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**Journal of Chemical and Pharmaceutical Research, 2014, 6(1):278-282**

**Research Article**

**ISSN : 0975-7384**
**CODEN(USA) : JCPRC5**

# Enzymatic hydrolysis of human-like collagen and molecular weight distribution of hydrolysates

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**ABSTRACT**

*Process parameters on enzymatic hydrolysis and molecular weight distribution of human-like collagen（HLC）hydrolysates were investigated. HLC was hydrolysed by trypsin and pepsin, the optimal process parameters were obtained by the single-factor and orthogonal experiments. The molecular weight distribution of hydrolysates (MW) was determined using both SDS-PAGE and Sephadex G25 partition. In the study, the maximum degree of hydrolysis (DH) of HLC hydrolysate obtained by trypsin (temperature 50°C, pH 8.0, HLC concentration 15mg/mL, E/S=80%, 8 h) was 70.9%, MW ranged from 500 to 1 400 Da. While that obtained by the pepsin (temperature 50° C, pH 2.5, HLC concentration=5mg/mL, and E/S=3%, 7 h) was 24.25%, MW ranged from 4000 to 6500 Da.*

**Keywords:** collagen, hydrolyze, molecular weight distribution, protease, peptides

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## INTRODUCTION

Collagen is a white opaque fibrous protein without branch, serving as the major protein component of skin, bone, tendon, and other forms of connective tissue [1]. It is the most common protein in mammal, which accounts for 25-30%. So collagen has been considered as a natural material with good biological compatibility and biological degradability [2]. However, the application of collagen is greatly limited due to its high molecular weight and water-insolubility. In contrast, collagen hydrolysates are attracting more and more attentions during the last two decades in food, pharmaceutical, and cosmetic industries [3], which show increasing water-solubility, better emulsification ability and gelatinization property.

Although some progresses and applications on collagen hydrolysates have been achieved, the functions of different collagen hydrolysates remain unclear and the relations between the molecular weight distribution of collagen hydrolysates and their function are not fully investigated [4].

HLC is a giant molecule bio-protein which was produced by gene engineering. In the study, HLC was used as substrate which is a recombinant protein produced by *E. coli*, containing human collagen's cDNA transcribed reversely from mRNA. The previous study demonstrated that it has several special characteristics which are significantly different from animal collagen, such as virus-free, chemically defined structure, biocompatibility, processability, little immunogenic reaction, and so on.

## EXPERIMENTAL SECTION

*Materials*

Human-like collagen (HLC, China patent number: ZL01106757.8, Mr=97,000) was supplied by our laboratory. Pepsin (nominal activity 3000U/mg) and trypsin (nominal activity of 250 U/mg) were provided by Amresco, American. They were stored at 4℃ until use. All other chemical reagents were of analytical grade.

*Process Parameters and Experimental Design*

The investigated process variables were substrate concentration (A), enzyme-substrate ratio (B), hydrolysis temperature (C), pH (D), and hydrolysis time. Single-factor experiments were performed to investigate the experiment parameter which significantly affected the DH. When the significant factor was determined, an orthogonal experiment was employed to optimize the definite experiment parameter. Table 1 and Table 2 gived the correspondence between coded levels of experiment factors. Experiment was designed according to 4 factors at 3 levels rectangular matrix. For each run, the response was DH.

**Table 1. Correspondence between Coded Levels of Trypsin Hydrolysis**

| Factor (Coded name) | Coded level | | |
|---|---|---|---|
| | 1 | 2 | 3 |
| HLC concentration(g/L) | 5 | 10 | 15 |
| ttypsin/HLC(%) | 60 | 70 | 80 |
| Temperature(℃) | 40 | 45 | 50 |
| pH | 7.5 | 8.0 | 8.5 |

**Table 2. Correspondence between Coded Levels of Pepsin Hydrolysis**

| Factor (Coded name) | Coded level | | |
|---|---|---|---|
| | 1 | 2 | 3 |
| HLC concentration(g/L) | 5 | 10 | 15 |
| pepsin/HLC(%) | 1.5 | 3 | 4.5 |
| Temperature(℃) | 40 | 45 | 50 |
| pH | 2.0 | 2.5 | 3.0 |

*Hydrolysis of HLC*

HLC was dissolved in deionized water and then was hydrolysed with trypsin and pepsin , respectively. After hydrolysis, the solution was then heated at 100℃ for 10 min to inactivate the enzyme. Each determination was performed in triplicate. DH of hydrolysed protein was determined using a ninhydrin colorimetric method.

*Measurement of DH*

DH is defined as the percentage of free amino groups cleaved from proteins, which was calculated from the ratio of α -amino nitrogen to total nitrogen. It was estimated according to ninhydrin colorimetric method with slight modifications [5]. L-glycine (20mg/mL) was used as standard. Each determination was performed in triplicate. DH was defined as:

$$DH = \frac{h}{h_{tot}} \times 100\% = \left[ \frac{A\left(\mu mol \cdot mL^{-1}\right)}{6.25 \cdot N\left(mg \cdot mL^{-1}\right)} - B\left(mmol \cdot g^{-1}\right) \right] \div h_{tot}\left(mmol \cdot g^{-1}\right) \times 100\%$$

Where:

h is the number of broken peptide bonds per unit weight

$H_{tot}$ is was the total number of bonds per unit weight

A is the $-NH_2$ in HLC hydrolysates

B is the $-NH_2$ in HLC.

