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Cloning, characterization and expression of escapin, a broadly antimicrobial FAD-containing L-amino acid oxidase from ink of the sea hare *Aplysia californica*

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Summary

A 60 kDa monomeric protein isolated from the defensive purple ink secretion of the sea hare *Aplysia californica* was cloned and sequenced, and is the first sea hare antimicrobial protein to be functionally expressed in *E. coli*. Sequence analysis suggested that this protein is a flavin-containing L-amino acid oxidase (LAAO), with one predicted potential glycosylation site, although the glycosylation could not be experimentally confirmed. This protein, which we call 'escapin', has high sequence similarity to several other gastropod proteins. Escapin was verified by NMR, mass spectroscopy and HPLC to have FAD as its flavin cofactor. Escapin's antimicrobial effects, bacteriostasis and bactericidal, were determined using a combination of two assays: (1) incubation of bacteria on solid media followed by assessment of inhibition by direct observation of zones of inhibition or by turbidity measurements; and (2) incubation of bacteria in liquid media followed by counting viable colonies after growing on agar plates. Native escapin inhibited the growth of Gram-positive and Gram-negative bacteria, including marine bacteria (*Vibrio harveyii* and *Staphylococcus aureus*) and pathogenic bacteria (*Staphylococcus aureus*, *Streptococcus pyogenes* and *Pseudomonas aeruginosa*). Escapin also inhibited the growth of yeast and fungi, with different efficacies. Escapin's antimicrobial activity was concentration dependent and did not decrease when stored for more than 5 months at room temperature. Escapin was bacteriostatic and not bactericidal in minimal media (e.g. salt media) with glucose, yeast extract, and a mixture of 20 amino acids each at 50 μmol l^{-1}, but was bactericidal in media enriched with Tryptone Peptone. Escapin was also strongly bactericidal in media with L-lysine at concentrations as low as 3 mmol l^{-1} and slightly bactericidal in 50 mmol l^{-1} L-arginine, but not in most other amino acids even at 50 mmol l^{-1}. Escapin had high oxidase activity (producing hydrogen peroxide) with either L-arginine or L-lysine as a substrate and little to no oxidase activity with other L-amino acids. Hydrogen peroxide alone (without escapin or amino acids) was strongly bacteriostatic but poorly bactericidal, similar in this respect to L-arginine but different from L-lysine in the presence of escapin. Together these results suggest that there are multiple mechanisms to escapin's antimicrobial effects, with bacteriostasis resulting largely or entirely from the effects of hydrogen peroxide produced by escapin's LAAO activity, but bactericidal effects resulting from lysine-dependent mechanisms not directly involving hydrogen peroxide. Recombinant escapin expressed in bacteria was also active against Gram-positive and Gram-negative bacteria, suggesting that glycosylation is not essential for antimicrobial activity.

Key words: toxin, chemical defense, flavin, gastropod, inking, Opisthobranchia.

Introduction

Several proteins isolated from different tissues and secretions of marine opisthobranch gastropod molluscs known as sea hares have been reported as antimicrobial and antitumor agents (Kamiya et al., 1986, 1989; Kisugi et al., 1989; Yamazaki et al., 1989a,b, 1990; Yamazaki, 1993; Iijima et al., 1995, 2003; Takamatsu et al., 1995; Melo et al., 1998, 2000; Petzelt et al., 2002; Jimbo et al., 2003; Butzke et al., 2004). All are derived from either albumen glands that package sea hare egg masses, the egg masses themselves, or ink secretions from defensive glands in the sea hare's mantle cavity. Achacin, a protein purified from mucus of the African giant land snail *Achatina fulica*, and apoxin I, a protein purified from venom of the western diamondback rattlesnake *Crotalus atrox*, also possess similar bioactivity (Iguchi et al., 1982; Kubota et al., 1985; Kamiya et al., 1986; Obara et al., 1992; Otsuka-Fuchino et al., 1992, 1993; Suhr and Kim, 1996; Torii et al., 1997, 2000;

Ogawa et al., 1999; Ehara et al., 2002; Kanzawa et al., 2004). Several of these proteins, including three from sea hares, are known to be L-amino acid oxidases (Iijima et al., 2003; Jimbo et al., 2003; Butzke et al., 2004), but the cofactors responsible for oxidase activity have not been experimentally demonstrated. These studies differ in their findings as to whether the proteins are bacteriostatic or bactericidal (Yamazaki et al., 1990; Otsuka-Fuchino et al., 1992; Melo et al., 1998; 2000). In addition to the interesting biological roles these proteins may play for their host organisms, some may also have practical industrial or medical uses, yet none have been successfully expressed in high output systems such as bacteria (Obara et al., 1999; Torii et al., 2000; Petzelt et al., 2002; Butzke et al., 2004; Cummins et al., 2004).

In this study, we have isolated, cloned and sequenced a 60 kDa monomeric antimicrobial protein from the purple ink of the sea hare *Aplysia californica* (Fig. 1A) and named it 'escapin', because of its potential role in sea hare defence, because it is only released when a sea hare is attacked by predators and it has cytotoxic effects against a predatory sea anemone (Johnson et al., 2001, 2003; Johnson, 2002). When attacked by natural predators, *A. californica* releases secretions from two glands in its mantle cavity: a purple secretion from its ink gland, and a sticky white secretion from its opaline gland (Johnson and Willows, 1999). Nearly 30% of the dry mass of *A. californica* ink is protein (Troxler et al., 1981; MacColl et al., 2000) while the remaining portion is algal-derived pigments from the sea hare's seaweed diet (Chapman and Fox, 1969). The ink-opaline secretion of *A. californica* is an effective deterrent against predatory sea anemones (Nolen et al., 1995; Kicklighter et al., 2005), and escapin is the major protein component of ink. Thus, as an abundant and potentially bioactive protein in ink, escapin may play a role in sea hare chemical defence.

We found that escapin contains flavin adenine dinucleotide (FAD) and is predicted to have one potential glycosylation site, although the glycosylation could not be experimentally confirmed. Escapin has a wide spectrum of antimicrobial activities, including against bacteria, yeasts and fungi, but with different efficacies. Escapin is highly stable, retaining its antimicrobial activity after storage for more than 5 months at room temperature. Escapin is bacteriostatic but not bactericidal under many growth conditions (e.g. in a mixture of amino acids each at 50 μmol l^{-1}, or 50 mmol l^{-1} L-valine), weakly bactericidal under some conditions (e.g. in yeast extract or in 50 mmol l^{-1} L-arginine or L-histidine), and strongly bactericidal under other conditions (e.g. in Tryptone Peptone or 50 mmol l^{-1} L-lysine). Hydrogen peroxide plays a prominent role in the bacteriostatic effect but only a weak role in the bactericidal effect. We successfully expressed in *E. coli* soluble recombinant escapin that was bioactive against both Gram-positive and Gram-negative bacteria.

Materials and methods

Animals

Sea hares *Aplysia californica* Cooper 1863 were collected in California by Marinus, Inc. (Long Beach, CA, USA) and Marinus Scientific (Garden Grove, CA, USA) or provided by the NIH National Resource for *Aplysia* Facility (http://www.rsmas.miami.edu/groups/sea-hares/). They were maintained in our laboratory in aquaria containing recirculating, filtered and aerated artificial seawater (Instant Ocean^{TM}: Aquarium Systems, Mentor, OH, USA), and fed red alga (*Gracilaria ferox* J Agardh).

Collection of purple ink

Purple ink from *A. californica* was collected by agitating the animals over a flask or by gently squeezing dissected ink glands in a Petri dish with the blunt end of a scalpel handle. The secretion was then frozen at –80°C until used.

Purification of proteins from purple ink

Proteins were isolated and purified using an ÄKTA 100 Automated FPLC (Amersham Pharmacia Biotech, Piscataway, NJ, USA). A preparative grade Hi-Load Superdex 200 16/60 column (Amersham Pharmacia Biotech) or an in-house-packed Sephacryl 300 HR 26/60 column was used for initial size separation with fractions collected in an automated fraction collector. Fractions identified to have activity by bacterial assay (described in the next section) were concentrated using a Biomax 5K NMWL membrane Ultrafree Centrifugal Filter Device (Millipore, Billerica, MA, USA). Active fractions were further purified on a cation exchange Mono S column, and fractions were collected, assayed, concentrated and frozen at –80°C. One purified protein of interest, which we call 'escapin', was bright yellow. Escapin's concentration was determined by Bradford (1976) assay using bovine serum albumen (BSA) as a standard. The molecular mass of purified escapin was determined by gel filtration using a Superose-6 10/30 column (Amersham Pharmacia Biotech) eluted with 50 mmol l^{-1} potassium phosphate buffer (pH 7.2) containing 150 mmol l^{-1} KCl at a flow rate of 0.5 ml min^{-1}. The molecular mass markers were BSA (67 kDa), ovalbumen (43 kDa) and chymotrypsinogen A (25 kDa).

