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Identification of lipolytic activity in a multitrophic population grown in wool–scour effluent

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SUMMARY

A multitrophic population established in wool–scour effluent produced esterase activity with specificity to steryl, wax and triacylglyceryl esters. The wax esterase activity peaked just prior to the esterase activity toward cholesteryl oleate and *p*-nitrophenol palmitate. The activity was present in both the extracellular and cell membrane fractions. Partial characterization revealed the presence of multiple esterase bands on native polyacrylamide gels. The bacterial esterase activity could be enhanced by the addition of mineral salts.

INTRODUCTION

Wool–scour effluent is the waste obtained after aqueous cleaning of raw wool. It contains emulsified wool grease, detergent, suint (sweat-salts from sheep), proteinaceous material, dirt and water and thus has a high COD level. After scouring of wool the scour liquor is centrifuged and less than half the wool grease is recovered for the preparation of lanolin and lubricants. The scour effluent still contains high levels of grease which is the major intractable component for biodegradation; it is a complex mixture of lipids composed primarily of wax esters and steryl esters of often branched and hydroxylated long-chain fatty acids and does not contain triglycerides [15].

One of the possible first steps in the biodegradation of wool grease may involve the hydrolysis of ester bonds and thus the involvement of esterases. This step may also be rate limiting for further biodegradation.

Steryl ester hydrolases which hydrolyse ester linkages in cholesteryl esters, such as cholesteryl oleate have been identified in both mammalian and microbial systems [13,14]. Wax ester hydrolases catalyse the hydrolysis of the ester bond in wax esters. Only a few sources of such enzymes have been identified; these include some plants [11] and marine organisms [4]. Limited information is available concerning microbial sources of wax esterases. A commercially available lipase produced by *Candida rugosa* was shown to possess wax esterase activity [5] and to contain several esterases of broad substrate specificity [3].

This study examines the production and substrate specificity of lipolytic activity produced by a multitrophic population of micro-organisms found previously [10] to be able to degrade wool grease and wool–scour effluent.

MATERIALS AND METHODS

Stock culture

A mixed microbial population was established in a chemostat running on dilute wool–scour effluent [10]. Bacterial strains were isolated by plating on horse blood agar [8] modified by the reduction of the NaCl content by half and by the addition of a trace metal mixture [10]. The sterile defibrinated horse blood was purchased from the Geelong Hospital Pathology Department. The mixed population was diluted in sterile phosphate-buffered saline, plated and colonies with different morphology, size or colour selected, streaked out and subcultured 10 times before testing. This population served as the starting point for a series of experiments reported here. Cultures devoid of protozoan were prepared by filtration using multiple layers of GFC filters and using only the first few ml of filtrate to prepare the stock culture.

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Media and culture conditions

An aliquot of stock culture (20 ml) was centrifuged (500 × *g*, 10 min) and the pellet washed twice with 20 ml of sterile water. After resuspension of the pellet in 20 ml sterile water it was added to an Erlenmeyer flask containing 300 ml of the following (per litre of water): NH_4NO_3, 1 g; $Na_2HPO_4 \cdot 2H_2O$, 3 g; KH_2PO_4, 1 g; $FeSO_4 \cdot 7H_2O$, 5 mg; $ZnCl_2$, 2 mg; $MgSO_4$, 0.2 g; $CaCl_2 \cdot 2H_2O$, 10 mg; $NaMoO_4 \cdot 2H_2O$, 2 mg; $CoCl_2 \cdot 4H_2O$, 2 mg; $MnCl_2 \cdot 4H_2O$, 2 mg; and 100–300 ml sterile (121 °C, 20 min) wool–scour effluent. Cultures were aerated by bubbling air through the medium and were incubated at room temperature (approx. 22 °C).