*SDS-PAGE Pattern*

To estimate the protein and peptide sizes in the hydrolysates, sodium-dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 12% precast gels. It was under reducing condition in the presence of 2-mercaptoethanol according the method of Leammli [6]. The electrode buffer was Tris/glycine/SDS running

buffer(25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3w $v^{-1}$, pH 8.3) and the gels were stained with Coomassie Blue R-250.

*Analyzing Molecular Weight Distribution by SephadexG25 Partition*

Molecular-weight distributions of HLC hydrolysate were analyzed using a gel chromatography equipped with a XK16/100 (mm/cm) Sephadex G-25 column(ATKA purifier, GE Healthcare, US) with a UV detector at 214 and 280 nm. The mobile phase (isocratic elution) was 0.02 M sodium phosphate buffer containing 0.25 M NaCl (pH 7.2), at a flow rate of 0.5 mL/min [7]. A molecular-weight calibration curve was prepared from the average elution volume of the following standards: cytochrome C (12,500Da), aprotinin (6500Da), vitamin B12 (1355Da), oxidized glutathione (612 Da) and potassium chromate (194.17 Da), as a control, HLC hydrolysate was also determined at the same condition.

## RESULTS AND DISCUSSION

*Optimization of Hydrolysis Parameters by Orthogonal Array Design*

Orthogonal experiments were carried out to obtain the optimal enzymatic hydrolysis condition. For hydrolysis by trypsin (HA) and hydrolysis by pepsin (HB), according to Table 3 and Table 4 the results showed that the major-minor order of factors affecting DH of HA was B, C, A, and D, so optimum process parameters were A3, B3, C3, and D2, it also showed that E/S significantly affected DH, and that other factors had also positive significant effects. While for HB the major-minor order of factors affecting DH was A, B, D, and C, so optimum process parameters were A1, B2, C3, and D2. On this basis the optimum conditions were determined as: trypsin (15 mg/mL HLC , trypsin/HLC 80% , 50℃, pH8.0) and pepsin(5mg/mL HLC, trypsin/HLC 3%, 50℃, pH2.5). Under these conditions, hydrolysates were produced by varying the hydrolysed time from 1 to 9h, the maximum DH of HA obtained was 70.9% (8h) , while the maximum DH of HB obtained was 24.25% (7h), which much less than that of HA, the result was shown in Fig 1.

**Table 3. The Orthogonal Test Results of Trypsin Hydrolysis**

| Number | A | B | C | D | DH |
|---|---|---|---|---|---|
| | (Substrate concentration) | (E/S) | (Temperature) | (pH) | |
| 1 | 5 | 60 | 40 | 7.5 | 36.71 |
| 2 | 5 | 70 | 45 | 8.0 | 54.22 |
| 3 | 5 | 80 | 50 | 8.5 | 65.70 |
| 4 | 10 | 60 | 45 | 8.5 | 48.46 |
| 5 | 10 | 70 | 50 | 7.5 | 61.31 |
| 6 | 10 | 80 | 40 | 8.0 | 63.65 |
| 7 | 15 | 60 | 50 | 8.0 | 60.58 |
| 8 | 15 | 70 | 40 | 8.5 | 59.70 |
| 9 | 15 | 80 | 45 | 7.5 | 66.59 |
| K1 | 52.210 | 48.583 | 53.353 | 54.867 | |
| K2 | 57.803 | 58.407 | 56.423 | 59.483 | |
| K3 | 62.290 | 65.313 | 62.527 | 57.953 | |
| R | 10.080 | 16.730 | 9.174 | 4.616 | |

**Table 4. The Orthogonal Test Results of Pepsin Hydrolysis**

| Number | A | B | C | D | DH |
|---|---|---|---|---|---|
| | (Substrate concentration) | (E/S) | (Temperature) | (pH) | |
| 1 | 5 | 1.5 | 40 | 2.0 | 19.63 |
| 2 | 5 | 3 | 45 | 2.5 | 23.94 |
| 3 | 5 | 4.5 | 50 | 3.0 | 22.06 |
| 4 | 10 | 1.5 | 45 | 3.0 | 15.96 |
| 5 | 10 | 3 | 50 | 2.0 | 19.44 |
| 6 | 10 | 4.5 | 40 | 2.5 | 18.03 |
| 7 | 15 | 1.5 | 50 | 2.5 | 15.85 |
| 8 | 15 | 3 | 40 | 3.0 | 14.89 |
| 9 | 15 | 4.5 | 45 | 2.0 | 16.98 |
| K1 | 21.877 | 17.147 | 17.517 | 18.683 | |
| K2 | 17.810 | 19.423 | 18.960 | 19.273 | |
| K3 | 15.907 | 19.023 | 19.117 | 17.637 | |
| R | 5.970 | 2.276 | 1.600 | 1.636 | |

*SDS-PAGE Pattern*

Fig 2.(A) and (B) showed SDS–PAGE patterns of HLC and its corresponding enzymatic hydrolysates, the results

indicated that the hydrolysate obtained from HA and HB wass quite small , they were less than 14400 Da, a further determination of the molecular weight distribution should be performed by SephadexG25 partition.

