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1 # Peptidomic analysis of bioactive peptides in zebra blenny (*Salaria basilisca*)
2 # muscle protein hydrolysate exhibiting antimicrobial activity obtained by
3 # fermentation with *Bacillus mojavensis* A21

4

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## ABSTRACT

The present study investigates the antibacterial activity of zebra blenny (*Salaria basilisca*) protein hydrolysates obtained by fermentation with a proteolytic bacterium, *Bacillus mojavensis* A21. The hydrolysate exhibited nutritional as well as antibacterial properties. The fermentative zebra blenny protein hydrolysate (FZPH) was fractionated by size exclusion chromatography on a Sephadex G-25 into six major fractions (F1-F6). Fraction F2, which exhibited the highest antibacterial activity against several Gram-positive and Gram-negative bacteria, was further fractionated by reversed-phase high performance liquid chromatography (RP-HPLC). The most active fractions named A and B which exhibited antibacterial activity, were analysed using nano ESI-LC-MS/MS to identify the sequences of the peptides. A total of 28 and 41 peptides, containing 8 to 31 amino acid residues, were identified in sub-fractions A and B, respectively. Further, identified peptides sharing sequences with previously identified peptides were reported. The results of this study suggest that FZPH are good source of natural antimicrobial peptides. Therefore, ZPH could be a beneficial ingredient for nutraceuticals.

**Keywords** : *Salaria basilisca;* Fermentation; *B. mojavensis* A21; Protein hydrolysates; Antibacterial; Peptide.

56 ## 1. Introduction

57 The excessive and uncontrolled use of antibiotics in medicine, animal production, and 58 preservation of food products has greatly contributed to the emergence of resistant pathogens 59 to conventional antibiotics which frequently leads in treatment failure, severe outcomes and 60 increasing expenditures. Thus, the discovery of new antimicrobial compounds has become a 61 major challenge of researchers and pharmaceutical industries. Therefore, there is great interest 62 in finding new and safe antibacterial compounds from natural sources.

63 Antimicrobial peptides (AMPs) are positively charged, usually have less than 50 amino 64 acids, of which nearly 50% are hydrophobic and they are generally amphipathic (Van't Hof, 65 et al., 2001). They serve as an ancient defense mechanism against pathogenic microorganisms 66 that easily come in contact with the host through the environment [1].

67 Antimicrobial peptides (AMPs) from marine organism hydrolysates are increasingly 68 isolated and reported during the last few years. Ennaas et al. [2] have recently isolated four 69 antibacterial peptides from Protamex hydrolysates of Atlantic mackerel (*Scomber scombrus*) 70 by-products. Among them SIFIQRFTT peptide totally inhibited tested Gram-positive 71 (*Listeria innocua*) and Gram-negative (*Esherichia coli*) bacterial strains, while the others 72 exhibited partial inhibition. A decapeptide (GLSRFTALK) inhibiting *Staphylococcus aureus* 73 was identified from anchovy cooking waste water by-products [3]. Similarly, antibacterial 74 peptides fractions isolated from proteolysed snow crab and Atlantic rock crab by-products 75 were found to exhibit inhibitory activity against several Gram-positive and Gram-negative 76 bacteria [4, 5]. Balakrishnan et al. [6] reported that fermented protein hydrolysates from 77 delimed tannery fleshings exhibited antioxidant and antibacterial activities.

78 Marine biofunctional peptides have been described to be obtained using three methods: 79 solvent extraction, enzymatic hydrolysis and microbial fermentation [7]. Although several 80 protein hydrolysates have been produced by from plant and animal sources using exogenous

81 proteases, few studies have been conducted on the generation of antibacterial peptides using
82 microbial fermentation [8].

83 In this study, the antibacterial activity of zebra blenny muscle protein hydrolysates
84 obtained by fermentation with *Bacillus mojavensis* A21 have been investigated for the first
85 time. The most active hydrolysate has been characterized and free amino acid composition
86 together with nucleotides and total amino acids were determined. The identification of the
87 sequences of generated peptides in most active RP-HPLC fractions was done using mass
88 spectrometry in tandem.

89

90 **2. Materials and methods**

91 **2.1. Reagents**

92 Acetonitrile was of HPLC grade from Scharlau (Scharlab SL, Barcelona). All solutions
93 were freshly prepared in bi-distilled water obtained from a Culligan system; the resistivity
94 was approximately 18 MΩ*cm. Other chemicals and reagents used were of analytical grade
95 (HCl, Ethanol, TFA…)

96 **2.2. Materials**

97 Zebra blenny (*Salaria basilisca*) was freshly purchased from the fish market of Sfax city,
98 Tunisia. The sample was packed in polyethylene bags, placed in ice with a sample/ice ratio of
99 approximately 1:3 (w/w) and transported to the laboratory within 30 min. Muscles were
100 separated and then rinsed with cold distilled water to remove salts and other contaminants.

101

102 **2.3. Zebra blenny meat flour (ZMF) preparation**

103 Raw muscles from zebra blenny (500 g) in 1000 ml bi-distilled water were cooked for 20
104 min at 100°C. The bones were removed from cooked fish and fillets were collected and dried
105 in an oven at 80 °C for 18 h. The dried fish preparation was minced to obtain fine powder.
106

107 **2.4. Production of fermented zebra blenny protein hydrolysate**

108 *B. mojavensis* A21 [9] was used to produce protein hydrolysate through fermentation of
109 zebra blenny meat proteins. Inoculum was routinely grown in Luria-Bertani broth medium
110 composed of 10 g/l peptone, 5 g/l yeast extract and 5 g/l NaCl [10]. For the production of
111 protein hydrolysate, *B. mojavensis* was grown in medium containing powdered zebra blenny
112 meat (30 g/l; pH 8.0) as only carbon and nitrogen sources. The media were autoclaved at 121
113 °C for 20 min. Cultivations were conducted in 1000 ml Erlenmeyer flask containing 100 ml of
114 culture medium. Incubations were carried out in a shaking incubator (Technico Ltd, Chennai,
115 India) at 200 rpm for 4 h to 48 h at 37 °C in order to select the best degree of hydrolysis (DH)
116 that allows maximum bioactivity. Cultures were then centrifuged at 8500×g for 30 min at 4
117 °C. Resulted cell-free supernatents, were freeze dried using freeze dryer (Bioblock Scientific
118 Christ ALPHA 1-2, IllKrich-Cedex, France). The freeze dried powders with a yield of about
119 0.3 g of dried powder/g of zebra blenny meat flour were then evaluated for compositions and
120 antibacterial activities. All experiments were carried out in triplicate.
121

122 **2.5. Determination of the degree of hydrolysis (DH)**

123 DH was measured using o-phthaldialdehyde (OPA) following the method referred by
124 Nielsen et al. [11]. An aliquot of the sample (400 µl), at a concentration of 1 mg/ml, was
125 added to 3 ml of OPA solution and homogenized for 5 seconds. The mixture's absorbance
126 was measured after 2 min at 340 nm. The same volume of distilled water instead of FZPH
127 sample was used as control. A serine standard was also prepared (0.1 mg/ml).

128 The DH was expressed in percentage and calculated using the following formulas :

129 $$DH = \frac{h}{h_{tot}} \times 100$$

130

131 $$h = \frac{Serine\text{–}NH_2 - \beta}{\alpha}$$

132

133 $$Serine\ \text{–}NH_2 = \frac{Abs_{sample} - Abs_{control}}{Abs_{serine} - Abs_{control}} \times \frac{0.9516 \times 0.1 \times 10}{W \times P}$$

134

135 where $Abs_{sample}$ is the absorbance of FZPH, $Abs_{serine}$ is the absorbance of serine standard,

136 $Abs_{control}$ is the absorbance of control, *w* is the weight in grams of FZPH sample in 100 mL

137 and *P* is the protein content (%) of FZPH. Constants values of the number of equivalent

138 peptide bonds per gram of protein ($h_{tot}$), α and β were assumed to be 8.6 meq $g^{-1}$, 1 and 0.4,

139 respectively, since these are the recommended values for fish by Nielsen et al. [11].

140 Each experiment was done in triplicate.

141

142 **2.6. Chemical analysis**

143 The moisture and ash content were determined according to the AOAC standard methods

144 930.15 and 942.05, respectively [12]. Total nitrogen content of FZPH and undigested protein

145 substrates was determined using the Kjeldahl method according to the AOAC method number

146 984.13 [12] and the equipment of BÛCHI Digestion Unit K-424, Switzerland. Crude protein

147 was estimated by multiplying total nitrogen content by the factor of 6.25. Fat content was

148 determined gravimetrically after Soxhlet extraction of dried samples with hexane for 2 h using

149 Nahita Model 655, Navarra. All measurements were performed in triplicate.

150

151 **2.7. Amino acid composition of FZPH and fractions from Sephadex G-25**

Amino acid composition was determined after samples hydrolysis with 6 N HCl at 110°C for 24 h under nitrogen atmosphere. After removing the HCl, freed amino acids were derivatized using phenylisothiocyanate according to Bidlingmeyer et al (1984) and analyzed by reversed-phase high performance liquid chromatography (HPLC) in a 1200 Agilent liquid chromatograph (Agilent Tech., CA, USA). Amino acids were separated using a Waters Pico Tag column (3.9 x 300 mm, 5 µm) by following the method described in Aristoy and Toldrá [13]. Detection was at 254 nm, and amino acids were identified by their retention times compared to standards. The amount of amino acids was calculated, based on the peak area in comparison with that of amino acids standard. The amino acid content was expressed as their respective percentage of number of residues in the sample. All analyses were performed in duplicate.

