applied Science Laboratories, State College, Pa.

All results presented in this paper are averages of 3 or more trials.

RESULTS

Factors affecting lipase production

pH of the medium. The organism was grown in the casitone medium adjusted at pH values varying from pH 5 to pH 10, at intervals of 0.5 pH unit, and the influence of pH of the medium upon the lipase production by the organism is shown in Fig. 1. The activity curve exhibited a sharp maximum in lipase production at pH 7.0.

Optimum temperature and time of incubation. The results of this phase of the study are also shown in Fig. 1. A temperature optimum for lipase production by the organism was exhibited at 21°. When the organism was grown at its optimum pH 7.0 and incubated at 21°, it produced the maximal amount of lipase after 36 h.

Addition of certain oils to the growth medium. In general, the addition of oil to the growth medium stimulated the lipase production (Table I). The lipase production was increased 25.0, 68.8, and 87.5% when 1% milk fat, corn oil, and olive oil, respectively, were added to the growth medium.

TABLE I

EFFECT OF THE ADDITION OF 1% OIL TO THE GROWTH MEDIUM UPON LIPASE PRODUCTION BY *Achromobacter lipolyticum*

Oils added	*Lipase activity**	*Increase in lipase production (%)*
Control**	16.0	—
Milk fat	20.0	25.0
Corn oil	27.0	68.8
Olive oil	30.0	87.5

* Expressed as net μmoles of free fatty acids extracted from 5 ml of the assay mixture.
** The control growth medium did not contain any oil.

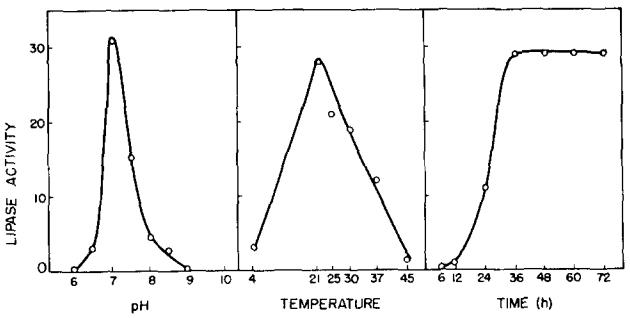

Fig. 1. Effect of pH of the growth medium, incubation temperature and time upon the extracellular lipase production by *A. lipolyticum*. Lipase activity is expressed as μmoles of fatty acids liberated under the assay conditions.

Stirring of growth medium during incubation. The effect of stirring of the medium by means of a magnetic stirrer during incubation upon cell and lipase production is shown in Table II. The organism produced considerably more cells and lipase when the medium was stirred slowly during incubation.

TABLE II

EFFECT OF STIRRING OF THE MEDIUM (CONTAINING 1% OLIVE OIL) DURING INCUBATION ON THE CELL WEIGHT AND LIPASE PRODUCTION

Conditions	*Cell production*		*Lipase production*	
	Dry cell wt. (mg/100 ml broth)	*Relative cell production* (%)*	*Free fatty acids (μmoles)*	*Relative lipase production* (%)*
No stirring	9.1	100	22.0	100
Stirring	19.5	214	68.0	309

* The cell weight and lipase production in the media incubated with and without stirring have been given on relative basis, considering 100% for the medium not stirred during incubation.

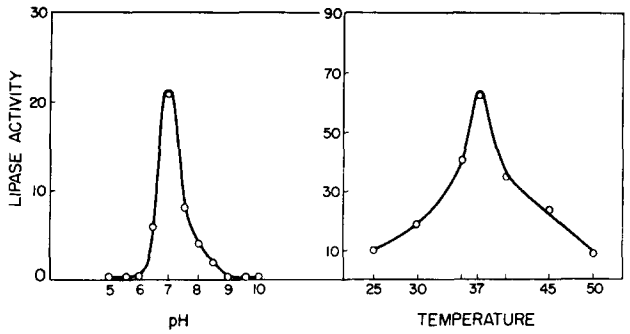

Fig. 2. Effect of pH and temperature upon the activity of the extracellular lipase of *A. lipolyticum*.

Factors affecting lipase activity

In order to determine the factors affecting the activity of the extracellular lipase produced by *A. lipolyticum*, the organism was grown under the conditions optimum for the production of the enzyme as established in the above section. Briefly, the organism was grown in casitone broth containing 1% olive oil, adjusted to pH 7.0, and incubated at 21° for 36 h. After incubation the cell-free broth was prepared by centrifugation and used as the enzyme source in all of the following studies.

