An Electron Microscopic Study of *Anaerovibrio lipolytica* (Strain 5s) and Its Lipolytic Enzyme

By C. HENDERSON

Rowett Research Institute, Bucksburn, Aberdeen, AB2 9SB

AND W. HODGKISS

Torry Research Station, Aberdeen, AB9 8DG

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SUMMARY

The extracellular lipolytic activity produced by *Anaerovibrio lipolytica* strain 5 s is associated with large particles composed of protein, lipid and nucleic acid. These particles have a membranous appearance when viewed by electron microscope and in the ultracentrifuge they have a sedimentation coefficient ($s_{20,w}$) of 13·1 S. Numerous 'blebs' are associated with the walls of *Anaerovibrio lipolytica* during the phase of logarithmic growth when lipase production is maximal. The lipolytic enzyme is released from the bacteria without visible bacterial lysis.

INTRODUCTION

Extracellular enzymes are more commonly produced by Gram-positive than by Gram-negative bacteria (Pollock, 1962) and the secretion of enzymes by Gram-positive bacteria has been linked with mesosomes (Beaton, 1968) or with organelles bearing a superficial morphological resemblance to mesosomes (Ghosh, Sargent & Lampen, 1968). Mesosomal material from Gram-positive bacteria has been widely studied. Popkin, Theodore & Cole (1971) examined by electron microscopy the mesosomal vesicles released from *Staphylococcus aureus* during protoplast formation. Ghosh & Murray (1969) found that mesosomal material from *Listeria monocytogenes* contained RNA, DNA, phospholipid and protein. A similar study of mesosomal material from *Micrococcus lysodeikticus* was made by Ellar & Freer (1969). Mesosomes are most prominent in Gram-positive bacteria but are not exclusive to such organisms and Ryter & Jacob (1966) and Pontefract, Bergeron & Thatcher (1969) have reported studies of mesosomes in *Escherichia coli*.

Anaerovibrio lipolytica strain 5 s, a Gram-negative rumen bacterium, produces an extracellular lipase of large molecular weight (Henderson, 1971). An electron microscopic study of the enzyme has been made and, in an attempt to observe the secretion of enzyme, the bacteria have also been examined by electron microscopy.

METHODS

Lipase activity was measured as described by Henderson (1971), using olive oil as substrate and employing the extraction and titration method of Cohen, Morgan & Hofman (1969).

Purification of the extracellular lipase of Anaerovibrio lipolytica. The bacteria were grown in a chemostat under carbon-limited conditions at 38 °C and a dilution rate of 0·120 h⁻¹ and the outflow medium collected in a glass vessel at 1 °C. Litre amounts of bacteria-free medium were half-saturated with ammonium sulphate and 2 % (w/v) of cellulose powder

added. The suspension was stirred for 3 h at 1 °C and the cellulose removed by centrifugation. A glass column (320 × 18 mm) was packed with the wet cellulose and the lipolytic activity eluted in 30 ml 0.1 M-phosphate buffer, pH 7.4. After overnight dialysis against distilled water the enzyme solution was concentrated to 6 ml by ultrafiltration through a collodion shell, applied to a Sephadex G 200 column (void volume 80 ml) and eluted with 0.1 M-phosphate buffer, pH 7.4. The lipase activity was detected in the protein fraction eluted in the void volume of buffer.

Ultracentrifugal separation. Samples of lipase from the Sephadex G 200 column were concentrated by ultrafiltration and solutions, containing 0.75 % (w/v) dry matter in 0.1 M-phosphate buffer, pH 7.4, were centrifuged at 59 780 rev./min in a Spinco Model E analytical ultracentrifuge (Beckman-RIIC Ltd, Glenrothes, Scotland). Two components were resolved, the major one having a sedimentation coefficient ($s_{20,w}$) of 5.0 S and the minor component a sedimentation coefficient 13.1 S.

To determine which of the two components separated by ultracentrifugation had lipase activity, preparative ultracentrifugal separations were performed on sucrose density gradients using an SW-50 L rotor in a Beckman L 2-50 ultracentrifuge. Sucrose density gradients were prepared by sequential pipetting into 5 ml cellulose nitrate tubes of 2 ml 30 % (w/v) sucrose in 0.1 M-phosphate buffer, pH 7.4, 1.5 ml 25 % sucrose solution and 1.0 ml 20 % sucrose solution. After the layers had interdiffused for 24 h at 1 °C, 0.5 ml of purified lipase solution containing 0.65 mg protein was layered above the sucrose gradient and the tubes were centrifuged at 50 000 rev./min for 6.0 h. The base of each tube was then punctured with a hypodermic needle and the contents pumped out at 4 ml/h and collected in 0.25 ml fractions. The $E_{280\text{ nm}}^{1\text{ cm}}$ and lipase activity of each fraction was assayed. The lipolytic activity was recovered in the fractions closest to the bottom of the tube which corresponded to the faster-moving minor component observed in the analytical ultracentrifuge.