**2.8. Free amino acid analysis**

ZMF and FZPH were deproteinized by adding 3 volumes ACN for 1h. After centrifugation at 12,000 rpm for 5 min, 300 µl of the supernatants were dried. The free amino acids were analyzed by RP-HPLC as previously described.

**2.9. Nucleotides content**

Nucleotides and derived compounds were analyzed in ZMF and FZPH by RP-HPLC and analysed with an 1100 Agilent liquid chromatograph equipped with a diode array detector using a C-18 Synergi MAX-RP 4 µm, 80A, 150×4.6 mm (Phenomenex, Torrance, CA, USA) column at 30 °C. The chromatographic separation was performed according to Hernández-Cázares et al. (2011). The separated compounds were monitored at a wavelength of 260 nm for ATP, AMP, UMP, GMP, uridin and NAD and 250 nm for IMP, inosine and hypoxanthine

176 and identified by their respective retention times and spectrum between 200 and 350 nm.
177 Quantification was performed by means of their respective calibration curves.
178

179 **2.10. Maldi-ToF mass spectrometry analysis**

180 The analysis was done in a 5800 MALDI-ToF/TOF instrument (AB Sciex) in positive
181 reflectron mode (3000 shots every position) in a range from 150 to 2000 Da; the laser
182 intensity was manually adjusted to maximize the S/N ratio. Plate model and acquisition
183 method were calibrated by AB SCIEX calibration mixture (des-Arg1-Bradykinin at 1 fmol/µl;
184 Angiotensin I at 2 fmol/µl; Glu1-Fibrinopeptide B at 1.3 fmol/µl; ACTH (1–17 clip) at 2
185 fmol/µl; ACTH (18–39 clip) at 5 fmol/µl; and ACTH (7–38 clip) at 3 fmol/µl) in 13 positions.
186 Dried hydrolysates were dissolved in 5% ACN containing 0.1% TFA, and 1 µl of every
187 sample was directly spotted on 10 positions in the MALDI plate and allowed to dry. Once
188 dried, 0.5 µl ofmatrix solution (5 mg/ml of α-Cyano-4-hydroxycinnamic acid (CHCA) in
189 0.1% TFA–ACN/$H_2O$ (7:3, v/v)) was spotted.

190 The analysis of data was done using mMass software (http://www.mmass.org/).
191

192 **2.11. Antibacterial activity**

193 Antibacterial activity assays were performed according to the method described by
194 Berghe and Vlietinck [14]. Antibacterial activities were tested against 6 Gram-negative (*E.*
195 *coli, P. aeruginosa, K. pneumoniae, S. enterica, S. typhi, Enterobacter sp.*) and 3 Gram-
196 positive (*S.aureus, B. cereus, M. luteus*) bacteria. Samples were dissolved in distilled water,
197 and sterilized by filtration through a 0.22 µm Nylon membrane filter. A 200 µl culture
198 suspension of the microorganism ($10^6$ colony-forming units (cfu)/ml of bacteria cells
199 estimated by absorbance at 600 nm) were spread on Muller–Hinton agar. Then, bores (3 mm
200 depth, 4 mm diameter) were made using a sterile borer and were loaded with 80 µl of

201 samples. The Petri dishes were kept, first during 1 h at 4 °C, and then during 24 h at 37 °C.
202 Antibacterial activity was evaluated by measuring the diameter of the growth inhibition zones
203 in millimeters.
204
205 **2.12. Fractionation and characterisation of peptides**
206 **2.12.1. Size-exclusion chromatography**
207 The freeze dried FZPH (1g) was suspended in 5 ml of bi-distilled water, and then was
208 subjected to Sephadex G-25 gel filtration column (2.9 cm × 54 cm) fractions of 5 ml were
209 collected at a flow rate of 30 ml/h and the absorbance was measeared at 280 nm.
210
211 **2.12.2. Reversed-phase high performance liquid chromatography**
212 The most active fractions was dissolved in distilled water, filtered through 0.22 µm
213 filters and then injected into an Agilent 1100 HPLC system for separation in a Symmetry
214 Prep™ C18 column (4.6×250 mm, 5µm) from Waters (Milford, MA, USA). Solvent A was
215 0.1% TFA in bidistilled water and solvent B consisted of ACN / bidistilled water (60:40, v/v)
216 containing 0.085% of TFA. The mobile phases were filtered through a 0.45 µm filter and
217 degassed. The elution was 100% solvent A for 2 min, followed by a linear gradient to 40% of
218 solvent B in 50 min and then taken to 100% of solvent B and maintaining for 10 min at a flow
219 rate of 1 ml/min. The chromatogram was monitored at 214 nm and collected fractions (1 ml)
220 were freeze-dried then assayed for antibacterial activity.
221
222 **2.12.3. Identification of peptides using nESI-LC–MS/MS**
223 Peptide identification was done using a nano-liquid chromatography system (Eksigent
224 of AB Sciex, CA) coupled to a quadrupole-time-of-flight (Q-ToF) system (TripleTOF®
225 5600+, AB Sciex Instruments, Framingham, MA) equipped with a nano-electrospray

226 ionization source (nano-ESI). Systems parameters were adjusted as previously published in
227 Mora et al. [15].

228 Regarding the spectra analysis, the peak list generation and database search for the
229 identification of the peptides were done using Mascot Distiller v2.4.2.0 software (Matrix
230 Science, Inc., Boston, MA).

231 The NCBInr protein database was used to identify the peptides with a significance
232 threshold $p < 0.05$ and a FDR (False Discovery Rate) of 1.5%. The tolerance on the mass
233 measurement was 100 ppm in MS mode and 0.3 Da in MS/MS ions.

234 BIOPEP (http://www.uwm.edu.pl/biochemia/index.php/pl/biopep) databases were used
235 in the search of similar sequences previously identified antibacterial activity.

236

237 **2.13. Statistical analyses**

238 Statistical analyses were performed with Stratgraphics ver. 5.1, professional edition
239 (Manugistics Corp., USA) using ANOVA analysis. Differences were considered significant at
240 $p< 0.05$.

241

242 **3. Results and discussion**

243 **3.1. Preparation of fermented zebra blenny protein hydrolysate**

244 In this study, previous tests were done in order to select the DH that allow to obtain the
245 most active protein hydrolysate. The antibacterial activity against *E. coli* was measured as a
246 function of time.

247 As shown in Table 1, the highest growth inhibition was obtained after 12 h of
248 incubation, thereafter the inhibitory activity decreased and then disappeared. Results
249 demonstrate that DH had a significant effect on bacteria growth inhibition activity, and that
250 the highest activity was reached with a DH of 17.35 % (12 h of incubation).

251 FZPH with a DH of 17.35 % was further tested against several Gram+ and Gram –
252 bacteria (Table 2). No antibacterial activity was detected with the undigested zebra blenny
253 muscle proteins (t = 0). FZPH was found to inhibit the growth of *M. luteus, E. coli, K.*
254 *pneumoniae, S. enterica* and *Enterobacter sp.,* and the highest antibaterial activity was
255 detected against *E. coli* and *K. pneumoniae*.
256
257 **3.2. Chemical composition, free amino acids and nucleotides content**
258 The chemical composition of freeze dried ZPH was determined (Table 3) and compared to
259 that of zebra blenny meat flour. The proximate composition of dried ZMF showed high
260 protein content (84.16% of dry matter basis) ($p<0.05$) whereas protein content was about 80%
261 in FZPH ($p<0.05$). The fat content level in the hydrolysate was 0.08%, which was
262 significantly lower than ZMF (5.44%) ($p< 0.05$). FZPH had high ash content (11.54%).
263 In Table 4, free amino acid compositions of ZMF and FZPH are expressed as
264 percentage of residues. The results revealed a wealth of Glu, Ala, Val and Ile. An important
265 amount of Taurine was detected (10.52 % in ZMF and 15.52% in FZPH).
266 Nucleotides and derived compound of undigested muscle and FZPH were determined
267 by HPLC and are shown in Table 5. Hypoxanthine is the major compound in the undigested
268 and FZPH samples (7.62 and 9 µmol/g, respectively), whereas ADP, ATP, and uric acid were
269 undetectable. AMP was not detected in FZPH; UMP and uridine were also not detected in
270 ZMF. Regarding this, it is well known that after death, ATP is rapidly split into ADP and
271 subsequently into AMP and IMP. IMP is then degraded to inosine and hypoxanthine which in
272 turn can be oxidized to uric acid [16]. So, the accumulation of hypoxanthine in the fish
273 muscle hydrolysate can reflect the enzymatic breakdown. Nucleotides and especially IMP and
274 GMP, which are abundant in seafood, are responsible for theumami taste sensation [17]. The
275 presence of these nucleotides in ZMF, contributes to the umami sensor. In fact, it has been

