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Study on the extraction technology of total flavonoids from *Ginkgo biloba* leaves by double enzyme

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Abstract: *In this study, Ginkgo biloba leaves were used as raw materials. Enzyme dosage, pH, temperature and time were used as the main factors. Single factor and orthogonal experiments were carried out. 70% ethanol was used for Soxhlet extraction. The results indicated that the optimal extraction conditions of total flavonoids in Ginkgo biloba* leaves *method double enzyme hydrolysis: adding amount of enzyme 4mg/g cellulose and 4mg/g pectinase, pH 4.5, temperature 50 C, time for extraction of total flavonoids from Ginkgo biloba 125min. rate of 5.48%, than the single with cellulase increased by 24.5%.*

Keywords: *Ginkgo biloba leaves, total flavonoids, double enzyme method, extraction rate*

1. INTRODUCTION

Ginkgo biloba L. is one of the oldest relics and rare plants in the Mesozoic Era. There is only one family, one genus and one species, and it is called the "living fossil" of gymnosperms [1]. As the main producing area of ginkgo in the world, China has been increasing every year, which provides a guarantee for the development of ginkgo industry [2]. As one of the provinces with the largest planting area of ginkgo, Jiangsu is blessed with unique research, and ginkgo has become the representative tree of Jiangsu Province. Taizhou is one of the regions with the most ginkgo production in Jiangsu, which provides us with a good research foundation for studying ginkgo. Ginkgo contains a large number of nutrients and functional substances, mainly including ginkgo leaf flavonoids, terpene lactones and ginkgolic acid, among which flavonoids and terpene lactones account for the largest proportion. In Europe, Japan, South Korea and other countries, the development and utilization of ginkgo has received great attention, especially the functional food based on ginkgo [3]. It has high medicinal value, and has significant curative effect in the treatment of cardiovascular and cerebrovascular diseases and bronchitis [4]. In addition, ginkgo biloba also contains active substances such as ginkgo protein, ginkgo polysaccharides and monosaccharides, vitamins, and ginkgo flavonoids. It has scavenging free radicals, anti-oxidation, and has a strong repairing effect [5]. According to literature records, ginkgo bilobahas been used as a medicinal material for the treatment of lung deficiency, cough and asthma, chest tightness, heartache, and ease of urination. Nowadays, relevant research shows that Ginkgo biloba can increase blood vessel permeability, reduce blood viscosity and other good effects of lowering blood

sugar and blood pressure. Therefore, it is effective in preventing cardiovascular and cerebrovascular diseases such as coronary heart disease, hyperlipidemia, hypertension, stroke and Alzheimer's disease. It can also promote metabolism and has a cosmetic effect. Therefore, the development of ginkgo biloba preparations has become the focus of research in many countries around the world. Ginkgo biloba, which is the homologous medicine and food, is widely found in natural plants, and the content of flavonoids in Ginkgo biloba is relatively high. So far, a variety of flavonoids have been identified, which can be roughly divided into three categories: monoflavonoids and their glycosides, diflavonoids and teas.

At present, there are many methods for extracting total flavonoids from ginkgo leaves, including organic solvent extraction, enzyme-assisted extraction, microwave-assisted extraction, ultrasonic-assisted extraction, and supercritical CO_2. The organic solvent extraction method is the most extensive method for extracting total flavonoids. This method is simple to operate but has low extraction rate, long operation time, waste of materials and many other drawbacks. Microwave-assisted extraction of total flavonoids from Ginkgo biloba leaves has the advantages of high efficiency, energy saving, and easy operation. However, due to the higher temperature, it will decompose flavonoids and dissolve more impurities; ultrasonic-assisted method is easier to control than microwave method, but the extraction It takes a long time. Enzymatic extraction has the characteristics of mild reaction conditions and environmental protection, and the activity of the extracted natural components is not easily destroyed [6]. Most of the active ingredients in plants exist in cells, and the cells are surrounded by a dense structure of cell walls. The main component of cell walls is cellulose. Using cellulase to break the cellulose structure can increase the permeability of the cell wall and improve the extraction efficiency of total flavonoids. The cell walls are connected by a small amount of pectin, so cellulase and pectinase are used to assist the extraction of ginkgo leaf flavonoids. Wu Meilin uses cellulase-assisted method, and the extraction rate obtained by enzymatic extraction is increased by about 19% on the basis of the original direct alcohol extraction method [7]. Enzymatic hydrolysis-assisted extraction of flavonoids from Ginkgo biloba leaves is a new research direction in the current study of flavonoid extraction. This method can not only make full use of the advantages of enzymatic hydrolysis but also avoid the disadvantages of other methods. Existing researches on the extraction of total flavonoids from Ginkgo biloba leaves mostly use a single enzymatic method, that is, cellulaseis used to break the fiber in the cell wall, but there are few research reports on pectin as a connection. This experiment uses cellulase and pectinase as the research idea of compound enzymes to explore the technology of extracting total flavonoids from Ginkgo biloba leaves by double enzyme method.

