Snakehead Fish, *Channa striata* (Bloch, 1973), Protein Concentrate: Excellent Recovery of Fish-Based Albumin Source and Its Possible Application for Sperm Capacitation

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Abstract

Snakehead fish protein concentrate (SFC) shows promising recovery of excellent protein content that has broad potential applications, including the future use of its albumin content as a sperm capacitation promoter. However, the quantity of snakehead fish albumin recovered depends on several variables, including the habitat in which the fish thrives, extraction procedures, and the assay employed. To optimise the albumin content retrieved from snakehead fish flesh with more reliable results, multiple protein extraction methods were undertaken through the protein concentrate preparation and analysed using the Bromocresol purple (BCP) albumin assay. Total soluble protein (TSP), albumin level, and albumin proportion were recovered at a significant reduction in an acid solvent when exposed to up to 50 °C heating compared to extractions undertaken without heating. Moreover, the small number of protein bands identified indicates that the combination of acid solvent and heating treatment was similarly damaging. In this study, we detected the presence of albumin in SFC at 32 %, the highest percentage ever recorded employing the BCP assay, following extraction with water solvent without thermal treatment. Using SDS-PAGE and densitometric analysis, the identified putative albumin-related bands from the two major bands at 40 kDa and 47 kDa, containing respective proportions of 57 % and 16 % of the total protein loaded. Although this exploratory work requires additional analysis and purification steps, further research employing the SFC is worth identifying its ability and how this alternative albumin source can replace serum albumin to modulate the capacitation process in mammalian sperm.

Keywords: fish processing, albumin content, bromocresol purple, SDS-PAGE, densitometric analysis

Introduction

Snakehead fish, *Channa striata* (Bloch, 1973), a member of the *Channidae* family, is a predatory freshwater fish endemic to South Asia (Adamson et al., 2012). It is a carnivorous fish that consumes a variety of fishes, frogs, insects, earthworms, and tadpoles and is renowned as a powerful and highly adaptable fish in marginal water with low oxygen and pH levels (Rahman et al., 2018; Khasani and Astuti, 2019). It has high economic value and is paramount in Southeast Asia aquaculture, with an annual production of 15.000 tonnes (Lefevre et al., 2012). According to the International Union for Conservation of Nature and Natural Resources (IUCN), the snakehead fish is currently assessed as Least Concern (LC) due to the large population in the wild (Chaudhry et al., 2019). The expansion of the snakehead farming industry and intensive culture either through traditional farming systems, aquaponic, culture buckets, and other captive breeding also helps sustain the population in nature (Hien et al., 2015; Bich et al., 2020; Kumar et al., 2022; Latifah et al., 2022).

Snakehead fish has been reported to contain amino acids, fatty acids, and other high-quality protein contents (Chasanah and Nugraheni, 2017). The excellent amino acid content of its flesh has identified them as one of the promising protein sources for human nutrition. It has become one of the leading food fishes in Thailand, Indochina, and Malaysia (Song et al., 2013). Due to its biochemical composition, snakehead fish extract

is now available as a commercial product that is not only used as a food supplement but also accepted in the medical field as traditional medicine and other pharmacological therapeutics (Rahman and Awal, 2016; Rahman et al., 2018). Several studies have demonstrated the promising outcomes of using this fish extract for wound healing: oral ingestion in humans, transdermal patch, or cream application (Tungadi et al., 2011; Sahid et al., 2018; Yuliana et al., 2022). Besides, snakehead fish extract has also been claimed to have antibacterial properties (Dhanaraj et al., 2009), antifungal, and antinociceptive (Mat Jais et al., 1997; Mat Jais et al., 2008).

According to their solubility, fish muscle proteins are generally differentiated into three types: i) Structural/myofibrillar (consisting of actomyosin, tropomyosin, actin, and myosin that make 70 – 80 % of total protein contents and are soluble in a salt solution), ii) Sarcoplasmic (including globulin, enzymes, and myoalbumin, comprising 25–30 % of total protein which are soluble in a low ionic salt solution), and iii) Muscle proteins (such as stroma protein that are insoluble) (Le Gouic et al., 2018; Khan et al., 2020). As one of the sarcoplasmic proteins, albumin from snakehead fish has previously been claimed to treat hypoalbuminemia, heal burns (Sahid et al., 2018), and post-surgical wounds (Nugroho, 2013). Therefore, this fish is widely consumed to accelerate recovery following vaginal or cesarean deliveries and other surgical procedures (Rahman et al., 2018). Concerning its albumin content, this composition also raises the prospect of further examining its potential use in assisted reproductive technologies (ARTs), particularly for promoting the capacitation process of mammalian sperm where albumin from serum is commonly required (Zhao et al., 2021).

Following this potential availability, the development of fish processing techniques has enhanced the recovery of functional and nutritious fish muscle proteins from snakehead fish to fish protein concentrate (FPC) – a stable fish preparation with a higher protein content than the fish from which it is derived and intended for human consumption (Saleh et al., 2021). This preparation process aims to turn fish from a perishable raw material into a less perishable product by using whole fish, or fish by-products, and employing physical processes such as temperature increase, agitation, ultrasonication, filtration, pressing, drying, or a combination of them, including the use of suitable solvent or enzymes to improve the efficiency of the process. The production of FPC allows the maximum recovery of functional and nutritious fish muscle protein and, thus, facilitates the specific properties of the recovered proteins (including albumin) and their biologically active peptides (BAPs) (Freitas et al., 2015; Kumoro et al., 2022). However, aside from the ecosystem in which the fish grows (Chasanah et al., 2015), the protein concentrations and composition rely on several factors, such as the extraction protocols and the assay used. The various extraction methods for snakehead fish protein concentrate (SFC) from independent studies resulted in different protein yields, which implicitly indicate that the solvent used, solvent-to-meat ratio, temperature treatment, and other extraction procedures influence the protein retrieval from the fish (Kumoro et al., 2022).

Moreover, most studies reporting the detection of albumin compound from snakehead fish utilised the assay with low specificity and affinity to albumin, such as Bradford (Alviodinasyari et al., 2019), Lowry method (Asfar et al., 2014; Romadhoni et al., 2016) and bromocresol green (BCG) (Asfar et al., 2019), resulting in substantial interference with the other soluble protein types. Meanwhile, a better assay with more reliable results to detect albumin using bromocresol purple (BCP) (Ueno et al., 2013; Moreira et al., 2018) has yet to be performed in snakehead fish. Considering the nutritional and functional demands for snakehead fish proteins and the importance of obtaining the highest protein uptake from this fish species, the present study aims to determine the operating conditions that maximise the recovery of protein content, primarily albumin, through FPC preparation by comparing existing extraction protocols using BCP assay. Regarding the albumin content, the possible utilisation of the SFC results as a capacitation promoter in mammalian sperm is also discussed in this study.

