



Assessment of the degree of hydrolysis of novel and conventional feed-stuffs for potential inclusion in *Octopus bimaculatus* aquafeeds

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Abstract: The California two-spot octopus, *Octopus bimaculatus*, stands out as a potential species for aquaculture; one of the main challenges in undertaking its culture is the formulation of aquafeeds that guarantee survival and optimal growth under controlled conditions. This study aimed at assessing the *in vitro* degree of hydrolysis of various feedstuffs using crude extracts from different sections of its digestive tract. The highest degree of hydrolysis exerted by acidic proteases from stomach and digestive gland was recorded for black soldier fly larvae meal (BS-FLM), followed by single-cell protein (SCP) bacterial meal, and fishmeal (FM), with 61.14, 60.91, and 60.91%, respectively; significantly lower values were recorded for *Arthrospira* sp. meal, krill meal, soy protein isolate, SCP yeast meal, and casein (58.11-59.34%). Thus, BSFLM and SCP bacterial meal can be considered novel and auspicious protein sources for the development of an aquafeed targeted for *O. bimaculatus*; *in vivo* evaluation is advised.

Keywords: Octopus; cleavage percentage; ingredients; formulated feeds; proteases.

Evaluación del grado de hidrólisis de ingredientes nuevos y convencionales para su potencial inclusión en alimentos acuícolas para *Octopus bimaculatus*. Resumen: El pulpo de dos manchas, *Octopus bimaculatus*, sobresale como una especie potencial en acuicultura; uno de los principales retos para su cultivo es la formulación de alimentos acuícolas que garanticen su sobrevivencia y crecimiento óptimo bajo condiciones controladas. Este estudio evaluó el grado de hidrólisis *in vitro* de varios ingredientes usando extractos crudos de diferentes secciones de su tracto digestivo. El mayor grado de hidrólisis fue ejercido por proteasas ácidas del estómago y glándula digestiva en harina de larva de mosca soldado negro (BSFLM), seguido de proteína unicelular (SCP) bacteriana y harina de pescado (FM) con 61.14, 60.91, y 60.91%, respectivamente; valores significativamente menores fueron registrados para la harina de *Arthrospira* sp., harina de krill, aislado de proteína de soya, SCP de levadura y caseína (58.11-59.34%). De modo que la BSFLM y la SCP bacteriana pueden considerarse fuentes de proteína nuevas y favorables para desarrollar un alimento acuícola para *O. bimaculatus*; la evaluación *in vivo* se recomienda como el siguiente paso necesario.

Palabras clave: Pulpo; porcentaje de escisión; ingredientes; alimentos formulados; proteasas.

Introduction

Cephalopods are of great commercial importance worldwide; the group includes about 800 species from which close to 300 belong to the order

Octopoda, with over 20 octopus' species having established commercial fisheries predominantly in countries like China, India, Morocco, and Peru (Arkhipkin *et al.* 2021). The largest octopus import

markets are in China, the European Union (Italy and Spain), Japan, and the Republic of Korea. In 2020, the significant increase in the demand for cephalopods led to exports valued at \$10.2 billion USD, representing 6.8% of the total aquatic product exports (FAO 2022). In Mexico, octopus catches have been inconsistent in size; in the last 10 years the lowest capture recorded was 25,416 t in 2020, while the largest, 56,754 t, was recorded in 2018. Generally, two states in the south, Yucatan and Campeche, contribute the largest volumes of these captures, followed by the northern states of Baja California Sur and Sonora (CONAPESCA 2023); thus, the limited supply to satisfy the increasing demand has driven the increased market prices in Mexico and around the world (FAO 2022).

Given their commercial importance, the interest in developing the commercial culture of octopus has expanded around the world (Bastos *et al.* 2020), and Mexico is no exception. The California two-spot octopus, *Octopus bimaculatus*, is a native species from the Gulf of California, whose commercial capture is local and permitted year-round (Marine Species Portal 2023). This species inhabits rocky reef habitats, reef flats, and sandy bottoms along the coasts of California, USA, to the state of Sinaloa, Mexico. The lifespan is estimated to be between 13 and 24 months, with the highest recorded adult weight being 3,400 g (average ~3.0 kg), larger than *O. bimaculoides* (~0.8 kg) in terms of weight and egg size (Jereb *et al.* 2016), which could represent an advantage for its culture. *O. bimaculatus* individuals reproduce only once in their lifetime, producing a large number (>20,000) of small eggs (4-7 mm) that develop into planktonic paralarvae, and right after hatching the mother dies (Hofmeister & Voss 2024). *O. bimaculatus* stands out as a promising aquaculture species because of the high market value in the regional market, its nutritional quality, and exquisite meat flavor. Hence, gathering information on biological aspects such as nutritional requirements, digestive physiology, and reproduction, among others, needs to be prioritized to achieve its successful culture.

Octopuses possess efficient proteases in their digestive tract that hydrolyze different sources of protein on account of their distinctive carnivorous feeding habits. To successfully transition cultured octopuses from live feed to a balanced diet, as has been achieved for *O. maya* (Martínez *et al.* 2014), studies focused in understanding their capacity to efficiently use specific feedstuffs that could be used in practical feed formulations are required. Evidence

suggests that the predominant digestive enzymes in octopuses possess higher activity in acidic pH environments, and that digestion is mainly carried out by aspartic proteases such as cathepsin D, which has been proposed as the most important enzyme in the digestive tract of *O. maya* (Martínez *et al.* 2011; Andrews *et al.* 2022). Proteases such as trypsin and chymotrypsin, generally show optimal activity in an alkaline pH range in vertebrates, but are also important contributors to protein digestion in octopuses and have been studied in species like *O. vulgaris* (Villanueva *et al.* 2002), *O. bimaculoides* (Solorzano *et al.* 2009; Ibarra-García *et al.* 2018), *O. maya* (Santiago *et al.* 2024), as well as in the paralarvae of *O. bimaculatus* (López-Peraza *et al.* 2018), for which enriched *Artemia franciscana* served as the primary food source. Although our understanding of the digestive physiology of *O. bimaculatus* remains limited, the afore mentioned study demonstrated acidic and alkaline proteolytic activities from the time of hatching, which increased with age; however, low survival rates limited the length of that study, lasting only 17 days in the group fed tuna-orbit-oil enriched *A. franciscana* (1.93% survival), and 11 days in the non-enriched control group (1.77% survival).