276 noted that the combination of nucleotides and umami-related amino acids (aspartic and
277 glutamic acids) would increase synergistically the umami response.
278
279 ### 3.3. Amino acid composition
280 The amino acid compositions of FZPH and its fractions, obtained after fractionation by
281 G25 gel filtration and expressed as percentage of residues, are reported in Table 6. The amino
282 acid compositions of the different samples revealed that they are rich in Lys, Glu, Ala and
283 Gly. The sum of these amino acids was between 36.13% for ZMF and 52.92% for FZPH-F6.
284 The obtained percentages were higher than previously published results in salmon (20.37%)
285 and cod (7.15%) protein hydrolysates [18]. Taurine, a sulfur-containing amino acid derived
286 from methionine and cysteine, was detected in all samples. The FZPH-F4 had the highest
287 taurine content (4.28%), whereas less taurine was found in ZMF (0.28%). Taurine contents
288 were similar to those reported by Lassoued et al. [19] in thornback ray protein hydrolysates
289 and by Silva et al. [20] in tilapia protein hydrolysates. Taurine may be a relevant amino acid
290 for use as a nutritional supplement to protect against oxidative stress, neurodegenerative
291 diseases or atherosclerosis [21].
292
293 ### 3.4. RP-HPLC profiles of ZMF and FZPH
294 RP-HPLC efficiently separates peptides generated in protein hydrolysates, and can give
295 some indication about hydrophobic/hydrophilic peptide ratio [22]. Several researchers have
296 employed this technique in studying protein hydrolysates from bovine β-lactoglobulin [23]
297 and thornback ray (*Raja clavata*) muscle hydrolysates [19]. The RP-HPLC elution profiles of
298 ZMF and FZPH are presented in Fig. 1. Several peaks are detectable by RP-HPLC confirming
299 the hydrolysis of the muscle protein and the generation of several peptides in FZPH. The
300 retention times of tyrosine and tryptophan (13.3 min and 26.03 min, respectively) were used

301 to divide the chromatograms into three zones. Zone 1 consisted of peptides eluted before
302 tyrosine (hydrophilic peptides). Zone 2 comprised of peptides eluted between tyrosine and
303 tryptophan (low hydrophobic peptides), while zone 3 comprised of those eluted after
304 tryptophan (high hydrophobic peptides). FZPH showed a higher a high content of
305 hydrophobic peptides.

306

307 **3.5. MALDI-ToF mass spectrometry analysis**

308 In this study, MALDI-ToF mass spectrometry was used to distribution of peptides
309 generated during fermentative hydrolysis from 600 to 1800 Da. As shown in Fig. 2, there is an
310 extensive distribution of peptides in FZPH in a wide range of molecular masses. In fact,
311 whereas no peptides were detected in ZMF (Fig. 2A), FZPH was mainly constituted of low
312 molecular weight peptides in the range of 600–1800 Da. Many studies reported the molecular
313 weight distribution of protein hydrolysates using the MALDI-ToF technique [24, 25, 26].

314

315 **3.6. Fractionation and identification of peptides sequences**

316 The hydrolysate FZPH, with a DH of 17.35% and produced by *B. mojavensis* A21 after
317 12 h of fermentation, contained a complex mixture of active and inactive molecules having
318 various sizes and different amino acid sequences. Potential peptides, which may exhibit high
319 antibacterial activity, are present at low concentration. Therefore, FZPH was fractionated by
320 size exclusion chromatography on a Sephadex G-25 column to isolate antibacterial peptides.
321 As reported in Fig. 3, six separated peaks, from F1 to F6 were obtained. The yields of F1, F2,
322 F3, F4, F5 and F6 fractions were 12.55%, 6.86%, 11.55%, 7.65%, 2% and 1.4%, respectively.
323 The fractions associated with each peak were pooled, freeze-dried and evaluated for
324 antibacterial activity. As reported in Table 7, except F5 and F6 which didn't display any
325 antibacterial activity against all the strains tested, F1, F2, F3 and F4 showed varying degrees

326 of antibacterial activities against at least one bacterium. Indeed, F1 was found to inhibit only
327 *E. coli*, while F2 inhibit five strains. However, none of the six fractions were found to inhibit
328 *S. aureus*, *B. cereus* and *S. typhi*. Therefore, F2 which displayed the highest antibacterial
329 activity was selected and then further fractionated by RP-HPLC. Fractions (1 ml) were
330 automatically collected, freeze-dried and their antibacterial activities were tested. The RP-
331 HPLC profile of F2 fraction, reported in Fig. 4, revealed a very large number of peaks relating
332 to the abundance of peptides generated. Antibacterial activity was only detected in fractions
333 eluted from 3 to 6 min (named sub-fraction A) and at 47 min (named sub-fraction B). These
334 two sub-fractions were selected for the identification.
335
336 **3.7. Identification of bioactive peptides**
337 Peptides from selected sub-fractions A and B were analysed by nESI-LC–MS/MS after
338 RP-HPLC separation. A total of 28 peptide sequences were identified in sub-fraction A and
339 41 peptide sequences were identified in sub-fraction B. The amino acid sequences of the
340 identified peptides were characterized as well as their position in the parent protein. The
341 observed (m/z), the experimental and the calculated molecular masses (Mr) are also shown in
342 Table 8. All the identified peptides were between 8 and 31 amino acids length, which is in
343 accord with the study of Brogden [27] reporting that the size of AMPs varies from 6 aas
344 residues to more than 59. Identified peptides have a substantial portion of hydrophobic
345 residues. The most hydrophobic peptides are GLPPYPYAG, ALPYDTPVPGY,
346 LAGNEDVILPVPA, VIISAPSADAPMF and FAGDDAPRAVFPS in sub-fraction A, and
347 AAVPSGASTGVHEALEL, WAAFPPDVAGNVDYKN and
348 LTVKEDQVFPMNPPKFDKIEDMAM in sub-fraction B. Wieprecht et al. [28] reported that
349 this hydrophobic character would let peptides enter the membrane, as the positive charge
350 would initiate the peptide interact with the surface of the bacteria (negatively charged). AMPs

351 can be further categorized based on their amino acids content and proportion, e.g., some have
352 a high content of one or two aas such as proline [29], arginine [30] and/or glycine [31]. If we
353 focused on identified peptides in sub-fraction A, we found that GLPPYPYAG has two
354 residues of glycine and three proline, ETPGGTPLAPEPD has four residues of proline and
355 two glycine, while AGCAGVGGAG has five residues of glycine. In sub-fraction B, three
356 residues of glycine were found in ISERLEEAGGATAA and four residues of arginine in
357 GYPDKIIIGMD. This high content of P, R and/or G could be attribuated to the antimicrobial
358 activity.

359 By using Biopep databases, several potential antibacterial or antimicrobial peptides
360 were detected in sub-fractions A and B (Table 9).

361 In sub-fraction A, P1 (GLPPYPYAG) contain GLP at the N-terminal, which is also
362 present at the N-terminal of GLPQE, an AMP derived from α S1-casein (10-14) [32]. Besides
363 of its potential antibacterial action, P1 could probably be hypotensive. Indeed, GLP, derived
364 from Alaska pollak skin, was found to exerce ACE-inhibitory activity [33]. Thus, this peptide
365 could be probably an antimicrobial and hypotensive. Further, GIL tripeptide found at the C-
366 terminal sequence of P2 peptide was also present at the C-terminal sequence of temporin A,
367 an antimicrobial peptide [34].

368 Comparaison revealed also the presence within the identified of peptides of tripeptides
369 or tetrapeptides present in previously identified antibacterial or antimicrobial peptides (Table
370 9).

371 In sub-fraction B, 13 peptides were found to share some homology with previously
372 identified antibacterial peptides (Table 9b). For example, peptide P6 contain IAK at the C-
373 terminal, which is also found at the C-ter of antibacterial and ACE inhibitor peptide
374 FSDKIAK derived from bovine *k*-casein [35, 36]. In another study, Gomez-Ruiz et al [37]
375 reported that the tripeptide IAK obtained from ovin milk protein exhibited a high ACE-

376 inhibitory activity. Similarly, QAA triprptide found at the C-ter op P16 peptide was also
377 found at the C-ter of Hadrurin, an antimicrobial peptide from the venom of the scorpion
378 *Hadrurus oztecus* [38]. This tripeptide could be implicated in the peptide action.

379 In addition to peptides sharing some homology sequences with previously identified
380 antibacterial peptides, potential ACE-inhibitor, DPP IV inhibitor and antioxidant peptides
381 were also reported in Table 9.

382 **4. Conclusion**

383 In the present study, ZPH obtained by fermentation was found to possess antibacterial
384 activity. The hydrolysate was fractionated by reversed-phase high performance liquid
385 chromatography (RP-HPLC) and sequences were identified using nESI-LC–MS/MS. Many
386 peptides were separated and the amino acid sequences were determined.

387 Therefore, this research provided a scientific basis for the generation of antibacterial
388 peptides from zebra blenny protein hydrolysates that could be utilized in nutraceutical.

