applied sciences

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Characterisation of seasonal *Mytilus edulis* by-products and generation of bioactive hydrolysates

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Received: date; Accepted: date; Published: date

Featured Application: Functional Foods for humans– potential to prevent diabetes through inhibition of the dipeptidyl peptidase IV (DPP-IV) enzyme.

Abstract: Mussel cultivation results in tons of by-product with 27% of the harvest considered as reject. In this study mussel by-products considered as undersized (mussel with a cooked meat yield <30%); mussels with broken shells and barnacle fouled mussels were collected from 3 different locations in the west, north-west and south-west of Ireland. Samples were hydrolysed using controlled temperature and agitation and the proteolytic enzyme Protamex® was added at an enzyme: substrate ratio of 1:50 (w:v). The hydrolysates were freeze-dried and analysed for protein content & amino acid composition, lipid content & fatty acid methyl ester (FAME) composition, ash, techno-functional and bioactive activities. The degree of hydrolysis was determined using the Adler-Nissen pH stat method and was found to be between 2.41%±0 - 7.55±0.6%. Mussel by-products harvested between February-May 2019 had protein contents ranging from 36.76%±0.41 to 52.19%±1.78. The protein content of mussels collected from July to October ranged from 59.07%±1.375 to 68.31%±3.42 - the spawning season. The ratio of essential to nonessential amino acid varied from 0.68-0.96 and it was highest for a sample collected in November from the west of Ireland. All the hydrolysate samples contained omega-3 polyunsaturated fatty acids (PUFA), especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) which are known anti-inflammatory agents. Selected hydrolysates

which had Angiotensin converting enzyme I (ACE-I; EC 3.4.15.1) and dipeptidyl peptidase IV (DPP-IV; EC 3.4.14.5) inhibitory activities were filtered using 3-kDa membrane filtration and the permeate fraction was sequenced using Mass spectrometry (MS). Identified peptides were > 7 amino acids in length. Following BIOPEP database mining, 91% of the by-product mussel peptides identified were found to be previously identified DPP-IV and ACE-I inhibitory peptides and this was confirmed using *in vitro* bioassays. The ACE-I inhibitory activity of the by-product mussel hydrolysates ranged from 22.23%±1.79 - 86.08%±1.59 and the most active hydrolysate had an ACE-I inhibitory concentration (IC_{50}) value of 0.2944 mg/ml compared to the positive control Captopril. This work demonstrates that by-product mussel hydrolysates have potential for use as health beneficial ingredients.

Keywords: Mussel by-products, *Mytilus edulis*, meat yield, protein hydrolysate, bioactivity, Dipeptidyl peptidase IV (DPP-IV); Angiotensin-I-converting enzyme (ACE-I) inhibition, Acetylcholinesterase (AChE)

1. Introduction

Mussel cultivation for human consumption has been practiced for years through rope or raft cultivation systems and the total global productivity of the common blue mussel (*Mytilus edulis*) is approximately 20,000 tons (9% of the total global mussel production) [1]. Of the total produced mussels, 27% is discarded as by-products [2]. By-product mussel can be edible mussels that are seed mussels, undersized mussels or mussels with broken shells, mussels that are fouled with barnacles or inedible due to the presence of toxins such as Azaspiracids (AZA) and Diarrhetic shellfish poisoning (DSP) toxin. However, mussel by-products are a rich source of protein, lipid and essential amino acids. Bioprocessing of by-product mussel for high value commercial ingredient development could contribute positively towards the circular economy. However, characterization of the raw material/by-products is necessary before initiating such work as there is a high degree of variability in the composition of mussel by-products which will result in variability in the end products. Season

of harvesting, by-product type, location and climate all have significant effects on the composition of mussel by-product. The spawning or the reproductive period for *Mytilus edilus* is spring to summer while gametogenesis occurs during the winter season when the mussels get bigger prior to spawning. Depending on when the mussels are harvested, the size, meat yield and composition vary. High meat yield, protein, lipid and pigment like carotenoid content are often associated with the gametogenesis phase of maturation in mussels [3, 4].

Meat yield is the amount of meat obtained per known quantity of whole wet mussel and when calculated after cooking it is referred to as the cooked meat yield [5]. Cooked meat yield values lower than 30% are not viable for sale and mussels with a cooked meat yield less than 30% are categorized as by-product. The whole mussel is made up of shell, meat, extrapallial fluid and byssus threads. Proximate composition analysis of mussel meat found that it contained 58.7% protein, 22.5% carbohydrates, 7% lipids and 11.8% ash on a dry weight basis apart from other minor components [6]. Lipids and proteins are the main components studied for health promoting benefits. A recent study revealed that consuming mussel meat three times a week for two weeks as the protein source in a personalized meal can moderately improve the omega-3 index and whole blood Docosahexaenoic acid (DHA) and Eicosapentanoic acid (EPA) content in young healthy adults [7]. EPA and DHA are known for their anti-inflammatory benefits and mussel lipids rich in these polyunsaturated fatty acids (PUFAs) have been used previously in formulating bone supplements [8]. Proteins in mussel can be hydrolysed using proteolytic enzymes to yield hydrolysates containing bioactive peptides. Several enzymes such as trypsin (EC 3.4.21.4), papain (EC 3.4.22.2), Alcalase® (EC 3.4.21.62), Protamex® (EC 3.4.21.62 and EC 3.4.24.28) and others have been used previously to generate bioactive peptides [9, 10]. The pH optima of the enzyme, temperature, batch time and ratio of enzyme: substrate can influence the degree of hydrolysis and resulting bioactivity of the peptides and hydrolysates. Several proteases such as papain (EC 3.4.22.2), Flavourzyme® (EC 3.4.11.1) and

Protamex® have been used to cleave mussel meat proteins for manufacturing bioactive peptides [11]. Protamex® a serine protease, is known for its ability to produce non-bitter tasting hydrolysates [12]. Bioactive peptides from *Mytilus edulis* have shown bioactivities previously including ACE-I inhibition, antioxidant potential, antimicrobial, anticoagulant, anti-inflammatory, osteogenic, hepatoprotective and anticancer activities [13-18]. The bioactivity of peptides depends on the parent protein, size of peptides, peptide hydrophobicity, and amino acid composition and location of amino acids within the peptide sequence [19]. Generally, bioactive peptides are known to be short sequences of between 2-30 amino acids in length with molecular weights less than 6 KDa [20]. Incorporation of bioactive peptide containing hydrolysates into food products requires analyses of techno-functional properties including water holding, oil binding, emulsion stability and activity [21]. Hydrolysates showing superior techno-functional properties and specific bioactivities can be incorporated into functional food products for human use or can be used to develop nutraceutical supplements with targets against specific diseases associated with cardiovascular diseases (CVD) and inflammation. One such novel food product approved by the European food safety authority (EFSA) recently is PreCardix®, a hydrolysate made from shrimp by-products which is effective against hypertension [22].There are many nutraceutical products on the market made from green lipped mussel lipids, but no such regulatory approved product exists from blue mussels currently.

The objective of the present work was to generate hydrolysates from by-product mussels and assess their functionality and bioactivity in relation to season and location of harvest and by-product type. This study will help mussel growers and processors to develop new markets for their by-products and provides information concerning the best season to harvest mussels for maximum hydrolysate yields and what process to use as well as information on what by-product type is most suited to this process.

2. Materials and Methods

2.1. *Chemicals:*

Protamex® enzyme (protease from *Bacillus* sp, Sigma-Aldrich, Dublin, Ireland), 3KDa spin tubes (Amicon® Ultra Centrifugal Filters, Millipore, Cork, Ireland) hexane and other solvents and the standard acotiamide dihydrochloride were purchased from Sigma Aldrich (Sigma-Aldrich, Dublin, Ireland), The ACE-I inhibitor captopril (the positive control used in the ACE-I inhibition assays) was supplied by Sigma Aldrich (Dublin, Ireland). The tris-tricine SDS-PAGE reagents were supplied by Fisher Scientific (Dublin, Ireland) and Bio-Rad Life Science Research (Dublin, Ireland). The ACE-I inhibition assay kit was supplied by NBS Biologicals, (Cambridge, England). The 3.5 kDa snakeskin dialysis membrane was supplied by the Medical Supply Company (MSC, Dublin, Ireland). All other chemicals used were of analytical grade (≥99% purity). Unless otherwise stated, all reagents were made using Milli-Q deionised water (18.2 ohms).

2.2. *Sampling:*

Mussel samples were collected over a period of 12 months from different sites; Mulroy bay, Donegal, Ireland (North West), Killary harbour, Galway Ireland (West); and Ardgroom, Co Cork (South West).The mussel by-products identified as seed mussels, undersized mussels, broken shells, byssus threads mixed samples, and toxin containing samples were characterised and their cooked meat yields determined.

