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Identification of soluble proteins present in giant squid (*Dosidicus gigas*) meal for human consumption[1]

Identificación de proteínas solubles presentes en la harina de calamar gigante (*Dosidicus gigas*) para consumo humano

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Abstract

Introduction. The giant squid (*Dosidicus gigas*) is a species of great abundance and considered as potential resource to meet the demand for protein in Peru. Giant squid meal represents an alternative to traditional proteins which can be used in food fortification. **Objective.** To optimize the extraction process and identification of soluble protein from giant squid meal (GSM). **Materials and methods.** This study was conducted at the Universidad Nacional Agraria La Molina, Lima, Peru, between 2018 and 2019. To obtain the highest yield of soluble protein (Ŷ) extracted, an alkaline extraction method using NaOH followed by acid precipitation at pH 4.5 was applied. Surface response methodology was used to determine the optimal parameters for protein extraction such as temperature, concentration of NaCl, time, and the ratio GSM: solvent. A 1D and 2D electrophoresis study was carried out to find the distribution of molecular weights and to identify the main proteins of GSM. **Results.** Values for optimal response were the concentration of NaCl 0 M, an extraction time of 35 min, the ratio GSM: solvent of 1:31.72, and a temperature at 71.9 ºC. The molecular weights of the proteins detected were in the range of 6.5 and 38.37 kDa, which would correspond to tropomyosins, troponins, and myosin light chain residues. **Conclusions.** This study allowed to optimize the extraction parameters and to identify soluble proteins corresponding to the sarcoplasmic fraction of the giant squid meal (GSM), which could be used in the food industry.

Keywords: non-protein nitrogen, alkaline extraction, sarcoplasmatic protein, fortified foods.

Resumen

Introducción. El calamar gigante (*Dosidicus gigas*) es una especie de gran abundancia y está considerado como un recurso potencial para satisfacer la demanda de proteína en Perú. La harina de calamar gigante representa una alternativa a las proteínas tradicionales, el cual puede ser usado en la fortificación de alimentos. **Objetivo.** Optimizar el proceso de extracción e identificación de proteínas solubles de la harina de calamar gigante (HCG). **Materiales y**

métodos. Este estudio se realizó en la Universidad Agraria La Molina, Lima, Perú, entre 2018 y 2019. Para obtener el mayor rendimiento de proteína soluble (Ŷ) extraída, se aplicó el método de extracción alcalina utilizando NaOH seguido de una precipitación ácida a un pH de 4,5. Se utilizó metodología de respuesta de superficie para determinar los parámetros óptimos para la extracción de proteínas como temperatura, concentración de NaCl, tiempo y la relación HCG: solvente. Se realizó un estudio de electroforesis 1D y 2D para encontrar la distribución de pesos moleculares e identificar las principales proteínas del HCG. **Resultados.** Los valores de respuesta óptima fueron la concentración de NaCl 0 M, un tiempo de extracción de 35 min, la relación HCG: solvente de 1:31,72 y una temperatura de 71,9 °C. Los pesos moleculares de las proteínas detectadas estuvieron en el rango de 6,5 y 38,37 kDa, lo que correspondería a tropomiosinas, troponinas y residuos de cadenas ligeras de miosina. **Conclusiones.** Este estudio permitió optimizar los parámetros de extracción e identificar las proteínas solubles las cuales corresponden a la fracción sarcoplasmática de la harina de calamar gigante (HCG), lo cuales pueden ser utilizados en la industria de alimentos.

Palabras clave: nitrógeno no proteico, extracción alcalina, proteína sarcoplasmática, alimentos fortificados.

Introduction

In Peru, giant squid (*Dosidicus gigas*) is the second most extracted species after anchovies. Despite that, this natural resource lacks significant added value for its use in industry. In fact, although frozen mantles, fins, tentacles, and napes represent the largest export volume, they certainly are minimally processed products (Luna, 2015; Rovegno, 2021). Total harvest of giant squid in this country reached a peak of 556 000 t in 2014, mostly done by local fishing fleets. Squads of giant squid in Peruvian waters are considered either underexploited or moderately exploited as determined by assessments of biomass estimates using acoustic surveys combined with modeling of surplus production (Csirke et al., 2018).

