Spheniscins, Avian β-Defensins in Preserved Stomach Contents of the King Penguin, *Aptenodytes patagonicus*

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During the last part of egg incubation in king penguins, the male can preserve undigested food in the stomach for several weeks. This ensures survival of the newly hatched chick, in cases where the return of the foraging female from the sea is delayed. In accordance with the characterization of stress-induced bacteria, we demonstrate the occurrence of strong antimicrobial activities in preserved stomach contents. We isolated and fully characterized two isoforms of a novel 38-residue antimicrobial peptide (AMP), spheniscin, belonging to the β-defensin subfamily. Spheniscin concentration was found to strongly increase during the period of food storage. Using a synthetic version of one of two spheniscin isoforms, we established that this peptide has a broad activity spectrum, affecting the growth of both pathogenic bacteria and fungi. Altogether, our data suggest that spheniscins and other, not yet identified, antimicrobial substances may play a role in the long term preservation of stored food in the stomach of king penguins.

Lack of food resources induced by climatic changes may impair the breeding success of wild animals. For king penguins, the female usually comes back from foraging at sea to feed the chick at hatching. However, in relation to the circumpolar wave, thought to be linked with the El Niño Southern Oscillation phenomenon (1), the return of the female may be delayed due to the necessity of foraging at a greater distance than usual. The survival of the chick can still be ensured by the male, as it can provide food preserved in its stomach during the final 3 weeks of incubation (2, 3). A remaining unanswered question is how can food be preserved from attack by microorganisms, when the rich 38 °C buffered growth medium (3, 4) and a long retention time in the stomach would be expected to favor microbial colonization (5, 6). The maintained mass and energetic value of the food throughout the fast (3) and the stressed-induced characteristics of the stomach bacteria (4) altogether suggest the existence of a protection mechanism against food degradation by microorganisms.

Like other mucosal surfaces, the gastrointestinal tract surface interacts directly with the external environment and therefore has to be protected from damage and invasion by ingested or indigenous microorganisms. One established facet of epithelial host defense is the synthesis and secretion of AMPs (7, 8). The role of these AMPs is not limited to epithelial protection through innate immune responses since they can attract human blood cells, alert the adaptive immune system and induce gastrointestinal secretions (8–10). It has also been assumed that some AMPs influence the resident microflora in the small intestine lumen (11, 12). An additional role could then be the participation of AMPs in the control of microbial proliferation, contributing to the preservation of retained food.

In the present study, we found numerous, strongly active antimicrobial substances in the stomach contents of male penguins that efficiently conserve food during their incubation fast. Among these substances, two isoforms of a novel 38-residue AMP were identified and named spheniscin. Spheniscin belongs to the β-defensin subfamily, a well documented AMP group in vertebrates. Its concentration is markedly higher in conserving than in digesting birds and, in the former, it increases during the period of food conservation up to the micromolar level. Using a synthetic version of spheniscin, we demonstrated that this peptide was active against Gram-positive and Gram-negative bacteria and filamentous fungi including some human pathogens. The results of this study therefore support the idea that the identified spheniscin, in addition or in synergy with other not yet identified AMPs, may play a role in the preservation of penguin stomach contents during the incubation fast.

**EXPERIMENTAL PROCEDURES**

**Birds and Food Sampling**

The study was carried out on Possession Island (46°25’S, 51°45’E), Crozet Archipelago, during the incubation period of the breeding cycle of the king penguin, *Aptenodytes patagonicus*, from December 2000 to March 2001. Stomach food sampling of seven male king penguins was performed at three points during the fast, which occurs over last part of incubation. The sampling time points were at the beginning and the middle (7 days after first sampling) of incubation, then at the end of incubation after relief by the female. Birds were handled with extreme care to minimize stress and potential injury. Handling did not result in breeding failure as the birds thereafter continued the incubation task.

