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Purification, Characterization, and Lytic Activity against *Naegleria fowleri* of Two Amoebicins Produced by *Bacillus licheniformis* A12

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***Bacillus licheniformis* A12 produces two amoebolytic substances (amoebicins A12-A and A12-B) in liquid media during sporulation. Both substances have been purified and characterized. They are heat- and protease-resistant peptides containing aspartic acid, glutamic acid, serine, proline, and tyrosine in a molar ratio of 5:2:2:2:2. No fatty acids or carbohydrates have been detected. Their molecular weight is 1,430. Purified amoebicins A12-A and A12-B exhibit amoebolytic action against *Naegleria fowleri*. They also exhibit antibiotic action against yeasts (*Saccharomyces heterogenicus* and *Cryptococcus neoformans*) and several fungal species (*Aspergillus niger*, *Microsporum canis*, *Mucor plumbeus*, and *Trychophyton mentagrophytes*). Their antibacterial spectrum appears to be restricted to *Bacillus megaterium*, *Corynebacterium glutamicum*, and *Sarcina* sp.**

The free-living amoeba *Naegleria fowleri* is the causative agent of primary amoebic meningoencephalitis, a fulminant and usually fatal illness affecting the central nervous system (7, 23). *N. fowleri* is ubiquitous and has been isolated from fresh or brackish warm waters and artificially heated aquatic environments worldwide (21, 30, 32–34). Most cases occur during the summer in patients with a recent history of swimming in water contaminated with this protozoan. The finding of pathogenic free-living amoebae in chlorinated domestic and swimming waters (9, 13, 15) has led to an expression of concern by public health authorities over the possible contraction of primary amoebic meningoencephalitis via these waters.

Also, because of the rapid time course and high mortality rate of primary amoebic meningoencephalitis, there is a need to establish a quick, effective treatment. Unfortunately, there is no safe, reliable chemotherapeutic remedy yet. A very few cases have been treated successfully with amphotericin B, alone or associated with miconazole and rifamycin (29). Since amphotericin B is toxic and unable to penetrate the brain-blood barrier, it must be administered by intraventricular or intrathecal means in order to produce a satisfactory therapeutic response (29). Many other chemotherapeutic agents have been tested on *N. fowleri*; some of these agents have an effect in vitro but not in vivo, while others are effective only at concentrations that are toxic to humans (29).

We have screened a number of bacteria living in the same habitat as *N. fowleri* to determine whether any of them produce a substance antagonistic to this organism, and we have found three strains of *Bacillus licheniformis* which exhibit a marked amoebolytic effect against it. The purification, characterization, and spectrum of inhibition of two amoebicidal substances produced by *B. licheniformis* A12 are discussed in this paper.

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MATERIALS AND METHODS

Microorganisms and culture conditions. Producer strain *B. licheniformis* A12 was isolated from a cave containing thermal vents in Cueva de los Murciélagos, Murcia, Spain, and was identified in our laboratory by using the method of Norris et al. (26). Other microbial strains used to establish a spectrum of inhibition are listed in Table 1. Bacterial strains were cultivated in brain heart infusion broth (BBL, Cockeysville, Md.) or Luria broth (BBL). Fungal strains were grown on CM broth (1% glucose, 0.5% yeast extract, 0.5% malt extract) or CM agar. *N. fowleri* S-3 (= ATCC 30809) and *Acanthamoeba* sp. strain Gr-1 (both supplied by C. Mascaró, Department of Parasitology, University of Granada, Granada, Spain) were cultivated at 37°C in Nelson medium (3, 24) or at 28°C in CGV medium (35). *N. fowleri* HB-1 (= ATCC 30174) (supplied by S. Kilvington, Public Health Laboratory, Royal United Hospital, Bath, United Kingdom) was grown on Cerva's medium (8) at 37°C. This strain was used as indicator throughout this study. *Naegleria gruberi* CCAP 1516/1e and *Naegleria lovaniensis* Aq/9/1/45D were supplied by P. Alonso, Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas, Madrid, Spain) and were maintained at 28°C in SCGYEM medium (14). Amoebae were cultivated axenically in 25-cm^2 Nunclon tissue culture flasks (Nunc, Roskilde, Denmark) containing 5-ml portions of the appropriate medium and were subcultured on fresh medium every 72 h.

