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FOOD TECHNOLOGY

Ultrasound applied at low temperature on the extraction and modification of proteins from by-products of the fishing industry

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ABSTRACT: This study explored the use only of ultrasound (US) for extracting proteins from grass carp (*Ctenopharyngodon idella)* backbones at low temperature and examined its impact on hydrophobicity, solubility, electrophoretic profile, and sulfhydryl levels. Backbones were treated with 35 and 130 kHz for 20, 30, and 40 minutes at 14 ºC, resulting in two protein fractions: solid (TPS) and liquid (TPL). US increased yield compared to the non-sonicated fraction. TPL (35 kHz) exhibited a 16% reduction in total and free sulfhydryl levels and a 25% increase in hydrophobicity. US induced protein conformation and band intensity alterations, particularly between 25 to 100 kDa for TPL at 130 kHz and below 30 kDa for TPS at 35 kHz. This study demonstrated the efficacy of US for protein extraction from fish by-products and its capacity to modify protein characteristics, facilitating the development of innovative food products.
Key words: fish backbone, protein, modification, by-product, reuse, electrophoresis.

Efeito do ultrassom aplicado a baixa temperatura na extração e modificação de proteinas de sub-produtos da indústria pesqueira

RESUMO: Este estudo explorou o uso exclusivo do ultrassom (US) para extrair proteínas das espinhas dorsais da carpa-capim (Ctenopharyngodon idella) em baixas temperaturas e examinou seu impacto na hidrofobicidade, solubilidade, perfil eletroforético e níveis de sulfidrilas. As espinhas dorsais foram tratadas com 35 e 130 kHz por 20, 30 e 40 minutos a 14 ºC, resultando em duas frações de proteínas: sólida (TPS) e líquida (TPL). O US aumentou o rendimento em comparação com a fração não sonificada. TPL (35 kHz) apresentou uma redução de 16% nos níveis totais e livres de sulfidrila e um aumento de 25% na hidrofobicidade. O US induziu alterações na conformação proteica e na intensidade das bandas, especialmente entre 25 e 100 kDa para TPL a 130 kHz e abaixo de 30 kDa para TPS a 35 kHz. Este estudo demonstrou a eficácia do US na extração e na modificação de algumas propriedades das proteínas de subprodutos de peixe, facilitando assim seu emprego no desenvolvimento de produtos alimentícios inovadores.
Palavras-chave: espinha dorsal de peixe, proteína, modificação, subproduto, reutilização, eletroforese.

INTRODUCTION

The substantial waste generated from fish processing (including heads, fins, viscera, skin, scales, and backbones), comprising around 70% of the animal's total weight, is a rich source of proteins, oils, and minerals (FAO, 2022). Thermal, chemical, autolytic, or enzymatic hydrolysis processes are commonly used to recover proteins. However, it involves high temperature, energy, organic solvents, and long times (HALIM et al., 2016). Ultrasound (US) is a green technology offering an alternative for obtaining higher-value protein compounds from fish by-products. US can favor the extraction and modification of protein structures. The recovery of proteins from animal products, such as from chicken (ZOU et al., 2017a) and duck liver (ZOU et al., 2017b) through alkaline extraction, as well as the extraction of collagen from bovine tendon (RAN & WANG, 2014) by enzymatic hydrolysis with pepsin, are some examples of traditional methods of protein recovery in which ultrasound (US) is used as a potentiating agent for these chemical and enzymatic processes (LIU et al., 2022) and not as the sole agent for promoting hydrolysis in these proteins. These processes have shown increased yield and protein content extracted and improved functional properties such as solubility, foaming capacity, and gelation (ZOU et al., 2017a). There is also a reduction in particle size and denaturation enthalpy, and improved

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enzymatic action due to the increased dispersion of enzyme aggregates and the opening of collagen fibers (RAN & WANG, 2014).

The production and collapse of microbubbles (cavitation) significantly alter the physicochemical properties of proteins by generating microjets, turbulence, and shockwaves that lead to mixing, heat, mass transfer, and particle size reduction (ZHANG et al., 2017). Additionally, chemical effects occur due to the formation of radicals such as ·OH and ·H, leading to the production of various other types of radicals that interact with other molecules and cause modifications (WANG et al., 2018).

Regarding fish slaughter residues, no prior separation of waste materials (head, backbones, fins, and viscera) impairs subsequent technological strategies. In addition, some processes use whole carcasses or only noble parts and pre-processed products (TANG & YONGSAWATDIGUL, 2020). Many studies are conducted on the properties of various protein-based foods; however, the relationships between structure, interactions, and properties in these systems are often limited (CHEN et al., 2022).

Thus, this study aimed to evaluate the unique effects of US at frequencies of 35 kHz and 130 kHz for 20, 30, and 40 minutes of exposure to the proteins of grass carp (*Ctenopharyngodon idella*) backbones at 14 ºC, taking into consideration previous studies (CICHOSKI et al., 2019; ALVES et al., 2020; NEHRING et al., 2022). The process yield, protein contents, hydrophobicity, total and free sulfhydryl levels, and solubility were determined, as well as possible changes in protein structures by electrophoresis aiming to investigate the characteristics of the proteins.

