Production of Microbial Lipases for the Study of Triglyceride Structure

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Abstract

Lipases from different microorganisms are known to differ in their site of attack on triglycerides, and recent evidence has indicated that they may be useful in studying triglyceride structure. This paper is concerned with the most recent developments in the production, recovery, and stability of lipases from three of these microorganisms. The lipase from *Staphylococcus aureus*, which attacks both the 1-, 2-, and 3-positions of triglycerides, is produced in an aerated, tryptic digest of casein at 30C in 1–2 days. The lipase from *Geotrichum candidum*, which attacks primarily unsaturated fatty acid linkages and shows some stereospecificity, is produced in a static culture grown on a mineral salts-glucose-protein hydrolysate medium incubated at 20C for 4–5 days. An improved method is described for preparing lyophilized preparations of these, and the *Pseudomonas fragi* lipase, which are quite stable when stored in a refrigerator.

Introduction

PREVIOUS STUDIES from this laboratory (2,4) have shown that lipases produced by microorganisms attack triglycerides in at least three different ways. The lipase from *Pseudomonas fragi* is similar to pancreatic lipase in that it attacks predominantly the 1-, 3-positions of triglycerides. The lipase from *Staphylococcus aureus* attacks both the 1-, 2-, and 3-positions, while the lipase produced by *Geotrichum candidum* attacks primarily unsaturated fatty acid linkages, regardless of the position of the attachment. More recent evidence has shown that the enzyme from *G. candidum* is stereospecific (5) and, therefore, can be used in studying the structure of triglycerides (6). Because of the interest in these enzymes as possible tools for determining triglyceride structure, particularly the one from *G. candidum*, the present study was undertaken to try to increase their yields, to standardize the recovery procedures, and to examine more closely the stability of the lipases under different conditions of storage.

Experimental Methods

Preparation of Media. The media for all comparisons, except for part of the investigation on *G. candidum*, consisted of: Nitrogen source 1.5% (w/v) and 1M phosphate buffer, 5% (v/v, pH 7.0 unless stated otherwise). For the latter part of the study on *G. candidum*, the salts-glucose-protein hydrolysate medium used was: Protein hydrolysate, 2–4%; NH_4Cl, 0.1%; KH_2PO_4, 0.15%; $MgSO_4 \cdot 7H_2O$, 0.012%; and 0.001% of $FeSO_4 \cdot 7H_2O$, $ZnSO_4 \cdot 7H_2O$, and $MnSO_4 \cdot H_2O$. After sterilization the pH was 5.8–6.0, and sterile glucose solution was added to give a final concentration of 0.25%. The sources of the media were as follows: BBL—Baltimore Biological Laboratories, Inc., Baltimore, Md.; Difco—Difco Laboratories, Inc., Detroit, Mich.; Case—Case Laboratories, Inc., Chicago, Ill.; Shef—Sheffield Chemical, Norwich, N.Y.; and NBC—Nutritional Biochemicals Corp., Cleveland, Ohio.

Assays for Lipase Activity. The substrates were prepared and the assays for lipolytic activity were carried out as previously described (3).

Stability Studies. After the cultures were grown under optimum conditions for lipase production, the cells were removed by centrifugation or, in the case of *G. candidum*, by filtration through coarse filter paper. Portions of this supernatant were stored at 20C and 2C, and samples were removed daily for assay. Additional amounts were shell-frozen in 3–4 ml quantities in test tubes in a dry ice-alcohol bath and stored in a dry ice chest. Tubes of the frozen enzyme were removed at weekly intervals, thawed, and assayed for activity. To avoid any effects of repeated freezing and thawing on the enzyme, none of the excess enzyme was refrozen.

Lyophilization of Enzymes. Supernatant solutions obtained as described above were placed in dialysis tubing (1.75 in. flat diameter) in approximately 300-ml quantities. Using the concentration method of Kohn (7), the tubes were placed in a tray, covered with polyethylene glycol (Carbowax 20 M, Union Carbide, N.Y.), and stored at 1–3C for 6–18 hr, during which time the solution was reduced to 10% or less of its original volume. The concentrated solution then was transferred to one or more small dialysis tubes (ca. 50 ml/tube) and dialyzed against at least 100 volumes of cold distilled water (1–3C) for 4–5 hr.

