IDENTIFICATION OF ANTIOXIDANT PEPTIDES OF HEN EGG WHITE LYSOZYME AND EVALUATION OF INHIBITION LIPID PEROXIDATION AND CYTOTOXICITY IN THE ZEBRAFISH MODEL

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Abstract

Hen egg lysozyme was hydrolyzed with pepsin in situ on a cation-exchange column to isolate antioxidant peptides. The most cationic fraction was eluted with 1 M NaCl. Five positively charged peptides f(109-119) VAWRNRCKGTD, f(111-119) WRNRCKGTD, f(122-129) AWIRGCRL, f(123-129) WIRGCRL and f(124-129) IRGCRL were identified using tandem mass spectrometry. Using ORAC-FL, all five peptides presented antioxidant activity with values of (1,970; 3,123; 2,743; 2,393 and 0.313 µmol Trolox/ µmol peptide) respectively. Using method TBARS in zebrafish larvae, all five synthetic peptides were found to efficiently inhibit lipid peroxidation (36.8; 51.6;55.56; 63.2; 61.0 % inhibition of lipid peroxidation) respectively. None of the five peptides were toxic in zebrafish eggs and larvae at concentrations lower than 50 µg/ml. Concentrations higher than 50µg/ml were toxic for both zebrafish eggs and larvae.

Keywords: lysozyme, antioxidant activity in zebrafish larvae, bioactive peptides, hydrolysate, cation exchange column and toxicity in zebrafish egg.

Introduction

Lysozyme is a basic protein consisting of 129 amino acids with a molecular weight of 14.3 kDa. These amino acid residues are cross-linked by four disulfide bridges, and have an isoelectric point of 10.7. Hen egg is the richest source of lysozyme, accounting for 3.5% of total egg white proteins [1]. Lysozyme belongs to a type of enzymes that lyses the cell wall of certain Gram-positive bacteria by splitting β (1-4) linkages between N-acetylmuramic acid and N-acetylglucosamine of the peptidoglycan [2]. As a well-known antimicrobial protein, hen egg white lysozyme has been commercialized for applications as a natural preservative to control lactic bacteria in meat products such as sausages, salami, pork, beef or turkey. Lysozyme has also been used to prevent growth of *Clostridium tyrobutyricum* in cheese production or to control lactic bacteria in wine and beer production [3, 4, 5, 6, 7]. Lysozyme can also be used in other pharmaceutical and cosmetics applications [3, 7]. Moreover, lysozyme is an enzyme widely used as food additive (E1105) due to its numerous properties [8]. Lysozyme has many other functions, including antiviral [9, 10], immune modulatory [11], anti-

inflammatory [12] and antitumor [13] activities. At pH 7.0, lysozyme is positively charged, whereas the rest of the proteins of the egg white are negatively charged. Many cationic proteins such as lactoferrin, lactoperoxidase and lysozyme may be purified using ion exchange, as this fact has already been demonstrated [14]. Lysozyme has also been purified with cation-exchange membranes and resins [15, 16].

Bioactive peptides have between 3-20 amino acid residues; their bioactivity depends on the sequence and amino acid compositions [17-19]. Recently, attention has mainly focused on the antioxidant peptides generated from food proteins, being these peptides safer and healthier than synthetic drugs [20]. Antioxidant peptides contain 5-16 amino acid residues. Their antioxidant activities can be related to ion chelating, radical scavenging and inhibition of lipid peroxidation. The importance of positively charged amino acids in determining the strength of peptides as antihypertensive and antioxidants has been indicated in different studies. Strong antimicrobial peptides are cationic charged. Those cationic charged peptides content amino acids as Lys, Arg and His [21, 3]. Lysozyme has an isoelectric point of 10.7, with a high content of positively charged amino acids. Lysozyme may be a great substrate for production of bioactive peptides with antioxidant activity. You et al., (2010)[3] have described two antioxidants fractions of hydrolysate of lysozyme with pepsin containing positively charged amino acids such as f(13-20)KRHGLDNY, f(14-23)RHGLDNYRGY and f(13-23)RHGLDNYRGY. Moreover, many researchers have reported that peptides and protein hydrolyzed from various food sources have significant antioxidant activity [22]. Furthermore, hen egg white lysozyme suppresses reactive oxygen species (ROS) generation and protects against acute and chronic oxidant injuries [20, 23]. Some peptides have shown to have multifunctional activities [3, 24]. Different bioactive peptides from lysozyme have been reported with antimicrobial, antioxidant and antihypertensive activities [25-29].

