JOURNAL OF BACTERIOLOGY, Mar. 1973, p. 1170-1176
Vol. 113, No. 3

Characterization of Esterases Produced by a Ruminal Bacterium Identified as *Butyrivibrio fibrisolvens*[1]

WAYNE W. LANZ[2] AND PHLETUS P. WILLIAMS

Department of Bacteriology, North Dakota State University, and the Metabolism and Radiation Research Laboratory, Animal Science Research Division, Agricultural Research Service, Fargo, North Dakota 58102

Received for publication 23 May 1972

An obligately anaerobic ruminal bacterial isolate was selected from 18 tributyrin-degrading isolates and identified as *Butyrivibrio fibrisolvens* strain 53. The culture in late exponential phase contained enzymes which could be released by sonic disruption. These enzymes degraded substrates at a rate in the order 1-naphthyl acetate (NA) > 1-naphthyl butyrate > 1-naphthyl propionate but did not degrade 1-naphthyl palmitate or 1-naphthyl phosphate. The enzymes on NA were neither stimulated nor inhibited by $CoCl_2$, $MgCl_2$, and MnCl (each varied from 10^{-6} to 10^{-4} M). CaCl at 10^{-3} M stimulated esterase activity by 16%. Aliphatic substrates were hydrolyzed at a rate in the order triacetin > tributyrin > tripropionin, and ethyl acetate > ethyl formate. Similarly, aromatic fluorescein diesters were degraded at a rate in the order acetyl > propionyl > caproyl > butyryl > capryl > lauryl. Polyacrylamide gel electrophoretic zymograms indicated that the enzyme composite contained cathodally migrating bands. By column chromatography, these enzymes were separated into six NA-degrading fractions. Fraction V contained an esterase which had an optimal temperature of 39 C, a K_m of 7.6×10^{-4} on NA, and a molecular weight of about 66,000. This enzyme was inhibited by paraoxon (41%, 10^{-4} M), eserine (17%, 10^{-2} M), NaF (17%, 10^{-2} M), and diisopropyl fluorophosphate (62%, 10^{-4} M) but not by 1-naphthyl *N*-methyl carbamate at 8.4×10^{-4} M.

Although microbial esterase activity has been the subject of investigation (28), there is little information available on the sources of production and characterization of the esterases. Such information is desirable, especially in view of the existence of nonenzymic proteins in milk and serum that degrade esters (12) and residual esterase activity associated with certain proteolytic enzymes (24).

There is evidence that ruminal contents contain hydrolases (lipases or esterases, or both) which degrade aliphatic esters to their constituents (19) and triglycerides to their component fatty acids and glycerol (10, 14, 15). In 1961, Hobson and Mann isolated gram-negative bacilli from ruminal contents that produced zones of hydrolysis in linseed oil emulsion medium. Some of these bacteria differed from presently characterized ruminal species in their limited ability to utilize alcohols and carbohydrates.

The purpose of this investigation was to isolate and identify a ruminal bacterium that degraded aliphatic and aromatic esters, and to study and characterize the esterases produced by such a bacterium.

A preliminary report was presented in part at the 71st Annual Meeting of the American Society for Microbiology at Minneapolis, Minn., 2-7 May 1971. This work was done as part of a thesis for the MS degree in bacteriology at the North Dakota State University, Fargo, 58102.

MATERIALS AND METHODS

Isolation and maintenance of bacteria. Samples of ruminal contents were obtained by stomach tube from calves maintained on a ration of pelleted

[1] Article no. 372 from the North Dakota Agriculture Experiment Station.

[2] Present address: Department of Bacteriology, Iowa State University, Ames, Iowa 50010.

alfalfa, pelleted grain, and wheat bran (10:5:1) (36). Serial dilutions of whole ruminal contents from 10^{-1} through 10^{-9} were each inoculated under CO_2 into triplicate tubes of 1% tributyrin-rumen fluid Trypticase yeast extract medium (tributyrin-RFTY; reference 13). The inoculated medium was solidifed as roll tube preparations (7) and incubated at 39 C for 2 weeks. Colonies showing zones of hydrolysis (20) at dilutions of 10^{-6} through 10^{-9} were picked and maintained in tributyrin-RFTY medium. In some instances tributyrin was replaced in the medium by an insecticide, di-*n*-butyl succinate (Tabutrex).

Cultural and biochemical characteristics. Unless otherwise stated, media, reagents, and procedures used to determine cultural and biochemical characteristics of ruminal bacteria have been reported (8, 9, 13). Test media for phenylalanine deamination and deoxyribonuclease activity (29, 33), extracellular proteinases (1), and hyaluronidase-chondroitin sulfatase activity (34) were modified by the addition of clarified ruminal fluid (30%, vol/vol) at *p*H 6.85 under CO_2. Cellular dimensions of phosphotungstic acid-negatively stained specimens were determined by electron microscopy (25). Volatile fatty acid concentrations in culture media were determined by gas-liquid chromatographic techniques (35). Lactic and succinic acids were determined according to a previous report (32).