2.3. *Cooked meat yield*

250 g of mussel by-products which were collected and transported to the laboratory on ice and subsequently frozen at -80 °C were thawed, dried with a paper towel, cooked in boiling water for 7 minutes and cooled. Meat was separated from shells and both empty shells and meat weights were determined. The cooked meat yield (MY_{cook}) was calculated using the following formula [5]:

MY_{cook} = [meat weight after cooking (g)/total weight (g)] X 100

2.4. *By-product mussel hydrolysis with Protamex®*

250 g of whole by-product mussels were ground using a table top cutter blender (Robot Coupe R2 table top cutter mixer, France) with 500 ml water for 1 minute. The slurry obtained was used for hydrolysis and endogenous enzymes were heat-deactivated for 10 min at 80 ˚C in a water bath prior to hydrolysis. Hydrolysis was carried out at 130 rpm, 35 ˚C, pH 7 with Protamex® added to the mussel by-product at the ratio of 1:50 (w:v). Hydrolysis was carried out for 1.5 h. 0.1 M NaOH was added to adjust the pH of the hydrolysates to 7. Protamex® was heat-deactivated at 95 ˚C for 10 min and hydrolysates allowed to cool to room temperature. Clean mussel shells were separated from the mixture, the hydrolysate slurries were poured into trays, frozen and then freeze-dried using a Labconco freeze-drier (Labconco corporation, USA) for 48 h. The freeze-dried hydrolysates were weighed to calculate yield and subsequently analysed for protein, lipid, ash and fatty acid methyl ester (FAME) content.

2.5. *Degree of hydrolysis*

The degree of hydrolysis was calculated using the pH stat method of Adler-Nissen [23]. Before starting the hydrolysis, pH was adjusted to 7 using 0.1 M NaOH and after completion of hydrolysis the pH was readjusted to 7. The volume of NaOH required to readjust the pH was noted, the protein content of the mussel substrate was determined from proximate analysis and the degree of hydrolysis (DH) was calculated using the following formula:

$$DH = B \times N_B \times 1/\alpha \times 1/M_P \times 1/h_{tot} \times 100\ \%$$

Where,

B = Volume of NaOH consumed

N_B = Normality of NaOH = 0.1 M

$1/\alpha$= 5.05 for Protamex®

M_P = amount of protein (g) in reaction mixture

h_{tot} = 8.6 for fish

2.6. *Protein and lipid composition*

The protein content of mussel by-products and hydrolysates was estimated using the AOAC 968.06 method, 15th Edition-Dumas method using the Leco FP628 analyser. A nitrogen factor of 6.25 was used to calculate the protein content in each sample. The lipid content was quantified using AOAC Method 2008.06 with an Oracle rapid NMR fat analyser. Samples were prepared according to standard procedures used for these analyses as described previously [24].

2.7. *Techno-functional properties of hydrolysate*

2.7.1. Emulsion Activity and Emulsion Stability assay of mussel hydrolysates

Hydrolysates at a concentration of 1% w/v were made in water and adjusted to pH 2, 4, 6, 8 and 10 using 1 M NaOH or 1 M HCl. The samples were homogenized for 30 s at 14,000 rpm. Emulsions were created using commercial vegetable oil which was added to the aqueous phase containing the hydrolysate at an oil: hydrolysate ratio of 3:2 (v:v). The addition was done in two steps where at first, half the volume of oil was added to the mixture, homogenized for 30 s and centrifuged at 14,000 rpm. This was followed by addition of the rest of the oil and homogenization at the same speed for 90 s. The formed emulsion was centrifuged at 1100 ×g for 5 min and the volume of the emulsion layer was measured. Emulsion Activity (EA) was calculated using the formula:

$$\text{EA } \% = \frac{V_E}{V_T} X100$$

Where; V_E is the volume of the emulsion layer after centrifuging and V_T is the volume inside the tube.

Further, to determine emulsion stability (ES), the previously prepared emulsions were heated at 85 °C for 15 min, cooled at room temperature for 10 min and centrifuged again at 1100 ×g for 5 min. The ES was expressed as the % of EA remaining after centrifuging as follows:

$$\text{ES } \% = \frac{\text{V}_{\text{emulsion after heating}}}{\text{V}_{\text{original emulsion}}} \text{X } 100$$

2.7.2. Solubility of protein hydrolysates

pH adjusted hydrolysate solutions at concentrations of 1% w/v were prepared as discussed above. The prepared solutions were agitated at room temperature for 45 min in a Multi Reax Vibrating Shaker. 1-2 ml aliquots of the full protein dispersion were dispensed in a separate tube. The hydrolysate solution was then centrifuged at 4000 ×g for 30 min. 1-2ml of the protein supernatant was dispensed in a second tube. BCA kit (Pierce™ BCA Protein Assay Kit, ThermoScientific, USA) was used to quantify soluble protein. The percentage solubility S (%) of the protein extract at different pH conditions is calculated using the formula:

$$\text{S\%} = \frac{\text{Protein Supernatant}}{\text{Protein in full Dispersion}} \text{X100}$$

2.7.3. Water activity

An aqualab 4TE bench-top water activity meter (Decagon Devices, Inc., Washington, USA) was used for water activity analysis.

2.8. *Amino acid composition of peptides*

Determination of the total amino acid composition of the individual hydrolysates was done by further hydrolysing hydrolysates using 6 M HCL at 110°C for 23 h [25]. The samples were then de-proteinized by mixing equal volumes of 24% (w/v) tri-chloroacetic acid and sample. These were allowed to stand for 10 min at room temperature before centrifugation at 14,400 x g for 10 min. The

supernatants were removed and diluted with 0.2 M sodium citrate buffer, pH 2.2 to give approximately 250 nmol of each amino acid residue. Samples were then diluted 1:2 with the internal standard nor-leucine to give a final concentration of 125 nm/ml. Amino acids were quantified using a Jeol JLC-500/V amino acid analyser (Jeol Ltd., Garden city, Herts, UK) fitted with a Jeol Na+ high performance cation exchange column.

2.9. *Sequencing using mass spectrometry (MS) and in silico analysis of peptides*

Hydrolysate samples were further purified prior to MS analysis and were passed through 3-kDa cut-off ultraspin tubes. The resulting permeates containing peptides less than 3 kDa in size were assessed using mass spectrometry. Nano-LC-MS/MS analysis was performed using an Eksigent Nano-LC Ultra 1D Plus system (Eksigent of AB Sciex, CA) coupled to the quadrupole-time-of-flight (Q-ToF) TripleTOF® 5600 system from AB Sciex Instruments (Framingham, MA),equipped with a nano-electrospray ionization source. After 5 min of pre-concentration, the trap column was automatically switched in-line onto a nano-HPLC capillary column (3 μm, 75 μm X 12.3 cm, C18) (Nikkyo Technos Co, Ltd., Tokyo, Japan). Mobile phase A contained 0.1% v/v formic acid in water, and mobile phase B, contained 0.1% v/v formic acid in 100% acetonitrile. A linear gradient from 5% to 35% of solvent B over 60 min at a flow rate of 0.3 μL/min and running temperature of 30 °C was used for chromatographic separations.

Sample was ionized applying 2.8 kV to the spray emitter. Scans were acquired from 350–1250 m/z for 250 ms. The quadrupole resolution was set to 'UNIT' for MS2 experiments, which were acquired 100–1500 m/z for 50 ms in 'high sensitivity' mode. Following switch criteria were used: charge: 1+ to 5+; minimum intensity; 70 counts per second (cps). Up to 25 ions were selected for fragmentation after each survey scan. Dynamic exclusion was set to 15 s. The system sensitivity was controlled with 2 fmol of 6 standard proteins (LC Packings). Automated spectral processing, peak list generation, and database search for the identification of the peptides were performed using Mascot Distiller v2.7.1.0

software (Matrix Science, Inc., Boston, MA).The UniProt protein database (https://www.uniprot.org/ accessed 10/1/2020) [26, 27] was used to identify the peptides with a significance threshold $p<0.05$. The tolerance on the mass measurement was 0.3 Da in MS mode and 100 ppm in MS/MS ions. *In silico* analysis of the sequenced peptides was done using the Thermo fisher peptide analysis online tool and BIOPEP-UWM databases (both accessed 10/01/2020) as described previously [28, 29].

2.10. *Gas Chromatography Mass Spectrometry for fatty acid composition analysis*

Lipid fractions were extracted from mussel hydrolysates using hexane and shaking (130 rpm, 6 h). Trans-esterification was carried out using the method of Araujo *et al*., with some modifications [30]. Approximately 10-20 mg of lipid sample along with 1 ml of 1 mg/ml internal standard (glycerol-tri-heptadecanoate) solution was mixed and dried under nitrogen. Then, 1 mL of BCl_3-methanol (12% w/w) was added for every 10 mg of dry sample and flushed with nitrogen. The mixture was heated to 90- 100 °C for 60-90 min, cooled to room temperature, followed by addition of 1 mL of 10% (w/v) NaCl solution in water and 1 mL of n-hexane (HPLC or GC-grade purity). The contents of the vial were mixed for 1 min to ensure extraction of FAMEs into n-hexane, settled for 10 minutes and the upper (hexane) layer was added to a tube containing anhydrous sodium sulphate to dry the hexane extract. Extracted FAMEs were separated and analysed using an Agilent 7890A GC/5975C MSD system. Separation was performed on the Agilent J&W DB-FastFAME GC column (30 m, 0.25 mm ID, 0.25 μm film thickness) using hydrogen, 8 psi, at constant pressure mode. A split ratio of 25:1 was used and the inlet was maintained at 250 °C while the oven was temperature programmed to 50 °C (0.5 min), 15 °C/min to 194 °C (4 min) and 4 °C/min to 240 °C (1 min). Supelco Fame 37 mix was utilized for external calibration by making series of appropriate dilutions with hexane, with individual compound peaks used to construct a calibration curve. The fatty acids in the sample were quantified with the calibration curve using MassHunter Quantitative Analysis software package (Agilent, USA).