The squid or giant squid is a species of high nutritional value, with low calories content, low fat, and high quality of proteins, among other nutrients. Squids are characterized by white flesh, a very attractive feature for the fishing industry for the production of similar products and *surimi* (Luna, 2015; Solari-Godiño et al., 2017). Moreover, the steady increase in human population worldwide makes necessary to find alternatives to traditional proteins. One of them is marine proteins which have a high digestibility component (Calvo et al., 2016; Zhang et al., 2017).

Giant squid meal (GSM) for human consumption represent an alternative for traditional protein used in food fortification. Is report GSM as a protein powder with a biological value of 90 % - 86 % protein, and 3 % of fat, from which 40 % are Omega 3 and 6 (Roldán Acero, 2007). In addition, consumption of the giant squid meal showed not to exhibit toxic effects or mortality in Sprague Dawley rats during a 90-day evaluation that included use of repeated doses (Rojas Hurtado, 2009).

In other studies, obtained a giant squid meal with 77.7 % total protein, 6.3 % crude fat with a ratio of saturated, mono-unsaturated, and polyunsaturated fatty acids of 1.66: 1: 1.08, and a ratio of n6: n3 of 1:1.35, so this was identified as a promising product (Calvo et al., 2016).

The GSM was used to elaborate extruded functional foods integrated in cereals which met the nutritional requirements of proteins and essential amino acids and had very good acceptability (93.3 %) among infants. Although there is information on the proteins that are part of the muscle structure in giant squids, information on soluble proteins that are present in the GSM is almost nonexistent (Espinoza et al., 2021; Roldán-Acero et al., 2021).

The most traditional method to obtain proteins from vegetable or meat sources is alkaline extraction and acid precipitation, to date, is the main scientific research and actual production of proteins, this method has been widely employed in protein extraction industry (Hou et al., 2017; Jangchud & Chinnan, 1999).

The objective of this research was to optimize the extraction process and identification of soluble protein from giant squid meal (GSM).

Materials and methods

The research was conducted between February, 2018 and January, 2019, at the Laboratory of Hydrobiological Resources Processes at the Department of Fishery and at the laboratories of Industrial Biotechnology and Bioprocesses which are branches of the Biotechnology Institute (IBT) at the Universidad Nacional Agraria La Molina (UNALM) in Lima, Peru.

Raw material

In the study, 2 kg of giant squid meal (GSM) produced from mantle tissue of giant squid (*Dosidicus gigas*) was used.

Chemical composition

Analysis of chemical composition in GSM were measure as moisture content by AOAC method 930.15, weigh the sample in a clean and dry Petri dish (10 g sample), place in the oven for 3 h at 105 °C ± 2 °C, cool for 30 min and weigh. The total protein content of the GSM was estimated by Kjeldahl procedure (N × 6.25) as described in AOAC method 981.10, approximately 1 g of raw material was hydrolyzed with 15 mL of concentrated sulfuric acid (H_2SO_4) containing catalyst in a heat block at 420 °C for 2 h. After cooling, H_2O was added to the hydrolysates before neutralization and titration. Crude fat content was obtained by AOAC method 948.16, samples were extracted with ether in a Soxhlet-type extractor for 16 h, then the lipid extract is evaporated to dryness at 95–100 °C and weighed. Moisture, total protein, and crude fat were expressed as percentages (Association of Official Analytical Chemists International, 2016). All analyzes were carried out in triplicate.

Alkaline extraction of protein from giant squid meal

An alkaline extraction protocol was applied in this study following the methodology described by Fritz et al. (2011) with modifications, which is detailed below.

Alkaline extraction: GSM protein was extracted using a combination of variables: NaCl concentration (M), ratio GSM: solvent (g mL^{-1}), temperature (°C), and extraction time (min). The protein extraction was carried out under alkaline conditions (a pH of 11 was calibrated with 10 N NaOH solution) which was applied to 2 g sample in a 500 mL flask. The mixture was then kept under constant agitation (200 rpm) in a shaking water bath (GFL Model 1083, Germany) under extraction conditions defined by the statistical design.

Centrifugation

After the alkaline extraction time was done, the solution obtained was centrifuged at 4000 g for 20 min at a temperature of 10 °C, using a centrifuge (Hettich Zentrifugen, Germany), then the supernatant was collected.

Filtration

The supernatant was filtered using a vacuum filtration system (Vacumbrand ME2C, Germany) and Whatman filter paper No. 1, this filter was used to recover floating particles in supernatant. The content of the soluble protein in mg/100 g, and the yield of the extracted protein in percentages (%), were determined.