Protein sequencing

Aplysia californica ink was analyzed for protein content using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 12% gels. A single dominant protein band was found at about 60 000 Da, and was named escapin. This band was blotted from the polyacrylamide gel to a PVDF membrane using CAPS (transfer buffer) and determined by protein microsequencing and proteomic mass spectrometry (University of Massachusetts Medical School Proteomic Mass Spectrometry Lab: http://www.umassmed.edu/proteomic/leszyk). After identifying peptide fragments from escapin, a BLAST search was conducted to find homologous protein fragments. N-terminal sequencing of escapin was carried out in GSU protein sequencing facility using a protein sequencer (Procise 492; Applied Biosystems, Foster City, CA, USA).

mRNA extraction and RT-PCR

To isolate intact mRNA from *A. californica* ink glands, animals were dissected in $MgCl_2$/diethylpyrocarbonate solution isotonic with seawater in a 4°C cold room. Ink glands were removed and immediately transferred to liquid N_2. Glands were ground with a mortar and pestle while still in liquid N_2. This material was transferred to an RNase-free tube, and mRNA was isolated following the manufacturer's protocols (Roche mRNA Isolation Kit, Cat. No.1-741-985, Indianapolis, IN, USA). Two primers from the N-terminal sequence (TTCGAGTTCTGCGACCGGGT) and C-terminal sequence (CCAAGGCTGGTCAAAGGTCA) of cyplasin L (GenBank accession no. AJ304802; Petzelt et al., 2002) were designed based on the results of homologous sequences recovered following the BLAST search. Primers were used for RT-PCR following the manufacturer's protocols (Roche Titan One Tube RT-PCR System, Best Nr.1-888-382) using an Eppendorf Mastercycler Gradient thermocycler. The resultant RT-PCR product, which was >900 bp, was then cloned in a pGEM T-vector (Promega, Madison, WI, USA), amplified and sequenced. This sequence was verified by alignment with homologous sequences from GenBank using MacVector 6.5.3 (Accelrys, San Diego, CA, USA).

5′/3′ rapid amplification of cDNA ends (RACE)-PCR

5′/3′ RACE-PCR was conducted to complete the cDNA clone of the >900 bp RT-PCR product described above. 1 μg mRNA was used with the 5′/3′ RACE Kit (Roche Cat. No. 1-734-792), and the manufacturer's protocol was followed. Three specific primers were designed from the original RT-PCR isolated fragment and used in 5′ RACE: one for first strand cDNA synthesis (SP1=GTTCACGTCGGGTGTGTTG-GGCAGC), one for dA-tailed cDNA amplification (SP2= TGGTAGGTGAACAGACGGCC), and one for nesting PCR (SP3=CCCGGTCGCAGAACTCGAAA). A final primer was used for the 3′ RACE reaction (SP4=ATCTACACCCTGGA-GGAAGG). All PCR products were analyzed on a 1% agarose gel. PCR products that appeared to be of appropriate size were subcloned into pGEM-T vector (Promega) and sequenced as described above.

Isolation and identification of the yellow pigment associated with escapin

Purified escapin was heated at 70°C for 15 min followed by centrifugation at 25 000 ***g*** for 20 min to separate the pigment from the protein. Yellow pigment in the supernatant was purified by high pressure liquid chromatography (HPLC) according to Light et al. (1980), using a Beckman system equipped with a 168 photodiode array set at 200–600 nm with a Phenomenex Luna C18 (0.46×250 mm) column (Phenomenex, Torrance, CA, USA). Isocratic reversed phase chromatography was performed using 5 mmol l^{-1} ammonium acetate and 20% methanol in water as a mobile phase with a flow rate of 1 ml min^{-1}. Retention times of a FAD standard (Sigma, St Louis, MO, USA) and the yellow pigment were compared by co-injection. ESI-TOF mass spectrometry was performed using an Applied Biosystems QSTAR XL and run in positive ion mode. ESI samples were injected into a flow of 50/50 water/acetonitrile containing 0.2% formic acid. NMR spectra were acquired on a 500 MHz Bruker Avance NMR (Rheinstetten, Germany) equipped with a triple resonance cryoprobe. 1 μmol l^{-1} of FAD standard was purified using the same method as for the yellow pigment. Spectra were recorded in D_2O at 309 K. Spectra for the FAD standard were obtained under identical conditions except the experimental time. Proton assignments for the FAD standard were based on established 2D NMR methods (COSY, ROESY). The amount of FAD in the supernatant of heated protein was calculated based on an extinction coefficient value, ϵ_{450}, for FAD of 11.3 mmol l^{-1} cm^{-1} (Whitby, 1953).

Detection of glycosylation of escapin

GelCode Glycoprotein Staining Kit (Pierce Biotechnology, Rockford, IL, USA) and DIG Glycan Detection Kit (Roche) were used to determine the carbohydrate component of escapin. 5 μg of escapin, BSA and *E. coli* protein (negative controls, since they lack glycosylation), and various concentrations of horseradish peroxidase (a positive control and standard, since it is 15% carbohydrate by mass) were analyzed by SDS-PAGE followed by staining for carbohydrates according to the manufacturers' protocols, as well as by Coomassie Blue labeling of proteins.

L-amino acid oxidase (LAAO) assay

LAAO activity of escapin was determined by an enzyme-coupled assay (MacHeroux et al., 2001). Purified escapin in 50 mmol l^{-1} phosphate buffer and 150 mmol l^{-1} KCl was added to a 100 μl reaction mixture containing 0.1 mol l^{-1} Tris-HCl, pH 7.6, 10 μg horseradish peroxidase, 0.2 mmol l^{-1} o-dianisidine, and indicated concentration of various L-amino acids. Reactions were performed at room temperature for 1–60 min; the activity was monitored by absorbance at 436 nm and the increase in absorbance was transformed into molar concentration of end product based on ϵ of o-dianisidine=8.31×10^3 mol l^{-1}. The K_m and V_{max} values were determined by Lineweaver–Burk plots.

Bacterial expression of the precursor of escapin

Primers were designed to amplify the whole coding sequence so that escapin could be over-expressed in *E. coli*. The 5′ primer included a *Bam*HI restriction site to allow in-frame insertion into the amplification and expression vectors (5′GGATCCCATGTCGTCTGCTTTCCTTC3′). The 3′ end included an extra *Hin*dIII restriction site (5′AAGCTTGAGG-AAGTAGTCGTTGATGA3′). PCR was conducted using Expand High Fidelity PCR System (Roche). The resultant whole gene fragment of expected size was cloned into pGEM-T vector (Promega), and the plasmids were amplified. The plasmids were then cut with *Bam*HI and *Hind*III, and the gene was subcloned into the pET-20b expression vector (Novagen, Madison, WI, USA) using the same enzymes. The sequence was confirmed by DNA sequencing using an ABI sequencer.

For over-expression, the plasmid was transformed into *E. coli* strain BL21 (λDE3). 26 liters of these cells were grown in Luria-Bertani (LB) medium in a Pilot Plant fermenter (New Brunswick Scientific, Edison, NJ, USA) at 37°C until reaching an A_{600} of 0.5, at which point they were induced with 0.5 mmol l^{-1} isopropyl-β-D-thiogalactopyranoside (IPTG) for 2 h. The cells were harvested and concentrated by centrifugation (5000 ***g*** at 4°C); a portion was resuspended in 0.1 mol l^{-1} phosphate buffer containing 1 mmol l^{-1} PMSF protease inhibitor, and broken on a Sim-Aminco French pressure cell at 16 000 psi. The resultant mixture was centrifuged at 127 000 ***g*** for 1 h in a Beckman Coulter Optima XL-100K ultracentrifuge. SDS-PAGE was used to identify the location of escapin, which formed an inclusion body and was found in the pellet. The inclusion body was first dissolved in denaturing buffer (8 mol l^{-1} urea, 20 mmol l^{-1} phosphate buffer, pH 7.2) and the supernatant was loaded onto an anion exchange column, Mono Q 10/10 (Amersham Pharmacia Biotech) using 8 mol l^{-1} urea, 20 mmol l^{-1} PPB, pH 7.2, and 1 mmol l^{-1} DTT (A buffer) and the same buffer plus 1 mol l^{-1} NaCl (B buffer) to elute escapin. Escapin was again identified by size using SDS-PAGE, and the resultant band was analyzed for MALDI-TOF MS (Emory University School of Medicine Microchemical and Proteomics Facility, http://corelabs.emory.edu/home.cfm#mcf) to verify the identity of the protein as escapin. Soluble escapin precursor could be obtained when protein was induced at a lower temperature (20°C) for 5–18 h, and tested for antimicrobial activities.

Antiserum preparation

An antiserum against escapin was obtained by injecting rabbits with denatured recombinant escapin purified from the *E. coli* expression extracts. The first injection was conducted using 1:1 mixed escapin and Freund's Complete Adjuvant (DIFCO, BD, Franklin Lakes, NJ, USA) followed by 4–5 injections using Freund's Incomplete Adjuvant (DIFCO).

Expression of escapin without signal peptide in E. coli

Similar methods to those described above were used to amplify and clone escapin in *E. coli*. The escapin gene without signal peptide was subcloned to *Nde*I and *Hin*dIII sites of pET 29a vector (Novagen). Plasmid was then transformed into BL21 (λDE3) strain, and proteins were induced by 0.5 mmol l^{-1} IPTG at A_{600}=0.8, followed by incubation at 20°C for 5–18 h.

Microbial species and strains

Eleven species or strains of microbes were examined for antimicrobial activity of escapin. These are the following: Gram-negative bacteria *Escherichia coli* (MC4100), *Pseudomonas aeruginosa* (PAO1), and *Salmonella typhimurium* (AA140), *Vibrio harveyi* BB170 (a marine species), Gram-positive bacteria *Bacillus subtilis* (2 strains, 168 and WB600), *Streptococcus pyogenes* (NZ131), and *Staphylococcus aureus* (6835; a pathogenic species); yeast *Candida krusei* and *Saccharomyces cerevisiae* (BY4761); and fungus *Cladosporium sphaerospermum*.