Materials and Methods

Ethical approval

No live animals were used in this study. Therefore, no Institutional Animal Care and Use Committee (IACUC) approval was required.

Chemicals

Unless otherwise stated, all chemicals used in this study were purchased from Sigma-Aldrich Chemical Company (St. Louis, USA).

Protein extraction methods

Snakehead fish was purchased from Mae Hia Fresh Market, Chiang Mai Province (18°44'52.4"N 98°56'30.9"E), Thailand. Snakehead fish protein used in this study was extracted from the flesh and converted to snakehead fish protein concentrate (SFC) through various methods (Asfar et al., 2019; Romadhoni et al., 2016). Briefly, snakehead fish fillets were washed, diced, blended with the solvents, and subjected to their respective treatments, which consist of the following different applications: T0 (Buffer solvent with ratio 4:1 to flesh + Heating to 50 °C + Hexane + Low temperature centrifugation + Supernatant recovery); T1 (Water solvent with ratio 4:1 to flesh + Room temperature centrifugation + Supernatant recovery); T2 (Acid solvent with ratio 4:1 to flesh + pH adjustment to 4.6 + Room temperature centrifugation + Pellet recovery); T3 (Acid

solvent with ratio 1:1 to flesh + Heating to 50 °C + Hexane + Low temperature centrifugation + Supernatant recovery); T4 (Water solvent with ratio 1:1 to flesh + Heating to 40 °C + Hexane + Room temperature centrifugation + Supernatant recovery); T5 (Acid solvent with ratio 4:1 to flesh + Heating to 50 °C + Hexane + pH adjustment to 7.4 + Low temperature centrifugation + Molecular filtration + Supernatant recovery) (Fig. 1). The obtained samples were freeze-dried using a freeze-dryer (DW-10, China) and subjected to protein analysis, including the total soluble protein using bicinchoninic acid (BCA) protein assay, bromocresol purple (BCP) albumin assay, and the screening of the protein profiles using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Each treatment was repeated three times.

Since each method has its approach to dissolving protein from the raw flesh, many factors, including the different solvents, their ratio to flesh mass, temperature, and any other extraction steps applied in each treatment, affect the level of dissolved protein, and thus define each method's ability to retrieve soluble protein from raw fish flesh. A comparative analysis was, therefore, conducted with a focus on treatment methods that shared similar concepts and had minimal differences on the abovementioned factors, as well as treatments with notable distinctions that resulted in significant value by carefully considering all variables of measurements.

Molecular weight cut-off (MWCO)

For the treatment involving molecular filtration, a membrane filter with a molecular weight cut-off (MWCO) <30 kDa was employed. The diafiltration was performed using an ultracentrifuge filter (Amicon, Germany) at 10,000 rpm under 4 °C for 20 min prior to freeze-drying.

Pierce bicinchoninic acid (BCA) protein assay

Total soluble protein was assayed using Pierce Bicinchoninic Acid (BCA) protein assay (ThermoFisher, USA) to establish the optimal approach for recovering the total amount of soluble protein from the flesh of snakehead fish. Its absorbance was measured at 540 nm in an ELISA plate reader. Bovine serum albumin (BSA) was used as the standard protein. The analysis was performed following the protocol provided by ThermoFisher Scientific. The ratio 1:8 of sample to working reagent was prepared, loaded into a 96-well plate, and incubated at 37 °C for 30 min. The total soluble protein concentration was quantified according to the standard curve obtained from the protein standard.

Bromocresol purple (BCP) assay

As albumin is a sarcoplasmic or soluble protein and is the primary focus of this study, it contributes to the total amount of soluble protein recovered after the extraction procedure. Therefore, the level is essential for determining the treatment's albumin level prior to its application in the in vitro capacitation medium. The albumin level from various extraction protocols was measured using BCP assay following its technical procedures with slight modification, according to Faizul et al. (2008). In summary, about 5.62 mg of BCP indicator (Loba Chemie, India) was dissolved in 100 mL 0.15PB8-NaCl (5 mM sodium phosphate buffer, pH 8.0 containing 0.14 M NaCl). Bovine serum albumin (BSA) and deionised water were prepared as the albumin standard and the blank, respectively. Ten milligrams of each SFC sample were dissolved in 1 mL of deionised water for sample preparation. Aliquot 60 µL of the sample solutions, standard, and blank were loaded into the 1.5 mL cuvette, added with 1000 µL of BCP solution, and incubated for 10 min at room temperature. The absorbance was measured with a spectrophotometer (Genesys 10 UV-Visible, USA) at 610 nm. The albumin level of SFC was calculated according to the protein standard curve. Meanwhile, the albumin proportion was quantified within 10 mg.mL^{-1} of SFC yield, following the equation below:

$$Albumin\ proportion\ (\%) = \frac{Albumin\ concentration\ (\mathrm{mg.mL^{-1}})}{SFC\ yield\ (\mathrm{mg.mL^{-1}})} \times 100$$

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

The equal concentration of 150 µg.mL^{-1} protein from different extraction methods based on BCA assay was subjected to sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) with 12 % polyacrylamide gel to screen the protein profiles based on their molecular weight (Hidayaturrahmah et al., 2019). Briefly, 25 µL of original (undiluted) SFC were added with 10 µL of 2× Laemmli buffer (Bio-Rad, USA) and incubated at 95 °C for 5 min. The electrophoresis was carried out at a constant voltage of 150 volts for 45 min of gel running, followed by Coomassie brilliant blue (CBB) gel staining, and washed with a de-staining solution. GelAnalyzer (Version 19.1, Hungary) was employed for the densitometric analysis of SDS-PAGE. Any uneven baseline or background staining distribution was corrected with "Background definition mode" prior to further quantitative analysis. The molecular weight of proteins was determined by comparing the protein band's retention factor (Rf) and standard protein markers (Haniffa et al., 2017). Following the equations below, the relative quantity of each band was estimated according to "Raw volume" of GelAnalyzer, which refers to the density of each band from the SDS-PAGE gel image (Eckard et al., 2018).