Assays

The following assays were carried out as previously described [4]: hydrolytic activities toward *p*-nitrophenyl acetate and *p*-nitrophenyl palmitate were determined spectrophotometrically at $A_{400\,nm}$, hydrolytic activity to α-naphthyl acetate was determined by spectroscopy at $A_{510\,nm}$ where released α-naphthol was coupled to the dye Fast Garnet. Tributyrin acylhydrolase activity was estimated with a pH–stat using tributyrin as substrate and cholesteryl esterase activity was determined using cholesteryl [1-^{14}C] oleate (Amersham) as substrate. The radioactively labelled wax ester, cetyl[1-^{14}C]palmitate (0.85 MBq/mmol) (hexadecyl[1-^{14}C]hexadecanoate) was synthesized and prepared for use as previously described [3,5]. Activities were expressed in units/ml where one unit is equivalent to one μmol of *p*-nitrophenyl or fatty acid equivalents released per min. Results are the average of triplicate determinations. Assays were performed on the supernatants (extracellular fraction) (centrifuged 11600 × *g*, 10 min) of an aliquot of culture media. For examination of the cellular location of the esterase activity, the pellet from 10 ml of culture was resuspended in 1 ml of H_2O, sonicated for 30 s, freeze-thawed twice and then centrifuged. The supernatant was referred to as the intracellular fraction. The pellet was then resuspended in 1 ml of 0.1% Triton X-100, sonicated for 30 s, freeze-thawed twice and centrifuged. The resultant supernatant was referred to as the cell membrane fraction. Protein was estimated by either the Bradford [2] or the Lowry et al. methods [9].

Polyacrylamide gel electrophoresis

Electrophoresis was performed as previously described [5]. Native gels were examined for enzyme activity by coupling of the hydrolysis products of α-naphthyl acetate to the dye Fast Garnet. Denaturing gels (SDS) were examined for protein by silver staining according to the method of Morrissey [12].

Thin-layer chromatography of culture lipids

Samples from 0, 24, 48 and 72 h were centrifuged (66000 × *g*, 15 min) and to 1.2 ml of the supernatant 7.5 ml of chloroform/methanol/H_2O (3 : 1 : 1, v/v/v) was added. The mixture was vortexed and shaken for one min and then centrifuged (1000 × *g*, 10 min). The upper aqueous phase was removed and the lower solvent phase was retained. The chloroform was evaporated off under N_2 and the residue resolubilized in 0.5 ml of chloroform/methanol (2 : 1, v/v). 20 μl was spotted onto a HPTLC (Merck), Silica Gel 60 F_{254} plate 10 × 10 cm with a concentrating zone and developed in heptane/ether/acetic acid (70 : 30 : 1, v/v/v). The plate was dried under N_2, dipped in 10% $CuSO_4$, 8% phosphoric acid for 30 s [1], drained, dried under N_2, heated to 140 °C for 5 min to reveal sterols and steryl esters. Further heating for one h led to charring of all lipid components.

Twenty μl of the following standards was also spotted: cholesterol (1 mg/ml; Sigma), lanosterol (1 mg/ml; Sigma), stearyl laurate (1 mg/ml) prepared as previously described [11], wool grease (5 mg/ml) from solvent scoured wool, wool grease steryl esters of non-hydroxylated acids and of hydroxylated acids (6 mg/ml) prepared by reverse phase HPLC.