MATERIALS AND METHODS

Materials

Backbones from the filleting of grass carp (*Ctenopharyngodon idella*) were obtained from slaughterhouses in Santa Maria, RS, Brazil. The backbones were milled through a 5 mm-diameter die (PJ22, Industrias Jamar). The resulting batter was packed (110 g) and stored at -20 ºC until analysis. All reagents used in this study were of analytical grade. The reagents 5,5-Dithio-bis 2-nitrobenzoic acid (DTNB), acrylamide, bromophenol blue (BPB), bis-acrylamide, Ultra-Low Range Molecular Weight Markers were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany) and BioRad Precision Plus Protein Dual Color Standards (BioRad, Hercules, CA, USA).

Ultrasound treatment (US)

Sample (100 g) and 400 mL of deionized water were mixed in a 1000 mL beaker and placed in a US bath. The US treatment was carried out for 20, 30, and 40 min (Elma, model TI-H-10) at 14 ºC, at 35 kHz (118 W) and 130 kHz (52 W), and 90% amplitude, in normal operating mode. The temperature was controlled to minimize alterations caused by the heating. This process was repeated three times. A control treatment was also performed under the same conditions as the US treatment, with the sonication switched off.

Afterward, the samples were passed through a mesh with a 2 mm sieve. The sieved material was centrifuged (Eppendorf 5810R) at 2800 g for 15 minutes at 4 ºC. After centrifugation, the white layer formed on top was mechanically removed. Centrifugation was carried out three more times until the presence of the white layer inside the tube was no longer detected. The supernatant (liquid) and the resulting sediment (solid) were frozen and freeze-dried to produce the liquid protein fraction (TPL) and the solid protein fraction (TPS) for the respective US exposure times (20, 30, and 40 minutes), which were stored at -20 ºC until analysis. Thus, the treatments generated the following samples: solid protein fractions subjected to 35 kHz (TPS35F20', TPS35F30', TPS35F40'), and subjected to 130 kHz (TPS130F20', TPS130F30', TPS130F40'), and samples not subjected to US treatment (ControlTPS20', ControlTPS30', ControlTPS40'). The liquid protein fractions subjected to 35 kHz (TPL35F20', TPL35F30', TPL35F40'), 130 kHz (TPL130F20', TPL130F30', TPL130F40'), and samples not subjected to US treatment (ControlTPL20', ControlTPL30', ControlTPL40') for 20, 30, and 40 minutes, as shown in figure 1.

Determinations

Acoustic Power

The acoustic power was determined using a calorimetric method (RAMSAY, 1996). It was calculated based on the variation in temperature increase caused by the ultrasonic energy converted into heat, as per equation 1.

$$Acoustic\ Power = mC_p \left(\frac{dT}{dt}\right)_o \quad (1)$$

Where m: mass of liquid, Cp: heat capacity of the liquid, and (dT/dt): initial slope of the temperature curve versus time.

Process yield and total protein

The yield was calculated using the ratio between the dry mass after freeze-drying (72 h, -

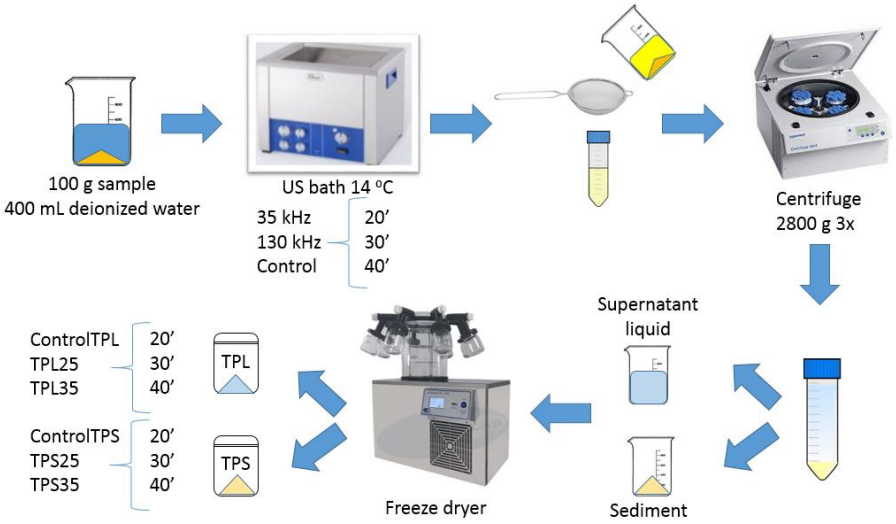

Figure 1 - Experiment treatments and variables. TPS: Solid protein fraction, TPL: Liquid protein fraction (supernatant).

42 ºC, -720 mmHg, Freeze-Drying SL-404, Solab, Brazil) and the dry matter of the backbones at the beginning of the process (BETTI & FLETCHER, 2005). The values were calculated according to equation 2. The total protein content was determined according to the micro Kjeldahl methodology (960.52 of AOAC, 1995).

$$\left(\frac{Pft}{Pi * MS}\right) * 100 \quad (2)$$

Where: Pft: weight after freeze-drying, Pi: weight of the initial raw sample (g), MS: % of dry matter in the raw sample, determined according to AOAC 925.45b (1995).