2.11. *Bioassays*

Various bioassays for detecting potential health benefits like heart-health and mental health maintenance, prevention of type-2-diabetes and anti-inflammation activities were performed.

2.11.1. Angiotensin-Converting Enzyme (ACE-I) *ACE-I inhibition*

The ACE-I inhibition assay was carried out to check for the heart health benefits of the hydrolysates. This was done using a kit supplied by Dojindo laboratories (Dojindo Laboratories, Kumamoto, Japan). It is an absorbance based assay where Captopril is used as the positive control. The percentage ACE-I Inhibitory activity was calculated for each sample and further the concentration of hydrolysate that inhibited ACE-I by 50% (IC_{50}) values were determined for selected samples.

2.11.2. Acetylcholinesterase (EC 3.1.1.7) *AChE inhibition*

The AChE assay kit (Fluorometric) from Abcam (Cambridge, UK) was used to study inhibition of AChE activity by mussel hydrolysates to understand their potential for improving mental health. Acotiamide dihydrochloride was used as the standard AChE inhibitor (Sigma Aldrich now Merck, Dublin, Ireland). Fluorescence was recorded at Ex/Em wavelength of 490/520 nm. Inhibition percentage for all the samples is estimated from the fluorescence values.

2.11.3. Dipeptidyl peptidase IV (DPP -IV) inhibition

DPP-IV Activity Fluorometric Assay Kit from BioVision Inc. (cambridge bioscience, UK) was obtained to study potential prevention of type-2 diabetes by the hydrolysates. Positive control for the assay was Sitagliptin. Fluorescence was recorded at E_x/E_m wavelength of 360/460 nm. Inhibition percentage for all the samples is estimated from the fluorescence values.

2.12. *In-silico analysis and statistics*

Two bioinformatics tools were used to analyse polarity profiles of the sequenced peptides along with their potential bioactivities. ThermoFisher peptide analysis tool (https://www.thermofisher.com/ie/en/home/life-science/protein-biology/peptides-proteins/custom-peptide-synthesis-services/peptide-analyzing-tool.html accessed on the 09/01/2020) and BIOPEP-UWM database (formerly BIOPEP) analysis (http://www.uwm.edu.pl/biochemia/index.php/pl/biopep- accessed on 09/01/2020) were the tools used. Within BIOPEP-UWM the option named "Profile of potential biological activity" was used to assess peptide fragments identified by MS for potential bioactivities. GraphPad prism 8 software (GraphPad Software, LLC, USA) was used to study analysis of variance using tukey method for Post Hoc analysis.

3. Results & Discussion

3.1. *Sampling*

Ten samples in total were collected from three different sampling sites - five samples were from Killary harbour and were labeled KHN18, KHM19, KHJ19, KHS19, KHO19 collected in the months of November 2018, March 2018, June 2019 and September and October 2019. Three samples were obtained from Mulroy bay and were labeled MBF19, MBMay19 andMBJuly19 as they were sampled in the months of February, May and July 2019, while two were obtained from Ardgroom - samples AJ19 and AJuly19 obtained in the months of June and July 2019. Most of the by-products were seed mussels except KHN18 which were broken shells. Samples labeled MBJuly19, KHS19 and KHO19 were undersized mussels by-products containing byssus and broken shells.

3.2. *Degree of hydrolysis and peptide length*

The degree of hydrolysis refers to the percentage of hydrolyzed peptide bonds and depends on several factors such as the initial amount of protein, type of enzyme used in the hydrolysis, the duration of the hydrolysis, temperature and pH. For all of the by-product mussel hydrolysis in this study, the degree of hydrolysis was between 2.41% - 7.55±0.6% as assayed using the pH stat method and shown in Fig.1. For samples KHN18, MBF19 & KHM19, the degree of hydrolysis values were positively correlated with hydrolysate yields. For other samples no such correlation was observed, which could be a result of these samples containing byssus threads or empty broken shells that were separated from the final hydrolysate product and did not contribute to the final yield. A lower degree

of hydrolysis is often associated with production of larger peptides having more than 7 amino acids while a greater degree of hydrolysis leads to smaller di and tri-peptides [31]. Similar results were reported by other researchers using Protamex® and hydrolysis of bone extract. Protamex® leads to a lower degree of hydrolysis and peptides with higher molecular weights compared to other proteases such as Flavourzyme® [32]. In the 3kDa fraction generated following filtration in this work about 57% of the peptides that were characterised consisted of 7 amino acids while the remaining 43% were larger peptides with more than 7 amino acids and up to 16 amino acids in length. No di or tri peptides were detected in the sequenced samples due to the mass tolerance setting and m/z range selected in the MS method.

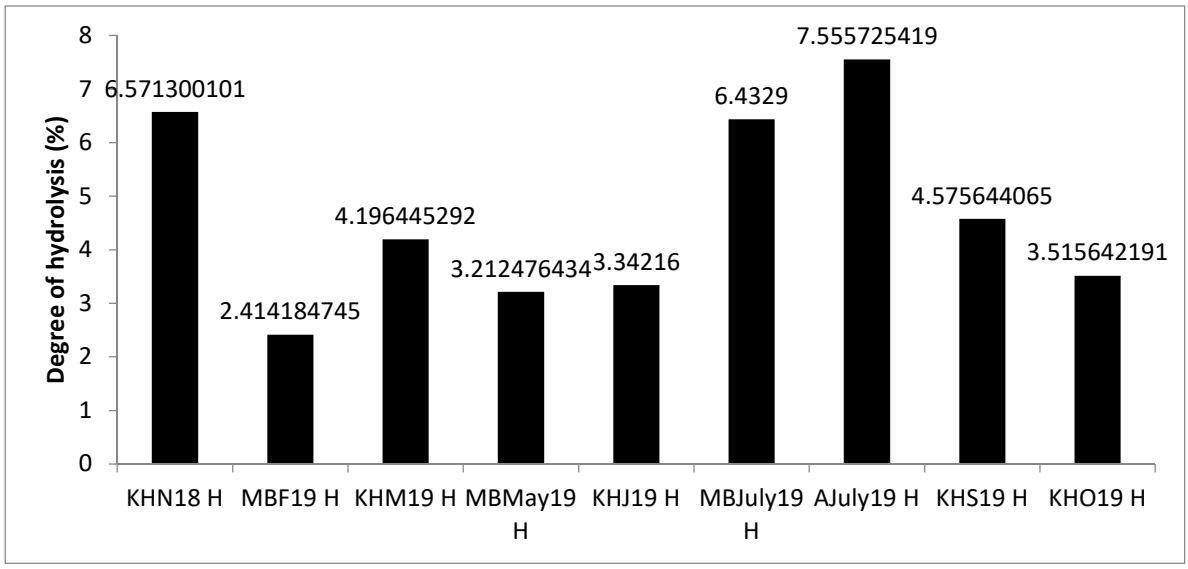


Figure 1. The Degree of hydrolysis (DH) of mussel by-product Protamex® hydrolysates (H) determined using the Adler-Nissen pH stat method. All the 9 samples from the three sampling sites Mulroy bay (MB), Killary harbour (KH) and Ardgroom (A) collected from November 2018 to October 2019 were used for hydrolysis. Analysis was performed in triplicate (n=3). Bars sharing the same letter are not significantly different according to Tukey's HSD test.

3.3. *Free amino acid content*

The free amino acid content of the generated hydrolysates ranged from 3.77% for sample MBF19 to 11.91% for sample MBMay19 of the total amino acid content. The free amino acid content depends on the enzyme used during hydrolysis, the time of hydrolysis and the resulting degree of hydrolysis. Amino acid contents of 30% were reported for marine samples with higher degrees of hydrolysis (35 - 40%) previously [33]. Taurine was the only amino acid found in higher concentration in the free amino acid fraction of the mussel hydrolysates when compared to the total amino acids fraction. It is

known that taurine, a free amino acid, is not part of the protein polypeptide chain and hence found in greater proportion in the free amino acid fraction. Fig.2. shows the relative abundance of free and total amino acids in the mussel by-product hydrolysates for sample KHN18 which had ACE-I inhibitory activity of 86.08%±1.59 when assayed at a concentration of 1mg/ml compared to the control.