The soluble protein obtained after the optimization process was precipitated at pH 4.5 using 1M HCl and then centrifuged at 4000 g for 12 min at 8 °C. The resulting precipitate was washed twice and then resuspended in water at a pH adjusted of 7.0 with 1N NaOH. To produce soluble protein powder, the solution was lyophilized (Labconco Freezone 4.5 Lyophilizer, USA).

Determination of moisture and total protein in the extracted soluble protein

Lyophilized soluble protein was used to determine moisture and total protein. The analysis of moisture was measure by AOAC methods 930.15, weigh the sample in a clean and dry Petri dish (10 g sample), place in the oven for 3 h at 105 °C ± 2 °C, cool for 30 min and weigh. Total proteins were carried out following AOAC method 981.10, approximately 1 g of raw material was hydrolyzed with 15 mL of concentrated sulfuric acid (H_2SO_4) containing catalyst in a heat block at 420 °C for 2 h. After cooling, H_2O was added to the hydrolysates before neutralization and titration. The amount of total nitrogen in the raw materials were multiplied by conversion factor of 6.25 (Association of Official Analytical Chemists Internationa, 2016). All the analyzes were carried out in triplicate.

Determination of non-protein nitrogen (NNP)

Non-protein nitrogen (NNP) was analyze followed the method proposed by Hashimoto et al. (1979) and was applied to both, the fresh giant squid sample and the soluble protein extracted. To accomplish this, 20 g of GSM was weighed and then 200 mL of 0.05 M phosphate buffer, pH 7.5 (a mixture of 5.6 mM Na_2HPO_4 and 3.5 mM KH_2PO_4) was homogenized under constant stirring at temperatures between 3 and 4 °C. The homogenate was centrifuged at 5000 g for 15 min in a centrifuge (Hettich Zentrifugen. Germany). An additional 200 mL of phosphate buffer was added to the precipitate and the previous procedure was repeated. To separate the sarcoplasmic protein from the NNP, 5 % trichloroacetic acid was added. NNP content was determined by the Kjeldahl method and results were reported in mg NNP/g sample.

Determination of soluble protein

This analysis was done in the soluble protein extracted applying the method proposed by Lowry et al. (1951) which is based on the reaction of copper in alkali, and the reduction of the reagent phosphomolybdic-phosphotungstic by a protein-copper complex. Hence, four solutions were prepared: Reagent A (2 % Na_2CO_3 and 0.1M NaOH), Reagent B1 (0.5 % $CuSO_4.5H_2O$), Reagent B2 (1 % sodium tartrate) and Reagent C (a mixture of Reagent B1, B2 with Reagent A). Folin-Ciocalteu 1N was used to prompt the reaction. For quantification, absorbance of the reaction at 750 nm was measured using a spectrophotometer (Thermo Spectronic, Genesys 20. USA), results were expressed in mg g^{-1} of sample considering a bovine serum albumin standard curve.

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Two-dimensional electrophoresis

The optimized soluble protein obtained after extractions was lyophilized and characterized using electrophoresis (Laemmli, 1970). Two separation dimensions were used in this analysis: IEF isoelectric focusing (first dimension) and SDS-PAGE (second dimension) following a procedure described by Pedreschi Plasencia (2009).

The first phase - isoelectric focusing (IEF) – was used to identify the type of proteins, which were immobilized in 18 cm dried strips (IPG) in a pH range of 3 to 10. The strips underwent passive rehydration overnight covered with mineral oil. After that the strips were washed with water from the Milli-Q water system (Merck-Millipore, Germany) and the wells were loaded with 50 µL of rehydration buffer [7 M urea, 2 M thiourea, (3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate)] (CHAPS) 4 % (v/v), bromophenol blue, DTT (Dithiothreitol) 75 mM, ampholyte buffer 40 % containing 5 µg of protein and covered with mineral oil to avoid dehydration caused by the heat generated from the electric field. The IEF100 Isoelectric Focusing system (Hoefer, Holliston MA, USA) was used and 4 ramps were programmed to increase voltage intensities (3 h at 250 V, 6 h at 1000 V, 3 h at 8000 V, and 2 h at 240 000 V). At the end of the IEF phase, the strips were equilibrated with equilibration buffer I (2 % DTT) for 15 min, then in equilibration buffer II (2.5 % iodoacetamide) for another 15 min.

The second phase - sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) – At this stage, three ramps with progressive voltage increases were applied (1st ramp 100 V for 70 min, 2nd ramp 120 V for 100 min, and 3rd ramp 140 V for 120 min). To visualize the proteins, silver staining according to Blum et al. (1987), and a GS-900 densitometer (Bio-rad, USA) were used.