To determine growth rates, a 15-h culture of strain 53 in RFTY broth medium was inoculated (0.1 ml/tube) into neoprene-stoppered tubes of sterile RFTY broth medium (9.9 ml) under CO_2. Inoculated medium and uninoculated controls were incubated at 39 C. At 0, 6, 12, 18, 24, 30, and 36 h, separate tubes of medium were filtered through preweighed membrane filters (0.45-μm pore size; Millipore Corp.). The cellular residues were each washed with distilled water and dried at 105 C for 18 h. Cell weights were determined by calculating the differences between inoculated and uninoculated medium filter weights at each prescribed incubation time.

Cell extracts and electrophoresis. *Butyrivibrio fibrisolvens* strain 53 was inoculated into 300-ml RFTY broth medium under CO_2 (neoprene-stoppered flasks) and incubated for 12 to 15 h at 39 C. The medium was centrifuged at 5 C (15,000 $\times$ *g*, 30 min). The supernatant fluid was removed, and the sedimented materials were suspended in Na_2HPO_4 buffer (0.1 M, *p*H 7.0), recentrifuged, and brought up to 5.0 to 7.0 ml in 0.05 M Na_2HPO_4 buffer at *p*H 7.0. The cell suspension was sonically treated at maximum intensity (Fisher Probe Model BP-5, Generator Model CW-5) for 5 min at 4 C. Cell debris was removed by centrifugation at 5 C (25,000 $\times$ *g*, 60 min). The cell extracts (and column fraction V) were applied (20 to 50 μliters) to polyacrylamide gels (7% in tris(hydroxymethyl)aminomethane [Tris]-glycine buffer; stacking gel at *p*H 8.9; and separating gel at *p*H 9.5) and run according to the procedure supplied with the Canalco model 6 electrophoresis system. The gels were developed with 1-naphthyl acetate (NA) as substrate with fast garnet GBC (4 amino-3:1'-dimethyl azobenzene) as a coupling agent (16, 27). Developed gels were preserved in 7% acetic acid.

Cell extracts were mixed with saturated ammonium sulfate (Sigma) solutions to 20, 30, 40, 50, and 60% of saturation at pH 5.2. The resulting precipitates were each collected by centrifugation at 5 C (10,000 $\times$ *g*, 30 min). The precipitates were then dialyzed against glass-distilled water for 2 h at 4 C. The precipitates were diluted in buffer and run on polyacrylamide gels as above. These gels were then incubated with NA, 1-naphthyl propionate, 1-naphthyl butyrate, 1-naphthyl palmitate, or 1-naphthyl phosphate (each at 0.84 mM) with fast garnet GBC (16, 27).

Protein and lipase/esterase assays. Protein from bacterial sources was determined by the method of Lowry et al. (26) with bovine serum albumin (Sigma, fraction V) as the standard. Samples eluted from diethylaminoethyl (DEAE) Sephadex A-25, and Sephadex G-200 gel columns (Pharmacia Fine Chemicals Inc.,) were monitored for protein by measuring absorbancy at 280 and 260 nm in matched quartz cuvettes. Lipase activity was determined by incubating 0.5 ml of the cell preparations with 3.0 ml of olive oil emulsion (Sigma), 1.0 ml of buffer (Sigma Trizma buffer, *p*H 8.0), and 1.5 ml of distilled water. The reaction mixture was incubated at 39 C for 6 h. Samples removed from the reaction mixture were mixed with ethanol and titrated with 0.05 N NaOH under N_2 to *p*H 9.0. Milliequivalents of free fatty acids were then calculated.

Cell extracts (with and without acetone treatment; reference 30) were assayed for esterase activity as described in a previous report (30). Substrates tested included tributyrin and tripropionin that were each prepared as emulsions in gum arabic-oleic acid solution (11). The rate of autohydrolysis of each substrate was determined by titrating for free fatty acids after 6 h of incubation at 39 C. Fluorescein diester substrates, dissolved in a methyl cellosolve-Na_2HPO_4 buffer system, were incubated with ammonium sulfate (30 to 60% of saturation) precipitates of cell extracts in buffer. These preparations were tested for esterase activity as previously reported (17).