RESULTS

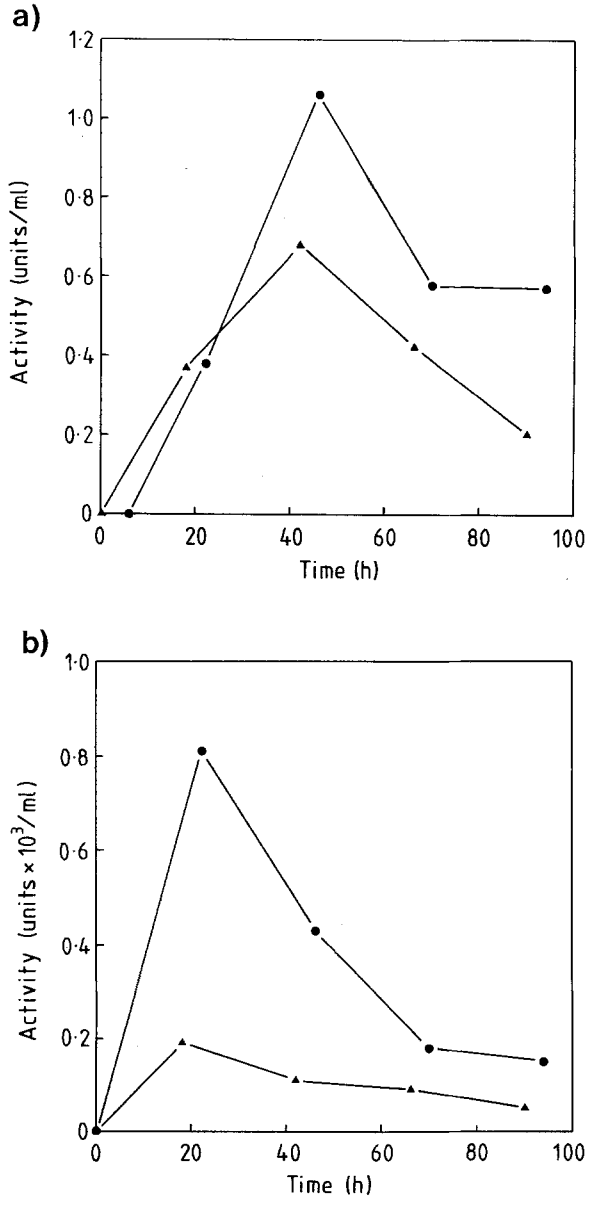
Description of the microbial population

The mixed microbial population was initially established in an air-lift tube continuous culture chemostat running on very dilute wool–scour effluent [10]. It consisted of multiple bacterial species as well as a single stalked-ciliate identified as *Opercularia coarctata* [6]. There were 11 initial isolates of which only eight proved different. Three strains were identified using standard tests and methods [7]. The rest did not readily fall into any taxonomic or identification scheme [7,8]. All isolates were Gram-negative [7]. An aliquot of the mixed population was fixed and Gram-stained, no Gram-positive bacterial strains were observed. Of the eight strains tested for hydrolytic activity toward *p*-nitrophenyl palmitate two strains, an *Acinetobacter calcoaceticus* and a *Pseudomonas aeruginosa* were found to produce detectable esterase activity in culture supernatants (Brahimi-Horn et al., manuscript in review). The individual strains showed similar growth patterns in wool–scour effluent cultures, entering stationary phase at similar times. There were however, differences in the actual growth or final titers at stationary phase.

General esterase activities

Duplicate cultures of the mixed population in scour effluent, culture 1 and 2, were set up and the supernatant

examined at different times for hydrolytic activity. The esterase activity (*p*-nitrophenyl palmitate) peaked at around 48 h after culture inoculation (Fig. 1a). The wax esterase activity (cetyl[^{14}C]palmitate) (Fig. 1b) peaked slightly earlier at around 24 h and declined after 42 h. The cholesterol esterase activity (Fig. 1c) also peaked later, at around 48 h, and remained stable for at least 72 h. Activity toward *p*-nitrophenyl acetate was also monitored but no activity was detected. Some variation in both the level of the esterase activity and the peak time for esterase production was observed between different cultures and with different batches of scour effluent. A low level (0.02 units/ml) of hydrolytic activity toward α-naphthyl acetate was also detected, peaking between 24 and 48 h. At 46 h,



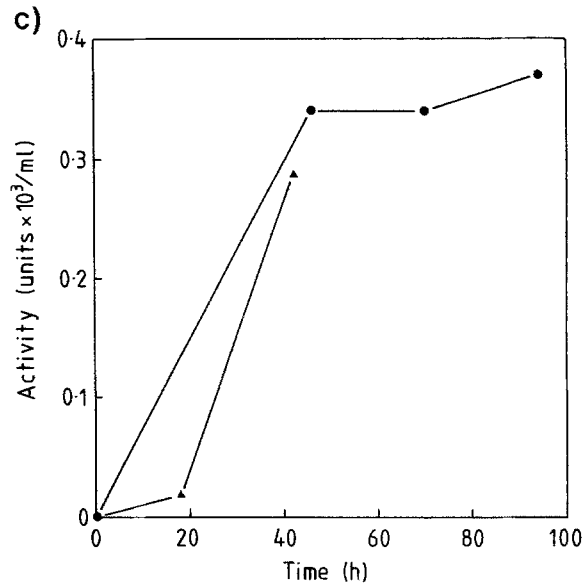


Fig. 1. Time course for the production of extracellular esterase activity toward: (a) *p*-nitrophenyl palmitate; (b) cetyl palmitate; and (c) cholesteryl oleate. Results for two individual batch cultures are presented, culture 1 (▲) and culture 2 (●).