389

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396

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671

672 **Table 1**

673 Comparison of antibacterial activity of fermented zebra blenny protein hydrolysate, elabored
674 using *B. mojavensis*, as function of time fermentation. The conditions of fermentation were
675 100 ml of reation volume, [substrate] 30 g/l, pH 8.0, temperature 37°C, agitation 200 rpm.
676 The indicator bacterium used was *E. coli*.

677

| **Time fermentation (h)** | **IZD (mm)** |
|---|---|
| **0** | – |
| **4** | – |
| **6** | – |
| **8** | 14 ± 1 |
| **12** | 20 ± 1 |
| **16** | 16 ± 1 |
| **20** | 14 ± 1 |
| **24** | 10 ± 1 |
| **28** | – |
| **32** | – |
| **48** | – |

678
679 The concentration of protein hydrolysate was 20 mg/ml.
680 IZD: Inhibition Zone Diameter
681 Values are given as mean±SD from triplicate determinations (n=3).
682 «–» No inhibition or no effect.
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**Table 2**

Antibaterial activity of fermented zebra blenny protein hydrolysate produced after 12 h of fermentation, against various indicator organisms.

| Indicator organism | ZMF | FZPH |
|---|---|---|
| **Gram (+)** | | |
| ***S.aureus*** | − | − |
| ***B. cereus*** | − | − |
| ***M. luteus*** | − | + |
| **Gram (-)** | | |
| ***E. coli*** | − | +++ |
| ***P. aeruginosa*** | − | − |
| ***K. pneumoniae*** | − | ++ |
| ***S. enterica*** | − | + |
| ***S. typhi*** | − | − |
| ***Enterobacter sp.*** | − | + |

Zone inhibition: +++: >1.5 cm; ++: 1.0-1.5 cm, +: <1.0 cm, and -: No activity.

725 **Table 3**

726 Chemical constituents of undigested zebra blenny protein and its hydrolysate.

727

| Composition (%) | ZMF | FZPH |
|---|---|---|
| **Protein** | 84.16 ± 0.41[a] | 80.00 ± 0.25[b] |
| **Fat** | 5.44 ± 0.30[a] | 0.08 ± 0.01[b] |
| **Moisture** | 8.67 ± 0.06[b] | 9.64 ± 0.44[a] |
| **Ash** | 9.68 ± 0.32[b] | 11.54 ± 0.21[a] |

734
735 Values are given as mean ± SD from triplicate determinations (n= 3).
736 ZMF : zebra meat flour.
737 FZPH : zebra blenny protein hydrolysate fermented using B. mojavensis A21 during 12h.
738 [a,b,c] Different letters in the same line indicate significant differences ($p < 0.05$).
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## Table 4

Free amino acid composition of zebra blenny meat flour and its hydrolysates.

| | Number of residues/100 | |
|---|---|---|
| | **ZMF** | **FZPH** |
| **Aspartic acid (Asx)[1]** | 2.91 ± 0.37[a] | 2.07 ± 0.27[b] |
| **Glutamic acid (Glx)[1]** | 7.97 ± 0.29[b] | 13.29 ± 0.14[a] |
| **Serine (Ser)** | 5.36 ± 0.08[a] | 1.40 ± 0.09[b] |
| **Histidine (His)[2]** | nd | nd |
| **Glycine (Gly)** | 3.97 ± 0.35[a] | 4.00 ± 0.24[a] |
| **Threonine (Thr)[2]** | 6.88 ± 0.11[a] | 3.84 ± 0.34[b] |
| **Alanine (Ala)** | 15.25 ± 0.22[a] | 9.60 ± 0.46[b] |
| **Arginine (Arg)** | 7.05 ± 0.04[b] | 7.72 ± 0.07[a] |
| **Taurine (Tau)** | 10.52 ±0.38[b] | 15.52 ± 0.28[a] |
| **Tyrosine (Tyr)** | 8.06 ± 0.41[a] | 4.47 ± 0.44[b] |
| **Valine (Val)[2]** | 5.20 ± 0.24[b] | 14.41 ± 0.07[a] |
| **Methionine (Met)[2]** | nd | nd |
| **Phenylalanine (Phe)[2]** | 4.02 ± 0.27[a] | 1.80 ± 0.33[b] |
| **Isoleucine (Ile)[2]** | 2.97 ± 0.20[b] | 11.81 ± 0.24[a] |
| **Leucine (Leu)[2]** | 6.19 ± 0.37[a] | 5.91 ± 0.25[b] |
| **Lysine (Lys)[2]** | 2.55 ± 0.32[a] | 1.78 ± 0.12[b] |
| **Hydroxyproline (Hyp)** | 2.70 ± 0.10 | nd |
| **Proline (Pro)** | 8.40 ± 0.37[a] | 2.36 ± 0.17[b] |
| **Total** | 100 | 100 |

Values are given as mean ± SD from duplicate determinations (n= 2).
[a,b] Different letters in the same line indicate significant differences ($p < 0.05$).
[1] Asx = Asp + Asn; Glx= Glu + Gln.
[2] Essential amino acids.
nd: not detected.

793 **Table 5**

794 Nucleotides composition of zebra blenny meat flour and zebra blenny protein hydrolysate.

795

| | µmole/g | |
|---|---|---|
| | **ZMF** | **FZPH** |
| **ATP** | nd | Nd |
| **AMP** | 1.43 ± 0.57 | Nd |
| **ADP** | nd | Nd |
| **IMP** | 0.92 ± 0.14[a] | 1.12 ± 0.25[a] |
| **Inosine** | 0.56 ± 0.08[b] | 0.74 ± 0.21[a] |
| **Hypoxanthine** | 7.62 ± 0.37[b] | 9.00 ± 0.15[a] |
| **Uridine** | nd | 1.30 ± 0.28 |
| **Uric acid** | nd | Nd |
| **UMP** | nd | 0.63 ± 0.23 |
| **GMP** | nd | 0.70 ± 0.15 |

796 797 798 799 800 801 802 803

804 Values are given as mean ± SD from duplicate determinations (n= 2).

805 [a,b] Different letters in the same line indicate significant differences ($p < 0.05$).

806 nd: not detected.

807 808 809 810 811 812 813 814 815 816 817 818 819 820 821 822 823 824

**Table 6**

Amino acid composition of zebra blenny meat flour, its hydrolysate and its G25 fractions.

