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

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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 mojavenensis* 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. mojavenensis* A21; Protein hydrolysates; Antibacterial; Peptide.

1. Introduction

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

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

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

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

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

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

2. Materials and methods

2.1. Reagents

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

2.2. Materials

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

2.3. Zebra blenny meat flour (ZMF) preparation

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

2.4. Production of fermented zebra blenny protein hydrolysate

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

2.5. Determination of the degree of hydrolysis (DH)

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

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

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

$$h = \frac{\text{Serine-NH}_2 - \beta}{\alpha}$$

$$\text{Serine -NH}_2 = \frac{\text{Abs}_{\text{sample}} - \text{Abs}_{\text{control}}}{\text{Abs}_{\text{serine}} - \text{Abs}_{\text{control}}} \times \frac{0.9516 \times 0.1 \times 10}{W \times P}$$

where $\text{Abs}_{\text{sample}}$ is the absorbance of FZPH, $\text{Abs}_{\text{serine}}$ is the absorbance of serine standard, $\text{Abs}_{\text{control}}$ is the absorbance of control, w is the weight in grams of FZPH sample in 100 mL and P is the protein content (%) of FZPH. Constants values of the number of equivalent peptide bonds per gram of protein (h_{tot}), α and β were assumed to be 8.6 meq g^{-1} , 1 and 0.4, respectively, since these are the recommended values for fish by [Nielsen et al. \[11\]](#).

Each experiment was done in triplicate.

2.6. Chemical analysis

The moisture and ash content were determined according to the [AOAC](#) standard methods 930.15 and 942.05, respectively [\[12\]](#). Total nitrogen content of FZPH and undigested protein substrates was determined using the Kjeldahl method according to the AOAC method number 984.13 [\[12\]](#) and the equipment of BÜCHI Digestion Unit K-424, Switzerland. Crude protein was estimated by multiplying total nitrogen content by the factor of 6.25. Fat content was determined gravimetrically after Soxhlet extraction of dried samples with hexane for 2 h using Nahita Model 655, Navarra. All measurements were performed in triplicate.

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

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

2.10. Maldi-ToF mass spectrometry analysis

The analysis was done in a 5800 MALDI-ToF/TOF instrument (AB Sciex) in positive reflectron mode (3000 shots every position) in a range from 150 to 2000 Da; the laser intensity was manually adjusted to maximize the S/N ratio. Plate model and acquisition method were calibrated by AB SCIEX calibration mixture (des-Arg1-Bradykinin at 1 fmol/μl; Angiotensin I at 2 fmol/μl; Glu1-Fibrinopeptide B at 1.3 fmol/μl; ACTH (1–17 clip) at 2 fmol/μl; ACTH (18–39 clip) at 5 fmol/μl; and ACTH (7–38 clip) at 3 fmol/μl) in 13 positions. Dried hydrolysates were dissolved in 5% ACN containing 0.1% TFA, and 1 μl of every sample was directly spotted on 10 positions in the MALDI plate and allowed to dry. Once dried, 0.5 μl of matrix solution (5 mg/ml of α-Cyano-4-hydroxycinnamic acid (CHCA) in 0.1% TFA–ACN/H₂O (7:3, v/v)) was spotted.

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

2.11. Antibacterial activity

Antibacterial activity assays were performed according to the method described by Berghe and Vlietinck [14]. Antibacterial activities were tested against 6 Gram-negative (*E. coli*, *P. aeruginosa*, *K. pneumoniae*, *S. enterica*, *S. typhi*, *Enterobacter sp.*) and 3 Gram-positive (*S. aureus*, *B. cereus*, *M. luteus*) bacteria. Samples were dissolved in distilled water, and sterilized by filtration through a 0.22 μm Nylon membrane filter. A 200 μl culture suspension of the microorganism (10⁶ colony-forming units (cfu)/ml of bacteria cells estimated by absorbance at 600 nm) were spread on Muller–Hinton agar. Then, bores (3 mm depth, 4 mm diameter) were made using a sterile borer and were loaded with 80 μl of

samples. The Petri dishes were kept, first during 1 h at 4 °C, and then during 24 h at 37 °C. Antibacterial activity was evaluated by measuring the diameter of the growth inhibition zones in millimeters.

2.12. Fractionation and characterisation of peptides

2.12.1. Size-exclusion chromatography

The freeze dried FZPH (1g) was suspended in 5 ml of bi-distilled water, and then was subjected to Sephadex G-25 gel filtration column (2.9 cm × 54 cm) fractions of 5 ml were collected at a flow rate of 30 ml/h and the absorbance was measured at 280 nm.

