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Research article

Structural and functional properties of dried fish protein isolates

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Abstract: Dried fish (DF) are rich in protein and widely available worldwide; however, they have long been limited to being used as a traditional food. This study investigated the structural and functional properties of dried fish protein isolates (DFPIs) extracted from seven commonly consumed DF species in Bangladesh, including both sun-dried and fermented varieties. The isolates were prepared via isoelectric precipitation. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis revealed that the DFPIs lacked intact muscle protein bands and were primarily composed of peptides of <70 kDa. Circular dichroism spectroscopy showed extensive protein unfolding and hydrolysis, with only the Ganges River sprat DFPI retaining some ordered tertiary structure. The drying and fermentation processes significantly disrupted the secondary structure, resulting in low α-helix content and high proportions of β-sheets and random coils. Consequently, protein yield during extraction was relatively low, with a maximum of 36%. At neutral pH (7.0), DFPIs exhibited low heat-induced coagulation (maximum 23%) but showed excellent oil-holding capacity (up to 20 g/g), likely due to exposed hydrophobic groups. They also demonstrated good gelation abilities (minimum gelling concentration of 3–7%) and emulsifying properties, with Bombay duck DFPI forming stable emulsions with droplet sizes as small as 2 μm. These findings highlight the potential of DFPIs as functional ingredients in heat-processed food formulations, particularly as heat-stable emulsifiers.

Keywords: dried fish; protein isolates; functional properties; emulsions; structural properties

1. Introduction

Fish is rich in protein; however, fish processing under modern industrial practices often results in the generation of protein-rich waste, as many by-products remain underutilized [1]. Extracting proteins from processed fishery products and exploring their potential use in the food industry could improve economic efficiency and alleviate the dilemma of unsustainable animal-based foods caused by overfishing [1]. Since fish protein isolate (FPI) has proven to be excellent in more than one aspect, for example, water retention capacity, gelling, foam stability and emulsion capacity, it is widely used in various food systems [2]. Multiple studies have reported on the applications of FPI as a binder in reconstituted meat [3] and as an emulsifier in muscle foods [4]. FPI has also been used to formulate food products such as puffed corn snacks [5], ice cream [6], bread [7], biscuits [8], mayonnaise [9], soup powder [10,11], ready-to-use fish cluster mix [12], sausages [13], and weaning foods [14]. In addition, FPI can serve as a binder for animal feed [15], as well as in the formation of edible films or coatings for fried foods [16].

The structures of fish protein fractions have been described with respect to amino acid content, molecular weight, isoelectric point, and tertiary conformation [17]. However, according to the solubility, fish muscle proteins include water-soluble sarcoplasmic proteins (20–50%), salt-soluble myofibrillar proteins (50–70%), and insoluble matrix proteins (~3%) [18]. Components of these protein fractions also contain different subspecies of proteins. For example, sarcoplasmic proteins contain glycolytic enzymes, creatine kinase, myoglobin, and parvalbumin; myofibrillar proteins contain myosin, actin, tropomyosin, troponin, and paramyosin (in many invertebrate species); and matrix proteins are primarily composed of the extracellular protein collagen [17]. In addition to protein composition, studies have been published on exploiting the functionality of fish proteins. For example, Feng et al. [19] reported that a gel made from fish myofibrils and myofibrillar protein can improve the freeze–thaw stability of food products and ensure the quality of frozen food. Xiong et al. [20] reported the formation of a stable emulsion with myofibrillar protein (and xanthan gum) after sonication, which can be used as a new delivery system for functional materials. Ding et al. [21] described the addition of sarcoplasmic proteins to surimi to improve the hardness and elasticity of surimi's colloids as well as its water-holding capacity.

Dried fish (DF) are long-term storage products of the fishery industry and have a long history of consumption for thousands of years [22]. DF are widely consumed around the world, especially in East Asia, South Asia, India, and Africa, where they are deeply loved by local people as an important part of traditional diets [22]. However, like other products from the fish processing industry, most uses for DF are currently limited to traditional cooking methods [23] and their protein-rich characteristic, which makes them potential raw materials for producing FPI has long been overlooked. Although a previous study explored DF as a super-supplement to alleviate micronutrient malnutrition [24], and others have optimized the production process to improve quality [25,26], the currently available information is not sufficient to bridge the gap with respect to value-added utilization of fish proteins. One of the reasons for the limited scientific information on the value-added utilization of DF is that production and consumption are restricted to regional areas and lack global recognition as a source of protein ingredients. In addition, improper storage of DF (leading to compression or animal gnawing) often results in brittle fragments [27]. Due to a lack of market recognition, these fragments are frequently excluded from the human food chain and sold at extremely low prices for use as animal feed [27]. Such handling not only results in economic losses for DF producers, but also, from the perspective of

the Food and Agriculture Organization's (FAO's) 'food-focused approach', contributes to food loss [28].

Aquaculture is a critical industry in several countries, including as a main contributor to Bangladesh's economy with a deep and extensive fish processing industry, which results in abundant DF that remain unstudied for potential use as sources of functional proteins [29]. Previous researchers have confirmed that most reported DF from Bangladesh contain more than 50% protein, namely, *Mola mola*, *Harpadon nehereus*, *Puntius sophore*, and *Corica soborna* [23,30,31], which lays a realistic foundation for the production of dried fish protein isolates (DFPIs). However, there is scant information on the physicochemical and functional characteristics of DFPIs, especially their potential to serve as food ingredients. Given their availability and prevalence, commonly sold DF from markets in Dhaka, Bangladesh, were selected for this study to analyze the structure (polypeptide composition, surface hydrophobicity, and conformation) and function (solubility, heat coagulation, water-/oil-holding capacity, gelling ability, and emulsifying properties) of DFPIs. Bombay duck (BD, *Harpadon nehereus*), ribbon fish (RF, *Trichiurus lepturus*), white sardine (WS, *Escualosa thoracata*), freshwater barb (FB, *Puntius* spp.), and Ganges River sprat (GR, *Corica soborna*) are commonly found species in the DF market of Bangladesh, and their nutritional profiles have been previously reported in several studies. Fermented DF products enjoy wide acceptance in Bangladesh due to their distinctive flavor and cultural significance. Therefore, two widely consumed fermented fish varieties—fermented barb (FM, *Puntius* spp.) and fermented anchovies (FA, *Setipinna* spp.) were selected to represent fermented fish in this study.

