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Production and Characterization of Protein Isolates and Hydrolysates from Slipper Cupped Oyster (*Crassostrea iredalei*)

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Proteins from oysters were extracted by the alkaline pH shift method and subsequently subjected to hydrolysis using pepsin. The physico-chemical characteristics of the protein isolate and hydrolysate – such as total soluble protein, protein solubility, water absorption capacity (WAC), oil absorption capacity (OAC), bulk density, hygroscopicity, water activity, and moisture – were determined. The results revealed that hydrolysis of oyster protein isolates (OPIs) significantly increased its functionality. Protein hydrolysates have 86–100% solubility at various pH levels. The WAC (5.65 g/g) and OAC (9.44 g/g) were also significantly higher after hydrolysis. However, the hydrolysates have very low bulk density and high hygroscopicity, which should be considered in the packaging and storage of the product. These findings indicate that oyster protein hydrolysates (OPHs) have the potential for food and nutritional applications as a natural additive or as a protein source in special diets.

Keywords: *Crassostrea iredalei*, enzyme hydrolysis, functional characterization, oyster protein

INTRODUCTION

Enzymatic hydrolysis has been employed in a wide range of foods to convert native proteins with limited functionalities into ingredients, with improved and novel functionalities for several food applications (Nyo and Nguyen 2017). It is an attractive process to improve food proteins' nutritional and functional properties by changing the proteins' physical properties. Protein hydrolysates are proteins that are chemically or enzymatically broken down, resulting in peptides of various sizes (Acquah *et al.* 2018). Hydrolysates are great nutritional supplements because they can be utilized for various metabolic activities of the human body compared to intact proteins and have easily absorbable bioactive components (Nesse *et al.* 2011). They have been used as alternative protein sources for the nutritional management of persons who have difficulty digesting intact proteins and for improvement of the health of malnourished individuals (Silvestre 1997; Nesse *et al.* 2011).

The physicochemical properties of proteins affect the functional behavior in food systems during the stages of the food's preparation, processing, storage, and consumption. For fish proteins to be valuable, they need to have several desirable functional characteristics (McWatters and Chhinnan 1985; Parks and Carpenter 1987). These functional and bioactive properties of peptides are linked to their molecular size, charges, amino acid sequence, and their interaction with water, which are influenced by the hydrolysis conditions used – such as hydrolysis time, pH, type of enzyme, and temperature (Klompong *et al.* 2007; Acquah *et al.* 2018).

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Recently, as an alternative to direct hydrolysis of the raw fish muscle, protein hydrolysates have been prepared from protein isolates obtained by the pH shift method. Hultin and Kelleher (2000) developed the method in which the protein is solubilized using acid or alkaline solutions, followed by isoelectric precipitation. The resulting protein isolates obtained have approximately the same water content as fish muscle but with higher protein content (Pires *et al.* 2012; Surasani 2018). The pH shift method has been applied to various underutilized fish species such as croaker (Kristinsson and Liang 2006; Choi *et al.* 2009), tilapia (Foh *et al.* 2012), and by-products from salmon, herring, and cod (Abdollahi and Undeland 2019) to recover functional and stable proteins.

Crassostrea iredalei, commonly known as slipper cupped oyster and locally known in the Philippines as "talaba," is an economically important shellfish that is widely cultured and harvested for human consumption. In 2019, the total volume of oysters produced in the country is 38,017.48 MT and continues to increase over the years. The highest producing regions are Central Luzon and Western Visayas – producing 19,964.08 and 14,302.25 MT, respectively (PSA 2020). However, the primary market for oysters is for domestic consumption only, and its potential for the international market is still yet to be realized (Andalecio *et al*. 2014).