2.12.2. Reversed-phase high performance liquid chromatography

The most active fractions were dissolved in distilled water, filtered through 0.22 µm filters and then injected into an Agilent 1100 HPLC system for separation in a Symmetry Prep™ C18 column (4.6×250 mm, 5µm) from Waters (Milford, MA, USA). Solvent A was 0.1% TFA in bidistilled water and solvent B consisted of ACN / bidistilled water (60:40, v/v) containing 0.085% of TFA. The mobile phases were filtered through a 0.45 µm filter and degassed. The elution was 100% solvent A for 2 min, followed by a linear gradient to 40% of solvent B in 50 min and then taken to 100% of solvent B and maintaining for 10 min at a flow rate of 1 ml/min. The chromatogram was monitored at 214 nm and collected fractions (1 ml) were freeze-dried then assayed for antibacterial activity.

2.12.3. Identification of peptides using nESI-LC–MS/MS

Peptide identification was done using a nano-liquid chromatography system (Eksigent of AB Sciex, CA) coupled to a quadrupole-time-of-flight (Q-ToF) system (TripleTOF® 5600+, AB Sciex Instruments, Framingham, MA) equipped with a nano-electrospray

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

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

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

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

2.13. Statistical analyses

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

3. Results and discussion

3.1. Preparation of fermented zebra blenny protein hydrolysate

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

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

FZPH with a DH of 17.35 % was further tested against several Gram⁺ and Gram⁻ bacteria (Table 2). No antibacterial activity was detected with the undigested zebra blenny muscle proteins (t = 0). FZPH was found to inhibit the growth of *M. luteus*, *E. coli*, *K. pneumoniae*, *S. enterica* and *Enterobacter sp.*, and the highest antibacterial activity was detected against *E. coli* and *K. pneumoniae*.

3.2. Chemical composition, free amino acids and nucleotides content

The chemical composition of freeze dried ZPH was determined (Table 3) and compared to that of zebra blenny meat flour. The proximate composition of dried ZMF showed high protein content (84.16% of dry matter basis) (p<0.05) whereas protein content was about 80% in FZPH (p<0.05). The fat content level in the hydrolysate was 0.08%, which was significantly lower than ZMF (5.44%) (p< 0.05). FZPH had high ash content (11.54%).

In Table 4, free amino acid compositions of ZMF and FZPH are expressed as percentage of residues. The results revealed a wealth of Glu, Ala, Val and Ile. An important amount of Taurine was detected (10.52 % in ZMF and 15.52% in FZPH).

Nucleotides and derived compound of undigested muscle and FZPH were determined by HPLC and are shown in Table 5. Hypoxanthine is the major compound in the undigested and FZPH samples (7.62 and 9 µmol/g, respectively), whereas ADP, ATP, and uric acid were undetectable. AMP was not detected in FZPH; UMP and uridine were also not detected in ZMF. Regarding this, it is well known that after death, ATP is rapidly split into ADP and subsequently into AMP and IMP. IMP is then degraded to inosine and hypoxanthine which in turn can be oxidized to uric acid [16]. So, the accumulation of hypoxanthine in the fish muscle hydrolysate can reflect the enzymatic breakdown. Nucleotides and especially IMP and GMP, which are abundant in seafood, are responsible for the umami taste sensation [17]. The presence of these nucleotides in ZMF, contributes to the umami sensor. In fact, it has been

noted that the combination of nucleotides and umami-related amino acids (aspartic and glutamic acids) would increase synergistically the umami response.

3.3. Amino acid composition

The amino acid compositions of FZPH and its fractions, obtained after fractionation by G25 gel filtration and expressed as percentage of residues, are reported in Table 6. The amino acid compositions of the different samples revealed that they are rich in Lys, Glu, Ala and Gly. The sum of these amino acids was between 36.13% for ZMF and 52.92% for FZPH-F6. The obtained percentages were higher than previously published results in salmon (20.37%) and cod (7.15%) protein hydrolysates [18]. Taurine, a sulfur-containing amino acid derived from methionine and cysteine, was detected in all samples. The FZPH-F4 had the highest taurine content (4.28%), whereas less taurine was found in ZMF (0.28%). Taurine contents were similar to those reported by Lassoued et al. [19] in thornback ray protein hydrolysates and by Silva et al. [20] in tilapia protein hydrolysates. Taurine may be a relevant amino acid for use as a nutritional supplement to protect against oxidative stress, neurodegenerative diseases or atherosclerosis [21].