Given these considerations, an initial, time-efficient approach to estimating the digestibility of formulated diets or feed ingredients is the *in vitro* assessment of the degree of hydrolysis, which reflects the percentage of cleaved peptide bonds during protein hydrolysis. This value is influenced by the amino acid profile of the protein source (Nielsen *et al.* 2001) and the characteristics of the enzyme system used, for instance, an extract containing digestive enzymes from a particular species. Extracts may be obtained from the complete digestive tract or from a specific section of the tract (e.g. digestive gland, intestine, stomach, or pyloric caeca), as digestive enzymes are typically synthesized by specific organs, glands or cells, either associated with or in specific regions of the digestive tract, where optimal conditions for their activity are provided.

Exploring alternative protein sources to fish-meal and identifying cost-effective and sustainable protein sources is a priority in aquaculture; consequently, several alternatives are actively being explored for the aquafeed industry, such as black soldier fly larvae meal (BSFLM), single-cell protein (SCP) meals from bacteria or yeast, microalgae meals, and soy protein concentrates, among others. In this context, this study aimed to generate knowledge on the ability of *O. bimaculatus* to utilize novel

and conventional feedstuffs by assessing the *in vitro* degree of hydrolysis, an initial step in evaluating their potential inclusion in aquafeeds for its culture. A supplementary objective was to estimate the molecular weight of specific proteases using conventional biochemical techniques such as sodium dodecyl sulfate-polyacrylamide gel electrophoresis and zymography, also as a preliminary approach.

Materials and methods

Collection of specimens: Ten wild *O. bimaculatus* specimens were caught by diving in the coastal zone of Kino Bay, Sonora, Mexico (28°48'59.99"N, 111°55'59.99"W) in October 2023. The organisms were immediately placed in iced seawater (≈8.0°C); once immobilization occurred, they were euthanized by an incision between the eyes, lethally piercing the brain; this was done in accordance with the Ethical and Welfare Considerations when Using Cephalopods as Experimental Animals (Moltschanivskyj *et al.* 2007). Animals were then transported in ice for optimal preservation to the Department of Scientific and Technological Research of the University of Sonora. On arrival, organisms were individually weighed; they were all adults (1.24-1.61 kg) in a non-reproductive stage. Subsequently, the stomach, pyloric caeca and digestive gland were dissected and kept at -20°C for 15 h, until the preparation of extracts.

***In vitro* degree of hydrolysis (DH):** Initially, a verification step to confirm the hydrolytic activity of enzyme extracts under different pH conditions was performed. Enzyme extracts from stomach (≈200 mg) combined with digestive gland (≈200 mg) from four different experimental organisms were evaluated under acidic hydrolytic conditions, whereas enzyme extracts from pyloric caeca (≈200 mg) combined with digestive gland (≈200 mg), also from four different experimental organisms, were evaluated under alkaline hydrolytic conditions. Each quadruplicate extract, along with a standard and a blank, was analyzed in duplicate. For the acidic hydrolytic conditions, the procedure by Anson (1938) for protease activity evaluation with a minor modification was followed; 20 µL of extract was combined with 1.5 mL of 0.5% hemoglobin and 0.5 mL glycine-HCl buffer of pH 2.0. Octopus stomach is generally less acidic (e.g., *O. vulgaris*: stomach pH is 3.0-4.0; Bastos *et al.* 2020), but pepsin-like enzymes and cathepsins show enzymatic activity *in vitro* at this pH. For the alkaline hydrolytic conditions, the method proposed by Kunitz (1947) and modified by Walter (1984) was followed; 20 µL of extract was

combined with 1.5 mL of 0.5% casein and 0.5 mL Tris-HCl 50 mM buffer of pH 8.5. Activity of alkaline proteases in octopus generally occurs at pH close to 6.0 (e.g., *O. maya* and *O. mimus*; Linares *et al.* 2015), but under *in vitro* conditions at this pH, enzymatic activity of serine proteases also shows adequate activity. Samples were incubated at 37°C in a water bath for 30 min, the reaction was then halted with 20% trichloroacetic acid (TCA), followed by centrifugation at 21,000 g for 15 min, then the absorbance was read in a UV/VIS spectrophotometer (Genesys 10S, Thermo Fisher Scientific, Madison, WI, USA) at 280 nm. Enzymatic activity was estimated as Activity (U mL⁻¹) = (*Δ absorbance ÷ total reaction volume) ÷ (coefficient of extinction molar × incubation time × extract volume) (Erlanger *et al.* 1961; Ásgeirsson & Bjarnason 1991; Anson 1938; *Δ absorbance = extract absorbance), with calibration curves for verification. Finally, the enzyme extracts were adjusted with the corresponding buffer to a concentration of 500 U mL⁻¹ of total proteolytic activity.

***In vitro* degree of hydrolysis of protein in the following feedstuffs** was then tested: microalgae meal from *Arthrospira* sp. (crude protein, CP: 65.6%; Alfa Delta S.A. de C.V., Naucalpan de Juárez, Estado de Mexico, Mexico), krill meal (CP: 65.6%; Aker BioMarine ASA, Lysaker, Norway), SCP bacterial meal (CP: 65.3%; MRD-Pro®; Meridian Biotech, Lexington, KY, USA), SCP yeast meal (CP: 56.9%; DY-Pro®; Meridian Biotech, Lexington, KY, USA), soy protein isolate (CP: 90.0%; Proce-sadora de Ingredientes, S.A. de C.V., Guadalajara, Jalisco, Mexico), defatted black soldier fly larvae meal (BSFLM) from *Hermetia illucens* (CP: 53%; Enterra, Langley, British Columbia, Canada), sardine fishmeal (FM; CP: 72.2%; Productos Pesqueros de Guaymas S.A. de C.V., Guaymas, Sonora, Mexico), and casein (CP: 80.0%; Sigma-Aldrich, St. Louis, MO, USA). The mean protein content of these feedstuffs was analyzed in duplicate samples by combustion (method 986.06; AOAC 2005) in a DUMAS Nitrogen Analyzer (Model 702 NDA, VELP Scientifica, Usmate, Italy).

