Ultrasonic extraction and identification of carp scale collagen

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Abstract. In this experiment, the carp scale as raw material, containing calcium and protein impurities in the scale and so on, respectively by two sodium EDTA (EDTA two sodium, EDTA) and sodium chloride solution (sodium chloride, NaCl) solution and its removal, scale collagen protein extraction and design. By using different single factor test the 4 factors determine the extraction of collagen during the experiment the optimum extraction process, the 4 factors are the enzyme (pepsin) dosage, ultrasonic time, solid-liquid ratio, extraction liquid (acetic acid) concentration of glue. The optimum extraction process for raw protein ultrasonic time 200Min, enzyme dosage 300mg/g, extraction concentration of 0.3 mol/L, the ratio of material to liquid 1:40 (g/mL), in this condition, the extraction rate was 28.7. By UV spectrophotometer identification, the material in the 230nm have obvious absorption peak, so the test the material of collagen.

1. Introduction

Freshwater fish production of china ranked the first in the world, used in aquatic products processing of raw materials mainly focus on fish [1-2]. Fish processing waste (scales, internal organs, bones) has not been fully utilized, general sold as low feed, waste, or random discharge, cause resource waste and environmental pollution.

Collagen is an extremely important structure of proteins, acting as a supporting organ, protecting our bodies [3-4]. In recent years, due to the serious pollution of the ecological environment and the outbreak of the land mammal, the safety of the mammals’ skin and collagen has been challenged. So the extraction of collagen from fish scales has also begun to be taken seriously in recent years. But fish phosphorus contains not only collagen, but also a lot of inorganic calcium salts and mixed proteins [5-7], which make the extraction of collagen directly and low yield. Therefore, we must Remove these substances before the test, the lower the calcium content, the better quality of the extracted collagen [8-10]. Calcium hydrochloric acid, EDTA and citric acid are often used to remove calcium. EDTA [11] was chosen in this experiment. And use NaCl to remove the protein.

2. Materials and methods

2.1. Test materials

Carp scales (market out of school), EDTA, NaCl, pepsin, acetic acid.

2.2. Test instruments

Xs-02 multi-function crusher: Shanghai zhaoshen technology co; LTD. Dhg-9420a-type electric thermostat drying oven: Shanghai yihe technology co; LTD. SH220M graphite digestion meter: Jinan sea energy instrument co; LTD. 85-2B digital display constant temperature magnetic stirrer:
2.3. Test method

2.3.1. Determination of basic components

Water and ash are determined by national standard; Determination of protein content:

1. Accurately weigh 2.5392g fish scales sample in a counter balance, put into the digestive tube washed and dried, the numbered. We added 6g of potassium sulfate, 0.2g of copper sulfate and 20ml concentrated sulfuric acid, and do parallel experiment and blank test at the same time.

2. Digestion: heat the digestive tube for 30 minutes, the temperature is set to 34 ℃, observes its color change, wait to turquoise, clear fluid, continue to digest the 0.5 h.

3. The sample digesting liquid which will be digested and cooled to room temperature is loaded into the fully automatic kjeldahl. manual operation: 40 ml boric acid dilution water 10 ml, add 40 ml alkali twice, 5 min of distillation and leaching, remove the conical flask under the tube.

4. Remove the conical flask, and then add two drops of methyl red - bromine formic acid green indicator, Titrate with the standard sulfuric acid solution until the blue-green turns to purplish and then stop, and write down the volume of the acid V1.

\[ X = \frac{(V_1 - V_2) \times C \times 0.0140}{m \times 0.1 \times F \times 100} \]

X — sample protein content (g / 100ml)
V1 — the volume of the standard titration liquid for the sample consumption (ml)
V2 — the volume of acid standard solution for blank consumption of reagents (ml)
C — the actual concentration of standard solution of hydrochloric acid (mol/L)
0.014—1.0 ml hydrochloric acid \([C(HCl)=1.000\text{mol/L}]\) standard titration solution is equivalent to the quality of nitrogen (g)
M — the mass or volume of the sample (g or ml)
F — nitrogen is the coefficient of protein

2.3.2. Extraction of collagen

Fish scale cleaning→drying→grinding→EDTA decalcification process→NaCl removing impurity protein→ultrasonic assisted extraction→collagen extract→purification→freeze drying→collagen

1. Pretreatment

Washing the fish scales multiple times, remove impurities, wash and dry them naturally, crush, make a flocculent powder, refrigerate reserve, use 0.18mol/L of EDTA solution 30.00mL/g oscillate 6h, remove the calcium in the fish scale. Use 3% NaCl solution 50.00mL/g soak 24h to remove the impure protein of fish scales [12-13].