Antimicrobial assay

Antimicrobial effects, which can be bacteriostatic and/or bactericidal, were determined for escapin using a combination of two assays. In the first assay, which measured inhibition, microbes were incubated on solid medium in the presence of escapin or controls, followed by assessment of escapin's effects either by direct observation of zones of inhibition or by turbidity measurements of cell density (measured as A_{600} in a spectrophotometer). In the second assay, bacteria were incubated in liquid medium in the presence of escapin or controls, followed by plating onto agar-filled Petri dishes and counting viable colonies. No effect on bacterial growth in the first assay indicated that the compound was not antimicrobial – neither bacteriostatic nor bactericidal. If there was inhibition of growth according to the first assay but no reduction in number of colonies according to the second assay, then bacteriostasis was indicated. If there was inhibition of growth according to the first assay and a reduction in number of colonies according to the second assay, then bactericidal effects were indicated.

In the first assay, growth inhibition plate assays, various bacteria species and strains were plated as a lawn of ca. $1–2\times10^8$ cells on Petri dishes with solid medium in 1.5% agar. Growth inhibition was examined by spotting 1 μl of escapin onto the plate, incubating overnight at 37°C or room temperature, and assaying for the presence of a clear zone around at the spot. In this assay, different microbes were cultured in an appropriate medium. *E. coli* cells were cultured in either minimal medium (e.g. M9+glucose) or enriched medium [e.g. Tryptone Peptone (Try) or LB]. Other bacteria species were cultured in LB medium, except for *Streptococcus pyogenes*, which was cultured in Todd Hewitt broth. Fungi were cultured in Sabouraud Dextrose medium. Yeast were cultured in YEPD solid medium (1% yeast extract, 2% peptone, 2% dextrose).

In the second assay, using liquid medium, bacteriostatic or bactericidal activity was determined by co-incubating bacteria in liquid medium with supplements and escapin, followed by measuring bacterial cell density either by turbidity measurement at A_{600} or by counting the number of viable colonies after incubating on Petri dishes with solid medium.

Assay of antimicrobial shelf-life at room temperature

Long-term stability of the antimicrobial activity of escapin was evaluated to aid in determining its potential as a practical antimicrobial agent. 250 μg ml^{-1} of escapin in buffer containing 50 mmol l^{-1} PPB (pH 8.0) and 150 mmol l^{-1} KCl was diluted at 1:1 ratio in the same buffer with or without 100% glycerol, separated into portions, and stored at room temperature for time intervals of more than 5 months. Control escapin was kept frozen at –80°C until used. The shelf-life of escapin was determined by twofold serial dilutions on a solid

LB medium antibacterial assay using *E. coli* or *B. subtilis*, as described elsewhere in Materials and methods.

Results

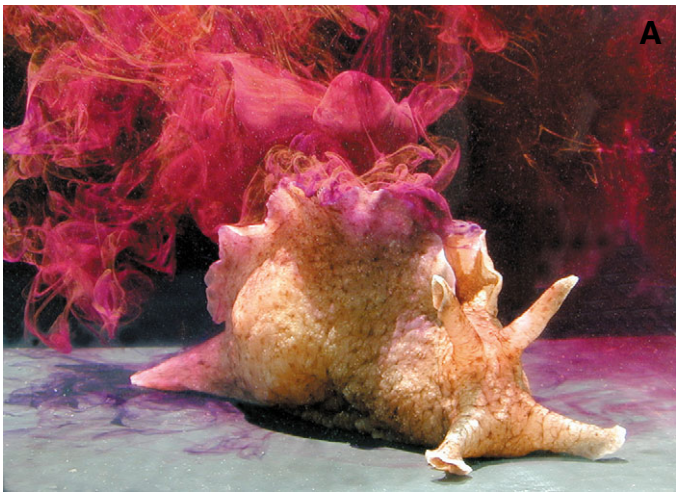
Purification and characterization of native escapin from purple fluid secretion of A. californica

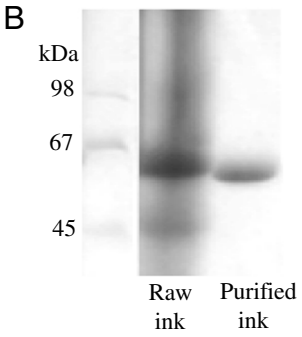
Raw purple from ink *A. californica* (Fig. 1A) run on a denaturing SDS-PAGE gel contains many proteins, including a dominant one at 60 kDa (Fig. 1B, left lane). The 60 kDa denatured protein can be purified to homogeneity using a MonoS column (see Materials and methods; Fig. 1B, right lane). Analytical gel filtration shows native (non-denatured) ink purified using the MonoS column contains a single molecule with a molecular mass of ca. 60 kDa (Fig. 1C). Furthermore, this purified 60 kDa protein has complete antibacterial activity (see later). Together, these results show that the 60 kDa protein occurs as a bioactive monomer in native ink. We called this 60 kDa protein 'escapin' because it is only released when a sea hare is attacked by predators and has cytotoxic effects against one predator (Johnson et al., 2001, 2003; Johnson, 2002). Escapin also has similarity to other proteins isolated from sea hares (Kamiya et al., 1989; Yamazaki et al., 1989a,b, 1990; Melo et al., 2000; Petzelt et al., 2002).

Cloning and sequence analysis of escapin cDNA

Two trypsin-digested fragments from purified escapin were determined by microsequencing, and the primers were made to probe the ink gland cDNA (see Materials and methods). The sequence of escapin cDNA was obtained by 5′ and 3′ RACE-PCR as described in Materials and methods. The cDNA of escapin was 1879 bp in length (GenBank accession no. AY615888) and had an open reading frame encoding 535 amino acid (aa) residues (Fig. 2). Based on the deduced aa sequence, a signal peptide cleavage site between the 18th and 19th aa residues was predicted by SignalIP (http://www.cbs.dtu.dk/services/SignalP-2.0/). This was verified by N-terminal aa sequencing of native escapin isolated from sea hare ink.

A BLAST search found that escapin shared identity with a number of L-amino acid oxidase (LAAO) flavoproteins (Fig. 2). Escapin had highest identity (93%) with APIT, a protein from the purple ink secretion of the sea hare *Aplysia punctata* (GenBank accession nos 442281, 442282, 4422883; Butzke et al., 2004). Escapin shared 61% identity with cyplasin L (Accession no. AJ304802; Petzelt et al., 2002), likely isolated from an ink-opaline secretion of *A. punctata*. Escapin shared 61% and 60% identity with aplysianin A precursor protein isolated from albumen glands of *A. kurodai* (Accession no. D83255; Kamiya et al., 1986) and *A. californica* (Accession no. AY161041; Cummins et al., 2004), respectively. Escapin also had 48% identity with achacin precursor, an antibacterial protein isolated from a land snail *Achatina fulica* (Accession no. X64584; Obara et al., 1992). It also showed 21% identity with other L-amino acid oxidases





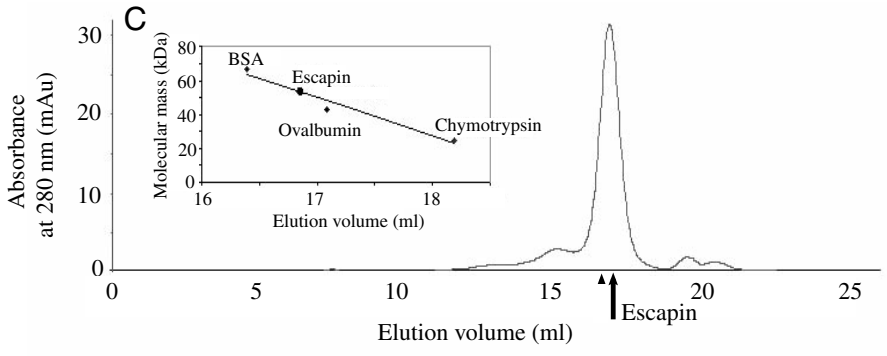


Fig. 1. (A) The ink of *Aplysia californica containing* escapin, a 60 kDa protein. (B) SDS-PAGE of raw purple ink (left lane) and ink purified as described in the Materials and methods to yield escapin, a 60 kDa protein (right lane). Molecular mass standards are also shown. (C) Analytical gel filtration shows that escapin elutes as a single peak. Absorbance is expressed on a relative scale; mAu, milliabsorbance units. Arrow and arrowhead indicate elution times for escapin and BSA, respectively. The inset shows elution volumes of molecular mass standards: BSA, 67 kDa; ovalbumen, 43 kDa; chymotrypsinogen A, 25 kDa, demonstrating that native (non-denatured) escapin has molecular mass of ca. 60 kDa, similar to that of the denatured escapin, as shown in B. The photograph in A is courtesy of Genevieve Anderson.

from various species, including apoxin I from the venom gland of the pit viper *Crotalus atrox* (Accession no. AF093248; Torii et al., 2000).

The alignment results indicate that the two characteristic sequence motifs of flavoproteins – 'GG' (RxGGRxxS/T) and βαβdinucleotide-binding (DMB) motifs – are well conserved among these proteins (Fig. 2). Glycosylation is commonly observed among LAAOs and is reported to be critical for the enzyme's activity (Ogawa et al., 1999; Torii et al., 2000; Ehara et al., 2002). However, only one possible N-glycosylation site (Thr 463) was predicted for escapin using the analytical program NetOGlyc (http://www.cbs.dtu.dk/services/NetOGlyc/). We directly examined the level of glycosylation of purified escapin using two glycoprotein staining methods. With the GelCode Glycoprotein Staining Kit, which has a sensitivity of 1.5% carbohydrate by mass, no carbohydrate was detected. Using the DIG Glycan Detection Kit, escapin contained <0.03% carbohydrate by mass and even less than dactylomelin-P (a homologue of escapin in *Aplysia dactylomela*, which has been reported to have <0.05% carbohydrate); however, this assay yielded false positives for our negative control *E. coli* proteins, so it is questionable whether any glycoprotein was present. We also attempted to identify glycoproteins in purified dactylomelin-P using a ConA-sepharose column (Amersham Pharmacia Biotech), and again we did not identify any glycoproteins. We also attempted to identify carbohydrates from purified dactylomelin-P, using the University of Georgia Complex Carbohydrate Research Center (http://www.ccrc.uga.edu/home.html); this assay, which is extremely sensitive, yielded negative results. Collectively, our results predict that escapin has one N-glycosylation site, but this prediction could not be experimentally confirmed. Thus, we conclude that escapin is minimally glycosylated, if at all. In addition, we found no evidence that the glycosylation is essential to escapin's antimicrobial activity (see Bacteria-expressed escaping, below).