2. Materials and methods

2.1. Materials

Seven DFs, namely BD (*Harpadon nehereus*), RF (*Trichiurus lepturus*), WS (*Escualosa thoracata*), FB (*Puntius* spp.), GR (*Corica soborna*), FM (*Puntius* spp.), and FA (*Setipinna* spp.) were purchased from local markets in Dhaka, Bangladesh, and kept at 4 ℃ during transportation to the laboratory. Upon arrival in the laboratory, the DF were stored at –20 ℃ pending further experiments. All chemical reagents used in this study were of analytical grade and purchased from Sigma-Aldrich (St Louis, MO, USA) and Fisher Scientific (Oakville, ON, Canada). Double-distilled water (DDW) was used for reagent preparation to guarantee the accuracy and repeatability of the results.

2.1.1. Raw material preparation

The frozen DF were thawed at 4 ℃ overnight (12 h) and then dried in a preheated oven at 50 ℃ for 24 h. The cooled-to-room-temperature DF were immediately ground into DF powders and stored in airtight sample bottles at –20 ℃. The fish powders were continuously defatted with acetone using a powder/solvent ratio of 1/10 (g of solute/10 mL of solution) for 30 min. The mixture was then left to stand at room temperature, and the supernatant was discarded once it became transparent. The solvent extraction was repeated three times, and the defatted flours were spread evenly on a clean tray in a fume hood for 12 h to dry, followed by grinding and storage in tightly capped bottles at –20 ℃.

2.1.2. Production of DFPIs

The DFPIs were prepared by a previously established protocol [32]. As shown in Figure 1, the defatted DF flour was mixed with DDW (5:100, g of solute/100 mL of solution) and adjusted to pH 10 through the addition of 1 M NaOH and stirred continuously for 1 h at room temperature. The aqueous mixture was centrifuged at 1600 × *g* for 30 min, after which the supernatant was filtered through a cheesecloth (grade 90, 40 × 36 thread count). The filtrate was adjusted to pH 4.5 using 1 M HCl and stirred constantly for 30 min at room temperature, after which it was centrifuged (1600 × *g* for 30 min). The precipitate was collected, washed with DDW twice (each washing was followed by centrifugation), and then adjusted to pH 7.0 before freeze-drying as the DFPIs. The DFPIs were transferred into airtight sample bottles and stored at –20 °C. Protein content was determined using the modified Lowry method [33] after complete solubilization in a 0.1 M NaOH solution. Protein yield was calculated as follows:

$$\text{Protein yield (\%)} = (\text{weight of freeze-dried DFPI} \times \text{DFPI protein content})/(\text{weight of DF flour used for extraction} \times \text{fish flour protein content}) \times 100\ \%, \quad (1)$$

where the weight of freeze-dried DFPI and the weight of DF flour are measured in grams (g), whereas DFPI protein content and fish flour protein content are expressed in percentages (%).

2.2. Structural properties of DFPIs

2.2.1. Surface hydrophobicity

Surface hydrophobicity (H_o) was determined using the method of Haskard and Li-Chan [34] with 1-anilino-8-naphthalenesulfonate (ANS) as the probe. Solutions of each DFPI (10 mg protein/mL) were prepared by dissolving the sample in a 10 mM phosphate buffer (pH 7.0). The solutions were then thoroughly vortexed and hydrated for 1 h at room temperature, followed by centrifugation at 11,200 × *g* for 10 min. The supernatants were diluted into a series of concentrations, ranging from 50 to 250 μg/mL, with a 10 mM phosphate buffer (pH 7.0). A 5-μL aliquot of 8 mM ANS prepared in a 10 mM phosphate buffer (pH 7.0) was added to every 200 μL of the diluted protein solution. The fluorescence intensity (FI) of each sample was measured in a Jasco FP-6300 spectrofluorometer (Jasco Inc., Tokyo, Japan) at an excitation wavelength of 390 nm and an emission wavelength of 470 nm. The H_o of each sample was calculated as the slope of the FI versus protein concentration plot.

2.2.2. Circular dichroism

The secondary and tertiary structures of the DFPIs were determined by obtaining the far- and near-ultraviolet (UV) spectra on a Jasco J-810 spectropolarimeter (Jasco Inc., Tokyo, Japan), respectively. Sample solutions (10 mg protein/mL) were prepared by vortexing and hydrating DFPIs in a 0.1 M phosphate buffer (pH 7.0). This was followed by centrifugation (11,200 × *g* for 30 min at room temperature) to obtain a clear supernatant. The supernatants were then diluted into 2 mg protein/mL and 6 mg protein/mL for far- and near-UV spectra measurements, respectively. The far-UV spectra were measured at 190–240 nm in a cuvette with a 0.05-cm path length, while the near-UV spectra were measured at 250–320 nm in a cuvette with a 0.1-cm path length. All circular dichroism (CD) spectra were obtained by calculating the average of three consecutive scans and subtracting the

corresponding buffer spectrum. The far-UV data were deconvoluted to obtain the secondary structure fractions using the DichroWeb SELCON3 algorithm [35] (http://dichroweb.cryst.bbk.ac.uk/html/home.shtml, accessed 10 June 2023).