Each feedstuff was subjected to independent analyses, beginning with the acid hydrolysis assay and followed by the alkaline assay, according to the procedure described by Nielsen *et al.* (2001) with minor modification. A 10 mL pepsin (P6887, Sigma-Aldrich, St. Louis, MO, USA) solution (0.1 mM) or a 10 mL solution (0.1 mM) of trypsin + chymotrypsin (T8003 + C4129, Sigma-Aldrich, St. Louis, MO, USA) in distilled water were used as

controls for the acid or the alkaline pH conditions, respectively. Samples for the acid condition assay were prepared by mixing 10 mL of the corresponding extract with 100 mL of glycine-HCl buffer 50 mM (pH 2.0) previously prepared that contained the powdered feedstuff at a concentration of 10 mg of protein mL⁻¹ kept in a water bath at 37°C for 90 min under constant agitation; this procedure was also used to produce the set of control samples, in which the enzyme extract was replaced by the control pepsin solution. Similarly, samples for the alkaline hydrolysis assay were prepared by combining 10 mL of the corresponding extract with 100 mL of 50 mM Tris-HCl (pH 8.5) containing the powdered feedstuff at 10 mg of protein mL⁻¹ kept in similar conditions as in the previous assay; for the set of control samples, the enzyme extract was replaced by the trypsin + chymotrypsin solution. A standard was prepared with a serine solution containing 50 mg serine (P7769, Sigma-Aldrich, St. Louis, MO, USA) diluted in 500 mL deionized water (0.9516 meqv/L), whereas distilled water was used as a blank. An ortho-phthalaldehyde (OPA) solution with 7.620 g of di-Na-tetraborate decahydrate and 200 mg of Na-dodecyl-sulfate (SDS) dissolved in 150 mL of deionized water was prepared; then, 160 mg of OPA ($\geq 99\%$, P0657, Sigma-Aldrich, St. Louis, MO, USA) were dissolved in 4 mL of ethanol and transferred to the above-mentioned solution by rinsing with deionized water. Next, 176 mg dithiothreitol (DTT: $\geq 99\%$, D9163, Sigma-Aldrich, St. Louis, MO, USA) were added to the solution and rinsed with deionized water; lastly, the solution was brought to 200 mL with deionized water. Absorbance was measured after mixing 750 μ L of OPA solution with either 100 μ L of the extract sample, 100 μ L of the control sample, 100 μ L of serine standard, or 100 μ L of distilled water (blank). Samples were vortexed for 5 s, and after 2 min of reaction, the absorbance of the extract samples, control samples, standards, and blanks were determined at 340 nm in a UV/VIS spectrophotometer (Genesys 10S, Thermo Fisher Scientific, Madison, WI, USA). Additionally, two samples of the serine standard were measured at the beginning and at end of the assay to verify the precision and consistency of the absorbance readings, though these measurements were not used in any calculation. The *in vitro* degree of hydrolysis (DH %; Adler-Nissen 1986) estimates the percentage of cleaved peptide bonds obtained from the ratio between the number of hydrolyzed bonds (h) and the total number of peptide bonds per protein equivalent (h_{tot}): $\text{DH (\%)} = h / h_{\text{tot}} \times 100$. Additional

calculations required are $\text{Serine-NH}_2 = (A_{340 \text{ sample}} - A_{340 \text{ blank}}) / (A_{340 \text{ serine}} - A_{340 \text{ blank}}) \times 0.9516 \text{ meqv/L} \times 0.1 \times 100 / X \times P$, where Serine-NH₂ = meqv serine NH₂/g protein; X = g of sample; P = % crude protein in sample; 0.1 is the sample volume (L). Then, $h = (\text{serine-NH}_2 - \beta) / \alpha \text{ meqv/g protein}$, where α and β for specific raw materials are provided by Nielsen *et al.* (2001). For fish, $\alpha = 1.00$ and $\beta = 0.40$, for casein $\alpha = 1.039$ and $\beta = 0.383$, etc. If a particular raw material has not been examined, then α and β are assessed as 1.00 and 0.40, respectively. Before statistical analysis, the absorbance of duplicate measurements from each quadruplicate extract samples, control samples, standards and blanks were averaged. **Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Zymography:** For the preparation of extracts containing cathepsin, among other enzymes, approximately 400 mg of each, stomach and digestive gland from ten individuals were independently processed using 50 mM Tris-HCl buffer (pH 7.5) with 150 mM NaCl, 1 mM CaCl₂, and 20 mM EDTA (Wilkesman *et al.* 2016). Cathepsins are active at acidic to neutral pH but are often extracted at pH 7.0-7.5 to maintain structural stability and prevent autolysis; this buffer is recommended to extract active cathepsins from tissue samples, minimizing the interference of other proteases and making it ideal for downstream applications like zymography or activity assays (Walker 2002; Creative Proteomics 2025). Trypsin and chymotrypsin, along with other enzymes, were obtained from nearly 400 mg of each, pyloric caeca and digestive gland from ten individuals, and were processed separately in 50 mM Tris-HCl buffer (pH 8.2) with 20 mM CaCl₂, a buffer commonly used as described or with minor modifications for extraction of alkaline proteases (Villanueva-Gutiérrez *et al.* 2020), including those of *O. maya* (Martínez *et al.* 2011). Samples were individually homogenized in 2.5 mL of the buffer at 4°C. The homogenates were centrifuged thrice at 4°C and 21,000 *g* for 30 min, and the supernatants collected and kept at -20°C for short-time storage, until further examination.