| | Number of residues/100 residues | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **ZMF** | **FZPH** | **F1** | **F2** | **F3** | **F4** | **F5** | **F6** |
| **Aspartic acid (Asx)[1]** | 7.00 ± 0.02[e] | 8.04 ± 0.05[c,d] | 9.20 ± 0.01[a] | 8.66 ± 0.09[b] | 8.80 ± 0.05[b] | 8.09 ± 0.08[c,d] | 8.22 ± 0.16[c] | 7.93 ± 0.05[d] |
| **Glutamic acid (Glx)[1]** | 7.60 ± 0.13[f] | 15.07 ± 0.02[c] | 16.75 ± 0.10[a] | 16.75 ± 0.06[a] | 16.51 ± 0.34[b] | 14.68 ± 0.03[d] | 13.42 ± 0.24[e] | 13.41 ± 0.18[e] |
| **Serine (Ser)** | 4.02 ± 0.10[a] | 3.70 ± 0.07[b,c] | 3.93 ± 0.02[a] | 3.81 ± 0.02[b] | 3.65 ± 0.05[c] | 3.62 ± 0.12[c] | 3.73 ± 0.09[b,c] | 3.60 ± 0.08[c] |
| **Histidine (His)[2]** | 2.94 ± 0.02[a] | 1.05 ± 0.17[d] | nd | nd | nd | 1.29 ± 0.29[c] | 1.09 ± 0.19[d] | 1.39 ± 0.22[b] |
| **Glycine (Gly)** | 7.12 ± 0.02[f] | 10.10 ± 0.04[d] | 12.22 ± 0.00[c] | 10.04 ± 0.01[d] | 8.21 ± 0.05[e] | 10.19 ± 0.06[d] | 16.84 ± 0.07[b] | 20.78 ± 0.03[a] |
| **Threonine (Thr)[2]** | 4.38 ± 0.02[b] | 3.00 ± 0.08[d] | 5.51 ± 0.04[a] | 5.60 ± 0.05[a] | 4.43 ± 0.05[b] | 3.43 ± 0.15[c] | 3.33 ± 0.09[c] | 3.08 ± 0.12[d] |
| **Alanine (Ala)** | 8.04 ± 0.02[c] | 9.94 ± 0.17[a] | 6.46 ± 0.07[e] | 6.15 ± 0.05[e] | 6.46 ± 0.14[e] | 8.57 ± 0.30[b] | 7.40 ± 0.23[d] | 7.60 ± 0.22[d] |
| **Arginine (Arg)** | 7.09 ± 0.03[d] | 7.84 ± 0.08[c] | 9.13 ± 0.04[b] | 9.74 ± 0.06[a] | 9.92 ± 0.04[a] | 8.02 ± 0.14[c] | 5.92 ± 0.07[e] | 5.17 ± 0.11[f] |
| **Taurine (Tau)** | 0.28 ± 0.02[e] | 2.13 ± 0.22[c] | 0.53 ± 0.02[e] | 1.37 ± 0.09[d] | 1.89 ± 0.23[c] | 4.28 ± 0.39[a] | 4.10 ± 0.20[a] | 3.35 ± 0.27[b] |
| **Tyrosine (Tyr)** | 3.46 ± 0.02[a] | 0.60 ± 0.16[e] | 0.86 ± 0.07[d] | 1.02 ± 0.07[d] | 0.96 ± 0.12[d] | 1.92 ± 0.30[c] | 3.22 ± 0.19[a] | 2.37 ± 0.23[b] |
| **Valine (Val)[2]** | 5.49 ± 0.02[a] | 2.61 ± 0.04[c] | 2.38 ± 0.01[d] | 2.77 ± 0.01[c] | 3.63 ± 0.03[b] | 3.47 ± 0.07[b] | 2.87 ± 0.06[c] | 2.79 ± 0.03[c] |
| **Methionine (Met)[2]** | 3.74 ± 0.02[a] | 2.24 ± 0.04[b] | 1.75 ± 0.02[b,c] | 1.97 ± 0.02[b] | 2.09 ± 0.03[b] | 2.09 ± 0.07[b] | 1.58 ± 0.04[c] | 1.58 ± 0.06[c] |
| **Phenylalanine (Phe)[2]** | 4.24 ± 0.01[a] | 2.41 ± 0.14[d,e] | 2.39 ± 0.06[d,e] | 2.32 ± 0.05[e] | 2.62 ± 0.10[d] | 3.16 ± 0.24[c] | 3.54 ± 0.17[b] | 3.17 ± 0.18[c] |
| **Isoleucine (Ile)[2]** | 5.32 ± 0.02[a] | 2.33 ± 0.04[b] | 1.50 ± 0.02[c] | 1.63 ± 0.02[c] | 2.18 ± 0.02[b] | 2.42 ± 0.07[b] | 2.88 ± 0.04[b] | 2.62 ± 0.03[b] |
| **Leucine (Leu)[2]** | 9.21 ± 0.03[a] | 4.50 ± 0.09[b] | 3.48 ± 0.03[d] | 3.91 ± 0.02[c] | 4.72 ± 0.07[b] | 4.94 ± 0.15[b] | 4.09 ± 0.11[c] | 3.92 ± 0.11[c] |
| **Lysine (Lys)[2]** | 13.37 ± 0.05[d] | 16.77 ± 0.11[b] | 13.11 ± 0.05[d] | 15.64 ± 0.08[c] | 17.73 ± 0.04[a] | 15.02 ± 0.18[c] | 11.48 ± 0.07[e] | 11.13 ± 0.14[e] |
| **Hydroxyproline (Hyp)** | 1.61 ± 0.05[e] | 2.91 ± 0.06[c] | 4.70 ± 0.03[a] | 3.49 ± 0.04[b] | 2.27 ± 0.04[d] | 1.52 ± 0.10[e] | 2.20 ± 0.07[d] | 2.12 ± 0.08[d] |
| **Proline (Pro)** | 5.08 ± 0.02[b] | 4.76 ± 0.07[c] | 6.10 ± 0.04[a] | 5.12 ± 0.04[b] | 3.93 ± 0.04[d] | 3.26 ± 0.12[e] | 4.10 ± 0.07[d] | 3.96 ± 0.10[d] |
| **Total** | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |

Values are given as mean ± SD from duplicate determinations (n= 2).

[a,b,c] Different letters in the same line indicate significant differences ($p < 0.05$).

[1] Asx = Asp + Asn; Glx= Glu + Gln.

[2] Essential amino acids.

nd: not detected.

845 **Table 7**

846 Antibacterial activity of FZPH fractions obtained from size-exclusion chromatography. The

847 concentration of fractions was 5 mg/ml.

| Organism indicator | Fractions G25 | | | | | |
|---|---|---|---|---|---|---|
| | F1 | F2 | F3 | F4 | F5 | F6 |
| **Gram (+)** | | | | | | |
| ***S. aureus*** | − | − | − | − | − | − |
| ***B. cereus*** | − | − | − | − | − | − |
| ***M. luteus*** | − | + | − | + | − | − |
| **Gram (-)** | | | | | | |
| ***E. coli*** | ++ | +++ | + | ++ | − | − |
| ***P. aeruginosa*** | − | − | − | − | − | − |
| ***K. pneumoniae*** | − | +++ | ++ | ++ | − | − |
| ***S. enterica*** | − | + | − | − | − | − |
| ***S. typhi*** | − | − | − | − | − | − |
| ***Enterobacter sp.*** | − | + | − | − | − | − |

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869 **Table 8**

870 Identification of peptides present in sub-fractions A (a) and B (b) by nano-liquid chromatography and mass spectrometry in tandem.

a

| Sequence | Observed[a] (m/z) | Expected[b] (Mr) | Charge state[c] (+) | Calculated[d] (Mr) | Modifications[e] | Protein origin |
|---|---|---|---|---|---|---|
| AGATGGSVP | 358.738 | 715.462 | 2 | 715.350 | Deamidated(N)@7 | REVERSED NAD-dependent L-serine dehydrogenase |
| AGCAGVGGAG | 360.221 | 718.427 | 2 | 718.307 | Oxidation(M)@10 | Chemotactic signal transduction system substrate-binding protein BasB |
| LSKNLAGA | 387.236 | 772.458 | 2 | 772.444 | | Trace amine-associated receptor 5 |
| DDFNPSVH | 465.705 | 929.395 | 2 | 929.388 | | Hemoglobin subunit alpha |
| GLPPYPYAG | 467.743 | 933.472 | 2 | 933.459 | | Regucalcin |
| LVDGLDVGIL | 507.278 | 1012.542 | 2 | 1012.580 | | Very-long-chain 3-oxoacyl-CoA reductase |
| LFDKPVSPL | 508.295 | 1014.576 | 2 | 1014.575 | Oxidation(M)@11 | Creatine kinase M-type |
| EADDWLRY | 534.241 | 1066.467 | 2 | 1066.472 | | Glycogen phosphorylase, liver form |
| TYDDYVEGL | 537.739 | 1073.464 | 2 | 1073.455 | | Myosin light chain 3, skeletal muscle isoform |
| VDPENFRLL | 551.802 | 1101.589 | 2 | 1101.582 | | Hemoglobin subunit gamma-1 |
| LEGDLKLSQE | 566.303 | 1130.592 | 2 | 1130.582 | | Myosin-13 |
| GVDNPGHPFIM | 592.284 | 1182.554 | 2 | 1182.549 | | Creatine kinase M-type |
| LSYEEAITTY | 595.356 | 1188.699 | 2 | 1188.555 | | REVERSED Phosphoenolpyruvate carboxylase |
| ALPYDTPVPGY | 596.801 | 1191.587 | 2 | 1191.581 | | Glycogen phosphorylase, liver form |
| GYPDKIIIGMD | 611.315 | 1220.616 | 2 | 1220.611 | | Beta-enolase |
| ETPGGTPLAPEPD | 640.801 | 1279.587 | 2 | 1279.593 | Arg-loss@C-term | Endoribonuclease YbeY |
| HHPDDFNPSVH | 434.529 | 1300.564 | 3 | 1300.558 | Deamidated(Q)@5; Oxidation(M)@10 | Hemoglobin subunit alpha |
| LAGNEDVILPVPA | 654.384 | 1306.754 | 2 | 1306.713 | Oxidation(M)@3 | Alpha-enolase |
| IDDLEDELYAQ | 662.310 | 1322.606 | 2 | 1322.588 | | Tropomyosin alpha-1 chain |

| | | | | | | |
|---|---|---|---|---|---|---|
| VIISAPSADAPMF | 667.850 | 1333.685 | 2 | 1333.659 | Oxidation(M)@12 | Glyceraldehyde-3-phosphate dehydrogenase |
| FAGDDAPRAVFPS | 675.332 | 1348.650 | 2 | 1348.641 | | Actin-2 |
| FDMFDTDGGGDIS | 688.772 | 1375.529 | 2 | 1375.524 | | Troponin C, skeletal muscle |
| RIEELEEEIEAE | 744.861 | 1487.708 | 2 | 1487.699 | | Myosin-13 |
| ISWYDNEFGYSN | 747.820 | 1493.6026 | 2 | 1493.610 | | Glyceraldehyde-3-phosphate dehydrogenase |
| ISWYDNEFGYSNR | 747.818 | 1493.622 | 2 | 1493.610 | Arg-loss@C-term | Glyceraldehyde-3-phosphate dehydrogenase |
| VTWYDNEFGYSNR | 747.818 | 1493.622 | 2 | 1493.610 | Arg-loss@C-term | Glyceraldehyde-3-phosphate dehydrogenase |
| VTWYDNEFGYSN | 758.809 | 1515.603 | 2 | 1515.592 | Cation:Na(E)@7 | Glyceraldehyde-3-phosphate dehydrogenase |
| LEKSYELPDGQVIT | 796.423 | 1590.832 | 2 | 1590.814 | Oxidation(M)@7; Oxidation(M)@10 | Actin-2 |