$$Relative\ quantity\ (RQ)(\%) = \frac{Raw\ volume\ of\ a\ band}{Total\ raw\ volume\ within\ a\ lane} \times 100$$

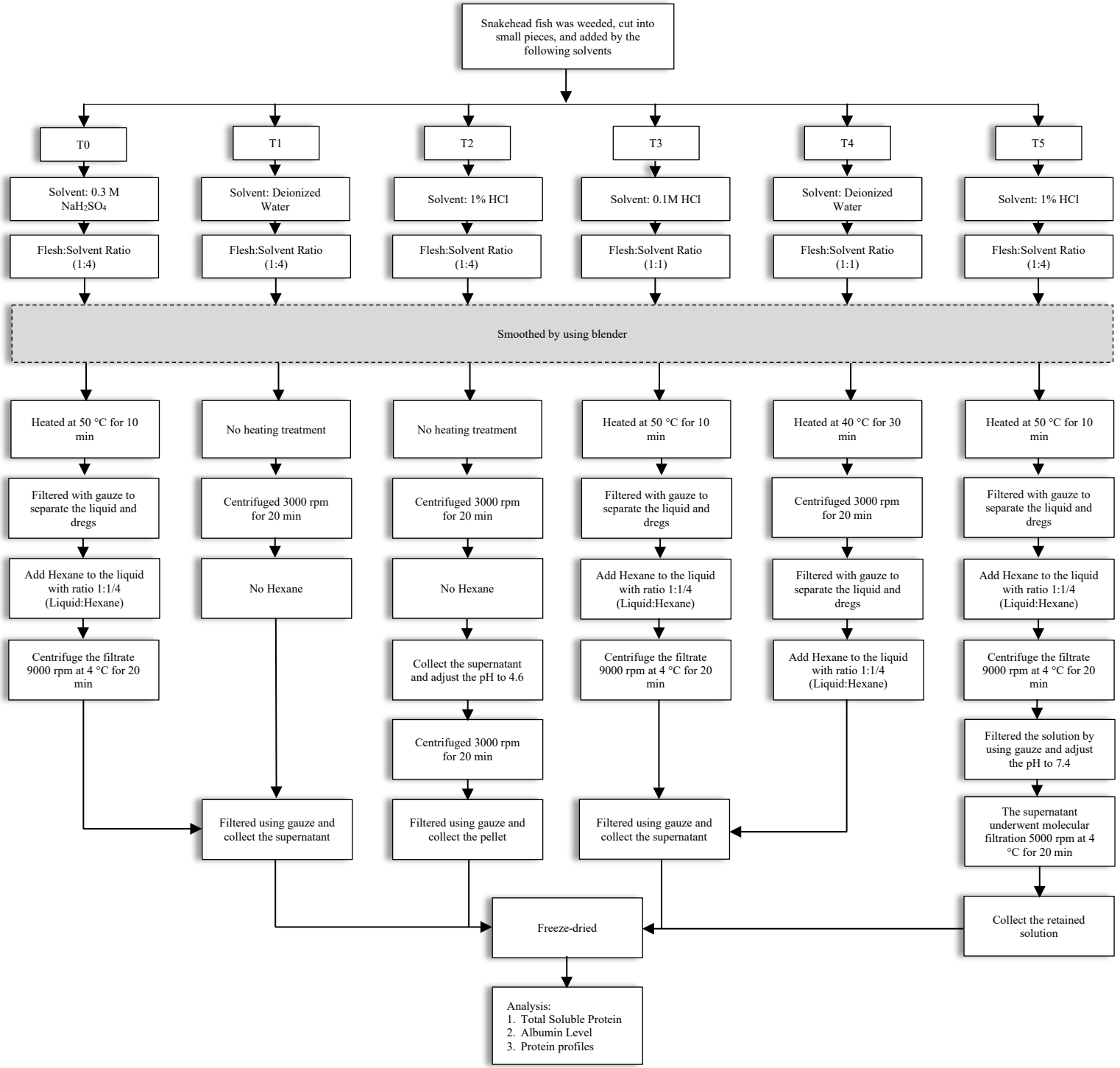

Fig. 1 .The flow diagram visualises the protein extraction protocols from the raw flesh of snakehead fish to the snakehead fish protein concentrate (SFC) from treatment 0 (T0), treatment 1 (T1), treatment 2 (T2), treatment 3 (T3), treatment 4 (T4), and treatment 5 (T5). Each protocol was repeated three times (n = 3). The diagram is modified from Romadhoni et al. (2016).

$$Relative\ quantity\ (\mu g.mL^{-1}) = \%RQ \times Amount\ of\ protein\ loaded\ (\mu g.mL^{-1})$$

Statistical analyses

All numerical data were presented as mean ± SEM. Data for the total soluble protein and albumin levels were analysed using a one-way analysis of variance (ANOVA) in RStudio (Version 2022.07.0, Austria) with the significance level $P \leq 0.05$ to compare the means. The Levene's test was used to check the homogeneity of variance assumption. All graphs were constructed in GraphPad Prism (Version 9.0.0, USA).

Results

Total soluble protein

The total soluble protein (TSP) obtained from SFC ranged from 194.250 ± 34.12 μg.mL^{-1} to 681.250 ± 51.52 μg.mL$^{-1,}$ with the highest level in treatment 2 (T2), and the lowest in treatment 5 (T5). This shows that the series of extraction procedures used in T2 – an acid-based protein extraction using 1 % hydrochloride (HCl) that recovered the protein from the pellet following precipitation at its isoelectric point (pH 4.6) – was prominent in retrieving the TSP compared to other treatments. However, this level did not significantly differ from the mean TSP for treatment 0 (T0), treatment 1 (T1), and treatment 4 (T4), which were recovered from the supernatant (Fig. 2a).

We omitted the possibility of a difference in the level of TSP resulting from the pellet (T2) and supernatant (T5) since the soluble protein content in T5's pellet also showed a significant difference as compared to T2 (data not shown). An identical result was also achieved when comparing the supernatant and pellet recovery between T1 and T2, which followed the same technique and could only be discriminated by the solvent employed (Water and 1 % HCl).

To narrow down the possibilities of the factors influencing TSP results, different applications of heating treatment and hexane addition existing in the same solvent within T1 and T4 were compared. The result showed no significant difference in TSP level was observed (both were measured from supernatant recovery). Also, the solvent-to-flesh ratio variation in both treatments did not appear to affect the outcome of the TSP level. It contradicts the finding that mentioned a significant statistical difference in TSP level between the flesh-to-solvent ratio of 1:1 and 1:4 (Asfar et al., 2019).