The molecular weights of cathepsin, trypsin, and chymotrypsin from *O. bimaculatus* were analyzed by SDS-PAGE using a 4-gel vertical electrophoresis system (Mini-Protean Tetra Cell, Bio-Rad®, Hercules, CA, USA). The extracts from the different sections of the digestive tract of ten individuals were run under similar conditions in at least eight different gels. A molecular weight standard (Precision Plus Protein Standard 161-0373, Bio-Rad®, Hercules, CA, USA) was employed for the de-

termination of molecular weights, while pepsin, trypsin and α -chymotrypsin were used as reference molecular markers. For cathepsin, a 4x sample loading buffer was prepared with 200 mM Tris-HCl pH 6.8, 4% SDS, 40% glycerol, and 0.02% bromophenol blue (Wilkesman *et al.* 2017), whereas for trypsin and chymotrypsin, a loading buffer with 124 mM Tris-HCl pH 7.5, 4% SDS, 50% glycerol, and 0.2% bromophenol blue was employed (Yasumitsu 2017), both mixed at a 1:1 ratio with the enzyme extracts; then, 20 μ L aliquots were loaded into 10% polyacrylamide gels and resolved in Tris/Glycine/SDS running buffer (#1610732, Bio-Rad Hercules, CA, USA) at 115 V for 2 h at 4°C. Gels were stained with QC Colloidal Coomassie stain (Bio-Rad®, Hercules, CA, USA) for 18 h, and destained by agitation in distilled water for 1 h. Gels were subsequently analyzed using a GS-900 densitometer and the Image Lab 5.0 software (Bio-Rad®, Hercules, CA, USA).

To discern which band corresponds to the specific enzyme, zymographic techniques were performed using the same extracts from ten individuals in at least eight different gels, under similar conditions. For cathepsin, 10% SDS-PAGE gels were copolymerized with 0.2% gelatin, in which 20 μ L aliquots were loaded and ran at 115 V for 2 h at 4°C and the same Tris/Glycine/SDS running buffer. After electrophoresis, gels were immersed in a renaturation buffer (50 mM Tris, 20% v/v glycerol, pH 7.4) with agitation for 40 min. Subsequently, the gels were submerged in an activation buffer (100 mM sodium acetate buffer pH 5.5, 1 mM EDTA, and 2 mM DTT) for 18 h at 37°C. This buffer optimizes the activity of cathepsins, whose maximal enzymatic activity occurs under mildly acidic (pH 5.0-6.0) and reducing conditions, as in *O. maya* (Martínez *et al.* 2011; Pineda-Suazo *et al.* 2024). DTT serves as a reducing agent to maintain the catalytic cysteine residue in its active thiolate form, while EDTA chelates divalent metal ions and inhibits metalloprotease activity, prevents metal-catalyzed oxidation reactions and interferes with substrate degradation. The conditions ensure proper refolding of the enzyme following electrophoresis and allow for sensitive and specific detection of cathepsin-mediated gelatinolytic activity (Platt *et al.* 2006, Platt 2017). Staining was carried out in a solution of 0.125% CBB-R250, 40% 2-propanol, and 10% acetic acid for 1 h, then washed with distilled water for processing (Platt *et al.* 2006). Trypsin and chymotrypsin zymograms consisted of 10% SDS-PAGE gels copolymerized with 2% casein from bovine milk (Sigma-Aldrich, St. Louis, MO, USA), ran under the same

electrophoretic conditions previously described. The resolved gels were kept in the renaturation buffer, consisting of 50 mM Tris-HCl pH 7.5, and 2.5% Triton X-100 under constant agitation for 40 min at room temperature; Triton X-100 (2.5%), a non-ionic detergent, removes residual SDS from the gel after electrophoresis, allowing the proteases to refold into their native conformation, whereas Tris-HCl at pH 7.5 provides the optimal environment for refolding and activity of serine proteases (Choi *et al.* 2005). Gels were then immersed in an incubation buffer (50 mM Tris-HCl pH 7.5, 1 mM CaCl_2) at room temperature with constant agitation for 10 min, followed by incubation at 37°C for 18 h. The staining procedure consisted of immersion in 100 mL of RAMA working solution (0.05% CBB, 10% acetic acid, 15% methanol, 3% ammonium sulfate) for 30 min, then washed with distilled water for processing (Yasumitsu 2017). Cathepsin, trypsin and chymotrypsin were evident in the zymograms by the contrast of a clear white band against the dark blue background; specifically for trypsin and chymotrypsin, the reference molecular markers helped elucidate the corresponding band.

Statistical analysis: One-way analysis of variance (ANOVA) with a significance level of $P < 0.05$ was used for the *in vitro* degree of hydrolysis data of the various protein sources analyzed, and of the control enzymes (pepsin, and trypsin + chymotrypsin), after verification of normality (Shapiro-Wilk's test) and homoscedasticity (Bartlett's test). In case significant differences were detected among feedstuffs, Tukey's-HSD test was used as the mean separation procedure. Molecular weight data obtained for the enzymes were analyzed using descriptive statistics. All statistical analyses were performed using the Statistical Analysis System (SAS Institute Inc. 2013, Software Release 9.4, Cary, NC, USA).

Results

Degree of hydrolysis: The *in vitro* degree of hydrolysis of combined stomach and digestive gland tissues from *O. bimaculatus*, evaluated under acidic conditions, showed significant statistical differences ($P < 0.0001$) among the different protein sources evaluated (Table I). The highest DH were recorded for BSFLM, SCP bacterial meal, and FM, with values of 61.14, 60.91, and 60.91%, respectively. The DH of feedstuffs carried under similar conditions but with pepsin showed numerically higher values, ranging from 99.61-99.90%, without significant differences among them ($P = 0.6953$). Under alkaline conditions, significant differences ($P < 0.0001$) were simi-

Table I. Degree of hydrolysis (DH, %) by acidic and alkaline proteases from different sections of the digestive tract of *O. bimaculatus* and from different control enzymes (pepsin and trypsin + chymotrypsin) over different novel and traditional protein sources.

