Article

Ovipin: a new antimicrobial peptide from chicken eggs *Gallus gallus*

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Abstract: The intensive and indiscriminate use of antibiotic has increased cases of microorganisms resistance and becoming a worldwide public health problem. In the last years, from natural sources such as hen's egg, have received special attention in the discovery of new bioactive compounds. This study aims to identify and characterize a new peptide from chicken egg of *Gallus gallus domesticus*. The chicken egg was subjected the extraction acid, the supernatant was prefractionated in Sep-Pak column and fractionated peptide by reverse-phase high-performance liquid chromatography (RP-HPLC). The antimicrobial activity of the fractions were evaluated through liquid growth inhibition assays. The molecular weight and amino acid sequence was determined by mass spectrometry (MS/MS), the characaterization performed by bioinformatics analysis with Peaks® tool and comparision with the NCBI and UniProt-SwisProt database. The physicochemical parameters of the samples were evaluated with online programs. One fraction named Ovipin peptide, showed antimicrobial activity against Gram-positive bacteria (*Micrococcus luteus* – MIC 1.94 μM) and Filamentous fungus (*Aspergillus niger* - MIC 31.01 μM). The minimum inhibitory concentration against *Cryptococcus neoformans* VNI (WM 148) Yeast was 22.75 μM, this microorganism an opportunistic yeast and mainly binds in immunosuppressed or immunocompromised people. Ovipin is a hydrophoic peptide and not cause significant hemolytic effect against human erythrocytes. Ovipin primary sequence is YVSPVAIVKGLNIPL and a molecular weight of 1,581.94 Da. Ovipin shows 100% and 93.3%, respectively, sequence similarity with the fragments Apolipoprotein B of *Gallus gallus* and Apolipoprotein B of five others species of Aves. Our data suggest that Ovipin peptide could be a potential therapeutic candidate to be used an alternative against infections developed by resistant microorganisms, mainly in the fight against *Cryptococcus neoformans* opportunistic yeast.

Keywords: 1: Ovipin; 2: chicken eggs; 3: antimicrobial; 4: peptide; 5: AMPs.

1. Introduction

Immunocompromised patients presents a high risk of developing infection because in this group there is a decrease or lack the basic mechanisms of cellular defense. These patients are more susceptible to attack by microorganisms, such as bacteria or fungal infection are mainly genus *Aspergillus*, and the genera as *Mucor*, *Fusarium*, *Rhizopus*, *Petriellidium*, *Cryptococcus*, *Histoplasma*, *Coccidioides* and *Candida* (**FRARE e SILVA, 2010**).

Cryptococcosis or cryptococcal infection is a relevant problem global because affect mainly immunocompromised and immunocompetent hosts with high incidence of morbidity and mortality. *Cryptococcus* species are encapsulated yeasts and as an opportunistic microorganism, it can trigger in patients with human immunodeficiency virus (HIV) infection, solid organ transplantation, corticosteroid use, diabetes and cirrhosis. Pulmonary and central nervous system (CNS) are the well-known clinical manifestations of cryptococcosis followed by cryptococcal meningoencephalitis (**GUSHIKEN et al, 2021; ZAVALA, BADDLEY, 2020**). *Cryptococcus spp.* (approximately 30 species) has wide geographical distribution – *Cryptococcus neoformans* and *Cryptococcus gattii* are two species relationship with human disease. (**GUSHIKEN et al, 2021; HENAO-MARTÍNEZ et al., 2018**). The polysaccharide capsule, melanin production, grow at body temperature and capacity to produce extracellular enzymes are the factors which associated with the health state host, confer the success and survival of these microorganisms against the complex responses of the host's immune system (**ZAVALA, BADDLEY, 2020; ALMEIDA et al., 2017**).

The resistance antibacterial or antifungals arising from selective pressure of clinical and agricultural antifungal agent use is a serious concern because limit the options treatment, research into new antibacterial or antifungal agents or new combinations is necessary to control the advancement of cryptococcal disease or others infections disease (**BEN-AMI, KONTOYIANNIS, 2021**).

In this regard, antimicrobial peptides (AMPs) are evolutionarily conserved innate immune defense system and are produced by many organisms (**Peters et al., 2010**), such as Prokaryotes (e.g., bacteria) and Eukaryotes (e.g., protozoan, fungi, plants, insects and animals) (**Izadpanah & Gallo, 2005; Bahar & Ren, 2013**). They are widely studied by presenting a fast and broad spectrum of action against Gram-positive and Gram-negative bacteria, fungi, protozoa and viruses (**Kang et al., 2014; Andreu & Rivas, 1998; Cruz et al., 2014**). Thus presenting the ability to inhibit growth or kill multi-resistant microorganism (**Peters et al., 2010**). Antimicrobial peptides (AMPs) are small (weight <10 kDa) an in general amphipathic molecules, positively or negatively charged with amino acids sequences diversity (**Bahar & Ren, 2013; Cruz et al., 2014**). They are selective for prokaryotic membranes and act on the cell membrane-disruptive of microorganisms through electrostatic interactions and show low natural resistance. Some peptides have no action on