Oysters are known to be rich sources of nutrients, including various amino acids, taurine, and trace elements (Wang *et al.* 2020b). Many studies have shown that species of oyster hydrolysates contain functional and biological activities, giving them the potential for nutritional or pharmaceutical applications. Protein hydrolysis is the disintegration of proteins, which enables the modification and improvement of its functional properties such as solubility, emulsifying and foaming properties, and gelling properties (Liu *et al.* 2014). Protein hydrolysates from oysters (*Crassostrea gigas*) have also exhibited antitumor activity and immunostimulant effects (Wang *et al.* 2010), antioxidant and antimicrobial activities (Zhang *et al.* 2014), antihypertensive activity (Lee *et al.* 2018), and zinc absorption capacities (Li *et al.* 2019a). In addition, there are reports on anticancer activities (Umayaparvathi *et al.* 2013), anti-fatigue activities (Miao *et al.* 2018), presence of immunoregulatory peptides (Li *et al.* 2019b), and effects on spatial learning and memory (Wang *et al.* 2018).

With the increasing production of oysters in the country, alternative forms of utilization can be undertaken to increase its economic value through its application in the food or pharmaceutical industries. One such option is the extraction of protein ingredients of high quality and functionality. Thus, the aim of this study is to extract OPIs using the alkaline pH shift method and prepare OPHs from oysters (*C. iredalei*) by enzymatic hydrolysis and then eventually evaluate their physico-chemical characteristics.

MATERIALS AND METHODS

Materials

Fresh live slipper cupped oysters (*Crassostrea iredalei*) were obtained from a market in Iloilo City. Oysters were transported live in gunny sacks to the Institute of Fish Processing Technology Processing Laboratory. The enzyme used was pepsin supplied by Sigma Aldrich Co (St. Louis, MO, USA). All reagents used were of analytical grade and commercially available.

Sample Preparation

The whole oysters were cleaned by brushing off mud and attached organisms and then blanched for 2 min and cooled directly in an ice water bath. Oyster meats were collected by shucking and were packed in polypropylene cups and kept at –20 °C until further use. The yield during the processing was measured.

Protein Isolation by Alkaline pH Shift Method

The OPIs were produced by alkaline pH shift based on the methods of Huang and co-authors (2015) and Kristinsson and Liang (2006), with some modifications. Frozen oyster meat was thawed in running water. After thawing, the sample was mixed with cold distilled water (1:6) and homogenized for 20 s in a food blender. The homogenates were kept in cold temperature (10–15 °C) in an ice bath, and the pH was adjusted to pH 11–13 with 2 N NaOH and was incubated with continuous stirring for 30 min. The homogenates were centrifuged at 3800 x g for 20 min. The middle protein supernatant layer was collected, while the upper layer and the bottom sediment were discarded. The recovered protein supernatant was adjusted to its isoelectric point of pH 5.5. The precipitated proteins were obtained by centrifugation at 3800 x g for 20 min. The protein isolate was collected, weighed, and freeze-dried (2–4 LDPlus Martin Christ freeze dryer, Germany). The freeze-dried OPI was kept at –20 °C for further processing.

Enzymatic Hydrolysis

The OPH was produced using the methods of Dong *et al*. (2010) and Jun *et al.* (2004) with modifications. About 1 g (protein basis) of the protein isolates were suspended in 100-mL distilled water. The pH of the mixture was adjusted to pH 2 at 37 °C, and the reaction was commenced with the addition of 1% pepsin. The hydrolysis proceeded for 6 h. The reaction was terminated by increasing the temperature to 95 °C for 10 min. The samples were immediately cooled down in an iced water bath and centrifuged at 3800 x g for 20 min. The pH of supernatant recovered after centrifugation was adjusted to pH 7 and stored at –20 °C. The samples were freeze-

dried (EYELA FD 550, Japan), and solid hydrolysates were stored at 4 °C.

Degree of Hydrolysis (DH)

The DH of oysters hydrolyzed using 1% pepsin was analyzed using *o*-phthalaldehyde (OPA) method based on Charoenphun *et al.* (2012). Briefly, 10-µL hydrolysates were mixed with 200-µL OPA solution. The mixture was incubated for 100 s at 37 °C. The absorbance was measured at 340 nm using a CLARIOstar microplate reader, and the total amino groups were quantified using the standard L-leucine. To calculate the DH, the following equation was used:

$$DH(\%) = \frac{L_t - L_o}{L_{max} - L_o} \times 100\% \quad (1)$$

where L_t is the quantity of α-amino acid released at time t, L_o is the quantity of α-amino acid in the original substrate, and L_{max} is the quantity of total amino acid in the original substrate obtained after acid hydrolysis at 6 M HCl 100 °C for 24 h. The L_{max} was based on the work of Adler-Nissen (1986), wherein the total number of peptide bonds is 8.6 mol equiv./kg for fish.

