APPLIED MICROBIOLOGY, Jan. 1971, p. 9-12
Vol. 21, No. 1

Glycerol Ester Hydrolase Activity of *Microbacterium thermosphactum*

D. L. COLLINS-THOMPSON, T. SØRHAUG, L. D. WITTER, AND Z. J. ORDAL
Departments of Food Science and Microbiology, University of Illinois, Urbana, Illinois 61801

Received for publication 5 October 1970

Microbacterium thermosphactum possesses a significant glycerol ester hydrolase (lipase, EC 3.1.1.3) activity and a weak but definite carboxylic ester hydrolase (esterase, EC 3.1.1.1) activity. Harvested whole cell preparations contained 53 units of lipase activity with tripropionin as the substrate. This activity decreased with an increasing chain length of fatty acid in the triglyceride to 13 units with trilaurin as the substrate and no activity with tripalmitin. Maximum lipase activity was found at a temperature of 35 to 37 C and at a *p*H of 7.1 to 7.3. Lipase activity was associated with three different protein peaks when the protein of cell-free extract was fractionated by polyacrylamide gel electrophoresis.

In 1953, McLean and Sulzbacher (8) identified *Microbacterium thermosphactum* as the organism repeatedly isolated from and associated with pork sausage flavor deterioration. This organism has since been thoroughly documented as a common isolate from fresh and spoiled meats (2, 3, 16, 20). Microbial lipase activity is frequently responsible for not only off-flavor development and definite spoilage in meats but also for the relatively subtle flavor changes characteristic of certain aged meats (1). Despite the possible participation of *M. thermosphactum* lipase in the flavor change in meats, the lipolytic activity of this bacterium has not yet been investigated and related literature was not very helpful in predicting the outcome. Other species of *Microbacterium* examined by Nashif and Nelson (11) were nonlipolytic, whereas some coryneform bacteria, apparently closely related to *M. thermosphactum*, tested by Jayne-Williams and Skerman (6) were lipolytic with butterfat. The purpose of this investigation was to show that *M. thermosphactum* possesses a glycerol ester hydrolase (EC 3.1.1.3) which forms monocarboxylic acids from triglycerides as a substrate and to characterize partially this enzyme.

MATERIALS AND METHODS

The strain of *M. thermosphactum* used was isolated from beef and identified by comparison tests with a known strain. Stock cultures were prepared by inoculating Trypticase soy broth (TSB) with freshly grown culture (1% inoculum) and then freezing and storing the inoculated broth at −20 C. These cultures were used to inoculate fresh broth as needed.

The objectives of the following methods were to determine the ability of *M. thermosphactum* to hydrolyze triglycerides in emulsion (lipase activity) and in solution (esterase activity). The methods were also designed to locate the protein bands which show lipase activity on polyacrylamide gel electrophoresis and to relate temperature, *p*H, and substrate specificity to the glycerol ester hydrolase activity.

Preparation of cells and cell-free extracts. Cells grown in TSB at 22 C were harvested by centrifugation at 2 C, washed twice with 0.01 M tris(hydroxymethyl)-aminomethane (Tris) buffer, *p*H 7.2, and resuspended in the same buffer. Cell-free extracts were prepared by the disruption of the cells by one passage through a French pressure cell at 8,000 psi. The cell debris was removed by centrifugation at 15,000 × *g* for 30 min at 2 C. The protein content of whole cells and that of cell-free extracts were determined by the biuret method (17). Crystalline serum albumin was used for the standard curve.

Esterase activity. The esterase activity of a cell suspension was determined by titration of the liberated acids with an automatic recording pH-stat (E. H. Sargent and Co., Chicago, Ill). The initial velocity of the hydrolysis of the substrate was followed by continuous titration. The reaction mixture of an aqueous solution of triacetin or methyl butyrate was assayed at 35 C. The mixture was maintained at *p*H 7.2 by continuous addition of 0.01 N CO_2-free KOH. Blanks containing boiled enzyme and the substrate alone were similarly treated.

Lipase activity. The lipolytic activity of *M. thermosphactum* cells or cell-free extracts was also determined by the continuous pH-stat method. The different substrates were emulsified with 10% gum arabic as described by Desnuelle et al. (5) by using ultrasonic treatment. The mixture was sonically treated with a Branson Sonifier (model SI25) by using full power for 2 min. During emulsification, the mixture was placed in an ice bath to prevent breakdown of the substrates.

