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# An Enzymolysis-Based Extraction Method to Obtain Collagen from Wood Frog Skin and Their Antioxidant Activities

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**Abstract:** The aim of the investigations was to extract collagen from Wood frog skins (WFS). WFS collagens were prepared and optimized in single factor experiments combined with ternary quadratic regression orthogonal combination design (TQROCD) optimization mathematical equation model. The optimal condition were peptide, pH 1.966, enzyme concentration of 5.42% and liquid-solid ratio of 145.2:1. Verification test average result in triplicate was 29.65%, which matches well with the predicted value. Spectrogram of reference standard and sample by High Performance Liquid Chromatography (HPLC) proved to be the existence of collagen in Wood frog skin.

**Keywords:** Wood frog skins, collagen, enzymolysis, HPLC

## 1. Introduction

The advantages of collagen are biomaterial nutritional supplements largely on the fact that it is a natural material which can contribute to anti-aging; therefore, it can be treated as the best choice to protect body skin [1]. The collagen obtained from pig bones cannot be used due to religious constraints. Fish can be the better choices, but it has been considered of low economic importance, because of their characteristic bad odour, taste and unpleasant appearance. It has been of great interest to find collagens from different sources with securities and profits [2]. The fact that bioactive peptides present in the amino acid sequence of food proteins have drawn great interest in food science currently [3]. The objective of the paper is to select the optimal conditions for the isolation of collagen from WFS. The physical and chemical properties of collagen from cattle skins are substantially different from those of WFS [4]. In the literature, there are only a few papers dealing with the practical utilization of connective tissue of abandoned WFS [5]. Other papers always focus on cognitive aspects, such as collagen types, amino-acid composition, collagen cross-linking, susceptibility to enzymes [6].

## 2. Materials and Methods

### 2.1. Chemicals

Hydroxyproline standard substance were purchased from Sigma Chemical Co. (St. Louis, Mo,USA). Other reagents were of analytical grade.

### 2.2. Test materials

The WFS were obtained in spring in the northeast of China. The WFS were mechanically separated by hand. The skin was cleaned after the adhering residue was removed. After freezing drying skins under the condition of -50℃ for 24h, all water in skins was completely dried. Disposed samples were then cut into small pieces (0.1×0.1cm2) with grinder, placed in polyethylene bags and stored at -40℃until used.

### 2.3. Quadratic regression orthogonal

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Ternary quadratic regression orthogonal combination was designed to optimize extraction route of WFS. Factor code was in Table.1. The nature factor level and codes were as followed.

Table 1. Independent variables and their levels used for ternary quadratic regression orthogonal combination design

| Xj(Zj) | z1 | z2 | z3 |
|---|---|---|---|
| | pH value | Enzyme Concentration | liquid-solid ratio |
| r(z2j) | 3 | 7% | 140:1 |
| 1(z0j+△j) | 2.74 | 6.47% | 130:1 |
| 0(z0j) | 2 | 5% | 100:1 |
| -1(z0j-△j) | 1.26 | 3.53% | 70:1 |
| -r(z1j) | 1 | 3% | 60:1 |
| △j | 0.74 | 1.47% | 30 |
| xj | 1.35(zj-2) | 0.68(zj-5%) | 0.033(zj-100) |

# 3. Results and Discussion

## 3.1. Effects of single factors to WFS extraction

Results showed that the collagen yield was influenced by enzyme concentration. Until enzyme concentration reached 7%, collagen yield was increasing. Much more enzyme that were added in solution made contact area larger. It suggested that excessive enzyme restrains the reaction proceeding to the positive direction. Furthermore, excessive enzyme caused cost waste. Thus enzyme concentration of 7% was chose for further experiments.

Enzymolysis time was investigated. The results showed the effects of enzymolysis time on collagen yield. The results suggested the increasing enzymolysis time extends reaction between WFS and enzyme. Therefore overmuch reaction time not only caused a waste of time, but also blocked reaction proceeding. In a certain range, enzymolysis time of 3h was better for extraction of collagen. Thus enzymolysis time of 3h was prepared for the following experiments step.

The extraction yield by different liquid-solid ratio from 60: 1 to 140:1 was investigated. Results indicated collagen yield ranged from 4.63% to 28.26%. Collagen yield approached its maximum when liquid-solid ratio was 120:1. At liquid-solid ratio of 60:1, collagen did not have enough contact area with enzyme leading to reaction insufficiency. But on the other hand, overmuch water (140:1) diluted concentration of reaction solution. Thus among the ratios, there must existed median which not only meets contract reaction needs but also be just right for optimal ratio.

## 3.2. Effects of enzyme species and pH to WFS extraction

Collagen has special structure of G-X-Y, particularly from the conformational viewpoint, the most prominent feature of amino acid composition, that is, the high glycine content. The amino acid glycine constitutes of one third of the total amino acid composition. Glycines favor the formation of β-turns and associated conformations. But the enzymolysis condition played a fatal role especially for pH effects. Contraposed various enzymes, their most suitable pH value were different.