Escapin is a member of the flavoprotein family

Amino acid sequence analysis suggested that escapin is a member of the flavoprotein family, and contains 'GG' (RxGGRxxS/T) and βαβdinucleotide-binding (DMB) motifs. Purified homogeneous escapin was bright yellow, and it was assumed that this yellow pigment was the flavin. Since flavin adenine dinucleotide (FAD) is the typical flavin cofactor for this protein family, we used NMR, ESI-TOF mass spectroscopy, and HPLC to detect for the presence of FAD. The aromatic region of the ^{1}H NMR spectrum of the yellow

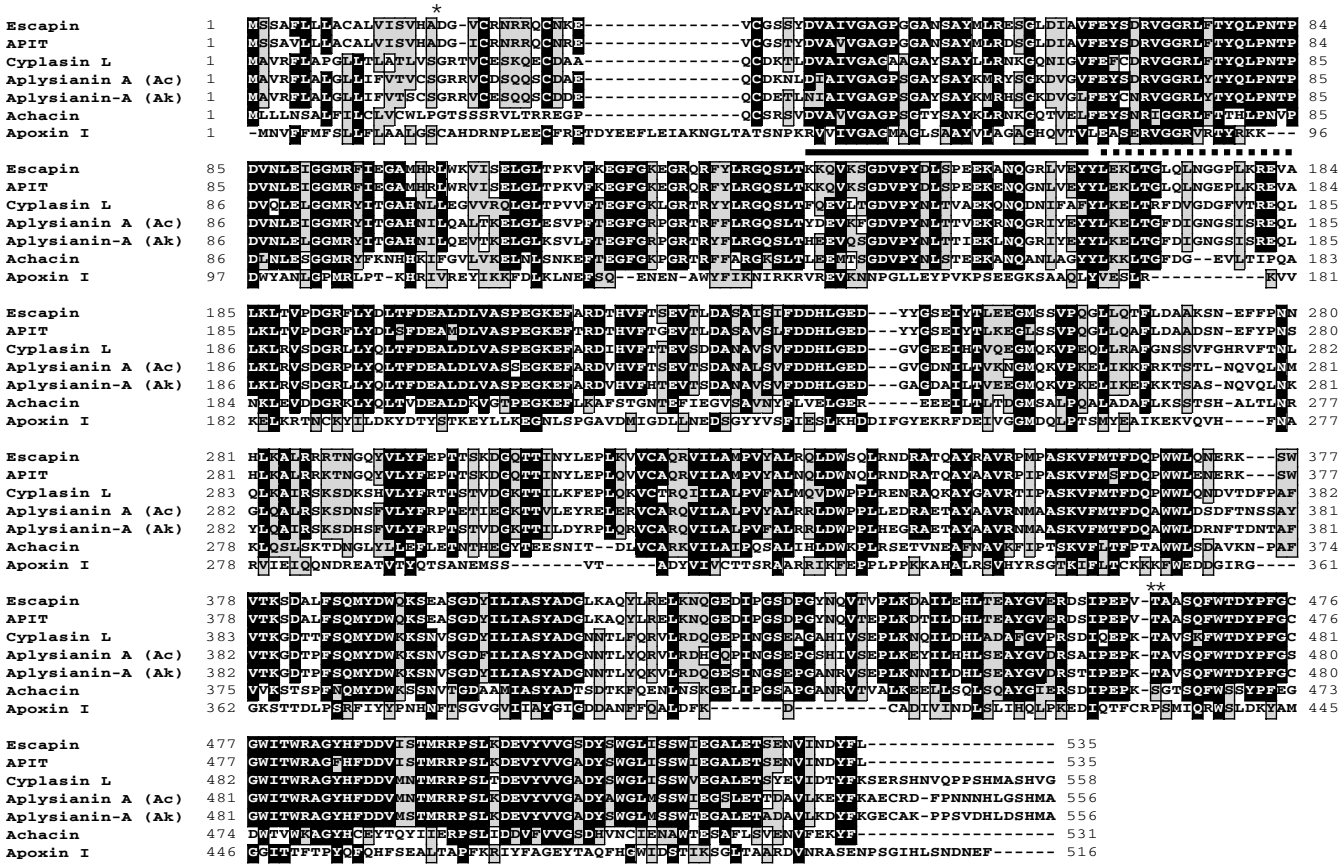


Fig. 2. Amino acid sequence alignment of escapin and related proteins. Solid and broken underlines indicate DMB and GG motifs, respectively (see text for details). *Predicted signal sequence cleavage site at A 18 and D 19. **Predicted glycosylation site at Thr 463. Boxed areas indicate regions of homology.

pigment isolated from escapin showed essentially identical resonances to the FAD standard, although the spectrum of the yellow pigment contained signals of impurity at 8.45 p.p.m. (Fig. 3A). Similar features were also obtained for the aliphatic region of the FAD standard and the yellow pigment (data not shown). Particularly noteworthy is the observation that two of the aromatic protons (7.8 p.p.m.) that have long T1 relaxation times in FAD are also observed for the pigment. A high-resolution ESI-TOF mass spectrum of purified yellow pigment from escapin showed a peak with an *m/z* value of 786.2 (Fig. 3B); this corresponds to the molecular formula $C_{27}H_{34}N_9O_{15}P_2$, which was designated as a $(M+H)^+$ ion of FAD. ESI-TOF mass spectrum of the yellow pigment also showed peaks at *m/z* of 808.1 and 830.1, which correspond to the $(M+Na)^+$ and $(M-H+2Na)^+$ ions of FAD (Fig. 3B). In this spectrum, signals below 400 *m/z* were due to solvents, and signals of 400–600 *m/z* were not identified. Ions corresponding to another flavin, FMN (molecular mass=478.3), were not found. In addition, in reversed phase HPLC, the yellow pigment had the same retention time (17.6 min) as FAD, and co-injection of the yellow pigment and FAD showed only one peak. The UV-visible absorbance spectrum of the peak of the yellow pigment showed absorbance at 263, 375 and 450 nm, which is characteristic for FAD. Thus, the yellow pigment released from escapin is FAD. Based on ϵ_{450} values, 17.2 nmoles FAD were extracted from 16.7 nmoles of purified escapin, yielding an escapin:FAD molar ratio of about 1:1.

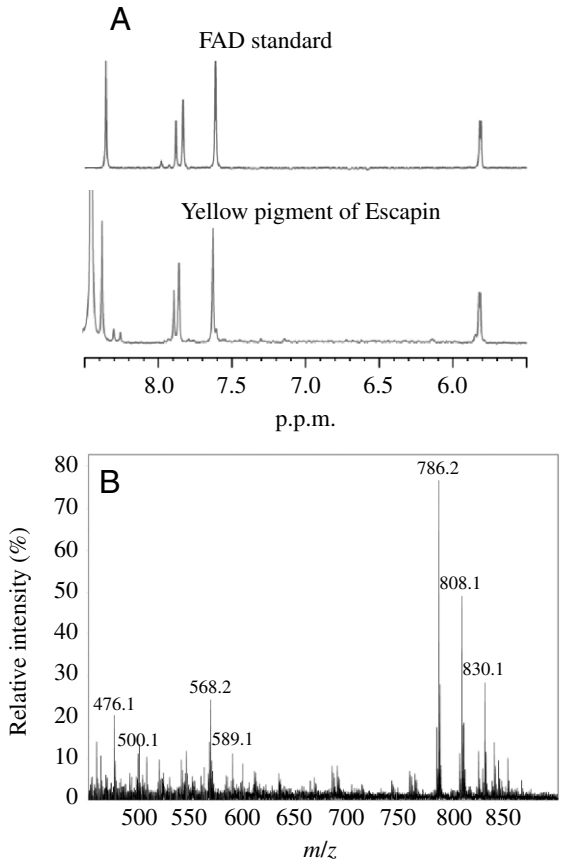


Fig. 3. The yellow pigment associated with escapin is FAD. (A) Aromatic region of ^{1}H NMR spectrum for FAD standard and the yellow pigment from escapin, showing identical signals. (B) Positive ion ESI-TOF mass spectrum of the yellow pigment from escapin. See text for explanation of these signals.