Physico-chemical Characterization

The protein content of the protein hydrolysates and isolates was determined based on the protocol of Lowry *et al.* (1951). The absorbance was measured at 660 nm (CLARIOstar microplate reader). The standard protein used was Bovine serum albumin. The moisture content was measured by oven drying at 102 °C until a constant weight was obtained (AOAC 2005). The water activity (A_w) of the hydrolysate powder was measured by water activity meter (AquaLab PawKit Water Activity Meter, Decagon Devices, Inc., USA) in triplicates. The solubility of hydrolysates was determined using the method of Liu *et al.* (2014). The protein concentration in the supernatant was determined using the Lowry assay (Lowry *et al.* 1951). The solubility of the sample was calculated by:

$$\text{Solubility (\%)} = \frac{\text{protein content in the supernatant}}{\text{total protein content in the sample}} \times 100\% \quad (2)$$

WAC was determined using the centrifugation method by Kang *et al.* (2014). WAC (g/g) was calculated using:

$$WAC\ \left(\frac{g}{g}\right) = \frac{\text{Initial solution volume} - \text{Final solution volume}}{\text{original weight of sample}} \times \text{water density} \quad (3)$$

Oil absorption capacity (OAC) was determined using the centrifugal method (Kang *et al.* 2014). Oil absorption capacity (g/g) was calculated using:

$$OAC\ \left(\frac{g}{g}\right) = \frac{\text{Initial solution volume} - \text{Final solution volume}}{\text{original weight of sample}} \times \text{oil density} \quad (4)$$

The hygroscopicity of the hydrolysate powders was determined using the method of Cai and Corke (2000) with modifications. Powdered hydrolysates and isolates (1-g sample) were placed in pre-weighed glass containers and exposed over a saturated NaCl solution having a relative humidity of 75.3% (Greenspan 1977) inside a desiccator at 25 °C. After 1 wk, the samples were weighed, and the hygroscopicity was expressed as g moisture/ 100-g solids.

The bulk density of the powders was measured, as per Goula and Adamopoulos (2004), by placing 2 g of sample into a 25-mL graduated cylinder. The cylinder was tapped by hand five times, and the bulk density was solved by dividing the weight of the powder with the volume occupied of the powder in the cylinder.

Statistical Analysis

All analyses were done in triplicates, and results were expressed as the mean with the standard deviation. The statistical analysis was performed using the software SPSS Statistics v24 (SPSS Inc., Chicago, IL, USA). Data were analyzed using one-way analysis of variance, followed by Tukey's test and independent t-test at a significance level of $p < 0.05$.

(1)

RESULTS AND DISCUSSION

Production of Protein Isolate and Hydrolysate from Oysters

Slipper cupped oysters were utilized to produce protein hydrolysates. The physico-chemical characteristics were determined. Table 1 shows the yield (%) for every processing step involved in the production of protein hydrolysates. The shucking of oysters yielded 8.48% meat.

The preparation of fish protein hydrolysate (FPH) from OPIs as an alternative to the direct hydrolysis of the raw fish has already been used by several researchers. Protein

Table 1. Processing yield during production of protein hydrolysates from oysters (*Crassostrea iredalei*).

Processing step	Yield (%); mean at n = 3
Shucking (removal of meat from shell)	8.48
Protein isolation (wet weight)	39.29
Protein isolation after freeze drying	10.95
Enzymatic hydrolysis	64.10

isolation *via* alkaline pH shift produced a yield of 39.29% (wet basis). The following hydrolysis of the OPI using pepsin, with subsequent freeze-drying, produced a yield of up to 64.1% protein.