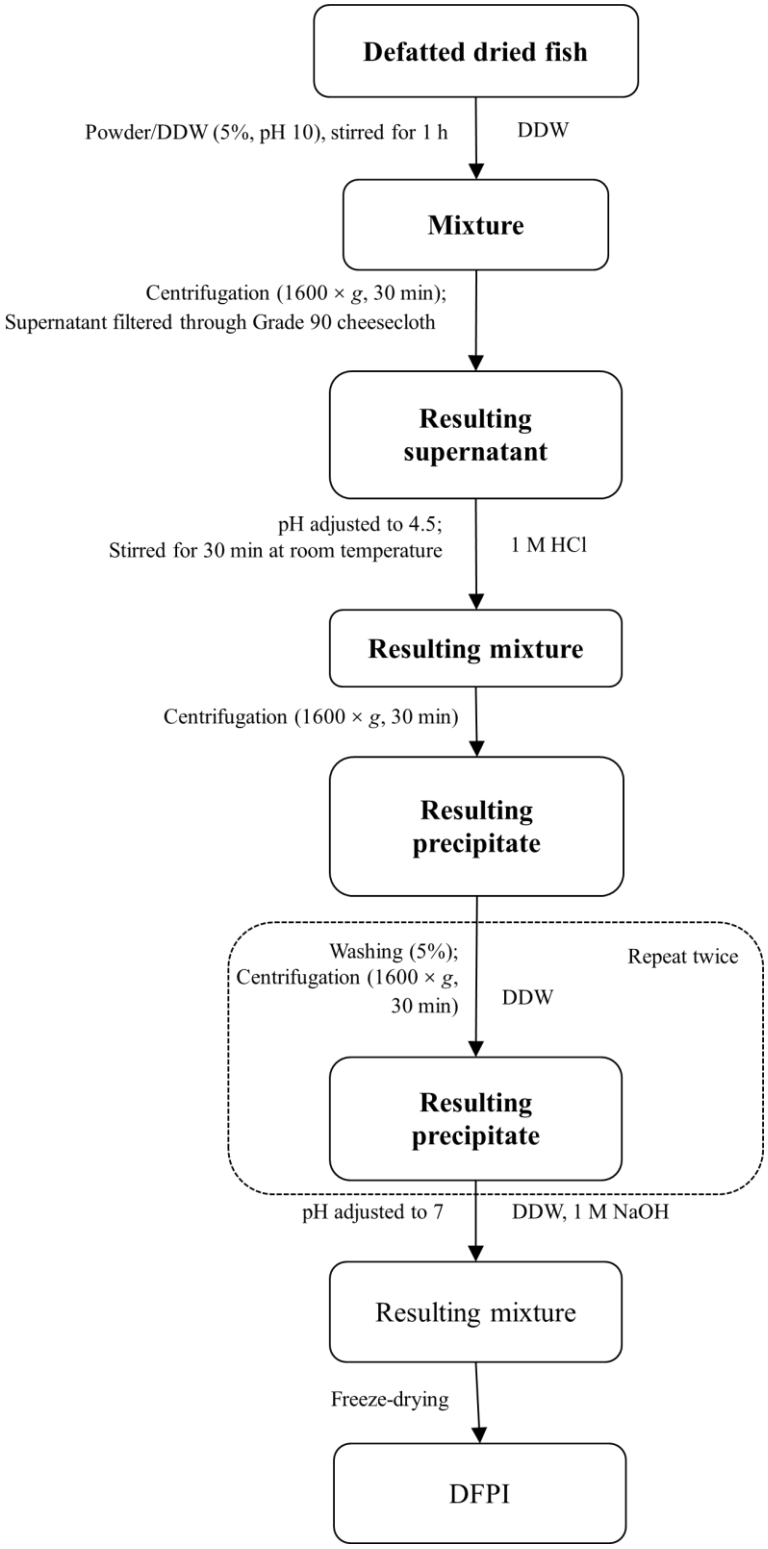

Figure 1. Preparation of DFPI by isoelectric point sedimentation method.

2.2.3. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

The polypeptide composition of DFPIs were determined by conducting sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on a Mini-Protean electrophoresis unit (Bio-Rad Laboratories, Inc., California, USA) according to the method described by Laemmli [36] and Raikos et al. [37] with slight modifications. The DFPI sample (6 mg protein/mL) was dispersed in a 5% sodium dodecyl sulfate (SDS) solution, heated in a water bath at 90 °C for 5 min, cooled to room temperature, and then centrifuged (5000 × *g*, 10 min). An equal volume of Laemmli buffer was mixed with the protein solution to obtain a protein concentration of 3 mg/mL. This was used as the nonreducing sample. Reducing samples were prepared in the presence of 2-mercaptoethanol (1:19:20, v/v/v, 2-mercaptoethanol–Laemmli buffer–protein solution) and incubated in a water bath at 90 °C for 5 min. After centrifugation, 5 μL of each sample and 10 μL of a standard protein mixture were loaded onto each lane on a 4–15% Mini-Protean® TGX™ precast gel and run in a Mini-Protean II electrophoresis tank at 150 V for 1 h. The gels were stained with Coomassie Brilliant Blue R-250 solution for 1 h, and destained using an aqueous solvent solution containing 50 mL/100 mL methanol and 10 mL/100 mL acetic acid aqueous solution for 2 h.

2.3. Functional proprieties of DFPIs

2.3.1. Protein solubility

The solubility of the DFPIs were determined using a previously published method with some modifications [38]. Each sample (10 mg) was vortexed and hydrated thoroughly in 5 mL of a 0.1 M phosphate buffer (pH 7.0) for 1 h. The resulting mixture was then centrifuged at 1600 × *g* for 30 min at room temperature. The supernatant was collected, and the protein content determined was using the modified Lowry method [33]. The total protein content of each DFPI was determined after hydrating the sample in a 0.1 M NaOH solution for 1 h to ensure total dissolution. The protein solubility (PS) of each DFPI was calculated as follows:

$$\text{PS (\%)} = (\text{protein content of supernatant})/\text{total protein content of sample}) \times 100\%. \quad (2)$$

2.3.2. Heat coagulability

The heat coagulability (HC) of the DFPIs were determined using a slightly modified method [32]. Briefly, the aqueous DFPI mixture (10 mg/mL in a phosphate buffer, pH 7.0) was heated at 100°C in a water bath for 15 min and then cooled to room temperature. The cooled mixture was centrifuged (1600 × *g* for 30 min at room temperature) and the protein content (PC I) of the supernatant was determined using the Lowry method [33]. Meanwhile, the total amount of protein (PC II) in the sample prior to heating was also determined by the Lowry method [33]. The HC of each DFPI was calculated as follows:

$$\text{HC (\%)} = (\text{PC II} - \text{PC I})/\text{PC II} \times 100. \quad (3)$$

2.3.3. Water- and oil-holding capacity

The water-holding capacity (WHC) and oil-holding capacity (OHC) of the DFPIs were determined using a previously outlined method with some modifications [38]. Each sample mixture (40 mg protein/mL liquid) was prepared with a phosphate buffer (pH 7.0) or pure canola oil in preweighed 15-mL centrifuge tubes. The samples were vortexed and then allowed to stand for 30 min at room temperature. The mixture was then centrifuged for 15 min at 1600 × *g*, followed by draining of the excess buffer or oil, after which the weight was obtained as WIII. The WHC and OHC of each DFPI were determined using the following equations:

$$\text{WHC (g of water/g of protein)} = (\text{WIII} - \text{WI})/\text{WII}; \quad (4)$$

$$\text{OHC (mL of oil/g of protein)} = (\text{WIII} - \text{WI}/0.92\ \text{g/mL})/\text{WII}, \quad (5)$$

where 0.92 g/mL is the density of pure canola oil, WI is the empty tube + sample weight, WII is the protein sample's weight, and WIII is weight of the tube + residue after draining the buffer or oil.