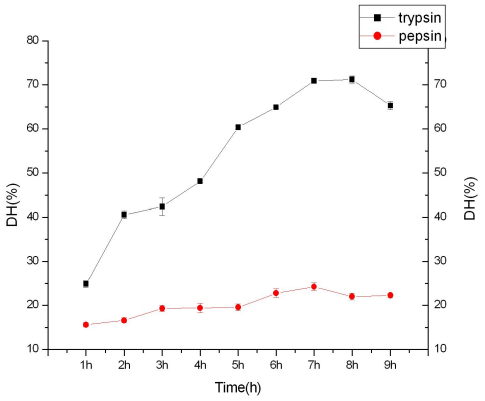


**Fig. 1: Trypsin and Pepsin Hydrolysis Time Curve**

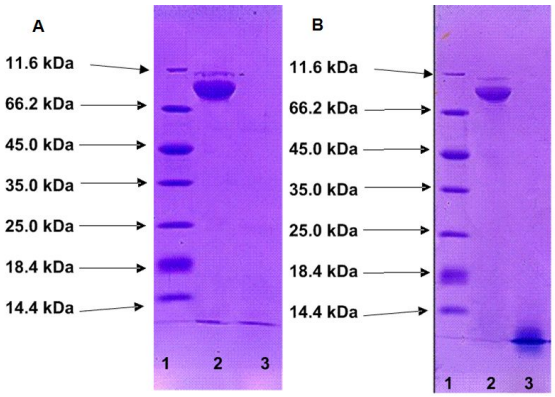


**Fig. 2: SDS-PAGE Patterns of (A)Trypsin Hydrolysis and (B) Pepsin Hydrolysis. Lane 1: protein markers; lane 2: HLC; lane3: HA (A) and HB (B)**

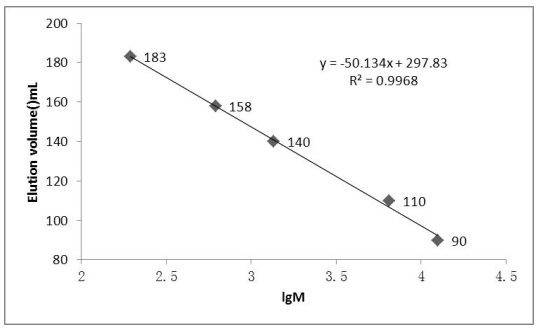


**Fig. 3: Control Sample Molecular Weight with Washing Volume (mL)**

*Analyzing molecular weight distribution by SephadexG25 partition*

The elution volume curve of control sample molecular weight was shown in Fig 3. Based on the washing volume of control sample molecular weight, the molecular weight (MW) distribution of HLC hydrolysates obtained by trypsin were ranged from 500 to 1 400 Da, and most of HLC peptide was under 1200 Da, all the hydrolysates were mainly composed of low molecular-weight peptides (<3000Da).While hydrolysates obtained by pepsin were ranged from 4000 to 6500 Da and most of the peptides were under 5000 Da. Owing to the molecular weight of HLC was 97000Da, it can be indicated that intra-chain cleavage of peptide bonds by the proteases had taken place.

## CONCLUSION

The DH is a very important parameter in monitoring of protein hydrolysis. It is an index of the size distribution of

the hydrolysates, due to the relationship between the DH and the peptide chain length. The content of the peptides obtained after hydrolysis is very important. The biological value of the peptides varies depending on the processing, the heat treatment, the enzymes used and the enzymatic hydrolysis conditions. Therefore, many kinds of collagen hydrolysates can be obtained on the conditions of the different hydrolysis process, but the physiological behavior of these hydrolysates is not clear.

In this study, HLC hydrolysis with trypsin and pepsin could be successful used for obtaining small and activity collagen peptide. SDS-PAGE combined with SephadexG25 partition methods could be successfully applied to determine the molecular weight distribution of HLC hydrolysates. According to these results, we can the contrast relationship between molecular weight distribution and the function of HLC hydrolysis peptides for further study.

**Acknowledgments**
This study was finacially supported by the National Natural Science Foundation of China( 21176200, 21276210, 21106112, 21206135, 31000019 and 21106111); the Scientific Research Program of Shaanxi Provincial Department of Education, China (2013JK0696, 12JK0449, 12JS099, 12JS100, 12JS101, 2012JC23, 11JS102, 2010JC21, 2010JS107, 2010JS108, 2010JK876 and 2010JS109); China Postdoctoral Science Foundation (20110490171), China Postdoctoral Science Special Foundation (2012T50815); Shaanxi Provincial Scientific Technology Research and Development Program (2011JE003, 2011JQ4026, 2012KJXX-28, 2012JM2014 and 2010JQ2012), and Shaanxi Key Subject Program, China.

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