The zebrafish (*Danio rerio*) has become a promising model organism for experimental studies in different biomedical areas. Zebrafish is an ideal animal model for laboratory research. These animals are inexpensive, low-maintenance, and abundantly produced all year round [30-33]. Zebrafish genes are highly conserved sharing a 70 – 80% homology to those of humans [34]. The transparent embryos rapidly develop externally. Organogenesis is completed within the first 48 hours of development. Since

zebrafish embryos develop externally, changes in development may be continuously monitored and observed, which greatly facilitates developmental time course studies. Zebrafish development has been well characterized and therefore results from zebrafish are comparable to mammalian developmental studies [35-37]. Moreover, zebrafish is a vertebrate model for modeling behavioral and functional parameters related to human pathogenesis and for clinical treatment screening. More recently, zebrafish has become also a valuable model to environmental and toxicological studies. Therefore, zebrafish model can be an interesting model to evaluate toxicology of new ingredients of functional foods such as antioxidant peptides.

In this study, ion-exchange chromatography has been used to isolate the bioactive peptides from hen egg white lysozyme. Lysozyme was hydrolyzed in situ with pepsin to generate positive charged peptides. Those peptides were separated on a cation-exchange column by selective elution. The objective was to identify new antioxidant peptides and evaluate their toxicity in the Zebrafish model *(Danio rerio*).

Materials and methods

Chemicals

Hen egg white lysozyme 58,000 U/ml, pepsin crystalline 3,440 U/mg obtained from porcine stomach mucus, 2,20-azobis (2-methylpropionamide)-dihydrochloride (AAPH), 6-hydroxy-2,5,7,8-tetramethylchroman- 2-carboxylic acid (Trolox), fluorescein disodium (FL), and dithiothreitol (DTT) were obtained from Sigma Chemical (Saint Louis, MO, USA). The rest of chemicals used were of HPLC grade.

Pepsin hydrolysis of lysozyme in situ on an ion-exchange column and isolation of peptides

Denaturation of lysozyme was performed as previously described by Carrillo et al., 2014 [38]. Lysozyme was denatured using heat treatment. Lysozyme at 5mg/ml was suspended in buffer phosphate pH 6.0 and heat at 95°C during 20 minutes. Then, lysozyme was lyophilized and stored at -20°C. To carry out the hydrolysis of column-bound native and denatured lysozyme, 1000 ml of a 0.1 mg/ml solution of protein in 10 mM NaCl, adjusted to pH 2.0 with HCl, were pumped through a column cation-

exchange column. The binding of native and denatured lysozyme was carried out at room temperature (25°C) with at a flow rate of 20 ml/min, which was generated by a peristaltic pump (Verder-Vleuten, Vleuten, The Netherlands). The process was monitored by a UV detector with a 2 mm light path flow cuvette (Model EM-1 Econo UV Monitor, Bio-Rad) at 280 nm. Prior to be used, the ion-exchange column was pre-equilibrated with water acidified with HCl (pH 2.0). The native and denatured lysozyme bound to the column were hydrolyzed at 37°C by recycling with 100 ml of an aqueous solution (pH 2.0) of porcine pepsin (25 mg/ml) at 20 ml/min during 6 hours. The column was washed sequentially with acidified water at pH 2.0. Solvent A was 10 mM ammonium hydrogen carbonate acidified to pH 7 with formic acid, and solvent B was 3M and 5M of ammonia solution and finally the column was treated with 1 M NaCl to remove more cationic peptides. The effluent was monitored at 280 nm. All fractions were collected with Fast Protein Liquid Chromatography (FPLC) of GE-Pharmacia, freeze-dried, and analyzed with a high-performance liquid chromatography-electrospray ionization-tandem mass spectrometry (HPLC-ESI-MS-MS).