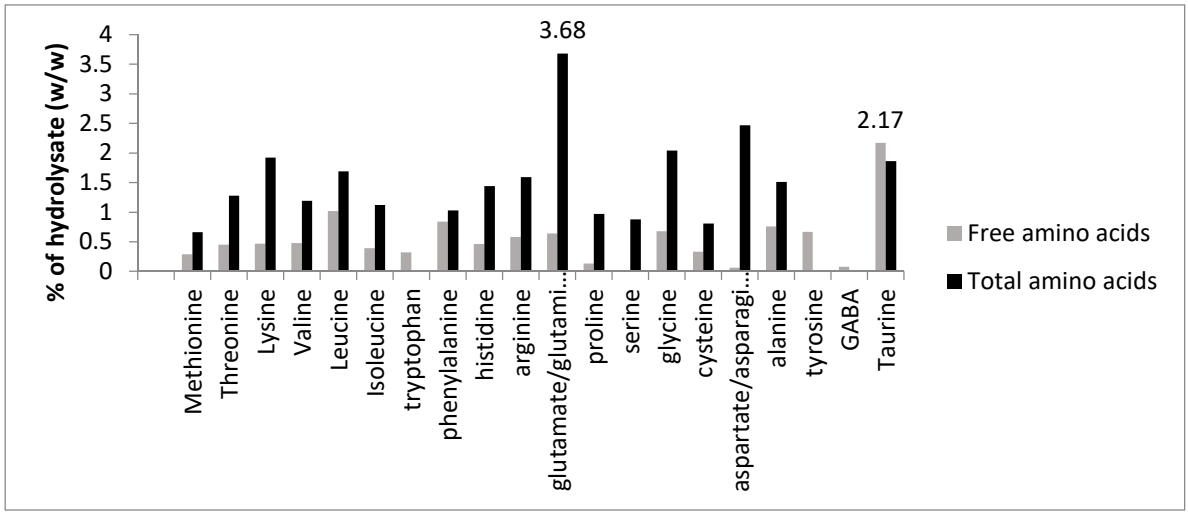


Figure 2. Free and total amino acid % composition (w/w) of mussel by-product Protamex® hydrolysate for Killary harbour November 2018 (KHN18) sample. The amino acids were quantified using a Jeol JLC-500/V amino acid analyser (Jeol Ltd., Garden city, Herts, UK) fitted with a Jeol Na+ high performance cation exchange column. Glutamate was the most abundant amino acid, while taurine was the most abundant free amino acid as seen from the average values displayed on the two bars. Analysis was performed in triplicate (n=1).

3.4. *Cooked meat yield, hydrolysate yield and protein content across different seasons and by-product types*

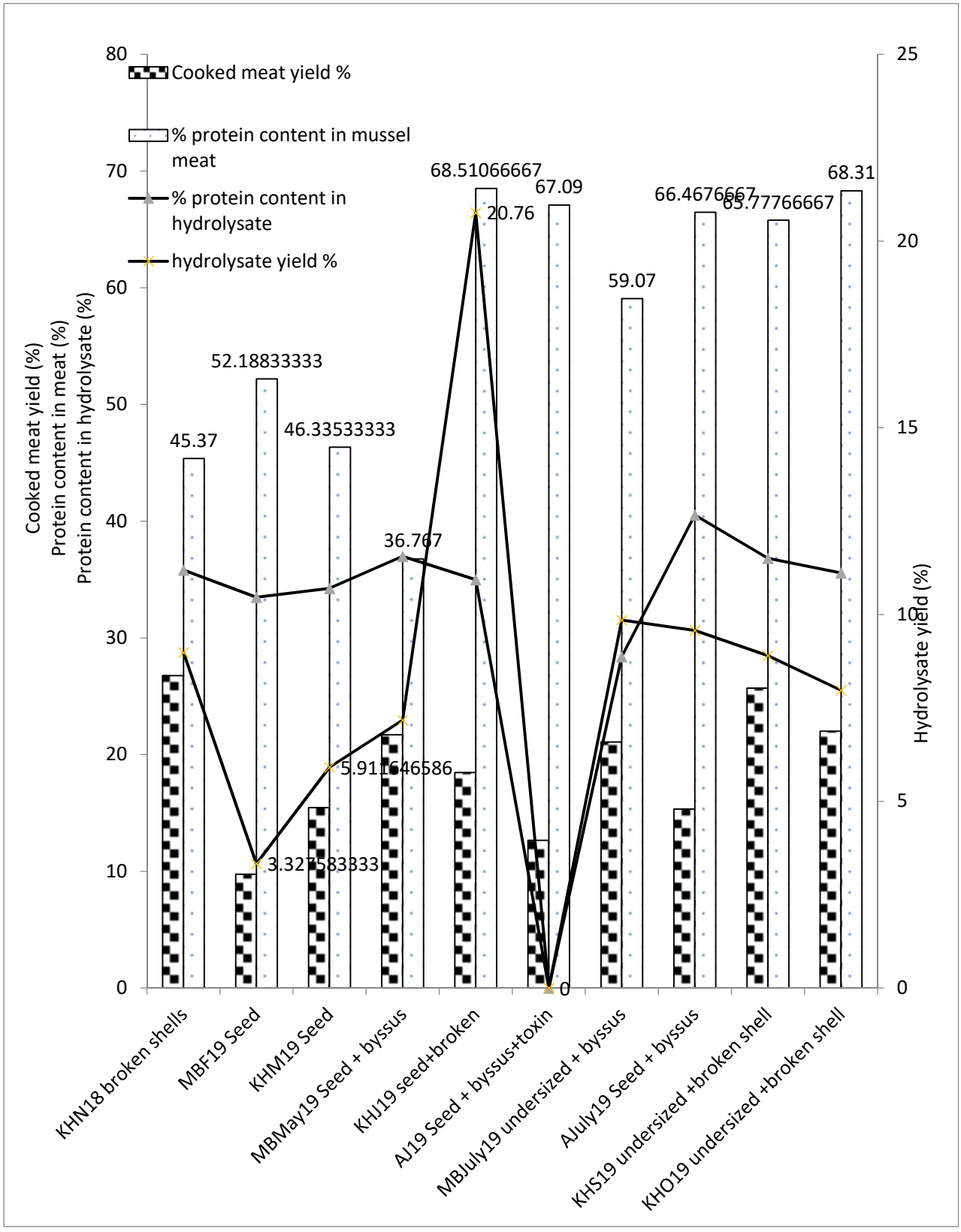
The meat yield and corresponding hydrolysate yield is dependent on the type of by-product mussel and the season of harvest. Seed mussels cooked meat yields ranged from 9.75% – 15.34% compared to undersized/broken shells or a combination of seed and broken sells which had meat yields ranging from 22% - 26.77%. Seed mussel samples in this study showed heterogeneity and some samples contained seed mussels < 1 cm in length, resulting in no retrievable meat and thus lower

meat yields/250 g of sample in comparison to larger seed mussels. The cooked meat yield for the by-products analysed varied between 9.75% (MBF19) to 26.77% (KHN18), always less than 30%. For *Mytilus edulis* mussels, the gamete development phase is between November to May during which the cells reach sexual maturation and there is a record drop in meat yield in the month of May [34].

In agreement to these published results, the present study showed relatively lower meat yields, hydrolysate yields and protein contents in mussel by-products harvested between February to May as shown in Fig. 3. The meat protein content is high between July to October, months associated with the spawning season. A higher amount of storage tissue accumulates during this season which corresponds to higher protein contents observed. Also in February and March lower meat yields were

found in comparison to samples harvested from September and November, though the effect was not as pronounced as the hydrolysate yields and protein contents observed.

Figure 3. Changes in meat yield (% w/w), hydrolysate yield (% w/w) and protein content (% w/w) for mussel by-products and Protamex® hydrolysates. All the 10 samples from the three sampling sites



Mulroy bay (MB), Killary harbor (KH) and Ardgroom (A) collected from November 2018 to October 2019 were used for analysis. Yields were determined gravimetrically while protein content was estimated using Dumas method (AOAC 968.06 method using Leco FP628). Analysis was performed in triplicate (n=3). Bars sharing the same letter are not significantly different according to Tukey's HSD test.

3.5. *Lipid content of mussels and hydrolysates*

The lipid content of mussel meat samples showed high standard deviation in comparison to hydrolysate samples, indicative of variability in any biological raw material. The lipid content of meat ranged from 0.52%±0.17 - 6.25%±1.19 while for hydrolysates it was between 0.966%±0.05 - 2%±0 as seen from Fig.4. The lipid content for mussel by-product meat samples varied based on mussel by-product type and season of collection. The lowest lipid content for mussel meat was observed between February to May (0.37%±0.07, 0.52%±0.17, 0.37%±0.11), i.e during the gamete development phase, a trend similar to protein content and the by-product type of these samples was seed mussel.

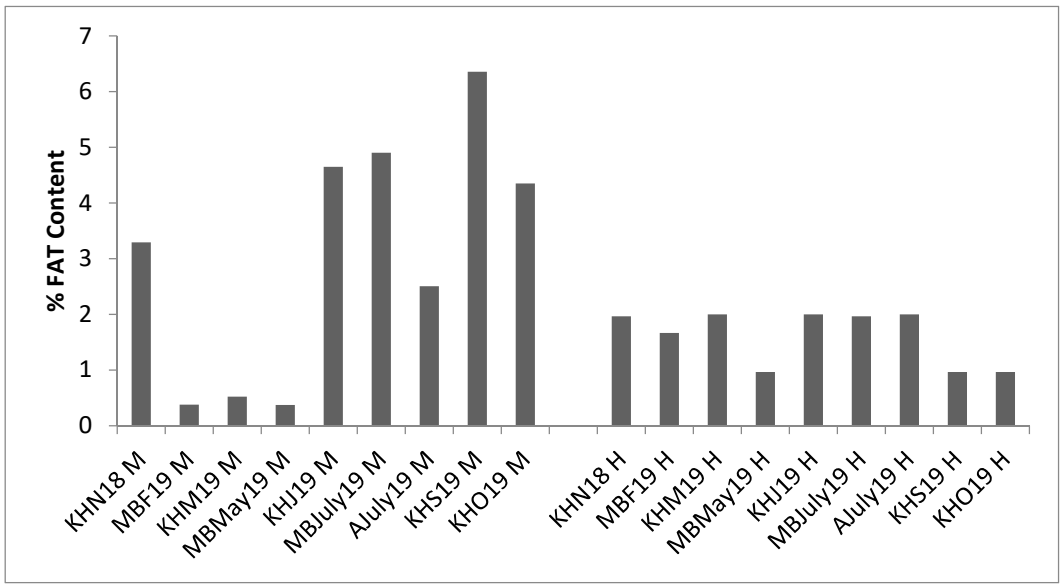


Figure 4. Changes in lipid content (%) for mussel by-products meat (M) and Protamex® hydrolysates (H). All the 9 samples from the three sampling sites Mulroy bay (MB), Killary harbor (KH) and Ardgroom (A) collected from November 2018 to October 2019 were used for analysis. The lipid content was quantified using AOAC Method 2008.06 with an Oracle rapid NMR fat analyser. Analysis was performed in triplicate (n=3).