2.3.4. Least gelation concentration

The least gelation concentration (LGC) was determined by the method of Malomo et al. [38]. Different sample concentrations (2–20 g/100 mL, protein weight basis) were dispersed in DDW and thoroughly vortexed in 5-mL glass tubes. The samples were heated in a 95 °C water bath for 1 h, cooled rapidly under tap water, and then refrigerated (4 °C) for 14 h. The sample concentration at which the gel did not slip upon inverting the tube was taken as the LGC.

2.3.5. Emulsion formation and stability

Oil-in-water emulsions were prepared by homogenizing 5 mL of 10, 15, and 20 mg protein/mL (prepared in 0.1 M phosphate buffer, pH 7.0) with 1 mL of pure canola oil at 20,000 rpm for 2 min as described by Chao et al. [39]. The homogenizer (Polytron PT 10-35, Kinematica AG, Lucerne, Switzerland) was equipped with a 12-mm generator. The oil droplet size ($d_{3,2}$) of the emulsion was determined in a Mastersizer 3000 (Malvern Instruments Ltd., Malvern, U.K.) with distilled water as the dispersant. The emulsified sample was added to the sample dispersion unit (Hydro 3000S, attached to the instrument) containing approximately 100 mL of water under constant shear until the desired level of obscuration was reached. The instrument was set to automatically measure the emulsion's oil droplet size in five replicates, with each sample prepared in duplicate. The mean oil droplet size ($d_{3,2}$) of each sample was used as an indicator of emulsifying capacity (EC). The emulsified sample was then allowed to stand at room temperature for 30 min, and the oil droplet size distribution and average particle size ($d_{3,2}$) of each sample was measured again as an indicator to evaluate the emulsion stability (ES). ES was calculated as follows:

$$\text{ES (\%)} = \text{oil droplet size at 0 min } (d_{3,2})/\text{ oil droplet size after 30 min } (d_{3,2}) \quad (6)$$

2.4. Statistical analysis

Duplicate replications were used to obtain mean and standard deviation. One-way analysis of

variance (ANOVA) was conducted in SPSS 28.0, followed by Duncan's multiple range test with significance accepted at $p < 0.05$ (IBM, Armonk, NY, USA).

3. Results and discussion

3.1. Protein content and yield

Except for BD (44.70 ± 0.01%), all the DFPIs had a ≥70% protein content (Table 1), which agrees with a previous report [2]. However, the protein content of BD obtained in present study is within the 36–53% range that was reported by Chen & Jaczynski [40] for rainbow trout protein isolates. The DFPI yield (Table 1) depended on the fish species, with the yield of BD and FM significantly ($p < 0.05$) lower than those of the other DF, while GR had the highest value. The low protein yield of FM may be due to the fermentation process, which could have converted the native proteins into soluble peptide fragments with reduced protein–protein aggregation capacity. The protein yield of GR is consistent with the 46% protein recovery of silver carp (*Hypophthalmichthys molitrix*) when extracted at pH 10 [41]. In addition to the extraction conditions (pH and temperature), factors such as the processing treatments, storage conditions, and protein oxidation level could also have affected the yield of DFPIs.

Table 1. Protein content, yield and surface hydrophobicity of DFPIs at pH 7.0.

Sample ID	Protein content (%)	Protein yield (%)	Hydrophobicity (H_o)
Bombay duck	44.70 ± 0.01^{g}	6.65 ± 1.33^{e}	13064.00 ± 1.33^{a}
Ribbon fish	87.32 ± 0.41^{a}	24.95 ± 1.47^{c}	3900.85 ± 1.33^{d}
White sardine	81.64 ± 0.68^{b}	36.94 ± 1.34^{b}	654.56 ± 1.33^{f}
Freshwater barb	69.57 ± 0.97^{f}	18.09 ± 1.42^{d}	5077.95 ± 1.33^{c}
Ganges River sprat	77.39 ± 0.79^{d}	51.18 ± 0.29^{a}	501.44 ± 1.33^{f}
Fermented barb	78.60 ± 0.05^{c}	5.64 ± 0.00^{e}	6502.80 ± 1.33^{b}
Fermented anchovies	73.65 ± 0.61^{e}	18.4 ± 1.27^{d}	2296.25 ± 1.33^{e}

Within each column, mean values with different letters are significantly ($p < 0.05$) different.

3.2. Surface hydrophobicity

BD had the highest H_o, which is significantly higher than those of the other DFPIs (Table 1). A higher H_o indicates that the BD DFPIs have more exposed hydrophobic groups, which suggests that the proteins may have become more unfolded after the drying process compared with the other DFPIs. On the other hand, WS and GR had significantly lower values, indicating that these DFPIs contained proteins with a more folded conformation than the other DFPIs. Tadpitchayangkoon et al. [42] reported an H_o of about 7000 for sarcoplasmic proteins from striped catfish (*Pangasius hypophthalmus*), which is slightly higher than the findings reported in our study, except for BD. Kobayashi and Park [43] suggested that in a FPI extracted at an alkaline pH, there is a resulting lack of restoration of the myosin head structure, which leads to increased exposure of hydrophobic clusters. More importantly, effects from factors such as salting, drying, and microbial digestion will also accelerate protein degradation and lead to the exposure of more hydrophobic clusters, thereby affecting the H_o of DFPIs [44].

3.3. SDS-PAGE

Figure 2 shows the distribution of molecular weight (MW) of the DFPIs' polypeptides. It can be clearly found that no myosin heavy chain (MHC) was detected at 200 kDa in all DFPIs; in addition, actin was also lacking at 48 kDa. These findings suggest that severe degradation occurred in myofibrillar proteins during the processing of DF [45]. In a study of ham peptides' MW distribution, which underwent similar treatments (salting and drying) as the DF, it was reported that heavy chains and actin were degraded into smaller peptide chains under the combined action of salt [46], endogenous muscle peptidases, and microbial enzymes [47]. In the current study, the MW of DFPIs mainly showed broad bands at 25–37 kDa and 10–15 kDa, which are very similar to the broad bands at 14–20 kDa found in sarcoplasmic proteins of ripened ham [47]. The findings of bands at 34 kDa and 25 kDa in the reducing gel agree with the bands at 34.9 kDa and 25.6 kDa reported in ham's sarcoplasmic proteins [47]. There was a notable band of about 250 kDa in the nonreducing gel, which could have been protein aggregates formed during the drying process. These aggregates are absent in the reducing gel, an indication that the polymeric units were held together by disulfide bonds.