Feedstuff	Stomach + digestive gland hydrolysis DH (%)	Pepsin DH (%)	Pyloric caeca + digestive gland hydrolysis DH (%)	Trypsin + chymotrypsin DH (%)
MRD-Pro®, SCP bacterial meal	60.91 ^a ± 0.27	99.90 ± 0.15	68.81 ^a ± 1.63	95.25 ± 0.32
Black soldier fly larvae meal	61.14 ^a ± 0.20	99.61 ± 0.50	59.27 ^b ± 0.45	95.63 ± 0.21
Fish meal (sardine)	60.91 ^a ± 0.27	99.90 ± 0.08	58.00 ^c ± 0.46	95.23 ± 0.39
<i>Arthrospira</i> sp. meal	59.17 ^b ± 0.11	99.67 ± 0.13	56.73 ^d ± 0.39	95.36 ± 0.23
Krill meal	59.30 ^b ± 0.12	99.69 ± 0.41	55.74 ^{de} ± 0.57	95.19 ± 0.79
Soy protein isolate	59.06 ^b ± 0.28	99.75 ± 0.33	56.14 ^{de} ± 0.56	95.38 ± 0.11
DY-Pro®, SCP yeast meal	59.34 ^b ± 0.35	99.75 ± 0.39	55.41 ^e ± 0.50	95.13 ± 0.42
Casein	58.11 ^c ± 0.06	99.79 ± 0.17	55.30 ^e ± 0.64	95.18 ± 0.34
ANOVA $P > F$	<0.0001	0.6953	<0.0001	0.7163

Values for DH are means ± standard deviation of four replicate samples; means under the same column with different superscripts are significantly different ($P \leq 0.05$).

larly observed for the DH of combined pyloric caeca and digestive gland, with SCP bacterial meal being the feedstuff with the highest DH, 68.81%, significantly higher than those of BSFLM (59.27%) and FM (58.00%), the feedstuffs with the next best DH values. The DH of the various feedstuffs evaluated with trypsin + chymotrypsin under alkaline conditions ranged between 95.13-95.63%, without statistical differences among them ($P = 0.7163$), which were also numerically higher values compared to those obtained with the extracts. On the other hand, casein showed the statistically lowest DH values in both assays with the enzyme extracts, under acidic (58.11%) and alkaline (58.00%) conditions (Table I). **SDS-PAGE and zymography:** Using conventional techniques like SDS-PAGE and zymography, the estimated molecular weights of cathepsin, trypsin, and chymotrypsin of *O. bimaculatus* were 37.6 ± 0.3 kDa (Fig. 1), 24.6 ± 0.6 , and 27.3 ± 0.6 kDa (Fig. 2), respectively. The presence of these enzymes was confirmed in the corresponding tissues and evidenced by the presence of clear bands in the blue-stained background of their respective zymograms, indicating proteolytic activity of the enzymes against their substrate. The white bands in the zymograms of each specific enzyme were aligned with the SDS-

PAGE gels; both sets of gels were run simultaneously under identical conditions in the 4-gel vertical electrophoresis system, allowing for specific matching of the band with the corresponding enzyme.

Discussion

Digestive proteases in aquatic organisms play a key role in the hydrolysis of dietary proteins; they are responsible for breaking peptide bonds in proteins, resulting in the production of small peptides or free amino acids (Villanueva-Gutiérrez *et al.* 2020). Proteases are essential for octopods because they are carnivores relying on highly efficient enzymes for protein digestion and assimilation. Protein is their main dietary macronutrient, and the metabolic substrate utilized for energy production, oxygen transport, osmoregulation, locomotion, and structural support (Lee 1995; López-Peraza *et al.* 2018); thus, a diet with considerable amounts of high-quality protein is required for proper development. As protein is generally the most expensive ingredient in aquafeeds, looking for cost-effective protein sources is imperative; moreover, understanding metabolic processes involved in protein digestion in octopuses could help formulate nutritionally balanced diets using ingredients with the highest digestibility, thereby

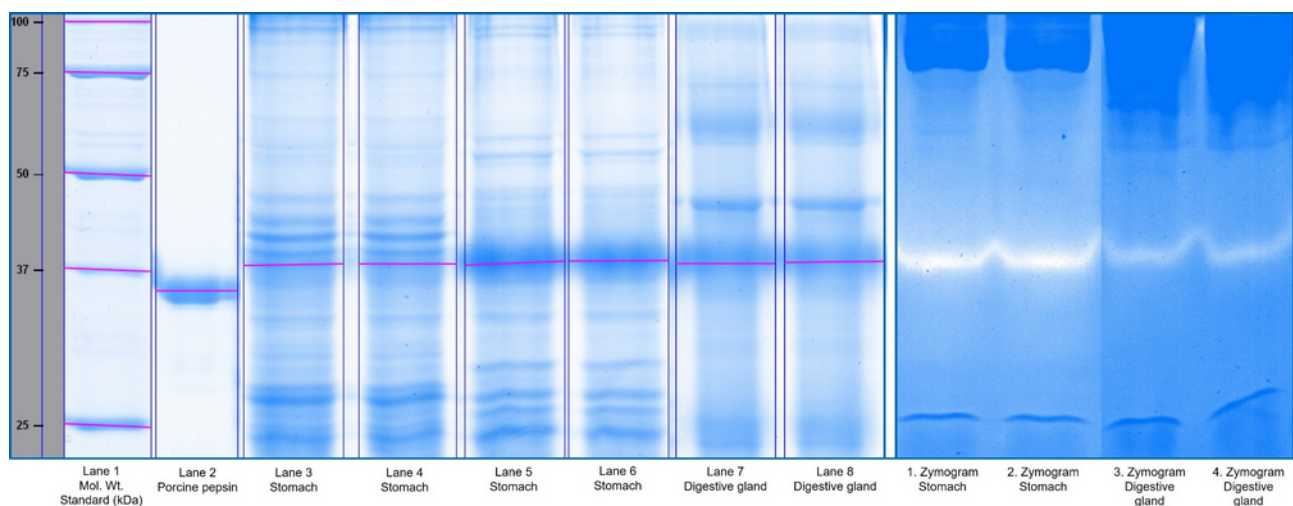


Figure 1. SDS-PAGE gel and zymogram from *Octopus bimaculatus* native extracts: Lane 1) molecular weight (Mol. Wt.) standard (kDa), Lane 2) pepsin standard from porcine gastric mucosa, Lanes 3-6) stomach of different organisms with cathepsin band, Lanes 7-8) digestive gland of different organisms with cathepsin band. Zymograms: Lanes 1-2) stomach of different organisms with cathepsin band, Lanes 3-4) digestive gland of different organisms with cathepsin band.