approximately 4.0 units/ml of tributyrin acylhydrolase activity were detected. Culture 1 showed less activity to all substrates but in particular to the wax ester. The starting protein concentration in the culture supernatant was approximately 0.6 mg/ml and declined linearly to approximately 0.3 mg/ml and stabilized at this level for at least 90 h.

Cellular localisation

Esterase activity to *p*-nitrophenyl palmitate was located in all cellular fractions (Fig. 2). The ratio of extracellular activity to cell membrane activity was greater than 1.0 early in the culture while at later times the ratio was approximately 1.0. The cell membrane fraction would also have contained enzyme strongly bound to suspended solids.

Partial esterase characterization

Polyacrylamide gel electrophoresis showed the presence of numerous protein bands in all fractions (Fig. 3a). A major band at 25 kD was found in all fractions while bands between 90 and 100 kD were predominantly associated with the cell membrane fraction and a band of 19 kD was prominent in the extracellular medium.

Electrophoresis under native conditions and staining for esterase activity with α-naphthyl acetate indicated the presence of several esterases of different mobility (Fig. 3b). Most species were present in all fractions except for band 1 which was absent from the cell membrane fraction.

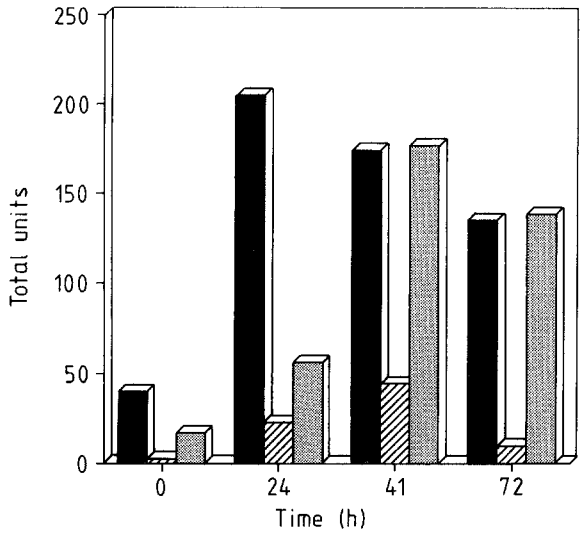


Fig. 2. Cellular location of esterase activity to *p*-nitrophenol palmitate of a 41 h culture. Extracellular (■), intracellular (▨), and cell membrane (▩) fractions.

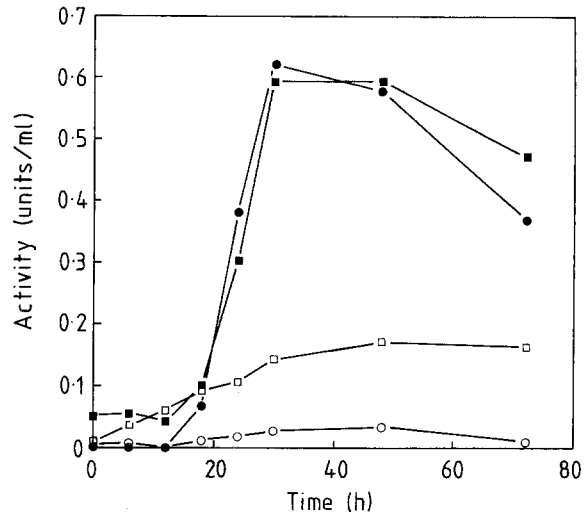


Fig. 4. Time occurs for the production of esterase activity by the mixed microbial culture including (●,○) or excluding (■,□) protozoa, grown in the presence (■,●) or absence (□,○) of mineral salts.