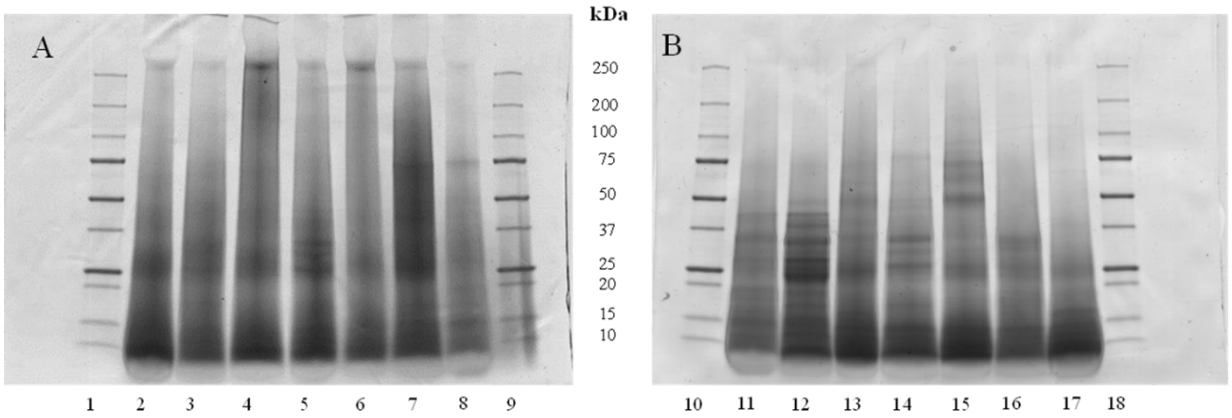

Figure 2. SDS-PAGE patterns of DFPIs. (A) Nonreducing gel; (B) reducing gel. Lanes 1, 9, 10, and 18: molecular weight standards; Lanes 8 and 11: BD; Lanes 7 and 12: RF; Lanes 6 and 13: WS; Lanes 5 and 14: FB; Lanes 4 and 15: GR; Lanes 3 and 16: FM; Lanes 2 and 17: FA. BD, Bombay duck; RF, ribbon fish; WS, white sardine; FB, freshwater barb; GR, Ganges River sprat; FM, fermented barb; FA, fermented anchovies.

3.4. Circular dichroism

Secondary and tertiary structure data are crucial to understanding proteins, as the values can reflect the degree of protein degradation and denaturation. Meanwhile, the content of each secondary fraction also affects the functional properties of the proteins. As shown in Table 2, the most predominant secondary structure detected in the DFPIs were random coils (35–40%), followed by β-sheets (20–35%), β-turns (16–25%), and α-helices (6–21%). Sun et al. [48] reported that in myosin extracted from *Decapterus maruadsi*, the contents of α-helices, β-structures (including β-turns and β-sheets), and random coils were approximately 47%, 27%, and 26%, respectively. In contrast, the secondary structures of the DFPIs in this study were markedly different, characterized by lower α-

helix content and higher random coil content, indicating a high degree of protein denaturation. Tan et al. [49] found that in tilapia–soy protein co-precipitates, solubility was negatively correlated with α-helix content and positively correlated with β-sheet content. This pattern aligns with the characteristics observed in the DFPI extracted from the BD sample in this study. However, DFPIs from samples such as FA and GR displayed the opposite trend: Relatively low α-helix and high β-structure content but extremely poor solubility. This suggests that factors such as random coil content and amino acid composition may also play important roles in determining the solubility of DFPIs. Additionally, given the highly denatured and hydrolyzed nature of the DFPIs, reflected in the SDS-PAGE results, where BD and FB showed weaker and less distinct bands compared with the other samples, it is speculated that the degree of protein hydrolysis may also contribute to the increased solubility. Beyond solubility, secondary structure also affects functional properties such as gelation. Liu et al. [50] reported a positive correlation between β-sheet content and both gelation ability and gel strength. This is generally consistent with the low LGCs observed in the BD, RF, and FB samples in this study, suggesting relatively stronger gel-forming capacities.

Table 2. Secondary structure fractions of DFPIs at pH 7.0.

Sample ID	α-helices	β-sheets	β-turns	Random coils
Bombay duck	9.30 ± 1.27^{bc}	30.40 ± 0.99^{a}	25.25 ± 8.13^{a}	35.00 ± 7.92^{b}
Ribbon fish	16.25 ± 0.64^{ab}	26.40 ± 0.57^{ab}	16.75 ± 0.64^{b}	40.65 ± 0.92^{ab}
White sardine	5.75 ± 3.89^{c}	35.00 ± 4.53^{a}	18.55 ± 0.78^{ab}	40.75 ± 1.48^{ab}
Freshwater barb	6.50 ± 3.68^{c}	30.70 ± 2.97^{a}	15.95 ± 0.35^{b}	46.85 ± 0.35^{a}
Ganges River sprat	8.60 ± 4.81^{c}	30.60 ± 4.10^{a}	18.05 ± 1.06^{ab}	42.70 ± 0.28^{ab}
Fermented barb	21.25 ± 0.78^{a}	19.80 ± 2.83^{b}	19.45 ± 0.21^{ab}	39.50 ± 1.70^{ab}
Fermented anchovies	5.75 ± 3.04^{c}	35.10 ± 5.52^{a}	18.25 ± 0.21^{ab}	40.95 ± 2.19^{ab}

Within each column, mean values with different letters are significantly ($p < 0.05$) different.

The near-UV CD signal originates from the chirality of the side chain environment of amino acid residues, reflecting the strength of the interactions between the amino acid residues, thereby revealing changes in the tertiary structure of proteins [49]. Each aromatic amino acid tends to show a typical peak range in the near-UV CD spectrum, such as tyrosine at 275–282 nm, phenylalanine at 255–270 nm, and tryptophan at 290–305 nm [51]. In the current study, the GR proteins showed the most intense positive tyrosine peak at 275–282 nm, which indicates a more folded tertiary conformation compared with the other DFPIs (Figure 3). BD and FB proteins had smaller negative tyrosine peaks, while those of FA, WS, RF, and FM (which overlapped with WS) had no distinct peak and lower ellipticity values that are close to zero. Therefore, the results suggest that FA, WS, RF, and FM proteins had negligible folded structures and exist mostly in disorganized conformations.