Growth conditions for amoebicidal activity production. Flasks containing glucose mineral base broth (12), Luria broth, tryptic soya broth, or brain heart infusion broth dissolved in distilled water or in 100 mM sodium phosphate buffer (pH 7.2) were inoculated with an overnight culture of the producer strain (4%, vol/vol). Incubation was carried out at 28 or 37°C with or without shaking. Aliquots (5 ml) were removed at regular intervals to make various determinations. Cell growth was estimated by measuring the optical density at 630 nm with a Spectronic 20 spectrophotometer. The number of viable cells was determined by plating serial dilutions on brain heart infusion agar. The number of spores was determined as described elsewhere (27). Samples of the culture were centrifuged at 5,000 × *g* for 20 min, and the

TABLE 1. Spectrum of inhibition for purified amoebicins[a]

Organism(s)	Titer of activity (AU/μg of protein)	
	Amoebicin A12-A	Amoebicin A12-B
Acanthamoeba sp. strain Gr-1	<0.02	<0.02
N. fowleri S-3 (= ATCC 30809)	15.42	17.23
N. fowleri HB-1 (= ATCC 30174)	8.33	10.55
N. lovaniensis Aq/9/1/45D	3.20	4.50
N. gruberi CCAP 1516/1e	2.85	3.30
Candida albicans CECT 1394	<0.02	<0.02
Cryptococcus neoformans CECT 1075	13.50	18.18
Saccharomyces heterogenicus	2.50	3.63
Aspergillus niger CECT 2089	2.45	3.63
Microsporum canis CECT 2797	3.50	4.38
Mucor mucedo CECT 2653	<0.02	<0.02
Mucor plumbeus CCM F443	2.50	3.63
Sporothrix schenchii CECT 2799	<0.02	<0.02
Trychophyton mentagrophytes CECT 2793	2.80	3.63
B. megaterium	4.50	6.82
Corynebacterium glutamicum CECT 78	28.40	36.30
Sarcina sp.	10.25	18.22
Other bacterial species[b]	<0.02	<0.02

[a] Preparations containing known protein concentrations were serially diluted and tested for activity as described in Materials and Methods.

[b] The other bacteria tested, which were resistant to the amoebicins, were *Alcaligenes faecalis* CCM 1052, *Bacillus cereus* CECT 131, *Bacillus circulans* CCM 2084, *Bacillus laterosporus* CCM 1612, *B. licheniformis* A12 (the producer strain), *B. licheniformis* D13, *B. licheniformis* M4, *Bacillus subtilis*, *Enterobacter cloacae* CECT 194, *Enterococcus faecalis* S-47, *Enterococcus faecium* S-29 and CECT 410, *Enterococcus durans* CECT 411, *Escherichia coli* Delbruck, *Klebsiella pneumoniae* M5a1, *Micrococcus luteus*, *Mycobacterium phlei*, *Planococcus citreus* CCM 316, *Proteus* sp., *Pseudomonas reptilivora* N5, *Salmonella typhimurium* SV3, and *Staphylococcus aureus* ATCC 8.

supernatants were filtered through 0.45-μm-pore-size filters (Millipore) before they were tested for amoebicidal activity.

Assay of amoebicidal, antibacterial, and antifungal activities. Amoebicidal activity assays were carried out in 96-well flat-bottom microtiter plates (diameter, 6.4 mm; Sterilin Ltd., Hounslow, United Kingdom) essentially as described elsewhere (22). About 6×10^3 amoebae (6-h-old trophozoites) were placed in each well, and the volume was brought to 150 μl with fresh medium. The plates were incubated at 37°C for 20 min to allow the amoebae to adhere to the bottom, and then 10-μl portions of different dilutions of the sample to be tested were added to each well. The preparations were examined at regular intervals with an inverted microscope (Carl Zeiss, Jena, Germany). Growth of amoebae was monitored by cell counting with a hemocytometer.

Antibacterial and antifungal activities were assayed by the agar spot test (19). Briefly, about 3×10^7 CFU of an exponential-phase culture of the strain being tested was mixed with 4 ml of melted brain heart infusion agar or CM agar, and the preparation was poured onto the surface of a brain heart infusion agar or CM agar plate. Standard amounts (10 μl) of serial dilutions of the sample to be tested were carefully placed on the seeded plate with a micropipette. After adequate incubation, the plates were examined for halos of inhibited growth.

One arbitrary unit (AU) was defined as the highest dilution which produced a clearly visible area of inhibited growth on a lawn seeded with bacteria, yeast cells, or fungi or produced lysis of ca. 50% of the amoebae placed in a well. The reciprocal of the dilution gave the titer of activity in arbitrary units per milliliter.