2. The single-factor experiment was performed to extract collagen

Accurately weigh 3 g fish scales power with electronic scales, respectively add the acetic acid concentration (0.1 mol/L, 0.3 mol/L, 0.5 mol/L, 0.7 mol/L, 0.9 mol/L) of the extract, the dosage of enzyme for (200 mg/g, 250 mg/g, 300 mg/g, 350 mg/g, 400 mg/g), the ultrasonic action time, respectively (140 min, 170 min, 200 min, 230 min, 260 min), and solid-liquid ratio control in (1:30, and 1:40, only 1, 1:50). At the frequency of 40Hz, then determine concentration of the optimum acetic acid, the dosage of pepsin, the time of ultrasonic action and the ratio of the material liquid, and calculated the extraction rate of collagen according to the formula.

3. Ultrasonic extraction

Weigh 3g frozen fish powder on electronic balance, put in the conical flask, according to the material liquid than respectively add several kinds of substance in the single factor experiment, to 300 mg/g of pepsin solution and 0.3 mol/L acetic acid solution[14-15]. Then adjust the time of the ultrasonic cleaning machine.

4. Freeze and dry collagen

Five clean petri dishes were prepared, and the ultrasonic oscillating samples were poured into a
petri dish, weighing on the electronic balance, recording the data and drying it in a freeze-dryer, 6h \cite{16-18}. Take the sample out and weigh it on the electronic balance and get the collagen powder.

### 2.3.3. Collagen determination

Dissolve collagen to the mixture of 300 mg/g pepsin solution concentration and 0.3 mol/L of acetic acid solution, join only mixture of acetic acid and pepsin solution as the reference \cite{19-20}. put the reference fluid into the uv-vis spectrophotometer, computer transferred to the connection state, in the 200-800 - nm wavelength, run of the baseline first, and then testing samples, Finally, the uv spectrogram is obtained. The peak of the general baseline is downward, and the peak of the sample should go up.

### 3. Results and analysis

#### 3.1. Determination of basic components

| Basic ingredient of fish scales | Quality percentage (%) |
|---------------------------------|------------------------|
| water content                   | 18.5                   |
| ash content                     | 26.6                   |
| protein                         | 49.6                   |

#### 3.2. Result of collagen extraction conditions optimization

##### 3.2.1. Analysis of the test results of collagen extraction single factor

As shown in figure 1, with the increase of enzyme dosage, the extraction rate of collagen in fish scales gradually increases, and when the enzyme dosage is 300mg/g, the peak value appears, then the extraction rate gradually decreases. When the enzyme dosage is too low, the enzyme activity is low, which can't occur relatively fast or sufficient reaction, but if the enzyme is too high, it can damage the collagen structure, thus reducing the extraction rate.

![Fig. 1 Effect of enzyme dosage on the extraction of collagen](image1)

As shown in figure 2, with the increase of solid-liquid ratio, the extraction rate of collagen in fish scales gradually increases, and when the solid-liquid ratio is 1:50, the peak value appears, then the extraction rate gradually decreases. When the solid-liquid ratio is too low, the enzyme activity is low, which can't occur relatively fast or sufficient reaction, but if the enzyme is too high, it can damage the collagen structure, thus reducing the extraction rate.

![Fig. 2 Effect of solid-liquid ratio on the extraction rate of collagen.](image2)
As shown in figure 2, the extraction rate of collagen in the fish scales gradually increases with the increase of the ratio of the material liquid. When the mixture ratio is 1:40, the peak value appears, then the extraction rate gradually decreases.

![Graph of extract concentration effect on collagen extraction rate](image)

**Fig. 3** Extract concentration effect on collagen extraction rate

As can be seen in figure 3, the extraction rate of collagen increased with the concentration of the extract (acetic acid). When the concentration of extract (acetic acid) was 0.5 mol/L, the peak appears. and the extraction rate gradually decreased. If the concentration is low, the collagen can not be completely extracted, and if the concentration is too high, it can destroy the structure of the collagen, neither of which can achieve the optimal extraction rate.