[a] Molecular ion mass observed in the nLC–MS/MS system in mass/charge (m/z).
[b] Expected molecular mass in Daltons calculated from the observed m/z.
[c] Charge of the ion as [M+ H]+.
[d] Calculated relative molecular mass in Daltons.
[g] Postrasductional modification oxidation of the methionine.

b

| Sequence | Observed[a] (m/z) | Expected[b] (Mr) | Charge state[c] (+) | Calculated[d] (Mr) | Modifications[e] | Protein origin |
|---|---|---|---|---|---|---|
| QQEISDLTEQL | 643.815 | 1285.615 | 2 | 1285.604 | Gln->pyro-Glu@N-term | Myosin heavy chain, fast skeletal muscle |
| QQEISDLTEQI | 643.815 | 1285.615 | 2 | 1285.604 | Gln->pyro-Glu@N-term | Myosin-2 |
| TDAETKAFLKAGD | 456.236 | 1365.686 | 3 | 1365.677 | | Parvalbumin beta 1 |
| ISERLEEAGGATAA | 687.865 | 1373.715 | 2 | 1373.679 | | Myosin heavy chain, fast skeletal muscle |
| VEDLMIDVERANS | 745.868 | 1489.721 | 2 | 1489.708 | | Myosin heavy chain, fast skeletal muscle |
| NVLSGGTTMYPGIAD | 748.367 | 1494.719 | 2 | 1494.702 | | Actin, alpha skeletal muscle |
| AEQELVDASERVGL | 758.366 | 1514.717 | 2 | 1514.757 | | Myosin heavy chain, fast skeletal muscle |
| AVPSGASTGVHEALEL | 769.405 | 1536.795 | 2 | 1536.778 | | Enolase |
| LTEEMASQDESIAK | 776.371 | 1550.728 | 2 | 1550.713 | | Myosin heavy chain, fast skeletal muscle |
| AAVPSGASTGVHEALEL | 804.924 | 1607.833 | 2 | 1607.815 | | Enolase |
| WAAFPPDVAGNVDYKN | 890.430 | 1778.847 | 2 | 1778.826 | Oxidation(W)@1 | Myosin regulatory light chain 2, skeletal muscle |
| TERAEDEEEINAELTAK | 659.344 | 1975.010 | 3 | 1974.902 | Formyl@N-term | Myosin-2 |
| GQKDSYVGDEAQSKRGILT | 513.763 | 2051.022 | 4 | 2051.028 | | Actin, alpha skeletal muscle |
| LRMDLERAKRKLEGDLK | 415.240 | 2071.165 | 5 | 2070.173 | | Myosin heavy chain, fast skeletal muscle |
| NRRIQLVEEELDRAQER | 539.292 | 2153.139 | 4 | 2153.130 | | Tropomyosin alpha-1 chain |
| YETDAIQRTEELEEAKKK | 546.031 | 2180.097 | 4 | 2180.096 | | Myosin heavy chain, fast skeletal muscle |
| AIQRTEELEEAKKKLAQRL | 564.327 | 2253.278 | 4 | 2253.280 | | Myosin heavy chain, fast skeletal muscle |
| TESGEFSRQLDEKEALVSQL | 770.374 | 2308.100 | 3 | 2308.118 | Carbamyl(R)@8 | Myosin-2 |
| KKKLAQRLQDAEESIEAVNSK | 597.083 | 2384.303 | 4 | 2384.302 | | Myosin heavy chain, fast skeletal muscle |
| KMEGDLNEMEIQLSHANRQAA | 601.032 | 2400.098 | 4 | 2400.116 | Oxidation(M)@9 | Myosin heavy chain, fast skeletal muscle |

| | | | | | | |
|---|---|---|---|---|---|---|
| IKVLTDKLKEAETRAEFAERS | 812.113 | 2433.318 | 3 | 2433.323 | | Tropomyosin alpha-1 chain |
| DLLIDLANEVKLQEKIDNLING | 621.340 | 2481.333 | 4 | 2481.321 | Deamidated(N)@8; Deamidated(Q)@13 | Glucose-6-phosphate isomerase |
| IDNLQRVKQKLEKEKSEYKM | 502.281 | 2506.369 | 5 | 2506.357 | | Myosin heavy chain, fast skeletal muscle |
| AAALRKEQADSVAELGEQIDNLQR | 875.841 | 2624.501 | 3 | 2624.352 | | Myosin heavy chain, fast skeletal muscle |
| DLQHRLDEAESLAMKGGKKQLQK | 663.357 | 2649.400 | 4 | 2649.391 | Delta:H(2)C(2)(K)@15; Deamidated(Q)@20 | Myosin heavy chain, fast skeletal muscle |
| LLREQYEEEQEAKAELQRGMSKA | 684.843 | 2735.343 | 4 | 2735.355 | | Myosin heavy chain, fast skeletal muscle |
| MEGDLNEMEIQLSHANRQAAEAQK | 687.065 | 2744.233 | 4 | 2744.249 | Oxidation(M)@1; Oxidation(M)@8 | Myosin heavy chain, fast skeletal muscle |
| LTKLEEAEKAADESERGMKVIENR | 461.240 | 2761.397 | 6 | 2761.391 | Oxidation(M)@18 | Tropomyosin alpha-1 chain |
| ARLQTENGEFSRQLEEKEALVSQL | 926.485 | 2776.434 | 3 | 2776.388 | Deamidated(N)@7; Deamidated(Q)@13 | Myosin heavy chain, fast skeletal muscle |
| LSKEKKALQETHQQTLDDLQAEED | 700.103 | 2796.383 | 4 | 2796.377 | | Myosin-8 |
| LTVKEDQVFPMNPPKFDKIEDMAM | 947.149 | 2838.424 | 3 | 2838.364 | Oxidation(M)@11 | Myosin-2 |
| MEGDLNEMEIQLSHANRQAAEAQKQ | 715.058 | 2856.204 | 4 | 2856.313 | Oxidation(M)@8 | 715.058 |
| VQGQLKDTQLHLDDALRGQEDLKEQ | 720.123 | 2876.465 | 4 | 2876.463 | | Myosin-3 |
| VDDLEGSLEQEKKLRMDLERAKRK | 481.931 | 2885.542 | 6 | 2885.539 | | Myosin heavy chain, fast skeletal muscle |
| VLAEWKQKYEESQAELEGAQKEARS | 585.694 | 2923.436 | 5 | 2922.436 | Oxidation(W)@5 | Myosin heavy chain, fast skeletal muscle |
| GEQIDNLQRVKQKLEKEKSEYKME | 984.195 | 2949.563 | 3 | 2949.523 | | Myosin heavy chain, fast skeletal muscle |
| ALRKEQADSVAELGEQIDNLQRVKQK | 593.936 | 2964.645 | 5 | 2965.594 | | Myosin heavy chain, fast skeletal muscle |
| AAALRKEQADSVAELGEQIDNLQRVKQK | 777.728 | 3106.883 | 4 | 3107.669 | | Myosin heavy chain, fast skeletal muscle |
| QQVDDLEGSLEQEKKLRMDLERAKRK | 524.610 | 3141.619 | 6 | 3141.656 | | Myosin heavy chain, fast skeletal muscle |
| MASQDESIAKLTKEKKALQEAHQQTLDDL | 1096.197 | 3285.569 | 3 | 3285.640 | Oxidation(H)@22; Deamidated(Q)@24 | Myosin heavy chain, fast skeletal muscle |
| RARLQTENGEFSRQLEEKEALVSQLTRGKQA | 715.803 | 3573.978 | 5 | 3573.850 | Deamidated(N)@8; Deamidated(Q)@14 | Myosin heavy chain, fast skeletal muscle |

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**Table 9**

Peptides identified from F2 in the sub-fractions A (a) and B (b) sharing sequences with previously identified bioactive peptides basing on Biopep data bases.

a

| | Sequence | Previously identified bioactive peptides | | |
|---|---|---|---|---|
| | | Sequence | Activity | Reference |
| P1 | **GLP**PYPYAG | **GLP** | ACE inhibitor | Byun and Kim [33] |
| | | **GLP**QE | Antimicrobial | Hayes et al. [32] |
| P2 | LVDGLDV**GIL** | FLPLIGRVLS**GIL**~ ; FLPLIGKVLS**GIL**~ ; FFPVIGRILN**GIL**~ | Antibacterial | Simmaco et al. [34] |
| P3 | EADDW**LRY** | **LRY** | ACE inhibitor | Meisel et al. [39] |
| P4 | LEGD**LKL**SQE | LKKLKKLKKKL**LKL** | Antibacterial | Appendini and Hotchkiss [40] |
| | | **LKL** | ACE inhibitor | Ukeda et al. [41] |
| P5 | LSYEEAI**TTY** | KVREG**TTY** | ACE inhibitor | Lee et al. [42] |
| P6 | ETPGGT**PLAP**EPD | QELLLNPTHQYPVTQ**PLAP**VHNPISV | Antimicrobial | Hayes et al. [32] |
| | | QELLLNPTHQIYPVTQ**PLAP**VHNPISV | Antibacterial | Minervini et al. [43] |
| P7 | **HHP**DDFNPSVH | **HHP ; HHP**L ; **HHP**LL | Antioxidant | Chen et al. [44] |
| P8 | FAGDDAPRA**VFPS** | **VFPS** | ACE inhibitor | Loponen [45] |
| P9 | FDMFDTD**GGG**DIS | NDAACAAHCLFRGRS**GGG** | Antibacterial | Lee et al. [46] |
| P10 | RIEE**LEEE**IEAE | LEE**LEEE**LEGCE | Antioxidative | Qian et al. [47] |

