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BIOACTIVE PEPTIDES GENERATED FROM THE BY-PRODUCTS OF THE WHELK PROCESSING INDUSTRY

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ABSTRACT

The worldwide fish and shellfish sector processes approximately one million ton of fish annually. However, only 43% of this is consumed, and important proteins are lost during product processing. The purpose of this research is to extract protein from industrial whelk processing byproducts and produce bioactive protein hydrolysates with marketable value. The whelk by-product extracted protein was hydrolyzed with Alcalase and α- Chymotrypsin. The resulting peptides were tested for their antioxidant ferric reducing ability (FRAP) and Angiotensin-converting enzyme (ACE) inhibitory activity using spectrophotometric testing kits. Bacterial growth inhibition for *e. coli* and *s.areus* was also tested using the disk diffusion test. The ACE inhibition lowest IC50 value belongs to the acid extracted protein at 3,44µg/mL, non-processed protein was shown to have higher ACE inhibition than hydrolyzed protein, all samples had much higher IC50 values for ferric reducing ability when comparing to the IC50 for the DPPH activity performed in a previous study. Antibacterial activity was shown to be lower than other studies, although not powerful bacterial growth inhibitors in conjunction with the other bioactivities the peptides exhibit, if incorporated in a food product. These by-products have the potential to generate a new generation of multifunctional bioactive peptides that can be valuable to the industry as supplement/fortified meals and nutraceuticals.

KEYWORDS: Shellfish; Bioactive peptides; Protein extraction; Protein hydrolysates, Waste management, Nutraceuticals; Antioxidants; ACE inhibitors; Antimicrobial.

1. INTRODUCTION

Marine and seafood industries are extremely important to coastal locations, particularly in Europe (Garcia-Garcia *et al.*, 2018). More than 70% of fish caught undergoes extra processing before being sold (la Caba *et al.*, 2019). The discarded by-product is mostly composed of shellfish shells, undersized specimens, or a mix of these with flesh and water (R. Olsen *et al.*, 2014).

Solid waste from fish and shellfish processing can be up to 60% of the harvested weight. This includes viscera, skin, and filet trims, as well as shell debris in the case of crabs and crustaceans (R. Olsen *et al.*, 2014). These waste products have a high quantity of organic content, such as protein, lipids, and enzymes, which constitute a significant environmental danger if simply discarded in landfills, such as the development of strong odors, air and soil pollution, and harm to marine species (Sila & Bougatef, 2016). Because of these factors, marine by-products are often incinerated after production, posing an environmental risk as well as a financial burden on fish and shellfish processors. Fish and shellfish by-products can have a crude protein composition ranging from 8% to 35% of its weight (Sila & Bougatef, 2016).

Sofrimar Ltd., Co Wexford, kindly contributed the shellfish waste for this study. It is mainly made up of whelk processing by-products. Whelk flesh contains mostly glutamic acid (13.11g/100g), with lysine, leucine, arginine, and isoleucine present in smaller but still considerable amounts. Whelk's total composition is made up of structural proteins (such collagen and elastin) that give the mollusk its high elastic properties (Ehrlich, 2019).

Nutraceuticals are compounds that offer physiological advantages or provide protection against chronic illness (DeFelice, 1995). Functional foods are foods that provide health advantages in addition to nutritional value functioning (Chakrabarti *et al.*, 2018). This category covers foods fortified with vitamins, minerals, probiotics, and fiber, as well as nutrient-rich components including fruits and vegetables. The International Life Sciences Institute (ILSI), Europe, and the European Parliament have all released declarations underlining the need for scientific guidelines to recognize the additional advantages of eating functional food products (Haleem *et al.*, 2013).

Beyond its established nutritional value, bioactive peptides are described as peptide sequences within a protein that have a favorable effect on physiological processes and/or

positively influence human health (Chakrabarti *et al.*, 2018). As a result, bioactive peptides have attracted a lot of attention as nutraceuticals and functional foods have attracted a lot of attention as nutraceuticals and functional foods, bioactive peptides with antihypertensive, antioxidant, antibacterial, and antiproliferative properties have been discovered in meat and fish protein hydrolysates to date (table 1).

Table I: Biological activity associated with different species derived protein hydrolysates and peptides.

Common name	Scientific name	Biological Activity	Reference
Chicken	*Gallus gallus domesticus*	ACE inhibitory	(Saiga *et al.*, 2008)
Dromedary colostrum	*Camelus dromedarius*	Antioxidant	(Oussaief *et al.*, 2020)
Flaxseed	*Linum usitatissimum*	Antioxidant	(Silva *et al.*, 2017)
Gilt-head bream	*Sparus aurata*	ACE inhibitory	(Fahmi *et al.*, 2004)
Green microalga	*Chlorella sorokiniana*	ACE inhibitory	(Lin *et al.*, 2018)
Mung bean	*Vigna radiata*	Antioxidant	(Guerra *et al.*, 2017)
Okara	*Abelmoschus esculentus*	Antioxidant	(Sbroggio *et al.*, 2016)
Pistachio Nuts	*Pistacia vera*	Antioxidant ACE inhibitory	(Dumandan *et al.*, 2014)
Red dulse	*Palmaria palmata*	ACE inhibitory	(Furuta *et al.*, 2016)
Red snapper	*Lutjanus campechanus*	Antioxidant ACE inhibitory Antioxidant	(Khantaphant *et al.*, 2011)
Red tilapia	*Coptodon rendalli*	Antioxidant	(Sierra-Lopera & Zapata-Montoya, 2021)
Rice bran	*Oryza sativa*	Antioxidant	(Thamnarathip *et al.*, 2016)
Salmon	*Salmo salar*	ACE inhibitory Dipeptidyl peptidase IV inhibitory Antioxidant	(Neves, Harnedy, O'Keeffe, Alashi, *et al.*, 2017)
Sheep (plasma)	*Ovis aries*	Antioxidant	(Hou *et al.*, 2019)
Shrimp	*Acetes vulgaris/*	Antioxidant	(Faithong *et al.*, 2010)
Snapper	*Lutjanus vitta*	Antioxidant ACE inhibitory	(Khantaphant *et al.*, 2011)
Southern laver	*Pyropia columbina*	Antioxidant	(Cian *et al.*, 2015)

Fish seems to be one of the most important and essential foods in the human diet. Fish and shellfish provide high-quality protein with all essential amino acids, as well as essential sources of dietary vitamins and minerals (Balami *et al.*, 2019). Protein, water, and lipids

make up the majority of the chemical makeup of fish and shellfish muscles (Abraha *et al.*, 2018).