Hydrophobicity

Hydrophobicity can be assessed by the binding capacity of bromophenol blue dye to hydrophobic groups present in protein molecules. Hydrophobicity was calculated (µg of BPB) according to equation 3, following the bromophenol blue (BPB) fixation method (CHELH et al., 2006). The absorbance of unbound BPB present in the supernatant was measured at a wavelength of 595 nm.

$$Bound\ BPB\ (\mu g\ BPB) = 200 * \frac{(ABS\ control - ABS\ sample)}{ABS\ control} * 100 \quad (3)$$

Where ABS control = absorbance of 200 µg of BPB in 1 mL of phosphate buffer; Abs sample: absorbance of the sample; 200 is the concentration of BPB of the control, in µg.

Total and free sulfhydryls

Total and free sulfhydryls are sulfur-containing groups that serve as indicators of structural modifications and functional characteristics of proteins, as they are responsible for interacting with each other or with other molecules in the environment, impacting protein conformation (ZOU et al., 2019). Total and free sulfhydryls were assessed following BEVERIDGE et al. (1974). For that, 25 mg of the freeze-dried material was suspended in 5 mL of tris-glycine-EDTA buffer (pH 8.0, 0.09 mol/L glycine, 0.086 mol/L Tris, 4 mmol/L EDTA) with 8 mol/L urea (total sulfhydryls) or without the addition of urea (free sulfhydryls). The mixture with 50µL of Ellman's reagent (4 mg de 5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB) em 1 mL tris-glycine-EDTA buffer) was centrifuged at 13600 x g for 10 minutes after 1 hour of incubation at 25 ºC. DTNB in an alkaline medium reacts with available SH groups, forming 2-nitro-5-thiobenzoate (NTB), which can be measured at 412 nm. The extinction coefficient of 2-nitro-5-thiobenzoate (NTB) at 412 nm (13600 $mol.L^{-1}\ cm^{-1}$) was used to calculate the results (µmol/g of protein).

Solubility index

The total protein content of each treatment and the soluble protein were used to calculate the percentage of solubility (MORR et al., 1985) according to Equation 4. First, the total protein concentration was assessed using the Kjeldahl method (method 960.52 of AOAC, 1995) through protein digestion in an acidic

medium and conversion of the nitrogen present in the sample into ammoniacal compounds, which are subsequently converted, under basic conditions, into ammonia. Finally, the nitrogen is quantified by titration. Following this, the sample underwent centrifugation at 2800 g for 15 minutes, and the protein content in the resulting supernatant was quantified using the BRADFORD method (1976) through the interaction between Coomassie Blue dye and the amino acid residues of basic or aromatic side chains.

$$Solubility\ (\%) = \frac{PS\ \left(\frac{mg}{mL}\right) * vol(mL) * 100}{Weight\ of\ the\ sample\ (mg) * \left(\frac{protein\ content(\%)}{100}\right)} \quad (4)$$

Where: PS – protein content of the supernatant; vol - volume used for solubilization; protein (%) - protein content of the sample (%).

SDS-PAGE and Tricine-SDS-PAGE

Electrophoresis separates protein molecules based on their molecular weights when subjected to an electric field. SDS-PAGE was performed according to LAEMMLI (1970), with the following modifications: the samples were solubilized in lysis buffer (150 mM NaCl, 1.5 mM MgCl2, 50 mM HEPES (pH 7.4), 1 mM EDTA, 10% glycerol, 1% IGEPAL, 1 mM DTT, and 1 mM PMSF) for 1 hour and centrifuged for 15 minutes at 2800 g (Eppendorf 5810R). The samples were standardized to a concentration of 3 mg/mL. After standardization, each sample was mixed with a loading buffer (0.130 M Tris–HCl, 2% SDS, 10% glycerol, 20% β-mercaptoethanol, 0.05% bromophenol blue, pH 6.8) for reducing and non-reducing conditions (20% β-mercaptoethanol). The samples were heated to 95 ºC for 5 min, and the electrophoretic run (120 V) was carried out using a 4% stacking gel and a 12% separating gel. Low-molecular-weight (LMW) electrophoresis was carried out according to SCHÄGGER (2006), using a 4% stacking gel and a 16% separation gel with urea. The ground grass carp backbones, raw samples (RS), were also subjected to electrophoresis analysis to determine the molecular weights at the beginning of the experiment.

Subsequently, the gels were subjected to staining with a solution containing Coomassie Brilliant Blue R-250 (0.075%), followed by destaining using a mixture of 25% methanol and 10% acetic acid. A molecular weight standard of 10-250 KDa (Precision Plus Protein Dual Color Standard, BioRad, Hercules, CA, USA) was employed for SDS-PAGE. Ultra-Low Range Molecular Weight Marker (Sigma-Aldrich, Missouri, USA) was used to LMW (3 – 27 kDa).

Statistical analysis

The experiment was carried out three times on different days. All analyses were done in triplicate (n = 9), using IBM SPSS Statistics software (SPSS, version 20, 2012). The results were submitted to analysis of variance (ANOVA) using the General Linear Model, considering the treatments as a fixed effect and the repetitions of the experiments as a random effect. Tukey's test analyzed significant effects between treatments at a significance level of 99.9% ($P < 0.001$).