67 the cell membrane, but rather they cross this membrane and act on intracellular targets, which can interfere in crucial
68 processes for the growth and development of microorganisms (**Brogden, 2005**).
69 On the other hand, in the last years has also growth the interest in the search for bioactive molecules in food sources.
70 The functional foods contain amounts and types of bioactive compounds different, which provide health benefits and
71 may decrease the development of chronic diseases (**Gil-Chávez et al., 2013; Rutherfurd-Markwick, 2012**). As a
72 natural source, the chicken eggs with their diversity of structure and variety of components are increasingly becoming
73 targets of research in health or biotechnology (**Hancock & Sahl, 2006**). Chicken eggs is an important dietary source
74 because it is rich in proteins, lipids, vitamins and minerals, but which are associated with other functions such as
75 defense to inhibit the invasion and proliferation of pathogens (**Kovacs-Nolan et al., 2005**).
76 The aim of this work is to performed the bioprospecting on chicken eggs of specie *Gallus gallus domesticus*, with focus
77 in the discovery of new antimicrobial peptides with great potential to act against microorganisms, especially
78 microorganisms that have already developed some resistance mechanism.

79

80 **2. Materials and Methods**

81 **2.1. Extraction acid and Pre-fractionated of Chicken eggs**

82 In this study chicken eggs without the shell of the species *Gallus gallus domesticus* were utilized. They were purchased
83 from the commercial market network in the city of São Paulo/SP.
84 The total chicken eggs crude were submitted to acid extraction in the presence of Acetic Acid 2 $mol.L^{-1}$, with constant
85 agitation for 60 min at 4° C. After the stirring time, the insoluble material was separated by centrifugation at 3113 x g
86 for 30 min and the supernatant obtained was injected into coupled Sep-$Pack_{18}$ cartridges (Waters Associates – 20 cc
87 vac cartridge, 5 g, and particle weight 55-105 μm) equilibrated in trifluoroacetic acid (0,1% TFA). The supernatant was
88 pre-fractionated in three different acetonitrile (ACN) concentrations (5%, 40%, and 80% of ACN), using vacuum
89 manifold system equipment (SPE 12 positions aho 6023 - Phenomenex) and then were lyophilized.

90 **2.2. Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC)**

91 The fractions, 5%, 40% and 80% of ACN with 2 g each, were reconstituted in 5 mL 0.1% TFA and subjected by
92 reverse-phase high-performance liquid chromatography (RP-HPLC) was carried out at room temperature on a
93 Shimadzu LC-8A system. The column was a Shim-Pack Preparative ODS C-18 (20 mm x 250 mm; particle weight 10
94 μm; pore weight 300 Å). The elution was performed with a linear ACN gradient, equilibrated in 0.1% TFA for 60 min

95 at a flow rate of 8.0 mL/min (0 to 20%) for the fraction eluted in 5%, 2 to 60% for the fraction in 40%, and 20 to 80%
96 fraction eluted in 80%. The ultraviolet (UV) absorbance of the effluent was monitored at 225 nm. The fractions were
97 manually collected, were vacuum-dried and suspended in 1 mL in ultrapure water for antimicrobial activity assays.
98 The molecule described in this work, fraction 21, was subjected to a second step of fractionation using linear gradient
99 of ACN (39 to 49%) with 0,05% TFA at a flow rate of 2 mL/min for 60 min on the same column Shim-Pack Preparative
100 ODS C-18 (20 mm x 250 mm; particle weight 10 μm; pore weight 300 Å). The fractions were hand-collected, were
101 vacuum-dried, suspended in 500 μL in ultrapure water and it was quantified based on absorbance 205 nm using a
102 NanoDrop 2000 spectrophotometer (Thermo Fischer Scientific, Waltham, MA, USA).
103
104 **2.3. Microorganisms**
105 Fungal and bacterial strains were obtained and stored in the freezer at -80°C in the Laboratory for Applied Toxinology
106 (LETA), of the Butantan Institute (São Paulo, Brazil). *Staphylococcus aureus* ATCC 29213 (Gram-positive bacteria),
107 *Escherichia coli* D31 (Gram–negative bacteria); *Aspergilus niger* (Filamentous fungus – isolated from bread); and
108 *Cryptococcus neoformans* yeast strain A serotype (CFP registration 55 (WM 148 = VNI) were used. The *Cryptococcus*
109 *neoformans* strain was granted by Dra. Marcia Melhem from the Adolfo Lutz Institute, Brazil - São Paulo.
110
111 **2.4. Antimicrobial Assays and Minimal Inhibitory Concentration** (**MIC**)
112 Antimicrobial activities of the fractions were evaluated by a liquid growth inhibition assay (**Bulet et al., 1993**). A
113 suspension of the microorganisms collected in the mid-logarithmic growth phase was used. Bacteria strains were
114 cultured in poor broth nutrient medium (PB: 1.0 g peptone in 100 mL of water containing 86 mM NaCl at pH 7.4; 217
115 mOsM); for the fungi and yeast strains were cultured in potato dextrose broth (1/2-strength PDB) (1.2 g potato
116 dextrose in 100 mL of H_2O at pH 5.0; 79 mOsM). Determination of antimicrobial activity was performed using 5-fold
117 microtiter broth dilution assay in 96-well sterile plates at a final volume of 100 μL. A mid-log phase culture were
118 diluted to a final concentration of 5 x 10^4 CFU/mL for bacteria and 5 x 10^5 CFU/mL for fungus and yeast (**Bulet, 2008;**
119 **Hetru and Bulet, 1997**). Sterile water and PB or PDB were used as growth control, and Streptomycin antibiotic (10.0
120 mg/mL) was used as growth inhibition control. Microtiter plate were incubated for 18 and 24 h for bacteria and
121 fungi/yeast, respectively, at 30°C; growth inhibition was determined by measuring absorbance at 595 nm. The
122 minimum inhibitory concentration (MIC) are expressed as the interval of the concentration [a]–[b], where [a] is the

highest tested concentration at which the microorganism grow, and [b] is the lowest concentration which causes a growth inhibition of 100% (**Bulet et al., 1993**). The assay was performed using a serial dilution and duplicate in 96-well sterile plate; at 20 μL stock solution was used in each microtiter plate well and added to 80 μL of the microorganism dilution (**Silva et al., 2000; Lorenzini et al., 2003; Riciluca et al., 2012**).