Samples of stomach contents were collected non-invasively by sucking up food with a sterile rubber tubing introduced via the bill down into the stomach. About 60 ml of food were retrieved and homogenized in a cold and sterile tube. Aliquots for AMPs study were maintained at

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**The amino acid sequences reported in this study have been deposited in the SwissProt Database (accession number(s) Sphe-1: Swiss-Prot P83429 and Sphe-2: Swiss-Prot P83430).**

* The abbreviations used are: AMP, antimicrobial peptide; ACN, acetonitrile; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MIC, minimal inhibitory concentration; ANOVA, analysis of variance; rm ANOVA, ANOVA for repeated measures; RP-HPLC, reverse-phase high performance liquid chromatography; Sphe-1, spheniscin-1; Sphe-2, spheniscin-2.*

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selected test organisms: Escherichia coli and Micrococcus luteus. The energetic value of food (through lipid content analysis). These criteria allowed the distinction to be made between birds that conserved (n = 3) or digested (n = 3) their stomach contents during the incubation fast. Another bird that conserved its stomach content during the first part of the fast and digested it during the second part was studied separately.

**Extent of Stomach Content Conservation**

To determine the level of food preservation three criteria were selected: (a) the presence of bile pigment coloration from duodenogastric reflexes, indicating active gastric digestion; (b) the evolution of the food pH during the fast, close to pH 2 being the optimum for gastric digestive enzymes in birds (13); (c) the energetic value of food (through lipid content analysis). These criteria allowed the distinction to be made between birds that conserved (n = 3) or digested (n = 3) their stomach contents during the incubation fast. Another bird that conserved its stomach content during the first part of the fast and digested it during the second part was studied separately.

**Cationic Peptides Extraction and Prepurification by Solid Phase Extraction**

Frozen food samples were ground on ice using an ultra-turrax and then sonicated (5 × 30 s bursts separated by 30-s cooling periods) in 0.2% trifluoroacetic acid (1:10 w/v) containing aprotinin (Sigma) as a protease inhibitor (22.5 μg/ml). Sample pH was adjusted to between 2.5–3.0, and the samples were left agitating overnight at 4 °C. Extracts were centrifuged at 10,000 rpm for 10 min at 6 °C, and the supernatants were prepurified by solid phase extraction on Sep-Pak C18 Cartridges (Waters). Peptides were eluted with 80% acetonitrile (ACN) acidified with 0.05% trifluoroacetic acid (acidified water) and freeze-dried.

**Sphensiscin Purification and Characterization**

**Sphensins Purification**—6 mg of extract were subjected to reverse-phase high performance liquid chromatography (RP-HPLC) on Aquapore RP-300 C18 column (250 × 7 mm, Brownlee™) using a 2–72% linear gradient of ACN in acidified water at a flow rate of 1.3 ml/min. The manually collected fractions were freeze-dried, then resuspended in 150 μl of ultrapure water, and aliquots were assayed for antimicrobial activity.

The RP-HPLC fraction with the broadest activity at the end of the fast (see Fig. 1C, star) was further subjected to RP-HPLC on an Aquapore OD-300 column (220 × 4.6 mm, Brownlee™), using a linear biphasic gradient of ACN in acidified water. Fractions were manually collected, then freeze-dried prior to resuspension in 70 μl of ultrapure water and assayed for antimicrobial activity. The active compounds were finally purified to homogeneity in a third step on the same column as above, using linear biphasic gradients of ACN in acidified water.

During the course of purification, the purity of the fraction was controlled by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDE-TOF-MS), performed on a Bruker Daltonics (Bremen, Germany) BIFLEX IIITM mass spectrometer operating in positive ion linear mode as described previously (14).

**Reduction and S-Pyridylethylation**—An aliquot of purified sphenins was subjected to reduction and alkylation as previously described (15). The S-pyridylethylated peptides were desalted by RP-HPLC on a narrow bore Delta Pak HIC18 column (150 × 2 mm, Waters™) with a 2–60% linear gradient of ACN in acidified water at a flow rate of 0.2 ml/min, analyzed by MALDI-TOF-MS, and sequenced using automated Edman degradation on a pulse liquid automatic sequencer (Applied Biosystems Inc., model 473A).