RESULTS AND DISCUSSION

Yield, acoustic power, and total protein of freeze-drying process

The process yield (Table 1) was close to 3.6% for all TPSControl, without significant difference among them. An increase was observed for TPS130F20 ($6.19^{c} \pm 0.27$), TPS130F30 ($8.96b^{c} \pm 0.15$), and TPS130F40 ($9.75^{bc} \pm 1.03$). The highest yield overall was for TPS35F40' ($16.86^{a} \pm 1.39$) and TPS35F30' ($14.44^{ab} \pm 1.04$). No significant differences ($P > 0.001$) were observed among the different frequencies and exposure durations for TPL. The yields ranged from 15.32 ± 0.19 (TPLControl20') to 17.63 ± 0.56 (TPL35F40'). The optimal application time for the 35 kHz frequency was 30 minutes for TPS, while no differences were observed in extraction yields at 130 kHz for all exposure times.

We observed that the acoustic power produced by the US at 35 kHz (110W) was more significant than that produced at 130 kHz (52 W). At lower frequencies (35 kHz), a smaller number of larger microbubbles is formed. The cavitation process releases more energy, and the formed microjets penetrate the solid phase, favoring the extraction and settling of proteins and organic materials. At the same time, higher frequencies result in lower energy release (ALARCON-ROJO et al., 2019). The higher yield of TPS or TPL using US shows the promising role of fish by-products.

The treatment TPS35F40' had the lowest protein value (69.65% ± 0.99), differing ($P < 0.001$) only from TPSControl40' (76.09 % ± 0.50). The reduction in protein concentration of the TPS35F40' sample may be related to the increase in yield recorded in this sample (16.86), indicating that more non-protein material may have been extracted, consequently decreasing the protein concentration. The protein from TPS probably did not solubilize in the extraction water, indicating fewer hydrogen bonds and less involvement by water (HALIM et al., 2016).

Table 1 - Yield (%) and protein concentration (%) in TPS and TPL treatments subjected to different frequencies (35 kHz and 130 kHz) and times (20', 30', 40') at 14 ºC, and their respective controls*.

	----------Yield (%)----------		----------Protein (%)----------	
Sample	TPS	TPL	TPS	TPL
Control20'	$3.76^{d} \pm 0.29$	15.32 ± 0.19	$72.13^{ab} \pm 0.89$	63.69 ± 0.95
Control30'	$3.60^{d} \pm 0.33$	15.64 ± 0.02	$72.99^{ab} \pm 1.05$	62.89 ± 0.89
Control40'	$3.72^{d} \pm 0.28$	16.04 ± 0.38	$76.09^{a} \pm 0.50$	65.89 ± 0.94
35F20'	$10.31^{bc} \pm 0.66$	16.42 ± 0.55	$70.23^{ab} \pm 1.24$	63.14 ± 0.77
35F30'	$14.44^{ab} \pm 1.04$	16.99 ± 0.59	$72.52^{ab} \pm 1.00$	62.86 ± 0.19
35F40'	$16.86^{a} \pm 1.39$	17.63 ± 0.56	$69.65^{b} \pm 0.99$	62.40 ± 0.66
130F20'	$6.19^{c} \pm 0.27$	15.81 ± 0.39	$73.20^{ab} \pm 1.27$	63.33 ± 0.46
130F30'	$8.96^{bc} \pm 0.15$	15.88 ± 0.23	$74.84^{ab} \pm 0.84$	64.08 ± 0.62
130F40'	$9.75^{bc} \pm 1.03$	16.72 ± 0.35	$73.30^{ab} \pm 0.92$	63.23 ± 0.48

TPS: Solid protein fraction, TPL: Liquid protein fraction (supernatant). Results are expressed as mean ± standard error of the mean. Different letters in the same column represent significant differences between treatments in the Tukey test ($P < 0.001$).

TPL presented lower protein levels, ranging from 62.40% ± 0.66 to 65.89% ± 0.94 for TPL35F40' and TPLControl40', respectively. There was no difference in the protein values of TPL when compared to the respective Controls.

Hydrophobicity

Hydrophobic forces within the quaternary structure are the primary driving force behind protein folding. Intense cavitation can bring these hydrophobic groups to the exterior of the molecule (CHEN et al., 2022).

The hydrophobicity (µg de BPB) of TPS (Figure 2A) ranged from 174.67 ± 1.34 to 182.60 ± 0.66 for TPS130F20' and TPS35F20', respectively, with significant differences between them ($P < 0.001$). The lowest value observed for TPS130F20' also differed ($P < 0.001$) from the controls, which presented 180.57 ± 0.93, 180.35 ± 1.00, and 180.17 ± 1.34 for ControlTPS20', ControlTPS30', and ControlTPS40', respectively. Regarding the TPL (Figure 2B), the highest hydrophobicity value was observed for TPL35F40' (55.58 ± 1.15), which differed ($P < 0.001$) from the other treatments and the Controls, while the lowest values were found for TPL130F30' (30.42 ± 0.41) and TPL130F40' (24.33 ± 1.01 µg).

Each frequency has an optimal duration for inducing alterations in protein structures and revealing hydrophobic groups. Thus, for TPL, the frequency of 35 kHz for 40 minutes provided greater exposure of the hydrophobic groups compared to 130 kHz, and in turn, only 20 minutes of US exposure at 130 kHz provided a lower hydrophobicity for TPS (Figure 2A).