Nutraceuticals derived from fish and shellfish can be used to enhance health in a variety of ways, including delaying the aging process, preventing acute and chronic illnesses, extending life expectancy, and supporting the body's fundamental structure and functioning (Nasri *et al.*, 2014). Fish and shellfish alike are a good source of high-quality animal proteins (particularly the essential amino acids like lysine and methionine). Fish and shellfish protein has been shown to have a higher satiety impact than other animal proteins such as beef and chicken (Balami *et al.*, 2019).

About 60% of individuals in developing nations rely on fish for more than 30% of their animal protein (Balami et al., 2019). Fish and shellfish proteins are extremely digestible and contain a high concentration of peptides and important amino acids, which are scarce in interplanetary meat proteins. Compared to land-based food, fish and seafood have a considerably lesser negative effect on the environment (Tsakiridis *et al.*, 2020). The by-products of fish/shellfish may possess between 7% and 23% w/w protein depending on species/development stage (R. L. Olsen *et al.*, 2014). This protein can be easily, efficiently, and cheaply extracted and hydrolyzed to yield bioactive and profitable compounds.

Oxygen is an essential molecule for living organisms, despite this its nature is very reactive and produces reactive oxygen species (ROS) (Vertuani *et al.*, 2004). Life forms have evolved a complex network of metabolites and antioxidant enzymes that work together to protect cellular components such as DNA, proteins, and lipids from oxidative damage. Antioxidant systems generally prevent the formation of ROS or eliminate them before they can damage vital cell components (Vertuani *et al.*, 2004). Oxidative stress is known to be a contributor to many neurodegenerative diseases, such as Alzheimer's disease (Butterfield & Boyd-Kimball, 2018; Nunomura *et al.*, 2006), Parkinson's disease and motor neuron diseases (Liu *et al.*, 2017; Trist *et al.*, 2019), diabetes-related pathologies (Oguntibeju, 2019), and rheumatoid arthritis (Butterfield & Boyd-Kimball, 2018; Fonseca *et al.*, 2019). Artificial antioxidants are compounds chemically synthesized in laboratory settings (Mahmoud *et al.*, 2019). However, the use of this synthetic chemicals has been connected to possible toxicity, and it has been suggested that it has some negative consequences such as carcinogenesis, therefore it should be used with caution (Caleja *et al.*, 2017). Natural extracts might be utilized to replace synthetic antioxidants and preservatives, while also providing bioactive/nutritive properties

and adding additional value to the final product. While research on bioactive peptides isolated from several types of shellfish and fish have been conducted (Bordbar *et al.*, 2018; Cunha Neves *et al.*, 2015a; Huang *et al.*, 2011; Jumeri & Kim, 2011; Ngo *et al.*, 2010). To the best of our knowledge, no research has been done on the issue in connection to industry by-products with more heterogeneous product combinations (mixed shellfish/fish species) than homogeneous mixtures (one species of shellfish/fish). Shellfish waste by-products might be a rich source of antioxidant peptides, resulting in a low-cost and long-term antioxidant dietary supplement.

High blood pressure, or hypertension, is a long-term medical condition defined by chronically high blood pressure in the arteries. It is connected to stroke, coronary artery disease, heart failure, atrial fibrillation, peripheral arterial disease, vision loss, chronic renal disease, and dementia (Mills *et al.*, 2020). In 90–95 percent of individuals, primary hypertension is caused by nonspecific lifestyle and hereditary factors such as diet, exercise, and stress, while 10–15% are caused by identifiable causes such as birth control pills or endocrine disorders (Kjeldsen, 2018).

The renin–angiotensin system (RAS) is a hormone system that controls blood pressure, fluid and electrolyte balance, and systemic vascular resistance. It was discovered in the mid-1950s and is the latest component of the renin-angioten system to be found (Skeggs Jr *et al.*, 1956). ACE is a component of the renin-angiotensin system that regulates blood pressure by converting angiotensin I to angiotensin II, a vascular constrictor. High blood pressure, heart failure, and type I and type II diabetes are all treated with ACE inhibitors, which are pharmacological medications (Hermida *et al.*, 2011). ACE inhibitory (ACE-I) peptides are one of the most investigated bio-peptides due to their influence on hypertension, the primary cause of cardiovascular disease (Wu *et al.*, 2017). During *in vitro* and/or *in vivo* enzymatic degradation, food proteins may create ACE inhibitory peptides. After being released, certain bioactive peptides, such as opioid peptides used to treat intestinal disorders, act locally in the gastrointestinal tract, whilst others act systemically after being absorbed by the intestinal mucosa and released into the circulation. Because of their effectiveness in preventing and treating hypertension, dietary peptides have attracted a lot of attention(de Leo *et al.*, 2009). Peptides with ACE inhibitory activity derived from hydrolysates of various types of shellfish and fish waste such as tuna muscle (Kohama *et al.*, 1988), Alaska pollack skin (Park *et al.*, 2009), sea bream scales(Fahmi *et al.*, 2004), oyster (Lin *et al.*, 2018), short-necked clam

(Suetsuna, 2002), freshwater clam (Tsai *et al.*, 2006), and sea cucumber (Ghanbari *et al.*, 2015), salmon (Neves, Harnedy, O'Keeffe, & FitzGerald, 2017) and mussel meat(Cunha Neves *et al.*, 2015b).