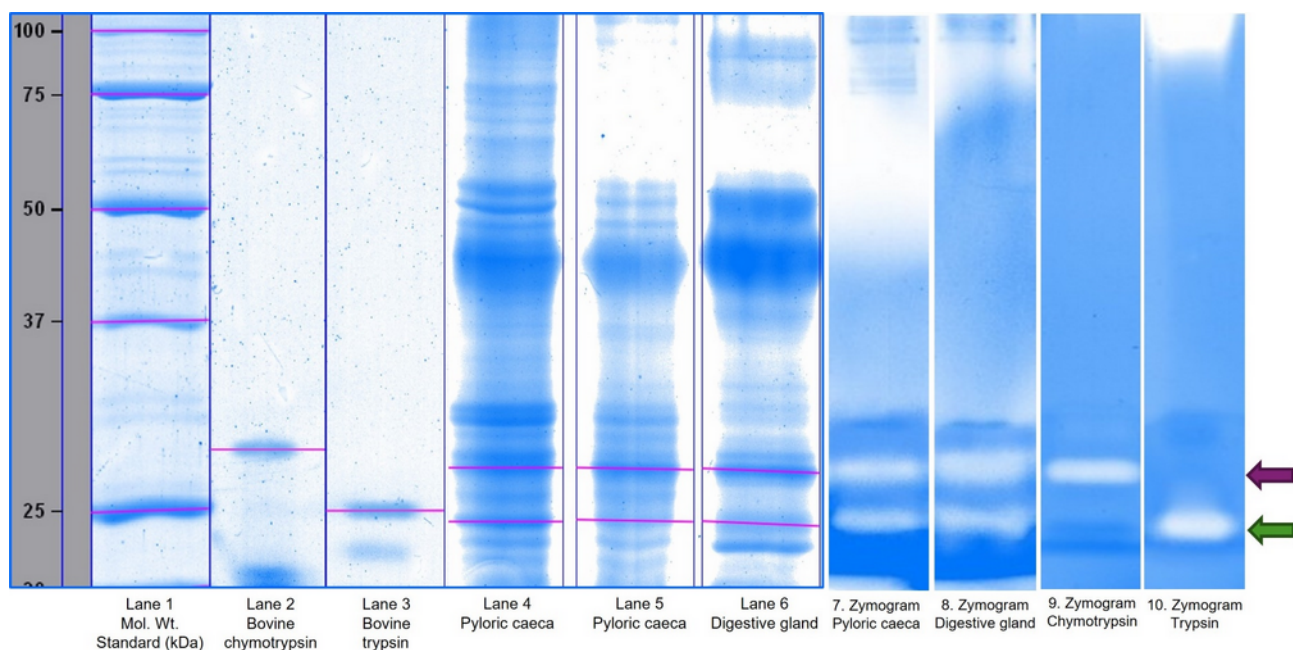


Figure 2. SDS-PAGE gel and zymogram from *Octopus bimaculatus* native extracts: Lane 1) molecular weight (Mol. Wt.) standard (kDa), Lane 2) chymotrypsin standard from bovine pancreas, Lane 3) trypsin standard from bovine pancreas, Lanes 4 and 5) pyloric caeca of different organisms with chymotrypsin and trypsin bands, Lane 6) digestive gland with chymotrypsin and trypsin bands. Zymograms: Lane 7) pyloric caeca with chymotrypsin and trypsin bands, Lane 8) digestive gland with chymotrypsin and trypsin bands, Lane 9) chymotrypsin standard (purple arrow), Lane 10) trypsin standard (green arrow).

optimizing nutrient absorption and promoting growth (Pineda-Suazo *et al.* 2024). Recently, *in vitro* digestibility studies have been used in cephalopods as an accurate, economic, and rapid process to simu-

late digestion and predict the quality of feedstuffs, by measuring the interaction between enzymes and these substrates (Hamdan *et al.* 2014; Rojo-Arreola *et al.* 2019). The DH estimates the percentage of

cleaved peptide bonds and significantly correlates to apparent protein digestibility (APD), which measures the apparent nitrogen assimilation by the organism; the DH is also a rapid method for the nutritional evaluation of feedstuffs or diets (Lemos *et al.* 2009). *In vitro* DH results in this study indicated that, under both acid or alkaline conditions, the SCP bacterial meal (MRD-Pro®), BSFLM and FM were the most digestible protein sources when subjected to enzyme extracts obtained from stomach + digestive gland or pyloric caeca + digestive gland, under these experimental conditions. Acid proteases appear to be more active than alkaline proteases in octopuses, and it has been suggested that digestion is predominantly acidic, mainly performed by aspartic proteases like cathepsin D, as proposed for *O. mimus* and *O. maya* (Martínez *et al.* 2011; Linares *et al.* 2015).

The amino acid composition of the protein is generally related to its digestibility, as digestive enzymes recognize and bind specific amino acids for hydrolysis of the polypeptide chain; cathepsin D hydrolyzes specific sites between leucine and proline (10%), tryptophan and isoleucine (30%), leucine and lysine (70%), and isoleucine and leucine (100%), primarily occurring among non-polar amino acids like leucine, isoleucine, and proline (Arnold *et al.* 1997). The protein feedstuffs with the highest DH in this study contain significant amounts of these amino acids, for instance, the SCP bacterial meal (MRD-Pro®) has 7.4 g of leucine, 4.3 g of isoleucine, 10.4 g of lysine, and 4.0 g of tryptophan per 100 g of CP on a dry weight basis (DWB). BSFLM includes 4.7 g of lysine, 2.6 g of isoleucine, 2.9 g of leucine, and 0.72 g of tryptophan per 100 g of CP on a DWB, while FM has up to 7.7 g of leucine, 4.7 g of isoleucine, 7.9 g of lysine, and 1.2 g of tryptophan per 100 g of CP on a DWB (Pérez-Velázquez *et al.* 2023), which explains the higher DH under acidic conditions for these feedstuffs. On the other hand, trypsin breaks peptide bonds at the C-terminal side of lysine and arginine, while chymotrypsin cleaves peptide bonds at the C-terminal side of the aromatic amino acids phenylalanine, tyrosine and tryptophan, and to a lesser extent of other amino acids like leucine and methionine (Nelson and Cox 2017); however, no clear pattern for the DH under alkaline conditions was observed, as the amino acid content of the feedstuffs did not correlate with the DH values. There is a high likelihood of the presence of additional alkaline proteases in these extracts, besides trypsin and chymotrypsin; hence, the combined action of all of them, cleaving a variety of