TABLE I

Effect of Medium and Aeration on Lipase Production by *Staphylococcus aureus*

Medium	Aerated[a]	Not aerated[b]
	ml[c]	ml
Peptone (Difco)	4.0	1.0
Phytone (BBL)	3.8	1.5
NZ Amine B (Shef.)	4.4	3.7
Trypticase (BBL)	6.0	3.0
Tryptone (Difco)	6.4	1.2
Trypticase soy broth (BBL)[d]	6.0	—
Peptone (Case)	5.5	1.0

[a] Aerated on reciprocating shaker (150–200 cycles/min) at 30C for 1–2 days.
[b] Incubated at 30C for 4 days.
[c] Ml 0.02 N NaOH required for titration of acid released from lard emulsion in 30 min at 35C.
[d] Prepared as described by manufacturer.

TABLE II

Comparison of Media for the Production of Lipase by *Geotrichum candidum* at 20C in 4 days

	Titratable acidity[a]
	ml
5% Phosphate buffer (pH 6.0) plus 1.5%:	
Edamin S (Shef.)	1.0
Trypticase (BBL)	4.0
Tryptone (Difco)	3.5
Peptone (Case)	3.2
Peptone (Difco)	3.5
Peptone I (NBC)	1.8
Salts glucose basal medium plus:	
Edamin S (2%)	6.8
Edamin S (4%)	7.3
Trypticase (2%)	6.8
Trypticase (4%)	7.1
Tryptone (2%)	4.8
Peptone (Case) (2%)	5.8

[a] Ml 0.02 N NaOH required for titration of acid released from lard emulsion in 30 min at 35C.

TABLE III

Effect of Temperature and Aeration on Production of Lipase by *Geotrichum candidum*

	Temperature and time of incubation[a]									
	20C			25C				31C		
	Days			Days				Days		
	3	4	5	2	3	4	5	2	3	4
	ml[b]	ml	ml	ml	ml	ml	ml	ml	ml	ml
Aerated—fast[c]				2.1	2.0	2.0	2.0			
Aerated–slow[d]	1.9	2.4	3.4	2.8	7.1	7.0	6.2	4.2	6.6	5.5
Not aerated	0.6	7.1	8.2	0.3	8.6	12.0	10.8	3.6	12.5	7.4

[a] 100 ml medium in 1000-ml Erlenmeyer flask.
[b] Ml 0.02 N NaOH required for titration of acid released from lard emulsion in 30 min at 35C.
[c] Incubated on a rotary shaker at 200 rpm.
[d] Incubated on a rotary shaker at 73 rpm.

TABLE IV

Effect of Surface-to-Volume Ratio of Medium on the Production of Lipase by *Geotrichum candidum*

Volume of medium[a] in 1000-ml Erlenmeyer flask	Method and time of incubation at 25C					
	Nonaerated			Aerated[d]		
	Days			Days		
	3	4	5	3	4	5
ml	ml	ml	ml	ml	ml	ml
100	8.6[c]	12.0	10.8	7.1	7.0	6.2
250	2.6	2.9	6.2	5.4	7.0	6.7
500	0.4	0.6	0.6	5.1	6.7	6.3

[a] 4% Edamin S-salts-glucose medium.
[b] Incubated on a rotary shaker at 73 rpm.
[c] Ml NaOH required for titration of acid released from lard emulsion in 30 min at 35C.

After dialysis, the contents of the tubes were transferred to suitable flasks, shell-frozen in a dry ice-alcohol bath, and dried under vacuum (<20 μ Hg) in a Virtis freeze-dryer. The dry powder was placed in glass vials, stoppered under atmospheric conditions, and stored in a refrigerator at 2–3C.