Experimental design and statistical analysis

To evaluate and optimize the GSM protein extraction process, the response surface methodology suggested by Aquino Méndez (2016) was implemented through two steps. The first was a two-level full factorial design used to assess the effect of four factors involved in the protein extraction process. The experimental factors and levels evaluated are shown in more detail in Table 1.

Table 1. Experimental factors and levels evaluated in the 2^K factorial design for the extraction of soluble protein from giant squid (*Dosidicus gigas*) meal conducted at the laboratories of Industrial Biotechnology and Bioprocesses at the Universidad Nacional Agraria La Molina (UNALM) in Lima, Peru, 2018.
Cuadro 1. Factores y niveles experimentales evaluados en el diseño factorial 2^K para la extracción de proteína soluble de la harina de calamar gigante (*Dosidicus gigas*), realizado en los laboratorios de Biotecnología Industrial y Bioprocesos de la Universidad Nacional Agraria La Molina (UNALM), Lima, Perú, 2018.

		Levels		
Independent variables	**Coded**	**-1**	**0**	**1**
NaCl Concentration (M)	A	0	1	2
Time (min)	B	10	35	60
Temperature (ºC)	C	20	42.5	65
GSM: solvent Ratio (g mL^{-1})	D	1:10	1:30	1:50

For the second step, once non-significant effects of GSM were determined, additional tests were added to the response surface method. To optimize the process, a Central Compound Design (CCD), as described by Ayala and

Pardo (1995), was applied (Table 2). This design considered the significant factors: temperature (°C), and the ratio GSM: solvent (D) while maintained NaCl concentration (A) as a constant because the best results were achieved when it has the lowest concentration (0 M). An upward scaling was used to get an approximation to the optimal region, and for that it was necessary to expand the range of significant factors temperature (C) and ratio GSM: solvent (D) between 40-80 °C and 1:40 - 1:80, respectively. The non-significant factor time (B) remained constant at 35 min. The baseline for optimization was yield of soluble protein (Y). This methodology was implemented using the statistical package Design Expert 10 (2016, MN - USA).

Table 2. Experimental factors and levels evaluated in the central composite design (CCD) for optimizing the giant squid (*Dosidicus gigas*) meal soluble protein extraction process conducted at the laboratories of Industrial Biotechnology and Bioprocesses at the Universidad Nacional Agraria La Molina (UNALM) in Lima, Peru. 2018.

Cuadro 2. Factores y niveles experimentales evaluados en el diseño central compuesto (DCC) para la optimización del proceso de extracción de proteína soluble de la harina de calamar gigante (*Dosidicus gigas*), realizado en los laboratorios de Biotecnología Industrial y Bioprocesos de la Universidad Nacional Agraria La Molina (UNALM) en Lima, Perú. 2018.

		Levels				
Independent variables	**Coded**	**-1.41**	**-1**	**0**	**1**	**1.41**
Temperature (ºC)	C	31.72	40	60	80	88.28
GSM: solvent Ratio (g mL^{-1})	D	1:31.72	1:40	1:60	1:80	1:88.28

Results

The results of the GSM analysis revealed that the contents of protein, moisture, and lipid were 88.05±0.03 %, 7.51±0.015 %, and 1.26±0.02 %, respectively. The complete factorial 2^4 design applied to evaluate the effects of the experimental factors: concentration of NaCl, time, temperature, and the ratio GSM: solvent, on the soluble protein extraction process are shown in Table 3. The maximum extraction process yielded was 11.41 %.

Results also revealed that temperature had a positive effect during extractions, this possibly in combination with other factors such as pH, the ratio GSM: solvent, and time, since all of them play important roles on protein solubility. According to the results obtained, only the linear term time was not significant ($p>0.05$), when compared to yield at the extraction process. An opposite behavior was observed in NaCl concentration, GSM: solvent ratio, and temperature, which were the most significant.

The soluble protein extraction yield model ($\hat{Y}$) explained 88.27 % of the variability (adjusted R^2) and was represented by the first order equation (1) which was transformed to a logarithm base 10 to meet assumptions of normality.

$$Log10\ (Y) = 0.4810 - 0.0950A + 0.0373B + 0.1165C + 0.1757D + 0.0587BD \quad (1)$$

The ANOVA showed that a curvature with a p-value of 0.0022. This result indicate that it is required to look for a second order model to get an approximation towards the optimal region.