Purification and estimation of the molecular weight of the active components. Crude supernatants were mixed with methanol (30%, vol/vol) and applied to a column (1 by 20 cm) filled with solid-phase extraction bulk packing (Supelclean LC-8; Supelco Inc., Bellefonte, Pa.). The gel was washed with 2 column volumes of methanol-water (3:7), and the retained material was further eluted with 2 volumes of methanol-acetonitrile (75:25, vol/vol). The eluate was lyophilized and redissolved in methanol-water (3:7) to 1:10 of the initial supernatant volume. Aliquots of this concentrate (10 ml) were applied to a Supelcosil LC-8 column (1 by 30 cm; Supelco) equilibrated with 50 mM ammonium acetate (pH 6.7) (solvent A). The retained material was first washed with 3 column volumes of solvent A and then eluted with the following two linear gradients of solvent B (50 mM ammonium acetate [pH 6.7] in methanol-acetonitrile-water [78:20:2]): 0 to 30% for 3 min and then 30 to 100% for 27 min (flow rate, 2.5 ml/min). The fractions collected were lyophilized, redissolved in 2 ml of methanol-water (3:7), and tested for inhibitory activity. Fractions from this column containing amoebicidal activity were loaded onto a Vydac 218TP54 column (4.6 by 25 mm; The Separation Group, Hesperia, Calif.) equilibrated with 10 mM trifluoroacetic acid. This column was eluted with two combined linear gradients (0 to 60% for 3 min and then 60 to 100% for 12 min) of isopropyl alcohol-acetonitrile (2:1) in 4 mM trifluoroacetic acid at a flow rate of 1 ml/min. The eluted fractions were lyophilized and tested for amoebicidal activity as described above.

The molecular weight of the purified amoebicins was estimated by gel filtration chromatography on a column (1 by 60 cm) packed with Bio-Gel P-4 (Bio-Rad, Hertfordshire, United Kingdom). The column was eluted with 100 mM ammonium bicarbonate–0.15% sodium dodecyl sulfate at a flow rate of 20 ml/h. Blue dextran (molecular weight, 2×10^6), the insulin β-chain (molecular weight, 3,400), bacitracin (molecular weight, 1,400), and polymixin B (molecular weight, 1,280) were used as markers.

Physicochemical analyses. Protein was determined as described by Bradford (4). Amino sugars were determined by a modified Morgan-Elson reaction (17) after hydrolysis in 3 N HCl for 4 h at 95°C. Phosphorus (2) and neutral sugar contents (16) were also determined. Lipids were determined with a Total Lipids test (Boehringer Mannheim, Barcelona, Spain). The optical absorbance spectrum of the purified substances was recorded with a Beckman model UV/VIS DU-70 spectrophotometer. Aliquots (ca. 10^{-3} optical absorbance unit at 280 nm) were analyzed for their amino acid compositions as described by Gimenez-Gallego and Thomas (18), and the protein content was correlated with the recorded absorbance. The N-terminal amino acid sequence was determined by modified Edman degradation (11).

A fatty acid analysis was carried out essentially as described elsewhere (1). The purified substances were hydrolyzed in 6 N HCl for 12 h at 120°C in vacuum-sealed ampoules under nitrogen. The hydrolysate was extracted with chloroform, lyophilized, and methylated with an excess of diazomethane in ether. The resulting fatty acid methyl esters were analyzed by gas chromatography-mass spectrometry with a Hewlett-Packard model HP5890 gas chromatograph equipped with a methyl-silicone capillary column (0.2 mm by 25 m) connected to a Hewlett-Packard model HP5988A mass spectrograph. The gas chromatograph was operated by using an injector temperature of 270°C and a column temperature gradient from 80 to 320°C over a period of 24 min, using helium as the carrier gas. The mass

spectrograph was operated in the electronic ionization mode at 70 eV, with a source temperature of 200°C.

The pH and heat stability of the amoebicidal substances were tested. Active samples were heated at pH 7.0 for 30 min at 50, 70, and 100°C and then tested for activity. Sample solutions were adjusted to pH 2.5 or 9.5, incubated for 30 min at room temperature, and then tested for remaining activity after the pH was readjusted to neutrality and the volumes were compensated.