Antimicrobial activity is a phrase used to describe all active principles (agents) that inhibit bacteria from developing and forming microbial colonies (Busch & Kadri, 2020). Humans and most animals contain millions of germs in and on their bodies (Wang *et al.*, 2017). The most common cause of bacterial mortality is respiratory infections. Pathogenic germs are responsible for diseases such as, cholera, syphilis, anthrax, leprosy, and bubonic plague. Antibiotics are used to treat bacterial infections as well as in agriculture, resulting in an increase in antibiotic resistance (Khurshid, 2018). *Escherichia coli* is a gram-negative bacillary bacterium that lives in warm-blooded (endothermic) animals' lower gastrointestinal tracts. Although the majority of *e. coli* strains are innocuous, certain serotypes can cause serious food poisoning in humans and are occasionally responsible for food product contamination (Panel *et al.*, 2020). Most *e. coli* strains are safe to eat however, some strains, such as Shiga toxin-producing *e. coli* (STEC), can cause serious food poisoning (Hosomi et al., 2019). *Staphylococcus aureus* is a spherical bacterium that belongs to the gram-positive cocci family and is commonly found in healthy people's skin and nasal passages. It can, however, induce a variety of ailments, from basic infections (acne, boils, and cellulitis) to more serious infections (pneumonia, meningitis, endocarditis, toxic shock syndrome, sepsis, and others)(Foster & Geoghegan, 2015). The disk diffusion technique is classified as an agar diffusion method because the extract being tested diffuses from its reservoir over agar medium (Fiebelkorn *et al.*, 2003). The diameter of the inhibition zone depicts individual compounds' antibacterial efficacy. Disk diffusion is one of the earliest methods of antimicrobial susceptibility testing and is still used in clinical laboratories today. It may be used to evaluate a wide range of items (Jorgensen & Turnidge, 2015).

Shellfish processing by-products might be a useful source of bioactive peptides, allowing for the development of a low-cost, long-lasting, and multifunctional component for use in fortified foods. The goal of this study is to determine whether waste products from the shellfish processing industry have the ability to extract valuable proteins as well as to generate natural, sustainable bioactive peptides.

2. MATERIALS AND METHODS

The shellfish by-products/waste from which the proteins were extracted was kindly provided by Sofrimar Ltd. Co Wexford. The by-product obtained is mainly composed of whelk processing by-products. The solutions in the methods section were made from stock solution of Hydrochloric acid reagent grade, 37% (Sigma-Aldrich, USA) and Sodium hydroxide ACS reagent, ≥97.0%, pellets (Sigma-Aldrich, USA. For the SDS-PAGE, a gel fixating solution made up of 500mL of USP-grade 95% (v/v) ethanol (VWR, USA) , 300 mL of distilled water and 100 mL of reagent grade acetic acid (VWR, USA) and volume adjusted to 1000 mL with water; a staining solution of 0.4g of Coomassie blue R350 in 200 mL of 40% (v/v) HPLC grade methanol (VWR, USA) and 200mL of 20% (v/v) acetic acid, destaining solution; 500mL of HPLC- grade methanol to 300 mL of distilled water, 100 mL of reagent grade acetic acid with the adjusted volume of 1000mL with water. For the disk diffusion assay, a 50µg/mL of ampicillin (Sigma-Aldrich, USA) in EtOH was prepared and Nutrient agar (Lab M Ltd, United Kingdom) petri dishes were prepared according to the manufacturer, the strains *Escherichia coli* ATCC 25922 (Lab M Ltd, United Kingdom) and Staphylococcus aureus ATCC 25923 (Lab M Ltd, United Kingdom) were used to test the hydrolysates antibacterial properties, these were stored in TSB broth (tryptic soy broth) (Lab M Ltd, United Kingdom) until use. For the DPPH assay, a 0.2mM solution of DPPH (Sigma-Aldrich, USA) was prepared in MeOH (VWR, USA).

2.1 Protein and peptide qualification

2.1.1 Fourier-transform infrared spectroscopy (FTIR)

To observe the type of amino acids that constitute our proteins, was performed on lyophilized protein samples (SP INDUSTRIES, USA). The IR spectra were acquired with an infrared spectrophotometer (PerkinElmer, model SPECTRA 65) in the 650–4000 cm^{-1} wave range.

2.1.2 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Protein sizes were extimated using SDS-PAGE, for this an electrophoresis chamber (Bio-Rad, USA) and Mini-PROTEAN 4-20% Precast Gels (Bio-Rad, USA), were used according to the protocol provided by the manufacturer. The protein samples were denatured in a water bath at 100ºC for 5 minutes. The gel had two wells loaded with 5µL of weight marker in addition with the loading buffer (Bio-Rad, USA) and the remaining wells were loaded with 5µL of each protein sample along with 5µL of loading buffer. The gel had a running time of 30 minutes at 200V, subsequently the gel was de-casted and incubated in a fixating solution

for 10 minutes, and then incubated in a Staining Solution overnight. After staining the gel was washed with the destaining solution until the bands were visible.

2.2 Bioactivity Assays

2.2.1 Disk-diffusion test

The strains *Escherichia coli* ATCC 25922 (Lab M Ltd, United Kingdom) and *Staphylococcus aureus* ATCC 25923 (Lab M Ltd, United Kingdom) were used to test the hydrolysates antibacterial properties. The disk-diffusion methodology was performed according to the NCCLS instructions (Thornsberry, 1983). With the aid of a bacteriological loop, some bacterial colonies were collected, with the same morphology as the fresh culture plate, and suspended in 5ml of TSB broth (in order to obtain a turbidity corresponding to 0.5 of the McFarland scale, a spectrophotometer (UV 1800 Shimadzu, USA)) was used to measure absorbance at 625nm. Mueller-Hinton agar (Lab M Ltd, United Kingdom) plates were prepared following the manufacturer's instructions. After homogenization of the inoculum, a sterile swab was introduced into the tube, Inoculation was made in the form of streaks on the agar surface in three directions, rotating the plate at an angle of 60° after each streak. Discs submerged with the sample solutions: intact protein (A, N and B), alcalase and α-Chymotrypsin hydrolyzed protein (A, N and B) were prepared, 3 replicas of each sample were tested. Ampicillin (50µg/mL) was used as positive control and distilled water was used as negative control. The disks were submersed in the sample and control solutions, following that, the application of the disks was done with the aid of sterile tweezers to avoid contamination. All discs were gently pressed into full contact with the agar surface. The plates were left in an incubator at 37ºC overnight. Subsequently, the halos of each sample were measured.