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b

| | Sequence | Similar sequence | | |
|---|---|---|---|---|
| | | Sequence | Activity | Reference |
| P1 | QQEISD**LTE**QI | KTK**LTE**EEKNRLNFLKKISQRYQKFALPQYLKTVYQHQK | Antibacterial | Zucht et al. [48] |
| P2 | **ISE**RLEEAGGATAA | **ISE**LGW | Antioxidative | Tsopmo et al. [49] |
| P3 | NVLSGGTT**MYPGIA**D | **MYPGIA** | ACE inhibitor | Escudero et al. [50] |
| P4 | AEQELVDASER**VGL** | **VGL** | DPP IV inhibitor | Nongonierma et al. [51] |
| P5 | **AVP**SGASTGVHEALEL | **AVP** ; **AVP**YP | ACE inhibitor | Meisel, [52] |
| | | **AVP**YPQR | ACE-inhibitor | Maruyama et al. [53] |
| | | **AVP**YPQR | Antioxidant | Rival et al. [54] |
| P6 | LTEEMASQDES**IAK** | **IAK** | ACE inhibitor | Gomez-Ruiz et al. [55] |
| | | FSDK**IAK** | ACE inhibitor<br>Antibacterial | Lopez-Exposito et al. [35]<br>Lopez-Exposito et al. [36] |
| P7 | **AA**VPSGASTGVHEALEL | **AAV**ALLPAVLLALLAPAAANYKKPKL | Regulating cell-permeability | Du et al. [56] |
| | | VPSGK | Antioxidative | Suetsuna, [57] |
| P8 | WA**AFPP**DVAGNVDYKN | **AFPP**PNVPGPRFPPPNFPGPRFPPPNFPGPRFPPPNFPGPRFPPPNFPGPPFPPPIFPGPWFPPPPPFRPPPFGPPRFP | Antibacterial | Harwig et al. [58] |
| | | QERGSIVIQGTKEGKSRPSLDIDYKQRVYDKNGMTGDAYGGLNIRPGQPSRQHAGFEFGKEYKNGFIKGQSEVQRGPGGRLSPYFGINGGFRF | Antibacterial | Casteels-Josson et al. [59] |
| P9 | **TER**AEDEEEINAELTAK | **TER**GY | Antioxidant | Liu et al. [60] |
| P10 | GQKDSYVGDEAQSKR**GIL**T | FLPLIGRVLS**GIL**~ | Antibacterial | Simmaco et al. [34] |
| P11 | LRMDLERAKRKLEG**DLK** | SIQ**DLK**VSMKLFRKQAKWKIIVKLNDGRELSLD | Antibacterial | Mayo et al. [61] |
| P12 | NRRIQLVEEELDRA**QER** | GKKIATY**QER** ; GHKIATF**QER** | ACE inhibitor | Kohama et al. [62] |
| P13 | YETDAIQRTEELEEA**KKK** | LIRLWSHLIHIWFQNRRLKW**KKK** | Membrane active | Ferrer – Miralles et al. [63] |
| P14 | AIQR**TEE**LEEAKKKLAQRL | KTKL**TEE**EKNRLNFLKKISQRYQKFALPQYLKTVYQHQK | Antibacterial | Zucht et al. [48] |
| P15 | **TES**GEFSRQLDEKEALVSQL | **TES**QSLT | ACE inhibitor | De Gobba et al. [64] |
| | | LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPR**TES** | Antibacterial | Larrick et al. [65] |
| P16 | KMEGDLNEMEIQLSHANR**QAA** | GILDTIKSIASKVWNSKTVQDLKRKGINWVANKLGVSP**QAA** | Antibacterial | Torres-Larios et al. [38] |
| P17 | IDNLQRVKQKLE**KEK**SEYKM | GLLQTI**KEK**LESLESLAKGIVSGIQAG | Antibacterial | Rozek et al. [66] |
| P18 | **AAA**LRKEQADSVAELGEQIDNLQR | **AAA**TP<br>**AAA**TP ; **AAA**AG | ACE inhibitor<br>DPP IV inhibitor | Escudero et al. [67]<br>Gallego et al. [68] |
| P19 | **LLR**EQYEEEQEAKAELQRGMSKA | **LLR** | Antioxidant | De Gobba et al. [64] |

| | | | | |
|---|---|---|---|---|
| P20 | **LTK**LEEAEKAADESERGMKVIENR | EQ**LTK** | Antibacterial | Pellegrini et al. [69] |
| P21 | **ARL**QTENGEFSRQLEEKEALVSQL | L**ARL** | Antioxidant | Bougatef et al. [70] |
| P22 | **LSK**EKKALQETHQQTLDDLQAEED | **LSK**AQSDFG | Antioxidative | Shen et al. [71] |
| P23 | VLAEWKQKYEESQAELEGAQKE**ARS** | **ARS**YGNGVYCNNKKCWVNRGEATQSI IGGMISGWASGLAGM | Antibacterial | Tichaczek et al. [72] |
| P24 | QQVDDLEGSLEQEKKLRMDLERA**KRK** | KKWH**KRK**K ; KGWH**KRK**K | Antibacterial | Mukhija et al. [73] |

**Figure captions**

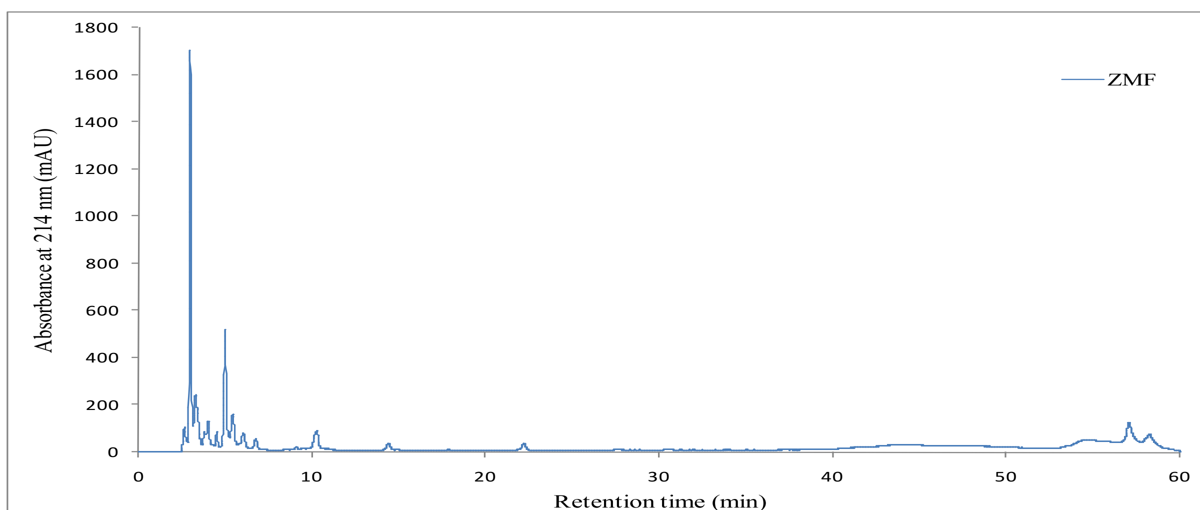
**Fig. 1.** RP-HPLC profiles of zebra blenny meat flour (ZMF) and its hydrolysate (ZPH). The column was equilibrated with solvent A (0.1% TFA in ultrapure water) and peptides were eluted with a linear increase in solvent B (0.085% TFA in acetonitrile:water (60:40 v:v)) from 0% to 100%.

**Fig. 2.** Maldi-Tof spectra of zebra blenny meat flour (ZMF) and its hydrolysate (ZPH), meseared from 600 to 2000 Da.

**Fig. 3.** Elution profile of zebra blenny protein hydrolysate separated by size exclusion chromatography on Sephadex G-25.

**Fig. 4.** Reversed-phase chromatographic separation of ZPH-F2 fraction obtained from size-exclusion chromatography. Fractions were automatically collected, freeze dried and then assayed for antibacterial activity. Fractions eluted from 3 to 6 min and fraction 47 min were pooled separately and named sub-fractions A and B, respectively.