3.4. RP-HPLC profiles of ZMF and FZPH

RP-HPLC efficiently separates peptides generated in protein hydrolysates, and can give some indication about hydrophobic/hydrophilic peptide ratio [22]. Several researchers have employed this technique in studying protein hydrolysates from bovine β -lactoglobulin [23] and thornback ray (*Raja clavata*) muscle hydrolysates [19]. The RP-HPLC elution profiles of ZMF and FZPH are presented in Fig. 1. Several peaks are detectable by RP-HPLC confirming the hydrolysis of the muscle protein and the generation of several peptides in FZPH. The retention times of tyrosine and tryptophan (13.3 min and 26.03 min, respectively) were used

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

3.5. MALDI-ToF mass spectrometry analysis

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

3.6. Fractionation and identification of peptides sequences

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

of antibacterial activities against at least one bacterium. Indeed, F1 was found to inhibit only *E. coli*, while F2 inhibit five strains. However, none of the six fractions were found to inhibit *S. aureus*, *B. cereus* and *S. typhi*. Therefore, F2 which displayed the highest antibacterial activity was selected and then further fractionated by RP-HPLC. Fractions (1 ml) were automatically collected, freeze-dried and their antibacterial activities were tested. The RP-HPLC profile of F2 fraction, reported in Fig. 4, revealed a very large number of peaks relating to the abundance of peptides generated. Antibacterial activity was only detected in fractions eluted from 3 to 6 min (named sub-fraction A) and at 47 min (named sub-fraction B). These two sub-fractions were selected for the identification.

3.7. Identification of bioactive peptides

Peptides from selected sub-fractions A and B were analysed by nESI-LC–MS/MS after RP-HPLC separation. A total of 28 peptide sequences were identified in sub-fraction A and 41 peptide sequences were identified in sub-fraction B. The amino acid sequences of the identified peptides were characterized as well as their position in the parent protein. The observed (m/z), the experimental and the calculated molecular masses (M_r) are also shown in Table 8. All the identified peptides were between 8 and 31 amino acids length, which is in accord with the study of Brogden [27] reporting that the size of AMPs varies from 6 aas residues to more than 59. Identified peptides have a substantial portion of hydrophobic residues. The most hydrophobic peptides are GLPPYPYAG, ALPYDTPVPGY, LAGNEDVILPVPA, VIISAPSADAPMF and FAGDDAPRAVFPS in sub-fraction A, and AAVPSGASTGVHEALEL, WAAFPPDVAGNVDYKN and LTVKEDQVFPMNPPKFDKIEDMAM in sub-fraction B. Wieprecht et al. [28] reported that this hydrophobic character would let peptides enter the membrane, as the positive charge would initiate the peptide interact with the surface of the bacteria (negatively charged). AMPs

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

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

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

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

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

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

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

4. Conclusion

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

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

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Table 1

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

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	—

The concentration of protein hydrolysate was 20 mg/ml.
IZD: Inhibition Zone Diameter
Values are given as mean±SD from triplicate determinations (n=3).
«—» No inhibition or no effect.

Table 2

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

Indicator organism	ZMF	FZPH
Gram (+)		
<i>S. aureus</i>	—	—
<i>B. cereus</i>	—	—
<i>M. luteus</i>	—	+
Gram (-)		
<i>E. coli</i>	—	+++
<i>P. aeruginosa</i>	—	—
<i>K. pneumoniae</i>	—	++
<i>S. enterica</i>	—	+
<i>S. typhi</i>	—	—
<i>Enterobacter sp.</i>	—	+

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

Table 3

Chemical constituents of undigested zebra blenny protein and its hydrolysate.

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

Values are given as mean ± SD from triplicate determinations (n= 3).

ZMF : zebra meat flour.

FZPH : zebra blenny protein hydrolysate fermented using *B. mojavensis* A21 during 12h.

^{a,b,c} Different letters in the same line indicate significant differences (p < 0.05).

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.

Table 5

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

	$\mu\text{mole/g}$	
	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

Values are given as mean $\pm$ SD from duplicate determinations (n= 2).^{a,b} Different letters in the same line indicate significant differences ($p < 0.05$).

nd: not detected.