Samples containing amoebicidal activity were incubated separately with trypsin, pronase, proteinase K, lipase, β-glucosidase, β-glucuronidase, and lysozyme (1 mg/ml each, in 50 mM Tris-HCl [pH 7.2]). Carboxypeptidase A (100 μg/ml, in 200 mM ammonium bicarbonate [pH 6.0]) and alkaline phosphatase (1 mg/ml, in 50 mM Tris-HCl [pH 8.5]–20 mM magnesium sulfate) were also tested. Most incubations with enzymes were carried out at 37°C for 2 h; the exceptions were the incubations with pronase (28°C for 2 h) and lysozyme (28°C for 18 h). As controls, active samples were incubated under the conditions described above without the enzymes. After incubation, the samples were tested for remaining activity. All of the enzymes were purchased from Sigma.

Electron microscopy examination of amoebae. Samples were prepared for electron microscopy as described elsewhere (5), with minor modifications. Aliquots (25 μl) from an exponentially growing culture were deposited onto coverslips (diameter, 4 mm), and the preparations were incubated at 37°C for 12 h to allow the cells to adhere to the glass. The amoebae were then treated with purified amoebicidal substances for different periods of time, washed with phosphate saline buffer (pH 7.0) to remove the culture medium, and fixed with 2% glutaraldehyde in 75 mM sodium phosphate buffer (pH 7.2) for 1 h. Any remaining fixative was removed by washing with ethanol. Dehydration was carried out by immersion in a graded ethanol series, and this was followed by lyophilization. After being dried off, the specimens were coated with a layer of gold by perpendicular ionic bombing for 2 min and then observed with a scanning electron microscope (model DSM 950; Zeiss).

RESULTS

Production of amoebicidal activity. The highest titers (65 AU/ml against *N. fowleri* HB-1 [= ATCC 30174]) were found in supernatants of cultures grown in buffered brain heart infusion broth for 72 h at 28°C with agitation. Therefore, this medium was used to produce amoebicidal activity. Supernatants from other buffered media contained much less activity. Cultures grown on glucose mineral base broth produced only 25 AU/ml after 24 h of incubation. The highest titers obtained for the rest of the media were 40 AU/ml in tryptic soya broth after 45 h of incubation and 45 AU/ml in Luria broth after 36 h of growth. Much lower production was obtained when the cultures were incubated without agitation. Likewise, production of amoebicidal activity was not satisfactory in cultures incubated at 37°C. The production of amoebicidal activity was also much lower if the growth media were dissolved in distilled water instead of phosphate buffer.

There was a correlation between sporulation and production of amoebicidal activity. The production of spores began when the cultures reached the stationary phase, and it stopped after 50 h of incubation (Fig. 1). Amoebicidal activity appeared in the culture supernatants late during the stationary phase of growth (36 h), and it increased to a titer of 50 AU/ml within the next 12 h. Thereafter, production continued slowly for another 24 h up to a maximum titer of 65 AU/ml (Fig. 1).

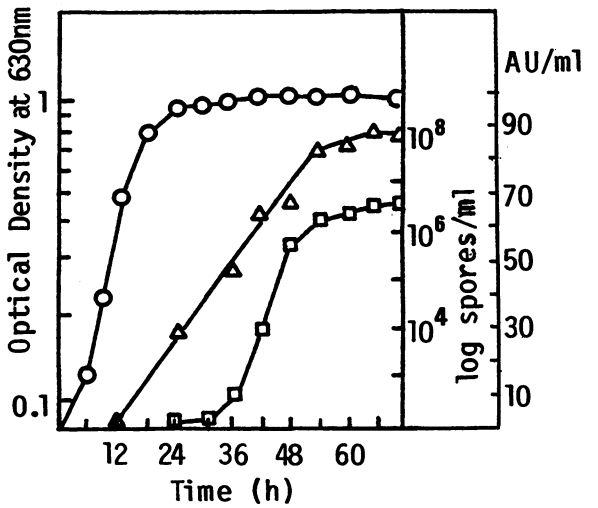

FIG. 1. Production of amoebicidal activity by *B. licheniformis* A12 grown in buffered brain heart infusion broth at 28°C with agitation. Growth of the producer strain was followed by measuring the optical density at 630 nm (○). Sporulation (△) and production of amoebicidal activity (expressed in arbitrary units per milliliter) (□) were also determined.