**Enzymatic Digestion**—The S-pyridylethylated peptides were treated with chymotrypsin (Roche Applied Science) at an enzyme/substrate ratio of 1:20 (w/w) and digestion products were characterized by MALDI-TOF-MS.

**Disulfide Array Assignment**—Purified sphenins (native or synthetic) were treated with trypsin (Roche Applied Science) at an enzyme/substrate ratio of 1:20 (w/w) and digestion products were characterized by MALDI-TOF-MS.

**Screening Bioassays**—Antimicrobial activity was determined against selected test organisms: Esherichia coli SBS 363 (Gram-negative bacteria), Micrococcus luteus (Gram-positive bacteria), and Neospora crassa (fungus). Activity was detected using liquid growth inhibition assays in microtiter plates as previously described (16).

**Sphensins Quantitation**—For all food samples, the RP-HPLC fractions initially collected between a 2 and 72% gradient of ACN were checked for the presence of both forms of sphenins by MALDI-TOF-MS. For each food sample, the fractions of interest were pooled and subjected to successive RP-HPLC until sufficient purification was achieved. The sphenins concentration was determined using capillary zone electrophoresis (details of the procedure in Ref. 17) by comparing peaks from the fraction analyzed and a calibrated solution of highly purified sphenins. The quantities were normalized to the amount of initial food sample collected. Two-way ANOVA for repeated measures (rm ANOVA) followed by multiple comparison (Tukey test) was performed using the SigmaStat software (Jandel-SPSS), and values quoted in results are means ± S.E.

**Activity Spectrum of Synthetic Sphinchen-2**—The activity spectrum of sphensins-2 was estimated using a synthesized version of the molecule (Altergen Laboratory, France). The integrity, purity, and correct refolding of the synthetic peptide were confirmed by MALDI-TOF-MS and treatment with trypsin (Roche Applied Science), as described above. The activity spectrum (minimal inhibitory concentration, MIC) of the synthetic peptide (concentration range, 0.75 μM up to 100 μM) was determined against bacteria, yeast, and fungi using liquid growth inhibition assays (16). Bactericidal or bacteriostatic effects were measured by colony forming unit counting at +24 h. When the antimicrobial activity was assayed in different pH conditions, the pH of the medium was adjusted with hydrochloric acid. Two positive controls MSL-94 (a broad spectrum linear amphiphatic magainin variant, Ref. 18) and chanatin (a broad spectrum antifungal peptide, Ref. 19) were used. MSL-94 was a gift from Dr. M. A. Zasloff (Magainin Scientific Institute, Plymouth Meeting, Philadelphia, PA). The MIC value corresponded to the interval of concentration [a] – [b], where [a] is the highest concentration tested at which the bacteria are growing and [b] is the lowest concentration that cause 100% inhibitory growth (20).

The strains used were those previously reported (21, 22) with the addition of the following strains: Bacillus cereus ATCC 11778 (Pasteur Institute Collection, Paris), Alcaligenes faecalis, Staphylococcus saprophyticus, S. haemolyticus, and Nocardioides asteroides (Dr. Montell and Piémont, Institute of Bacteriology, University of Strasbourg, France), E. coli SBS 363 (Dr. Bouquet, Centre d’Études Nucléaires, Saclay, France), VibrioMetchnikovi, and V. anguillarum (Dr. Bachère, IFREMER, Montpellier, France), Candida albicans IHEM 8060 (EnToMed, Strasbourg, France), and C. tropicalis (Dr. Koening, Hôpital Civil, Strasbourg, France).