Identification of peptides by ESI-MS/MS

The selected fractions separated and collected from FPLC were analyzed by RP-HPLC-ESI-MS/MS, on an Agilent 1100 HPLC System (Agilent Technologies, Waldbron, Germany) connected on-line to an Esquire 3000 ion trap (Bruker Daltonik GmbH, Bremen, Germany) and equipped with an electrospray ionization source, as described by López-Expósito et al. (2006) [39]. The variable-wavelength detector was set at 214 nm. A C18-guard column (Nova-Pak® 20 mm × 2826 x 3.9 × 4 µm of particle size; Waters Corp., Milford, MA, USA) was used to protect the analytical column (HiPore® RP318 C18 column 250×4.6 mm and 5 µm of particle size; Bio-Rad, Richmond, CA, USA). The samples were eluted at 0.8 mll/min with a linear gradient from 0 to 45% of solvent B (acetonitrile and TFA, 1,000:0.270, v/v) in solvent A (water and TFA, 1,000:0.370, v/v) in 60 minutes. The injection volume was 50 µl and duplicate of injection was made for each point of the standard curve and the samples. The flow from HPLC was divided approximately 1:3 previous to ionization source, and the first 6 min of the eluent flow was directed to waste to reduce salt deposit on the transfer capillary of the MS instrument and to reduce interferences. For HPLC-MS, spectra were

recorded over the mass-to-charge (m/z) range of 100 to 1,500. Helium was used as collision gas with an estimated pressure of 5×10−3 bar. About 15 spectra were averaged in the MS analyses and about five spectra in the tandem MS analyses. Using Data AnalysisTM (version 3.0; Bruker Daltoniks), the m/z spectral data were processed and transformed to spectra representing mass values. The acquired MS/MS spectra were interpreted using BioTools (version 2.1; Bruker Daltoniks).

Peptide synthesis

The synthetic peptides from lysozyme VAWRNRCKGTD, f(109-119), WRNRCKGTD, f(111-119) AWIRGCRL, f (122-129), WIRGCRL, f (123-129) and IRGCRL, f (124-129) were prepared using a conventional FMOC solid-phase synthesis method with a 431A peptide synthesizer (Applied Biosystems Inc. Überlingen, Germany).

Oxygen radical absorbance capacity-fluorescein (ORAC-FL) assay

The ORAC-FL assay was based on the assay proposed by Ou, Hampsch- Woodill, & Prior (2001) and Dávalos et al., 2004[40, 41]. The reaction was made at 40°C in 75 mM phosphate buffer (pH 7.4). The final assay mixture (200 mL) contained FL (70 nM), AAPH (14 mM), and antioxidant [Trolox (0.2-1.6 nmol) or samples of the five synthetic peptides (at different concentrations)]. The fluorescence was recorded during 137 min (104 cycles). A FLUOstar OPTIMA plate reader (BMG Labtech, Offenburg, Germany) with 485 nm excitation and 520 nm emission filters was used. The equipment was controlled by the FLUOstar Control software version (1.32 R2) for fluorescence measurement. Black polystyrene 96-well microplates (Nunc, Denmark) were used. AAPH and Trolox solutions were prepared daily and FL was diluted from a stock solution (1.17 mM) in 75Mm phosphate buffer (pH 7.4). All reaction mixtures were prepared in duplicate and at least three independent runs were performed for each sample. Final ORAC-FL values were expressed as µmol of Trolox equivalent/ µmol of peptide [42].

Thiobarbituric acid reactive substances (TBARS)

The thiobarbituric acid reactive species method was used as described by Westerfield, 1995 [43]. The Zebrafish colony was established in the laboratory, in a glass aquarium,

containing an internal filter and an activated carbon aerator for water oxygenation. The population of animals was fed three times a day with food chips for fish. Adult fish were kept on 16 hours light and 10 hours dark cycles. Embryos were obtained by photo-induced spawning over green plants and cultured at 28°C in a fish tank water. 5 days post fecundation (dpf) larvae were then incubated in 24-well plates, 30 larvae per well, with 50 µg/ml of lysozyme peptide in each well. Lipid peroxidation was initiated by adding 1 ml 500 µM H_2O_2 and incubated during 8 hours at 28°C. Groups from 30 larvae/well in aquarium water were used as controls. Then, H_2O_2 was removed with a micropipette and 500 µl of Tween 0.1% was added. All groups were mixed and homogenized with a T25 Ultra turrax IKA. Then, absorbance of the solution of zebrafish larvae and peptides was measured at 532 nm. The decrease of absorbance indicates an increase of antioxidant activity. The values of antioxidant activity were expressed as the percentage inhibition of lipid peroxidation in larvae homogenate as follows:

The total antioxidant activity % Inhibition of lipid peroxidation = $[(A_b - A_s)/A_b = X\ 100]$

where A_b is the absorbance of blank and A_s is the absorbance the sample.