Purification of the amoebicidal compounds. Two amoebicidal substances were purified from 72-h culture supernatants by performing three steps of reversed-phase chromatography. The results are summarized in Table 2. Amoebicidal activity was retained in reversed-phase Supelclean LC-8 bulk packing with 30% methanol. The solvent was added directly to the supernatants before the preparations were applied to the column in order to prevent the adsorption of most of the components of the broth. The amoebicidal activity recovered after this step was 3.47-fold purified, and it represented 58.46% of the initial activity. The active concentrate obtained from this step was fractionated on a semipreparative reversed-phase high-performance liquid chromatography (RP-HPLC) column into several peaks. Components 1 and 2 exhibited amoebicidal activity (Fig. 2A). In addition, component 3 strongly inhibited *Bacillus megaterium*, but did not inhibit amoebae and therefore is not discussed further in this paper. The specific activities against *N. fowleri* HB-1 (= ATCC 30174) were 6.51 AU/μg of protein for component 1 and 7.69 AU/μg of protein for component 2.

Components 1 and 2 were further purified on an analytical RP-HPLC column, as described in Materials and Methods; each of them eluted as a single peak of activity, and in each case the elution position coincided with that of the UV light absorbance peak (Fig. 2B and C, respectively). The two pure compounds differed slightly in retention time; compound 2 was more hydrophobic and also more abundant. Components 1 and 2 are referred to below as amoebicins A12-A and A12-B, respectively. The specific activities of the pure amoebicins against *N. fowleri* HB-1 (= ATCC 30174) were 8.33 AU/μg of protein for amoebicin A12-A and 10.55 AU/μg of protein for amoebicin A12-B. Pure amoebicins A12-A and A12-B contained 12.30 and 23.8%, respectively, of the initial amoebicidal activity present in culture supernatants (Table 2).

TABLE 2. Purification of amoebicins A12-A and A12-B from 50-ml portions of 72-h culture supernatants of *B. licheniformis* A12 grown in buffered brain heart infusion broth at 28°C with agitation

Prepn	Total amt of protein (μg)	Total amt of activity (AU)[a]	Sp act (AU/μg of protein)	% Recovery[b]	Purification (fold)
Crude supernatant	12,500	3,250	0.26	100.00	1.00
Bulk Supelclean LC-8	2,100	1,900	0.90	58.46	3.47
Supelcosil LC-8					
Component 1	66	430	6.51	13.23	7.23
Component 2	104	800	7.69	24.61	8.54
Vydac 218TP54					
Amoebicin A12-A	48	400	8.33	12.30	32.00
Amoebicin A12-B	72	760	10.55	23.38	40.57

[a] Activity is expressed in arbitrary units against *N. fowleri* HB-1 (= ATCC 30174).
[b] Percentage of initial activity.

The molecular weight of the purified amoebicins was estimated by gel filtration on Bio-Gel P-4. The two amoebicins eluted at an identical volume, corresponding to a molecular weight of 1,600.

Chemical composition. Aliquots of the purified amoebicins containing 100 μg of protein each were used in various experiments to establish their chemical composition. Neither of the amoebicins contained any detectable amounts of phosphate, neutral sugars, amino sugars, or lipids. No fatty acids were detected by gas chromatography-mass spectrometry.

The two amoebicins had identical amino acid compositions (Table 3). Each of them contained only five different amino acids: aspartic acid, glutamic acid, serine, proline, and tyrosine. The molar ratio of aspartic acid to the other amino acids was 5 to 2. On the basis of their amino acid compositions both amoebicin A12-A and amoebicin A12-B have a molecular weight of 1,430. An amino acid sequence determination was attempted by manual Edman degradation. No amino acid derivatives were found after three consecutive cycles, suggesting that amoebicins A12-A and A12-B are either N terminal blocked or cyclic.

The values of extinction coefficients E_{210} and E_{280} for amoebicin A12-A were 14.66 and 2.23 $(mg/ml)^{-1} \cdot cm^{-1}$, respectively; the values of these extinction coefficients for amoebicin A12-B were 15.33 and 2.11 $(mg/ml)^{-1} \cdot cm^{-1}$, respectively. Neither of the amoebicins absorbed light at wavelengths greater than 400 nm.