2.2.2 Ferric reducing ability of plasma (FRAP) antioxidant capacity assay

The FRAP assay was performed using the MAK369-1KT kit (Sigma, USA). The procedure was performed according to the manufacturer's instructions. Three replicas were performed for each sample. After the samples was added into each microplate's well, the assay buffer and the working solution were subsequently added, and then incubated at 37ºC for 1h. After this the FRAP probe solution was added to the wells and the absorbance was measured at 590nm wavelength in a spectrophotometric plate reader (VersaMax ELISA, USA).

2.2.3 ACE inhibition assay

The ACE inhibition test was done using the "ACE Kit-WST™" (Dojindo, Japan). The procedure was performed according to the manufacturer's instructions. Three replicas were performed for each sample. After each sample was added into the well, the substrate buffer and the enzyme working solution were also added and then incubated at 37ºC for 1h. After this the indicator working solution was added to the wells and incubated at room temperature for 10 min. Subsequently, the absorbance was measured at 450nm wavelength in a spectrophotometric plate reader (VersaMax ELISA, USA).

2.3 Statistical Analysis

The results from the FRAP and ACE inhibition assay and antimicrobial test were tested using one-way ANOVA and the Tukey post-hoc test using Excel 2019 (Microsoft office, Microsoft, USA).

3. RESULTS AND DISCUSSION

3.1 Reverse-Phase High Performance Liquid Cromatography (RP-HPLC)

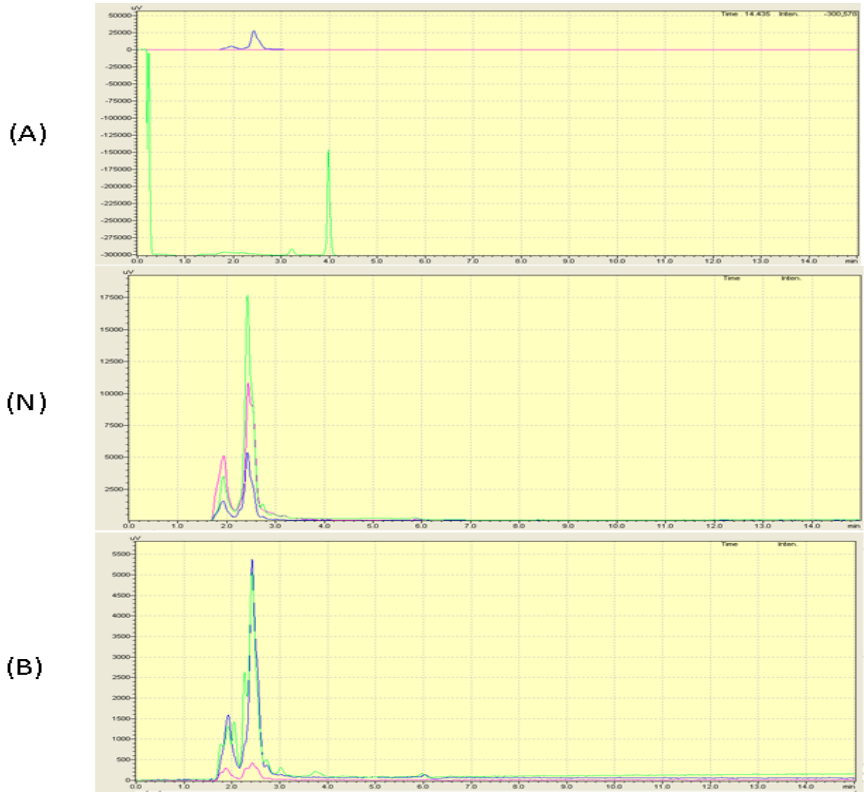

Figure 1: RP-HPLC graphical results of the extracted ang hydrolysed protein fror acid (A), neutral (N) and basic (B). The green, pink and blue lines correspond to the intact protein, α-chymotrypsin, and alacalase hydrolysed prtoein, respectivley, at a 280nm absorption.

Proteolytic enzymes like alcalase and α-chymotrypsin hydrolyze proteins and often target particular peptide cleavage bonds, resulting in the digestion of amino acids and peptides of varied sizes (Bender & Kezdy, 1964; Tacias-Pascacio *et al.*, 2020). Proteins hydrolysed by proteolytic enzymes vary in terms of deamidation, surface hydrophobicity, and molar mass which will result in different RP-HPLC profiles from protein to hydrolysate (Silvestre *et al.*, 2012).

Figure 1 shows the peptide and free amino acid contents of the protein hydrolysates, where a substantial difference in the peptide and proteins RP-HPLC profile can be seen. Alcalase acid extracted protein's hydrolysates show a small peak in the hydrophilic phase while α-chymotrypsin shows now peaks in the spectrum. . Neutral and basic protein and hydrolysates display identical peaks at various intensities, with non-hydrolyzed protein peaks having the maximum intensity. For neutral proteins samples, α-chymotrypsin peaks are more intense than alcalase peaks, and for basic samples, the opposite is true. Hydrolysis induces significant structural changes in protein, most notably in the amino acid sequence, molecular weight profile, and protein shape, as seen by the RP-HPLC profiles of the protein and related hydrolysates.

3.1 Fourier-transform infrared spectroscopy (FTIR)

Extracted and hydrolyzed protein samples infrared spectra is presented in Figure 1.