Protein yield (Y) obteined by central compound design was within the range of 2.56 and 13.10 %, and that the best combination of variables was a temperature of 80 °C and, a GSM: solvent of 1:40 which provided a maximum extraction yield of 13.10 % (Table 4).

The mathematical model using in the second order revealed a relationship between yield of soluble protein (Y) and the independent variables. This is presented in equation (2).

Table 3. The complete factorial 2^K design with replications in the center, coded variables (-1, 0 1), experimental and estimated yield of the giant squid (*Dosidicus gigas*) meal soluble protein conducted at the laboratories of Industrial Biotechnology and Bioprocesses at the Universidad Nacional Agraria La Molina (UNALM) in Lima, Peru, 2018.

Cuadro 3. Diseño factorial completo 2^K con repeticiones en el centro, variables codificadas (-1, 0 1), rendimiento experimental y estimado de la proteína soluble de la harina de calamar gigante (*Dosidicus gigas*), realizado en los laboratorios de Biotecnología Industrial y Bioprocesos de la Universidad Nacional Agraria La Molina (UNALM) en Lima, Perú, 2018.

Concentration NaCl (M)	Time (min)	Temperature (°C)	GSM: Solvent Ratio (mg mL^{-1})	Yield (Ŷ)	
				Experimental	Estimated
1 (0)	35 (0)	42,5 (0)	1:30 (0)	5.58	4.29
0 (-1)	10 (-1)	20 (-1)	1:10 (-1)	1.82	1.96
2 (1)	60 (1)	20 (-1)	1:10 (-1)	2.06	1.68
2 (1)	10 (-1)	65 (1)	1:10 (-1)	2.28	3.11
0 (-1)	10 (-1)	20 (-1)	1:50 (1)	3.37	3.66
2 (1)	60 (1)	65 (1)	1:50 (1)	5.35	6.20
0 (-1)	60 (1)	65 (1)	1:10 (-1)	3.05	4.38
2 (1)	10 (-1)	20 (-1)	1:50 (1)	2.98	2.64
0 (-1)	10 (-1)	65 (1)	1:10 (-1)	5.98	5.00
0 (-1)	60 (1)	65 (1)	1:50 (1)	11.41	10.52
1 (0)	35 (0)	42.5 (0)	1:30 (0)	5.02	4.29
0 (-1)	60 (1)	20 (-1)	1:10 (-1)	1.25	1.58
2 (1)	60 (1)	65 (1)	1:10 (-1)	2.20	1.84
2 (1)	10 (-1)	65 (1)	1:50 (1)	4.52	4.12
2 (1)	60 (1)	20 (-1)	1:50 (1)	4.12	4.94
0 (-1)	60 (1)	20 (-1)	1:50 (1)	6.47	6.62
0 (-1)	10 (-1)	65 (1)	1:50 (1)	6.42	7.78
2 (1)	10 (-1)	20 (-1)	1:10 (-1)	1.89	2.71
1 (0)	35 (0)	42.5 (0)	1:30 (0)	5.95	4.29

Table 4. Central compound design (CCD) with repetitions in the center, coded variables, experimental and estimated yield of extraction of soluble protein from giant squid (*Dosidicus gigas*) meal conducted at the laboratories of Industrial Biotechnology and Bioprocesses at the Universidad Nacional Agraria La Molina (UNALM) in Lima, Peru, 2018.

Cuadro 4. Diseño central compuesto (DCC) con repeticiones en el centro, variables codificadas, rendimiento experimental y estimado de extracción de proteína soluble de la harina de calamar gigante (*Dosidicus gigas*), realizado en los laboratorios de Biotecnología Industrial y Bioprocesos de la Universidad Nacional Agraria La Molina (UNALM) en Lima, Perú, 2018.

Temperature (°C)	GSM: Solvent ratio (g mL^{-1})	Yield	
		Experimental (Ŷ)	Estimated (Ŷ)
60 (0)	1:60 (0)	9.82	9.70
60 (0)	1:31.71 (-1.41)	10.10	11.39
88.28 (1.41)	1:60 (0)	11.19	10.93
31.71 (-1.41)	1:60 (0)	4.20	3.64
60 (0)	1:60 (0)	9.57	9.70
80 (1)	1:80 (1)	8.17	9.67
40 (-1)	1:80 (1)	2.56	2.56
80 (1)	1:40 (-1)	13.10	12.20
60 (0)	1:88.28 (1.41)	9.57	7.47
40 (-1)	1:40 (-1)	7.98	7.29

Yield(Ŷ)= -4.79715+0.471408C+0.0478097D+0.00300781C2+0.00030625CD-0.000332811D2 (2).