Albumin level

Of the total soluble protein obtained, the level of albumin and its proportion were also measured. The highest mean albumin level of 3.230 ± 0.706 mg.mL^{-1} was recorded in treatment 1 (T1), followed by T2 (1.689 ± 0.088 mg.mL^{-1}), T5 (0.604 ± 0.06 mg.mL^{-1}), T0 (0.407 ± 0.129 mg.mL^{-1}), T3 (0.333 ± 0.149 mg.mL^{-1}), and T4 (0.321 ± 0.012 mg.mL^{-1}) (Fig. 2b). Although the highest TSP level was recorded in T2, only 17 % albumin was

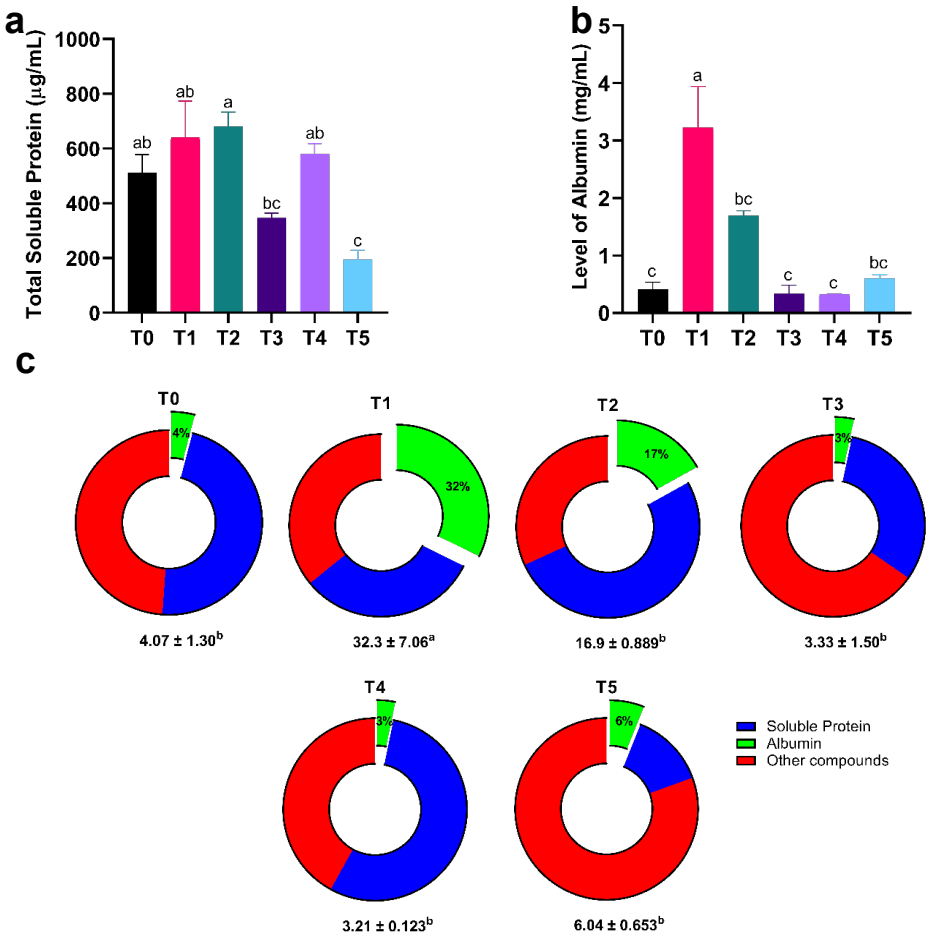

Fig. 2. Mean (± SEM) total soluble protein (a), albumin level (b), and albumin proportion of snakehead fish protein concentrate (SFC) contents (c) from treatment 0 (T0), treatment 1 (T1), treatment (2), treatment 3 (T3), treatment (T4), and treatment 5 (T5) (n = 3). Albumin proportion was calculated within the SFC concentration of 10 mg.mL^{-1}. Means that do not share a letter differ significantly ($P < 0.05$).

achieved in this treatment of SFC. The highest albumin proportion was recovered in T1, making up to 32 % following the extraction using water solvent without heating treatment and hexane addition (Fig. 2c).

Protein profiles

Given the resulting discrepancy in total soluble protein concentration, albumin level, and SFC proportion, the yield from all the extraction treatments was loaded to SDS-PAGE to determine whether the distinction of the protein profiles also occurred within the extraction methods. The densitometric analysis and protein bands of SFC under different conditions are shown in Figures 3a-h. All extraction protocols mainly delivered a good band separation with small indistinct bands. As displayed by the electrophoretic gel, a total number of 2 – 8 bands with molecular weight (MW) ranging from 17 kDa to 159 kDa were identified. Detailed observation of the densitometric evaluation of SDS-PAGE revealed that the use of water or buffer solvent (T0, T1, and T4) showed a more complex pattern in terms of the number of separated protein bands. In contrast, acid-based extraction using HCl (T2, T3, and T5) generated a lower number of protein bands recovered after extraction, with the protein bands mostly dispersed to the molecular weights between 17 kDa and 32 kDa; only a band was detected at 159 kDa.

There were also differences in the distribution of the high-intensity protein bands, henceforth referred to as major bands, recovered following the electrophoresis. Two major bands with molecular weights of 18 kDa and 47 kDa were detected in T0 with the respective quantities of 35 % (51.8 $\mu g.mL^{-1}$) and 28 % (41.8 $\mu g.mL^{-1}$) from the total protein loaded. A band with an identical molecular weight of 47 kDa was also seen in T1 as the major band, with another band appearing at 40 kDa. These two major bands possessed 16 % (24.4 $\mu g.mL^{-1}$) and 57 % (85.1 $\mu g.mL^{-1}$) proportions of protein loaded, respectively. Similar to that condition, T2 and T5 shared the same band of 32 kDa as the only recovered major protein with a proportion of 73 % (109.6 $\mu g.mL^{-1}$) and 93 % (137.3 $\mu g.mL^{-1}$) each. Meanwhile, relatively homogeneous distributions in the quantity of protein bands appeared at T3 and T4, although they differed in the number of total protein bands retrieved. Of all SDS-PAGE lanes analysed, T3 becomes the only treatment with the lowest number of protein bands recovery, accounting for only two bands with low intensity.