3.6. *Emulsion activity and Emulsion stability*

The emulsion activity is the potential of a substance to form emulsions and is useful in formulation of products like mayonnaise or in the preparation of foods where prevention of phase separation is required i.e. in preparation of beverages having both an oil and water phase. As seen in Fig.5, the % emulsion activity of all the hydrolysates was between 56%±0-65%±1.4. Similar emulsion activity ranges were reported by other authors working on marine based protein hydrolysates [35]. Proteins in the hydrolysate are the chief component responsible for the emulsion activity seen. However the thermal stability of these emulsions was noted to be very poor and there was complete phase separation on heating the emulsions. The results show that the mussel hydrolysates have appreciable emulsion activity that can be explored for cold temperature applications like cold soups and blended smoothies.

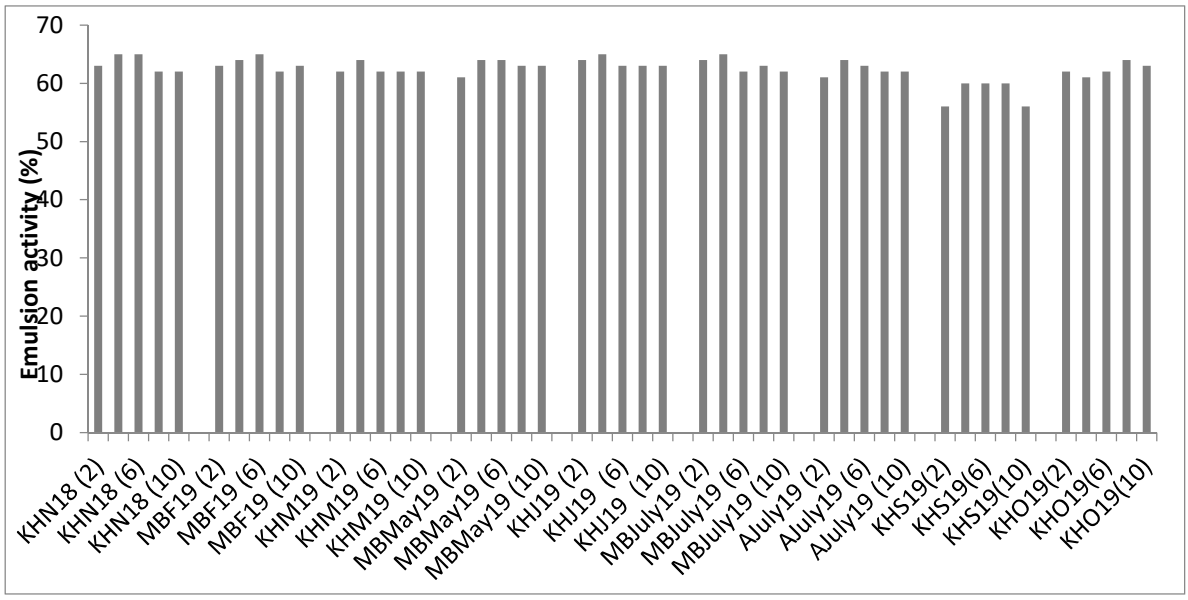


Figure 5. Emulsion activity (%) of various mussel Protamex® hydrolysates (H) across a pH range of (2) to (10). All the 9 samples from the three sampling sites Mulroy bay (MB), Killary harbor (KH) and Ardgroom (A) collected from November 2018 to October 2019 were used for analysis. No significant difference was found between the average solubility values after Tukey posthoc analysis. Analysis was performed in triplicate (n=3).

3.7. *Water activity*

As seen in Table 1, the water activity (a_w) readings for the freeze dried mussel hydrolysates were between 0.25-0.44 indicating little scope for microbial growth and showing that freeze-drying was appropriate to maintain product quality.

Table 1: Water activity (a_w) measurements of mussel Protamex® hydrolysates. Nine samples from the three sampling sites Mulroy bay, Killary harbour and Ardgroom collected from November 2018 to October 2019 were used for analysis. Analysis was performed in triplicate (n=3).

Sample	Average a_w	Standard deviation (±)
KHN18	0.35	0.0028
MBF19	0.44	0.0035
KHM19	0.33	0.004
MBMay19	0.33	0.0005
KHJ19	0.35	0.0028
MBJuly19	0.37	0.008
AJuly19	0.30	0.0005
KHS19	0.25	0.0047
KHO19	0.29	0.0115

3.8. *Solubility of mussel by-product hydrolysates*

Selected mussel by-product hydrolysates representative of the different seasons and sampling sites were analysed for solubility. Solubility of the mussel by-product hydrolysates ranged between 60-100% as seen in Fig. 6. Change in pH had a differential effect on the solubility of different hydrolysates. Some hydrolysates showed highest solubility at pH 2 (KHN18: 95.21%±3.47) while others at pH 10 (MBF19: 99.63%±4.5) and most showed very little change with change in pH. This shows pH has very little influence on the solubility of mussel hydrolysate samples, thereby providing wider applicability in selection of target food matrices. Similar results have been reported by other researchers for protein hydrolysates having low degree of hydrolysis similar to our results [34]. Enzymatic treatment often leads to unfolding of proteins exposing amino acids buried within the protein structure [37]. Since Protamex® is a relatively nonspecific serine protease, protein unfolding will differ resulting in varied solubility of the samples as seen in our study. Protein solubility of the samples is a pre-requisite techno-functional property for designing many food products and also to ensure higher digestibility and bioavailability of the ingredients. A similar range of solubility was reported previously by authors working on octopus hydrolysates [38].

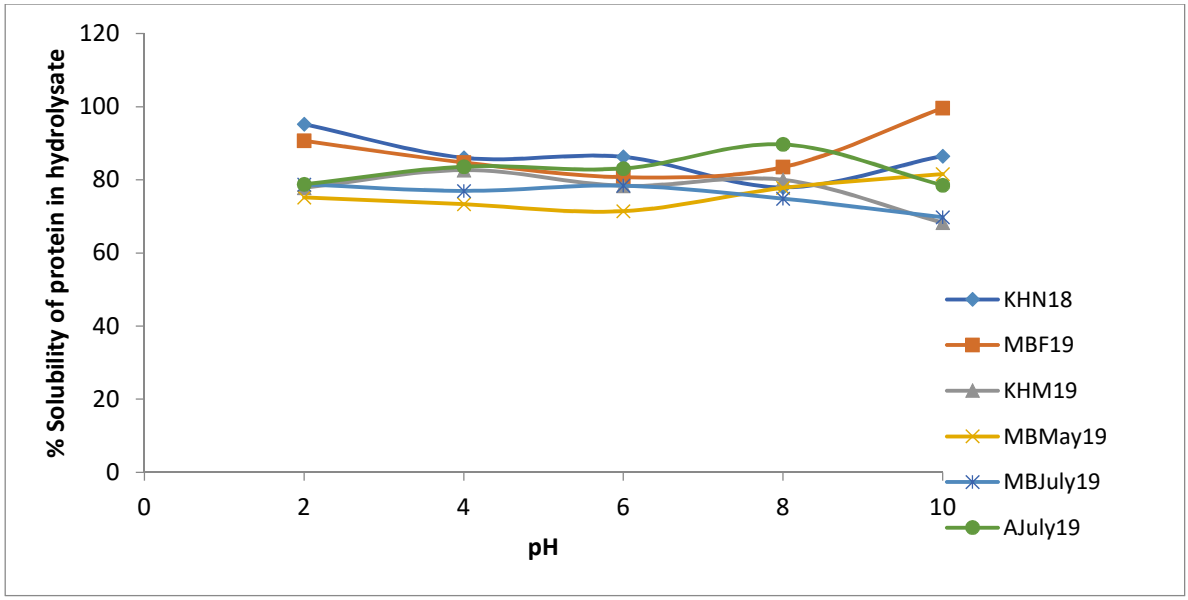


Figure 6. The percentage solubility of various mussel Protamex® hydrolysates across a pH range of 2 to 10. Six representative samples from the three sampling sites Mulroy bay, Killary harbour and Ardgroom collected from November 2018 to July 2019 were used for analysis. Analysis was performed in triplicate (n=3).