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 ^c	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 ^c	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 ^c	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 (+)						
<i>S. aureus</i>	—	—	—	—	—	—
<i>B. cereus</i>	—	—	—	—	—	—
<i>M. luteus</i>	—	+	—	+	—	—
Gram (-)						
<i>E. coli</i>	++	+++	+	++	—	—
<i>P. aeruginosa</i>	—	—	—	—	—	—
<i>K. pneumoniae</i>	—	+++	++	++	—	—
<i>S. enterica</i>	—	+	—	—	—	—
<i>S. typhi</i>	—	—	—	—	—	—
<i>Enterobacter sp.</i>	—	+	—	—	—	—

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Table 8
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
	LFDKPV SPL	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
	GYPDKIIIIGMD	611.315	1220.616	2	1220.611		Beta-enolase
	ETPGGTPLAEPD	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

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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
RIEEEEIEAE	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.
[#] Postrasductional modification oxidation of the methionine.

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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
TDAETKAFKAGD	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
LTEEMASQDESIK	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
YETDAIQRTEEEAAKKK	546.031	2180.097	4	2180.096		Myosin heavy chain, fast skeletal muscle
AIQRTEEEAAKKKLAQRL	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

IKVLTDKLEAEETRAEFAERS	812.113	2433.318	3	2433.323		Tropomyosin alpha-1 chain
DLLIDLANEVKLQEIDNLING	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
AAALRKEQADSV AELGEQIDNLQR	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
MEGDLNEMEIQLSHANRQAEEAQK	687.065	2744.233	4	2744.249	Oxidation(M)@1; Oxidation(M)@8	Myosin heavy chain, fast skeletal muscle
LTKLEEAEEKADESERGMKVIENR	461.240	2761.397	6	2761.391	Oxidation(M)@18	Tropomyosin alpha-1 chain
ARLQTENGEFSRQLEEKEALVSQ L	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
LTVKEDQVFPMPNPPKFDKIEDMAM	947.149	2838.424	3	2838.364	Oxidation(M)@11	Myosin-2
MEGDLNEMEIQLSHANRQAEEAQKQ	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
ALRKEQADSV AELGEQIDNLQRVKQK	593.936	2964.645	5	2965.594		Myosin heavy chain, fast skeletal muscle
AAALRKEQADSV AELGEQIDNLQRVKQK	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
MASQDESIAKLTKEKKALQEAHQTLDDL	1096.197	3285.569	3	3285.640	Oxidation(H)@22; Deamidated(Q)@24	Myosin heavy chain, fast skeletal muscle
RARLQTENGEFSRQLEEKEALVSQ LTRGKQA	715.803	3573.978	5	3573.850	Deamidated(N)@8; Deamidated(Q)@14	Myosin heavy chain, fast skeletal muscle

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	GLPPYPYAG	GLP	ACE inhibitor	Byun and Kim [33]
		GLPQE	Antimicrobial	Hayes et al. [32]
P2	LVDGLDVGIL	FLPLIGRVLSGIL~ ; FLPLIGKVLSGIL~ ; FFPVIGRILNGIL~	Antibacterial	Simmaco et al. [34]
P3	EADDWLRY	LRY	ACE inhibitor	Meisel et al. [39]
P4	LEGDLKLSQE	LKKLKKLKKKLLKL	Antibacterial	Appendini and Hotchkiss [40]
		LKL	ACE inhibitor	Ukeda et al. [41]
P5	LSYEEAITTY	KVREGTTY	ACE inhibitor	Lee et al. [42]
P6	ETPGGTPLAPEPD	QELLLNPTHQYPVTQPLAPVHNPIV	Antimicrobial	Hayes et al. [32]
		QELLLNPTHQIYPVTQPLAPVHNPIV	Antibacterial	Minervini et al. [43]
P7	HHPDDFNPSVH	HHP ; HHPL ; HHPLL	Antioxidant	Chen et al. [44]
P8	FAGDDAPRAVFPS	VFPS	ACE inhibitor	Loponen [45]
P9	FDMFDTDGGGDIS	NDAACAAHCLFRGRSGGG	Antibacterial	Lee et al. [46]
P10	RIEELEEEIEAE	LEELEEELEGCE	Antioxidative	Qian et al. [47]