## 3.3. Results of quadratic regression orthogonal optimization

The collagen extraction yield results with quadratic regression orthogonal optimization method were in Table.2.

In this study, a total of 17 experiments were performed for ternary quadratic regression orthogonal combination design (TQROCD) optimization mathematical equation model, together with the three essential individual parameters and their interaction. The results (in Table.2) indicated the significance (a) of pH(X1), enzyme concentration(X2) and liquid-solid ratio (X3) were 0.05, 0.05, 0.1 respectively. By data analysis, ternary quadratic equation was as followed. Y is the collagen extraction yield. The coefficient of equation (R) is 0.943 which represented high degree and reliability of the equation.

Y(%)=0.1611-0.0314X1+0.0372X2-0.018X3-0.0037X1X2+0.0211X2X3-0.0226X1X3-0.04092 X12-0.01172 X22-0.01004 X32.

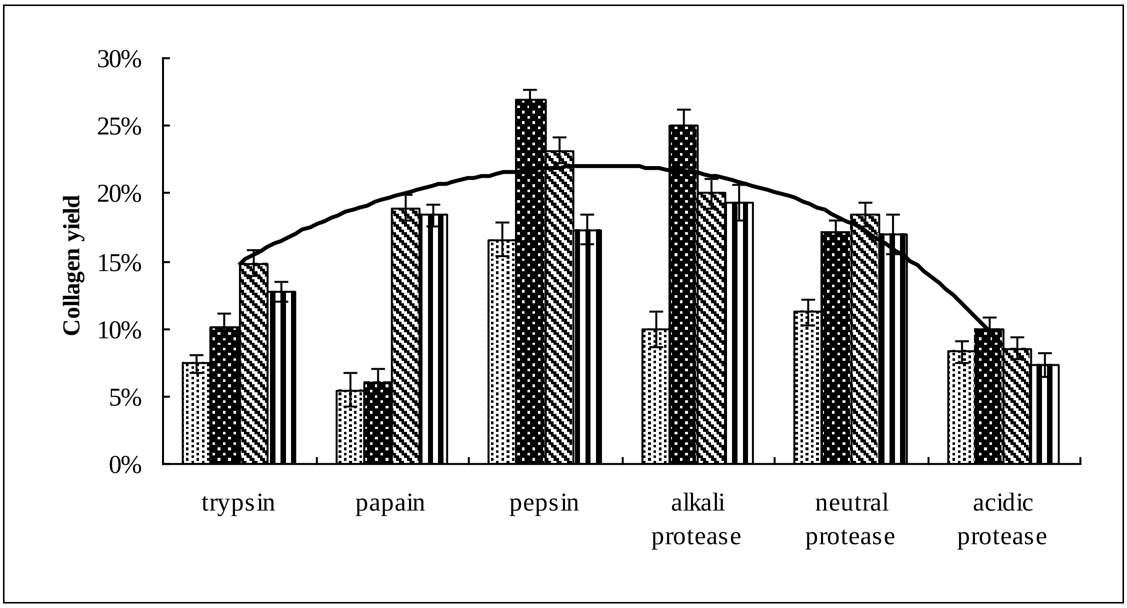


Fig. 1: Effects of enzyme species and pH-value to collagen extraction yield

Table 2. Extraction yield results with quadratic regression orthogonal optimization method