Esterase assay. Esterase activity of ruminal bacteria was determined by observing the conversion of 1-naphthyl acetate (NA) to 1-naphthol. Each NA assay reaction mixture contained 0.1 ml of whole cells or cell extracts and 1.5 ml of NA (0.42 mM), 0.5 ml of Na_2HPO_4 buffer (0.2 M, *p*H 7.0), and 0.4 ml of glass-distilled water. The mixture was incubated for 10 min at 39 C. One-half milliliter of 10% lauryl sulfate (Sigma) solution containing 2.5 mg of fast garnet GBC was added to the mixture (16, 17). The mixture was then incubated for 15 min at room temperature for development of color. Absorbancy was measured at 560 nm and compared with the absorbancy of a 1-naphthol curve (linear from 0 to 0.08 μM). In some experiments, NA was replaced by 1-naphthyl propionate, 1-naphthyl butyrate, 1-naphthyl palmitate, or 1-naphthyl phosphate (each at 0.42 mM).

Test compounds used in the NA reaction mixture in place of the distilled water (0.4 ml) included: $CoCl_2$, $MgCl_2$, $CaCl_2$, or NaCl (each at 10^{-6} to 10^{-3} M); eserine (Sigma) in 2% ethanol; carbaryl (1-naphthyl *N*-methyl-carbamate) in 10% acetone; paraoxon; diisopropyl fluorophosphate; ethyl formate; ethyl acetate; and NaF. Each of these compounds was preincubated for 10 min at 39 C with the enzyme source before the addition of NA.

Column chromatography. Sephadex (DEAE) A-25 was washed sequentially with glass-distilled water for 1 h followed by 0.5 N NaOH, 0.5 N H_3PO_4, glass-distilled water, and 0.05 M Na_2HPO_4 (*p*H 7.2). After standing overnight in the Na_2HPO_4 buffer at 6 C, the fines were decanted and the gel was packed in a 2.5- by 45-cm Pharmacia column to a height of 35 cm. A sample (8 to 10 ml containing about 175 mg of protein) was applied to the column gel and subsequently eluted with 200 ml of Na_2HPO_4 buffer (0.05 M, *p*H 7.0–7.2) and a NaCl gradient at a rate of 30 ml/h (31). The eluate was collected in 4-ml fractions, and the fractions were stabilized by precipitating them with ammonium sulfate (80% of saturation) at 4 C under N_2.

A Sephadex G-200 gel column was prepared as follows. Sephadex G-200 was heated to about 98 C for 5 h in 0.05 M Na_2HPO_4 buffer (*p*H 7.2), cooled, decanted of fines, and poured into a 1.5- by 75-cm Bio-Rad column. The column was packed under a hydrostatic pressure head of 12 cm and inverted and calibrated with bovine serum albumin, catalase, cytochrome *c*, gamma globulin, and ovalbumin (10 mg each in 0.6 ml of buffer) at a flow rate of 4 ml/h. Fraction V from the DEAE Sephadex column was dissolved in buffer and applied to the G-200 column. Fractions collected (1.5 or 2.0 ml) were assayed for NA esterase activity and protein as noted above.

RESULTS

Isolates. One hundred fourteen bacterial isolates at 10^{-6} through 10^{-8} dilutions of ruminal contents showed zones of hydrolysis in tributyrin-RFTY medium. These isolates included gram-positive streptococci, gram-positive and gram-variable bacilli, and gram-negative bacilli. After four 72-h transfers, 20 of the isolates grew slowly or not at all. Thirty-seven of the isolates failed to show tributyrin hydrolysis on repeated testing. Of the remaining isolates, 18 gram-negative bacilli consistently showed degradation of tributyrin and also showed degradation of di-*n*-butyl succinate in RFTY medium. One such culture (strain 53, isolated at 10^{-7} dilution) was chosen for further experimentation.

Identification of strain 53. Gram-stain preparations of the culture indicated that strain 53 was a gram-negative, slightly curved bacillus with bluntly tapered ends, occurring singly or in chains. By negative stain preparations, the bacilli were observed as monotrichous (0.5–0.6 μm by 1.4–3.2 μm) and surrounded with mucoidal debris. The flagella were helicoidal and 0.05 μm in diameter. Surface colonies in media with agar were entire, convex, translucent, tan colored, and 2 to 4 mm in diameter. Deep colonies were thin and lens-shaped with filamentous margins. In broth media, growth was mainly a flocculent sediment. The culture grew at 30, 39, and 45 C but not at 3, 21, and 65 C. Strain 53 was observed to be an obligate anaerobe that requires CO_2. The culture did not hemolyze bovine erythrocytes or degrade casein, gelatin, chondroitin sulfate, deoxyribonucleate, hyaluronic acid, indole, or phenylalanine to phenyl-pyruvic acid. Carbohydrates utilized by strain 53 included dextrin, esculin, fructose, galactose, glucose, guar gum, lactose, mannose, pectin, salicin, starch, xylan, and xylose. Minimum pH in glucose medium was 5.2. Strain 53 in the glucose medium produced (in mM/100 ml of medium) acetic (0.86), butyric (1.17), and lactic (0.18) acids. Formic acid was not determined. Succinic acid was not produced. Strain 53 showed no medium *p*H change with L-arabinose, cellobiose, cellulose, gum arabic, inulin, melezitose, melibiose, raffinose, L-rhamnose, ribose, L-sorbose, sucrose, trehalose, or turanose. No growth was observed on arabitol, adonitol, L-arabitol, dulcitol, erythritol, glycerol, inositol, mannitol, or sorbitol. On the basis of the above characteristics, the organism was identified as *B. fibrisolvens* (9).