Degree of Hydrolysis (DH)

DH is defined as the proportion of cleaved peptide bonds during hydrolysis. It has an important correlation with yield (Adler-Nissen 1986; Rutherford 2010). Results of OPI hydrolyzed with 1% pepsin are shown in Figure 1. As the hydrolysis progresses, there is a significant increase in the DH. The maximum DH reached after 6 h was at 40.32%. A higher DH also indicates the production of lower molecular weight peptides, thereby improving its functional properties (Pacheco-Aguilar *et al.* 2008).

Functional Characteristics of Protein Products from Oysters

Solubility is an essential functional property of protein hydrolysates. The solubilities of OPH and OPI at pHs 3, 5, 7, 9, and 11 are shown in Figure 2. OPH had consistently high solubility at various pH values ranging from 86–100%. Meanwhile, the relative solubility of OPI has lower solubility at acidic pH and was significantly increasing when pH was increased. The results suggest that this might be due to the protein isolates being produced through solubilization at alkaline pH. The hydrolyzed protein has significantly increased the solubility of the protein isolates over a wide range of pH. This is one of the desirable functional properties of the OPH (Raymundo *et al.* 2000).

The protein isolates have high solubility at extreme pHs (2–3 and 11–12), which exhibit a U-shape behavior, and have solubilities higher than 60% (Foh *et al.* 2012; Abdollahi and Undeland 2019). Furthermore, the low solubility of OPI is probably induced by a pH shift caused by the denaturation of muscle proteins (Foh *et al.* 2012). Meanwhile, the solubility of OPH is similar to the results of Klompong *et al.* (2007) and Chalamaiah *et al.* (2010), where protein hydrolysates have consistently high solubilities of ≥ 85% and ≥ 72%, respectively.

The enzymatic hydrolysis alters the molecular size, hydrophobicity, including polar and ionizable groups of the resulting protein hydrolysates (Mutilangi *et al.* 1996). In general, the degradation of proteins to smaller peptides due to hydrolysis has made the hydrolyzed products more soluble (Klompong *et al.* 2007). The hydrolyzed myofibrillar proteins produce smaller peptides, which are expected to be more polar and are able to bond with water, thereby increasing their solubility (Gbogouri *et al.* 2004). Thus, the size of hydrolyzed peptides, the hydrophobic-hydrophilic balance, and also the charge of the peptides will dictate the differences in the solubility of the hydrolysates (Nalinanon *et al.* 2011).

The ability of food to hold water during processing, expressed as the WAC, is also an important characteristic of proteins. The interaction of food protein with water determines the nature of hydration, solubility, viscosity,