Results

Production of Lipase by S. aureus. In our first work on *S. aureus* (2), a buffered 1% peptone broth was employed; in later work (4), buffered 1% Trypticase (BBL) was used because slightly higher yields were obtained. Statically grown cultures were employed in these earlier studies because aeration had been shown to cause a definite decrease in lipase production by *P. fragi* (1). In the present investigation, the effect of aeration on the production of lipase by *S. aureus* was examined. The data in Table I show that higher yields of lipase could be obtained in a shorter time when the culture was aerated and that the highest yields were produced in the tryptic digest of casein.

Production of Lipase by G. candidum. The data in Table II show that the highest yields of lipase produced by *G. candidum* were obtained with the salts-glucose medium containing either Edamin Type S (Shef.) or Trypticase (BBL) as the nitrogen source. (Edamin Type S (Shef.) is a lactalbumin hydrolysate.) It should be emphasized that no attempt was made to maintain the pH at 6.0 during growth, and the final pH of the medium was 7.5–8.0. When the initial pH was adjusted to pH 7.0, yields were slightly lower, although the final pH was similar.

The effect of aeration and temperature on the production of *G. candidum* lipase is shown in Table III. The maximum yield of lipase per milliliter of culture was obtained at 31C in 3 days, or at 25C in 4 days in static culture. Destruction of the lipase was faster at the higher temperature, however. Since appreciable lipase was produced with very slow agitation, the effect of increased volumes of culture in flasks of the same size was determined. The results are shown in Table IV. When the surface-volume ratio of static cultures was decreased, there was a sharp drop in the yield of lipase per milliliter of culture. With the slowly agitated culture, however, yield per milliliter of culture remained essentially unchanged as the volume increased.

Production of Lipase by P. fragi. The media and cultural conditions for the production of *P. fragi* in both synthetic and partially defined media have been previously described (3). No further increase in yield has been obtained by substituting other protein hydrolysates.

Stability of Lipase. The stability of the lipases from the three microorganisms under various conditions of storage is shown in Table V. These data indicate that the lipases are relatively stable, and lyophilized preparations, after an initial loss of up to 50% during processing, can be held for long periods with relatively little change in activity.

Discussion

By manipulation of the media, temperature, and aeration of the cultures, the production of lipases by *S. aureus* and *G. candidum* has been improved in a manner similar to that previously reported for *P. fragi* (3). It is apparent that the nutritional and oxygen requirements for maximum lipase production vary widely among microorganisms. One must also be aware that, in addition to a varying response depending upon the nitrogen source, variations will occur between products with the same designation (e.g., peptone) but obtained from different manufacturers.

Although the amount of lipase activity per milliliter of medium produced by *G. candidum* in a static culture with a large surface-to-volume ratio was almost twice that obtainable with slow aeration, the total amount of lipase obtainable per flask was nearly three times as great with the aerated culture when the volume of medium per flask was increased. Thus, when large lots of enzyme are to be produced and incuba-

TABLE V

Stability of Lipolytic Enzymes under Different Conditions of Storage

Preparation of enzyme	Loss during preparation			Storage life		
	P. fragi	*S. aureus*	*G. candidum*	*P. fragi*	*S. aureus*	*G. candidum*
	%	%	%			
Supernatant stored at 20C	N.A.[a]	N.A.	N.A.	2 days[b]	—[c]	3 days[d]
Supernatant stored at 2C	N.A.	N.A.	N.A.	3 days[b]	—	10 days[d]
Supernatant frozen; stored in dry ice cabinet	20	——	5	>2 mo[e]	—	>2 mo[e]
Lyophilized; stored in refrigerator	25–50	25–50	25–50	>6 mo	>1 yr	>1 yr

[a] Not applicable.
[b] Turbidity due to cell growth appeared.
[c] Test not done.
[d] Time at which approximately 20% of activity was lost.
[e] Less than 20% loss of activity by time shown.

tion space is at a premium, use of mild aeration should be considered.