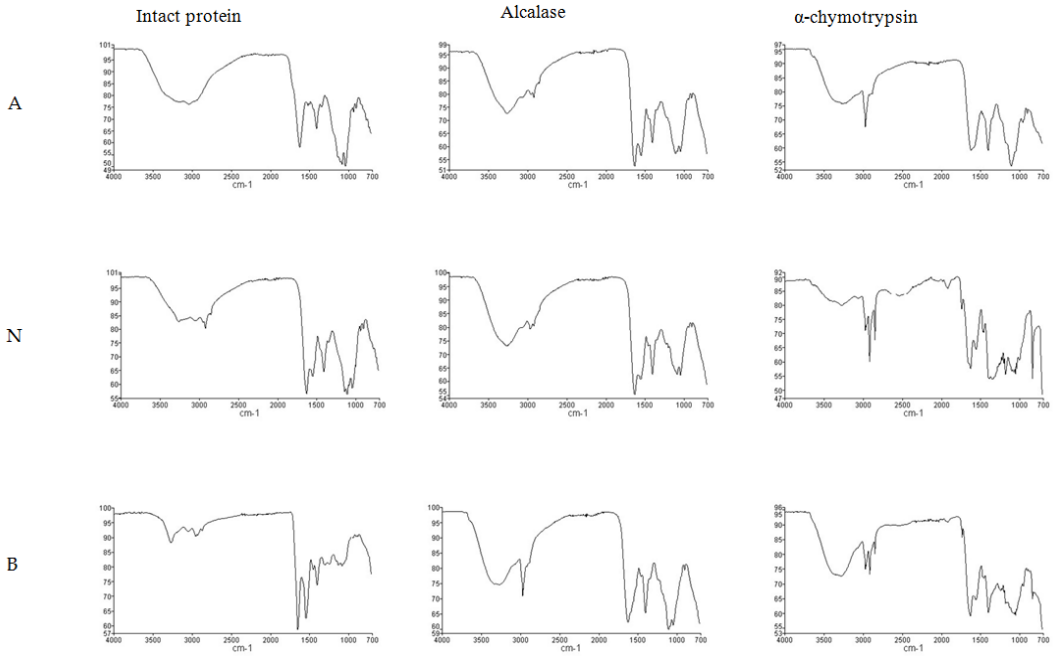

Figure 2: Fourier-transform infrared spectroscopy results for Extracted and hydrolyzed protein samples, for all three categories> acid, neutral and basic (A, N and B).

All three protein and hydrolysate show peaks fingerprint region, this region is defined as the area between 400 cm^{-1} and 1500 cm^{-1}. It usually has a lot of peaks, which makes identifying individual peaks challenging. In the functional group region (4000 cm^{-1} to 1450 cm^{-1}) all extracted proteins and hydrolysates show peaks at 1668-1687 cm^{-1}, which is characteristic of C=O bond(Barth, 2007). The basic and neutral protein spectrum show faint peaks in the primary and secondary amine stretch (3500-3100 cm^{1}). The α-chymotrypsin hydrolysates all show significantly stronger peaks primary and secondary amine stretch than their non/hydrolyses counterparts. α-Chymotrypsin hydrolyzed protein show also faint aromatic overtones (a series of small peaks, usually three or four, typically found in the ~2000 cm^{-1} to ~1700 cm^{-1} range) caused by overtones of the benzene ring vibrational modes α-Chymotrypsin is a digestive enzyme produced in the pancreas that aids in the breakdown of proteins and polypeptides, a process known as proteolysis(Bender & Kezdy, 1964). Chymotrypsin prefers aromatic residues such as phenylalanine, tyrosine, and tryptophan, although it may also hydrolyze other peptide bonds at a slower rate(Bender *et al.*, 1967). Hydrolysis with Chymotrypsin may have released aromatic amino acid residues that may be responsible for the aromatic overtones seen in the spectrum. α-Chymotrypsin samples also show stronger peaks in the sp3 alkanes stretch (3000-2850 cm-1) and alkene stretch (300-3000 cm-1), than the non-hydrolyzed extracted protein, that may be due to the fact that peptide alkane and alkene vibrational stretches might be stronger than those in a whole protein molecule. The same peak strength increase of the sp3 alkanes and alkene stretches can be observed from the intact extracted protein to the alcalase hydrolyzed, although not as strong and evident to the α-Chymotrypsin ones.

Alcalase is a serine type endo-protease that can hydrolyze most of the peptide bonds within a molecule. In the active site of serine proteases, a serine group is required for substrate binding and cleavage. Serine proteases have a broad substrate specificity, and their activity includes esterase and amidase activities in addition to peptidase activity. A catalytic core including serine as a nucleophile, aspartate as an electrophile, and histidine as a base is the most prevalent reaction mechanism. Through acylation, a covalently bonded enzyme substrate intermediate is formed, which results in the loss of the matching amino acid or peptide fragment (Tacias-Pascacio *et al.*, 2020).

3.2 SDS-PAGE – Hydrolysis

Figure 2 shows the SDS-PAGE gel run with the unprocessed extracted protein samples as well as the alcalase and α-Chymotrypsin hydrolyzed samples.

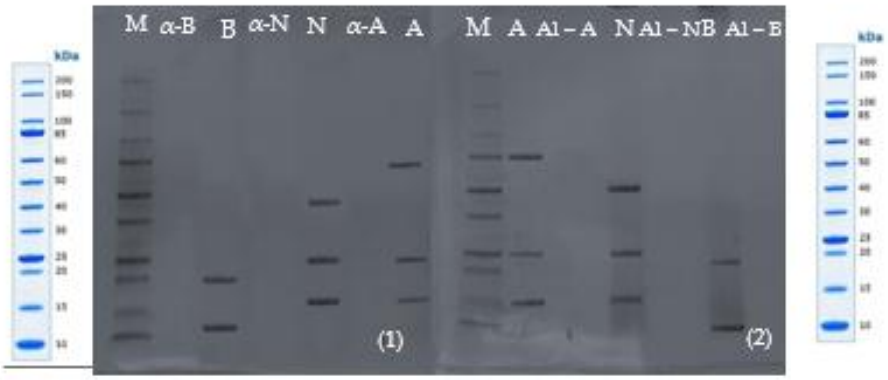

Figure 3: SDS-PAGE gel of the extracted protein samples and corresponding hydrolysis with α-Chymotrypsin (1) and Alcalase (2). The bands in each well correspond to the weight marker (M), the intact protein acid, neutral and basic (A, N and B) the acid, neutral and basic hydrolyzed protein with α-Chymotrypsin (α-A,α-N and α-B) and Alcalase (al-A, Al-N and Al-B).

Overall, the sizes of unprocessed proteins range from 85 to 15 kDa, with the largest belonging to acid proteins and the smallest to basic proteins. The hydrolysates do not appear in the gel. This may be due to the fact the peptide molecules being too small for the gel., i.e., less than 10 kDa.