2.5. Determination of Hemolytic Activity

The hemolytic activity of the fractions with antimicrobial activity was assessed against human erythrocytes from a health adult donor. The Ethics Committee was the University of São Paulo School of Medicine (USP - FMUSP), n° 18794419.7.0000.0065. The erythrocytes were collected in 0.15 M citrate buffer, pH 7.4, and washed three times by centrifugation (700 x g, 10 min, 4°C) with 0.15 M phosphate buffer saline (PBS – 35 mM phosphate buffer, 0.15 M NaCl, pH 7.4), and ressuspended in PBS to a final 3% (v/v) concentration.

Aliquots of 50 μL of each fraction were added to 50 μL of 3% (v/v) suspension erythrocytes in the well of U – shaped bottom plates and incubated for 1h at 37° C (with a final volume of 100 μL). The supernatant was first collected and hemolysis was measuring the absorbance at 405 nm of each well in a Victor³ (1420 Multilabel Counter/ Victor³, Perkin Elmer). The hemolysis percentage was expressed in relation to a 100% lysis control (erythrocytes incubated with 0.1% triton X-100) (Sigma-Aldrich, St. Louis, MO, USA); PBS was used as negative control. The fractions was tested serial dilution and duplicate, and calculation was made according to the fallowing equation: %hemolysis = (Abs sample – Abs negative)/(Abs positive – Abs negative) (**Riciluca et al., 2012; Díaz-Roa et al., 2018; Segura and Silva Junior,2018**).

2.6. Sodium Dodecyl Sulfate-Polyacrylamide Gel (SDS-PAGE)

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-Page) was carried out. This experiment was performed, with modifications, based on the protocol described by **Laemmli** (**1970**). The fraction Ovipin (455 μg) were analyzed under non-reducing conditions (without the presence β-mercaptoetanol) and diluted in four times concentrated buffer (250 $mmol.L^{-1}$ Tris-HCl pH 6.8; 300 $mmol.L^{-1}$ SDS; 1 $mmol.L^{-1}$ bromophenol blue; 40% glycerol), and heated at 90° C for about 2 minutes. The stacking gel and the running gel were prepared in the concentrations 4.5% and 12% of the polyacrylamide, respectively. The constant voltage and temperature utilized was 120 V at 4° C,

150 and the total running time was 3 h. As a molecular weight marker was used See Blue (Thermo Fisher Scientific Inc.).
151 And the proteins were stained with Coomassie Blue R-250 (**Laemmli,1970**).
152
153 **2.7. Mass Spectrometry**
154 The fractions that showed antimicrobial activity were analyzed by positive mode mass spectrometry LC-MS/MS on a
155 LTQ XL (Thermo Scientific). The fractions (10 µL of which) were concentrated and ressuspended in 10 µL Formic
156 Acid 0,1%. The liquid chromatography was perfomed in a C18 column (13 cm; 75 µm, i. d.x 100 mm – 3 µm from
157 Waters), using a 5 to 95%B in 25 minutes with solvents A: 15% in acidified water (0,1% TFA) and B: 95% in acidified
158 water (0.1% TFA) for 2 min; with flow of 300 *n*L/min, voltage of 2.5 kV at 200°C. The full scan was performed with
159 m/z of 100 a 2000, occurring MS/MS–CID, and parameters of five most intense ions, with isolation width of 2 m/z at
160 oCID (collision energy at 35).
161
162 **2.8. Database Search**
163 The resulting spectra in "*.RAW*", format were collected and processed using MSconvert software (**Chambers et al.,**
164 **2012**). Thus, they were converted to the mascot generic format ("*.MGF*") and thus analyzed into database searches
165 using the Mascot tool (**Perkins et al., 1999**). The MS/MS peak list files were submitted to an in – house version of the
166 Mascot server (Matrix Science, United States) and screened against the Uniprot database. PEAKS Studio Software
167 (version 8.5, Bioinformatics Solutions Inc., Waterloo, Ontario, Canada) *de novo* sequencing/database search used for
168 establishing sequence. Analysis involved 10 ppm error tolerance for precursor ions and 0.6 Da for fragment ions.
169 Oxidation was considered a variable modification.
170 The physicochemical parameters of which the fraction (such as the total number of positively and negatively charged
171 residues, molecular weight, and theoretical pI) were calculated using the ProtParam tool, available through the portal
172 ExPASy of the Swiss Institute of Bioinformatics website (https://web.expasy.org/protparam/) (**Gasteiger et al., 2005**).
173
174
175 **3. Results**
176 **3.1. Chicken Eggs Fractionation and Antimicrobial Activity**

177 After acid extraction of the crude extract from the chicken eggs *Gallus gallus domesticus*. The acid extract was applied
178 to Sep-Pak® C18 cartridges for pre-fractionation. The supernatant were eluted by three successive concentrations of
179 acetonitrile (ACN) (5%, 40% and 80%) in acidified water (0.1% TFA). All the material at 80% ACN was subjected and
180 analyzed by RP-HPLC (**Figure 1A**), and the fractions obtained was lyophilized, suspended in ultrapure water (1 mL)
181 and tested against *Staphylococcus aureus*, *Escherichia coli*, *Aspergillus niger*, and *Cryptococcus neoformans* in a liquid
182 growth inhibitory assay. The searched for the eluted 80% ACN showed the presence of six fractions (9, 11, 20, 21, 22
183 and 24) with antimicrobial activity (**Table 1**).