The analysis of variance for this stage determined a high significance ($p<0.05$) for temperature, identifying this parameter as the one with the greatest influence in the extraction of soluble protein from GSM. The relation GSM: solvent was not significant. Shows the variability of the results, explained by an adjusted R^2 of 85.43 %.

The relationship between experimental and estimated values calculated by the model shows in Figure 1. In the graph, the association between the experimental values and estimated values, determined with the mathematical model, had an R^2 value of 89.68 %.

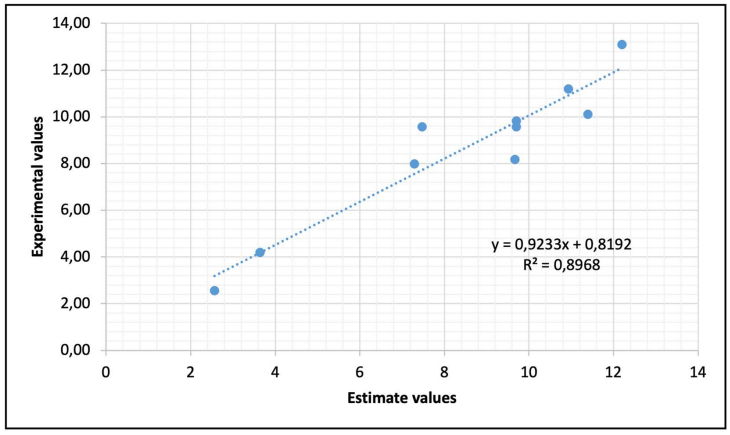

Figure 1. Relationship between experimental and estimated values, obtained to calculate yield of protein extracted from giant squid (*Dosidicus gigas*) meal conducted at the laboratories of Industrial Biotechnology and Bioprocesses at the Universidad Nacional Agraria La Molina (UNALM) in Lima, Peru, 2018.

Figura 1. Relación entre los valores experimentales y estimados, obtenidos para calcular el rendimiento de proteína extraída de la proteína de la harina de calamar gigante (*Dosidicus gigas*), realizado en los laboratorios de Biotecnología Industrial y Bioprocesos de la Universidad Nacional Agraria La Molina (UNALM) en Lima, Perú, 2018.

The surface response showed that the solvent: GSM ratio exhibits a direct effect on decreasing protein concentration, which was directly related to the quantity of solvent and feedstock (Figure 2).

Thus, the maximum yield (Ŷ) for a desirability level of 0.92 was 12.59 % and the optimized parameters were 79.98 °C and 1:31.72 for temperature and for the ratio of GSM: solvent, respectively.

When analyzing the protein content of the lyophilized protein extract collected from GS, an amount of 86±0.1 % was determined in dry matter.

Determination of non-protein nitrogen content (NNP)

Results obtained from non-protein nitrogen content (NNP) in fresh giant squid muscle and GSM were 8.2 and 1.14 mg NNP/g of sample, respectively. The result obtained for the lyophilized optimized soluble protein was 1.0 ± 0.021 mg NNP/g, thus this confirmed that the content of this compound decreased after the processes of washing and cooking those raw materials underwent during the preparation of GSM.

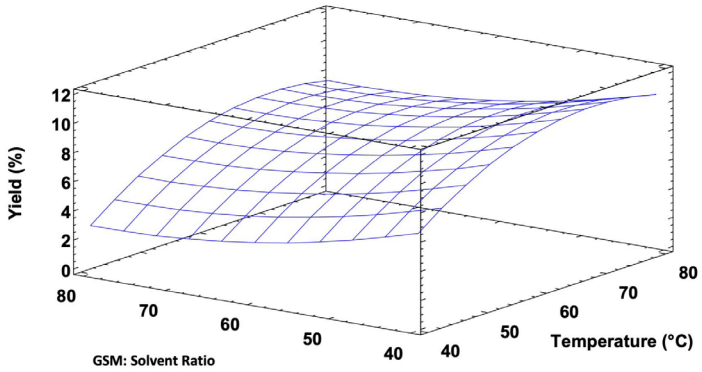

Figure 2. Surface response of the giant squid (*Dosidicus gigas*) meal soluble protein extraction yield, conducted at the laboratories of Industrial Biotechnology and Bioprocesses at the Universidad Nacional Agraria La Molina (UNALM) in Lima, Peru, 2018.