Rapid freezing of the culture supernatant with storage at a low temperature affords a simple procedure for prolonged storage of small quantities of enzyme. However, since the concentration, dialysis, and lyophilization procedure will reduce several liters of material to a few grams, it has obvious advantages for any extensive study.

It is reasonable to expect that further study on the interrelationships of pH, aeration, and supply of nutrients during the entire growth cycle would bring about additional increases in yield of these lipases. However, with the media and conditions of growth described here, coupled with proper recovery and storage procedures, stable preparations can be routinely prepared which are suitable for the study of the structure of triglycerides.

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The Unsolved Problems of Triglyceride Analysis[1]

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Abstract

The modern techniques of countercurrent distribution between solvents, GLC and TLC (especially on silver nitrate impregnated silica gel), and enzymic hydrolysis have greatly simplified and speeded up the analysis of triglyceride mixtures, such as are found in natural fats. The application of these methods has provided data for the formulation of a number of new theoretical descriptions of the fatty acid distributions found in fats.

But it is clear that neither the experimental techniques, nor the theories of glyceride structure arrived at with their aid, are entirely satisfactory. On the one hand analytical methods are inadequate for such fats as the nut oils; and in no case can the accuracy of the analysis be regarded as complete. On the other hand present theories have yet to accommodate minor components, enantiomorphic forms, and recent evidence concerning the biosynthesis of natural triglycerides.

Attention is drawn to some of the outstanding problems still facing those concerned with the analysis of triglyceride mixtures; and an attempt it made to indicate the direction in which solutions to these problems may be sought.

Introduction

NATURAL FATS ARE COMPOSED of triglycerides, the esters of a variety of fatty acids with the trihydric alcohol, glycerol. The consequence of the trihydric character of glycerol is that triglycerides may be simple (containing one fatty acid species) or mixed (containing two or three fatty acid residues per molecule). The presence of two primary and one secondary hydroxyl group in glycerol gives rise to positional isomers of the same fatty acid composition: two in the case of diacid triglycerides, and three in the case of triacid triglycerides. If the two primary hydroxyls are esterified with different fatty acids, the central glycerol C atom becomes asymmetric; and such triglycerides can exist in two enantiomorphic forms. For the complete analysis of a mixture of mixed triglycerides of the kind encountered in natural fats, it is therefore necessary to know: (a) the overall fatty acid composition; (b) the distribution of these acids between the various triglycerides present; (c) the distribution of the acids between the primary and secondary hydroxyls, within each mixed triglyceride molecule; (d) the distribution between the two primary hydroxyls of each asymmetric, mixed, triglyceride molecule.

The methods for making each of these determinations will be considered separately, as far as present techniques allow.

Experimental Methods Available

(a) To Determine Overall Fatty Acid Composition

It is not proposed to consider here the identification of unusual fatty acids by chemical means, although much remains to be done. This work of Korn (1) in identifying and analysing the fatty acids of *Euglena gracilis* may be cited as an example of the kind of careful work required in a comprehensive analysis.

There are a number of methods available for fatty acid analysis (see Ref. 2), but for most purposes the speed and high resolution of gas-liquid chromatography (GLC) renders it superior to other methods. It is very versatile, and is probably the easiest method to operate for quantitative analyses: and in biological work the small size of the sample required is a further advantage. A comprehensive review (3) of the application of GLC to fatty acid analysis, renders detailed consideration of the technique unnecessary here.

Sources of error or difficulty with this method are now well documented. Thus it is necessary to make sure that samples for analysis contain only fatty acids: a preliminary purification by thin-layer chromatography (TLC) may be advisable. Samples are usually run as methyl esters, and care should be taken to ensure that methylation is complete (4) and to avoid artifacts (1,2,5). It may be desirable to purify the methyl esters (6). For identification of the emergent peaks it is unwise to rely on retention volume alone; Fontell et al. (7) have advocated the use of two stationary phases, or two column temperatures, to be sure of identification. Certain classes of fatty acids are subject to alteration during the process of GLC (8,9).

[1] Presented at the AOCS meeting in Houston, Texas, 1965.