Test of toxicity in the Zebrafish model

Zebrafish of the AB strain (wild-type, wt) embryos were obtained from natural spawning. Embryos were raised and fish were maintained as described by Westerfield, (1995) [43]. After collection and disinfection, eggs were placed in 24-well microplates with 1 mL of water. To study the in vivo toxicity of all peptides coming from lysozyme with the zebrafish model, the FET test was employed.

FET Test

The assay was based on the OECD draft guideline on Fish Embryo Toxicity (FET) Test [44] and is described in detail by (Domingues et al., 2010) [45]. The Test Guideline is based on chemical exposure of newly fertilized zebrafish eggs for up to 48 hours and is expected to reflect acute toxicity in fish in general. After 24 and 48 hours of exposure to the peptides, four apical endpoints were recorded as indicators of acute lethality in fish: coagulation of fertilized eggs, lack of somite formation, lack of detachment of the

tail-bud from the yolk sac and lack of heart-beat. The eggs were considered dead when they exhibit at least one of the previous mentioned indicators.

In the control wells, there should be less than 10% of the eggs with one of the mentioned indicators after 48 hours, (29) [46]. Ten eggs per treatment (3 replicates) were selected and distributed in 24-well microplates. The test started with newly fertilized eggs exposed to the nominal concentrations of 50; 156; 312; 625; 1250; 2500 and 5000 µg/ml of peptides and run during 2 days. Embryos were observed at 24 and 48 hours under a stereomicroscope (magnification used in the stereomicroscope for observations was 40X).

Results and discussion

Hydrolysis in situ from lysozyme with pepsin in a cation-exchange column.

Hen egg white lysozyme was subject to hydrolysis in situ with pepsin in a cation-exchange column (Figure 1). The objective was to obtain, in one step, rich peptides with positively charged amino acids derived from the hydrolysis of lysozyme and, to assess whether denatured lysozyme could generate peptides other than native lysozyme. It is known that lysozyme has resistance to the hydrolysis with pepsin, but it has been recently described that lysozyme at pH 1.2 has total susceptibility to the hydrolysis with pepsin [39, 47-49]. Fu, Abbott, and Hatzos (2002) [50] have reported that lysozyme resisted more than 60 minutes at pH 1.2, at an E: S of (13:1) wt:wt. Thomas et al., (2004) [49] described that hen egg white lysozyme is resistant to hydrolysis with pepsin at pH 2.0. Ibrahim et al. (2005)[51] found that 40% of the original lysozyme was hydrolyzed after 120 minutes of digestion at an E: S of 1:50 (wt:wt) and pH 4.0. There is then controversy about the hydrolysis of hen egg white lysozyme and this can be due to the different methods used. In this study, lysozyme was hydrolyzed at pH 2.0 with an excess of pepsin.

Many antimicrobial and antioxidant peptides contain positively charged amino acids thus determining the strength of their activity [21, 52, 53]. Lysozyme has an isoelectric point of 10.7 with a high content of positively charged amino acids. Hen egg white lysozyme has 17 positively charged (6 Lys, 11 Arg) and nine negatively charged residues (7 Asp, 2 Glu), thus leading to a net positive charge at pH below the isoelectric point

(10.7). This positive charge makes hen egg white lysozyme even more attractive for investigation with the negative charged peptides [54].

Therefore, lysozyme may be a good substrate for production of antimicrobial and antioxidant peptides. You et al., 2010 [53] have reported antioxidant hydrolysates from lysozyme obtained with alcalase. They found that the fractions were rich in cationic peptides with high percentage of Arg and Lys (positively charged amino acids). Samples of native and denatured lysozyme were loaded in the cation-exchange column. Then, those samples were treated over night at 37°C with recirculation of pepsin solution. Immediately after, the hydrolysate was eluted with a gradient of 3 M and 5 M of ammonia. Two different fractions were successively collected respectively, and then a third fraction was eluted with sodium chloride (NaCl) 1 M. This fraction contained the peptides with maximum net positive charge and, therefore, those with the highest affinity for the cation-exchange column (Figure 2).