As mentioned in the introduction, whelk's overall composition is structural proteins, manly collagen. Although collagen is practically insoluble in water, it can be made more soluble by lowering the pH of the solution, taking this into consideration it is plausible to assume that there is collagen proteins dissolved in the acid protein extraction samples, these are also shown to have the molecules with the highest molecular weight (León-López *et al.*, 2019).

Because of its low molecular weight (MW), which ranges between 3 and 6 KDa, hydrolyzed collagen would not show a band in the SDS-PAGE gel.

3.3 Antibacterial Activity

Table 1 shows the results of antibacterial activity of all hydrolyzed and non-hydrolyzed samples for the *e.coli* and *s.aureus* strains.

Table II: Radius of antibacterial activity halo of intact and hydrolyzed protein for all three categories: acid (A), neutral (N) and basic (B). Ampicillin was used as a positive control and distilled water was used as a negative control.

	Bacterial strain	
Antibacterial agent (20 µg/mL)	***E. coli***	***S. areus***
Intact Protein		
A	-	-
N	-	-
B	-	-
Alcalase Hydrolysed		
A	-	-
N	-	-
B	++	-
α-Chymotrypsin Hydrolysed		
A	+++	+++
N	++	++
B	+	++
Ampicillin (5µg/mL)	+++	+++
dH20	-	-
Inhibition zone: +++: 6-7 mm; ++: 5-6 mm; +4-5 mm		

All α-Chymotrypsin peptides samples showed some inhibition of bacterial growth, perhaps, Chymotrypsin preference towards aromatic residues, produces peptides with similar molecular structures to the carboxylic acid and ketone present in ampicillin that interact with β-lactam. The only Alcalase sample that showed any kind of inhibition of bacterial growth was the basic alcalase peptides which, coincidentally, is the sample with the highest DH. Intact protein shows no inhibition of bacterial growth, thus hydrolysis with α-Chymotrypsin does significantly increase bacterial growth inhibition. The majority of antimicrobial peptides have a significant proportion of hydrophobic residues (up to 50%). As proved by model peptides versus target membranes, increasing peptide's hydrophobicity, which are measured by the percentage of hydrophobic amino acids, leads to higher bacterial membrane permeability (Cheng *et al.*, 2013). α-Chymotrypsin hydrolyses the C-terminal side of aromatic amino hydrophobic acids like phenylalanine and tryptophan, that can explain why its peptides all show some sort of activity.

In Guillén *et al.*, 2013 (Guillén *et al.*, 2010) the same technique was used to test Tuna and quid alcalase hydrolysed gelatine's antibacterial properties, for *e. coli* and *s. areus* both had halos around 0.25cm. In Wald *et al.*, (2016)(Wald *et al.*, 2016) pepsin hydrolyzed trout extracted protein was shown to have an inhibition halo between 0.8–1.5 cm for *e. coli*.

These results show significantly less antibacterial activity than the studies cited above, although not powerful bacterial growth inhibitors in conjunction with the other bioactivities the peptides exhibit, if incorporated in a food product the small antibacterial activity is a positive attribute as it will aid in preservation.

3.4 FRAP Antioxidant Activity

The average IC50 values ferric reducing activity of non-hydrolyzed and hydrolyzed proteins are shown in Figure 4.

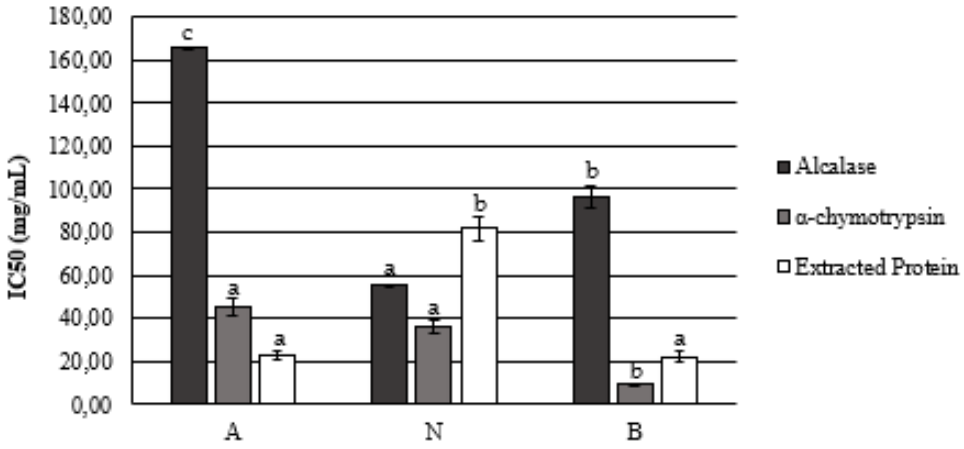

Figure 4: IC50 for FRAP antioxidant activity of non-processed protein (Extracted Protein) and alcalase and α-Chymotrypsin hydrolyzed protein for all three categories: acid (A), neutral (N) and basic (B). Values represent averages of n=3. For each category (extracted protein, alcalase and α-chymotrypsin) different superscript letters are significantly different (P<0.05).

The Ferric Reducing Antioxidant Powe (FRAP) assay is based on the production of the Fe^{2+} (ferrous state) from the reduction of the Fe^{3+} ion (ferric state) present in the 2,4,6-tripyridyl-s-triazine (TPTZ). When this reduction occurs, there is a change of color in the solution to an intense blue, whose absorbance intense blue, whose absorbance can be measured at 595 nm wavelength (Benzie & Strain, 1996). It is worth to note that this particular reaction is biologically relevant since the reaction of giving off electron in order to reduce Fe (III) is similar to the quenching of free radical in the body, based on the supposition that water soluble antioxidant molecule's ability to reduce ferric ions translates into their capacity of ROS reduction (Benzie, et al. 2017).