Lower frequencies produce cycles of bubble rarefaction with greater intensity, leading to transient cavitation, which causes the collapse of the microbubbles formed, releasing a large amount of energy and pressure (FU et al., 2020). This energy release favors the opening of the protein structure and exposure of hydrophobic groups. Conversely, higher frequencies present more stable rarefaction cycles, where the microbubbles formed do not always collapse, resulting in a lesser impact on the proteins, which may enhance the molecular structure reformation by internalizing the hydrophobic groups. This provides higher hydrophobicity values in samples exposed to 35 kHz compared to samples exposed to 130 kHz, and both concerning the control samples. JUN et al. (2020) studied egg whites treated with US (40 kHz, 375 W) at 25 ºC for 40, 50, and 60 minutes. They observed significant differences in hydrophobicity values when comparing the control group to the treatment exposed for 50 minutes. The rise in hydrophobicity may impact viscosity because of the increased presence of hydrophobic amino acids. These compounds enable antioxidant activity by chelating metal ions or by the scavenging effect (ZHANG et al., 2023). It can also influence proteins in emulsification processes, potentially enhancing emulsifying activity, stabilizing formed emulsions, and modifying viscoelasticity during gelling and foaming (AMIRI et al., 2021).

Total sulfhydryls (total SH) and free sulfhydryls (free SH)

The total and free sulfhydryls (µmol/g) indicate alterations in the functional attributes

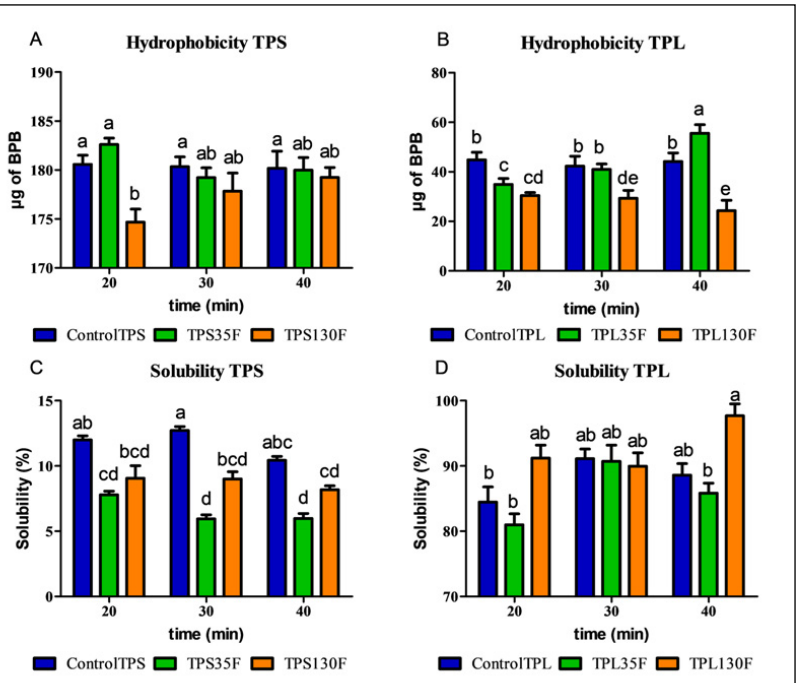

Figure 2 - Hydrophobicity (µg of BPB) and Solubility (%) of liquid protein fraction (TPL) and the solid protein fraction (TPS) subjected to different US frequencies (35 kHz and 130 kHz) and exposure times (20, 30, and 40 minutes) at 14 ºC and the respective controls. Results are expressed as mean ± standard error. Different letters represent significant differences between frequencies and exposure times for each sample according to Tukey's test ($P < 0.001$).

of proteins, denaturation processes, and protein composition (ZOU et al., 2019). Concerning the TPS, the treatment TPS130F20' showed the highest total SH (41.34 ± 0.63), differing ($P < 0.001$) from TPS35F30' (37.48 ± 0.25), with the lowest total SH, and the ControlTPS40' (38.39 ± 0.33) (Figure 3A).

Cavitation forms free radicals (•OH, •H, hydroperoxyl (HO2-), superoxide (O2-). The •H radical can destabilize disulfide bonds, which are then reduced, forming available SH groups. On the other hand, radicals (•OH and $HO2^-$) can bind to SH groups (previously available or new), leading to the formation of sulfonic (R'-SO3H) or sulfinic acid (R'-SO2H), rendering these groups inaccessible to new disulfide bridge (S-S) formation (FENG et al., 2017). For TPL (Figure 3C), the highest total SH was reported for the ControlTPL20' (39.41 ± 0.64), ControlTPL30' (40.36 ± 0.79), ControlTPL40' (39.12 ± 0.79) and TPL130F20' (39.46 ± 0.63) which did not differ ($P > 0.001$) from each other. The lowest total SH values were observed for all treatments exposed to 35 kHz (34.38 ± 0.69, 34.42 ± 0.36, and 32.79 ± 0.35), with no significant differences ($P > 0.001$) between them. TANG & YONGSAWATDIGUL (2020) evaluated the effect of ultrasound at 20 kHz and different intensities (7 and 13.37 W/cm^2) on tilapia (Oreochromis niloticus) actomyosin, finding a 31% reduction in total sulfhydryl groups.