Stability. Both amoebicins retained 100% of their activity after being heated at 100°C for 30 min at pH 7.0. They were also resistant to incubation at room temperature under acidic conditions (pH 2.5), but lost 75% of their activity upon incubation at pH 9.5 for 30 min. The crude supernatants, as well as the purified substances, retained 100% of their activity after storage for 1 month at 4°C or for 6 months at −20°C. Amoebicins A12-A and A12-B were resistant to the enzymes trypsin, pronase, proteinase K, alkaline phosphatase, lipase, lysozyme, β-glucosidase, and β-glucuronidase. They were also resistant to carboxy peptidase A, suggesting that a free carboxyl terminus was not present. None of the enzymes mentioned above had any visible effect on the test strain (*N. fowleri* HB-1 [= ATCC 30174]) at the final concentrations used in our experiments.

Antimicrobial spectrum. Preparations of the purified amoebicins were tested with different human pathogenic and nonpathogenic amoebae (Table 1). *N. fowleri* S-3 (= ATCC 30809) and HB-1 (= ATCC 30174) were the most sensitive pathogenic strains. Neither of the amoebicins showed any activity against *Acanthamoeba* sp. strain Gr-1 at final concentrations up to 50 μg/ml. The purified amoebicins were less active against the nonpathogenic strains *N. lovaniensis* Aq/9/1/45D and *N. gruberi* CCAP 1516/1e.

Since pure amoebicins A12-A and A12-B exhibit low solubility in water but are freely soluble in mixtures of water and polar solvents (30% methanol, 40% ethanol), the effect of solvent concentration on amoebae was also tested. We found that, while ethanol was less well tolerated, methanol did not have any visible effect on the morphology of amoebae or cell growth up to a concentration of 10% of the final culture volume. Nevertheless, we never exceeded a final concentration of methanol of 4% in the assays.

Both amoebicins had a narrow antibacterial spectrum (Table 1). *Corynebacterium glutamicum* CECT 78, *Sarcina* sp., and *B. megaterium* were the only species inhibited. The rest of the bacteria tested, as well as producer strain *B. licheniformis* A12, were resistant (Table 1). Among the fungal species tested, *Aspergillus niger* CECT 2089, *Mucor plumbeus* CCM F443, *Microsporum canis* CECT 2797, and *Trychophyton mentagrophytes* CECT 2793 were inhibited by amoebicins A12-A and A12-B. Other species (*Mucor mucedo* CECT 2653 and *Sporothrix schenckii* CECT 2799) were resistant. Several yeast species (*Saccharomyces heterogenicus* and *Cryptococcus neoformans* CECT 1075, a human pathogen) were also sensitive, while *Candida albicans* CECT 1394 was resistant (Table 1).

Amoebicidal and amoebolytic activities. The effect of the amoebicins on sensitive amoebae was easy to observe with

TABLE 3. Amino acid analysis of purified amoebicins[a]

Amino acid[b]	Amt in:			
	Amoebicin A12-A		Amoebicin A12-B	
	Observed	Integer	Observed	Integer
Asp	2.48	5	2.54	5
Glu	1.10	2	1.07	2
Ser	0.98	2	0.90	2
Pro	1.00	2	1.00	2
Tyr	1.05	2	0.99	2
Lan	ND[c]		ND	
Orn	ND		ND	
D-Abu	ND		ND	
DhAla	ND		ND	

[a] Amino acid compositions were determined as described in Materials and Methods.
[b] Lan, lanthionine; D-Abu, D-aminobutyric acid; DhAla, dehydroalanine.
[c] ND, not detected.