Results from an NCBI conserved domain search predicted that escapin is an L-amino acid oxidase (LAAO), which oxidizes L-amino acids to produce hydrogen peroxide. To test this prediction, we performed an enzyme-coupled oxidase assay. Our results confirmed the prediction, showing that amino acids serve as a substrate for escapin, resulting in generation of hydrogen peroxide (Fig. 4). Unlike LAAOs from African snail and snake venom (Torii et al., 1997; MacHeroux et al., 2001; Du and Clemetson, 2002; Ehara et al., 2002; Lu et al., 2002), but similar to aplysianin A isolated from the albumen gland of *Aplysia kurodai* (Jimbo et al., 2003), escapin preferentially utilized positively charged amino acids. The most effective amino acids were L-lysine and L-arginine. When escapin and 2 mmol l^{-1} amino acids were incubated for 1 min, L-lysine and L-arginine proved excellent LAAO substrates, L-tyrosine and L-histidine were low quality substrates, and other amino acids were poor substrates (Fig. 4). K_m (μmol) and V_{max} (μmol s^{-1}) values of were 31–85 μmol and 1.92–2.70 μmol s^{-1} for lysine and 25–120 μmol and 1.56-3.30 μmol s^{-1} for arginine (Fig. 4). Similar efficacies for the substrates were seen for 60 min incubation times. The reactions were

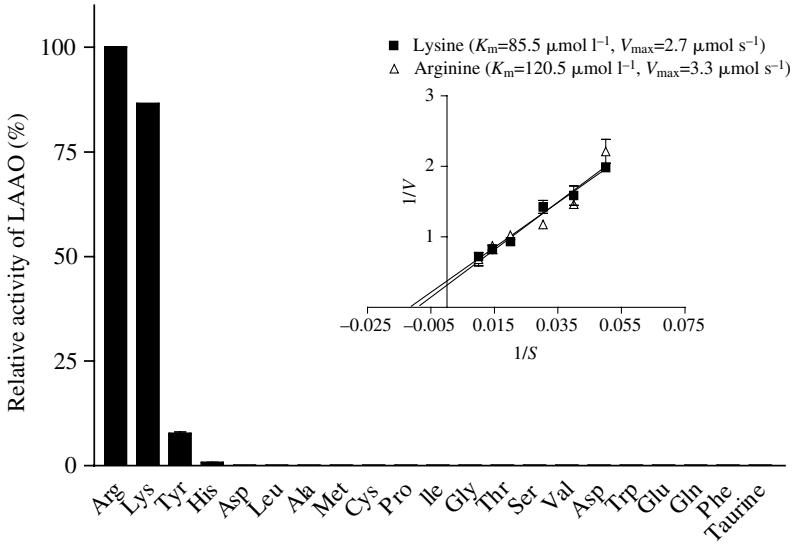


Fig. 4. LAAO enzyme activity of escapin, and its substrate specificity. 0.6 μg escapin in 100 μl was incubated at 22°C for 1 min in 2 mmol l^{-1} of each L-amino acid and taurine. LAAO activity was measured by absorbance at 436 nm and normalized to the value for arginine. Values are mean ± S.E.M., *N*=2. Inset shows a Lineweaver–Burk plot of data for LAAO activity at different concentrations for lysine and arginine. Values are means ± S.E.M., *N*=2. K_m and V_{max} values calculated from this experiment are shown.

Table 1. *Antimicrobial activity of wild-type escapin*

Microbial species	MIC (μg ml^{-1})
Gram-negative bacteria	
Escherichia coli (MC4100)	0.62
Salmonella typhimurium AA 140	0.62
Pseudomonas aeruginosa PAO1	0.31
Vibrio harveyi BB170	0.25
Gram-positive bacteria	
Staphylococcus aureus 6835	0.31
Streptococcus pyogenes NZ131	0.62
Bacillus subtilis 168	2.50
Bacillus subtilis WB600	2.50
Yeast	
Candida krusei	5.0
Saccharomyces cerevisiae BY4761	5.0
Fungus	
Cladosporium sphaerospermum	
in SD medium with 0.8 mmol l^{-1} lysine	62
in SD medium with 4.9 mmol l^{-1} lysine	15

The minimum inhibition concentration (MIC) of escapin against various microbes was determined by measuring inhibition of growth on plates of solid media.

completed within 30 s at room temperature for lysine or arginine concentrations of 0.02–2 mmol l^{-1} (data not shown). LAAO assays for lysine and arginine in the presence of *E. coli* produced similar effects (data not shown), demonstrating that hydrogen peroxide is produced from escapin's oxidation of lysine or arginine under the conditions of the antimicrobial assays (described later).

Antimicrobial activity of escapin

Escapin inhibited the growth of several types of bacteria including Gram-negative (*Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Vibrio harveyi*) and Gram-positive (*Bacillus subtilis*, *Streptococcus pyogenes* and *Staphylococcus aureus*) (Table 1). *Vibrio harveyi*, which is a marine bacterium, was most sensitive to escapin, with a minimum inhibitory concentration (MIC) of 0.25 μg ml^{-1}. The pathogenic species *Staphylococcus aureus* and *Pseudomonas aeruginosa* were the next most sensitive to escapin, with MIC values of 0.31 μg ml^{-1}. Bacillus of both wild-type and protease-deficient strains showed the highest resistance to escapin, with MIC=2.5 μg ml^{-1}. Escapin also exhibited anti-fungal and anti-yeast activity, though it was less effective against them than bacteria. The MIC values of escapin against a common pest mold *Cladosporium sphaerospermum* and a pathogenic yeast strain *Candida krusei* were 62 and 5 μg ml^{-1}, respectively, which is 10–100 times higher than against *E. coli* (Table 1).

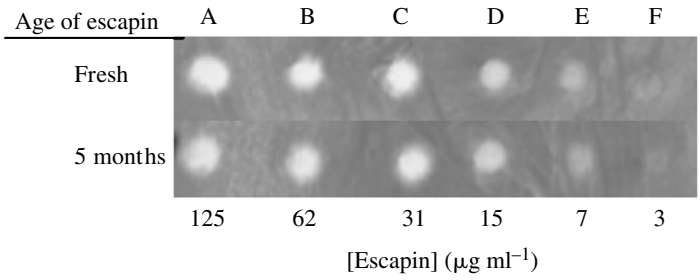


Fig. 5. Escapin has a very long antimicrobial shelf-life at room temperature. *E. coli* cells in stationary phase were grown as a lawn on solid medium and tested in a plate assay for ability of escapin to inhibit growth. Escapin was tested at concentrations of 3–125 μg ml^{-1}, under two conditions: 'Fresh': freshly isolated; '5 months': after storage for 5 months at room temperature.

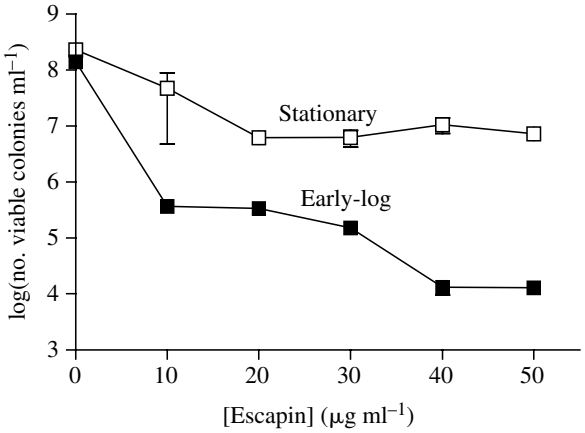


Fig. 6. Escapin preferentially kills *E. coli* cells in their growing but not in their resting state. 2×10^8 cells ml^{-1} in early-log growth or stationary phase were centrifuged, and the pellet was resuspended in fresh LB medium. The absorbance reading was adjusted to that of early-log phase cells (=0.5). Cells were then coincubated in escapin at 0–50 μg ml^{-1} at 37°C for 2 h. Cultures were then placed on LB agar plates and incubated at 37°C for 18 h, at which time the number of viable colonies was counted. Values are means ± S.E.M., *N*=3.

Escapin has a very long shelf life at room temperature (Fig. 5). Escapin with or without 50% glycerol retained the same antibacterial activity over a period of more than 5 months stored at room temperature. Unlike other LAAOs found in snake venom (MacHeroux et al., 2001), escapin is also highly stable when taken through several freeze–thaw cycles (data not shown).

Escapin preferentially killed bacteria in early-log growth phase over resting cells (Fig. 6), and therefore acts on growing rather than stationary phase cells. Escapin also killed bacteria in a concentration-dependent manner (Fig. 6).

Escapin can be either bacteriostatic or bactericidal against *E. coli*. The bacteriostatic effect occurs in either minimal medium (e.g. M9+glucose) or in enriched medium (e.g. LB). Fig. 7A shows this effect, in which growth in the absence of escapin ('Control') is indicated as a line with a positive slope, and an absence of growth in the presence of escapin ('Esc') is indicated by a flat line. Hydrogen

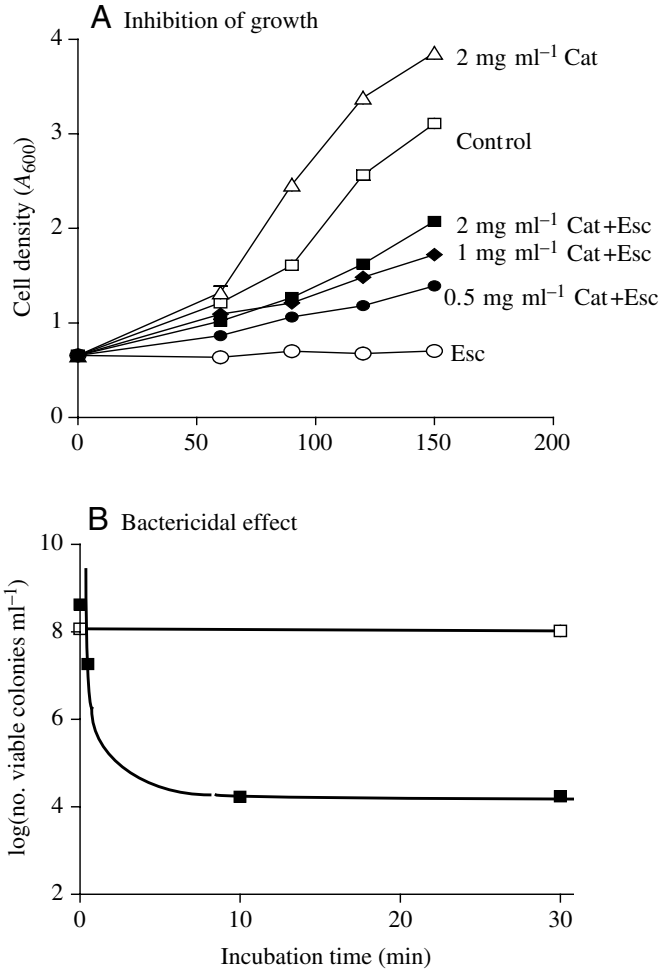


Fig. 7. Escapin can be bacteriostatic or bactericidal. (A) Inhibition of growth. *E. coli* cells were incubated in LB medium at 37°C with 10 μg ml^{-1} escapin (Esc) or without escapin (Control: buffer added instead), and with or without 0.4–1.6 mg catalase (Cat). Absorbance at 600 nm was measured at the indicated incubation times to determine cell density. This experiment was repeated twice more with similar effects. (B) Bactericidal effect. *E. coli* cells were grown in LB medium containing 3×10^8 cells ml^{-1}, then incubated in escapin (10 μg ml^{-1}) in LB medium at 37°C (closed squares) or 0°C (open squares). Values are means ± S.E.M., *N*=3.