![Graph of effect of the time of ultrasonic treatment on the extraction of collagen](image)

**Fig. 4** Effect of the time of ultrasonic treatment on the extraction of collagen

As can be seen from figure 4, as the time of ultrasound increases, the extraction rate of collagen increases gradually, and when the time is 200min, the peak value appears, then the extraction ratio shows a downward trend. Too short ultrasonic time can make the collagen not very well extracted, and if the ultrasound time is too long, it will be the loss of collagen, which will reduce the extraction rate.
3.2.2. response surface test results

Table 2

| Run | A: The amount of enzyme /mg/g | B: Solid liquid ratio | C: Extract concentration | D: Ultrasonic action time /min | Extraction rate |
|-----|-------------------------------|-----------------------|--------------------------|-------------------------------|----------------|
| 1   | 0.00                          | 0.00                  | 0.00                      | 1.00                          | 27.70          |
| 2   | 0.00                          | 0.00                  | 0.00                      | 0.00                          | 28.70          |
| 3   | -1.00                         | -1.00                 | 1.00                      | -1.00                         | 26.70          |
| 4   | 1.00                          | -1.00                 | 1.00                      | -1.00                         | 27.39          |
| 5   | 1.00                          | 1.00                  | 1.00                      | 1.00                          | 27.72          |
| 6   | 0.00                          | 0.00                  | 0.00                      | 2.00                          | 27.67          |
| 7   | 1.00                          | 1.00                  | 1.00                      | -1.00                         | 27.35          |
| 8   | 1.00                          | 1.00                  | -1.00                     | -1.00                         | 27.13          |
| 9   | 0.00                          | 0.00                  | 0.00                      | -2.00                         | 26.89          |
| 10  | 1.00                          | 1.00                  | -1.00                     | 1.00                          | 27.80          |
| 11  | 0.00                          | -2.00                 | 0.00                      | 0.00                          | 26.76          |
| 12  | 0.00                          | 0.00                  | 0.00                      | 0.00                          | 28.70          |
| 13  | 2.00                          | 0.00                  | 0.00                      | 0.00                          | 27.56          |
| 14  | -1.00                         | 1.00                  | -1.00                     | -1.00                         | 21.72          |
| 15  | 0.00                          | 0.00                  | 0.00                      | 0.00                          | 28.70          |
| 16  | -2.00                         | 0.00                  | 0.00                      | 0.00                          | 26.72          |
| 17  | -1.00                         | -1.00                 | 1.00                      | -1.00                         | 26.72          |
| 18  | 1.00                          | -1.00                 | -1.00                     | 1.00                          | 27.45          |
| 19  | 0.00                          | 0.00                  | -2.00                     | 0.00                          | 26.97          |
| 20  | 1.00                          | -1.00                 | -1.00                     | -1.00                         | 27.40          |
| 21  | 0.00                          | 0.00                  | 0.00                      | 0.00                          | 28.70          |
| 22  | 0.00                          | 2.00                  | 0.00                      | 0.00                          | 27.89          |
| 23  | 1.00                          | -1.00                 | 1.00                      | 1.00                          | 28.13          |
| 24  | -1.00                         | 1.00                  | 1.00                      | -1.00                         | 27.35          |
| 25  | -1.00                         | -1.00                 | 1.00                      | 1.00                          | 27.28          |
| 26  | 0.00                          | 0.00                  | 0.00                      | 0.00                          | 28.70          |
| 27  | 0.00                          | 0.00                  | 2.00                      | 0.00                          | 27.78          |
| 28  | -1.00                         | 1.00                  | -1.00                     | 1.00                          | 27.32          |
| 29  | -1.00                         | -1.00                 | -1.00                     | 1.00                          | 26.66          |

The Design Expert V 8.0.4 response surface software was used for quadratic multivariate regression fitting. It is concluded that the carp fish scales in the extraction yield of collagen of enzyme dosage, solid-liquid ratio, the concentration of the extract, ultrasonic action time of quadratic regression model:

\[ Y = +28.70 + 0.22A + 0.17B + 0.15C + 0.18D - 0.15AB - 0.024AC - 0.038BC + 0.014BD + 0.068CD + 0.051AD - 0.38A^2 - 0.34B^2 - 0.32C^2 - 0.35D^2 \]

The variance analysis of response surface test was performed on the response surface design model of the collagen response surface, and the results were shown in table 3.
Table 3  Response interview variance analysis