3.5. Protein solubility

The PS of the DFPIs also varied significantly according to the DF species, with the highest value found in BD and lowest in FA (Table 3). Previous reports have pointed out that in the range of pH 2–12, the plot of solubility versus pH for most animal and plant proteins has a V-shaped distribution, with the lowest solubility being previously reported in the pH 5–6 range [40,41,52–54]. In the acidic pH range, solubility of FPI is relatively low and will significantly increase when the pH value increases

beyond that range [55]. With exception of the BD and freshwater barb (FB and FM) samples, the other species had protein isolates with low solubility at pH 7. While the yield of FM was low, the higher protein solubility compared with the unfermented equivalent (FB) suggests that fermentation may have produced more soluble proteins because of the proteolytic action of endogenous enzymes. It is also possible that the extracted proteins from BD, FB, and FM did not experience severe adverse denaturing effects to their structures during processing compared with the other species.

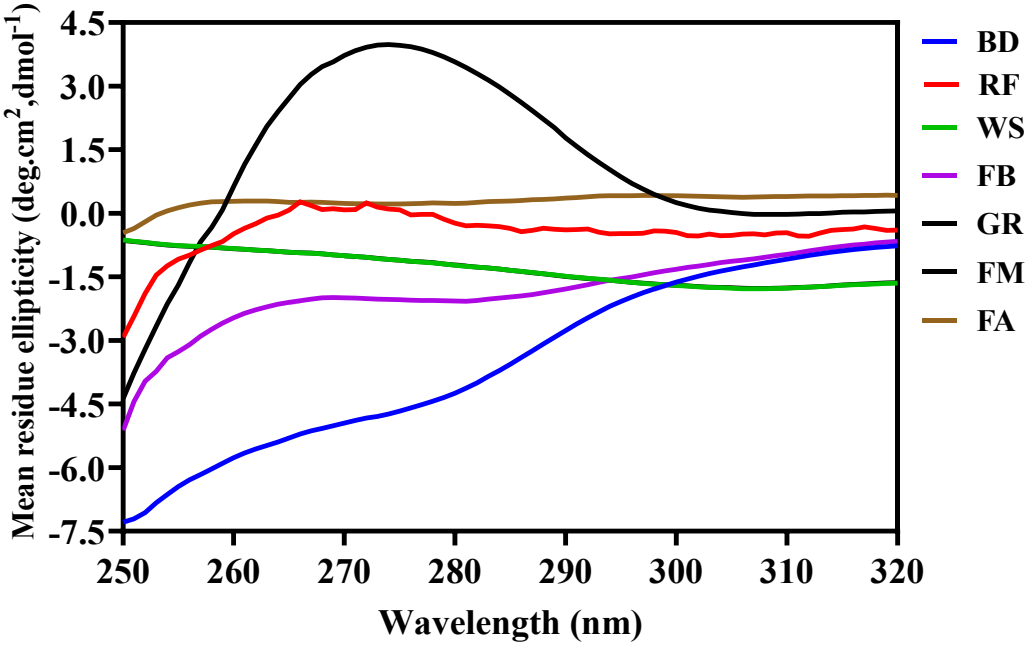

Figure 3. Near-UV circular dichroism spectra of DFPIs at pH 7, BD, Bombay duck; RF, ribbon fish; WS, white sardine; FB, freshwater barb; GR, Ganges River sprat; FM, fermented barb; FA, fermented anchovies.

3.6. Heat coagulability

Overall, the heat coagulation (HC) values of all DFPIs were below 25%, with GR exhibiting the highest HC value at 23.33% and FM the lowest at 0.76% (Table 3). The relatively high HC of GR-DFPI corresponds with its higher content of ordered protein structures, as indicated by the near-UV spectra (Figure 2). Upon heating, these ordered structures in GR tend to unfold, leading to the aggregation of polypeptides into insoluble complexes. In contrast, other DFPIs contain fewer folded structures, as shown by the absence of characteristic peaks in the near-UV spectra, making them less sensitive to heat and less likely to form protein-protein aggregates. In addition, increasing the degree of protein hydrolysis contributes to enhanced thermal stability. Hidalgo & Gamper [56] reported that trypsin treatment significantly improved the heat stability of whey proteins. In this study, the presence of a large proportion of low-molecular-weight peptides (under 15 kDa) observed in SDS-PAGE suggests that the DFPIs possessed moderate to high thermal stability. Furthermore, FM and FA were derived from fermentation processes, which likely increased the degree of hydrolysis and thereby reduced the potential for protein-protein interactions. Thermal treatment is a common procedure in

food processing, with significant influence on protein functionality within food systems. During heating, native proteins undergo denaturation and subsequently aggregate via intermolecular β-sheet interactions to form insoluble high-molecular-weight complexes [57]. Therefore, maintaining thermal stability is essential. The low HC values observed in this study at pH 7.0—particularly for FM and FA—suggest that heat treatment had minimal effects on protein solubility. This makes DFPIs promising ingredients for formulating heat-processed liquid food products [57].

3.7. WHC and OHC

In the present study, at pH 7.0, the highest WHC was found in BD, while the lowest was in FM (Table 3). A previous study [58] reported a significant positive correlation ($p < 0.001$) between the WHC of muscle proteins and the thermal stability of myosin tails and sarcoplasmic proteins. However, this finding is inconsistent with our current observation in Section 3.6, where FM displayed both the highest thermal stability and the lowest WHC. This contradiction further supports the conclusion that the DFPIs in this study have undergone extensive hydrolysis and no longer retain the structural characteristics of native myofibrillar or sarcoplasmic proteins. The WHC values of the other DFPIs fell between those of BD and FM. These differences likely stem from substantial variations in their degree of hydrolysis, solubility, conformation, and amino acid composition—factors that inherently affect water retention. For example, FA showed a WHC of 5.18 ± 0.15 g/g, which is consistent with the 6 g/g at pH 7.0 that was reported in a previous study on Argentine anchovy (*Engraulis anchoita*) protein isolates [55]. Similarly, in a previous study [59], the WHC of four FPIs (catfish, *Batrachocephalus mino*; Indian mackerel, *Rastrelliger kanagurta*; ponyfish, *Aurigequula fasciata*; and sardine, *Sardinella brachy-soma*) were reported to be 4.23 ± 0.15 to 4.80 ± 0.05 g/g, which is slightly lower than some of our findings. The WHC of proteins plays a crucial role in sensory aspects such as mouthfeel, flavor retention, and texture when developing a new product [59]. Therefore, DFPIs obtained from BD, FB, and GR with a higher WHC may find uses as ingredients to formulate foods with a moist texture. The poor WHC of FM is consistent with the high protein solubility, which indicates that the proteins formed a solution rather than a solid matrix that binds to or holds water molecules.