Identification of peptides sequences

After treatment with dithiothreitol all fractions from native and denatured lysozyme were analyzed with RP-HPLC-ESI-MS-MS to characterize their molecular mass and amino acid sequences. Table 1 shows the identified peptides eluted with NaCl 1 M. The peptides from 3 M and 5 M ammonia fractions were discarded as those peptides are less cationic. Both fractions were very complex with high content of peaks. As expected, this fraction contained peptides with abundant positively charged amino acids (Arg and Lys). For this reason, it was decided to work with this fraction. All sequences are located in the C-terminus of lysozyme, in the α-dominium in the zone of helix 90-129. It can be seen that there was no difference between the peptides identified in both hydrolysates, indicating that the process of heat denaturation of lysozyme results in a lack of production of new hydrolysis sites in the protein. Ibrahim et al., (2001, 2005) [51, 55] have reported antimicrobial peptides with high activity present in the α-dominium (1-40 and 90-129), specifically in the regions 1-38 and 87-114 from the lysozyme. However, we only found peptides located in the α-dominium C-terminal 90-129. This could be caused by the ionic separation performed, where cationic peptides have predominantly been recovered. The peptides identified were synthesized to be used in the antioxidant assay.

Antioxidant peptides sequences

Five peptides from the fraction NaCl were assayed for their antioxidant activity, against peroxyl radicals, by using ORAC-FL assay. Table 2 shows results of antioxidant activities for the five peptides assayed. ORAC-FL values of peptides from hen egg white lysozyme were very high, indicating very high antioxidant activity. Four peptides f(109-119) VAWRNRCKGTD, f(111-119) WRNRCKGTD, f(122-129) AWIRGCRL and f(123-129) WIRGCRL (1,970; 3,123; 2,743 and 2,393 μmol Trolox/ μmol peptide) respectively were more active than vitamin C (1.65 μmol Trolox equivalents/μmol vitamin C). Peptides f(122-129) AWIRGCRL and f(123-129) WIRGCRL were more active than synthetic antioxidant peptide named butylated hydroxyanisole (BHA) (2,430 μmol Trolox equivalents/μmol BHA) used in food industry for its high antioxidant activity [56,42]. Only the peptide f(124-129) IRGCRL presents low activity with 0.313 μmol Trolox/ μmol peptide, this might be due to the absence of Trp in its sequence. As shown in the ORAC database prepared by Li and Li (2013) [57], the length of peptides derived from food sources with peroxyl radical scavenging activity, ranges from 4 to 20 amino acids. Peptides described in our study with peroxyl radical scavenging activity have between 1 to 11 amino acids. Peptides of our study are small peptides with high antioxidant activity using ORAC-FL. On the other hand, Hernández-Ledesma et al., (2005) [58] have described a peptide from soybean named lunasin with high antioxidant activity 3.44 ± 0.07 μmol Trolox equivalents/μmol lunasin. Potent activity of lunasin was attributed to the presence of amino acids Trp, Cys, and Met in its sequence. All peptides in our study contain in their sequence Trp, Cys or both. Possibly, the higher antioxidant activity of peptides in this study is explained by the presence of Trp and Cys amino acids in a particular site of their sequence. Peptide number 2 IRGCRL was compared to peptide number 3 WIRGCRL. We observed that IRGCRL peptide has not Trp (W) in its sequence, and a value of ORAC-FL of 0.311 μmol Trolox/ μmol peptide was detected. On the other hand, peptide number 3 WIRGCRL presented antioxidant activity with a value of 2,393μmol Trolox/ μmol peptide. The difference in the antioxidant activity can be related to the presence of Trp in peptide number 3 WIRGCRL sequence.

The molecular weight of the identified peptides are in the range of most of the antioxidant peptides derived from food sources isolated previously of 4 to 20 residues amino acids [59]. Moreover, antioxidant peptides often possess hydrophobic amino

acid residues such as Pro, His, Tyr, Trp, Met, or Cys in their sequences and Val or Leu at the N-terminus [60]. One of our peptides showed Val at the N-terminus.