3.9. *Amino acid composition of hydrolysates*

LC-MS analysis of mussel by-product Protamex® hydrolysates showed them to contain most essential amino acids except tryptophan which isn't measured by the method used. The ratio of essential to nonessential amino acid varied from 0.68-0.96 and this ratio was 0.96 for sample KHN18 as shown in Table 2. At 0.96 the ratio was close to the ideal ratio of 1 and was better than plant sources like soy, pea and hemp protein [39] and also marine sourced salmon hydrolysate generated by other researchers [40]. Glutamate was the most abundant amino acid present, which is known to be responsible for the umami taste of food products, followed by aspartate and glycine in the total amino acid fraction of the hydrolysate.

GC-MS analysis of mussel by-product hydrolysates showed the presence of minor components such as benzaldehydes that are known to be flavour compounds. Benzaldehyde is thought to be a product of maillard reaction and was reported to be present in volatiles of other protein hydrolysates . Hydrolysates from other mussel varieties were explored previously for development of flavour ingredients [41] and the presence of these compounds in the hydrolysates would favour use of these for flavour ingredients. Certain amino acids are associated with specific taste such as L-histidine (essential amino acid) which is bitter, L-glutamate (non-essential amino acid) which imparts a umami

flavour and L-threonine (essential amino acid) which gives a sweet taste [42]. Thus the relative abundance of these amino acids in the peptides dictates the final taste of the hydrolysate product. Further, taurine, a sulphur containing amino acid was found abundantly (0.86%-3.25% w/w) in the mussel hydrolysate samples with maximum amount detected in KHM19 at 3.25% w/w of hydrolysate. Taurine is considered a critical nutrient for humans and diet represents its main source. Amongst several functions of taurine, it is known to be anti-oxidant and also effective against cardiovascular disorders [43].

3.10. Fatty acid analysis of hydrolysates

All the hydrolysate samples generated contained the omega-3 polyunsaturated fatty acids, eicosapentaenoic acid (EPA) (AJuly19: 313.41±11.59 mg/gm lipid) and docosahexaenoic acid (DHA) (KHN18: 538.28±10.03 mg/gm lipid) as seen in Table 3. These fatty acids are known to be anti-inflammatory and are implicated in reducing symptoms of several inflammatory disorders such as asthma, arthritis and even cardiovascular diseases. The content of fatty acids in mussels harvested in summer usually have decreased levels of saturated fatty acids (SFAs) indicating the low energy requirement of the blue mussels during this season as SFAs like palmitic (C16:0) or myristic (C14:0) acids, are associated with energetic-type functions [4]. A similar seasonal trend was observed in our work with respect to SFAs in by-product mussel hydrolysates generated during the summer period. The recommended ratio of omega 6: omega 3 in the human diet for anti-inflammatory benefit has been established as 10:1-4:1. Most of the hydrolysates showed a ratio of 0.1, which translates as 10 times more omega 3 in the hydrolysates compared to omega 6 content. The hydrolysates can be considered a rich source of both essential amino acids & anti-inflammatory omega-3 fatty acids.

3.11.Sequence, physicochemical nature and bioactivity of identified peptides

Most of the peptides identified in the 3-kDa permeate fraction were medium to large sized peptides having > 7 amino acids as shown in Table 4. Table 4 provides biological activity of fragments of peptides identified by mass spectrometry, but not activity of entire peptides. The average molecular weight of a 7 amino acid long peptide was 650 Da. The solubility profile of the peptides varied from being moderately hydrophobic (27% of the peptides analysed) to highy hydrophilic (73% of the peptides analysed) nature (ThermoFisher peptide analysing tool,https://www.thermofisher.com/ie/en/home/life-science/protein-biology/peptides proteins/custom-peptide-synthesis-services/peptide-analyzing-tool.html). This tool does not state how hydrophobicity is calculated but absolute amino acid based scores are either based on Kyte and Doolittle [44] or the Woolfenden et.al [45] methods. Using BIOPEP-UWM (formerly BIOPEP) analysis (http://www.uwm.edu.pl/biochemia/index.php/en/biopep -accessed 20/01/2020), 91% of the

peptides found in the 3 –kDa permeate for hydrolysate KHN18 had previously identified DPP-IV and ACE-I inhibitory activities (eg: EDGKNPDDDE, HGCGMHS, DPKGGGA).Correlations between ACE-I and DPP-IV inhibitory activity has been previously reported by scientists working on bioactive peptides [46]. Other bioactivities predicted for the mussel by-product hydrolysates using in silico analysis included antioxidant (eg: VDDHHDDHHD), anti-amnestic (eg: DHPLPGTD), anti-thrombotic (eg: GPPGEPGEPGSS), regulation of stomach mucosal activity (eg: VGEPGPPGP), immune-modulating (eg: ATASILGY) and chemotactic activity (eg: KPGPSHPGDSKA).

3.11.1. *DPP (IV) inhibition*: The DPP-IV inhibitory activity at 30 min for the generated mussel by-product hydrolysates ranged from 0% to 90.51%±0.18 while it was 81.14%±0.47 for sitagliptin when samples were assayed at a concentration of 1mg/ml compared to the positive control sitagliptin which was assayed at a concentration of 18 nM (IC_{50} value) (Fig.7.).

3.11.2. *AChE inhibition*: A trend similar to DPPIV inhibition was seen where AJuly19, KHS19 and KHO19 displayed AChE inhibition (Fig.8.), though the % inhibition (KHO19: 29.59%±1.88) of the samples (1 mg/ml) was low as compared to positive control acotiamide dihydrochloride (94.201%±0.89) assayed at 100uM concentration (IC_{50} 3uM). Researchers have found AChE, ACE-I and DPPH inhibitory activity in subcritical extracts of blue mussels [45]. The obtained results warrant further studies to explore possible potential of some of these hydrolysates in promoting mental health.

3.11.3. *ACE-I inhibition*: All the samples showed appreciable ACE-I inhibition as compared to un-hydrolysed mussel broth as seen in Fig.9. % ACE-I inhibition was seen to vary between 22.23%±1.79 - 86.08%±1.59 for hydrolysates as compared to 97.31% ±0.255 by positive control captopril. The KHN18 sample inhibited ACE-I by 86.08%±1.59 when assayed at a concentration of 1 mg/ml compared to captopril which was assayed at a concentration of 0.5 mg/ml. Further, the IC_{50} value for KHN18 was 0.2944 mg/ml which is lower than reported values of other marine hydrolysates (1.50-2.54 mg/ml) [48] highlighting the superior ACE-I inhibitor potential of the mussel hydrolysates. An interesting study conducted on *Mytilus edulis* based fermented sauce also established formation of ACE-I inhibitory peptides from blue mussel source [14]. The findings from the *in vitro* assay also confirm the results obtained from bioinformatics mining of the sequenced peptides as 92% of the peptides identified in the 3-kDa permeate fraction and assessed using BIOPEP had ACE-I inhibitory activity.

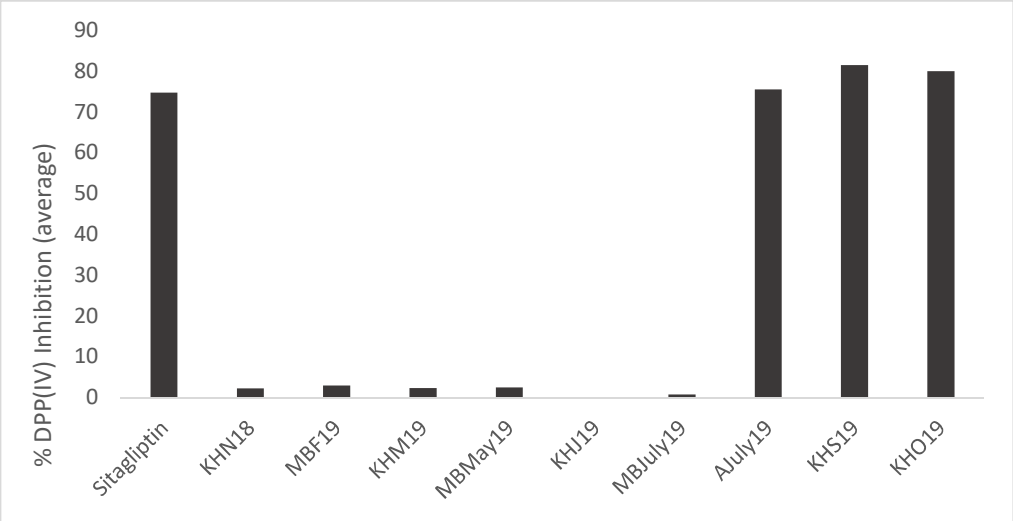


Figure 7. DPP(IV) inhibition (%) by mussel Protamex® hydrolysates. Nine samples from the three sampling sites Mulroy bay, Killary harbour and Ardgroom collected from November 2018 to October 2019 were used for analysis at a concentration of 1mg/ml. Analysis was performed in triplicate (n=3).

No significant difference was found between positive control Sitagliptin and samples AJuly19, KHS19, KHO19 after Dunn Bonferroni (nonparametric) posthoc analysis.