Optimum pH and temperature. The lipase activity was determined at pH values ranging between 5.0 and 10.0 (Fig. 2), and its pH optimum was found to be 7.0. Fig. 2 also illustrates the effect of temperature, ranging between 25° and 50° upon the lipase activity at its optimum pH of 7.0, and its optimal temperature was observed to be 37°.

Various buffers. The lipase activity of the cell-free broth was measured in the presence of several commonly used buffers at its optimum pH 7.0 (Table III). The enzyme displayed maximal activity in potassium phosphate buffer. In the other buffers used, the enzyme displayed decreasing activity in the order in which the buffers are listed in the table. The phosphate buffer was therefore used throughout the work.

Heat stability of the enzyme. Studies were conducted to determine the heat stability of the lipase. The cell-free broth containing the enzyme was heated at 71° and 99° for various lengths of time and was autoclaved at 121° for 15 min, and the lipase activity was determined before and after heating (Fig. 3). The enzyme was

TABLE III

ACHROMOBACTER LIPASE ACTIVITY IN VARIOUS BUFFERS

Buffers (0.025 M, pH 7.0)	*Lipase activity**
Potassium phosphate	54
Sodium citrate	43
Sodium borate	39
Tris-HCl	35
Tris-maleate	33
Sodium barbital	29

* Expressed as net μmoles of free fatty acids extracted from 5 ml of the assay mixture.

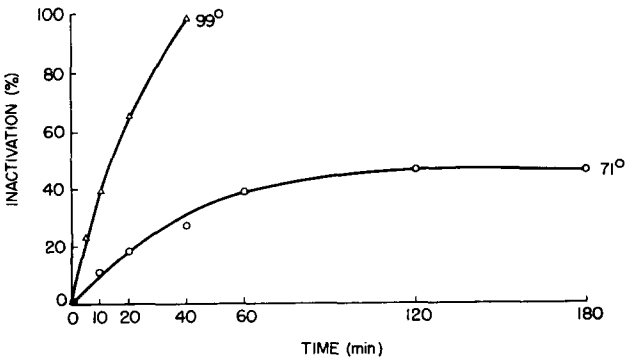

Fig. 3. Heat inactivation of the microbial lipase at 71° and 99°.

found to be fairly heat-stable. It lost only 47% of its activity upon heating at 71° for as long as 180 min, but its activity was lost completely when heated at 99° for 40 min. Also, it lost its activity completely when autoclaved.

Certain salts. The effect of several salts on the lipase activity are presented in Table IV. In general, all the salts used stimulated the lipase activity. At the lowest concn. ($1 \cdot 10^{-3}$) of all the salts tested, $MgCl_2$ had the most stimulatory effect and $CaCl_2$ the least stimulatory effect upon the activity of the enzyme. However, at the highest concn. of the salts ($1 \cdot 10^{-2}$), MgO was the most stimulatory.

Substrate concentration. The substrates selected for study were corn oil, olive oil, milk fat, and triolein and tributyrin. Emulsions of each were prepared separately in

TABLE IV

EFFECT OF VARYING CONCENTRATIONS OF SEVERAL SALTS UPON THE ACHROMOBACTER LIPASE ACTIVITY

Salt	*Salt concn. (moles)*					
	$1 \cdot 10^{-3}$		$5 \cdot 10^{-3}$		$1 \cdot 10^{-2}$	
	*Lipase activity**	*Stimulation (%)*	*Lipase activity**	*Stimulation (%)*	*Lipase activity**	*Stimulation (%)*
Control (no salt)	3.0	—	3.0	—	3.0	—
$MgCl_2$	7.8	160	5.1	70	3.5	17
Na_2SO_4	7.3	143	6.9	130	5.4	80
$MgSO_4$	7.2	140	6.5	116	5.7	90
K_2SO_4	7.0	133	6.4	113	5.3	77
KH_2PO_4	7.0	133	6.3	110	5.2	73
NaH_2PO_4	6.8	126	5.4	80	4.6	53
Potassium citrate	6.6	120	6.1	103	5.3	77
KCl	6.6	120	4.9	63	3.5	17
MgO	6.6	120	7.5	150	5.9	96
$Ca(OH)_2$	6.6	120	6.1	103	5.0	67
NaCl	6.5	117	5.0	66	4.2	40
Sodium citrate	6.4	113	5.1	70	4.9	63
$CaCl_2$	5.9	96	5.1	70	3.5	17

* Expressed as net ml of 0.01 M ethanolic KOH required to neutralize the free fatty acids produced under the assay conditions.