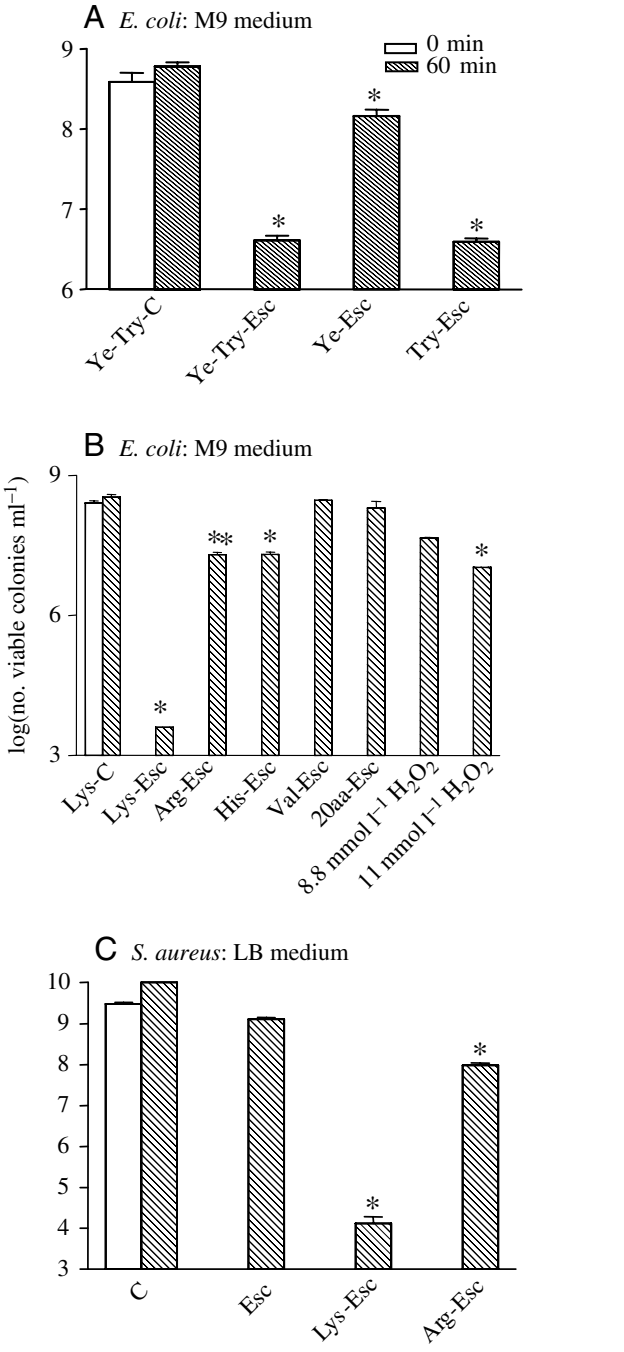


Fig. 8. Escapin's bactericidal effect was greatest in the enriched growth medium and L-lysine. (A,B) *E. coli* and (C) *Staphylococcus aureus* cells in early-log growth phase were cultured in M9-glucose medium (for *E. coli*) or LB (for *S. aureus*), and 4 samples of equal cell density were resuspended in different growth media in the presence of escapin (Esc: 50 μg ml^{-1}) or (C) buffer control. Cells were then incubated at 37°C for 60 min. Ye, 1% yeast extract; Try, 1% Tryptone Peptone; Lys, 50 mmol l^{-1} L-lysine; Arg, 50 mmol l^{-1} L-arginine; His, 50 mmol l^{-1} L-histidine; Val, 50 mmol l^{-1} L-valine; aa, amino acid mixture containing 20 L-amino acids each at 50 μmol l^{-1}. In a follow-up experiment, a 10 times higher concentration of Ye or aa was used, and similar results were observed. Values are means ± S.E.M., *N*=3. An asterisk indicates a significant reduction in the number of viable clones (P<0.05, *t*-test).

peroxide, which is produced under the conditions of this assay, appears to be necessary for bacteriostasis, since the addition of catalase, a scavenger of hydrogen peroxide and other free radicals, strongly reduced bacteriostasis in a concentration-dependent manner (Fig. 7A). The observation that catalase alone (in the absence of escapin) actually enhanced bacterial growth (Fig. 7A: compare '2 mg ml^{-1} Cat' *vs* 'Control') might result from the catalase scavenging naturally produced hydrogen peroxide or other free radicals. Escapin's bactericidal effect in enriched medium is shown in Fig. 7B. Killing occurred rapidly, within 10 min, and was maintained for up to 2 h when cells were incubated at 37°C (Fig. 7B). No killing occurred at 0°C (Fig. 7B). Hydrogen peroxide was also produced under these assay conditions (see earlier results).

The bactericidal effect of escapin occurred in enriched media but not minimal media, even though minimal media supported growth inhibition. When *E. coli* cells were grown in minimal medium (M9) alone or M9 plus yeast extract, escapin was either weakly or not at all bactericidal (Fig. 8A). However, when *E. coli* cells were grown in M9 plus Tryptone Peptone, escapin was strongly bactericidal (Fig. 8A).

The strong enhancement of escapin's bactericidal effect on *E. coli* also occurred by adding L-lysine to minimal medium. L-Lysine at 50 mmol l^{-1} dramatically enhanced escapin's bactericidal effect (Fig. 8B). L-Arginine and L-histidine at 50 mmol l^{-1} produced a small enhancement of escapin's bactericidal effect, whereas L-valine or other amino acids did not affect escapin's bactericidal effects (Fig. 8B). A similar large enhancement of escapin's bactericidal effect by L-lysine but not by L-arginine or other L-amino acids was also seen for *Staphylococcus aureus* incubated in LB medium. The killing effect by escapin on *S. aureus* in LB medium was not apparent, presumably because of the presence of a naturally occurring catalase (Fig. 8C).

To examine the concentration dependency of the ability of L-amino acids to mediate escapin's antimicrobial effects, we performed both types of assays with *E. coli*, using amino acid concentrations up to 50 mmol l^{-1}. In the growth inhibition plate assay, escapin inhibited bacterial growth with a similar concentration dependence and threshold in L-lysine and L-arginine (Fig. 9A). Escapin did not inhibit growth of *E. coli* in the presence of up to 50 mmol l^{-1} L-tyrosine, L-histidine, or any other amino acid (Fig. 9A). Hydrogen peroxide alone (without either escapin or amino acids) had a concentration dependence and threshold to bacterial growth inhibition that was similar to that of lysine and arginine in the presence of escapin (Fig. 9A), demonstrating that hydrogen peroxide is sufficient to mediate growth inhibition. Together with the observation that hydrogen peroxide is also largely or completely necessary for growth inhibition (Fig. 7A), these results are consistent with the idea that escapin exerts its inhibition of bacterial growth through hydrogen peroxide, which is a product of escapin's oxidation of either L-lysine or L-arginine.

In contrast, the bactericidal effect of escapin occurred in a concentration-dependent fashion only for L-lysine (Fig. 9B). L-arginine at any concentration did not mediate escapin's bactericidal effect, even though L-arginine is a good substrate for escapin's LAAO activity. L-arginine's inability to mediate escapin's bactericidal effect is similar to L-tyrosine, which unlike L-tyrosine is a poor substrate for escapin's LAAO activity (Fig. 9B). In addition, hydrogen peroxide at 8–11 mmol l^{-1} had no to little bactericidal effects on *E. coli* (Fig. 8B), even though L-lysine is an excellent substrate for escapin's generation of hydrogen peroxide at 8–11 mmol l^{-1} greatly enhanced escapin's bactericidal effects (Fig. 9B).

