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Research Article Araştırma Makalesi

Optimization of Collagen Isolation from Carp (*Carasobarbus luteus*) Scales using Response Surface Methodology

Sazan (*Carasobarbus luteus*) Pulundan Kolajen İzolasyonunun Yanıt Yüzey Yöntemiyle ile Optimizasyonu

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Abstract: The scale, which is separated during the mechanical processing of fish, can be used in in many other different ways besides the production of collagen products. To obtain high-purity natural collagen, it is necessary to remove non-collagenous proteins and perform demineralization of fish scales. Therefore, the aim of this study is to achieve collagen isolation from common carp (*Carasobarbus luteus*) scales for the production of collagen products with higher efficiency and quality. The demineralization of the scales was optimized using response surface methodology. Experimentally, approximately 89.92±1.10% of the mineral was removed, which closely matched the value predicted by the model. The protein content of the fish scales was increased from 23.12% to 86.16% after the applied procedures. At the end of all the processes, 20.54% of the protein and 3.56% of hydroxyproline (Hyp) were lost. However, the Hyp concentration in the fish scale samples increased from 23.85 to 56.13 mg/g scale, and the Hyp/pro ratio increased from 4.49% to 6.51%. The effective removal of mineral matter, the increase in the amount of Hyp in fish scales, and the increase in the Hyp/pro ratio demonstrate that the applied procedures successfully separate non-collagenous components and achieve collagen isolation.

Keywords
- Fish scale
- Collagen isolation
- Optimization
- Hydroxyproline

Özet: Balıkların mekanik işlenmesi sırasında ayrılan pullar kolajen ürünleri üretiminin yanı sıra birçok farklı şekilde kullanılabilmektedir. Yüksek saflıkta doğal kolajen elde etmek için, balık pullarının kolajen olmayan proteinlerden arındırılması ve demineralizasyonunu gerçekleştirmek gerekmektedir. Bu nedenle, bu çalışmanın amacı kolajen ürünlerinin üretimi için daha yüksek verim ve kalite için sazan balığı (*Carasobarbus luteus*) pullarında kolajen izolasyonunu sağlamaktır. Pulların demineralizasyonu yanıt yüzeyi metodolojisi ile optimize edilmiştir. Deneysel olarak mineralin yaklaşık 89.92±1.10'i uzaklaştırılmış ve bu, model tarafından tahmin edilen değerle oldukça tutarlı olmuştur. Balık puluna uygulanan işlemler sonucunda protein oranı %23.12'den %86.16'ya kadar çıkarılmıştır. Tüm işlemlerin sonunda proteinin %20.54'ü ve hidroksiprolinin %3.56'sı kaybedilmiştir. Ancak balık pul örneklerinde Hyp konsantrasyonu 23.85'ten 56.13 mg/g pula, Hyp/pro oranı ise %4.49'dan %6.51'e yükselmiştir. Mineral maddenin etkili bir şekilde uzaklaştırılması, balık pulundaki Hyp miktarı ve Hyp/pro oranının artması, uygulanan işlemlerin sonucunda kolajen olmayan bileşenlerin etkili bir şekilde ayrılmasının ve kolajen izolasyonunun sağlandığını göstermektedir.

Anahtar kelimeler
- Balık pulu
- Kolajen izolasyonu
- Optimizasyon
- Hidroksiprolin

1. INTRODUCTION

Collagen is an important animal-derived connective tissue protein, and its use is increasing in the fields of health, food, cosmetics, and biomaterial industries. The growing demand for collagen in various industries has led to a focus on alternative sources (Jaziri et al., 2022). Collagen isolation from fish processing by-products is considered one of these alternative sources. Approximately 30% of fish processing by-products consist of skin, scales, and bones, with fish scales accounting for about 5% of these by-products. These by-products are rich sources of collagen. Improper disposal of these by-products not only leads to serious environmental pollution but also primarily results in their use as animal feed or fertilizer (Yang et al., 2022). Therefore, it is necessary to optimize the utilization of these by-products, especially in the production of value-added products. In recent years, these fish by-products have gained increasing attention as potential sources of collagen and gelatin. Additionally, fish scales have emerged as bio-composites containing highly regular type I collagen (Fengxiang et al., 2011; Zhang et al., 2020).