**RESULTS**

**Detection of Antimicrobial Substances in the Stomach Contents of Penguins**

**Detection of Several Antimicrobial Substances**—Following solid-phase extraction of the acidified extracts from stomach contents of king penguins, RP-HPLC revealed the presence of numerous fractions with antimicrobial activity mostly in the elution zone between 20 and 40% ACN (Fig. 1). Some were active against one, two, or all of the three tested microorganisms, namely M. luteus, E. coli, and N. crassa. Fractions with antimicrobial activity were more frequent in the stomach contents of birds conserving food, particularly at the onset and middle of the fast (Fig. 1). Furthermore, most recorded antimicrobial activity corresponded to full growth inhibition in samples from conserving birds, while growth delay was predominant in digesting birds, suggesting higher concentrations of AMPs in samples from the conserving birds (Fig. 2). In conserving birds, fractions active against the three tested microorganisms were present for each fasting period (Fig. 2A). Conversely, a fraction active against E. coli was present in only one single sample from the digesting birds (Figs. 1C and 2B).

**Isolation and Identification of the Antimicrobial Substances from the Most Active RP-HPLC Fraction Observed in Conserving Birds**—As the strongest activities were observed in the fraction eluted at 29% ACN in conserving birds (Fig. 1C, star), an extensive purification was performed on this fraction. Two compounds with molecular masses of 4482.8 MH+ and 4501.7 MH+ were obtained (Fig. 3A). Mass spectrometry analysis after reduction and S-pyridylethylation indicated that the two compounds contained six cysteine residues involved in the formation of three internal disulfide bridges (Fig. 3B, mass excess of 6 × 105 Da). Sequencing by Edman degradation on the pyridylethylated peptides yielded two sequences of 38 residues: SFGLC*RLRGRFPC*AH/RGRG*C*RFPSIPIGRC*SRRFXQC*C*R RXWX, where C* stands for a pyridylethylated cysteine, H/R.
stands for two residues found at position 14, and X for unidentified residues. To solve the identity of residues at position 31 and 37, the pyridylethylated peptide was subjected to chymotrypsin digestion and the proteolytic fragments were analyzed by MALDI-TOF-MS. The resulting mass fingerprint established that residues 31 and 37 corresponded to valines. The calculated molecular masses of the peptides with histidine or arginine residues in position 14 were 4482.4 and 4501.4 Da, respectively. Comparison of the calculated molecular masses with those measures by MALDI-TOF-MS (Fig. 3A) confirmed the two sequences (Fig. 3C) obtained by automatic Edman degradation. The disulfide array of the peptide was investigated using trypsin digestion and analysis of the products by MALDI-TOF-MS. One fragment, containing cysteine residues in positions 12 and 27 linked by a disulfide bridge, was observed at MH$/H_1 / H 11001^{915.4}$. A second fragment containing the four other cysteine residues, and including two disulfide bridges, was observed at MH$/H_1 / H 11001^{1709.6}$. These data agreed with folding typical for the $\beta$-Defensin family. In addition, data bank searches (FASTA-genome, NCBI-TBLASTN) revealed that the two peptides isolated were homologous to $\beta$-Defensins from chicken and turkey. The $\beta$-Defensins isolated from the king penguin were named spheniscins-1 (Sphe-1, synonym pBD-1; histidine as residue 14; molecular mass, 4481.8 Da), and spheniscin-2 (Sphe-2, synonym pBD-2; arginine at position 14; molecular mass, 4500.7 Da), after the order name of penguins. Quantification of Spheniscins in the Stomach Content During the Incubation Fast—Sphe-2 was detected in all birds, while Sphe-1 was detected only in the three samples from one of the conserving birds. The concentration of spheniscins in the stomach contents was markedly higher in conserving compared with digesting birds (Fig. 3D; rm ANOVA, $F_{1,17} = 31.2, p = 0.005$), whatever the fasting period (Tukey test, $p < 0.05$). The concentration of spheniscins increased 13-fold from the onset to the end of the fast in conserving birds (74 ± 48 nM and 943 ±
In contrast, spheniscins concentration remained at a low level in digesting birds at all time points (maximum: middle fast, 24 ± 14 nM). Interestingly, a drop in spheniscin concentration was observed during the second part of the fast in one particular bird where food was initially stored then digested (Fig. 3D).