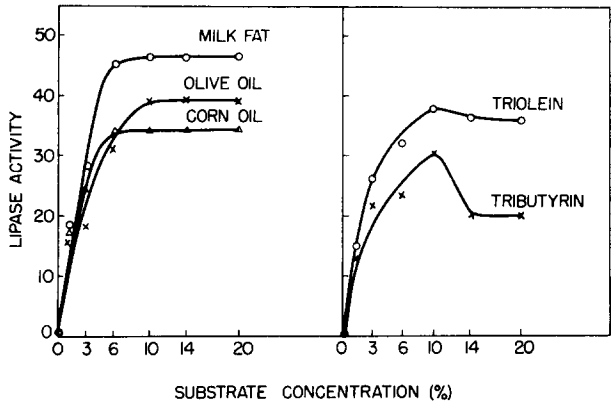

Fig. 4. Effect of substrate concn. on the hydrolysis of natural oils and synthetic triglycerides by the microbial lipase.

concn. of 2, 6, 12, 20, 28, and 40% in buffered gum arabic solution (pH 7.0). Equal amounts of these emulsions and cell-free broth were mixed bringing the concentrations of the substrate in the assay mixtures down to 1, 3, 6, 10, 14, and 20%, respectively. The enzyme lipolyzed all the substrates (Fig. 4). In the case of the natural glycerides, corn oil, olive oil, and milk fat, maximum lipolysis occurred at 6–10% substrate concentration, after which no further increase or decrease in the rate of enzymic hydrolysis was observed. With synthetic glycerides, however, the activity curve showed a peak at 10% substrate concentration followed by a reduction in the extent of lipolysis at the higher substrate concentrations.

Rate of hydrolysis. Ten % emulsions of natural and synthetic glycerides were used to study their rate of lipolysis by the Achromobacter lipase at 37° for 60 min (Fig. 5). The lipase hydrolyzed a maximum amount of substrate in 20–30 min, after which no significant increase in lipolysis was observed. Also, the rates of reaction of the enzyme for oleyl glycerides were consistently higher than those for butyryl glycerides, revealing the enzyme to be possibly more active towards glycerides containing the long-chain unsaturated fatty acids.

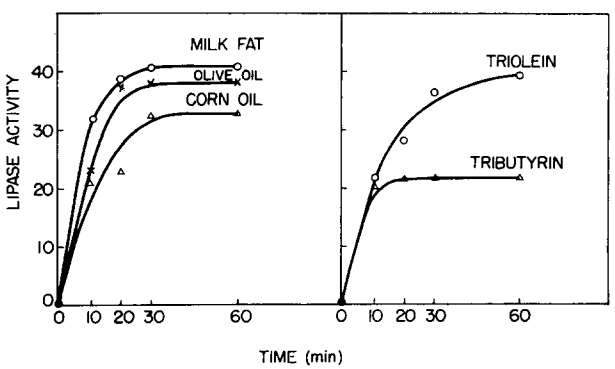

Fig. 5. Relative rates of hydrolysis of different substrates by the microbial lipase.

TABLE V

RATE OF HYDROLYSIS OF TRIOLEIN AND TRIBUTYRIN BY ACHROMOBACTER LIPASE AND MILK LIPASE

Enzyme source	*Triolein* hydrolysed after*				*Tributyrin* hydrolysed after*			
	10 min	*20 min*	*30 min*	*60 min*	*10 min*	*20 min*	*30 min*	*60 min*
Microbial lipase	26	38	44	49	11	22	25	27
Milk lipase	13	15	27	29	41	85	121	127

* Results expressed as net μmoles of free fatty acids liberated under the conditions of the assay.

Substrate and fatty acid specificity. Lipases from different sources split off various fatty acids from natural and synthetic glycerides at different rates[19-21]. A study was undertaken to investigate the substrate reactivity and fatty acid specificity of the microbial lipase and to compare it with such characteristics of milk lipase (Table V). The ratio of fatty acids liberated from tributyrin and those from triolein by the 2 lipases revealed that the microbial lipase lipolyzed triolein more rapidly than tributyrin. On the other hand, milk lipase hydrolyzed tributyrin more rapidly than triolein.