As for OHC, BD exhibited the highest value, while FA showed the lowest (Table 3). The DFPIs in this study demonstrated relatively high OHC values, exceeding those previously reported for protein hydrolysates from grass carp skin (3.6 mL/g), Nile perch skin (3.4 mL/g), and Nile tilapia skin (3.8 mL/g) [60]. One possible explanation is the photo-oxidation of fish proteins during sun-drying in this study, which may have led to the unfolding of protein structures, thereby exposing more hydrophobic groups and lipophilic amino acids that can interact with oil molecules [60]. In addition, near-UV spectroscopy (Figure 2) revealed that DFPIs possess loose tertiary structures, which could further contribute to the enhanced OHC [60]. Similar to WHC, significant differences in OHC among the various DFPIs are likely attributed to differences in the degree of hydrolysis, solubility, protein conformation, and amino acid composition. Compared with previous findings, the OHC values in this study also surpass those of protein isolates from white croaker (8 mL/g) and Argentine anchovy (7.5 mL/g) [55]. OHC is an important functional property in the food industry, particularly in the formulation of meat products and confectionery, and is closely linked to flavor retention, as most flavor compounds are soluble in the lipid phase of food systems [60]. From this perspective, DFPIs such as BD and FB, which exhibit excellent OHCs, may perform well as functional ingredients in food formulations where oil retention is crucial for product quality and consumer acceptance.

Table 3. Functional properties of DFPIs at pH 7.0.

Sample ID	Heat coagulability (%)	Water-holding capacity (g/g)	Oil-holding capacity (g/g)	Least gelation concentration (%)	Protein solubility (%)
Bombay duck	6.23 ± 0.41 ^{bc}	7.00 ± 0.47 ^{a}	20.13 ± 0.14 ^{a}	3	85.03 ± 1.06^{a}
Ribbon fish	7.36 ± 1.26 ^{bc}	2.79 ± 0.04 ^{d}	15.38 ± 0.72 ^{c}	3	58.90 ± 1.76^{d}
White sardine	6.60 ± 2.94 ^{bc}	3.82 ± 0.05 ^{c}	18.35 ± 0.76 ^{ab}	5	28.13 ± 0.79^{e}
Freshwater barb	11.18 ± 2.16 ^{b}	5.36 ± 0.05 ^{b}	17.85 ± 1.15 ^{ab}	3	68.53 ± 0.51^{c}
Ganges River sprat	23.33 ± 1.16 ^{a}	5.48 ± 0.08 ^{b}	16.43 ± 0.18 ^{bc}	5	28.36 ± 0.11^{e}
Fermented barb	0.76 ± 1.07 ^{d}	0.01 ± 0.01 ^{e}	17.23 ± 1.43 ^{bc}	7	72.71 ± 1.15^{b}
Fermented anchovies	2.89 ± 4.08 ^{cd}	5.18 ± 0.15 ^{b}	12.28 ± 1.49 ^{d}	6	19.35 ± 0.34^{f}

Within each column, mean values with different letters are significantly ($p < 0.05$) different.

3.8. Least gelation concentration

The gelling ability of the DFPIs, measured as the LGC, differed according to the type of DF, with proteins from fermented fish (FM and FA) having the highest LGC, which indicates poorer gel formation when compared with the nonfermented fish proteins (Table 3). The lower the value, the stronger the gelling ability of the protein. The lower gelling ability of the FA and FM proteins may be due to polypeptide fragmentation during fermentation, which reduced the strength of the protein–protein interactions needed to form a three-dimensional network. The negative effect of fermentation on protein gelation is further supported by the stronger gelling ability of FB, the nonfermented equivalent of FM. In general, the gelling ability of the DFPIs is better than those of the isolates extracted from legumes, as the reported LGCs of soybean, pea, faba bean, and lentil ranged between 12% and 15% [61], which are higher than the values found in the present study. The current findings of the LGC of the DFPIs agree with the finding of 6% LGC in alkali-extracted saithe (*Pollachius virens*) isolates [11]. The LGC of DFPIs are comprehensively affected by different factors, including the degree of denaturation of myofibrillar proteins and the ratio of myofibrillar to sarcoplasmic proteins [62]. In addition, factors such as the molecular weight distribution, the effective volume fraction, and the chemical and physical interactions formed by proteins during the thermal treatment will also affect the LGC of DFPIs [61]. The high gelling ability of most of the DFPIs suggests that they may be used to make fish protein gels like surimi, which are important ingredients in the formulation of imitation shellfish products.

3.9. Emulsion formation and stability

In the food industry, protein isolates are widely used as emulsifiers in multiple food systems, such as beverages, sausages, salad dressings, cakes, and soups, because of their ability to reduce the interfacial tension between water and the lipid phase and form a protective coating that prevents the coalescence of oil droplets [61]. Droplet size reduction is the key target when it comes to the ability to form an emulsion. In the present study, the DFPIs at all concentrations showed a relatively good emulsion-forming ability, as the maximum droplet size at pH 7.0 was found in RF (~4.5 μm), while the minimum was as small as ~2 μm for the emulsion formed with 20 mg/mL BD (Figure 4A). The emulsion oil droplet sizes obtained in current study with values of about 3.6 μm are consistent with a

previously reported oil droplet size of 20 mg/mL for hydrated raw sardine protein (3.672 ± 0.179 μm) at pH 2.0 [63]. However, at pH 2.0, fish proteins are more soluble than at pH 7.0 and are consequently easier to adsorb at the oil–water phase, which may also explain the smaller droplet size found in BD (which had the highest solubility among all DFPIs; see Table 3). Within the same pH and protein concentration, an even smaller oil droplet size of DFPI emulsions can be expected. Ma et al. [61] reported that in addition to solubility, protein concentration also significantly affected the droplet size, as a significant decrease in the droplet size of emulsions formed with soy protein isolate was obtained at a 10 mg/mL concentration (2.5 μm) compared with 19 μm for 1 mg/mL. A less severe but statistically significant ($p < 0.05$) decrease in droplet size with an increase in protein concentration was also observed in the present study, which may suggest that the DFPI concentrations used here were sufficient to form good emulsions.