**Activity Spectrum of Sphe-2**—To investigate the antimicrobial activity of spheniscin, a synthetic version of properly folded Sphe-2 was used. Sphe-2 affected the growth of Gram-positive and Gram-negative bacteria, yeast and filamentous fungi (Table I). When used at a range of concentrations identical to that of the broad spectrum control antibiotic peptide (MSI-94), Sphe-2 was highly effective against all the Gram-positive bacteria tested. It had a bactericidal effect against all the Gram-positive bacteria, with the exception of *S. saprophyticus*. Sphe-2 showed activity against most of the Gram-negative bacteria tested. This activity was mainly bacteriostatic, except against *E. coli* 1106 and *V. metshnikovii*. No activity could be detected against the enteropathogenic Gram-positive bacteria *Enterobacter cloacae* and *A. faecalis* at up to 100 μM peptide.

Sphe-2 was also active against yeast and filamentous fungi. While moderately active against *C. albicans* and *C. glabrata* (MIC 50–100 μM and MIC > 100 μM, respectively), Sphe-2...
was highly effective against *C. tropicalis* (MIC = 1.5–3.0 μM). The two filamentous fungi tested were found to be susceptible to Sphe-2 in the same range of concentration as the control antibiotic, thanatin (< 6 μM). Interestingly, Sphe-2 was even more active against human pathogenic strain *Aspergillus fumigatus* (MIC = 3–6 μM) than thanatin (MIC close to 20 μM), and it was even found to inhibit fungal sporulation at 6 μM (Fig. 4).

In order to see if the moderate acidity of the preserved stomach content (pH 4–6) (4) might impair the efficacy of spheniscins in *vivo*, the effect of Sphe-2 on the bacterial growth was tested at pH ranging from 4.2 to 6.1. Sphe-2 is highly effective against *E. coli* throughout this pH range (MIC = 6–12, 25–50, and 6–12 μM at pH of 4.2, 5.2, and 6.1, respectively).

**DISCUSSION**

This study reports the presence of antimicrobial activity in the stomach contents of those king penguins that efficiently conserve food for more than 2 weeks (Fig. 1). This activity was found throughout the RP-HPLC profile, suggesting the existence of several antimicrobial substances. Among the active RP-HPLC fractions, we identified two isoforms of a 38-residue AMP, spheniscin-1 and spheniscin-2. The presence of both isoforms in only one of the birds analyzed suggested a gene polymorphism. Comparison of the primary spheniscin sequences with others in databanks revealed high similarities to vertebrate β-defensins (Fig. 5). The closest similarity was with β-defensins from chicken (50% identity versus 63% including conservative residues) and turkey (47% identities versus 58% including conservative residues) (23). The quantitation of the spheniscins in two types of penguin, those conserving and those digesting stomach contents, initially allowed us to demonstrate that the concentration of spheniscins was markedly higher in penguins with well-preserved stomach contents. Second, it was shown that the maintenance of food preservation throughout the incubation period was associated with an increase in the concentration of spheniscins. This increase could be due to an increase of *de novo* synthesis or to a decrease in peptide degradation. However, as the level of spheniscin increased in the stomach contents whereas the pH was maintained at a constant and high level from the beginning of the fast in preserving birds, it would appear that *de novo* synthesis is the most likely hypothesis. In addition, the observed stability of peptide activity within the 4.2–6.1 pH range strongly suggests that the peptide is not affected by conserving conditions in the stomach.

The stomach contents are conserved in anticipation of feeding the newly-hatched chick in cases where the mate is not back at eclosion (2). Consequently, to remain a valuable nutrient resource, the stomach contents must be protected from degradation by microorganisms during storage. We had previously shown that most of the bacteria in the well preserved stomach food exhibit morphological characteristics of stressed bacteria (sporulated, deformed, or dead cells (4)). These data suggested factors inhibiting bacterial growth in penguins with well preserved stomach contents. It is therefore tempting to assume that spheniscins, among other antimicrobial substances detected, may play a pivotal function in the protection of food storage and protection from digestion by microorganisms introduced with the food. This would not exclude a potential contribution from non-pathogenic outgrowing microorganisms preventing the growth of others.