Growth and esterase activity. Under the stated condition, the exponential phase of growth of *B. fibrisolvens* strain 53 was between 6 and 18 h. Cells collected in late exponential phase (12 to 18 h) degraded aliphatic substrates as shown in Table 1. Tributyrin was degraded more rapidly than tripropionin but not as rapidly as triacetin. Ethyl formate and ethyl acetate were very slowly hydrolyzed. NA was degraded more rapidly than the aliphatic esters. Olive oil emulsions were degraded at a rate of 4.7×10^{-5} meq per ml per min. 1-Naphthyl esters were degraded at a rate in the order 1-naphthyl acetate > 1-naphthyl butyrate > 1-naphthyl propionate, but the enzymes did not degrade 1-naphthyl palmitate or 1-naphthyl phosphate in 10 min at 39 C. Addition of $CoCl_2$, $MgCl_2$, $MnCl_2$, or NaCl at 10^{-6} to 10^{-3} M to cell extracts degrading NA (0.42 mM, rate of degradation, 4.6×10^{-4} meq per ml per min) showed no stimulatory or inhibitory effects. CaCl at 10^{-3} M stimulated esterase activity by 16%.

Degradation of fluorescein diesters by esterases of strain 53 is shown in Table 2. Rate of

TABLE 1. *Degradation of aliphatic esters and 1-naphthyl esters by strain 53*

Substrate	Concn (mM)	Rate of hydrolysis (meq per ml per min)
Triacetin	2.4	2.9×10^{-4}
	4.2	4.6×10^{-4}
Tributyrin	1.4	8.5×10^{-5}
	4.4	2.8×10^{-4}
Tripropionin	4.4	2.5×10^{-5}
Ethyl acetate	60	5.0×10^{-6}
Ethyl formate	50	1.0×10^{-6}
1-Naphthyl acetate	0.42	4.6×10^{-4}
	0.84	7.0×10^{-4}
1-Naphthyl propionate	0.42	1.7×10^{-4}
1-Naphthyl butyrate	0.42	2.1×10^{-4}

degradation was in the order of acetyl > propionyl > caproyl > butyryl > capryl > lauryl. These results indicated that esterases of strain 53 remained active when precipitated by ammonium sulfate and dissolved in buffer. By the NA assay method, it was found that ammonium sulfate at 30 to 60% (of saturation) concentration precipitated 70.5% of the total activity in cell extracts of strain 53. Only 1.3% of the activity was detected at the 0 to 30% ammonium sulfate concentrations. The medium supernatant, freed of whole cells of strain 53 cultures (12 to 18 h), showed no NA esterase activity. A unit of esterase was defined as the amount of enzyme that degrades NA to form 1 μmol of 1-naphthol in 10 min at *p*H 7.0 and 39 C.

Column chromatographic separation of esterase fractions. Esterases of strain 53 separated into six fractions by column chromatography as shown in Fig. 1. Fraction I was eluted before the NaCl gradient was applied to the column. Fractions II through IV were eluted with the NaCl gradient. In preliminary runs, the column buffer was at *p*H 7.0 rather than *p*H 7.2. In these runs, fractions II and III were eluted before the NaCl gradient was on the column. This indicated that the esterases in these fractions had isoelectric points near *p*H 7.0. Specific activity, percent recoveries, and purification values for each of the esterase fractions eluted from DEAE Sephadex columns are given in Table 3. All eluted fractions were found to be stable at 5 C in ammonium sulfate (80%) in neoprene-stoppered flasks flushed with N_2.

Zymograms. Polyacrylamide gel electrophoretic zymograms of the column fractions are shown in Fig. 2. All esterase bands degraded NA and migrated toward the cathode. Esterases at R_f 0.47 and R_f 0.26 of fractions II and III degraded 1-naphthyl butyrate. Esterases at R_f 0.46 to 0.49 of fractions I, III, and IV (and the homogenate) showed traces of esterase activity on 1-naphthyl propionate. None of the esterase bands degraded 1-naphthyl palmitate or 1-naphthyl phosphate. Fraction V possessed only one esterase band at R_f 0.81, and an attempt was made to characterize the esterase activity of this fraction.