Fish scales exhibit variations among species but generally consist of connective tissue proteins, various minerals, and trace amounts of fat. The protein composition of scales, including collagen, keratin, and mucin, ranges from 41% to 84%, while the remaining portion mostly consists of calcium phosphate compounds (hydroxyapatite and calcium carbonate). The amount of hydroxyapatite in fish scales ranges from 38% to 46% (Sankar et al., 2008; Wang & Regenstein, 2009). Fish scales form a highly organized three-dimensional structure primarily composed of type I collagen fibers and hydroxyapatite within the extracellular matrix. The scales consist of two distinct regions: an outer (bone) layer and an inner fibrous plate. The demineralization of scales, particularly the scales located in the outer layer, is a critical step in the production of collagen and its derivatives from fish scales (Fengxiang et al., 2011; Yang et al., 2022). The presence of Ca^{+2} ions in the spiral structures of collagen causes relaxation, leading to the breaking of intra- and intermolecular hydrogen bonds in collagen molecules. Moreover, the presence of minerals adversely affects the quality of gelatin, which is the thermal denaturation product of collagen (Feng et al., 2015). In addition, the demineralization pretreatment of fish scales plays a critical role in the isolation efficiency, purity, and quality of collagen products. In the demineralization process of collagen-rich materials like fish scales and bones, EDTA (Ethylene Diamine Tetraacetic Acid) and commonly used strong acids such as HCl (Hydrochloric acid) are employed. HCl acid reacts with Ca^{+2} ions in mineral substances, resulting in the formation of $CaCl_2$, which is a water-soluble compound. Therefore, HCl acid can be used to react with the calcium salts in scales, enabling their dissolution. However, it is known that strong acids can cause damage to certain protein components in the scales during the demineralization process (Wang & Regenstein, 2009; Cansu & Boran, 2015). To achieve the highest yield and quality in collagen production, it is essential not only to remove minerals but also to minimize collagen loss (Cansu & Boran, 2015).

The objective of this study is to design a procedure for the removal of mineral impurities, the most significant impurity in collagen isolation from fish scales and optimize the conditions to isolate the highest amount of collagen. To achieve this goal, a two-step approach was followed. Firstly, a standard method using 0.1 M NaOH treatment for one hour was employed to remove non-collagenous proteins from fish scales. In the second stage, the demineralization process of scales from the species (*C. luteus*) was optimized using the response surface methodology in HCl solution. A central composite design (CCD) was adopted to investigate the effects of various conditions on demineralization. Protein, hydroxyproline (Hyp), and mineral contents were monitored, and the losses incurred during the applied processes were.

2. MATERIAL and METHODS

2.1. Material

Carp (*Carasobarbus luteus*) scales were used for collagen isolation. The skin of carp was obtained from local markets in Şanlıurfa, Türkiye. The scales were separated from the fish skin and frozen at -22°C. The frozen scales were then dried using a freeze-dryer (Armfield Limited-FT33, England) operating at a condenser temperature of -35 to -44°C and a vacuum pressure of 20 mbar for approximately 18 hours. The dried scales were stored in sealed plastic bags at -22°C. All chemicals

used in the study were of analytical grade.

2.2. Methods

2.2.1. Biochemical composition of fish scales

The moisture, protein, fat, and mineral content of the fish scale samples were determined according to AOAC (2000) methods. The protein content of the scale samples was calculated by determining the total nitrogen values using the Kjeldahl method (AOAC method 984.13) and converting them to protein content using a conversion factor of 5.7. The moisture content was determined by drying the scales in a oven (Nüve, NF500, Türkiye) at 105°C until a constant weight was achieved (AOAC method 927.05). The mineral content was determined in both dried scale samples and solutions in a muffle furnace at approximately 550°C for 16 hours (AOAC method 942.05). The fat content of the scales was determined using the solvent extraction method (Soxhlet) with n-hexane as the solvent (AOAC, 2000).

2.2.2. Removal of non-collagenous proteins in fish scales

To remove non-collagenous proteins and impurities from fish scales, the scale samples were treated with 0.1 M NaOH at a ratio of 1:25 (w/v) of scales to solution at room temperature (24°C) for 1 hour. The scale samples in the alkaline solution were gently agitated at 15-minute intervals. After the treatment, 25 mL of the alkaline solution containing the dissolved proteins (Pro), hydroxyproline (Hyp), and mineral content was collected and stored at -22°C. For neutralization of the fish scales, the scale samples were washed three times with tap water (1:5, w/v) from the alkaline solution and then drained. To remove excess moisture from the scales, they were frozen at -22°C for 16 hours and subsequently freeze-dried (Boran & Regenstein, 2009).