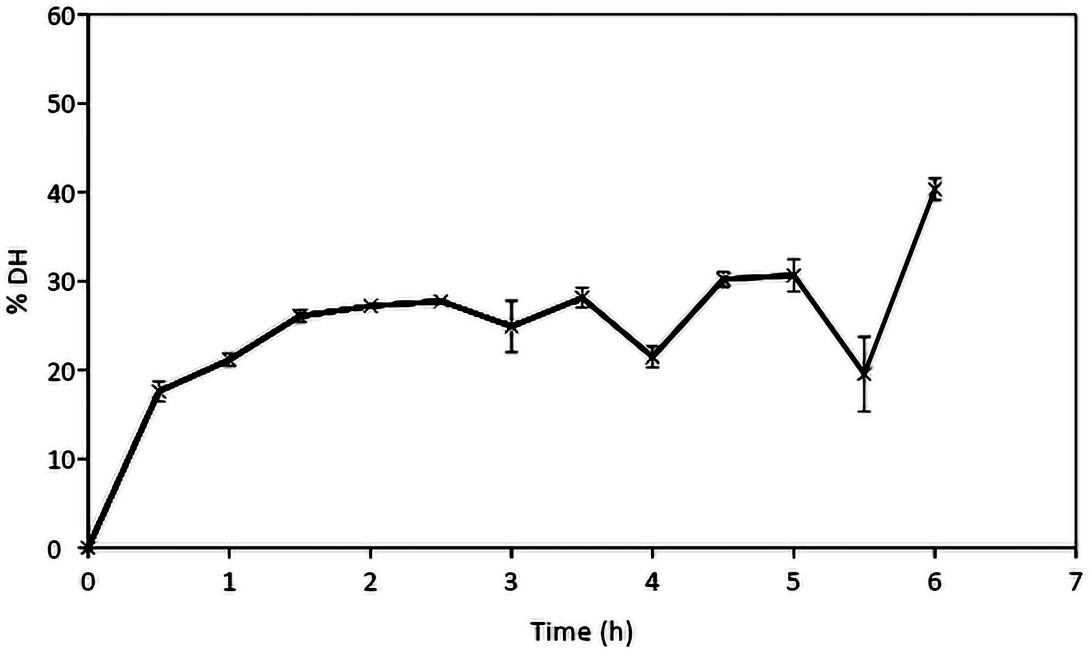

Figure 1. DH of oyster hydrolysates using 1% pepsin. Value represents the mean ± SD (n = 3).

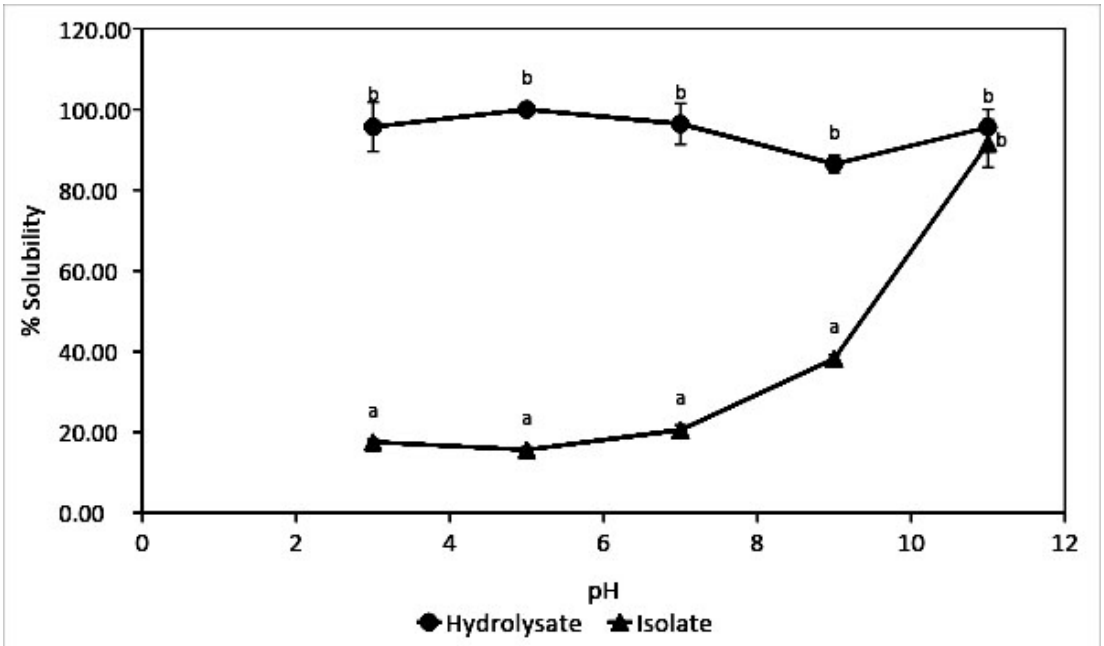

Figure 2. Solubility of protein isolates and hydrolysates at pH 3, 5, 7, 9, and 11. Value represents the mean ± SD (n = 3). Mean with different superscript letters are significantly different at $p < 0.05$.