Fraction V. This fraction eluted from a G-200 Sephadex gel filtration column as shown in Fig. 3. By comparing the elution characteristics of this fraction with those of the protein standards, its molecular weight was calculated as 66,000. The K_m was calculated as 7.6×10^{-4} with NA as substrate from a Lineweaver-Burk plot. The optimal temperature for fraction V esterase was 39 C. The enzyme was denatured when heated at 60 C for 10 min, as only 13% of the enzymatic activity remained.

Effects of potential inhibitors on fraction V esterase are shown in Table 4. Carbaryl was not inhibitory at 4.2×10^{-4} M and 8.4×10^{-4} M. When carbaryl was tested as a substrate at the above concentrations but without NA, no 1-naphthol was produced from carbaryl in 30 min at 39 C. Both diisopropyl fluorophosphate and paraoxon inhibited fraction V esterase at all levels tested. Eserine and sodium fluoride showed low inhibitory responses.

DISCUSSION

Strain 53 in relation to other bacteria. *B. fibrisolvens* strain 53 differs morphologically and physiologically from the lipolytic selenomonads isolated by Hobson and Mann (20). It also differs from the extracellular lipase-pro-

TABLE 2. *Degradation of fluorescein diesters by strain 53*

Fluorescein ester[a]	Fluorescein produced in 10 min[b] (M)
Diacetyl	3.85×10^{-9}
Dipropionyl	1.45×10^{-9}
Dicaproyl	0.56×10^{-9}
Dibutyryl	0.50×10^{-9}
Dicapryl	0.15×10^{-9}
Dilauryl	0.10×10^{-9}

[a] Fluorescein 3',6'-diacetyl, etc.; each ester was tested at 23.9×10^{-7} M.

[b] Each fluorescein value was corrected for nonenzymatic hydrolysis (17). The assay mixture contained esterase (ammonium sulfate, 30 to 60% of saturation; 0.68 mg of protein) and fluorescein diester (substrate) in a methyl cellosolve-phosphate buffer up to 2.1 ml (total volume) at pH 7.0 and 39 C (17).

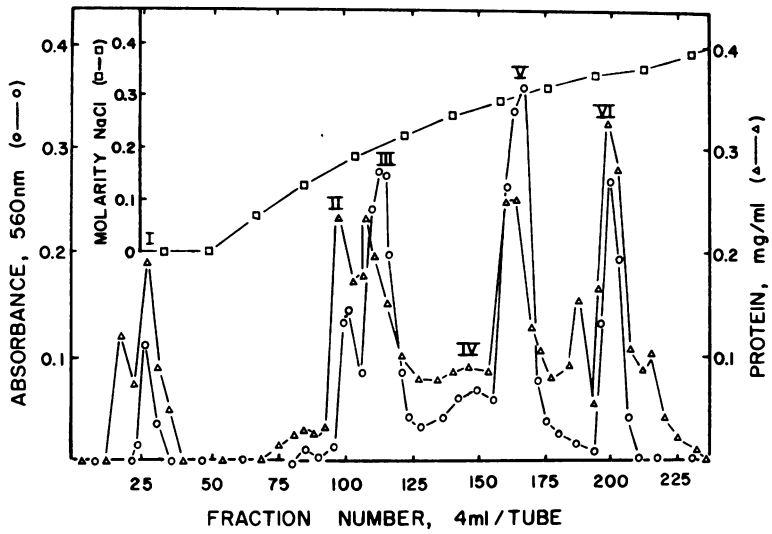

FIG. 1. *DEAE Sephadex column chromatography of esterases from B. fibrisolvens strain 53. Each 4-ml fraction was collected at 6 C. Column flow rate was 30 ml/h. Numbers I through VI refer to fractions with esterase activity on 1-naphthyl acetate. Absorbance readings at 560 nm detected esterase activity on 1-naphthyl acetate in the presence of fast garnet GBC (16, 27). Absorbance readings at 280 nm detected protein in the fractions. The NaCl gradient used is shown as an insert in the figure.*

TABLE 3. *Specific activity and percent recovery of Butyrivibrio fibrisolvens strain 53 esterases eluted from DEAE Sephadex columns*

Fraction[a]	Total units[b]	Total protein (mg)	Specific activity (units/mg of protein)	Percent recovery of esterase	Fold purification
I	0.60	0.24	2.50	0.9	13.2
II	0.88	0.65	1.35	1.3	7.1
III	0.91	0.26	3.50	1.3	18.8
IV	0.86	0.25	3.44	1.2	18.4
V	5.87	2.51	2.34	8.4	12.3
VI	ND[b]	2.03	ND	ND	ND
Supernatant[c]	70.20	369.00	0.19	100.0	1.0
Dialysate[d]	38.88	190.35	0.20	56.0	1.1

[a] Numbers I through VI refer to fractions collected by column chromatography as shown in Fig. 1.

[b] A unit is defined as that amount of enzyme that produces 1 μmol of 1-naphthol from 1-naphthyl acetate in 10 min at pH 7.0 and a temperature of 39 C. ND, not determined.