Source of esterase and effect of mineral salts on production

The production of esterase with time was the same when cultures devoid of protozoa were examined (Fig. 4). This suggests that the source of the esterase activity lies with the bacteria rather than with the protozoa. The addition of mineral salts to the culture medium enhanced

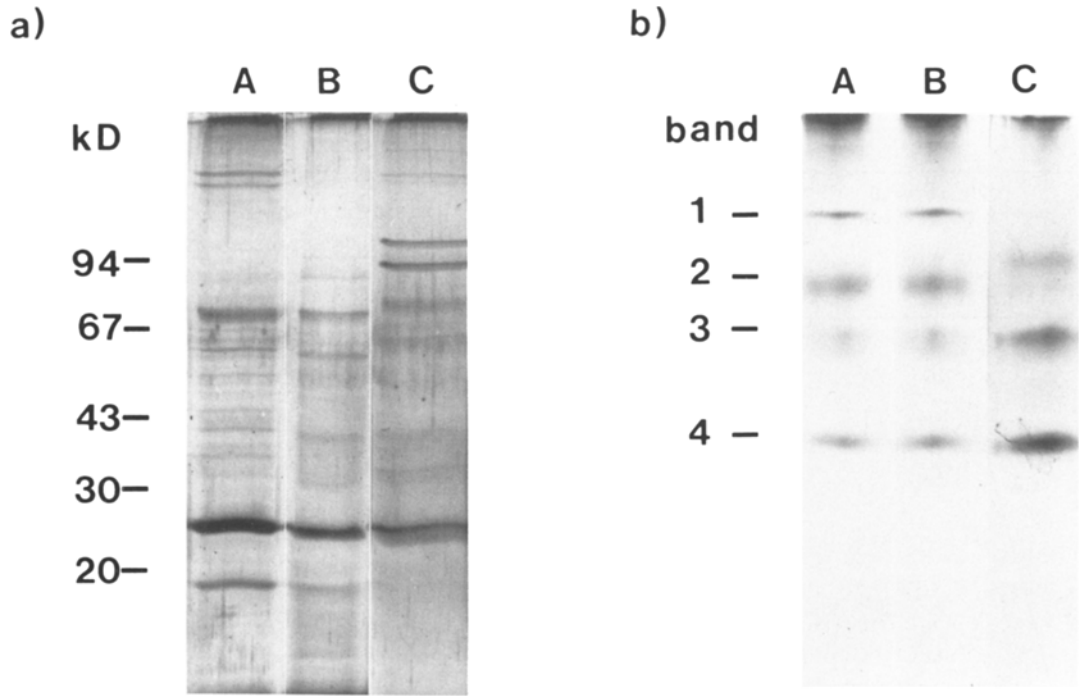


Fig. 3. Polyacrylamide gel electrophoresis of cellular fractions from a 41 h culture. (a) Samples were run under SDS denaturing conditions and examined for protein; 0.04, 0.02 and 0.10 μg of extracellular, intracellular and cell membrane protein was loaded. (b) Samples were run under native conditions and examined for esterase activity; 0.04, 0.53 and 0.83 μg of extracellular, intracellular and cell membrane protein was loaded. 15 μl of sample was applied: A, extracellular; B, intracellular; and C, cell membrane fractions.

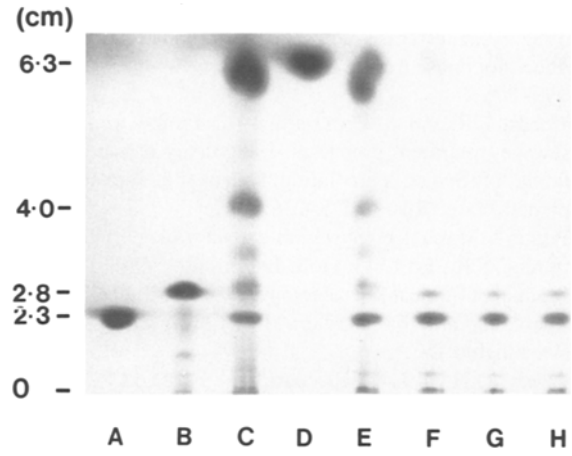


Fig. 5. Thin-layer chromatography of sterols and steryl esters in culture supernatants during incubation. The following standards: A, cholesterol; B, lanosterol; C, wool grease; D, cholesteryl laurate; and extracted culture supernatants from 0, 24, 48 and 72 h cultures; E, F, G and H, respectively, were applied. Migration distances (cm) from the origin are given.