**Fig. 1.**



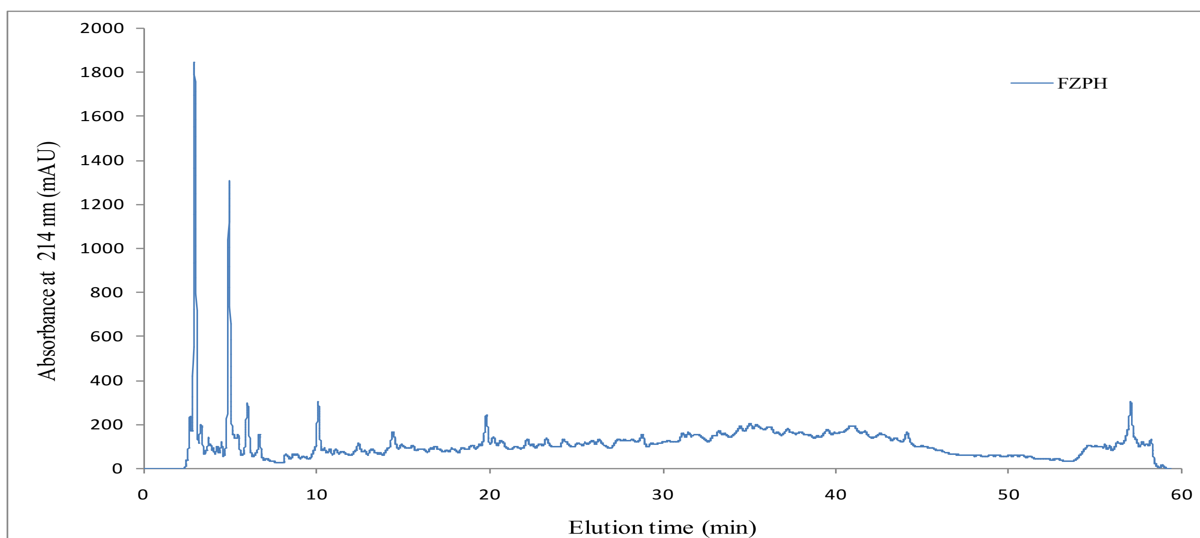
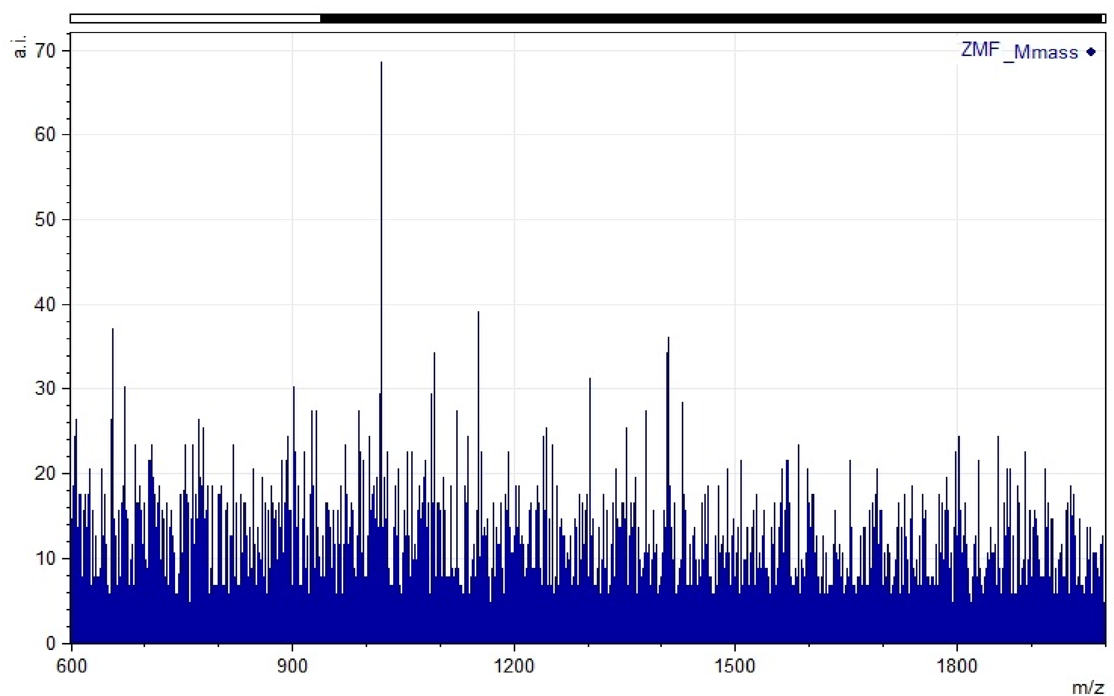



**Fig. 2.**



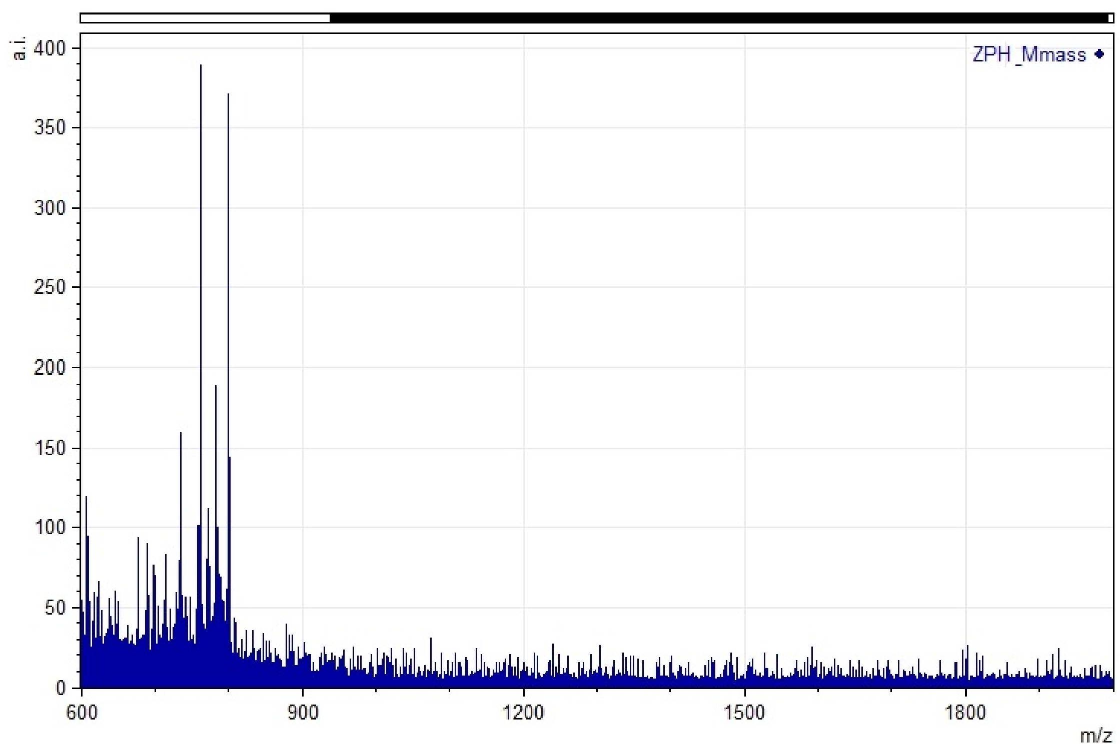



**Fig. 3.**

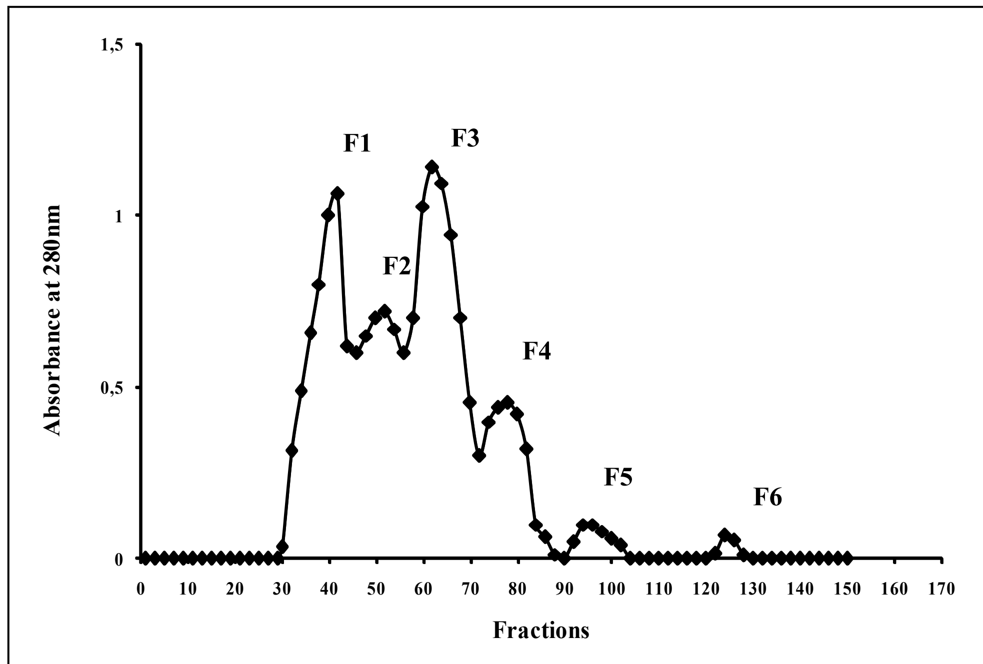


**Fig. 4.**