184

185 **Table 1.** Antimicrobial activity fractions from elution 80% ACN Chicken eggs.

186

Microorganisms				
	Fungus		Bacteria	
	Filamentous	Yeast	Gram-positive	Gram-negative
Fractions	*A. niger*	*C. neoformans*	*S. aureus*	*E. coli*
9	-	+	-	-
11	+	+	-	-
20	+	+	+	+
21	+	+	+	+
22	+	+	+	+
24	+	+	-	-

187

188 Symbol (+) fraction showed antimicrobial activity; Symbol (-) fraction do not showed antimicrobial activity.

189

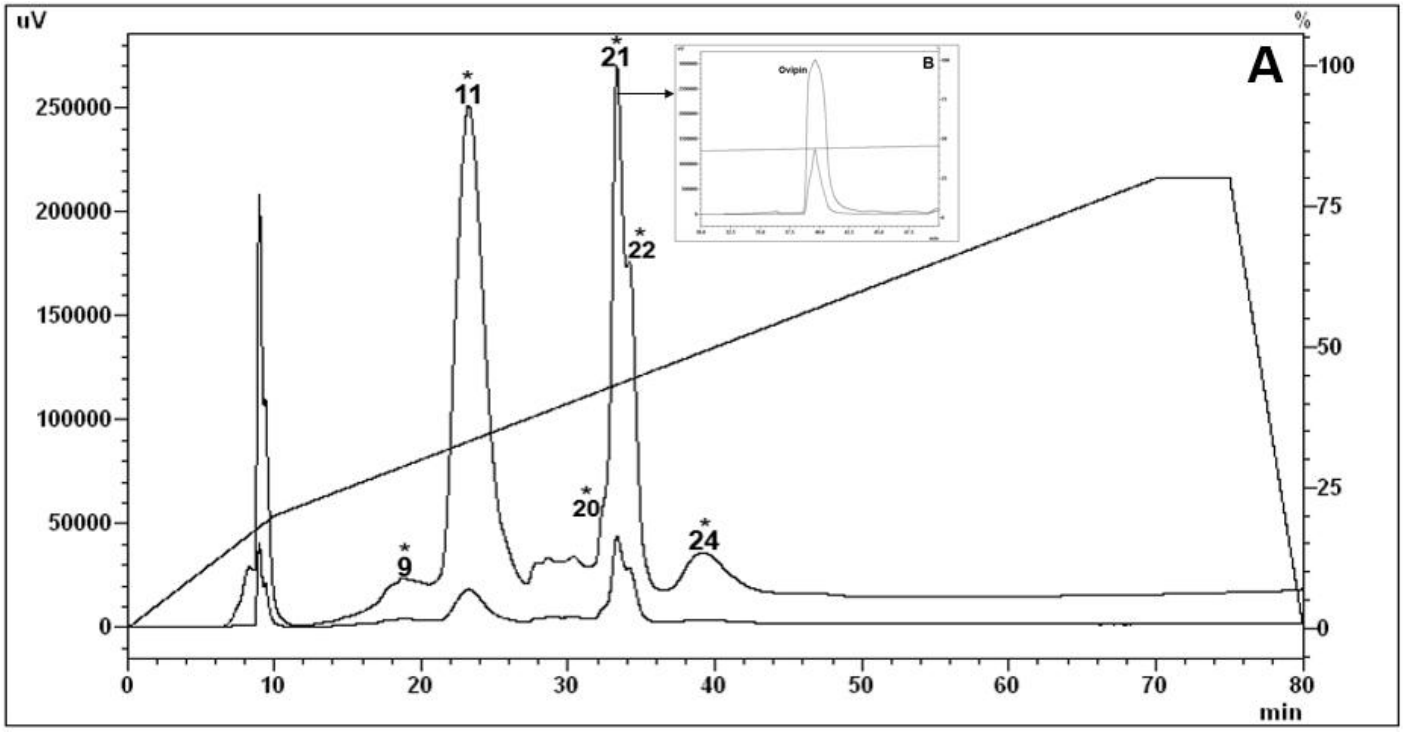


FIGURE 1 | Reversed-phase high-performance liquid chromatograph (RP-HPLC) of the Chicken eggs *Gallus gallus domesticus* extract eluted with 80% acetonitrile (ACN). After the pre-fractionation in a Sep-Pak C18 column, the fraction was eluted with 80% ACN were subjected to a Shim-Pack ODS C18 (20 x 250 mm, 300 Å, 10 μm); Solvents System: Solvent A: 0.1% TFA/H_2O and Solvent B: 0.1%TFA/ACN with a linear gradient 20% to 80% ACN, 60 min at 8 mL/min flow rate. (**A**)The fraction 21 indicated by an asterisk (*) had antimicrobial activity against microorganisms tested. (**B**) Ovipin, indicated with an arrow, using same column and solvents system: Solvent A: 0.05% TFA/H_2O and Solvent B: 0.05%TFA/ACN. Ovipin was eluted from linear gradient 39% to 49% ACN, for 60 min at 2 mL/min flow rate. Both runs the absorbance was monitored at 225 nm.