Figura 2. Superficie respuesta del rendimiento de extracción de proteína soluble de la harina de calamar gigante (*Dosidicus gigas*), realizado en los laboratorios de Biotecnología Industrial y Bioprocesos de la Universidad Nacional Agraria La Molina (UNALM) en Lima, Perú, 2018.

Electrophoretic profile

The electrophoretic pattern of a broad range of molecular weights and the GSM protein extract are shows in Figure 3A. A band of 38.37 kDa observed in the gel could be tropomyosin, a protein of regulatory function in the giant squid. The bands with greater intensity ranged between 6.5 and 21.5 kDa was observed.

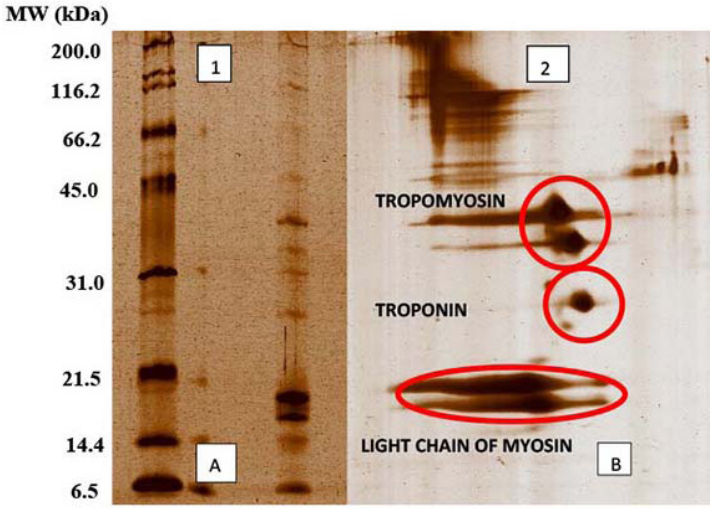

Figure 3. Electrophoretic Profile of soluble protein obtained in giant squid (*Dosidicus gigas*) meal. (A) Electrophoretic pattern (SDS-PAGE): (1) molecular weight for wide range standard ladder. (2) Protein extract of giant squid protein concentrate. (B) Two-dimensional electrophoretic profile. Laboratories of Industrial Biotechnology and Bioprocesses at the Universidad Nacional Agraria La Molina (UNALM) in Lima, Peru, 2019.

Figura 3. Perfil electroforético de proteína soluble obtenida de la harina de calamar gigante (*Dosidicus gigas*). (A) Patrón electroforético: (1) estándar de pesos moleculares de amplio rango. (2) Extracto proteico del concentrado proteico de calamar gigante. (B) Perfil electroforético bidimensional. Laboratorios de Biotecnología Industrial y Bioprocesos de la Universidad Nacional Agraria La Molina (UNALM) en Lima, Perú, 2019.

A two-dimensional electrophoretic profile presented in Figure 3B shows protein migrations according to their isoelectric points. The proteins observed had isoelectric points in the acid range, between a pH of 3 and 5.4. Based on the molecular weight of the proteins, it was possible to determine the presence of tropomyosin (TM), troponins (T) or myosin light chain residues (MLC).

Discussions

Chemical composition results were similar to those reported by Roldán Acero (2007), using similar materials, where percentages of total protein, moisture, and fat were 86.04 %, 6.30 %, and 2.70 %, respectively. Also, have been reported that for giant squid meal, the protein content was 77.76 %, the ethereal extract was 6.33 %, and a final moisture level of 3.46 %. These differences may be explained by the fact that only one part the mantle of the giant squid, was used (i.e., viscera, fins, feather, or nape were not included), so that this could have influenced the percentage of moisture, protein, and final fat (Calvo et al., 2016).

Values of protein of 85.42 %, fat of 2.65 %, and humidity of 5.34 % in giant squid meal utilized in the preparation of extruded functional foods was reported (Roldán-Acero et al., 2021). Differences with the present research can also be attributed to the type of squid species, the time of the harvest or the way the samples were handled as suggested by Calvo et al. (2016).