The A, N and B non-hydrolyzed protein showed an IC50 of ferric reducing ability of 76.91± 6.30 mg/mL, 91.82±7.66 mg/mL and 106.51±1.04 mg/mL respectively. Alcalase acid hydrolysates showed to have significantly lower FRAP antioxidant activity then the other samples with an IC50 of 165.89± 0.22mg/mL. Chymotrypsin hydrolysates showed better antioxidant activities then then the hydrolysates generated with alcalase with IC50 of 45.04± 4.11 mg/mL, 36.08± 2.91 mg/mL and 9.79± 0.19 mg/mL for A, N and B protein, respectively. All samples had much higher IC50 values for ferric reducing ability when comparing to the IC50 for the DPPH activity studied in Varela *et al.*, (2021). In the DPPH technique, the ability to contribute an electron or a hydrogen radical to the stable DPPH free radical is being tested, while the FRAP technique assesses antioxidants based on their capacity to donate an electron to reduce ferric (Fe^{3+}) to ferrous (Fe^{2+}) ions (Thaipong *et al.*, 2006). These are different types of antioxidant capacity and even though *in vitro*, the DPPH reducing ability of the peptides and proteins appears to be higher, although, *in vitro* studies could potentially show the samples having an increase in ferric reducing activity when comparing to *in vitro* ones.

Table III: Ferric-reducing antioxidant power (FRAP) of intact and hydrolyzed protein. FRAP (mM Ferrous equivalents) of samples.

Ferric reducing agent (2 mg/mL)	Ferric Reducing Antioxidant Power (nM)
Intact Protein	
A	5.133
N	1.091
B	5.212
Alcalase Hydrolysed	
A	0.147
N	2.066
B	1.056
α-Chymotrypsin Hydrolysed	
A	3.166
N	6.118
B	10.294

The sample's ferric reducing antioxidant power was calculated by multiplying the ferrous ammonium sulphate amount from standard curve to the sample dilution factor and dividing that by the sample volume added into the reaction well, the result is in nmol/µL or mM Fe^{2+} equivalents. The FRAP assay was used in other studies studying the antioxidant capacity of protein hydrolysates. Alcalase-hydrolysate prepared from sheep plasma showed 59.8 ± 0.10 mM $FeSO_4$ equivalent per g of sample (Hou *et al.*, 2019), alcalase hydrolyzed flaxseed

protein showed 93.0 ± 0.04 nmol/µL (Silva *et al.*, 2017). Alcalase-hydrolyzed Soybean protein showed FRAP activity from 54.7 ± 1.2 to 79.0 ± 0.6 mM Fe^{2+}/µM (Zhang *et al.*, 2019). Alcalase hydrolysed rice bran (Thamnarathip *et al.*, 2016) and stone fish (Bordbar *et al.*, 2018) protein were shown to have 139 ± 6.44 mM Fe^{2+}/µM and 39.18 ± 0.6 mM Fe^{2+}/µM, respectively. Protein hydrolysates from shrimp head waste showed 8.21 µM Fe (II)/g of sample (da Rocha *et al.*, 2018), dried squid head protein hydrolysed with Alcalase 60.20 mM Fe^{2+}/µM (Sukkhown *et al.*, 2021) and red tilapia scales showed 13.1 ± 0.7 mM Fe^{2+}/µM (Sierra-Lopera & Zapata-Montoya, 2021). Studies performed with α-chymotrypsin showed 25.00 mM Fe2+/µM for Camel milk casein powder (Kumar *et al.*, 2016)and 0.66 ± 0.02 mM Fe^{2+}/µM for enzymatic-hydrolyzed proteins of dromedary (Camelus dromedarius) colostrum (Oussaief *et al.*, 2020). The ferric reducing antioxidant power of all samples are significantly lower than the other peptides used in different studies in which the same enzymes, but different sources of protein was used.

3.5 ACE inhibitory activity

The ACE inhibitory activity of the samples was determined with a colorimetric assay that detects the quantity of 3-hydroxybutyric acid (3HB) generated from 3-hydroxybutyryl–Gly–Gly–Gly by ACE (Figure 5).

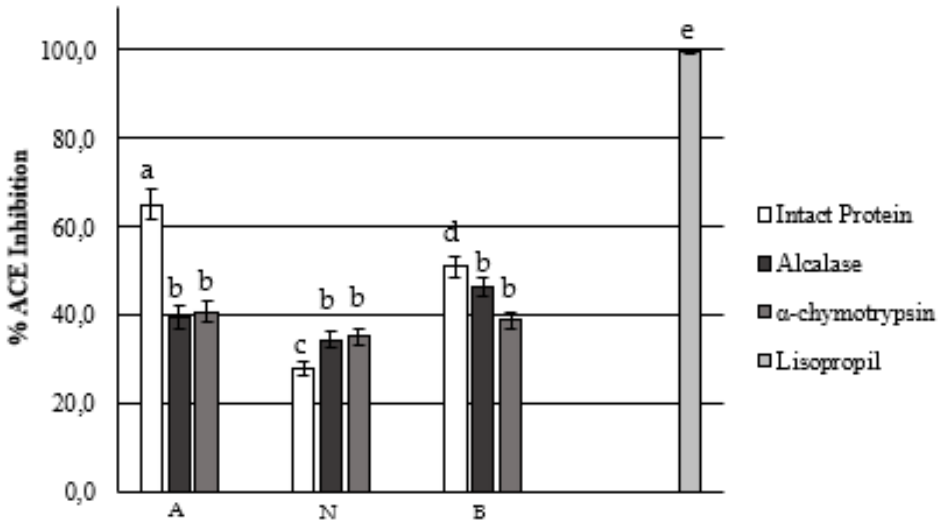

Figure 5: ACE activity at 5mg/mL of non-processed protein (Extracted Protein) and alcalase and α-Chymotrypsin hydrolyzed protein for all three categories: acid (A), neutral (N) and basic (B). Lisinopril 5µg/mL was used as a positive control. Values represent averages of n=3±sd. For each sample type (extracted protein, alcalase and α-chymotrypsin) different superscript letters are significantly different (P<0.05).

The IC50 values of inhibition of the ACE enzyme were calculated using the percentage of inhibition values of the assay at the concentration of 5µg/mL (Figure 6).