	Sequence	Similar sequence		
		Sequence	Activity	Reference
P1	QQEISDLTEQI	KTKL TEEE KNRLN FL KKISQRYQKFAL PQYLKTVYQH QK	Antibacterial	Zucht et al. [48]
P2	ISERLEEAGGATAA	ISELGW	Antioxidative	Tsopmo et al. [49]
P3	NVLSGGTTMYPGIAD	MYPGIA	ACE inhibitor	Escudero et al. [50]
P4	AEQELVDASERVGL	VGL	DPP IV inhibitor	Nongonierma et al. [51]
P5	AVPSGASTGVHEALEL	AVP ; AVPYP	ACE inhibitor	Meisel, [52]
		AVPYPQR	ACE-inhibitor	Maruyama et al. [53]
		AVPYPQR	Antioxidant	Rival et al. [54]
P6	LTEEMASQDESI AK	IAK	ACE inhibitor	Gomez-Ruiz et al. [55]
		FSDKIAK	ACE inhibitor	Lopez-Exposito et al. [35]
			Antibacterial	Lopez-Exposito et al. [36]
P7	AA V PSGASTGVHEALEL	AAVALLPAVLLALLAPAAANYKKPKL	Regulating cell-permeability	Du et al. [56]
		<u>VPSGK</u>	Antioxidative	Suetsuna, [57]
P8	WAA F PPDVAGNV <u>DYKN</u>	AF PPPNVPGPRFPPPNFPGPRFPPPNFPG PRFPPPNFPGPRFPPPNFPGPPFPPPIFPGP WFPPPPFRPPPFPPPRFP	Antibacterial	Harwig et al. [58]
		QERGSIVIQGTKEGKSRPSLD <u>IDYK</u> QRV YDKNGMTGDAYGGLNIRPGQPSRQHA GFEFGKEYKNGFIKQSEVQRGPGGRL SPYFGINGGFRF	Antibacterial	Casteels-Josson et al. [59]
P9	TERAEDEEEINAELTAK	TERGY	Antioxidant	Liu et al. [60]
P10	GQKDSYVGDEAQSKRGILT	FLPLIGRVLS GIL ~	Antibacterial	Simmaco et al. [34]
P11	LRMDLERAKRKLEG DLK	SIQ DLK VSMKLF RK QAKWKIIVKLNDG RELSLD	Antibacterial	Mayo et al. [61]
P12	NRRIQLVEEELDRA QER	GKKIATY QER ; GHKIATF QER	ACE inhibitor	Kohama et al. [62]
P13	YETDAIQRT EE EE AKKK	LRLWSHLIHWFNRR LKWKKK	Membrane active	Ferrer – Miralles et al. [63]
P14	AIQR TEE EE AKKK LQRL	KTKL TEEE KNRLN FL KKISQRYQKFAL PQYLKTVYQH QK	Antibacterial	Zucht et al. [48]
P15	TESGEFSRQLDEKEALVSQL	TESQSLT	ACE inhibitor	De Gobba et al. [64]
		LLGDFFRKSKEKIGKEFKRIVQRIKDFL RNLVPR TES	Antibacterial	Larriek et al. [65]
P16	KMEGDLNEMEIQLSHANR QAA	GILDTIKSIASKVWNSKTVQDLKRKGIN WVANKLGVSP QAA	Antibacterial	Torres-Larios et al. [38]
P17	IDNLQRVKQKLE KEK SEYKM	GLLQTI KEK LESLESLAKGIVSGIQAG	Antibacterial	Rozek et al. [66]
P18	AAALRKEQADSV AE LGEQIDNLQR	AAATP	ACE inhibitor	Escudero et al. [67]
		AAATP ; AAAAG	DPP IV inhibitor	Gallego et al. [68]
P19	LL REQYEEEQEAKAELQRGMSKA	LLR	Antioxidant	De Gobba et al. [64]

P20	L TKLEEA E KAADESERGMKVIENR	EQL T K	Antibacterial	Pellegrini et al. [69]
P21	A RLQTENGEFSRQLEEKEALVSQL	L A RL	Antioxidant	Bougatef et al. [70]
P22	L SKEKKALQETHQQTLDLQAEED	L SKAQSDFG	Antioxidative	Shen et al. [71]
P23	VLA E WKQKYEESQAELEGAQKE A RS	A RSYGNGVYC N NKKCWVNRGEATQSI IGGMISGWASGLAGM	Antibacterial	Tichaczek et al. [72]
P24	QQVDDLEGSLEQEKKLRMDLER A KRK	KKWH K RKK ; KGWH K RKK	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), measured 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.