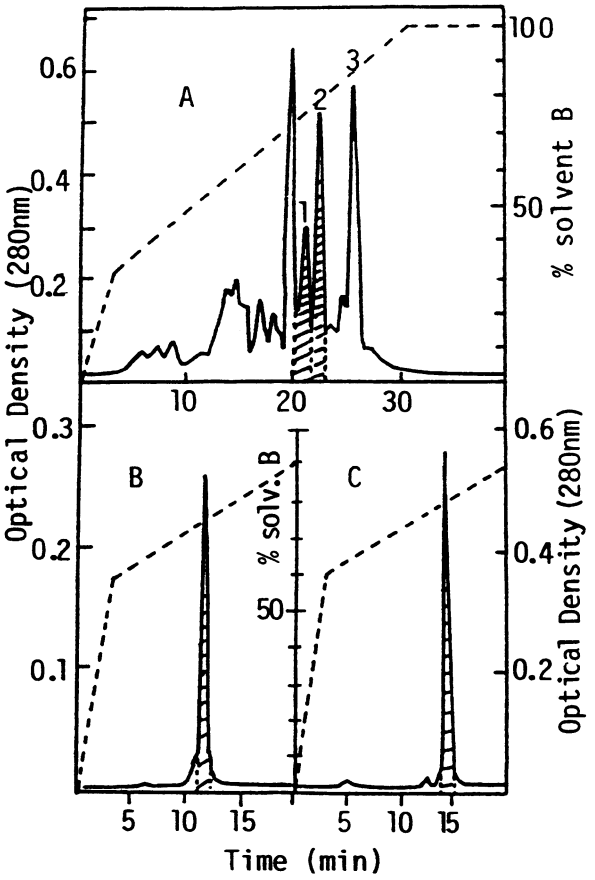

FIG. 2. Purification of the amoebicidal substances produced by *B. licheniformis* A12 by RP-HPLC. (A) Partially purified concentrated material from bulk packing Supelclean LC-8 was applied to a Supelcosil LC-8 column. The cross-hatched areas (peaks 1 and 2) represent the fractions containing amoebicidal activity. Fraction 3 was active against *B. megaterium*. (B) Fraction 1 was repurified on a Vydac 218TP54 column. The cross-hatched area represents the fraction containing amoebicin A12-A. (C) Fraction 2 from the experiment shown in panel A was resolved on a Vydac 218TP54 column. The cross-hatched area represents amoebicin A12-B. The dashed lines represent the percentages of solvent B. The solvents and elution conditions used are those described in Materials and Methods.

an inverted light microscope. The cells became rounded after 30 min of treatment and lost adherence to the wells. Complete lysis of the amoebae occurred within the following 15 min.

The amoebicidal effect of amoebicins A12-A and A12-B on *N. fowleri* HB-1 (= ATCC 30174) was quantified. Addition of either of the two compounds (final concentration, 1.5 AU/ml) caused a rapid decrease in the number of intact amoebae within the following 1 to 2 h (Fig. 3). The reduction in cell numbers caused by addition of amoebicin A12-B was somewhat more pronounced than the reduction in cell numbers caused by amoebicin A12-A at the same concentration.

The amoebolytic effect was studied by electron microscopy. Figure 4 shows the effect of amoebicin A12-B on *N. fowleri* HB-1 (= ATCC 30174). At 10 min after addition the characteristic shape of the cells changed. First they developed abnormal globular pseudopodia, and then they became rounded (Fig. 4B). After 30 min of incubation the cell

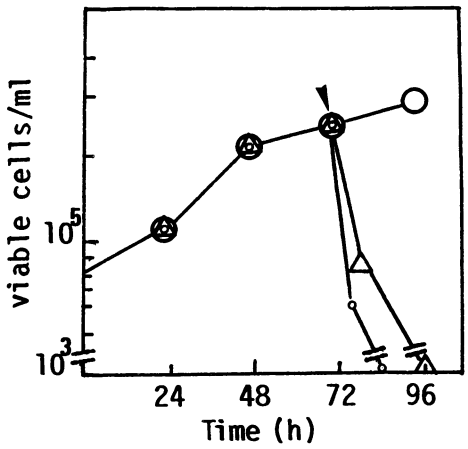

FIG. 3. Effect of purified amoebicins A12-A (△) and A12-B (○) on the number of cells in an axenic culture of *N. fowleri* HB-1 (= ATCC 30174). The amoeba was cultivated at 37°C in Cerva's medium (○). The arrowhead indicates the time of addition of amoebicins (final concentration of each, 1.5 AU/ml). The number of cells was counted with a hemocytometer after regular intervals of incubation.

membrane ruptured, with the release of abundant cytoplasmic material (Fig. 4C). All of this was followed by complete cellular destruction within 1 h. Identical effects were observed with amoebae treated with amoebicin A12-A (data not shown).

DISCUSSION

The most serious and yet unresolved problems concerning primary amoebic meningoencephalitis include the immediate identification of the etiological agent and an efficient therapeutic treatment (28). We chose therefore to evaluate new antiamoebic substances from bacterial sources, to establish the best conditions for production, and to isolate and characterize these substances.

B. licheniformis A12 releases substances with high amoebolytic activity into culture media during sporulation. This is consistent with the general observation that secondary metabolites are produced in response to nutrient deprivation. The culture supernatants of this strain seem to contain more than one type of antibiotic. Although we have focused on purification of the fractions containing relatively high antiamoebic activity, these fractions represent only 35% of the total fractions. Therefore, there may be other low-activity components that we have not detected in our assays. It is known that most antibiotics of the genus *Bacillus*, as well as other bacterial species, are produced as families of related substances with slight chemical differences, and this seems to be the case with amoebicins A12-A and A12-B.