The bactericidal effect of escapin did not depend directly on

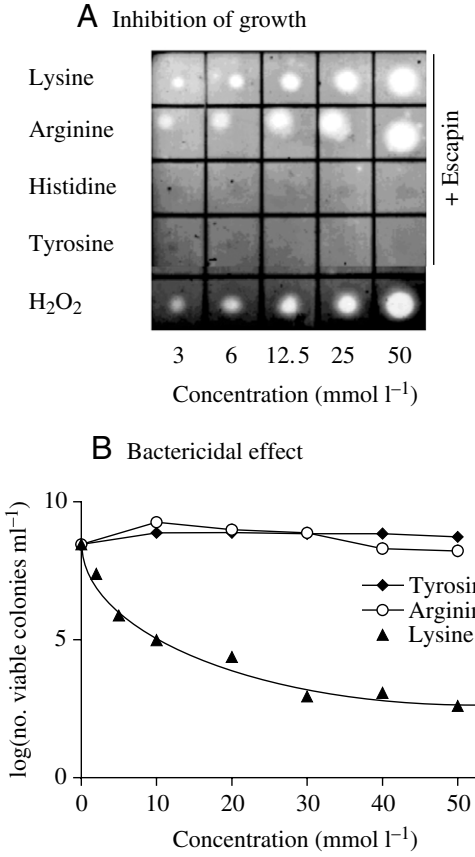


Fig. 9. Concentration dependence of the effects of amino acids on escapin's antimicrobial activity. (A) Plate assay of growth inhibition. *E. coli* cells were grown at 37°C on a plate in minimal medium, (M9+glucose) in the presence of amino acids and escapin, or in hydrogen peroxide alone (without escapin) at concentrations from 3 to 50 mmol l^{-1}. (B) Bactericidal assay. *E. coli* cells were grown in M9+glucose containing 3×10^8 cells ml^{-1}, then incubated at 37°C with escapin (60 μg ml^{-1}) in M9+glucose with L-lysine, L-arginine, or L-tyrosine at 3–50 mmol l^{-1}. Values are means ± S.E.M., N=3.

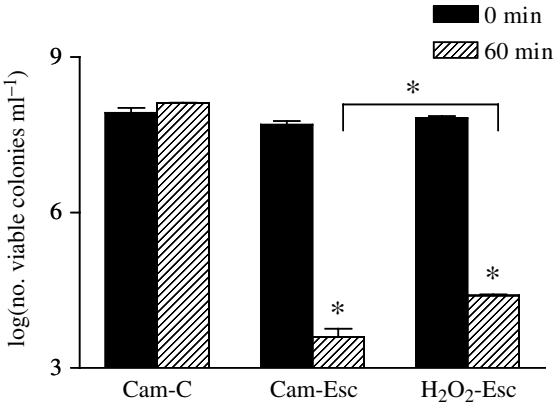


Fig. 10. Escapin's bactericidal effect does not require protein synthesis. *E. coli* at 2×10^8 cells ml^{-1} were incubated with or without 50 μg ml^{-1} chloramphenicol (Cam), an inhibitor of protein synthesis, in the presence of 50 μg ml^{-1} escapin (Esc) or buffer control (C) in LB medium at 37°C. Values are means ± S.E.M., N=3. An asterisk indicates a significant reduction in the number of viable clones (P<0.05, t-test).

protein synthesis, since addition of chloramphenicol, a protein synthesis inhibitor, did not affect escapin's bactericidal effects in LB medium. In contrast, it enhanced the killing effect by at least one-log unit (Fig. 10).

Finally, since some of escapin's antibacterial effects depend on the concentration of L-lysine in the growth medium, and since the microbes tested in this study (Table 1) used different growth media, we wanted to know whether the different inhibitory efficacies of escape were dependent on the lysine concentration in the media. So, we analyzed the concentration of lysine and other free amino acids in the media, using an ion exchange, post-column ninhydrin detection system (Beckman Model 6300/7300 Amino Acid Analyzer, The Scientific Research Consortium, Inc.: www.aminoacids.com). We found that the media contained the following concentrations of lysine: tryptone peptone medium (for bacteria), 2.636 mmol l^{-1}; YEPD (for yeast), 4.980 mmol l^{-1}; and Sabouraud Dextrose (SD) medium (for fungus), 0.812 mmol l^{-1}. Thus, the lysine concentration was different in the media. But even so, lysine concentration cannot explain the lower inhibitory efficacy of yeast compared to bacteria because yeast has lower efficacy even though yeast medium has a higher lysine concentration: yeast MIC of 5 μg ml^{-1} at 4.980 mmol l^{-1} lysine, *E. coli* MIC of 0.62 μg ml^{-1} at a lysine concentration of 2.636 mmol l^{-1}. We increased the concentration of L-lysine in SD medium to 4.980 mmol l^{-1}, and found that its inhibitory efficacy against fungus increased (MIC of 62 μg ml^{-1} in 0.812 mmol l^{-1} lysine, and 15 μg ml^{-1} in 4.980 mmol l^{-1}). Thus, the inhibitory efficacy of escapin against different types of microbes is dependent on lysine concentration, with greater inhibition at higher lysine concentrations (results not shown). But nonetheless, escapin has different efficacies against different microbes that are independent of lysine concentration, with the relatively efficacies being bacteria>yeast>fungi for the species tested.

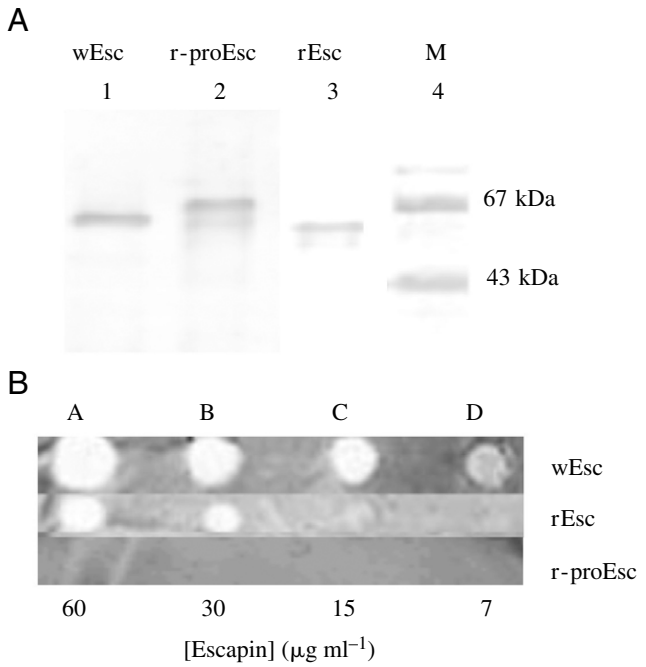


Fig. 11. Recombinant escapin and its antibacterial activity. (A) Western blots demonstrating successful expression of recombinant escapin. (B) Plate assay of inhibition of growth of *E. coli* by wild-type escapin and recombinant escapin. *E. coli* cells in stationary phase were grown at 37°C as a lawn on LB solid medium and tested against wild-type escapin and recombinant escapin at 7–60 μg ml^{-1}. Concentrations of wild-type and recombinant escapin were estimated from western blots. WEsc, wild-type escapin purified from ink; r-proEsc, recombinant escapin, with 18 amino acid signal peptide, in *E. coli* cell lysate; rEsc, recombinant in *E. coli* cell lysate, escapin without signal sequence (i.e. lacking 18 amino acids at N terminus); M, molecular mass markers.

Expression of bioactive recombinant escapin in E. coli *system*

Since escapin is at most minimally glycosylated, we determined whether it is possible to use prokaryotic systems to produce bioactive escapin. We used two recombinant sequences: full-length escapin (rEsc) and escapin precursor (r-proEsc). r-proEsc is rEsc plus the 18-amino acid signal peptide and 29 additional amino acids from the vector added to the N terminus. This construction is the only one that can be stably expressed, because the clone without the extra amino acids from the vector in the signal peptide results in a truncated protein (data not shown). The r-proEsc and rEsc genes were cloned into pET20b and pET 29a, respectively, and both were transformed into *E. coli* BL21 (λDE3) strain and expressed as described in the Materials and methods. Soluble recombinant proteins with lower expression levels were obtained when proteins were induced at lower temperature (20°C) and at a shorter time. Approximately 0.2 mg of soluble rEsc was expressed per liter of bacterial culture medium. In contrast, inclusion bodies with higher expression levels were obtained under induction at 37°C. Thus, denatured recombinant escapin precursor (r-proEsc) was used for production of antiserum. Expressed r-proEsc and rEsc were confirmed by western blot to be present in the *E. coli* cell lysate (Fig. 11A). As expected, r-proEsc containing a signal peptide showed the highest molecular mass. However, rEsc ran slightly lower on the gel than did native or wild-type escapin (wEsc). Antibacterial activity of the recombinant proteins was determined using plate assays with lawns of cells grown on solid LB medium. For the Gram-negative *E. coli*, rEsc had antibacterial activity (Fig. 11B), although its activity was 3–4 times less than that of wEsc. r-proEsc had no activity on *E. coli*. Similar results were obtained when these expression products were tested against the Gram-positive bacterium *Staphylococcus aureus* (data not shown).

Discussion

This study is the first time in which a sea hare antimicrobial protein has been functionally expressed in a bacterial system. These proteins have been of interest to biomedical researchers for more than a decade, largely for the promise they show both as antitumor agents and as a new class of broad antimicrobial

compounds. They may also be of use as antifouling agents in aquatic environments. They are also of interest to chemical ecologists for the potential role that they may play in protecting sea hares from microbial infections or from predators, and in protecting their egg masses from both predators and biofoulers. The research presented here focuses primarily on the practical antimicrobial activity of escapin. However, there are implications for how escapin, as the dominant protein in the defensive ink secretion of *Aplysia californica*, may also function as an antipredatory or antiseptic compound.