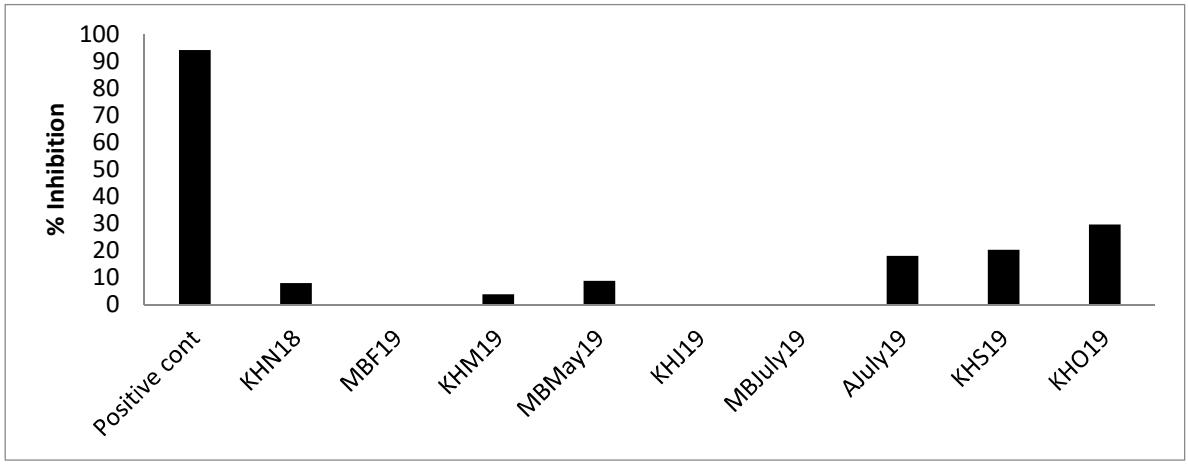


Figure 8. AChE enzyme inhibition (%) by inhibition by mussel Protamex® hydrolysates. Nine samples from the three sampling sites Mulroy bay (MB), Killary harbor (KH) and Ardgroom (A) collected from November 2018 to October 2019 were used for analysis at a concentration of 1mg/ml. *The positive control showed significantly higher AChE inhibition as compared to all the samples. Assays were performed in triplicate (n=3).

*The positive control showed significantly higher AChE inhibition as compared to all the samples

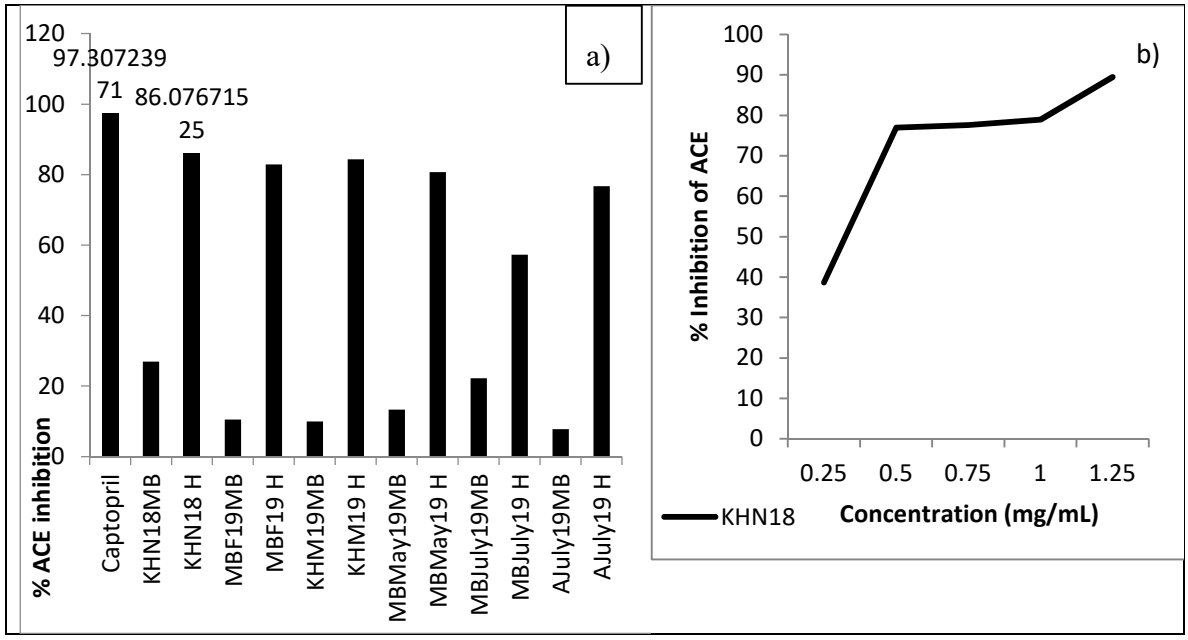


Figure 9a. The % ACE-I enzyme inhibition (%) by mussel Protamex® hydrolysates. 6 representative unhydrolyzed mussle broth samples (MB) and corresponding hydrolysates (H) from the three sampling sites Mulroy bay (MB), Killary harbor (KH) and Ardgroom (A) collected from November 2018 to July 2019 were used for analysis at a concentration of 1mg/ml against Captopril - the positive standard. **Figure 9 b.** The IC_{50} curve for Killary harbour November 2018 sample (KHN18).The positive control showed significantly higher ACE inhibition as compared to all the samples and all the hydrolysates showed significantly higher ACE inhibition as compared to unhydrolyzed samples. Assays were performed in triplicate (n=3).

4. Summary

The mussel meat yield and resulting hydrolysate yields showed a season specific trend in terms and this related to the fat and protein content of generated hydrolysates. Mussel meat yields were 9.75% to 21.69%, 0.37% ±0.11 to 0.52%±0.17, 36.77%±7.17 to 52.19%±3.33 for mussel meat and 3.33% ±0.93 to 7.17%±0.65, 0.97%±0.06 to 2%±0, 33.48%±1.45 to 36.99%±1.27 for hydrolysate harvested during the period February to May - during – the gamete development season. This compared to values of 15.34% to 22, 2.5%±1.32 to 6.36±1.2, 65.78%±0.54 to 68.31%±1.02 for mussel meat and 7.97%±0.39 to 9.57%±0.94, 0.96%±0.05 to 2±0, 35.57%±1.02 to 40.54%±2.12 for hydrolysates generated with July-

October samples - during the blue mussel spawning season. All the hydrolysates were rich in the essential amino acids, taurine, polyunsaturated fatty acids EPA and DHA and contained several unique bioactive peptides with ACE-I inhibitory activity. Few hydrolysates had DPP-IV inhibitory activities (AJuly19,KHS19 and KHO19) and hydrolysates generated with these samples also inhibited AChE. The in silico results were in agreement with the results seen through in vitro enzyme inhibition assays. Peptides obtained from the 3-kDa fraction of the hydrolysates were medium to large sized having > 7 amino acids. The hydrolysates showed good emulsion activity and excellent protein solubility profiles over a range of pH and can thus be incorporated in further product formulation studies targeting cardiovascular diseases and obesity. The study enabled characterization of raw material and hydrolysate product through a period of one year sampling. In future, inhibitory peptides from marine source need not be limited to over exploited green lipped mussels The bio-actives rich composition of the resulting hydrolysates from lesser studied *Mytilus edulis* mussels opens doors for nutraceutical products.. Further, *in silico* studies can be undertaken using blue mussel derived sequenced peptides to dock critical enzymes such as Prolyl endopeptidase, Cyclooxygenase 1 & 2, along with Dipeptidyl peptidase, Acetylcholinesterase and Angiotensin converting enzyme-1. The enzymes have been implicated in several disorders such as mental degeneration, inflammation, diabetes, cardiovascular diseases and other metabolic diseases.

Amino Acids		KHN18pro	MBF19 H	KHM19 H	MBMay19H	Soy protein	Whey protein	Icelandic scallop hydrolysate	Atlantic salmon hydrolysate
Essential amino acids	Methionine	0.6607	0.1135	0.1799	0.3687	0.3	1.8	0.457	1.3
	Threonine	1.2815	0.2494	0.2855	0.2355	2.3	5.4	0.856	2.9
	Lysine	1.9237	0.2726	0.7896	0.4005	3.4	7.1	3.778	0
	Valine	1.1967	0.0063	0.4275	0.7775	2.2	3.5	1.681	2.5
	Leucine	1.6869	0.2679	0.2968	0.8958	5	8.6	0	2.4
	Isoleucine	1.1182	0.1956	0.2297	0.6471	1.9	3.8	0	1.2
	tryptophan	0	0	0	0.0989	0	0	0.812	
	phenylalanine	1.0326	0.2275	0.347	0.565	3.2	2.5	2.811	3.1
	histidine	1.4382	0.3174	0.5225	0.5066	1.5	1.4	0.229	16.4
	arginine	1.5961	0.1854	0.2495	0.1888	4.8	1.7	4.611	1.3
Non essential amino acids	glutamate/glutamine	3.6814	0.5535	1.5894	1.9325	12.4	15.5	2.678	3.1
	proline	0.9729	0.1508	0.2229	0.5169	3.3	4.8	0.741	0
	serine	0.8765	0.2015	0.2646	0.2063	3.4	4	0.941	4.8
	glycine	2.0381	0.2704	0.5315	1.5489	2.7	1.5	1.6	13.7
	cysteine	0.8134	0.0385	0.643	0.5137	0.2	0.8	0	
	aspartate/asparagine	2.4696	0.6104	0.7903	0.66	0		4.116	0.4
	alanine	1.5091	0.2281	0.7916	0.8975	2.8	4.2	2.03	24.7
	tyrosine	0	0.0124	0.0078	0.021	2.2	2.4	1.909	1.9