TABLE 1. *Lipase activity of whole cells and fractions of Microbacterium thermosphactum measured by the pH-stat procedure with tripropionin as the substrate*

Fraction	Lipase activity (units)
Whole cells	53
Broken cells	40
Cell-free extract	40
Cell debris	0
Growth medium supernatant	20[a]

[a] Activity expressed as units per milliliter of medium.

Blanks containing the boiled enzyme, substrate without enzyme, and enzyme alone were similarly titrated. In initial studies, the agar-well technique (14) was used to screen for possible substrates. Plain unbuffered 1% agar gels were used. Zones of hydrolysis were noted after 24 hr of incubation at 30 C.

For both esterase and lipase, 1 unit of activity is defined as that amount of enzyme catalyzing the formation of one nano-equivalent of acid per minute per milligram of protein.

Polyacrylamide gel electrophoresis. A system was developed on the basis of the principles of Ornstein (13) and of Davis (4) for the detection of glycerol ester hydrolase activity in electrophoretically separated protein components of bacterial extracts. Disc gel electrophoresis was carried out on 6% polyacrylamide gel (*p*H 8.5) at 4 C with a current of 3.5 ma per 6.5-mm plexiglass tube for 2 hr on a Canalco model 6 electrophoresis cell. Gel containing 6% acrylamide, 0.3% *bis*-acrylamide (*N,N'*-methylenebisacrylamide; Canalco, Inc.), 0.0008% *N,N,N',N'*-tetramethylethylenediamine (Canalco, Inc.), and 0.0008% ammonium persulfate (Fischer Scientific) was made in 0.12 M Tris-hydrochloride buffer, *p*H 8.5. The bath buffer was 0.04 M phosphate buffer, *p*H 7.2. Gels were allowed to solidify for 30 min before use, and no spacer or stacking gel was used. The gels were stained for 45 min in 7% glacial acetic acid containing 0.5% aniline black. Destaining was done by repeated washing in 7% glacial acetic acid on a shaker.

After development and staining, the gels were scanned by using a Gilford gel scanning attachment to a Beckman DU spectrophotometer converted with a photomultiplier apparatus attached to a Sargent model SR recorder. Scan speed was 4 cm/min with the recorder set on 5 inches/min.

Lipase detection in gels. Developed polyacrylamide gels were cut in the frozen state by use of razor blades taped in a pile to yield slices of 1.2 mm in thickness. These were layered separately on the agar of plates prepared by the agar-well technique described above. Lipase activity was observed as clearing zones around gels in 48 hr.

RESULTS AND DISCUSSION

A preliminary demonstration of the lipolytic activity of *M. thermosphactum* was made by using the agar-well technique. Clear, wide zones were formed with tripropionin or tributyrin as the substrate, a definite but smaller clear zone was formed with triacetin, and no clear zone was formed with tricaproin, tricaprylin, trilaurin, and tripalmitin. Identical results were obtained

TABLE 2. *Substrate specificity of the lipase from whole cells of Microbacterium thermosphactum measured by the pH-stat procedure*

Substrate[a]	Lipase activities (units)
Tripropionin	53
Tributyrin	40
Tricaproin	40
Tricaprylin	20
Trilaurin	13
Tripalmitin	0

[a] Total volume of 6 ml of the reaction mixture contained 60 mmoles of substrate sonically dispersed in 10% gum arabic and 1 ml of whole cells of *M. thermosphactum* at a final *p*H of 7.2.

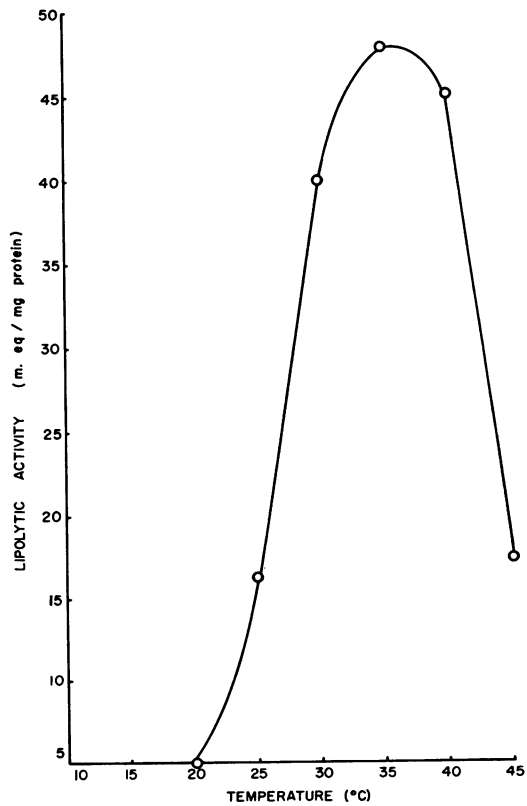

FIG. 1. *Effect of temperature on the lipolytic activity of whole cells of Microbacterium thermosphactum with tripropionin as the substrate.*