2.2.3. Demineralization Optimization of Fish Scales

Dependent variables and their levels determined for the removal of mineral matter, which is the component with the second-highest proportion in fish scales, are given in Table 1. The dependent variables for the removal of mineral matter in scale samples, namely HCl acid and treatment duration, were selected based on similar studies in the literature and preliminary trial results. The aim of the mineral separation process was to remove the targeted component while minimizing damage to collagen and/or protein and isolating collagen. Accordingly, the dependent variables were defined as the maximum removal of mineral matter and the minimum protein loss. The response surface methodology was employed to determine the conditions that result in the highest removal of mineral matter and the lowest protein loss. For the optimization of mineral matter removal, a central composite design with 2 factors, 3 levels, 2 center points, and 2 replications, requiring a total of 20 experiments, was adopted.

Table 1. Dependent variables and their levels selected for collagen isolation from fish scales.

Dependent Variables	Levels		
	-1	**0**	**1**
HCl acid concentration (M)	0.1	0.15	0.2
Treatment duration (min) (min)	60	90	120

The dried scale samples obtained after the removal of non-collagenous proteins were used for mineral separation optimization. 10 g of the purified scale samples were transferred to covered erlenmeyer flasks, and HCl acid solution (1/20, w/v) prepared at different concentrations as specified in the experimental design was added to the flasks. The prepared samples were placed in a shaking incubator (Lab-Line, USA) at 25°C and 150 rpm for varying durations as specified in the experimental design. At the end of the process, the volumes of the filtered solutions were measured using a graduated cylinder, and 25 mL of each solution was taken and stored at -22°C until further analysis to determine the protein, Hyp, and removed mineral content. The scale samples from which the solution was removed were washed three times with tap water, left overnight at -22°C, and then dried by freeze-drying.

2.2.4. Determination of protein and hydroxyproline content

The protein content (protein loss) in the solutions obtained after both the removal of non-collagen proteins and each trial of demineralization optimization was determined using the Biuret method, as

described by Gornall et al (1949). The protein content of the prepared samples was calculated based on the absorbance values measured at 540 nm after appropriate dilutions were made (Gornall et al., 1949).

The hydroxyproline (Hyp) content was determined in the solutions obtained after the removal of non-collagen proteins and at the optimal mineral separation point, as well as in the dried scale samples obtained at each stage. The method described by Woessner (1961) with some modifications was used to determine the Hyp content in both the solutions and dried scale samples. For the solutions, 2 mL was taken, and for the dried scales, 1 g was taken and hydrolyzed using 5 mL of 6N HCl acid in Pyrex tubes at 130°C in an oven (Nüve, NF500, Turkey). After hydrolysis, appropriate dilutions were made, and the absorbance of the samples was measured at 547 nm using a spectrophotometer (UV-Mini 1240 UV-VIS, Shimadzu, Kyoto, Japan). The Hyp values were calculated based on the absorbance readings (Woessner, 1961).

2.2.5. Calculation of protein, hydroxyproline, and mineral substance losses

Protein, Hyp, and mineral losses were calculated throughout the process of collagen isolation from fish scales. The changes in protein and Hyp content were tracked in both the solutions and the extracted samples. The amounts of Hyp and protein transferred to the solutions were determined and compared with the initial amounts present in the samples, resulting in the calculation of percentage losses. The Hyp/pro (%) ratios in the extracted samples were also calculated using equation 1.

$$Hyp/pro\ (\%) = \frac{Hydroxyproline\ (mg/\ g\ scale)}{Protein\ amount\ (mg/\ g\ scale)} * 100 \quad (1)$$

These calculations allow for the assessment of protein, Hyp, and mineral losses during the collagen isolation process and provide valuable information on the efficiency of the procedure. During the mineral separation stage, the mineral content of the scale samples was monitored, along with the extracts, to verify the removal of mineral substances. This monitoring aimed to confirm that the extracted minerals were effectively removed and that the desired level of separation was achieved (Wang & Regenstein, 2009).

2.3. Statistical analyzes

The analyses conducted in the study were performed with a minimum of 3 replications, and the results were presented along with standard deviation rates. The differences between the means of the analysis results were determined using variance analysis (ANOVA) within a 95% confidence interval. JMP 8.0 statistical software (SAS, USA) was used for optimization design, data analysis, regression models, regression terms, and surface graph plotting using the response surface method.