L-amino acid oxidase (LAAO) activity of antimicrobial and antineoplastic agents from a variety of species has been reported as a major (Iijima et al., 1995; Suhr and Kim, 1996; Torii et al., 1997; Ehara et al., 2002; Lu et al., 2002; Wei et al., 2003; Butzke et al., 2004) or partial (Jimbo et al., 2003; Kanzawa et al., 2004) mechanism for their effects. Escapin also possesses LAAO activity: it shows strong and rapid activity under our assay conditions when using arginine or lysine as a substrate, with reactions being completed within 30 s at room temperature (Fig. 4). Similar to other LAAOs from snails and snakes, escapin is a flavoprotein. We show that the flavin in escapin is FAD (Fig. 3). Escapin has one potential glycosylation site, but this is probably not essential for its antimicrobial activity, since bacterial-expressed escapin is bioactive (Fig. 11). Dactylomelin-P, a homologue of escapin in *Aplysia dactylomela*, was likewise found to have no or minimal glycosylation (<0.05% carbohydrate by mass; Melo et al., 2000) and was still fully functional. This is in contrast to other reports that for related LAAOs, the sugar moiety is necessary for antimicrobial effects (Obara et al., 1992; Takamatsu et al., 1995; Ogawa et al., 1999; Torii et al., 2000; Petzelt et al., 2002).

Escapin has a broad antimicrobial spectrum compared to many known antimicrobial agents, and thus far escapin has been effective against all tested microorganisms (Table 1). It was most effective against the bacteria found in the marine environment (*Vibrio harveyi* and *Staphylococcus aureus*), so it can be highly active against microbes that occur in the environment in which escapin normally acts. Escapin was also effective against pathogenic species (*Staphylococcus aureus*, *Streptococcus pyogenes*, and *Pseudomonas aeruginosa*), demonstrating its potential in use against medically important species.

Escapin is an effective inhibitor of many microbes (Table 1), but we mostly used *E. coli* in our characterization of antimicrobial effects of escapin. Escapin is highly stable, having no or little loss of activity after multiple freeze–thaw cycles and after more than 5 months storage at room temperature (Fig. 5). Its activity is concentration dependent (Fig. 6). Escapin exerts its bactericidal effects preferentially against fast growing cells (Fig. 6) and does so very quickly – within 10 min (Fig. 7).

Escapin is bacteriostatic in minimal media and bactericidal in enriched media (Fig. 7). Unlike previous reports of similar proteins isolated from sea hares (Kisugi et al., 1989; Yamazaki et al., 1990; Melo et al., 1998, 2000), we demonstrate that substrates determined the bactericidal or bacteriostatic effects of escapin. Bacteria cultured in M9 and glucose medium without high levels (50 mmol l^{-1}) of selected amino acids or without Tryptone Peptone supplements were not killed (Figs 7, 8). Escapin's bacteriostatic effect seems to be mediated through its oxidation of the L-amino acids lysine and/or arginine and the consequent subsequent production of hydrogen peroxide. Both lysine and arginine are substrates for escapin's oxidase activity (Fig. 4). Hydrogen peroxide is sufficient to cause bacteriostasis, and does so with a concentration dependency and threshold (ca. 3 mmol l^{-1}) similar to that of amino acids as substrates for escapin (Fig. 9A). Thus, hydrogen peroxide appears to be both necessary (Fig. 7) and sufficient (Fig. 9) for bacteriostasis under our conditions, and likely mediates escapin's bacteriostatic effects.

We have identified L-lysine as a major cofactor in escapin's bactericidal effect. Lysine is much more effective than arginine in enhancing escapin's bactericidal activity. Lysine's effect is over three-log units greater than that of arginine at the same concentration (50 mmol l^{-1}; Fig. 8B), and lysine mediates the bactericidal effect with a threshold of ca. 3 mmol l^{-1} whereas arginine is ineffective in killing bacteria at concentrations as high as 50 mmol l^{-1} (Fig. 9B). This is true even though arginine and lysine have similar LAAO activities (Fig. 4) and similar thresholds for bacteriostasis (Fig. 9A). In addition, arginine's limited enhancement of escapin's bactericidal effect is similar to that of histidine (Fig. 8), even though arginine has much greater LAAO activity than histidine (Fig. 4). Thus LAAO activity alone, and the resultant production of hydrogen peroxide, cannot explain escapin's pronounced killing effect in the presence of lysine as compared to arginine. Hydrogen oxide production thus plays little to no direct role in escapin's bactericidal effect. We are currently examining escapin's bactericidal mechanisms.

Analysis of the free amino acids found in the opaline secretion of *Aplysia californica* show that it has a very high lysine concentration (65 mmol l^{-1}), while the purple ink secretion, containing escapin, has none (Kicklighter et al., 2005). Only small amounts of arginine (<0.4 mmol l^{-1}) are found in opaline and none in ink (Kicklighter et al., 2005). This raises the possibility that the lysine is mixed with escapin, which is only in ink, when ink and opaline are released and mixed by sea hares following attack by predators. In fact, ink and opaline are normally co-released and mixed in the sea hare's mantle cavity, then pumped toward the attacking predator (Johnson and Willows, 1999). It should be noted, however, that the lack of any detectible lysine or arginine in the ink could also be a result of stores being used as substrates by escapin by the time we collect the ink and thus prior to analysis of free amino acids. We are currently conducting experiments to evaluate this and related hypotheses. Understanding the natural roles that escapin plays for sea hares will likely aid our understanding of how it may function as a practical antimicrobial or antineoplastic agent.

Given our finding of a lysine-dependent antimicrobial effect

for *E. coli*, it may be that the different inhibitory efficacies of escapin against the species of microbes tested (Table 1) were due to the concentration of lysine in their growth media (which are necessarily different). Although the concentration of lysine did influence the efficacy of escapin against a fungus to a small degree (Table 1), nonetheless escapin had different efficacies against different microbes when tested in media with similar lysine concentrations, with escapin being most potent against bacteria and least against fungi for the species tested.

Although the mechanisms of antimicrobial activity of escapin are not yet known in detail, our results give some suggestions. Escapin preferentially kills bacteria that are metabolically active (i.e. in log-growth phase *vs* stationary growth phase; Fig. 6). An interesting observation was that although escapin exerted its bactericidal effect in a concentration-dependent manner, the killing never reached 100% as the protein concentration increased or over longer time periods (Figs 6, 7). In addition, these cells were not resistant to escapin, since they were sensitive to more cycles of killing when re-inoculated with fresh medium (data not shown). These results suggest the existence of persister cells, which neither grow nor die in the presence of microbial antibiotics (Keren et al., 2004), and that more persisters were produced in stationary phase than in log-growth phase at the same protein concentration (as in other studies: Balaban et al., 2004; Keren et al., 2004). Thus, it appears that escapin preferentially kills cells that are metabolically active. In this way, escapin is similar to other antimicrobial agents such as penicillin, streptomycin, ampicillin and O^{-}oxacin in being bactericidal on log-growth phase cells and not stationary cells, in contrast to bismuth, which is bactericidal on stationary cells but not log-growth phase cells (Davis et al., 1990; Coudron and Stratton, 1995; Herbert et al., 1996).

The existence of persister cells in our cultures of *E. coli* may explain why several sea hare homologues of escapin, including aplysianin-E (Kisugi et al., 1989), aplysianin-P (Yamazaki et al., 1990) and dactylomelin-P isolated from purple fluid of *Aplysia dactylomela* (Melo et al., 2000), have been characterized as bacteriostatic and not bactericidal, whereas we found escapin to be bactericidal. To test this idea, we extracted and purified dactylomelin-P from *Aplysia dactylomela* and found that it was also bactericidal under our assay conditions; K.-C. Ko and H. Yong, unpublished data). These differences in our results with escapin and dactylomelin-P *vs* those of the earlier studies of related proteins could be due to the presence of persister cells in our culture conditions; other explanations could include methodological differences such as the concentrations of lysine in culture media or the species and strains of microbes used.

In addition to escapin's preferential killing of bacteria that are metabolically active, escapin also kills cells in the presence of chloramphenicol to stop protein synthesis (Fig. 10), and it does so without lysing the cells. Comparison of the mechanisms of action of escapin with other antimicrobial agents can give some clues as to escapin's mode of action. Streptomycin, which affects ribosomes and cell membrane, is similar to escapin in that it kills bacteria without lysing cells but dissimilar in requiring protein synthesis for its bactericidal effect (Davis et al., 1990; Coudron and Stratton, 1995). Penicillin, which inhibits cell wall formation, is more dissimilar to escapin in that it kills bacteria through lysis and requires protein synthesis for its effect (Davis et al., 1990; Coudron and Stratton, 1995).

In summary, escapin is both bacteriostatic and bactericidal, and its bactericidal effect does not require protein synthesis or cytolysis. A more complete understanding of the cellular mechanism of escapin's bactericidal effects awaits further investigation.

Finally, we expressed bioactive recombinant escapin in a bacterial system, the first reported success of this for snail and snake LAAOs. Our expression level for soluble recombinant escapin was ca. 0.2 mg l^{-1} culture medium. This level may be relatively low for two reasons. First, this level is for soluble escapin, while much of the escapin is present in insoluble inclusion bodies. Second, the escapin inhibits growth of *E. coli* and other bacteria at doses below 1 mg l^{-1} (Table 1), and this would likely inhibit the levels of bacterial expression. The fact that bacterially expressed recombinant escapin is bioactive shows that glycosylation of escapin, which is at best minimal, is not essential for its antimicrobial activity. The fact that recombinant escapin is 3–4 times less effective against bacteria than is wild-type escapin might be because the recombinant form contains inactive escapin, which could also explain the double band for recombinant escapin in Fig. 11A. This could be tested by examining the specific activity of the recombinant and wild-type forms, but this will require higher levels of bacterial expression of recombinant escapin than we presently have. We are currently working on further improving expression of bioactive recombinant escapin in *E. coli* and *S. aureus*, and hope that this will enhance its viability as a new antimicrobial agent.

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