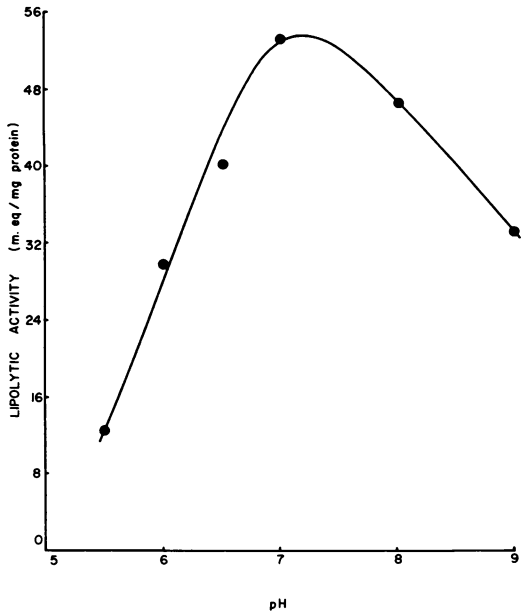

FIG. 2. *Effect of pH on the lipolytic activity of whole cells of Microbacterium thermosphactum with tripropionin as the substrate.*

with both whole cells and cell-free extracts. These findings were sufficiently encouraging to suggest the use of the more sensitive and dependable pH-stat method of measuring glycerol ester hydrolase (lipase) activity.

The lipolytic activities on tripropionin of whole cells, cell fractions, and the growth medium supernatant of *M. thermosphactum* by using the pH-stat method are given in Table 1. Although no attempt was made to define carefully the enzyme location, the lipase appears to be both extracellular and intracellular. It was unlikely that the rather high extracellular activity resulted from the artifact of cell death and autolysis, because the cultures used were harvested during the exponential growth phase. Since whole cells showed the highest activity, they were used in all subsequent experiments.

The substrate specificity of *M. thermosphactum* lipase was again determined by using the pH-stat method, and the results are given in Table 2. This more sensitive method extended the substrate specificity of *M. thermospactum* lipase to include tricaproin, tricaprylin, and trilaurin but still showed no activity towards tripalmitin. This lipase appears to catalyze preferentially the hydrolysis of short-chain fatty acid triglycerides, and the activity is progressively reduced with increased chain length.

The temperature-activity and *p*H-activity profiles of the lipase of *M. thermosphactum* whole cells by using tripropionin as the substrate are given in Fig. 1 and Fig. 2, respectively. Maximum lipase activity was found at 35 to 37 C and at *p*H 7.1 to 7.3. These values correspond to those found for lipases from other microorganisms, despite the use of a wide variety of assay methods and substrates. The majority of microbial lipase show an optimum temperature within the range of 35 to 40 C (7, 10, 12, 18) and an optimum *p*H within the range from 7.0 to 7.8 (10, 15, 18, 19).

The ability of whole cells of *M. thermosphactum* to hydrolyze ester bonds in solution (esterase activity) was definite but weak. The esterase activity was 16 units with triacetin as the substrate and 10 units with methyl butyrate as the substrate. Both substrates were at a concentration of 10 mM/ml, which was well below their limit of solubility and excluded lipase activity being confused with esterase activity as a result of hydrolysis of these substrates at concentrations exceeding their solubility.

The separation of the protein in cell-free extracts of *M. thermosphactum* by polyacrylamide gel electrophoresis is shown in Fig. 3. Lipase activity was found associated with the protein peaks located at migration distances of 1.2, 2.2, and 4.8 cm. Separable bands of lipase activity have been previously observed as discussed by Mencher and Alford (9), who also resolved the purified lipase of *Pseudomonas fragi* into two distinct peaks by electrophoresis and by sucrose density gradient centrifugation. No conclusion could be drawn as to whether the three separate lipase fractions found in our study were true isoenzymes or merely a single lipase species complexed with other components of the cell, but they were consistent and reproducible.