Additional studies, employing gas–liquid chromatographic techniques, were made to determine the individual fatty acids released from milk fat by the 2 enzymes (Table VI). The composition of free fatty acids liberated by milk lipase from milk fat was similar to the composition of the original milk fat. In contrast, from the same substrate which contained 23.0% oleic acid, 2.2% linoleic acid, and 1.0% linolenic acid, the microbial lipase liberated free fatty acids consisting of 47.4% oleic acid,

TABLE VI

FREE FATTY ACIDS LIBERATED FROM MILK FAT BY ACHROMOBACTER LIPASE AND MILK LIPASE

Lipolysis conditions: 4.5 ml 10% milk fat substrate in 0.025 M phosphate buffer (pH 7.0) and 0.5 ml enzyme, incubated at 37° for 30 min.

*Fatty acid**	*Fatty acid content of milk fat*	*Free fatty acids liberated by lipase action*	
		Milk lipase	*Microbial lipase*
4:0	3.3	2.9	Trace
6:0	3.8	3.1	1.2
8:0	1.9	1.8	1.9
10:0	4.1	3.8	2.2
12:0	3.7	3.4	1.0
14:0	10.2	9.2	4.2
16:0	24.2	25.0	12.9
18:0	12.3	15.1	8.0
18:1	23.0	27.3	47.4
18:2	2.2	2.6	6.5
18:3	1.0	1.3	8.1

* First figure refers to number of carbon atoms; second figure refers to number of double bonds.

6.5% linoleic and 8.1% linolenic acid. These data revealed that the Achromobacter lipase selectively liberated a higher proportion of C_{18} unsaturated fatty acids from milk fat than did milk lipase. These results signified the divergent specificities of various fat-hydrolyzing enzymes.

DISCUSSION

A. lipolyticum produced a maximum amount of lipase when it was grown in a medium at pH 7.0 and when incubated for 36 h at 21°. ALFORD AND ELLIOTT[26] observed that there was a significant increase in the number of cells of *Pseudomonas fragi* when it was grown with shaking, but the amount of lipase decreased considerably. However, *A. lipolyticum* produced a maximum amount of lipase when it was incubated with slow stirring.

The results reported in this paper are in general agreement with the observations of NASHIF AND NELSON[22]. They noticed that *A. lipolyticum* lipase exhibited a pH optimum at 7.0 and an optimum temperature of 32° when 10% coconut oil emulsion was used as substrate and the reaction mixture was incubated for 24 h. The lipase reported herein was very active in hydrolyzing natural and synthetic glycerides at 37° which is a higher temperature than the optimum temperature for growth of the organism and its lipase production (21°). This disparity in the optimum temperature for lipase production and for the lipase activity may be due to the fact that at 21° possibly the physical state of the substrate (milk fat) is not as conducive for interaction with the active sites of the enzyme.

Achromobacter lipase exhibited maximum activity in the presence of magnesium ions (10^{-3} mole), whereas $CaCl_2$ (10^{-3} mole) did not stimulate the enzyme activity to the same degree. The pancreatic lipase displays a maximum activity in the presence of calcium ions[23]. However, these ions inhibited milk lipase[24].

Generally, enzymes are heat-labile, but one of the most significant properties of the bacterial lipases is their marked stability to heat[14,22]. *A. lipolyticum* lipase was not completely inactivated at pasteurization temperatures and times commonly used (62° for 30 min or 71° for 15 sec) in the dairy industry. This lipase lost only 47% activity when heated at 71° for as long as 180 min. This stability forms the outstanding feature of this lipase and emphasizes the necessity of avoiding the post-pasteurization contamination of dairy products with *A. lipolyticum*.

FUKOMOTO, TWAI AND JSUJISAKA[25] reported that the crystalline lipase of *Aspergillus niger* hydrolyzed olive oil almost completely, and it did not attack methyl butyrate at all. Achromobacter lipase was more active toward unsaturated C_{18} fatty acid glycerides than short-chain fatty acid glycerides. This lipase hydrolyzed several synthetic glycerides and natural oils to varying degrees, but it hydrolyzed milk fat emulsion faster than any glyceride emulsions under similar conditions.

ALFORD and co-workers[26] studied the patterns of free fatty acids liberated from corn oil, lard, and coconut oil by fungal and microbial lipases. They noticed that fungal and microbial lipase released 66–99% C_{18} acids. *A. lipolyticum* lipase liberated a significant amount of long-chain acids in comparison to a small amount of short-chain fatty acids from milk fat.

The results presented in this paper show that the Achromobacter lipase is quite

different in its behavior than milk lipase and pancreatic lipase. In its specificity this lipase resembles the lipase of *Geotrichum candidum*[27] and is the first bacterial lipase reported to have such a specificity.

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