amino acids, makes it difficult to correlate the higher DH of SCP bacterial meal (MRD-Pro®), BSFLM, and FM to their amino acid profile, although it is noteworthy that the same three ingredients showed higher DH under alkaline conditions as well. Numerically lower values for the DH under alkaline conditions as compared to the acidic conditions were observed for all feedstuffs, except for SCP bacterial meal (MRD-Pro®), which partly supports the observation that acid proteases appear to be more active than alkaline proteases in octopuses (Martínez *et al.* 2011, Linares *et al.* 2015). Similar observations were recorded for the DH using pepsin solution (99.61-99.9%) as compared to the trypsin + chymotrypsin solution (95.13-95.63%) (Table I); both solutions showed higher hydrolytic activity than the extracts, an expected result due to the higher concentration (0.1 mM) and presumably, their overall activity.

An important remark is that, despite the high protein content of some feedstuffs like soy protein isolate (90.0%) and casein (80%), they were among the feedstuffs with the lowest DH when using enzyme extracts. This may be attributed to different factors, including their solubility and charge, which depend on pH. Carboxyl and amino groups in proteins can be ionized and have different charges depending on the pH, and the pH at which the molecule has no net charge is the isoelectric point (pI). The protein molecule is positively charged if its pI is greater than the pH and negatively charged if its pI is smaller than the pH. Enzyme and substrate should not be both positively or negatively charged at a given ambient pH, or they will repulse each other (Xia 2007). For both casein and soy protein isolate, their pI is close to pH 4.6, the pH at which these proteins carry no net charge, and their molecules have minimal electrostatic repulsion leading to their lowest solubility and to aggregation or precipitation. The pH values in this DH study, under acid and alkaline conditions, were 2.0 and 8.5, respectively, allowing increased solubility, but differences in amino acid composition (primary structure) and structure (secondary, tertiary and quaternary structures) could make these feedstuffs less accessible to certain proteolytic enzymes, while for others, more cleavage sites in their structure would enable a more extensive hydrolysis under these pH conditions. For example, casein contains the highest levels of lysine and arginine (7.4 and 3.7 g per 100 g of CP on a DWB, respectively) (Rasmussen 2008) but showed the lowest DH (55.30%), while the SCP bacterial meal had the highest DH (68.81%), but contains only 3.5 and 2.8 g per 100 g of CP of lysine and

arginine on a DWB. Casein also possesses weak hygroscopic properties, which makes it less soluble due to the formation of hydrophobic aggregations, eliciting a less efficient surface contact ratio between the casein molecules and the digestive enzymes, despite the high levels of lysine and arginine (Tonheim *et al.* 2007), possibly contributing to the low DH value recorded.

The origin of the protein source also plays a role in the efficiency of digestive enzymes. Generally, animal proteins are more digestible than plant proteins for marine organisms. FM is considered a nutritionally ideal ingredient for aquatic organisms, with very high digestibility (Cho & Kim 2011); therefore, its high DH is not an unexpected result, but the fact that it has been equaled or even surpassed by the SCP bacterial meal and BSFLM meals is noteworthy, highlighting a promising application of these meals in diets for *O. bimaculatus*. The SCP bacterial meal contains 60-70% CP with high levels of lysine and methionine, essential amino acids usually limited in plant protein sources, and has produced good growth results for Nile tilapia *Oreochromis niloticus* (González-Félix *et al.* 2024) and Pacific white shrimp *Penaeus vannamei* (Felix *et al.* 2023). On the other hand, BSFLM has been successfully used for various marine and freshwater fish species; it is characterized by a high protein content, bioavailability, and relatively low levels of chitin, a polymer that may inhibit digestion (Surendra *et al.* 2020). In krill meal, another feedstuff tested in this study, chitin is present in even larger quantities compared to BSFLM, which may explain its relatively lower DH, possibly resulting from interactions between the chitin-rich exoskeletons and muscle proteins during the milling and drying stages of processing (Sáenz de Rodríguez *et al.* 2011). However, krill meal was evaluated *in vivo* in pelleted extruded diets for *O. vulgaris* with positive results, confirming that krill meal, alone or in combination with FM, improved growth performance (9.0-10.16% increment of the initial weight) and feed efficiency (0.95-1.17) in a short-term study (15 days) (Querol *et al.* 2014); one should consider that *in vitro* trials may underestimate digestibility because of the difficulty of an accurate simulation of *in vivo* conditions (Moyano *et al.* 2015), explaining the difference in the efficiency of krill meal utilization.

Recently, a trial with *O. maya* juveniles fed dry-pelleted diets based on a mixture of squid and crab meals, showed similar growth but higher survival and activity of alkaline proteases compared to juveniles fed diets with the same formulation pro-

vided as a semi-humid paste; this is a promising result for the development of future aquafeeds, but in spite of this positive outcome, the use of squid and crab meals is an unsustainable practice (Santiago *et al.* 2024). Other commercially available feedstuffs, like those evaluated in the present study, need to be considered to develop more profitable aquafeeds for octopuses. Between 2023 and 2024, the cost was approximately \$1.60-1.70 USD/Kg for FM, and \$2.00-3.50 USD/Kg for BSFLM. The price of soy protein isolate ranged between \$2.21-2.68 USD/Kg (Perez-Velazquez *et al.* 2023; Nunes *et al.* 2023). Commercial prices for the SCPs used in this study have not yet been established, whereas casein and *Arthrospira* sp. are more costly due to their demand in other markets. Beyond cost, availability and sustainability play crucial roles and largely influence feed formulation in the aquaculture industry. Therefore, assessing the DH for different feedstuffs provides a valuable insight into their potential use in aquafeeds for *O. bimaculatus*. Nonetheless, future studies should include *in vivo* digestibility analysis to confirm their nutritional value for this species. Although research on cephalopod nutrition suggests that they appear to require foods presented in the form of semi-moist pastes with fresh/raw elements like crustacean and mollusk meats (Rosas *et al.* 2012; Cerezo-Valverde & García 2016), exploring the use of novel and traditional feedstuffs is desirable as they allow precise feed formulation, aquafeeds can be produced year-round and are suitable for automated delivery methods, which is not feasible with fresh foods with a limited shelf life that require manual labor for preparation and delivery.