| | $X_0$ | $X_1$ | $X_2$ | $X_3$ | $X_1X_2$ | $X_1X_3$ | $X_2X_3$ | $X_1$^2 | $X_2$^2 | $X_3$^2 | Y |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 1 | 1(2.74) | 1(6.47%) | 1(130:1) | 1 | 1 | 1 | 1(0.314) | 1(0.314) | 1(0.314) | 13.64% |
| 2 | 1 | 1 | 1 | -1(70:1) | 1 | -1 | -1 | 1(0.314) | 1(0.314) | 1(0.314) | 18.39% |
| 3 | 1 | 1 | -1(3.53%) | 1 | -1 | 1 | -1 | 1(0.314) | 1(0.314) | 1(0.314) | 10.29% |
| 4 | 1 | 1 | -1 | -1 | -1 | -1 | 1 | 1(0.314) | 1(0.314) | 1(0.314) | 4.96% |
| 5 | 1 | -1(1.26) | 1 | 1 | -1 | -1 | 1 | 1(0.314) | 1(0.314) | 1(0.314) | 17.03% |
| 6 | 1 | -1 | 1 | -1 | -1 | 1 | -1 | 1(0.314) | 1(0.314) | 1(0.314) | 29.22% |
| 7 | 1 | -1 | -1 | 1 | 1 | -1 | -1 | 1(0.314) | 1(0.314) | 1(0.314) | 11.18% |
| 8 | 1 | -1 | -1 | -1 | 1 | 1 | 1 | 1(0.314) | 1(0.314) | 1(0.314) | 15.30% |
| 9 | 1 | r(3) | 0(5%) | 0(100:1) | 0 | 0 | 0 | $r^2$(1. 145) | -0.686 | -0.686 | 7.00% |
| 10 | 1 | -r(1) | 0 | 0 | 0 | 0 | 0 | $r^2$(1. 145) | -0.686 | -0.686 | 15.14% |
| 11 | 1 | 0(2) | r(7%) | 0 | 0 | 0 | 0 | 0(-0.686) | $r^2$(1. 145) | -0.686 | 18.96% |
| 12 | 1 | 0 | -r(3%) | 0 | 0 | 0 | 0 | 0(-0.686) | $r^2$(1. 145) | -0.686 | 13.86% |
| 13 | 1 | 0 | 0 | r(140:1) | 0 | 0 | 0 | 0(-0.686) | 0(-0.686) | $r^2$(1. 145) | 14.77% |
| 14 | 1 | 0 | 0 | -r(60:1) | 0 | 0 | 0 | 0(-0.686) | 0(-0.686) | $r^2$(1. 145) | 18.66% |
| 15 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0(-0.686) | 0(-0.686) | 0(-0.686) | 19.27% |
| 16 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0(-0.686) | 0(-0.686) | 0(-0.686) | 21.77% |
| 17 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0(-0.686) | 0(-0.686) | 0(-0.686) | 24.56% |

Warinda Suphatharaprateep studied production and properties of two collagenases from bacteria and their application for collagen extraction. When collagenase was applied, the yields of collagen extracted by collagenases from a Gram positive Bacillus cereus CNA1 and a Gram negative Klebsiella pneumoniae CNL3 were 18.1% and 16.5% (dry weight basis), respectively. Sourour Addad did research on isolation, characterization and biological evaluation of Jellyfish collagen for use in biomedical applications.

## 3.4. Qualitative analysis by HPLC

Spectrogram of reference standard and sample was in Fig.2. The consistency of the retention time proved to be existence of collagen. The peak area indicated reference standard had higher content of collagen than sample. Spectrogram determined by HPLC was at wavelength of 360nm.

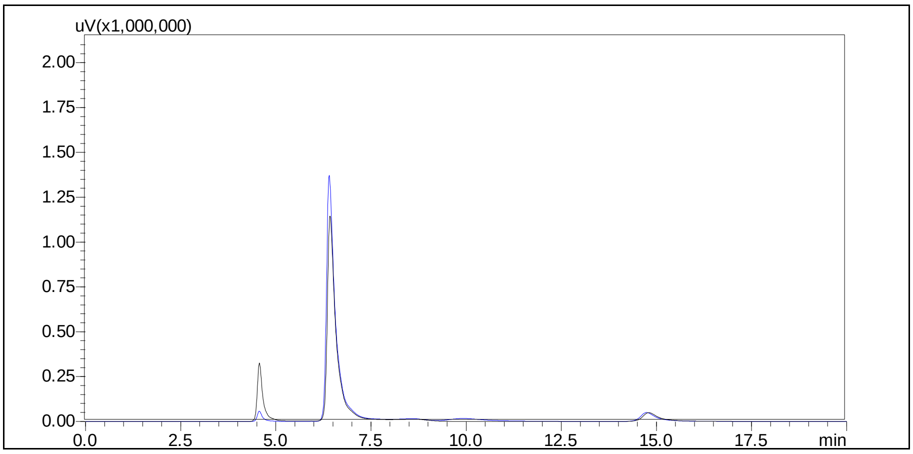


Fig. 2: Sample by high Performance Liquid Chromatography (HPLC)

The first peak showed the existence of collagen. The second peak showed the reagent peak. Reference standard had higher peak in the black line. Sample had lower peak in the blue line. They had the same retention time.

## 4. Conclusion

The effects of different factors for collagen extraction from WFS were studied. The results showed that collagen extracted from WFS contained a higher yield. Single factor experiments (enzyme species, pH-value, enzyme concentration, enzymolysis time and liquid-solid ratio) and ternary quadratic regression orthogonal combination design (TQROCD) optimization mathematical equation model optimization with three individual parameters (pH-value, enzyme concentration and liquid-solid ratio) and their interaction were combined to optimize collagen extraction condition. Though calculating quadratic regression orthogonal optimization mathematical equation model, optimized condition was as followed: peptide, pH 1.966, enzyme concentration 5.42%, liquid-solid ratio 145.2:1. Verification tests average results in triplicate was 29.65% which is similar to predicted value. Spectrogram of reference standard and the sample was proved the existence of collagen by HPLC.

## 5. Acknowledgement

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