3.2. Minimum Inhibitory Concentration (MIC) of Ovipin peptide

The antimicrobial activity occurred in the fraction 21 eluted with retention time range of 32.7-34.1 min (**Figure 1A**). This fraction was submitted to a second step of fractionation and showed only one a pronounced antimicrobial fraction: named Ovipin (**Figure 1B**). The name to choice for which fraction referring the chicken eggs species *Galus gallus domesticus*. The concentration of Ovipin peptide was determined based on the Lambert-Beer Law using the molar extinction coefficient at 205 nm absorption (**Anthis and Clore, 2013**), obtained using the Protein Parameter Calculator tool available at the Nick Anthis website (http://nickanthis.com/tools/a205.html; accessed on 15 September 2021). The antimicroial activity was performed by liquid growth inibition in liquid medium. The native Ovipin peptide was capable of inhibited the growth of *M. luteus* A270 1.94 μM (2.84 μg/mL), *A. niger* 31.01 μM (45.5 μg/mL), *C. neoformans* VNI (WM 148) 22.75 μM (15.51 μg/mL). And the *E. coli* D31 was the least sensitive to this peptide (**Table 2**).

211

212 **Table 2.** Antimicrobial activity spectrum of Ovipin peptide.

Microorganisms	MIC (μM (μg/mL))[1]
Gram-positive bacteria	
Micrococcus luteus A270	1.94 (2.84)
Gram-negative bacteria	
Escherichia coli D31	ND
Fungi	
Aspergillus niger	31.01 (45.5)
Yeast	
Cryptococcus neoformans VNI (WM 148)	22.75 (15.51)

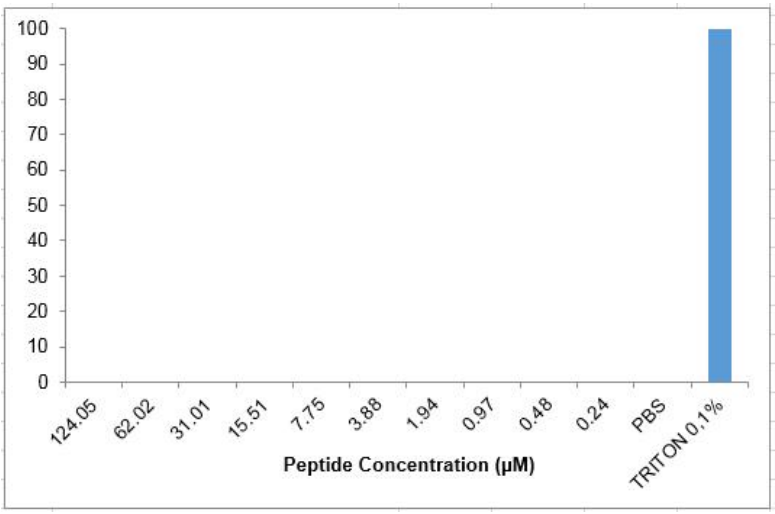
213

214 MIC, minimum inhibitory concentration (μg/mL): μM. ND, not detectable (antimicrobial activity not detected in the
215 concentrations assayed). [1]The MIC refers to the concentration necessary to achieve a growth inhibition of 100%.

216

217 **3.3. Hemolytic Activity**

218 To determine the effect of the Ovipin peptide on human erythrocytes at the antimicrobial concentrations, its hemolytic
219 activity was tested. After incubating red blood cells from a healthy donor with Ovipin (124.05 μM up to 0.24 μM)
220 concentrations for 1 h at 37 °C. No hemolytic activity was observed, demonstrated that Ovipin does not cause
221 significant hemolytic effect against of human erythrocytes within these concentrations (**Figure 2**) (**Yacoub et al., 2014**).
222 And the electrophoretic profile (data not show) of this peptide (up to 455 μg) could not observe bands in this gel
223 concentration (12% polyacrilamide).



224

225 **FIGURE 2** | Hemolytic Activity of the Ovipin peptide incubated with human erythrocytes in different concentrations of
226 the peptides. Serial dilution and duplicated was performed, for 1 h at 37°C. The hemolysis percentage was expressed

in relation to 0% hemolysis with PBS and 100% lysis control (0.1% triton X-100)

3.4. Mass Spectrometry and Characterization of Ovipin Peptide

After the eletrophoresis, where it was not possible observe bands in the gel concentration (12% polyacrilamide), the Ovipin peptide was analyzed using the MASCOT® software to search the *Gallus gallus* database. This tool revealed a sequence with 15 amino acid – YVSPVAIVKGLNIPL- and the molecular weight of 1,581.9494 Da with high identity with a fragment from the Apolipoprotein B (Apo-B) of *Gallus gallus* (**Figure 3**). By the deconvolution of the peptide and analysis of the data in Peaks software, the PEAKS *de novo* sequencing showed, as well as the Mascot tool, 15 amino acid sequence do not having post-translational modification (PTM) (**Figure 4**). Collision-induced dissociation (CID) spectrum from mass/charge (m/z) of its double charged ion give $[M + 2H]^{2+}$, m/z 792.3484 (**Figure 5**). Both tools confirmed the amino acid sequence of Ovipin peptide.