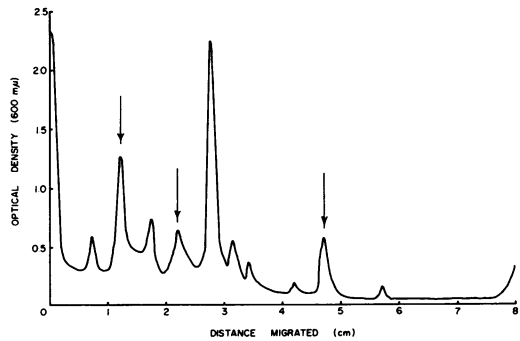

FIG. 3. *Protein fractions of cell-free extracts of Microbacterium thermosphactum separated by polyacrylamide gel electrophoresis. Protein peaks showing lipolytic activity with tributyrin as the substrate are indicated by arrows.*

LITERATURE CITED

1. Alford, J. A., D. A. Pierce, and W. L. Sulzbacher. 1964. Microbial lipases and their potential importance to the meat industry, p. 11–16. Proc. 15th Conf. on Research Council on Research Amer. Meat Inst., University of Chicago, Chicago.
2. Ayres, J. C. 1960. Temperature relationships and some other characteristics of the microbial flora developing on refrigerated beef. Food Res. **25**:1–18.
3. Barlow, J., and A. G. Kitchell. 1966. A note on the spoilage of prepacked lamb chops by *Microbacterium thermosphactum*. J. Appl. Bacteriol. **29**:185–188.
4. Davis, B. J. 1964. Disc electrophoresis. II. Method and application to human serum proteins. Ann. N.Y. Acad. Sci. **121**:404–427.
5. Desnuelle, P., M. J. Constantin, and J. Baldy. 1955. Technique potentiometrique pour la mesure de l'activite de la lipase pancreatique. Bull. Soc. Chim. Biol. **37**:285–290.
6. Jayne-Williams, D. J., and T. M. Skerman. 1966. Comparative studies on coryneform bacteria from milk and dairy sources. J. Appl. Bacteriol. **29**:72–92.
7. Kirsh, D. 1935. Factors influencing the activity of fungus lipase. J. Biol. Chem. **108**:421–430.
8. McLean, R. A., and W. L. Sulzbacher. 1953. Microbacterium thermosphactum, spec nov; a nonheat resistant bacterium from fresh pork sausage. J. Bacteriol. **65**:428–433.
9. Mencher, J. R., and J. A. Alford. 1967. Purification and characterization of the lipase of *Pseudomonas fragi*. J. Gen. Microbiol. **48**:317–328.
10. Nashif, S. A., and F. E. Nelson. 1953. The lipase of *Pseudomonas fragi*. I. Characterization of the enzyme. J. Dairy Sci. **36**:459–470.
11. Nashif, S. A., and F. E. Nelson. 1953. Some studies on microbacteria from Iowa dairy products. Appl. Microbiol. **1**: 47–52.
12. O'Leary, W. M., and J. T. Weld. 1964. Lipolytic activities of *Staphylococcus aureus*. I. Nature of the enzyme producing free fatty acids from plasma lipids. J. Bacteriol. **88**:1356–1363.
13. Ornstein, L. 1964. Disc electrophoresis. I. Background and theory. Ann. N.Y. Acad. Sci. **121**:321–349.
14. Oterholm, A., and Z. J. Ordal. 1966. Improved method for detection of microbial lipolysis. J. Dairy Sci. **49**:1280–1284.
15. Oterholm, A., Z. J. Ordal, and L. D. Witter. 1970. Purification and properties of a glycerol ester hydrolase (lipase) from *Propionibacterium shermanii*. Appl. Microbiol. **20**:16–22.
16. Pierson, M. D., D. L. Collins-Thompson, and Z. J. Ordal. 1970. Microbiological, sensory and pigment changes of aerobically and anaerobically packaged beef. Food Technol. **24**:1171–1175.
17. Robinson, H. W., and G. G. Hogden. 1940. The biuret reaction in the determination of serum proteins. I. A study of the conditions necessary for the production of a stable color which bears a quantitative relationship to the protein concentration. J. Biol. Chem. **135**:707–725.
18. Rottem, S., and S. Razin. 1964. Lipase activity of Mycoplasma. J. Gen. Microbiol. **37**:123–134.
19. Shah, D. B., and J. B. Wilson. 1965. Egg yolk factor of *Staphylococcus aureus*. II. Characterization of the lipase activity. J. Bacteriol. **89**:949–953.
20. Wolin, E. F., J. B. Evans, and C. F. Niven. 1957. The microbiology of fresh and irradiated beef. Food Res. **22**:682–696