3. RESULTS and DISCUSSION

3.1. Optimization of the demineralization process

The experimental results obtained during the mineral separation stage were analyzed using JMP 8.0 software to obtain regression models for each dependent variable. The predicted results based on these regression models, along with the obtained experimental data, are presented in Table 2. The response surface graphs for the experimental results are shown in Figure 1.

With an increase in HCl concentration, the amount of dissolved mineral substance also increased, independent of the treatment time, and the highest mineral separation was observed at 0.2 M HCl concentration (Table 2, Figure 1). In a study conducted using the scales of mirror carp (*Cyprinus carpio haematopterus*), it was determined that an increase in HCl concentration led to an increase in the removed minerals. However, it was found that there was no statistically significant increase in removed minerals after 1M HCl concentration (Feng et al., 2015). The effect of treatment time, which is the dependent variable, on mineral separation was relatively limited compared to HCl concentration. It was observed that the mineral substance slightly increased with an increased treatment time at a constant HCl concentration, but this increase was not as influential as the HCl concentration variable (Figure 1). In a demineralization study conducted on carp scales, it was found that more than 90% of the existing minerals on the scales were removed when the HCl concentration was as high as 0.1 M (Wang & Regenstein, 2009). The study conducted by Skierka et al. (2007) highlighted that the impact

of treatment time was limited in the demineralization of Atlantic cod (*Gadus morhua*) fish spine bones using HCl. This suggests that the acid solution quickly encounters the mineral layer due to its location on the outer surface of the fish scales, as also noted by Feng et al. (2015).

Table 2. Response surface design for collagen isolation from fish scales, experimental and predicted responses.

			Mineral Removal (%)		Protein Loss (%)	
Trial	**HCl Concentration (M)**	**Treatment Duration (min)**	**Experimental**	**Estimated**	**Experimental**	**Estimated**
1	-1	-1	54.63	54.13	10.07	9.93
2	-1	1	58.37	59.27	19.26	18.37
3	1	-1	91.50	89.87	14.79	14.42
4	1	1	93.36	93.16	23.99	24.58
5	-1	0	56.80	55.55	15.38	15.82
6	1	0	88.50	90.37	22.23	21.17
7	0	-1	77.18	77.07	11.22	10.99
8	0	1	81.02	81.29	20.19	20.29
9	0	0	72.66	78.03	17.68	17.31
10	0	0	83.40	78.03	17.08	17.31
11	-1	-1	53.12	54.13	10.56	9.93
12	-1	1	59.14	59.27	18.00	18.37
13	1	-1	90.03	89.87	13.28	14.42
14	1	1	94.25	93.16	24.16	24.58
15	-1	0	55.84	55.55	14.97	15.82
16	1	0	89.14	90.37	21.90	21.17
17	0	-1	75.68	77.07	10.76	10.99
18	0	1	81.28	81.29	20.86	20.29
19	0	0	75.98	78.03	17.03	17.31
20	0	0	81.65	78.03	16.99	17.31

With the increase in HCl concentration and extension of treatment duration, the protein loss in the scales has also increased, and the highest protein loss was observed to be approximately 24% (Figure 1). In a study conducted by Wang & Regenstein (2009), it was stated that increasing HCl concentration during demineralization with HCl in carp fish scales resulted in an increase in protein loss (Wang & Regenstein, 2009). Since minimizing protein loss and maximizing mineral removal are the desired outcomes in the separation of minerals from fish scales, using a higher concentration of HCl solution and prolonging the treatment duration may seem more effective in removing minerals; however, it also leads to higher protein loss. Therefore, a relatively short duration and high concentration of HCl solution appear to be preferable for the mineral separation process.