Figure 3: Result obtained in searches databases using the tool Mascot® (Matrix Science). The Ovipin peptide is similar with a fragment from the *Gallus gallus* Apolipoprotein B (APO B)

Figure 4: Peptide spectrum match indicated by a blue line below the sequence of the Apolipoprotein B of *Gallus gallus*. Ovipin covered 2% of the whole protein sequence.

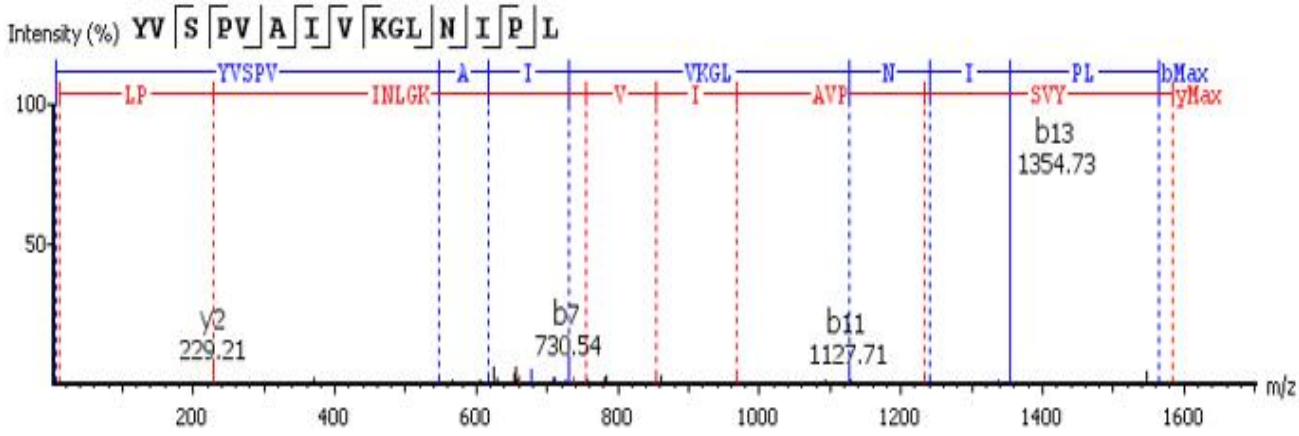


Figure 5: Collision-induced dissociation (CID) spectrum of *de novo* sequencing antimicrobial peptide, Ovipin. The ions belonging to –y (red) and –b (blue) series indicated in the spectrum correspond to the amino acid sequence of the peptide: YVSPVAIVKGLNIPL. The fragments of the sequenced peptide are represented by standard amino acid code letters.

Knowing the sequence, some tools were used predicting Ovipin significant physicochemical characteristics. ExPASY's (SIB Bioinformatics Resource Portal) PepDraw and Pep-Calc.com sequence analysis yielded a potential peptide isoelectric point (pI), molar extinction coefficient and net (**Table 3**).

The peptide was predicted to have one Lys positively-charged aa residue and six of the 15 aa were predicted to be hydrophobic (2 Ile, 2 Leu and 3 Val), showed hydrophobic ratio of 53% and suggesting poor water solubility for Ovipin. ExPASy's ProtParam tool predicted that the peptide would intact for up 2.8 hours in mammalian reticulocytes (*in vitro*), 10 min in yeast (*in vivo*) and 2 min in *Escherichia coli* (*in vivo*). This was likely due to the presence of a Tyr (Y) residue at the N-terminus.

Table 3: Ovipin theoretical physicochemical properties.

Peptide Properties	
Sequence:	YVSPVAIVKGLNIPL
Lenght:	15
Mass:	1581.9494
Isoelectric point (pI):	9.49
Net charge at pH:	1
Hydrophobicity:	+ 7.11 Kcal*mol^{-1}
Estimated solubility:	Poor water solubility
Extinction coefficient:	1280 M^{-1} cm^{-1}

Physicochemical parameters were calculated using ExPASy PepDraw and Pep-Calc.com

A BLAST (basic local alignment search tool) was used to identify a multiple alignment analysis of the peptide sequence Ovipin using Clustal Omega program. The BLAST searches and the alignments showed 100% sequence similarity with the fragment Apolipoprotein B of *Gallus gallus*, and 93.3% of similarity with fragments Apolipoprotein B with others five species of Aves, being *Patagioenas fasciata monilis*, *Amazona aestiva*, *Tinamus guttatus*, *Aptenodytes forsteri* and *Egretta garzetta* (**Figure 6**).