TPS's highest free SH values (Figure 3B) were found for the treatments TPS130F40' (29.30 ± 0.37) and TPS130F20' (30.66 ± 0.40). Low free SH values were observed for TPS35F30' (24.53 ± 0.32). During the treatment, the free SH groups can be exposed and come into contact, forming disulfide bonds (S-S), a phenomenon influenced by cavitation and turbulence effects (JUN et al., 2020).

Regarding the TPL, the highest free SH were found for TPL130F20' (40.84 ± 0.36), TPL130F30' (39.49 ± 0.83), TPLControl20' (38.83 ± 0.70), and TPLControl30' (39.63 ± 0.81). The lowest values were reported for TPL35F30' (32.37 ± 0.47) and TPL35F40' (31.29 ± 0.48), which differed ($P < 0.001$) from the other treatments and the Controls (Figure 3D).

The sonolysis promoted by US can form -OH and -H radicals, which, when combined, result in the formation of H_2O_2 in the presence of oxygen,

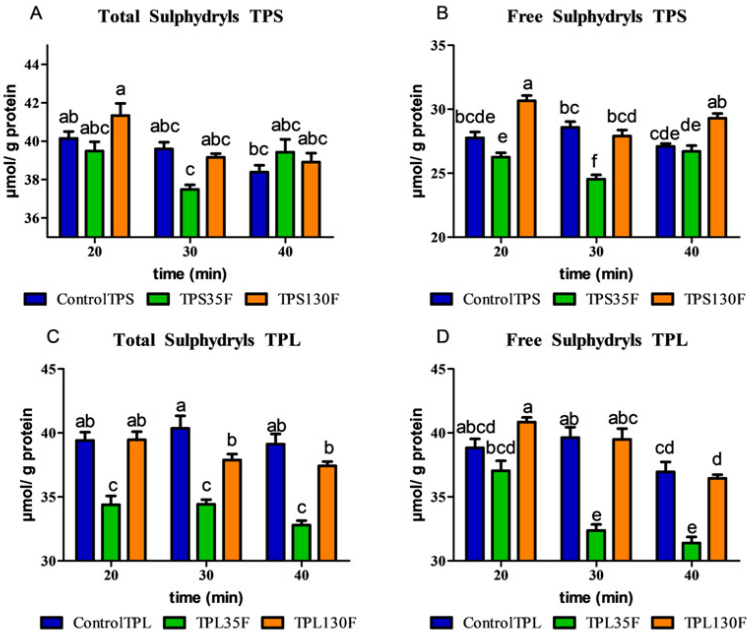

Figure 3 - Total and free sulfhydryls values (µmol/g protein) of liquid protein fraction (TPL) and the solid protein fraction (TPS) subjected to different US frequencies (35 kHz and 130 kHz) and exposure times (20, 30, and 40 minutes) at 14 ºC and the respective controls. Results are expressed as mean ± standard error. Different letters represent significant differences between frequencies and exposure times for each sample according to Tukey's test ($P < 0.001$).

thus producing the hydroperoxyl radical (HO^{2-}) and the superoxide radical ($O_2^{\cdot-}$) and singlet oxygen ($^1O_2$) (FENG et al., 2017). These radicals have a strong oxidizing power and form sulfonic acid (R'-SO_3H) or sulfinic acid (R'-SO_2H) when in contact with free SH groups, potentially inhibiting protein aggregation. However, the transformation of free SH groups into disulfide bonds is responsible for the development of aggregates (LIU et al., 2022).

A significant reduction of free SH groups ($P < 0.001$) was noticed following 30 minutes of ultrasonic exposure for both investigated frequencies (35 and 130 kHz). ZOU et al. (2019) observed a significant decrease in free SH values after subjecting chicken plasma proteins US (20 kHz, 30 minutes, 200 W), similar to our findings. Contrary findings were documented by WU et al. (2019), who studied protein from *Chlamys farreri* subjected to US treatment (20 kHz, 60 minutes, 200 – 950 W) and found an increase in free SH values, indicating greater exposure of SH groups.

Different results were observed for total and free SH of the TPS and TPL fractions, with higher total SH for TPS compared to the free SH values (Figure 3A and 3B), while TPL exhibited similar total and free SH values (Figure 3C and 3D). Free SH values similar to or greater than total SH indicate exposure of all SH groups despite the low interaction with oxidizing reactive species. These distinct behaviors are due to the type of protein molecules in TPL and TPS, which undergo different effects from the hydrodynamic forces produced by US (LIU et al., 2017).

Free SH groups have a significant role in emulsification processes and the production of viscoelastic films, contributing to forming disulfide bonds, improving film elasticity, stabilization, shear stress, and forming relatively firm structures. The interaction with hydrophobic groups strengthens the protein interfacial layer and greater emulsion stability upon heating. Free SH groups and disulfide bonds can interconvert depending on the conditions, contributing to the stabilization of the foaming process. In addition, free sulfhydryl groups can act as antioxidant agents in proteins once they are vulnerable to free radical attack, forming disulfide bonds (YAO et al., 2023). In this study, the proteins

from the treatments TPS130F40' and TPS130F20' (Figure 3B) and the proteins from TPL130F20' and TPL130F30' (Figure 3D) have characteristics that allow them to be used in food products that are more susceptible to oxidation, which may have antioxidant activity due to their higher free SH values.