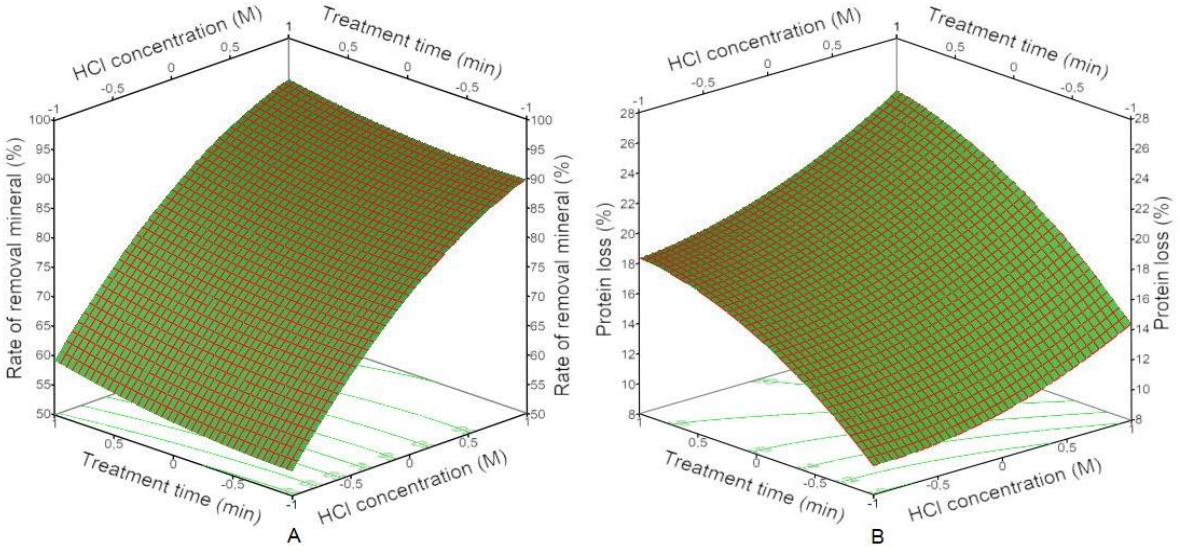

Figure 1. Surface plots of mineral removal (A) and protein loss (B) in collagen isolation.

The surface plots (Figure 1) for mineral removal and protein loss in collagen isolation show the relationship between HCl acid concentration (M) and treatment duration (min) on the x and y axes, respectively. The z-axis represents the corresponding values for mineral removal (%) and protein loss (%). These plots help visualize how different combinations of acid concentration and treatment duration affect the efficiency of mineral removal and the extent of protein loss during the collagen isolation process.

The statistical data for the model of mineral separation optimization from fish scales is presented in Table 3. The statistical results indicating the model's fit include determination coefficient (R^2) and adjusted determination coefficient (R^2_{adj}), which range between 0.97 and 0.98. Additionally, the mean square error (RMSE) and the high significance level of the *p*-value have been identified. The statistical results regarding the effect of HCl acid concentration as an independent variable on the rate of mineral removal and protein loss reveal that HCl concentration has a significant impact on both mineral removal ($p<0.001$) and protein loss ($p<0.001$). The effect of treatment duration on mineral removal ($p<0.01$) and protein loss ($p<0.001$) has also been determined.

Table 3. Model coefficients and analysis of variance (ANOVA) results for collagen isolation from fish scales.

Model Coefficients	**Mineral Removal (%)**	**Protein Loss (%)**
β_0	78.03	17.31
Linear		
β_1 *(HCl acid concentration)*	17.41^{***}	2.67^{***}
β_2 *(Treatment duration)*	2.11^{**}	4.65^{***}
Intercept		
B_{12} *(HCl acid concentration × Treatment duration)*	-0.46	0.43
Quadratic		
β_{11} *(HCl acid concentration)*^{2}	-5.07^{***}	1.19^{**}
β_{22} *(Treatment duration)*^{2}	-	-1.67^{***}
R^2	0.9770	0.9819
R^2_{adj}	0.9688	0.9755
RMSE	2.5303	0.6914
p-value	$<0.001^{***}$	$<0.001^{***}$
Desirability	0.88	

Model: *Mineral removal (%) = 78.03* + 17.41×β_1 + 2.11×β_2-5.07×β_{11}
Protein loss (%) = 17.31+2.67×β_1*+4.65×*β_2*+1.19×*β_{11}*-1.67×* β_{22}
Significance levels: $^{**}P<0.01$; $^{***}P<0.001$

The conditions for achieving the highest mineral removal and the lowest protein loss were determined during the optimization of the demineralization process. According to the statistical results obtained from the experimental data during the demineralization stage, the optimal conditions were determined to be an HCl concentration of 0.2 M and a treatment duration of 60 minutes. The desirability value for these conditions was found to be 0.88. Under these specified conditions, the estimated mineral removal rate was found to be 89.86% ± 3.42, and the estimated protein loss rate was 14.41% ± 0.94.