Proteases have been barely studied for *O. bimaculatus*, and related information is scarce; total alkaline and acidic proteolytic activities have been reported in newly hatched paralarvae, showing an increasing trend in activity with age, regardless of the type of food consumed (López-Peraza *et al.* 2018). However, identification of the proteases in the digestive tract or their molecular weights has not been reported. In the present study, the activity of the proteases cathepsin, trypsin, and chymotrypsin in adult *O. bimaculatus* is confirmed for the first time by zymography, a gel-based electrophoretic method. There are over 1,000 methods published for the detection of over 400 different enzymes that provide high sensitivity and specificity, allowing the analysis of unpurified enzyme preparations (Manchenko 2003). Several types of cathepsins have been reported for cephalopods, for example A, B, C, D, E, H and L (Martínez *et al.* 2011; Kim *et al.* 2012;

Ibarra-García *et al.* 2018). In this study, the estimated molecular weight of cathepsin corresponded to 37.6 kDa. Cathepsins generally have low molecular weights; cathepsin B is approximately 37 kDa, while cathepsins H and L have molecular weights around 23 kDa (Aranishi *et al.* 1997; Wilson *et al.* 1998; Cárdenas-López & Haard 2005). A purified cathepsin B from the squid *Dorytheuthis bleekeri* was 13.6 kDa (Inaba *et al.* 1976). Molecular weights reported for cathepsin D in octopods include the pelagic squid *Todarodes sagittatus* at 38 kDa (Gildberg 1987), the Japanese common squid *Todarodes pacificus* at 36.5 kDa (Komai *et al.* 2004), the cuttlefish *Sepia officinalis* at 37.5 kDa (Balti *et al.* 2010), the jumbo squid *Dosidicus gigas* at 38.5 kDa (Cadena-Cadena *et al.* 2019), and the neon flying squid *Ommastrephes bartramii* at 45 kDa (Zhang *et al.* 2019). Based on similarity, there is a strong likelihood that the identified cathepsin in this study is cathepsin D. Supporting this consideration, for *O. bimaculoides*, a sympatric sibling species of *O. bimaculatus* inhabiting the same geographical range, the highest peak of acid protease activity has been reported in the digestive gland at pH 3.0, and a substantial amount of the catalytic activity has been attributed to cathepsin D (Ibarra-García *et al.* 2018). Although this 37.6 kDa enzyme is likely cathepsin D, further confirmation through enzyme purification and molecular characterization is still pending.

Trypsin and chymotrypsin are the most studied alkaline proteases in cephalopods (Ezquerro-Brauer *et al.* 2002). In this study, the activity of both enzymes was confirmed by zymography in the pyloric caeca and digestive gland of adult *O. bimaculatus*, and their molecular weights, 24.6 kDa for trypsin and 27.3 kDa for chymotrypsin, are reported for the first time for this species. Activity of both enzymes has been reported in early life stages of various cephalopods, for example, in the paralarvae of *O. vulgaris*, *Robsonella fontaniana*, *Enteroctopus megalocyathus*, *S. pharaonis*, and *Sepioteuthis lessoniana* (Villanueva *et al.* 2002; Pereda *et al.* 2009; Tito *et al.* 2016; Farías *et al.* 2020; Jiang *et al.* 2022; Satjarak *et al.* 2024), as well as in juveniles of *O. bimaculoides* (Solorzano *et al.* 2009) and *O. maya* (Santiago *et al.* 2024). There are no molecular weight reports for octopuses thus far, but for *O. maya*, a mixture of proteases has been reported to range from 30 to 60 kDa (Martínez *et al.* 2011), although the enzymes were not identified. Previous reports on trypsin molecular weight in cephalopods refer to *S. officinalis* with 22 kDa (Balti *et al.* 2009) and *D. gigas* with 23.5 kDa (Villalba-Villalba *et al.*

2017). On the other hand, chymotrypsins from aquatic animals have molecular weights ranging from 22 to 35 kDa (Satjarak *et al.* 2024), but data on cephalopods are also scarce, among them are the reports for *D. gigas* of 31 kDa (Marquez-Rios *et al.* 2016) and for *S. lessoniana* of 43 kDa (Satjarak *et al.* 2024). Molecular weights of trypsin and chymotrypsin in *O. bimaculatus* closely relate to reported values for other cephalopods, which were confirmed with zymographic techniques in the present study, although conducting their molecular characterization is essential.

Conclusion

The *in vitro* DH analysis of various protein sources for aquafeeds showed significantly higher DH values for SCP bacterial meal, BSFLM, and FM, highlighting their potential as candidate ingredients for inclusion in aquafeeds formulated for *O. bimaculatus*. Nevertheless, further validation through *in vivo* digestibility trials is necessary. Additionally, the present study suggests the presence of cathepsin, trypsin, and chymotrypsin activities in the digestive tract of *O. bimaculatus*. These proteases were tentatively characterized in terms of their molecular weights (37.6, 24.6, and 27.3 kDa, respectively); however, further confirmation through enzyme purification and molecular characterization is required.

Ethical statement

The collection of biological samples was conducted in accordance with the Ethical and Welfare Considerations when Using Cephalopods as Experimental Animals provided by Moltschaniwskyj *et al.* (2007), and the Procedures for the Husbandry and Handling of Organisms established under the Official Mexican Norm (NOM-062-ZOO-1999).

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Disclosure statement

The authors report there are no competing interests to declare.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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