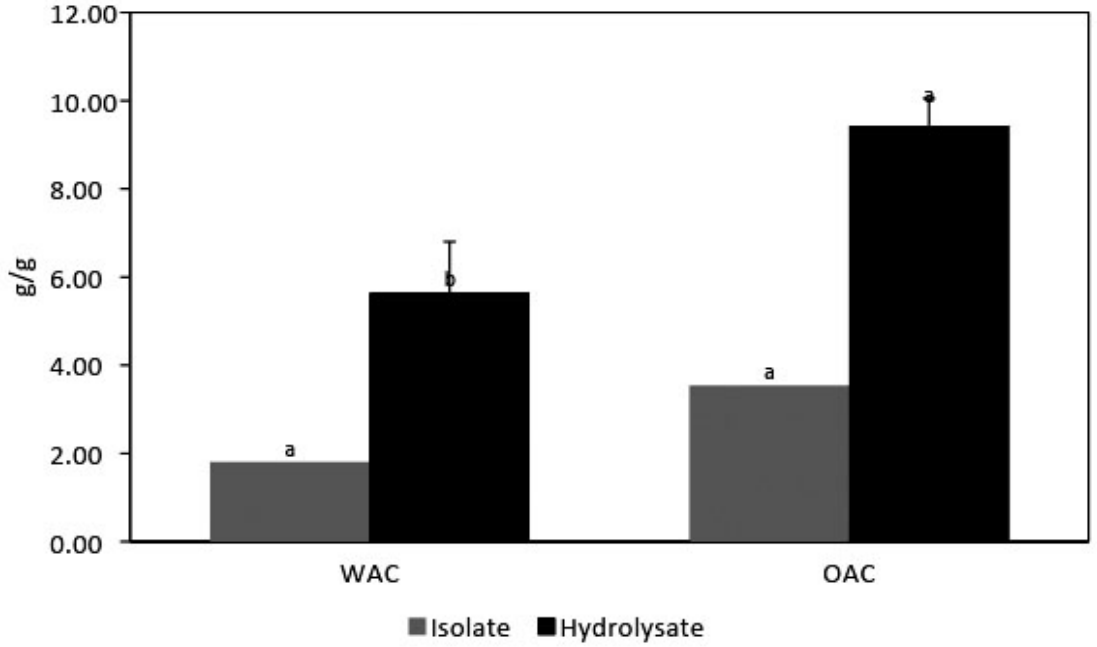

Figure 3. Water and oil absorption capacity of OPI and OPH. Value represents the mean ± SD (n = 3). Mean with different superscript letters are significantly different at $p < 0.05$.

gelation, and product development (Haryati *et al.* 2020). The results shown in Figure 3 shows that OPH had a significantly higher WAC at 4.99 g/g sample than OPI. The high absorption capacity of the hydrolysates may have indicated high porosity of the powder, such that water is trapped in the interstices of particles, and also suggests the presence of polar amino acids that binds water molecules. Hydrolysis of proteins increases the terminal carboxyl and amino groups, which substantially affect the amount of absorbed water and the strength of the sorption bond, making them hygroscopic. Due to this, protein hydrolysates are shown to have a good ability to bind and hold water, which is particularly useful in many formulations (Kristinsson and Rasco 2000).

On the other hand, protein-fat interaction greatly affects fat absorption. The OAC property indicates the amount of oil that can directly bind with the protein (Shahidi *et al.* 1995; Gbogouri *et al.* 2004). Other functional attributes such as protein bulk density, DH, and the specificity of the hydrolyzing enzyme affect the ability of the hydrolysates to bind fat. Results found in Figure 3 shows that OPH has a high OAC of 9.44 g/g sample, which is significantly higher than the OAC of OPI. The study of Chalamaiah *et al.* (2010) reported a range of 0.91–1.05 g/g sample in fish egg protein hydrolysates, while a decrease in oil absorption capacity with an increase in DH was reported by Diniz and Martin (1997).

Oil binding could be influenced by the presence of non-polar chains, which can form hydrophobic interactions with hydrocarbon chains of lipids (Wani *et al.* 2013). The high absorption capacity could also be related to the type of enzyme used, resulting in more hydrophobic groups to the oil interface (Barbut 1999; Narikimelli *et al.* 2019). It is expected that if the OPHs have a high WAC, they will not have similar results for OAC.

Physico-chemical Characterization of Protein Products from Oysters

The characterization of protein products from oysters is displayed in Table 2. The total soluble proteins significantly increased after hydrolysis. The solubilization of proteins during enzyme hydrolysis and the subsequent removal of insoluble, undigested non-protein substances resulted in higher protein content of the OPH (Benjakul and Morrissey 1997). The results of the OPH protein content are consistent with the results of other fish hydrolysate products, which are ≥ 80% (Benjakul and Morrissey 1997; Choi *et al.* 2009).