[c] Supernatant fluid of cell extracts of strain 53.

[d] Ammonium sulfate fraction (30 to 60% of saturation) of strain 53 that was dialyzed for 2 h at 4 C against glass-distilled water.

ducing rumen bacterium *Anaerovibrio lipolyticum* of Henderson (18, 20) in degrading tributyrin and producing esterases that are cell bound. Since *B. fibrisolvens* strain 53 showed no proteolytic activity on casein or gelatin, it appears unlikely that the organism degrades aromatic and aliphatic esters by nonspecific proteinases. A ruminal strain, *Bacteroides amylophilus* H18, has recently been shown to degrade denatured casein by a protease that also degrades an aromatic ester, *p*-toluenesolphonyl-L-arginine methyl ester, but unlike strain 53 enzymes, does not degrade NA (5).

B. fibrisolvens strain 53 produces esterases that may be cell bound similar to nonruminal bacteria. *Bacillus cereus* produces esterases associated with the plasma membrane and the outer surface of the cell wall (6). Lipase is absent in ruminal contents that have been freed of microorganisms (10). This suggests that lipase (esterase) activity is cell bound and not released into the surrounding medium. This finding appears to need further study, for a ruminal bacterium, *A. lipolyticum*, has recently been reported to produce an extracellular lipase (18).

Natural substrates for esterases. Natural substrates for esterases in ruminal contents are not known. In pasture-fed ruminants, most of the neutral lipids in feedstuffs are in the form of galactosyl-diglycerides, which could be first degraded by galactosidases to yield diglycerides (18). These diglycerides could then be degraded by esterases or lipases, or both (18, 19). Other suggested substrates found in feedstuffs or in rumen microorganisms, or both, include plant sterols, lecithin, lyolecithin (21),

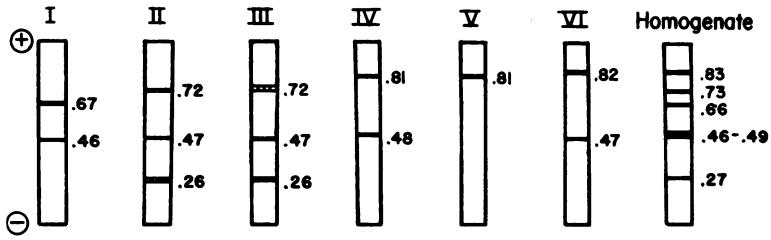

FIG. 2. *Polyacrylamide gel electrophoresis zymograms of strain 53 esterases. Numbers I through VI refer to DEAE Sephadex esterase fractions. Homogenates refers to a zymogram of the esterases in cell extracts of strain 53.*

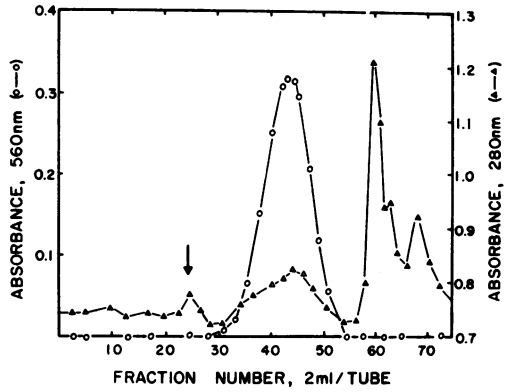

FIG. 3. *Sephadex G-200 column chromatography of DEAE Sephadex fraction V esterase of strain 53. Each 2-ml fraction was collected at 4 C. Column flow rate was 4 ml/h. The arrow indicates the void volume for blue dextran. Absorbance readings at 560 nm detected esterase activity on 1-naphthyl acetate in the presence of fast garnet GBC (16, 27). Absorbance readings at 280 nm detected protein in the fractions.*

TABLE 4. *Effect of potential inhibitors on Sephadex G-200 fraction V esterase*

Inhibitor[a]	Concn (M)	Percent inhibition[b]
Carbaryl	8.4×10^{-4}	0
	4.2×10^{-4}	0
Diisopropyl fluorophosphate	10^{-4}	62
	10^{-5}	49
	10^{-6}	33
	10^{-7}	7
Eserine	10^{-4}	17
	10^{-5}	0
Paraoxon	10^{-4}	41
	10^{-5}	11
	10^{-6}	11
	10^{-7}	9
NaF	10^{-2}	17
	10^{-3}	3

[a] Carbaryl (1-naphthyl *N*-methylcarbamate), Eserine (physostigmine), Paraoxon (diethyl *p*-nitrophenyl phosphate).