the production of enzyme (Fig. 4), although the overall bacterial titres were the same under both conditions. Addition of mineral salts also leads to considerably greater reductions in the grease content, COD and suspended solids [10].

Wool grease degradation

Wool grease steryl and wax esters disappeared from the supernatant within 24 h of incubation of the microbial population in the presence of wool–scour effluent. Fig. 5 shows a thin-layer chromatogram stained for sterols and sterol esters. The hydroxylated and the non-hydroxylated steryl esters in wool grease show migration distances from the origin of 6.3 and 4.0 cm, respectively, while cholesterol and lanosterol give values of 2.3 and 2.8, respectively. After charring of the plate for a further hour to reveal all lipid components it appeared that only cholesterol and lanosterol predominated in the culture supernatant after 24 h.

DISCUSSION

The detection of cholesterol and wax esterase activities in a multitrophic population of micro-organisms growing on wool–scour effluent together with the observed decrease in the grease content and COD [10] suggests that esterases play a major role in the biodegradation of wool grease components. The detection of tributyrin acyl hydrolase activity is unexpected since wool grease does not contain triacylglycerols. This activity however, may be associated with the esterase activities to give esterases with broad substrate specificities. Alternatively, activity may be induced by the presence of lipids other than triglycerides. The variable peak times for the wax, cholesterol and *p*-nitrophenyl palmitate activities suggest that esterases of different substrate specificities may be present at different stages in the culture. This may be the result of different microbial species predominating at different times and producing different esterases. Some variation in the level of esterase produced and the time for optimal production was observed between cultures. This variation was not due to variations in the amount or make-up of the scour effluent or bacterial inoculum or to different batches of substrate since both cultures were set-up and assayed in parallel. For this reason results for individual cultures rather than averages are presented.

Hydrolytic activity to two short chain esters α-naphthyl acetate and *p*-nitrophenyl acetate was also monitored. Low levels of activity were detected with the former but no activity or very low levels were detected with the latter. In early cultures the esterase activity was predominantly extracellular while in older cultures a large proportion of the activity was found associated with the cell membrane fraction. The terms extracellular, intracellular and cell membrane as defined by the methodology are employed loosely since the presence of extracellular enzyme tightly bound to suspended solids arising from bioflocculation could not be excluded. Such enzyme would precipitate with the first pellet fraction and probably be detected in the so-called cell membrane fraction after solubilization in Triton X-100. Alternatively, in older cultures the synthesized enzyme is not secreted to the same extent or different enzymes are present. Preliminary studies suggest that the bacteria, and not the protozoa, in the multitrophic population are responsible for the synthesis of esterase activity. The optimal conditions for the production of esterase activity as well as the partial purification and characterization of the esterases produced by the individual bacteria should provide further insight into the biodegradation of the lipid components (Brahimi-Horn et al., manuscript in review).

The observation that addition of mineral salts enhanced the production of esterase suggests either an induction of certain enzymes, the stimulation of the production of an activating substance, removal or inactivation of an inhibiting substance, a stabilizing effect on produced enzyme or a selective encouragement of the growth of the esterase producing bacteria over non-producers.

The disappearance of the steryl and wax esters from the culture supernatant after 24 h, as shown by thin-layer chromatography, was due to a combination of ester hydrolysis and absorption onto biomass flocs during emulsion destabilization. A detailed analysis of total wool

grease breakdown products and intermediates is in progress (Mickelson et al., manuscript in preparation).

Identification of the enzymes involved in biodegradation of components of an effluent and the examination of their production should lead to a better understanding of treatment processes and thus improvement to such processes. The isolation of the major enzymes responsible may also lead to alternative biocatalyst-based treatments.

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