3.2. Hydroxyproline, protein losses and removal mineral rate

The mineral substance ratio, protein, and Hyp losses monitored throughout all the processes applied to fish scales are provided in Table 4. The experimental results of the expelled mineral substance and protein loss rates were determined as 89.92±1.10% and 13.32±0.30%, respectively (Table 4). After mineral substance optimization, no statistically significant difference was found between the predicted values of the model and the experimental data.

The results of the scale samples obtained after the alkaline treatment and optimal mineral separation process have shown that minerals were effectively removed. In the alkaline treatment applied for the removal of non-collagen proteins and other impurities, a trace amount of mineral substance was removed, resulting in a loss of 7.22±0.42% protein and 1.16±0.10% collagen. During the optimal mineral separation stage, there was a protein loss of 13.32±0.30% and a Hyp loss of 2.40±0.07%. Duan et al. (2004) reported that a 0.075 M EDTA solution removed approximately 68% of the minerals in carp scales. Treatment of carp scales with HCl acid (>0.1 M) resulted in the removal of 90% of the minerals, with protein and Hyp losses reported to be around 2% and 5.3%, respectively (Wang & Regenstein, 2009). However, Feng et al. (2015) found that carp scales needed to be soaked in 1M HCl acid for at least 90 minutes to remove over 90% of the minerals.

Table 4. Hyp, protein and mineral losses during collagen isolation.

Örnek	Removal mineral (%)	Protein loss (%)	Hyp loss (%)
After alkaline treatment	0.15±0.00	7.22±0.42	1.16±0.10
After optimal HCl treatment	89.92±1.10	13.32±0.30	2.40±0.07

3.3. Biochemical composition change and collagen isolation

In the production of collagen products, impurities such as minerals and fats in the raw material can hinder the diffusion of the target protein. As a result, the extraction yield of protein decreases, and the high concentration of impurities adversely affects the quality of the final product (Duan et al., 2004; Cansu & Boran, 2022). Fish scales consist of a hard surface layer composed mainly of calcium-based salts and a fibrous inner layer predominantly made of collagen. Therefore, in the production (extraction) of collagen products, a demineralization step is performed to expose the collagen fibers and facilitate solubility (Pati et al., 2010).

The images and biochemical composition of dried fish scale samples obtained after alkaline treatment and optimal mineral separation are presented in Figures 2 and 3, respectively. It was observed that the protein concentration in fish scales increased from 54% to 86% after all the applied processes. Additionally, the mineral content was successfully reduced from 26% to 1.2%. This indicates the effective removal of the most significant impurity, minerals, from fish scales (Pati et al., 2010). Furthermore, the alkaline treatment and demineralization processes applied to the untreated scale samples resulted in a cleaner appearance and improved processability of fish scales (Figure 2).

Figure 2. Morphological change of fish scales during collagen isolation.

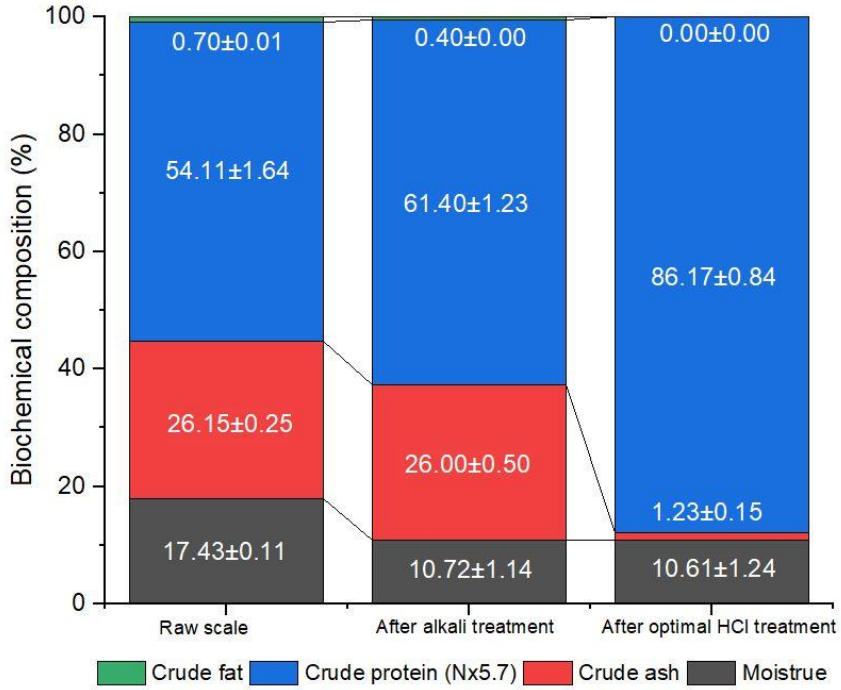

Figure 3. Biochemical composition change during collagen isolation.