Solubility

Protein solubility is the primary physicochemical attribute and can reflect protein denaturation and aggregation in solutions, affecting gelatinization and foaming properties (JUN et al., 2020). The TPS subjected to frequencies of 35 and 130 kHz (Figure 2C) showed lower solubility values ($P < 0.001$) than the TPS control, regardless of the time utilized. JUN et al. (2020) found that the exposure of eggs to ultrasound (20 kHz, 20-30 minutes) increased the solubility of proteins. However, longer exposure times decreased, likely due to protein aggregation and denaturation caused by the unfolding of the protein structure and exposure of hydrophobic groups.

Concerning the solubility of TPL (Figure 2D), TPL130F40' (97.69 ± 2.76 %) exhibited the highest value, differing ($P < 0.001$) from the ControlTPL20' (84.45 ± 2.45 %), TPL35F20' (80.98 ± 2.23 %), and TPL35F40' (85.85 ± 1.81%). All TPL presented higher solubility values when compared to TPS (Figure 2C and 2D).

The yield (section 3.1) was also higher (15.32 to 17.63%) than those reported for TPS (3.60 to 14.44%). In contrast, lower hydrophobicity (Figures 2A and 2B) was observed for TPL (24.33 to 55.58 %) when compared to TPS (174.67 to 182.60 %). These results showedthat the proteins from the TPL fraction have technological characteristics that allow them to be used in various products, including food formulations. Soluble proteins have better functional attributes, which provide a greater number of applications in food preparation processes. These technological characteristics are due to the effect of cavitation through the exposure of hydrophilic amino acids, which increases protein solubility (ZHANG et al., 2017).

SDS-PAGE

The SDS-PAGE pattern of TPS (Figure 4A) showed bands between 75 and 50 kDa only for the raw sample (RS), with a reduction in intensity for the TPSControl, TPS35, and TPS130 (rectangle A). A reduction in the intensity of the bands between 49 and 37 kDa (squares 1 and 2) was also detected for the TPS35F and TPS130F treatments when compared to RS and Controls. BASHASH et al. (2022) reported that egg white and gelatin hydrogels subjected to US (20 kHz, 150W) demonstrated a reduction in the intensity of the protein bands. The decrease in free sulfhydryl groups (Figure 3) and solubility (Figure 2) indicate a greater functional performance of these proteins to form hydrogels, with better hardness, gumminess, chewiness, and elasticity when compared to the control samples. The intermediate structure formed by a protein molecule depends on its ability to aggregate and its interaction with the molecules in the medium, affecting their functionality (LI et al., 2023). Regarding the electrophoretic profile of TPL (Figure 4B), a lower intensity of the bands at 100, 65, 40, and 25 kDa (rectangles F, G, H, I, respectively) was observed for TPL130F20', TPL130F30', and TPL130F40' when compared to the other treatments. The bands at 139 and 79 kDa (arrows 4 and 5) in RS were not detected in the other treatments, indicating the low water solubility of these proteins, which were extracted using another methodology. Modifications in protein structure were observed after ultrasound treatment (20 kHz, 200-400 W), enhancing protein digestibility by providing hydrolytic sites for digestive enzymes (BHAT et al., 2022).

Tricine-SDS-PAGE

The electrophoretic profile of TPS (Figure 4C) at 35 kHz showed the disappearance of bands between 23 and 25 kDa (rectangle A) as well as a reduction in the band intensity at 14 kDa (square C) when compared to RS (square B), this can be attributed to the breakdown of the protein molecules. The band's intensity increased at 21 kDa (arrows 1, 2, and 3) for TPS130F20', TPS130F30', and TPS130F40'. The frequencies 35 and 130 kHz led to changes in the protein structures, shown by the lower intensity of some bands for the different Controls and RS.

All TPL treatments (Figure 4D), both the US-treated samples and the controls, showed the absence of bands at 17 kDa, also observed in RS (arrow 4). In turn, bands ≤ 14 kDa were identified in the different Controls and US treatments.

Other authors have reported no protein breakdown, although they have detected physical changes in structure, with modifications in hydrophobicity, solubility, total and free sulfhydryls levels, including chicken proteins extracted at alkaline pH and subjected to 24 kHz and 300 W (ZOU et al., 2017a), and *Amaranthus cruentus* protein isolate at 30 kHz and 100 W (TOMÉ & GARCIA-ROJAS, 2020).

In contrast, ZHANG et al. (2022) reported that US at 600 W and 800 W led to protein breakdown during the co-fermentation of shrimp shells by *Bacillus subtilis* and *Acetobacter pasteurianus*.