Potential of synthetic peptides from lysozyme to inhibit lipid peroxidation

The antioxidant action is assessed by inhibiting the damage caused by free radicals and the mechanisms involved in many human diseases such as hepatotoxicities, hepatocarcinogenesis, diabetes, and skin cancer to include lipid peroxidation as a main source of cellular damage. Lipid peroxidation in biological systems has been thought to be a toxicological phenomenon leading to various pathological consequences. MDA formed from lipid peroxidation of unsaturated phospholipid reacts with TBA to produce a pink MDA-TBA adducts. MDA is reactive and active in crosslinking with DNA and proteins and damages liver cells [61]. Phospholipids are believed to be present in high amounts in cell membranes [62]. Lipid peroxidation has been a major contributor to the loss of cell function under oxidative stress [63, 64]. To determine oxidative stress, inhibition of lipid peroxidation in zebrafish larvae model was used to determine damage cellular in vivo. Figure 3A presents the inhibition of lipid peroxidation by synthetic peptides from lysozyme at a concentration of 50 µg/ml. This assay confirmed that these synthetic peptides were not toxic for zebrafish larvae. Zebrafish larvae presented normal aspect after 24 hours of assay. When zebrafish larvae were examined, no morphological abnormalities are shown such as crooked bodies, spinal deformities or any significant effects in the growth of the body (Figure 3B). The values of percentage inhibition of lipid peroxidation indicated that all synthetic peptides were efficient to inhibit the lipid peroxidation in zebrafish larvae. For example peptide P4 (AWIRGCRL) had a result of 63.2 % TBARS inhibition, (Figure 3A). The antioxidant results showed the higher activity of peptides 3, 4 and 5 in both assays. The presence of tryptophan seems important for the ORAC activity. However, in the case of the TBARS inhibition, the peptide size is probably contributing to the increased values of activity in peptides P2 to P5.

Test in Zebrafish embryo

Based on the in vitro studies antioxidant activity described above, we decided to evaluate the toxicity of the peptides in a model of zebrafish eggs. Zebrafish has become a widely used model organism for studies of developmental biology and drug discovery. This model helps drug development by combining the tools of medicinal chemistry and zebrafish biology.

Figure 4 shows a representative curve doses-response result of FET test for AWIRGCRL peptide, this peptide presents the highest TBARS inhibition percentage. This sample was only taken as an example as all samples presented identical results. The test was carried out for all peptides of this study, however, no significant differences were observed in the rest of peptides. Again, as in the zebrafish larvae test, the AWIRGCRL peptide does not present toxicity at a concentration of 50 μg/ml for zebrafish eggs. However, concentrations higher than 50 μg/ml of peptides were cytotoxic to zebrafish egg after 24 hours of incubation. Mortality was identified with an absence of embryonic development and coagulation of nuclear material of eggs. The control eggs were totally normal in their development (Figure 5A). However, eggs treated with the peptides of this study presented no embryonic development and coagulation total of nuclear material of eggs. Around the eggs, material of the chorion due to ruptures of the eggs was observed (Figure 5B).

As a conclusion, hen egg lysozyme was hydrolyzed with pepsin in situ using a cation-exchange. Hen egg white lysozyme is a good source of antioxidant peptides using pepsin for hydrolysis at low pH. The zebrafish model was efficient to measure the inhibition of lipid peroxidation and cytotoxicity of synthetic peptides from lysozyme. Development of zebrafish is sensitive to the exposure to all lysozyme synthetic peptides used in this study at concentrations higher than 50μg/ml. However, further investigations would need to be carried out to evaluate the death mechanisms of these peptides on zebrafish embryos, for eventual pharmaceutical and medical applications.

Conflict of interest

The authors declare that they have no conflict of interest.

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List of figures and tables

Table 1. Identification of the molecular mass and amino acid sequence of the fraction eluted with 1 M NaCl using LC-ESI-MS/MS.

N°	Fragment	Mass Obss.	Mass Calc.[a]	m/z[b]	Sequence[c]	NLZ	LZ95°C
1	f(109-119)	1306,0	1306,0	653,5 (2)	VAWRNRCKGTD	+	+
2	f(124-129)	716,6	717,6	717,6 (1)	IRGCRL	+	+
3	f(123-129)	902,7	903,7	903,7 (1)	WIRGCRL	+	+
4	f(122-129)	973,7	974,7	974,7 (1)	AWIRGCRL	+	+
5	f(111-119)	1134,7	1135,7	1135,7 (1)	WRNRCKGTD	+	+

Table 2. In vitro antioxidant activity of peptides from lysozyme.