During the processes applied to the fish scales, the change in Hyp concentration and the Hyp/pro ratio, calculated using protein and Hyp concentrations, were provided (Figure 4). Hydroxyproline (Hyp) is an unique imino acid in collagen and is used for estimating collagen concentration in animal-derived materials (Fratzl, 2008; Tümerkan et al., 2019). Despite the losses in both protein and Hyp during the processes applied to fish scales, Hyp concentration gradually increased. It was determined that the Hyp concentration in fish scales successfully increased from 23.85 to 56.12 mg/g scale through the performed processes. Additionally, the Hyp/pro ratio, which is an indicator of collagen within the total protein after the removal of non-collagen proteins and minerals from the scale samples, increased from 4.49% to 6.51%. These increases in Hyp concentration and Hyp/pro ratio indicate the successful isolation of collagen.

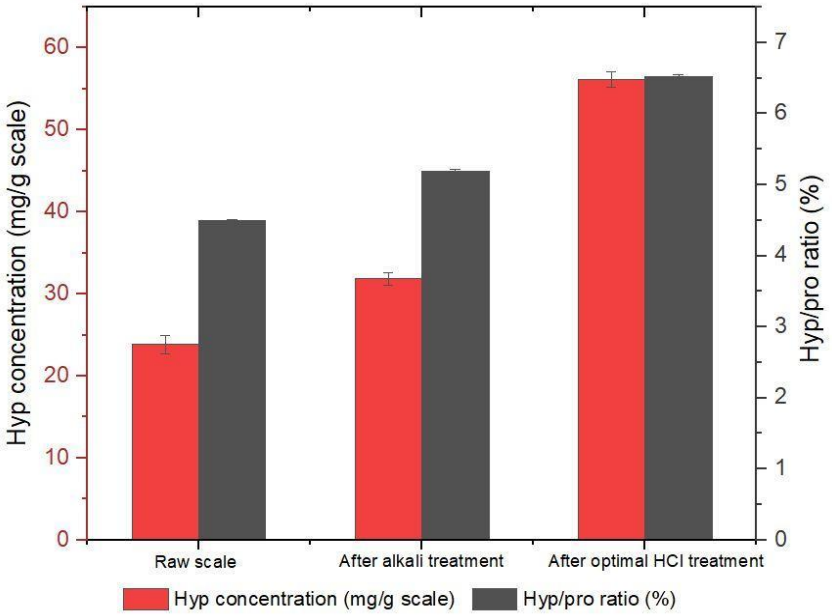

Figure 4. Hyp content and Hyp/pro ratio change during collagen isolation.

5. CONCLUSION

This study focused on the isolation of collagen from fish scales, which are considered as by-products of fish processing. The aim was to increase the protein content and remove impurities from the fish scales before collagen and its products' production. The conditions for removing mineral substances, which are the second most abundant component in fish scales, were optimized. The mineral separation process of the scales subjected to alkali treatment using a standard method was predicted and optimized using response surface methodology (RSM) by experimental variables of HCl acid concentration (M) and treatment time (minutes). The high correlation of the model demonstrated that the linear model could be used to optimize the demineralization of carp scales. Under the conditions of alkali treatment and optimal mineral separation, it resulted in a 90.07% mineral removal along with 20.55% protein and 3.56% Hyp loss. The total protein in the scale was successfully increased to 86%, and the amount of Hyp in the total protein was raised to 56.12 mg/g scale. This indicates that collagen in fish scales was effectively isolated. Further studies are needed to determine the quality and functional properties of the isolated collagen.

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CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

AUTHOR CONTRIBUTIONS

This article was written by a single author.

ETHICAL STATEMENTS

There are no ethical issues with the publication of this manuscript.

DATA AVAILABILITY STATEMENT

Data supporting the findings of the present study are available from the corresponding author upon reasonable request.

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