| source of variation | Quadratic sum | DOF | Mean square | F      | P        | significance |
|---------------------|----------------|-----|-------------|--------|----------|--------------|
| model               | 13.4           | 14  | 0.93        | 36.26  | <0.0001  | ***          |
| A-enzyme dosage     | 1.15           | 1   | 1.15        | 44.70  | <0.0001  | ***          |
| B- material liquid   | 0.66           | 1   | 0.66        | 25.56  | 0.0001   | **           |
| C-extract concentration | 0.55       | 1   | 0.55        | 21.37  | 0.0003   | **           |
| D- ultrasonic time   | 0.81           | 1   | 0.81        | 31.54  | <0.0001  | ***          |
| AB                  | 0.38           | 1   | 0.38        | 14.60  | 0.0017   | **           |
| AC                  | 1.95           | 1   | 1.95        | 0.37   | 0.5521   | *            |
| AD                  | 0.041          | 1   | 0.041       | 1.60   | 0.2257   |              |
| BC                  | 0.023          | 1   | 0.023       | 0.91   | 0.3565   |              |
| BD                  | 3.30           | 1   | 3.30        | 0.13   | 0.7248   |              |
| CD                  | 0.074          | 1   | 0.074       | 2.89   | 0.1097   | *            |
| A²                  | 4.02           | 1   | 4.02        | 156.46 | <0.0001  | ***          |
| B²                  | 3.11           | 1   | 3.11        | 120.94 | <0.0001  | ***          |
| C²                  | 2.88           | 1   | 2.88        | 112.12 | <0.0001  | ***          |
| D²                  | 3.32           | 1   | 3.32        | 129.16 | <0.0001  | ***          |
| residual term       | 0.39           | 15  | 0.026       |        |          |              |
| Lack of fit         | 0.39           | 10  | 0.039       |        |          |              |
| The error term      | 0.000          | 5   | 0.000       |        |          |              |
| sum                 | 13.43          | 29  |             |        |          |              |
| R²                  | 0.9713         |  | 0.9445      |  | 0.8347  |              |

Table 3 shows that the model has a significant difference (P < 0.0001), indicating that the regression equation is consistent with the actual situation, so the error term is not significant and the experimental error is relatively small. The variance analysis shows that the difference in the regression model is A, D, A², B², C² and D², and the differences are highly significant in terms of B, C and AB. Quadratic polynomial correlation coefficient $R^2 = 97.13\%$ ($R^2$ is the adjustment factor for the model), $R^2_{adj} = 94.45\%$ ($R^2_{adj}$ is the correction decision coefficient of the model)

$R^2_{pred} = 83.47\%$ ($R^2_{pred}$ is the modified determination coefficient for the model), $R^2_{adj}$ and $R^2_{pred}$ are close to, through the model can explain the changes response value reached 83%, equations have better fitting degree. The value of $R^2_{adj}$ and $R^2_{Pred}$ are reasonable. By the P value and F value, you can see that the four factors affecting the extraction yield of collagen in carp fish scales order: the amount of enzyme > ultrasonic action time > solid-liquid ratio > extract concentration. The response surface analysis is shown in figure 5 to 10 below.

Fig. 5  Enzyme dosage and solid-liquid ratio on the extraction rate of collagen interaction effect  

Fig. 6  Effect of extraction concentration and enzyme dosage on the extraction rate of collagen
From the above 6 diagrams, it can be seen that the rate of enzyme dosage is the most significant effect on the extraction rate of fish scales collagen. The other three factors, including ultrasonic time, liquid ratio and extract concentration, were all flat and consistent with the analysis of variance. The shape of contour line reflects the interaction strength of the two factors, and the ellipse indicates that the interaction between two factors is obvious, the extraction rate varies greatly, and the circle indicates that the two interactions are not significant. As can be seen from figure 5, the enzyme dosage and the liquid ratio are elliptical and the interaction is significant.

According to the results of the response surface optimization software can see, the best extraction technology conditions of carp fish scales is extract concentration is 0.3 mol/L, ultrasonic action time is 20 min, material liquid than 1:40 mg/L, the dosage of enzyme is 300 mg/g, in these conditions the collagen extraction yield is 28.7%
3.3. Collagen identification

![Fig. 11](image)

Extracted from collagen in ultraviolet scanning results

It can be seen from figure 11 that the collagen ultraviolet spectrogram shows that the protein has the maximum absorption peak at 230nm, and the absorbance is 0.5835, which corresponds to the absorption peak of collagen. It is said that the material extracted from fish scales is collagen, so the method of ultrasonic assisted extraction of collagen is feasible.

4. Conclusion

The fish scales are extracted by ultrasound to help extract the collagen. The extract is purified and the color is yellowish. The collagen is the porous foam after freeze-drying, and gently knead and form the powder. In this experiment to carp fish scales as the main raw material to extract collagen, the collagen protein extracted by single factor experiments the optimum experimental conditions: extract concentration (acetic acid) 0.3 mol/L, 200 min, ultrasonic action time material liquid ratio of 1:40 mg/L, enzyme dosage is 350 mg/g, collagen extraction yield of 28.7%. Compared with the extraction rate of the literature [7], the extraction rate is slightly lower, which may be due to the unadjustable frequency of the laboratory instruments and the low frequency of ultrasonic vibration. The second is that the fish scales are not fully pulverized, causing the collagen to not dissolve completely. Through ultraviolet spectrophotometer, the obtained material has obvious absorption peak at 230nm, so the material obtained from the experiment is collagen.

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