Nº	Peptides	ORAC (micromol Trolox equivs/micromol peptide) ± SD
1	VAWRNRCKGTD	1.970 ± 0.171
2	IRGCRL	0.313 ± 0.029
3	WIRGCRL	2,393 ± 0.280
4	AWIRGCRL	2,743 ± 0.193
5	WRNRCKGTD	3,123 ± 0.266

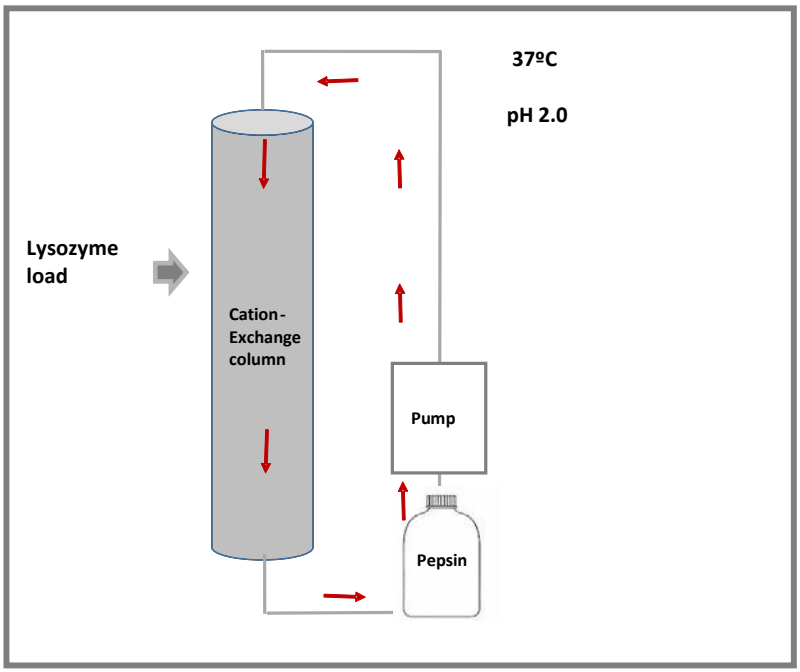

Figure 1. Hydrolysis in situ from lysozyme with pepsin in a cation exchange column inside of oven at 37˚C over night.

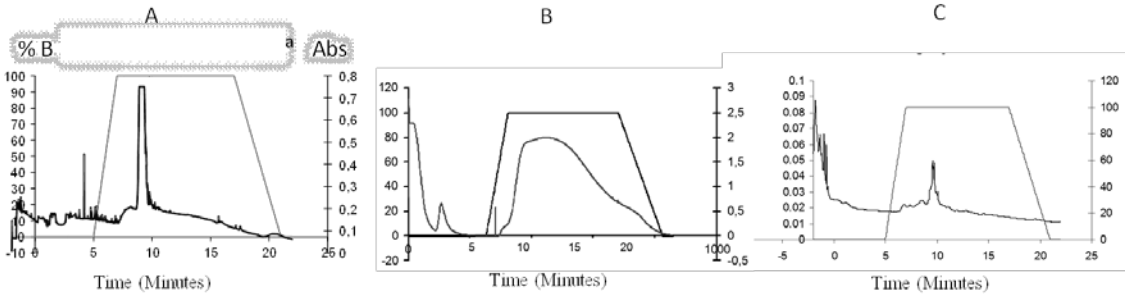

Figure 2. Fractions of FPLC obtained with: A) 3 M ammonia, B) 5 M ammonia, C) NaCl 1 M