[b] The esterase contained 0.25 mg of protein with 0.6 unit of activity per NA assay. Refer to Table 3 for the definition of a unit.

and aliphatic esters (19, 21). It seems that microbial esterases play an important role in the biohydrogenation of dietary lipids. No hydrogenation of unsaturated fatty acids takes place unless the constituent fatty acids are first released in free form (10). At the present time, *B. fibrisolvens* is apparentl the only rumen bacterium on which detailed studies of fatty acid hydrogenases have been made (23). *B. fibrisolvens* strain 53 degraded an aliphatic insecticide, di-*n*-butyl succinate, and also a carbamate insecticide, benzo(b)-thien-4-yl-methylcarbamate (38). These findings suggest that the bacterial esterases play a role in the degradation of agricultural chemical esters.

Esterases. Throughout these experiments, the ester-degrading enzymes of *B. fibrisolvens* strain 53 were referred to as esterases (hydrolases), as described by the International Union of Biochemistry nomenclature. This is a broad classification that includes lipases, cholinesterases, and simple esterases. Strain 53 showed evidence of lipase activity in degrading lipoidal emulsions and fluorescein dibutyrate, and being activated by calcium ion (11, 17). The sequence for the rate of hydrolysis of fluorescein diesters was similar to that reported for porcine pancreatic lipase (17). The calcium ion activation was low and similar to the activation levels of *A. lipolyticum* lipase (18). However, the ester-degrading enzymes of strain 53 showed no increase in esterase activity when treated with acetone. The bacterial enzymes, therefore, differ from rat adipose tissue lipases (30).

The inhibitor experiments indicated that G-200 fraction V esterase was not a cholinesterase, A-esterase (2), or C-esterase (4). Cholinesterases were eliminated on the basis that they are inhibited by 10^{-5} M of eserine. The

A-esterases of Aldridge (2) and C-esterases of Bergmann (4) were eliminated because they were not inhibited by organophosphates. In the classification of Holmes and Masters (21), fraction V esterase does not fit into any category. Since fraction V esterase rapidly hydrolyzed an aromatic ester (NA) and was inhibited by organophosphates, it was classified as a B-esterase as described by Aldridge (2).

ACKNOWLEDGMENTS

We thank George Gassner III for helpful discussions and assistance in electron microscopy. We also thank Paul P. Hipps and Rosa L. Stolzenberg for their suggestions and technical assistance.