The two amoebicins are small peptides with the same amino acid composition. The slightly different retention times obtained for the two substances by RP-HPLC indicate that there must be some small difference in their chemistry or structure, but we have not found any. The molecular weight calculated from the amino acid composition is 1,430, which is very close to the value obtained by gel filtration (1,600). The first value seems more accurate, since the elution profiles of small peptides may not correspond to their exact molecular weights. Amoebicins A12-A and A12-B differ in amino acid composition from other peptide antibi-

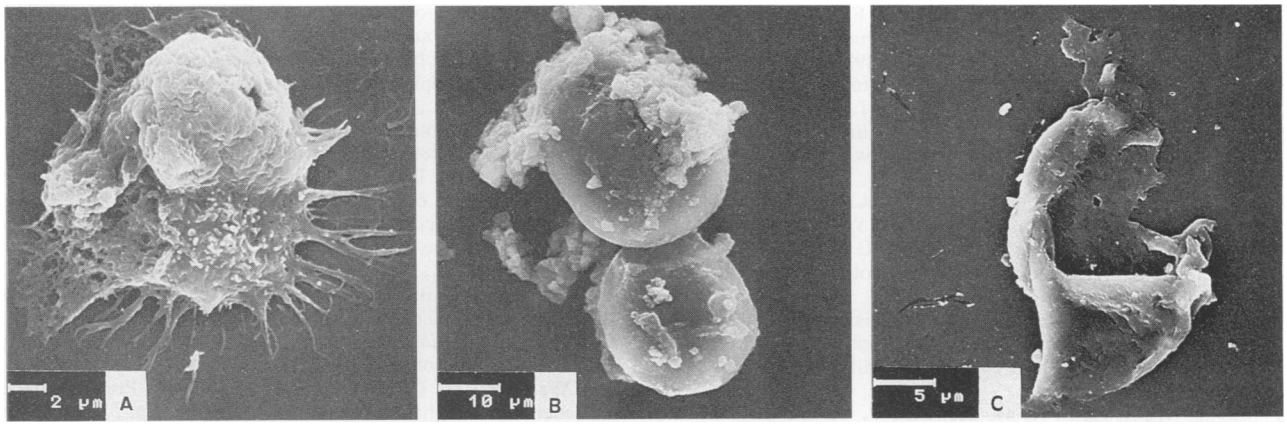

FIG. 4. Scanning electron micrographs of *N. fowleri* HB-1 (= ATCC 30174) treated for different periods of time with purified amoebicin A12-B. (A) Control. (B and C) Amoebae treated with 1 AU of amoebicin A12-B for 10 and 30 min, respectively.

otics produced by *B. licheniformis*, such as the licheniformins (6), bacitracin (20), and the phosphorus-containing triene proticin described in *B. licheniformis* subsp. *mesentericus* (25).

Of particular interest is the preferential action of amoebicins A12-A and A12-B against eukaryotic microorganisms, such as yeasts, fungi, and amoebae, and their narrow spectrum against bacteria. The fact that both amoebicins cause lysis of sensitive cells in a short period of time, together with scanning electron microscope observation of cell damage to amoebae, suggests that the primary site of action of the amoebicins is the cell membrane.

Also noteworthy are the different sensitivities of the various amoebae tested. Resistance of *Acanthamoeba* sp. is not surprising since this protozoan, especially its cysts, exhibits greater resistance to physical and chemical agents, such as chlorine-containing disinfectants (13) and amphotericin B (31), than *Naegleria* spp. Different *Naegleria* species also exhibit various levels of sensitivity to chemical drugs, as is the case in the higher level of resistance of *N. fowleri* to trimethroprim and other antifolates (10) or econazole (28) or its higher level of sensitivity to amphotericin B (28). Such differences have been attributed to variations in cell membrane composition and, in the case of antifolates, to a deviation in dihydrofolate reductase chemical structure or thymine dependency (10).

In future studies we will determine the usefulness of amoebicins A12-A and A12-B as therapeutic agents against pathogenic amoebae, as well as the possible practical use of these compounds as antifungal agents. The extent of amoebicin production in natural habitats and the possible role of amoebicins in controlling populations of free-living amoebae also deserve further investigation.

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