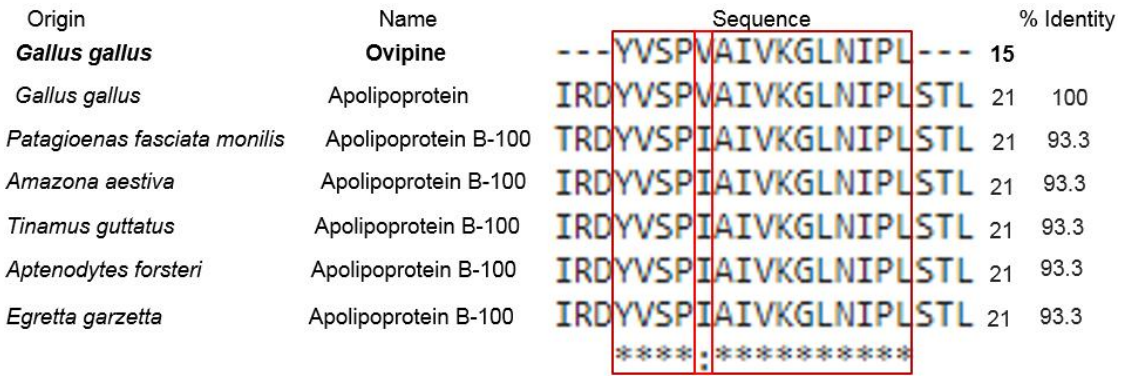


Figure 6: Alignment of the amino acid sequence of Ovipin. Using the Clustal Omega alignment, the Ovipin sequence was aligned with fragments of the Apolipoproteins of the *Gallus gallus* and others Aves species. Regions that show identical amino acid among all species are identified in dark red or (*), and minor modifications are show in light red (**:**). Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/; accessed on 24 January 2020) modified manually.

Table 4. Known antimicrobial peptides having similarity with Ovipin, as identified in the Antimicrobial Peptide Database (APD2) (**Wang et al., 2009**).

Peptide Name	Sequence Alignment	Source Organism	APD Identifier	Percentage Similarity
Temporin-1SKa (17 aa)	F L P V I L P V + I G K L L N G I + L + + Y V + S P V A I V K G L N + I P L	Frog *Rana sakuraii*	AP01924	47,36
Temporin-1GY (13 aa)	+ V I P + + I V S G L L S S L L Y V S P V A I V K G + L N I P L	Frogs *Pelophylax nigromaculatus*	AP02223	43,75
Temporin-1TGc (17 aa)	F L P V I L P V + I G K L L S G I + L + + Y V + S P V A I V K G L + N I P L	*Rana sakuraii*	AP01268	42,1
Peptide AN5-1 (12 aa)	Y S K S L P + L S V + + L N + P + Y + V S + P V A I V K G L N I P L	*Paenibacillus alvei* AN5	AP02193	41,17

The Antimicrobial Peptide Database (APD) prediction tool was used to align Ovipin

281 **4. Discussion**

282 The abundance of multidrug-resistance of the microorganisms has emerged, increasing the spectrum of untreatable
283 infections and triggering a worldwide public health problem (**Gaglione et al., 2011; Blair et al., 2015**). The accelerated
284 growth of antimicrobial resistance has created a great interest in the discovery for new compounds more effective,
285 mainly with antimicrobial action, to be used as tools for antimicrobial therapies (**Diaz-Roa et al., 2018; Chernysh et al.,**
286 **2015**)

287 During the hatched time, eggs are constantly attacked by a variety of microorganisms that decrease their chances of
288 survival and viability. (**Packard & Packard, 1980**). The compounds isolated from eggs showed a broad spectrum of
289 antimicrobial activity, mainly against bacteria's (**D'Alba & Shawkey, 2015**). In the present work we isolated and
290 characterize a peptide, denoted Ovipin, from chicken eggs *Gallus gallus domesticus*, its antimicrobial activity,
291 biochemical and structural properties were evaluated.

292 The antimicrobial activity Ovipin (MIC = 22.75 μM (15.51 μg/mL)) against *C. neoformans* was very interesting, because
293 this yeast has a strong relationship with human infections with highly incidence in immunocompromised and
294 immunocompetent individuals. *Cryptococcus* virulence factors protect it from host immune responses. According to
295 the host's immune response, this microorganism can make changes in the metabolism itself as well as induce changes
296 in the structure of its capsule, decreasing its sensitivity to conventional antifungals. The production of melanin
297 protects the microorganism from oxidative stress, the urease enzyme is able to neutralize the acidic pH of
298 phagolysosomes, as well as being able to carry out non-lytic exocytosis. *Cryptococcus* fuses its phagosomal membranes
299 with plasma membranes to escape the phagocytosis process. Since it can survive inside the phagolysosome without
300 being destroyed, it can more easily cross the blood-brain barrier. And finally, cryptococci can manipulate the adaptive
301 immune system response and remain in a latent state of infection (**GUSHIKEN et al, 2021**). Thus, as Ovipin was able
302 to inhibit the growth of *Cryptococcus neoformans*, could this peptide be interfering in any way with the virulence factors
303 of this microorganism?