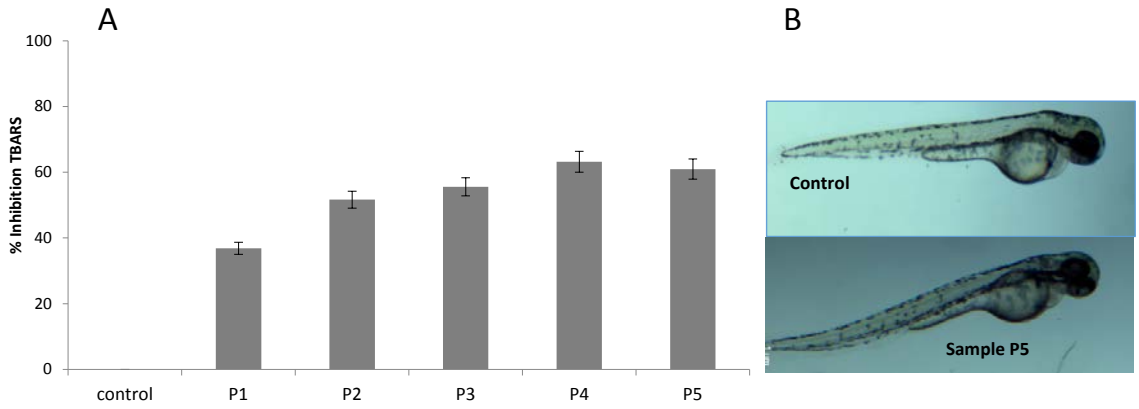

Figure 3. A) TBARS result of synthetic peptides from lysozyme. Data is expressed as % TBARS inhibition compared to positive control (error bars expressed as ± SD). P1= VAWRNRCKGTD;

P2= IRGCRL; P3= WIRGCRL; P4= AWIRGCRL; P5= WRNRCKGTD. B) Photography of zebrafish larvae with peptide and without peptide after assay. All peptides were assay at 50 µg7ml.

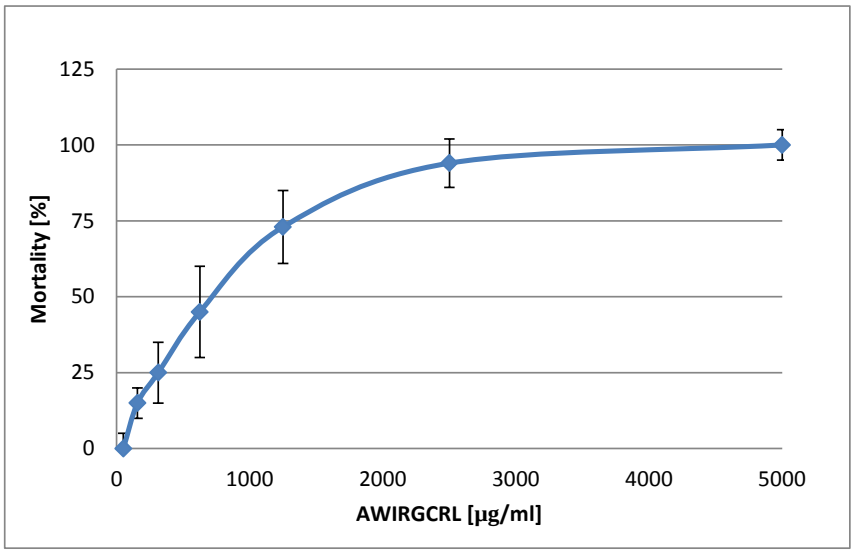

Figure 4. Mortality percentage of Zebrafish embryo treated with AWIRGCRL peptide from lysozyme at different concentrations at 48 hours for three replicates.

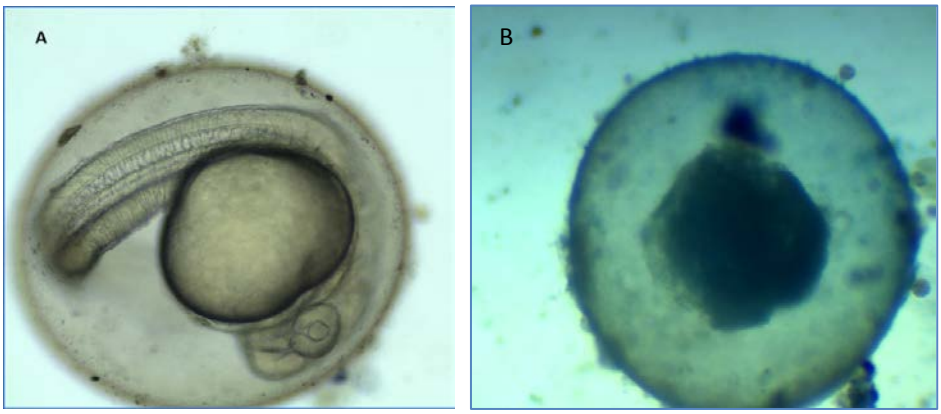

Figure 5. Peptides from lysozyme induced inhibition cellular in embryo Zebrafish. A) Control without peptides and B) embryo with AWIRGCRL peptide from lysozyme. Magnification was of 40X. AWIRGCRL peptide was incubated with zebrafish eggs during 24 hours at 26°C.