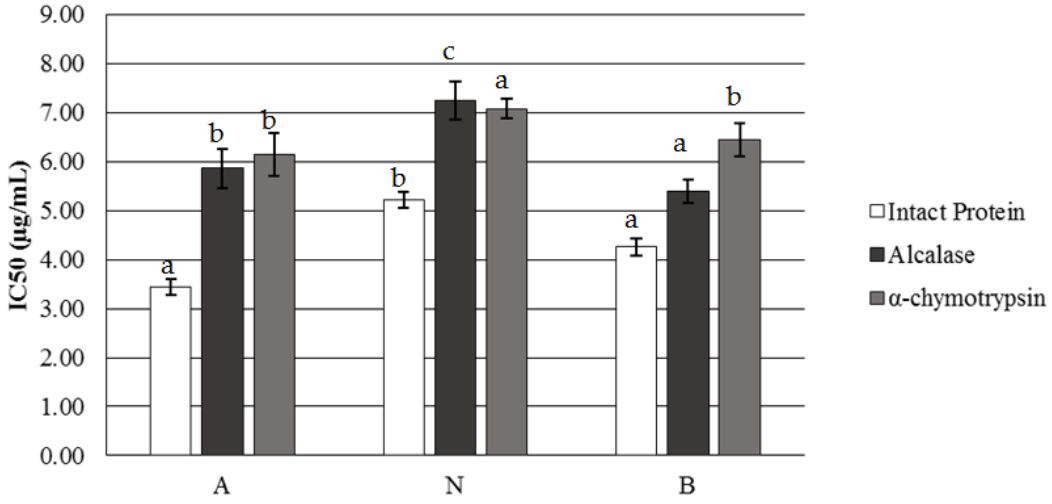

Figure 6: IC50 for ACE inhibitory activity of non-processed protein (Extracted Protein) and alcalase and α-Chymotrypsin hydrolyzed protein for all three categories: acid (A), neutral (N) and basic (B). Values represent averages of n=3. For each sample type (extracted protein, alcalase and α-chymotrypsin) different superscript letters are significantly different (P<0.05).

The *in vitro* ACE inhibitory efficacy is expressed in % inhibition of ACE in an *in vitro* experiment, as illustrated in Fig. 4. In this case the non-hydrolyzed protein shows a higher degree of activity than the hydrolyzed protein in comparison with the other bio-activity assays. The percentage of inhibition of alcalase and α-Chymotrypsin peptides at 5mg/mL were not significantly different from each other, however all three intact protein samples were considerably different. When performing the assay, a buffer (pH≥5) was used that could affect protein configuration in the neutral and basic non-processed extracts. Using the percentage of ACE inhibitory activity, the IC50 for each sample was calculated as the concentration required to inhibit 50% of ACE activity. The results as illustrated in Fig. 5 show that the lowest IC50 value belongs to the acid extracted protein at 3,44µg/mL followed by basic and neutral protein with 5.22µg/mL and 4,.6µg/mL respectively. Alcalase and α-Chymotrypsin IC50's fall between 5.00 µg/mL and 7.00 µg/mL. Lisinopril, a known potent ACE inhibitor, IC50 value is 2.27E-03µg/mL (Udupa & Rao, 1998)

In Byun & Kim, (2001) ACE inhibitory activity was tested for hydrolysates from protein extracted from Alaska pollack skin, alcalase and α-Chymotrypsin peptides were found to

have IC50's of 759µg/ml and 5375µg/mL, respectively. However, it was found that ACE inhibitory activity increased when using hydrolysates prepared by the hydrolysis combination of two proteases. When combining alcalase and α-Chymotrypsin for the hydrolysis of Alaska pollack skin extracted protein the resulting peptides showed an IC50 of 0.952µg/mL (Byun & Kim, 2001). These results contrast with ours since they imply higher degree of hydrolysis of protein increases ACE inhibition while our results imply the opposite. In Tiengo *et al.*, (2009), alcalase hydrolyzed Amaranth extracted protein IC50 was almost 100 times smaller than the non-hydrolyzed protein. Peptides with dicarboxylic amino acids at the N-terminal position and branched-chain amino acid residues, such as valine and isoleucine, are favoured ACE inhibitors(Tiengo *et al.*, 2009). In Jung *et al.*, (2006), the IC50 of α-Chymotrypsin hydrolyzed protein extracted from yellowfin sole (*Limanda aspera*) was determined to be 883µg/mL. Both alcalase and α-Chymotrypsin's peptide IC50's, although higher than non-processed protein, showed significantly lower IC50s from peptides generated by the same enzymes reported from different protein sources.

4. CONCLUSIONS

The protein characterization results (SDS-PAGE and FTIR), show that proteins of different degrees of solubility (hydrophobic and hydrophilic) are present in the extract as well as different sizes. Most samples didn't show any kind of bacterial growth inhibition except α-Chymotrypsin and basic alcalase hydrolyzed, which also happens to have the highest DH, therefore smaller peptides. Chymotrypsin prefers aromatic residues, resulting in peptides that have comparable molecular structures to the carboxylic acid and ketone found in ampicillin. Although not powerful bacterial growth inhibitors in conjunction with the other bioactivities the peptides exhibit, the minimal antibacterial activity is a favorable quality in a food product since it aids in its preservation.

When compared to the IC50 for the DPPH activity studied previously in Varela et al., (2021), all samples had substantially IC50s for ferric reducing ability than DPPH scavenging ability. These are different types of antioxidant capacity. Even though the antioxidant power tested using the FRAP's reaction is comparable to the body's quenching of free radicals, in vivo testing could reveal that the samples have a higher ferric reduction activity. The ACE results reported in other studies with different fish/shellfish protein sources contradict ours since they imply that a larger degree of protein hydrolysis promotes ACE inhibition, but our findings suggest the contrary. Although greater than non-processed protein, the peptide IC50s

of alcalase and α-Chymotrypsin revealed considerably lower IC50s from peptides synthesized by the same enzymes reported from various protein sources. Regardless, the activity is substantially good for food product incorporation.

To the best of our knowledge there is no reported literature studying bioactive properties of whelk by-products hydrolyzed extracted protein. These by-products show potential to be a new generation of multifunctional bio active peptides that can be valuable to the industry as novel and sustainable ingredients for the food industry as supplement/fortified foods and nutraceuticals. Further studies need to be completed to assess the antioxidant capacity of these hydrolysates using other in vitro antioxidant assays, as well as *in vitro* studies to assess the bio availability of these peptides. Furthermore, there is potential for other bio activities linked with the hydrolysates generated in the current study.

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