Discussion

The snakehead fish in this study was prepared as the fish protein concentrate (FPC) – a stable fish preparation with a more excellent protein content than from which it is derived and is usually intended for human consumption. According to the definition, FPC is generally yielded from fish simply by removing the water content from the flesh and, in some instances, by discarding the oil content, the bones, and drying the final product (Bárzana and Garía-Garibay, 1994; Shaviklo, 2015). The manufacturing of FPC is particularly efficient from a technological and economic standpoint since it has a substantially lower protein loss (<4 %) than other fish processing procedures such as freezing, filleting, or canning, which can range from 40 % to 60 % (Kumoro et al., 2022). The excellent recovery of nutritional value from whole edible-grade fish, low calorific content and antinutritional component, long shelf-life, and good storage stability, particularly during shipping, are among the benefits of FPC preparation (Pires et al., 2012). In the present investigation, the protocols for preparing FPC placed greater emphasis on extracting the soluble protein from snakehead fish, specifically sarcoplasmic protein, in an effort to isolate albumin to the greatest extent possible.

The snakehead fish protein extraction breaks the raw fish flesh and retrieves some bands of soluble protein such as myofibrillar and sarcoplasmic. Since FPC is also generated from primarily insoluble fish by-products, its potential is limited mainly by the lack of solubility and the poor functional properties (Bárzana and Garía-Garibay, 1994). Therefore, protein extractions are often carried out in acidic (pH≤3.5) or alkaline (pH ≥10.5) solutions to aid the protein solubilisation (Batista et al., 2007). For acid-based hydrolysis of fish protein, the fish raw material, including skin, scales, bone, cartilage, and swim bladder (Saleh et al., 2021), is often treated with strong hydrochloric acid (HCl) or sulphuric acid (H_2SO_4) and then extensively hydrolysed the proteins at a high temperature, sometimes under high pressure (Bárzana and Garía-Garibay, 1994; Kristinsson et al., 2006).

In the present study, we are limited in comparing each treatment thoroughly because each follows a distinct protocol. Nevertheless, we drew attention to several significant treatment-related differences that might influence the retrieval of TSP levels from snakehead fish flesh. By comparing the similarity in terms of the solvent used (1 % HCl) and the flesh-to-solvent ratio (1:4) between T2 and T5, the varied result of TSP could be attributed to the application of the heating treatment, hexane addition, pH adjustment (pH 4.6 vs. 7.4), and the molecular filtration which were different between the two protocols. Hexane, however, is a commonly used solvent to eliminate lipids. It has lower toxicity yet effective extraction capabilities. Its combination with water or iso-propanol has efficiently extracted lipids from freshwater and marine fish (Haedrich et al., 2020; Kumoro et al., 2022). To the best of our knowledge, little research has been done on how hexane exposure affects the level of TSP.

The previous study reported that an acidic solvent increased protein solubility as evidenced by the high levels of total soluble protein (TSP) obtained when HCl was combined with a heating treatment at 50–60 °C. This result was prominent compared to water, salt, or