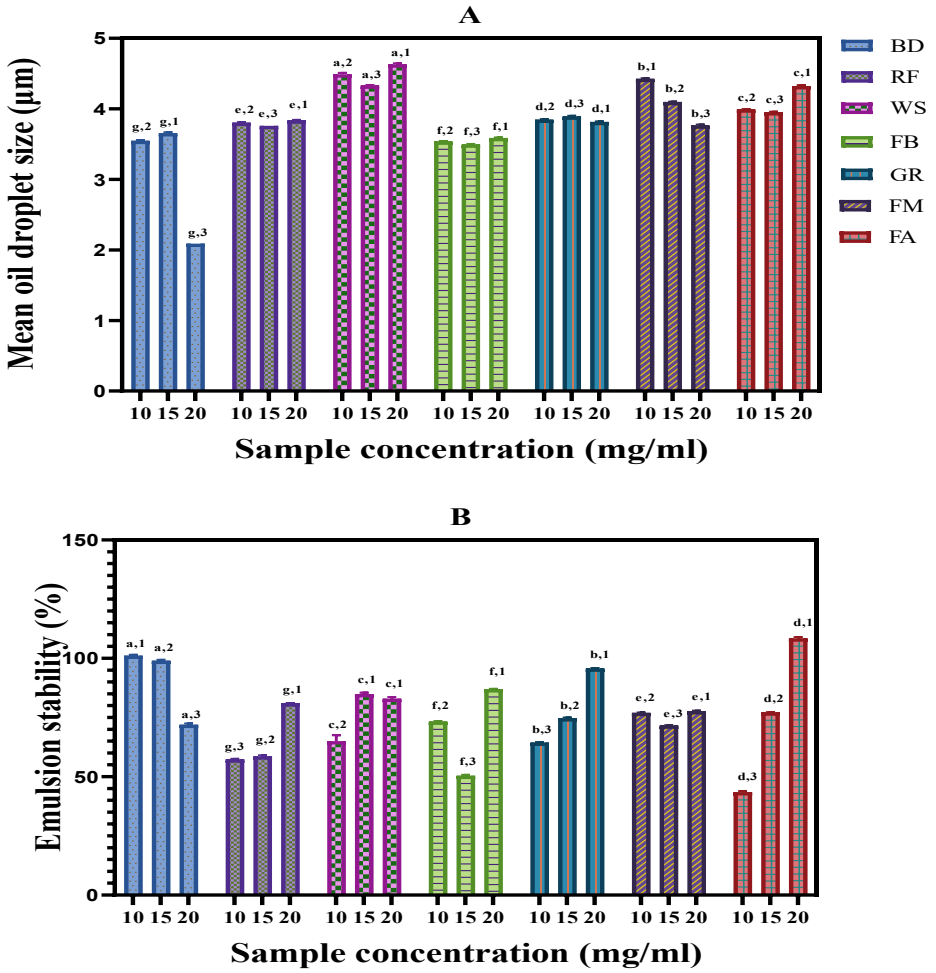

Figure 4. Emulsification (A) and emulsion stability (B) properties of DFPIs at pH 7.0. (A) Emulsion-forming ability; (B) ES. BD, Bombay duck; RF, ribbon fish; WS, white sardine; FB, freshwater barb; GR, Ganges River sprat; FM, fermented barb; FA, fermented anchovies. Different letters (a, b, and c) represent significant ($p < 0.05$) differences between fish types via two-way ANOVA. Different numbers (1, 2, and 3) represent significant ($p < 0.05$) differences within the same fish type and different protein concentrations (mg/mL) determined by one-way ANOVA.

Figure 4B provides information on emulsion stability (ES), which generally indicates that BD had the best ES overall, as reflected in the 100% value at 10 and 15 mg/mL. The underlying reason for this may be related to the higher solubility of BD, which would have enabled the formation of packed and strong interfacial oil–water membranes. In the current study, the findings of ES values close to 70% at 10 mg/mL protein content are consistent with a previously reported ES of 71.3% [62], which was observed for saithe (*Pollachius virens*) emulsions. Apart from the solubility, protein concentration may be another critical factor in maintaining a stable emulsion, as detected in RF, WS, GR, and FA, where ES increased significantly ($p < 0.05$) with increases in the protein concentration. The results are consistent with the findings of Rajasekaran et al. [64], who applied an ultrasonic treatment to form fish protein-coated shrimp oil emulsions, and the ES increased from 61.54% to 84.75% with increasing concentrations of 15–45 mg/mL after 15 days of storage. This can be explained by the presence of more protein molecules at the oil–water interface, which interact together to form strong membranes around the oil droplets, providing stability against oil droplet coalescence.

4. Conclusions

SDS-PAGE structural analysis revealed that the DFPIs are primarily composed of denatured, unfolded, and fragmented myofibrillar proteins, which accounts for the typically low extraction yield via isoelectric precipitation. Additionally, the drying process significantly altered the protein structure, resulting in a predominance of random coils and reduced α-helix content. At a neutral pH (7.0), DFPIs demonstrated low sensitivity to heat-induced coagulation, indicating their suitability for formulating heat-processed liquid foods. Their OHC, excellent gelling ability, and relatively strong emulsifying properties suggest that DFPIs hold promise as heat-resistant emulsifiers in the food industry. Future work should focus on optimizing the DFPI extraction process to improve yield and further evaluate their emulsifying performance under varying pH and high-temperature conditions.

Use of AI tools declaration

The authors declare they have not used artificial intelligence (AI) tools in the creation of this article.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

Huan Sun: Methodology, investigation, data curation, writing—Original draft and editing. Rotimi E. Aluko: Conceptualization, methodology, supervision, funding acquisition, writing—Review and editing. Derek S. Johnson: Project administration, supervision, conceptualization, writing—Review and editing, funding acquisition.

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