LITERATURE CITED

1. Ahearn, D. G., S. P. Meyers, and R. A. Nichols. 1968. Extracellular proteinases of yeasts and yeastlike fungi. Appl. Microbiol. **16**:1370-1374.
2. Aldridge, W. N. 1953. Serum esterases. I. Two types of esterase (A and B) hydrolyzing p-nitrophenyl acetate, propionate and butyrate, and a method for their determination. Biochem. J. **53**:110-117.
3. Baillie, A., R. O. Thomson, I. Batty, and P. D. Walker. 1967. Some preliminary observations on the location of esterases in *Bacillus cereus*. J. Appl. Bacteriol. **30**:312-316.
4. Bergmann, F., R. Segal, and S. Rimon. 1957. A new type of esterase in hog-kidney extract. Biochem. J. **67**:481-486.
5. Blackburn, T. H. 1968. The protease liberated from *Bacteroides amylophilus* strain H-18 by mechanical disintegration. J. Gen. Microbiol. **53**:37-51.
6. Brown, J. P. 1970. Cytochemical electron microscopic localization of esterase activity in *Lactobacillus casei*. Appl. Microbiol. **19**:1001-1004.
7. Bryant, M. P., and L. A. Burkey. 1953. Cultural methods and some characteristics of some of the more numerous groups of bacteria in the bovine rumen. J. Dairy Sci. **36**:205-217.
8. Bryant, M. P., and R. N. Doetsch. 1954. A study of actively cellulolytic rod-shaped bacteria of the bovine rumen. J. Dairy Sci. **37**:1176-1183.
9. Bryant, M. P., and N. Small. 1956. The anaerobic monotrichous butyric acid-producing curved rod-shaped bacteria of the rumen. J. Bacteriol. **72**:16-71.
10. Clarke, D. G., and J. C. Hawke. 1970. Studies on rumen metabolism. VI. *In vitro* hydrolysis of triglyceride and isolation of a lipolytic fraction. J. Sci. Food Agr. **21**:446-452.
11. Desnuelle, P., and P. Savary. 1963. Specificities of lipases. J. Lipid Res. **4**:369-384.
12. Downey, W. K., and P. Andrews. 1965. Esterase activity of non-enzymic proteins of milk and serum. Biochem. J. **96**:21C.
13. Fulghum, R. S., B. B. Baldwin, and P. P. Williams. 1968. Antibiotic susceptibility of anaerobic ruminal bacteria. Appl. Microbiol. **16**:301-307.
14. Garton, G. A., P. N. Hobson, and A. K. Lough. 1958. Lipolysis in the rumen. Nature (London) **182**:1511-1512.
15. Garton, G. A., A. K. Lough, and E. Vioque. 1961. Glyceride hydrolysis and glycerol fermentation by sheep rumen contents. J. Gen. Microbiol. **25**:215-225.
16. Gomori, G. 1953. Human esterases. J. Lab. Clin. Med. **42**:445-453.
17. Guilbault, G. G., and D. N. Kramer. 1964. Fluorometric determination of lipase, acylase, alpha-, and gamma-chymotrypsin and inhibitors of the enzymes. Anal. Chem. **36**:409-412.
18. Henderson, C. 1971. A study of the lipase produced by *Anaerovibrio lipolytica*, a rumen bacterium. J. Gen. Microbiol. **65**:81-89.
19. Hill, F. D., J. H., Saylor, R. S. Allen, and N. L. Jacobson, 1960. *In vitro* lipolysis by rumen ingesta. J. Anim. Sci. **19**:1266-1270.
20. Hobson, P. N., and S. O. Mann. 1961. The isolation of glycerol-fermenting and lipolytic bacteria from the rumen of the sheep. J. Gen. Microbiol. **25**:227-240.
21. Holmes, R. S., and C. J. Masters. 1967. The developmental multiplicity of isoenzyme status of cavian tissues. Biochim. Biophys. Acta **132**:379-399.
22. Hungate, R. E. 1966. The rumen and its microbes. Academic Press Inc., New York.
23. Kepler, C. R., and S. B. Tove. 1967. Biohydrogenation of unsaturated fatty acids. III. Purification and properties of a linoleate Δ^{12}-cis, Δ^{11}-trans-isomerase from *Butyrivibrio fibrisolvens*. J. Biol. Chem. **242**:5686-5692.
24. Kimmel, J. R., and E. L. Smith. 1954. Crystalline papain. I. Preparation, specificity, and activation. J. Biol. Chem. **207**:515-531.
25. Langenberg, K. F., M. P. Bryant, and R. S. Wolfe. 1968. Hydrogen-oxidizing methane bacteria. II. Electron microscopy. J. Bacteriol. **95**:1124-1129.
26. Lowry, H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265-275.
27. Markert, C. L., and R. L. Hunter. 1959. The distribution of esterases in mouse tissues. J. Histochem. Cytochem. **7**:42-49.
28. Murphy, P. M., and C. L. Masterson. 1970. Determination of multiple forms of esterases in *Rhizobium* by paper electrophoresis. J. Gen. Microbiol. **61**:121-129.
29. Oberhofer, T. R., and L. Maddox. 1970. Combined medium to determine deoxyribonuclease activity and phenylalanine deamination by *Enterobacteriaceae*. Appl. Microbiol. **19**:385-386.
30. Okuda, H., and S. Fujii. 1966. Lipase and esterase in adipose tissue and liver. J. Biochem. **61**:313-319.
31. Peterson, E. A., and H. A. Sober. 1959. Variable gradient device for chromatography. Anal. Chem. **31**:857-862.
32. Rumsey, T. S., C. H. Noller, J. C. Burns, D. Kalib, C. L. Rhyderd, and D. L. Hill. 1964. Gas chromatographic resolution of pyruvic, lactic, glyoxylic, oxalic, malonic, fumaric, malic, and alpha-ketoglutaric, and citric acids in forage and rumen fluid. J. Dairy Sci. **47**:1418-1421.
33. Smith, P. B., G. A. Hancock, and D. L. Rhoden. 1969. Improved medium for detecting deoxyribonuclease-producing bacteria. Appl. Microbiol. **18**:991-993.
34. Smith, R. F., and N. P. Willett. 1968. Rapid plate method for screening hyaluronidase and chondroitin sulfatase-producing microorganisms. Appl. Microbiol. **16**:1434-1436.
35. Williams, P. P., K. L. Davison, and E. J. Thacker. 1968. *In vitro* and *in vivo* rumen microbiological studies with 2-chloro-4,6-bis(isopropyl-amino)-*s*-triazine (Propazine). J. Anim. Sci. **27**:1472-1476.
36. Williams, P. P., and W. E. Dinusson. 1972. Composition of the ruminal flora and establishment of ruminal ciliated protozoal species in isolated calves. J. Anim. Sci. **34**:469-474.
37. Williams, P. P., and V. J. Feil. 1971. Identification of trifluralin metabolites from rumen microbial cultures. Effect of trifluralin on bacteria and protozoa. J. Agr. Food Chem. **19**:1198-1204.
38. Williams, P. P., and R. L. Stolzenberg. 1972. Rumen bacterial degradation of benzo(b)thien-4-yl methylcarbamate (Mobam) and effect of Mobam on ruminal bacteria. Appl. Microbiol. **23**:745-749.