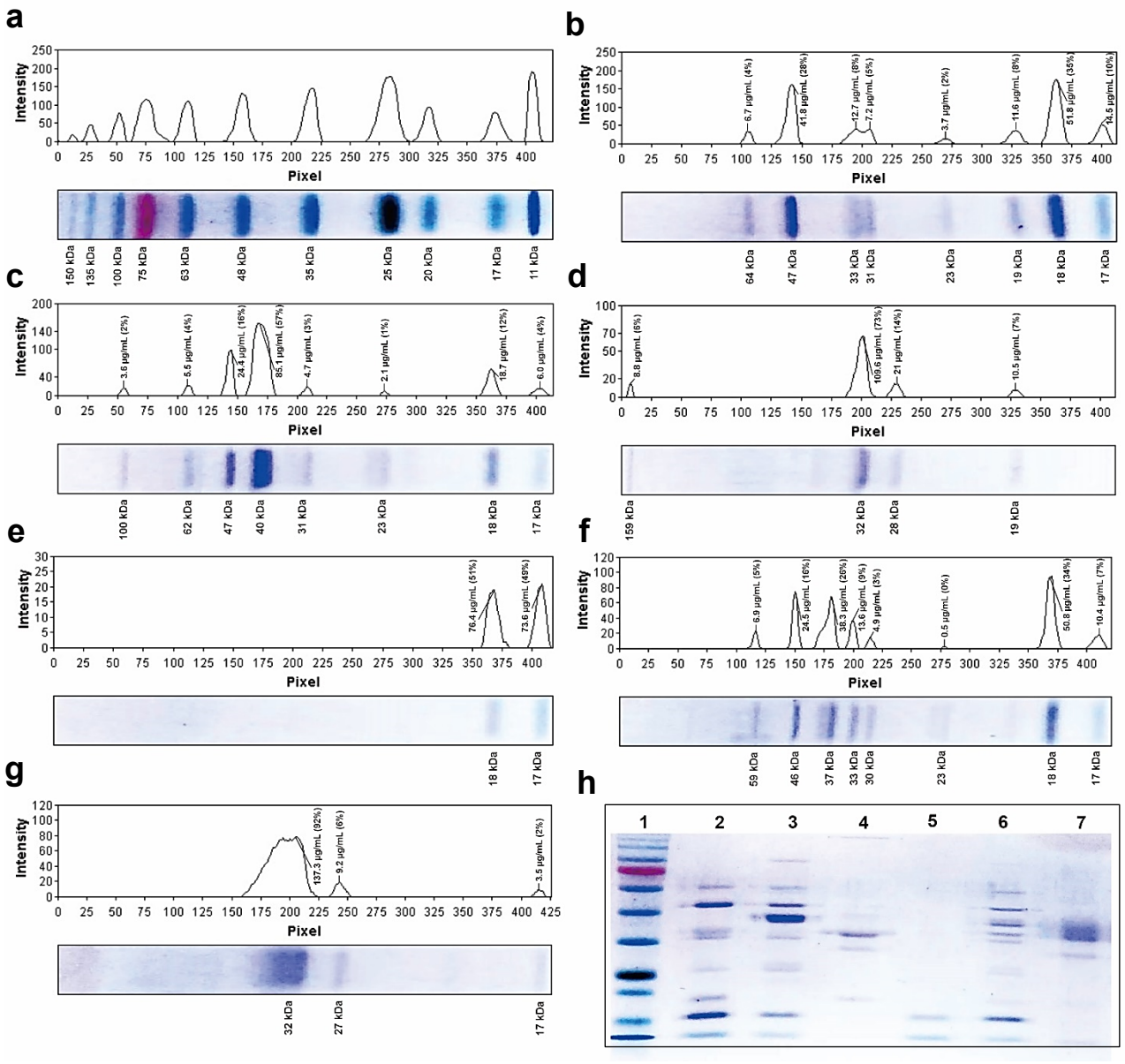

Fig. 3. Densitometric analysis (a-g) and Protein bands of SDS-PAGE (h) from Snakehead Fish Protein Concentrate (SFC). (Lane 1 and a) Protein marker; (Lane 2 and b) Treatment 0; (Lane 3 and c) Treatment 1; (Lane 4 and d) Treatment 2; (Lane 5 and e) Treatment 3; (Lane 6 and f) Treatment 4; and (Lane 7 and g) Treatment 5. The number of 8 bands were retrieved following water or buffer-based protein extraction (b, c, and f), while the acid-based protein extraction recovered 2 – 3 bands (d, e, and g). Two major bands were observed with the respective molecular weight of 18 kDa, 40 kDa, and 47 kDa (b and c).

ethanol solvent (Asfar et al., 2014; Romadhoni et al., 2016). A similar result was observed in this study when HCl was utilised in treatment 2 (T2), resulting in the highest total soluble protein compared to other treatments. This finding justifies that most proteins are barely soluble in water solvents due to the existence of hydrophobic functional groups and disulfide connections among their molecules (Asakura et al., 1978).

Regarding the heating treatments, thermal exposure to the sample up to 50 °C for 10 min still maintained the TSP level at a considerably high concentration when 0.3 M NaH_2SO_4 with 1:4 of solvent to flesh ratio was employed (T0). Furthermore, extending the heating time to 30 min with a lower temperature application of 40 °C (T4) in water solvent generated no significant difference compared to T0. However, a notable result was found when acid solvent combined with heating treatment (T3 and T5) consistently resulted in lesser soluble protein retrieval than buffer solvent (T0) and water solvent (T1 and T4). This notion is supported not only by the result of T2, which demonstrated the highest TSP level when heating treatment was abandoned but also by the initial discovery stating that heating treatment up to 50 °C resulted in a much lower TSP level than no heating treatment in the same HCl solvent (Asfar et al., 2014).

Moreover, a significant difference in TSP levels between T2 and T5 shared a comparable albumin level but significantly differed in the respective albumin

proportion of 17 % and 6 % from SFC yield. Conversely, the equivalent results of TSP levels seen in T1 and T2 significantly differed in both albumin levels and their proportions (32 % and 17 %) from the SFC yield. This study thus revealed that T1 had the most significant amount of albumin, accounting for approximately 32% of the SFC yield, indicating the ability of water solvents to extract the albumin from fish flesh better than acid and buffer solvents if it is applied without any heating treatment. In contrast, the remaining treatments (T0, T3, and T4) made up an albumin portion below 5 % from the yield of SFC (4 %, 3 %, and 3 % each) (Fig. 2c). These results demonstrate that the application of heating treatment lowered the albumin levels and their proportion regardless of the solvents used. Also, it denotes that the highest albumin content does not always in line with the highest TSP level measured by BCA. They make up a proportion that relies on the capability of each treatment to retrieve the albumin content within the extracted soluble protein.

Essentially, the selection of solvent used for extraction must rely on the solvent's ability to dissolve the lipids quickly and break any interactions between the lipids and the tissue matrices, as well as remove co-extracted non-lipid components from the extracts so that the lipids and water content can be eliminated from fish flesh tissue to obtain high-quality FPC. According to the like-dissolve-like principle, which states that polar compounds are only soluble in polar solvents and vice versa (Haedrich et al., 2020), the ideal solvent for the preparation of FPC should be a combination of polar and non-polar solvents, as this may disturb both polar and non-polar interactions in the extraction system. However, the removal of lipids and water content can also be enhanced by chopping the flesh. This size reduction increases the breakage of cell structure which improves the permeability, promotes effective solvent penetration, accelerates solvent diffusion inside the fish material, and elevates the exposure of fish particles to the solvent; a fish particle of 1 mm is considered the optimum size for this extraction (Jayasinghe et al., 2013; Kumoro et al., 2022).

Likewise, the applied temperature also synergises with the solvent in eliminating the oil content. Shear mixing cannot separate oil at the lowest temperatures of 40 °C due to the formation of an emulsion, the less volatile mixture of oil and solvent, and the limited capacity of the solvent to dissolve in the oil. Meanwhile, the high-temperature application between 60–90 °C improves the molecular interaction between the solvent and oil, increasing the solvent's dissolving power and therefore elevating the penetration and diffusion of solvent's molecules into the tissue matrix, resulting in a high yield of oil extraction (Jayasinghe et al., 2013). Following centrifugation, the heated mixture of minced flesh and solvent separates into three distinct phases, i.e., (1) Lower phase: a semi-solid phase carrying insoluble protein, (2) Middle phase: a heavy liquid phase containing soluble proteins, and (3) Upper phase: a light liquid phase with crude lipids. The middle phase is then separated from insoluble proteins and lipids for further drying or pH adjustment at the isoelectric point prior to the drying process (Shaviklo, 2015).

Other significant factors for solvent selection in FPC production include probable protein denaturation, chemical reactivity, safety, and solvent toxicity (Breil et al., 2016). In the present study, the lower number of bands regained following the acid-based extraction method compared to water or buffer solvent indicates that low pH is often harmful to proteins, causing denaturation and the loss of functional characteristics. Seemingly, acid solvent applied to fish by-products increased their solubility. Still, its exposure to fish flesh caused extensive hydrolysis, resulting in products with very high solubility and dispersibility and products in which other functional properties are largely destroyed. Furthermore, the acid solvent is destructive for sarcoplasmic protein, evidenced by the lower albumin level, its proportion to SFC yield, and low protein band retrieval when HCl was employed. Thus the bands displayed following gel electrophoresis might represent the myofibrillar proteins such as myosin light chain (17–22 kDa) and actin (~43 kDa) (Le Gouic et al., 2018). It is known that sarcoplasmic proteins, found in the sarcoplasm of muscle fibres, are soluble in water or solutions with low ionic strength. Meanwhile, myofibrillar proteins demand the denaturing conditions in the solution with high ionic strength to be solubilised and extracted, whereas stromal proteins (collagen and elastin) are reported to remain insoluble in high-salt solutions (Malva et al., 2018).