GABA	0.0065	0.0243	0.0507	0	0	0	0	0
Taurine	1.8639	2.3182	2.5595	2.3964	0	0	0	0
Total	26.166	6.2437	10.7793	13.3776	0	0	0	0
TEAA	11.9346	1.8356	3.328	4.6844	24.6	35.8	15.235	31.1
TNEAA	12.361	2.0656	4.8411	6.2968	27	33.2	14.015	48.6
Ratio of E:NE	**0.9655044**	**0.8886522**	**0.68744707**	**0.743933427**	**0.911111**	**1.078313**	**1.08704959**	**0.639917695**

Table 2. Total amino acid composition (%, w/w) of selected mussel by-product Protamex® hydrolysates compared to other marine and non-marine protein hydrolysates

		mg fatty acid/g lipid								
Name (methyl ester)		**KHN18**	**MBF19**	**KHM19**	**MBMay19**	**KHJ19**	**MBJuly19**	**AJuly19**	**KHS19**	**KHO19**
Butyric acid	C4:0	15.06	14.02	14.83	14.13	15.33	39.19	12.378	14.065	20.340
Caproic acid	C6:0	16.26	15.10	15.98	15.20	15.45	18.05	13.369	15.130	21.986
Caprylic acid	C8:0	30.58	39.55	34.01	30.57	28.85	35.12	19.442	48.533	61.002
Capric acid	C10:0	22.59	31.65	27.53	24.54	22.93	25.86	15.243	38.067	44.464
Undecanoic acid	C11:0	6.98	6.49	6.85	6.53	6.63	7.71	5.731	6.514	9.458
Lauric acid	C12:0	9.26	8.16	8.66	8.42	8.86	8.91	7.886	8.605	12.316
Tridecanoic acid	C13:0	7.13	6.54	6.93	6.64	6.75	7.85	5.886	6.613	9.576
Myristic acid	C14:0	179.19	9.01	12.39	25.42	31.37	19.00	41.669	27.279	25.037
Myristoleic acid	C14:1	5.58	3.00	3.17	3.03	3.25	3.73	2.914	3.066	4.441
Pentadecanoic acid	C15:0	10.66	7.18	7.99	7.82	8.95	8.63	7.825	8.662	11.53
cis-10-Pentadecenoic acid	C15:1	3.15	3.10	3.28	3.07	3.16	3.62	2.676	2.932	4.249

Palmitic acid	C16:0	395.73	32.17	66.60	115.13	175.60	99.48	173.312	132.21	114.37
Palmitoleic acid	C16:1	210.43	3.61	16.28	56.53	70.06	53.58	92.557	55.937	43.761
cis-10-Heptadecenoic acid	C17:1	2.11	0.56	1.40	2.10	2.50	1.30	1.892	1.878	1.521
Stearic acid	C18:0	34.27	20.56	22.26	23.38	39.42	28.38	30.469	29.365	34.148
Elaidic acid	C18:1 t	45.37	0.92	2.64	4.55	3.73	2.36	5.749	2.964	2.751
Oleic acid	C18:1 c	90.50	1.81	5.25	9.07	7.42	4.69	11.455	5.896	5.468
Linolelaidic acid	C18:2 t	4.74	3.50	3.95	3.95	2.64	4.11	4.353	3.489	5.418
Linoleic acid	C18:2 c	95.81	5.56	10.18	12.73	14.12	8.66	16.439	13.031	13.276
γ-Linolenic acid	C18:3 n6	5.24	3.61	3.96	4.22	4.13	4.64	4.714	4.199	5.533
***α*-Linolenic acid**	C18:3 n3	43.98	5.99	12.26	19.32	23.31	9.61	22.633	19.865	20.183
Arachidic acid	C20:0	16.47	14.80	15.62	14.94	15.24	17.83	13.191	14.866	21.402
cis-11-Eicosenoic acid	C20:1	18.18	10.10	9.75	6.08	15.54	6.77	11.152	7.211	8.213
cis-11,14-Eicosadienoic acid	C20:2	9.22	1.42	4.03	3.43	5.01	1.74	4.835	4.634	4.964
Henicosanoic acid	C21:0	9.26	8.08	8.54	7.98	8.18	9.44	7.213	8.026	11.619

cis-8,11,14-Eicosatrienoic acid	C20:3 n6	1.24	1.13	1.19	1.58	1.42	1.19	2.133	1.400	2.117
Arachidonic acid	C20:4 n6	20.77	20.56	14.82	10.03	21.76	16.83	22.099	17.688	19.010
cis-11,14,17-Eicosatrienoic acid	C20:3 n3	3.82	2.92	3.56	3.56	3.96	3.66	3.342	4.251	4.687
cis-5,8,11,14,17-Eicosapentaenoic	C20:5 n3 (EPA)	141.49	47.02	54.62	128.90	212.28	73.04	313.410	193.973	163.444
Tricosanoic acid	C23:0	8.26	7.73	8.17	7.77	7.90	9.15	6.808	7.693	11.178
Lignoceric acid	C24:0	15.80	14.75	15.62	14.76	15.12	17.38	12.969	14.710	21.319
cis-4,7,10,13,16,19-Docosahexaenoic acid	C22:6 (DHA)	538.28	81.33	92.31	82.71	158.53	53.75	173.005	150.713	131.364
omega 6		137.01	35.78	38.13	35.94	49.09	40.27	54.58	44.44	50.32
omega 3		727.57	137.26	162.75	234.49	398.08	140.05	512.39	368.80	319.68
omega 6:omega 3 ratio		0.188	0.260	0.234	0.153	0.123	0.287	0.106	0.120	0.157

Table 3. Fatty acid composition (mg/g lipid) of mussel Protamex® hydrolysates. All the 9 samples from the three sampling sites Mulroy bay, Killary harbour and Ardgroom collected from November 2018 to October 2019 were used for GCMS based fatty acid analysis.

Peptide sequence	mol wt	no of AA residues	hydrophobicity	GRAVY	Nature	pI	Potential bioactivities (BIOPEP)
VDDHHDDHD	1240.44	10	0.57	-2.61	very high hydrophilic	4.4	DPP-IV inhibition & antioxidant
PVDDHHDDHD	1337.50	11	2.2	-2.52	very high hydrophilic	4.4	DPP4-IV inhibition & antioxidant
NPVDDHHDDHD	1451.53	12	3.57	-2.6	very high hydrophilic	4.4	DPP4-IV inhibition & antioxidant
KPEAPKVP	864.54	8	8.9	-1.26	high hydrophilicity	9.9	ACE-I inhibitor, DPP-IV &DPP-III inhibition & antioxidant
NLGAIGH	680.36	7	13.74	0.37	moderate hydrophobicity	7.8	ACE-I inhibitor, DPP-IV inhibition
KGLVDGAPAN	940.47	10	16.48	-0.17	moderate hydrophobicity	6.8	ACE-I inhibitor, DPP-IV inhibition & glucose uptake stimulating
FNAEKGFGF	1015.47	9	26.99	-0.17	moderate hydrophobicity	6.9	ACE-I inhibitor, DPP-IV&DPP-III inhibition
DHPLPGTD	850.38	8	14.49	-1.34	moderate hydrophobicity	3.9	ACE-I inhibitor, DPP-IV &DPP-III inhibition, antiamnestic, antithrombotic, regulates stomach mucosal activity

SSDVPGV	659.32	7	11.81	0.19	moderate hydrophobicity	3.1	ACE-I inhibitor, DPP-IV inhibition, antiamnestic, antithrombotic, regulates stomach mucosal activity
GPPGEPGEPGSS	1066.45	12	6.37	-1.38	very high hydrophilic	3.1	ACE-I inhibitor, antioxidative, DPP-IV & DPPIII inhibition, antiamnestic, antithrombotic, regulates stomach mucosal activity

Table 4. Sequence, physicochemical nature and bioactivity of peptide fragments identified using mass spectrometry in the 3-kDa permeate of Killary harbour November 2018 (KHN18) mussel by-product Protamex® hydrolysate as analyzed by BIOPEP and ThermoFisher peptide analysis *in silico* tools.

Author Contributions: Conceptualization, M.H. ; methodology, A.N.; validation, A.N.., M.H..; formal analysis, A.N. L. M-S.; investigation, M.H. and A. N; writing—original draft preparation, A.N.; writing—review and editing, A. N. and M.H.; supervision, M.H.; project administration, M.H.; funding acquisition, M.H.

Funding: Please add: This research was funded by Bord Iascaigh Mhara (BIM) and the European Maritime Fisheries Fund (EMFF), grant number 17/SRDP/002 2018-2020.

Acknowledgments: The authors would like to acknowledge the contribution of Dr Ivan Milovanovic for assistance with Fatty Acid Methyl Ester (FAME) analysis.

Conflicts of Interest:

The authors declare no conflict of interest.

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