304 In the work **Candido-Ferreira *et al.*** (**2017**) was identified and characterized the Oligoventin peptide (8 amino acid
305 residues and MW 1,061.4 Da) from the eggs of the Brazilian armed spider *Phoneutria nigriventer* (Ctenidae,
306 Araneomorphae). Oligoventin showed antimicrobial activity (MIC range at 47 to 188.9 μM) against Gram-positive and
307 Gram- negative bacteria, as also yeast (*Candida albicans* MDM8). The molecule shows 4,3% of hemolysis against
308 human erythrocytes at 188.9 μM and 2% at 94.5 μM . Therefore, it is worth pointing out that both Ovipin and

309 Oligoventin, although islolated from eggs of different animals, have antimicrobial action against opportunistic
310 pathogens of great medical importance.
311 Ovipin presented antimicrobial activity (MIC = 1.94 μM (2.84 μg/mL)) against *Micrococcus luteus* (Gram-positive
312 bacteria). In the work of **Memarpoor-Yazdia and Chamania (2012**), was identified from hydrolyzed lysozyme, the F2
313 peptide (MW 1753.98 Da) that was active against *Leuconostoc mesenteroides* (Gram-positive bacteria) and *Escherichia coli*
314 (Gram-negative bacteria). **Mine et al. (2004**) also isolated and identified from hydrolyzed lysozyme, the peptide F1d
315 (MW 1185 Da), with antimicrobial activity against *Escherichia coli* K-12, and the peptide F3b (MW 875 Da) that was
316 active against *Staphylococcus aureus* 23-394 (Gram-positive bacteria).
317 Through searches carried out in online databases, Ovipin demonstrated a high percentage of similarity with frog
318 peptides. Temporin-1SKa (47%), Temporin-1GY (43%) and Temporin-1TGc (42%), peptides from *Rana sakuraii* and
319 *Pelophylax nigromaculatus*, respectively, both peptides with antimicrobial activity against gram-positive and
320 gram-negative bacteria (**SONG et al, 2013; SUZUKI et al, 2007**). Amphibians, like chicken eggs, are frequently
321 exposed to many pathogens, and studies have shown several isolated and identified antimicrobial peptides or
322 pharmacological peptides from frogs with diverse function, mainly against multi-resistant microorganisms
323 (**HAYASHIDA, SILVA JUNIOR, 2021; XIAO et al., 2011**)
324 Ovipin showed to have 1,581.9494 Da and similarity of 93.3 to 100% with Apoliproteins B of the five groups of Aves.
325 Very low-density lipoproteins (VLDL) are precursors low-density lipoproteins (LDL) of eggs yolk. VLDL II and apo B
326 are mainly proteins that constituted VLDL. The molecular weight of apo B is 500 kDa, showed only one structural unit
327 and 64% of the similarity with human apo B-100. When transferred into the yolk, the apo B undergoes enzymatic
328 cleavages, forming news fragments (**Jolivet *et al.*, 2006**), which may have new functions in the yolk.
329 The apolipoproteins are recognized as source bioactive-peptides showed broad antimicrobial or antiviral activity
330 (**Gaglione *et al.* (2017**). In the work **Zambrovicz et al.,** (**2015**) was identified four peptides with molecular weight
331 ranging from 1,210.62 to 1,677.88 Da obtained from Apoliproteins B, Vitellogenin-2 and Apovitelenin-1, all proteins
332 localized in chicken egg yolk. In relation the peptides identified from Apolipoprotein B, one peptide showed amino
333 acid sequence YINQMPQKSRE (MW: 1,393.774 Da) and the other peptide showed amino acid sequence
334 YINQMPQKSREA (MW: 1,464.813). In this study not was evaluated the antimicrobial activity this peptides, but was
335 demonstrated the antioxidant activity, ACE inhibitory and/or antidiabetic activities. The peptide YINQMPQKSRE
336 demonstrated the strongest antioxidant, and the DPPH free radical scavenging and iron chelating activities reached

337 2.3 μM Trolox eq/mg and 37.4 μg Fe^{2+}/mg, respectively. The peptide showed YINQMPQKSREA the highest ferric
338 reducing activity (76.0 μg Fe^{2+}/mg), an activity very strong ACE (IC_{50} = 10.1 μg/mL) and DPP-IV (IC_{50} = 222.8 μg/mL)
339 inhibitory activity.
340

341 **5. Conclusions**

342 Whereas antimicrobial resistance has become a serious concern and a public health issue because it limits treatment
343 options, the identification and characterization of new antibiotics agents can lead to the development of new
344 compounds that act against resistant pathogenic microorganisms. In this work we reported one peptide that was
345 extracted from chicken eggs *Gallus gallus domesticus*, purified by RP-HPLC and characterized by amino acid
346 sequencing. The Ovipin, a small antimicrobial peptide that is a fragment of Apolipoprotein B of *Gallus gallus*; it
347 contains 15 amino acid sequence and is active against Gram-positive (*Micrococcus luteus*), Filamentous fungi
348 (*Aspergillus niger*) and Yeast (*Cryptococcus neoformans*), and which in the future as possible multifunctional candidates.
349 The results of this work show that chicken eggs, over time, have become increasingly important not only as a food to
350 meet nutritional needs, but also the chicken egg has become a great source for the discovery of new peptides, as the
351 literature has showed, and as has been effective against microorganisms that developed resistance to conventional
352 antibiotics. In searches carried out in the literature, it was not yet possible to find fragments of Apolipoprotein B,
353 whether from birds or humans, that have antimicrobial action, mainly against fungi. Perhaps the Ovipin peptide
354 could be the first fragment of Apolipoprotein B, was also able to inhibit the growth of *C. neoformans*, an important
355 yeast for medicine because act mainly imunossupressed patient. Further studies aimed at evaluating the activity of the
356 Ovipin peptide against others multidrug-resistance bacteria, fungi, viruses, and parasites are needed, as well as
357 evaluating its mechanism of action against *C. neoformans*, to understand in which possible virulence factor of this yeast
358 the Ovipin peptide may be acting and that inhibit its growth, and it can be used as a new peptide in the biotechnology
359 or pharmaceutical industry.
360

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