Another report claimed that acid hydrolysis also destroyed tryptophan, a partial loss of methionine, and the conversion of glutamine into glutamate and asparagine into aspartate (Kristinsson et al., 2006; Nowsad, 2007; Hou et al., 2017). As the secondary, tertiary, and quaternary structures of proteins, as well as their biological and functional properties, are determined by the primary sequence of amino acids (Hou et al., 2017), any changes in environmental factors such as pH, ionic strength, and temperature result in the unfolding of proteins and the alteration of their native form due to the breaking of weak bonds, causing denaturation and the loss of their original properties (Nowsad, 2007). As reported in the previous study, the application of heating treatment at 50–70 °C may raise the risk of protein denaturation and destruction, evidenced by a substantial difference in TSP level between protein extraction with and without heating treatment at 50 °C using the same solvent (Asfar et al., 2019). A similar result was identified in this study when acid solvent (HCl) was used in combination with heating treatment to 50 °C for 10 min (T3 and T5), lowered the retrieval of TSP level than in buffer solvent (T0) and water solvent (T1 and T4). Contrarily, acid solvents without thermal treatment resulted in the highest TSP level in T2. Apart from that, the thermal

application also resulted in a low proportion of albumin content, making up for just below 6 % of SFC yield, evidenced by T0, T3, T4, and T5. During thermal processing, protein degradation begins with denaturation and is followed by aggregation, resulting in myofibril shrinkage and moisture removal. Also, excessive exposure to heat can cause the denaturation of amino acids in FPC.

Moreover, unlike the result obtained from the acid solution, the application of heating treatment in water and buffer solvent did not affect the TSP level, as evidenced by the result of TSP levels from T0, T1, and T4. Considering the isolation of albumin and any other high protein content that should be fully functional from the SFC, the extraction procedure should, therefore, not be undertaken at over 50 °C to avoid denaturation of essential protein components (Freitas et al. 2015; Samson et al., 2016). In addition, the performance of FPC manufacture using solvent extraction techniques was also influenced by the solvent volume to fish mass ratio (Le Gouic et al., 2018). The finding of a previous study demonstrated that the treatment with a ratio of 1:1, 2:1, and 3:1 (solvent:flesh) did not show a significant difference in the extraction of soluble protein from snakehead fish. Nonetheless, a significantly higher outcome was obtained when the ratio of 4:1 was applied (Asfar et al., 2019).

In contrast, our results indicated that the ratios of 1:1 and 4:1 did not influence the TSP level; thus, the considerable variation in albumin concentration and its proportion shown in T1 and T4 may have resulted from the heating treatment. Theoretically, the solvent volume ratio to fish mass (v/w) promotes the extensive interaction between both, resulting in significant lipid and moisture removal (Kumoro et al., 2022). Further study using a central composite rotatable surface response design involving different solvents, solvent-to-flesh ratio, and temperature variation needs to conduct to clarify the variation in TSP and albumin levels.

Up to this point, the albumin level of snakehead fish concentrate produced by the T1 protocol in this study is the highest ever recorded from the bromocresol purple (BCP) assay. However, the level of 35 % and 62.9 % snakehead fish albumin from the yield were previously detected using bromocresol green (BCG) following the surimi preparation (Syukroni and Trilaksani, 2017) and protocol given in T2 (Asfar et al., 2019), respectively. This difference may hypothetically stem from the assays used and the diversity of the environments in which snakehead fish were sourced. Moreira et al. (2018) reported that BCG was affected by positive interference from globulin. The BCG method is not specific for albumin and therefore overestimates the albumin measurement at the low albumin level and high globulin condition. Although there is also a limitation for BCP, this assay is considered a superior method for evaluating albumin due to the higher specificity compared to BCG. Besides, the distinctions of biochemical compositions were also reported not only between native and cultured snakehead fish but also between the regions in which the fish were obtained (Chasanah et al., 2015).

The preparation of snakehead fish protein concentrate (SFC) in this study recovered protein bands ranging from 17 kDa to 159 kDa with various bands and the pattern depending on the extraction methods. Regarding the molecular weight of albumin, some previous studies claimed that the molecular weight of about 65 kDa corresponds to the snakehead fish albumin (Alviodinasyari et al., 2019). Other studies from *Channa striata* and the species of *Channa pleurophthalma* – another species within the genus of *Channa* – have also identified a protein band of 59.08 kDa (Haniffa et al., 2017), 67.741 kDa (Syukroni and Trilaksani, 2017) and 67.610 kDa (Aryani et al., 2020), respectively. The latter also estimated the protein band ranging from 37.031–95.948 kDa as albumin (Aryani et al., 2020). Meanwhile, according to the extraction protocol in the present study, the minor protein band of 62 kDa to 64 kDa was observed. However, the molecular weight of albumin differed depending on the organism and the source from which the proteins are extracted, e.g., bovine serum albumin (BSA) and human serum albumin (HAS) possess a molecular weight of 66.4 kDa (Babcock and Brancaleon, 2013) and 66.7 kDa (Lee and Wu, 2017), respectively. Meanwhile, albumin from African catfish (*Clarias gariepinus*) derived from serum has a molecular weight of 70 kDa (Hasnain et al., 2004), whereas Masu salmon (*Oncorhynchus masou*) serum contains 75 kDa albumin (Ura et al., 1993).

On the other hand, as the protein bands of 40 kDa to 47 kDa were also identified in this study, snakehead fish flesh might also possess ovalbumin – the major protein constituting egg white protein bearing 45 kDa molecular weight (Arzeni et al., 2015) – similar to giant mudskipper (*Periphtalmodon schlosseri*) (Hidayaturrahmah et al., 2019). Additionally, the protein bands of 15 kDa and 23.50 kDa have been identified as the novel fish myoglobin (Mb) from snakehead which might be present as two-type Mbs (Chotichayapong et al., 2016) while the bands ranging from 17–20 kDa and 43 kDa, respectively belong to myosin and G-actin (Le Gouic et al., 2018). Therefore, further studies need to be conducted to detect the molecular weight of snakehead fish albumin accurately and can clarify the scattered presumptions.