Examination, by thin-layer chromatography, of chloroform-methanol (2:1, v/v) extracts of purified lipase preparations showed that the major lipid component was phosphatidyl ethanolamine. Phosphatidyl ethanolamine was also the major phospholipid in lipid-solvent extracts of washed suspensions of *Anaerovibrio lipolytica*.

As with the lipase preparations described by Henderson (1971), the purest preparations obtained in this study contained a high proportion of nucleic acid.

Electron microscopy. Enzyme preparations were dialysed overnight against distilled water and stained by addition of 0.1 ml aqueous phosphotungstic acid (1 %, w/v; adjusted to pH 7.2) to 1 ml of enzyme preparation. A drop of this mixture was placed on a carbon-stabilized, formvar-coated copper grid (mesh 200), air-dried, and examined in the electron microscope.

Bacteria were obtained from carbon-limited continuous cultures growing at a dilution rate of 0.120 h⁻¹, or from batch cultures during the early logarithmic phase of growth (Henderson, 1971).

For negatively stained and metal-shadowed specimens the bacteria were fixed for 4 h at 4 °C by the addition of formaldehyde (final concentration 2.5 %, v/v) to a sample of culture medium. After fixation, bacteria were washed three times by centrifugation in sterile, distilled water. Negatively stained specimens of fixed bacteria were prepared in the same way as negatively stained enzyme preparations. For metal-shadowed specimens, 1 drop of washed bacterial suspension was placed on a formvar-coated copper grid (200 mesh) air-dried and shadowed with gold + palladium (40:60) at an angle of 20°.

For ultramicrotomy, bacteria were fixed for 4 h at 4 °C by adding 0.5 g osmium tetroxide

to 50 ml of culture. The fixed bacteria were washed with veronal buffer, dehydrated with alcohol and embedded in Araldite (Geigy Ltd, Duxford, Cambridge). Sections were cut with glass knives in an LKB Ultratome III and stained with lead oxide (Karnovsky, 1961).

Specimens were examined in a Siemens Elmiskop I electron microscope, using double condenser illumination, a 200 μm condenser aperture, 50 μm objective aperture and an accelerating voltage of 60 kV. Micrographs were recorded on Kodak Electron Image plates.

RESULTS AND DISCUSSION

The enzyme preparations used in this study were broadly similar to those described by Henderson (1971) in a study of lipase produced by *Anaerovibrio lipolytica* grown at a lower growth rate.

Samples of enzyme purified by gel filtration on Sephadex G 200 were shown to be membranous in appearance (Fig. 1). The particles were of fairly uniform size, and although usually circular, more irregular shapes suggestive of fragmented membranes were occasionally found. In specimens of lipase purified by ultracentrifugation in sucrose density gradients (Fig. 2), particles were uniformly circular and varied in size depending on whether or not sucrose was removed by dialysis.

The possibility that the lipase was released from *Anaerovibrio lipolytica* by autolytic fragmentation of the bacterial membrane was discounted since an electron microscopic study of whole cultures gave no evidence of lysed bacteria. Fig. 3 shows a typical field on a grid. Also, in batch cultures the lipase appears early in the logarithmic phase of growth when little lysis would have occurred and no lipase is associated with the whole or fragmented bacteria. (Henderson, 1971).

Bacteria harvested from cultures in the early logarithmic phase (Fig. 3, 5) and bacteria grown in continuous culture at dilution rate 0.120 h^{-1} (Fig. 4) showed the presence of 'blebs' on the surface. It is suggested that these produce the particles which are obtained when the lipolytic activity is isolated and purified. The blebs were not associated with degenerated bacteria; on the contrary they were most frequently found attached to the area of the cross-wall in dividing bacteria.

Thin sections of *Anaerovibrio lipolytica* were examined to look for possible involvement of mesosomal structures in the secretion of lipase (Fig. 6). However, in a large number of studies no structures were observed in the bacterial membranes which correlated with the 'blebs' observed in examination of whole bacteria. Ryter & Jacob (1966) indicated that the mesosomes of Gram-negative bacteria were very difficult to observe.

In conclusion we believe that the lipase of *Anaerovibrio lipolytica* is associated with membranous material of similar phospholipid composition to that of the whole bacteria and that the enzyme is a truly extracellular enzyme, being extruded, by an unknown mechanism, from bacteria which do not lose their structural integrity.

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Fig. 1. Negatively stained preparation of enzyme purified by gel filtration on Sephadex G 200. The marker bar in each photograph is 1 μ m.

Fig. 2. Negatively stained preparation of enzyme purified by ultracentrifugation in sucrose density gradient. Sucrose was removed by dialysis against distilled water prior to staining.

Fig. 3. Negatively stained *Anaerovibrio lipolytica* harvested in the early stages of the phase of logarithmic growth in a batch culture.

Fig. 4. Negatively stained *Anaerovibrio lipolytica* from a carbon-limited continuous culture growing at a dilution rate of 0.120 h⁻¹.

Fig. 5. Metal shadowed *Anaerovibrio lipolytica* harvested in the early stages of the phase of logarithmic growth in a batch culture.

Fig. 6. Thin section of *Anaerovibrio lipolytica* harvested in the early stages of the phase of logarithmic growth in a batch culture.