Regarding to the optimization of the extraction process by surface response, the yield of protein presented a very small value when compared to others, like those reported in Cortés-Ruiz et al. (2008), Dihort-Garcia et al. (2011), and Maza and Rosales (2004), where extraction yields, with alkaline solubilization of ground meat of giant squid, were 77.2 %, 80.86 %, and 72.2 %, respectively.

Difference in yield can be attributed to denaturation events generated by the heat treatments used during the GSM production. In fact, similar results were pointed out in Azagoh et al. (2016) when soluble protein of larvae and larval meal of *Tenebrio molitor* were contrasted and found that the meal had lower yield.

The solubility of proteins, when exposed to temperatures of about 90 °C, was inferior to those that were not treated (Ma et al., 2010). Protein denaturation by heat was directly proportional to a decrease in solubility (Alais et al., 2020). Hence, this extraction type has the advantage of increasing yields in protein extractions because of the decomposition of the protein matrix (Deleu et al., 2019). It is also known that sarcoplasmic proteins influence humidity levels in the protein matrix and that when temperatures go above 90 °C, they tend to be released (Elizondo-Garza et al., 2017). According to that this would depend on the species and the properties of their proteins, while higher yields in the methods using alkaline conditions are largely attributed to the recovery of sarcoplasmic proteins (Gehring et al., 2011; Momen et al., 2021).

Considering the comparison between yield and recovery process, these results agreed with Reddy Surasani et al. (2017) that an uneven dispersion of non-protein substances, in the homogenate used for recovery at different extraction times, could be the reason behind the differences in solubility and recovery obtained.

The conformational changes in the sarcoplasmic protein are influenced by pH and the concentration of NaCl. Thus, proteins exhibit high solubility and hydrophobicity in the absence of this compound (Lopez-Enriquez et al., 2015). This can be explained by a decrease in yield when large amounts of solvent are used. Consequently, a low GSM: solvent ratio would have inverse effect on extraction. Temperature showed a positive effect at ~70 °C, then the effect was negative on the protein extraction yield (Y) (Oomah et al. 1994; Rivas et al. 1981).

Respect to non-protein nitrogen, it is necessary to know that nitrogen that comes from food is found in two forms, either as part of the protein molecule (dietary protein) or as non-protein nitrogen (NNP) (Galindo et al., 2017). NNP values in fresh giant squid were 7.3 and 8.5 mg NNP/g, those results were similar (Maza et al., 2003;

Pedreschi, 1993). The low concentration of NNP in giant squid meal (GSM) would suggest that these compounds are reduced during the processes of washing and cooking.

NNP compounds were responsible of acid-bitter taste and distinctive smell in raw materials. Reduction and decrease take place as a consequence of consecutive washes that the raw material is subjected during the process of obtaining GSM. NNP could be derived from free amino acids, small peptides, nucleic acids, urea, and ammonium ions. Gel formation, including denaturation and irreversible aggregation of proteins to form large aggregates, plays an important role in the reduction of myofibrillar protein fractions (Zhao et al., 2019).

To optimize the extraction process, based on the mathematical model proposed, the optimal combination of factors to get the desired product was estimated. To attain maximum yield of protein extraction, a desirability function method described by Gutiérrez and De la Vara (2008) was applied. This was defined as a function of a number of factors which allow to estimate the global desirability of the product at each point, then desirability is maximized to the optimal.

In the latter, some main proteins in giant squid muscle tissue such as myosin, paramyosin, and actin were observed in the gel as low intensity bands as heat treatment reduces myosin heavy chain (200 kDa) (Murphy & Marks, 2000). Other types of proteins were recognized as troponin C (~18 kDa), troponin I (~22 kDa) or troponin T (~38.5 kDa), as well as to myosin light chain (De La Fuente-Betancourt et al., 2008; Dublán García, 2001; Pedreschi, 1993). Also was reported molecular weights around of 35 kDa for mollusks like scallops, mussels, squid, and fish such as herring (Mignino & Paredi, 2006; Shahidi & Venugopal, 1994).

Conclusions

In this study, the response surface method turned out to be a very useful tool because it allowed the optimization of the variables and processes, thus determining the appropriate parameters to obtain the maximum yield of the extraction of alkaline soluble proteins from the giant squid meal, for the investigation it was possible to obtain the highest yield of 12.59 % using the temperature parameters 79.98 °C, ratio of GSM -solvent 1: 31.72 and extraction time of 35 minutes. The use of the 1D and 2D electrophoresis system allowed the identification of the main soluble proteins such as tropomyosins, type I, and C troponins, as well as myosin light chain residues.

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