Albumin is the principal constituent of uterine and oviductal fluid, accounting for 34 $mg.mL^{-1}$ (Zhao et al., 2021) and 29–35 % of the total protein each (Gonçalves et al., 2008), which is recognised as a transporter for hormones, metabolites, and drugs, and serves as an antioxidant (Quinlan et al., 2005). In the in vivo milieu, albumin acts as a cholesterol acceptor which is capable of mediating the removal of cholesterol from sperm membrane, initiating sperm capacitation, and thus modulating the subsequent processes, including

the hyperactivation of sperm motility, the induced-acrosome reaction, sperm-zona pellucida (ZP) binding, and fertilisation (Kumaresan et al., 2019). As cholesterol is the dominant component located in the interstitial spaces of the plasma membrane lipid bilayer of mammalian sperm (about 90 %), cholesterol redistribution and depletion become a crucial part of sperm preparation for fertilisation (Leahy and Gadella, 2015). Albumin can also chelate seminal zinc from the sperm surface, which is associated with acquiring a capacitated state, as revealed by zinc signature changes between fresh and capacitated sperm (Lishko et al., 2010; Kerns et al., 2018) (Fig. 4).

From the in vitro viewpoint, albumin is an essential compound whose role is commonly replaced by serum-dependent albumins such as BSA and HSA contained in the capacitation and fertilisation medium (Aitken and Nixon, 2013). Along with bicarbonate which induces the formation of oxysterol, the synergistic effect of albumin and bicarbonate presented in the capacitation medium has triggered the hyperactivation of sperm, protein aggregation (flotillin) to the apical ridge of the sperm head, maintained the acrosome integrity, generated sperm with higher affinity to ZP, and high fertilisation rate output (Boerke et al., 2008; Boerke et al., 2013). A recent study in

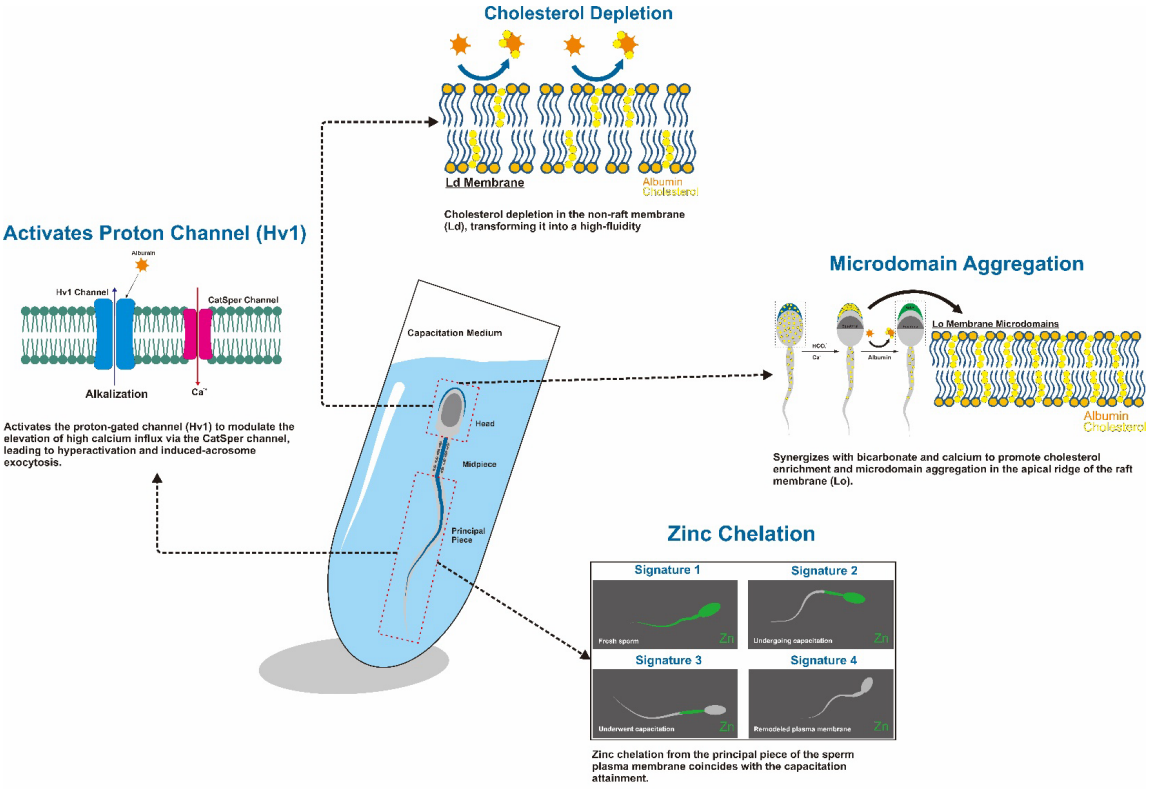

Fig. 4. Several identified mechanisms of action from serum albumin in modulating the capacitation process in sperm. The illustration was constructed according to the following publications (Lishko et al., 2010; Leahy and Gadella, 2015; Kerns et al., 2018; Zhao et al., 2021)

human sperm also reported that albumin directly binds and activates the human voltage-gated proton channels (hHv1), which increase the proton current (H^+) efflux, induce changes in pH_i (sperm intracellular alkalisation), elevate Ca^{2+} influx via CatSper channel, and acrosome exocytosis (Zhao et al., 2021). Considering those albumin functions, this study proves the presence of albumin in the snakehead fish protein concentrate through BCP assay, which is worth attempting as an alternative source of non-serum-based albumin in modulating the sperm capacitation process.

Conclusion

This preliminary study established the optimum method for protein extraction from raw flesh of snakehead fish, which yielded the most significant albumin compound following the extraction with water solvent without thermal application (T1) at 32 %, the highest albumin level ever reported employing bromocresol purple assay. According to the densitometric analysis, the present research evidenced that acid solvent generated the highest total soluble protein with a low retrieval of protein bands, while water or buffer solvent recovered more complex protein bands. The potential albumin-related bands were also identified from the two major bands shown at 40 kDa and 47 kDa, sharing for 57 % and 16 % of the total protein loaded, respectively.

By referring to the mechanism of serum albumin,

application and further evaluation studies to provide definitive evidence of the capabilities and to understand the mechanism of action from this compound in promoting the capacitation process in sperm are highly encouraged. Moreover, the molecular weight of albumin, purification, and amino acid sequence are also among the limitations of this study. Therefore, approaches are needed to confirm the molecular weight of albumin and unravel the albumin structure from snakehead fish to carry out the protein-ligand binding.

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