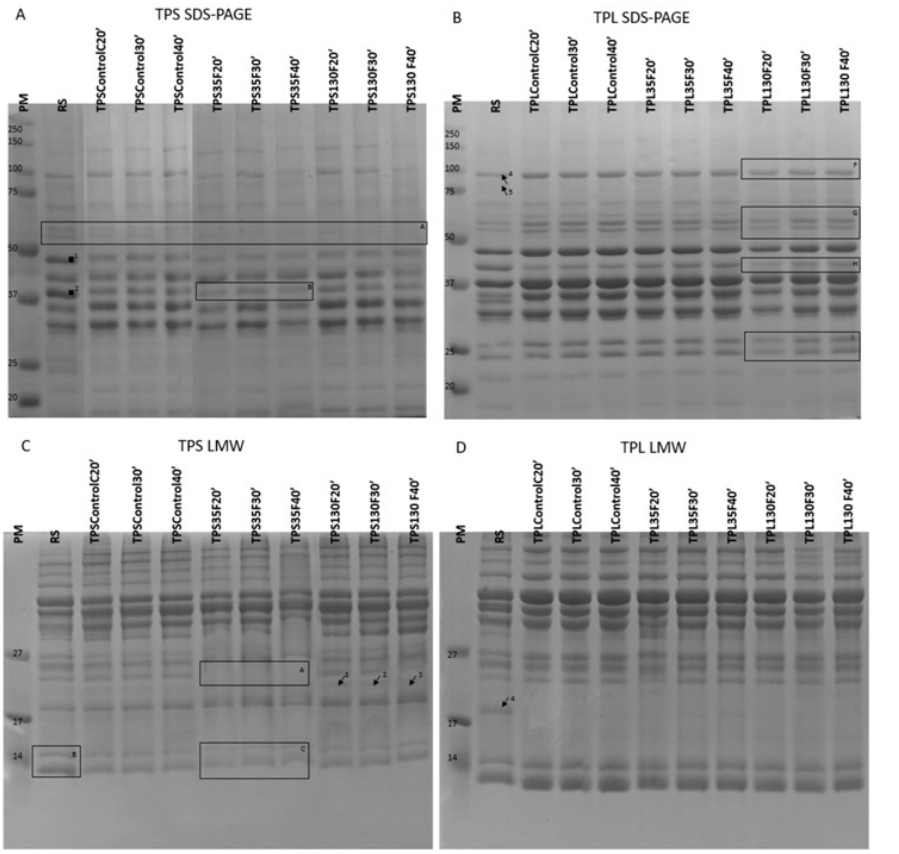

Figure 4 - SDS-PAGE and Tricine-SDS-PAGE (LMW) profile of liquid protein fraction (TPL) and the solid protein fraction (TPS) subjected to different US frequencies (35 kHz and 130 kHz) and exposure times (20, 30, and 40 minutes), the respective controls, and the raw sample (RS).

However, those studies used higher potencies when compared to the grass carp by-product of the present study (50 W and 130 W), and US acted as an effect enhancer. Therefore, the utilization of US alone results in diverse effects on proteins, and these effects are correlated with the protein chain size and the specific US conditions applied.

The treatments performed with a frequency of 130 kHz showed a more pronounced effect on modifying band characteristics in the TPL treatments. Although, no protein breaks were observed, the sum of all modifications caused by the US treatment resulted in a decrease in band intensity for these samples. In contrast, in the TPS samples, the 35 kHz frequency promoted more significant modifications, as demonstrated by the absence of the 23 and 25 kDa bands (Figure 3C, rectangle A). One of the factors to be considered is the origin of the material, where the TPL samples originate from the liquid fraction of the treatment, exhibiting higher solubility and, consequently, a protein conformation susceptible to modifications caused by the lower intensity of the effects of the 130 kHz US. Meanwhile, the TPS samples originated from the solid fraction and exhibited insoluble characteristics, which were more impacted by the intense cavitation forces produced by the 35 kHz frequency. Both TPS and TPL samples showed the presence of low molecular weight proteins (< 30 kDa). The proteins in TPL stood out, as all bands were more intense, indicating a higher concentration of protein sequences. Studies performed on this molecular weight range have found a large concentration of bioactive compounds of protein origin (SAFARI & YAGHOUBZADEH, 2020), which can be used to formulate various products.

CONCLUSION

US technology led to extraction and changes in protein fractions from grass carp backbones to low

temperature. The most pronounced changes in TPS and TPL were detected at 35 kHz and an exposure time of 30 minutes. Although, protein breaks were not detected in the performed electrophoresis, different effects were observed in the shape and intensity of the protein bands as a function of the molecular weight. Even though the frequency of 130 kHz did not provide protein breakdown, it had greater effects on TPL proteins with molecular weights between 100 and 25 kDa, while the frequency of 35 kHz had greater effects on TPS proteins with low molecular weight bands (< 30 kDa). The protein fractions of this study (TPS and TPL) open new perspectives for their use as raw materials with modified characteristics, allowing their application in new food formulations. The different characteristics in terms of solubility, hydrophobicity, and sulfhydryl groups, as well as the changes in the electrophoretic profile promoted by US, can contribute to the improvement of food formulations and bring health benefits to the consumer due to the presence of compounds with low molecular weight. The modified characteristics can be useful in the development of emulsified products or may even exhibit antioxidant properties. Therefore, more studies are needed to identify the impacts of adding TPS and TPL to food formulations and the effects of bioactive compounds due to the presence of proteins with a molecular weight below 30 kDa.

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DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHORS' CONTRIBUTIONS

All authors contributed equally to the conception and writing of the manuscript. All authors critically revised the manuscript and approved the final version.

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