Water activity (Aw) is a significant index for powdered products used to predict the shelf life. It is defined as the ratio of the vapor pressure of water in a food system to the vapor pressure of pure water at a similar temperature (Fennema 1996). It affects the rate and degree of non-enzymatic browning reaction and lipid oxidation of food products (Uzzaman *et al.* 2018).

The water activity of OPH is at 0.57, well within the microbiologically stable range of below 0.75 where most bacteria, molds, and yeast could not grow (Majumdar *et al.* 2018). The a_w of OPH is not significantly different from the a_w of OPI. The secondary measure of moisture; the moisture content of OPH ranged from 9.4–11.0%. OPHs had a higher moisture content than the protein isolates.

The ability of powdered materials to take in moisture from the surroundings is termed hygroscopicity. It is a property of a product that needs to be greatly considered as it influences the product's handling, storage, and shelf stability. Less hygroscopic samples are easier to handle and pack (Wang *et al.* 2020a). The hygroscopicity of the OPH and OPI, expressed as g moisture/ 100 g solids, is shown in Table 2. The results showed that OPH has a significantly higher hygroscopicity (13.67 g/ 100 g solid) compared to OPI (3.80 g/ 100 g solids). Based on the hygroscopicity classification by Callahan *et al.* (1982), the protein hydrolysates produced from the protein isolates are very hygroscopic. Studies show that most FPHs are hygroscopic in nature. Hygroscopicity is important during powder reconstitution since it can lead to caking. Thereby, reducing the ability of the powder to be uniformly distributed in a solution (Fernandes *et al.* 2013).

Bulk density is the ratio between the weight of the material and the volume of space occupied. The smaller the density of the sample, the bulkier the material is (Haryati *et al.* 2020). It is important in the determination of the packaging requirements of the product. The cost of packaging and transport is less for food powders with higher bulk density. It also signifies the behavior of a product in dry mixes, varying with particle fineness (Foh *et al.* 2012). Table 2 shows the bulk density of the protein isolates and hydrolysates from oysters. The freeze-dried OPH had a significantly lower density at 0.057 g/mL than OPI (0.407 g/mL), indicating it is bulkier. The lower bulk density of OPH may be due to its larger particle size, with an ensuing increase in interparticle spaces leading to smaller contact surface areas per unit volume (Walton 2000). Results of

Table 2. Physico-chemical characteristics of OPH and OPI.

Characteristic	Hydrolysate	Isolate
Moisture, %	10.978 ± 0.095^a	9.405 ± 0.204^a
Total soluble protein, %	82.339 ± 1.392^a	73.707 ± 4.315^b
Water activity	0.573 ± 0.049^a	0.573 ± 0.006^b
Hygroscopicity, g moisture/100 g solid	13.677 ± 0.743^a	3.799 ± 0.221^b
Bulk density, g/ml	0.057 ± 0.002^a	0.407 ± 0.012^b

Values expressed are mean ± SD (n = 3). Mean with different superscript letters in each row are significantly different at $p < 0.05$.

Foh *et al.* (2012) showed that alkaline-assisted tilapia isolate has a bulk density of 0.34 g/mL, which has similar results to the study.

Overall, the results revealed that enzymatic hydrolysis had significantly altered the solubility and absorption properties plus various physico-chemical properties of the protein isolates.

CONCLUSION

The results of the study revealed that oysters are a very good source of protein and protein hydrolysates that are highly soluble over a wide range of pH, have high WAC and OAC, as well as total soluble proteins. However, OPHs have high hygroscopicity and bulk density, which need to be considered during the handling and storage of the hydrolysates. There are other functional properties and potential bioactivities, which could be further assessed. Having these desirable characteristics, OPH could potentially be used in various food and pharmaceutical applications, as a functional ingredient, or as an alternative protein source.

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CONFLICTS OF INTEREST

There are no conflicts of interest.

ETHICAL APPROVAL

This report did not involve the conduct of any human or animal test.

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