With regard to the potential for spheniscins to inhibit bacterial and/or fungal growth, synthetic Sphe-2 was found to be active against most of the microorganisms tested. Highest potency was seen against Gram-positive bacteria with significant activity against Gram-negative bacteria and fungi. AMP activity greatly depends on electrostatic interactions (24), and with a large number of positively charged amino acids (9 arginine residues and 1 histidine for Sphe-1; 10 arginine for Sphe-2) and no negatively charged residues present, the spheniscins are highly cationic. Under physiological conditions, the isoelectric points of Sphe-1 and Sphe-2 are pH 12.90 and 12.95 (see Fig. 5). These characteristics could all contribute to the wide activity spectrum observed for synthetic sphenisin. Moreover, penguin β-defensins have a higher proportion of positively charged amino acids as compared with other avian β-defensins, possibly reflecting a greater antimicrobial activity for the penguin molecules *in vivo*. It is important to note that synthetic Sphe-2 was functionally active, *in vitro*, at pH conditions found in the penguin stomach contents.

The inhibition of *A. fumigatus* sporation by sphenisin is similar to that described for stomoxin, an AMP from the anterior midgut of the blood-feeding insect *Stomoxys calcitrans* (25), and for termicin, an antifungal defensin from the termite *Pseudacanthotermes spiniger* (26). *A. fumigatus* is the most frequently isolated strain in invasive aspergillosis found in immunocompromised humans and animals. Birds, and particularly penguins, are known to be especially sensitive to the high activity of sphenisin against this fungus and other potentially pathogenic microorganisms, such as *S. aureus*, *P. aeruginosa*, or *Listeria monocytogenes*, indicates a potential therapeutic capacity of such peptides.

In the mammalian intestinal host defense model presented by Bevins *et al.* (11), an AMP concentration in the micromolar range is estimated to be sufficient for the defensins present in the intestinal lumen to influence the resident microflora. Such a level of sphenisin was found in stomach content samples (see Fig. 3D). At the intestinal epithelial level the estimated concentrations of α-defensin in humans and the cryptdins of mice approach the millimolar level, *i.e.* up to 1,000-fold higher than the estimated concentrations in the intestinal lumen (11, 27). Therefore, the local concentration of sphenisin in the penguin upper digestive tract may easily reach higher levels than those

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**Fig. 5. Sequence alignment of β-defensins from birds (king penguin (Sphe-1, Sphe-2), chicken (Gal-3), and turkey (GPV-1)) and mammals (human (hBD-3) and bovine (EBD, enteric β-defensin)).** Identical or conserved residues are shaded. The conserved cysteine pairings, and the isoelectric points (pI) of these molecules are marked.
found in food samples. The possibility also exists that the additional antimicrobially active fractions found in preserved stomach content samples may complement the in vivo efficacy of the spheniscins through a synergistic effect (28) or act in an additive way (29). The exact mode of action of spheniscins and the other antimicrobial substances found within the stomach contents necessitates further studies, which are underway. In conjunction with our data, these observations made in other studies tend to support the hypothesis that the antimicrobial substances detected in the stomach contents of king penguins are participants in food preservation.

The presence of exogenous microorganisms induces a local immune response at the epithelial surface, which includes the rapid secretion of AMPs (8). In the king penguin stomach, it is possible that such an immune response is induced by exogenous microorganisms which reach the stomach cavity during feeding or as a consequence of pecking behavior of the incubating adults, as well as through the irritant contact of food with the gastric epithelial surface. At this juncture it is also tempting to make the parallel with blood-sucking insects where, following feeding, production of AMPs is thought to aid in the conservation of the blood meal prior to digestion (25, 30). Whatever the direct or indirect effect of AMP secretion on the stomach contents of king penguins, it is critical for the reproductive success of these birds, as conservation of undigested food during the key breeding stage of incubation determines the survival of the newly-hatched chick. Our data further illustrate the general interest that lie in physiological adaptations of free-ranging wild animals, a situation that cannot easily be mimicked in the laboratory (31).

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