2. MATERIALS AND METHODS

2.1. Materials and Equipment

Ginkgo biloba, 95% rutin standard, cellulase (400U/mg), pectinase (500U/mg) provided by Shanghai Enzyme-Linked Biological Co., Ltd.; analytical pure ethanol, HAc-produced by Sinopharm Chemical Reagent Co., Ltd. NaAc buffer solution, sodium nitrite, aluminum acid, sodium hydroxide. Ginkgo biloba, 95% rutin standard, cellulase (400U/mg), pectinase (500U/mg) provided by Shanghai Enzyme-Linked Biological Co., Ltd.; analytical pure ethanol, HAc-produced by Sinopharm Chemical Reagent Co., Ltd. NaAc buffer solution, sodium nitrite, aluminum acid, sodium hydroxide. Chinese herbal medicine plant pulverizer, centrifuge, refrigerator, freeze dryer, electric thermostatic water bath, analytical balance, ultraviolet spectrophotometer, Sox-type extractor, pH (EL20K).

2.2. Method

2.2.1. Extraction process of total flavonoids from Ginkgo biloba leaves

Ginkgo powder→ add buffer solution (HAc-NaAc) →enzymatic hydrolysis→ethanolreflux→recover

ethanol solution→ get ginkgo flavonoid crude extract.

2.2.2. Key points of operation for extraction of total flavonoids from ginkgo

(1) After the ginkgo leaves are dried at low temperature, they are crushed with a Chinese herbal medicine pulverizer and passed through a 60-mesh sieve, and 1 g of ginkgo powder is accurately weighed; the ginkgo is dried and broken into shells and crushed.

(2) Accurately weigh 1g of Ginkgo biloba powder, add 4mg of cellulase and 4mg of pectinase into 20ml of buffer solution at an enzymatic hydrolysis temperature of 50℃ and a pH of 4.5. After acting for 125 minutes, it will be dried by a freeze dryer.

(3) Add the dried powder after enzymatic hydrolysis to 70% ethanol solution and extract at 70℃ for 2h[8].

(4) The ethanol solution is recovered and centrifuged to obtain a crude extract of ginkgo flavonoids.

(5) Reference method for determination of extraction rate of total flavonoids from Ginkgo biloba leaves

2.2.3. Single factor test of extracting total flavonoids from Ginkgo biloba leaves by double enzyme

The orthogonal experiment design for the extraction of total flavonoids from Ginkgo biloba leaves by double enzyme hydrolysis is shown in Table 1.

Table1. *Factor and level design table*

	Factor			
	enzyme amount （A）	pH（B）	temp（C）	time（D）
1	4mg/g：0mg/g	4.0	40℃	75min
2	4mg/g：4mg/g	4.5	45℃	100min
3	6mg/g：4mg/g	5.0	50℃	125min

(1) The effect of the amount of enzyme added on the extraction rate of total flavonoids from Ginkgo biloba leaves

Accurately weigh 7 parts of 1g ginkgo leaf powder by mass, under the condition of pH 4.5, temperature 50 ℃, time 100 min, enzyme addition (mg/g of cellulase: mg/g of pectinase) respectively: Group A (0 mg/g: 4 mg/g), group B (4 mg/g: 0 mg/g), group C (4 mg/g: 4 mg/g), group D (6 mg/g: 4 mg/g) g), E group (6 mg/g: 6 mg/g), F group (0 mg/g: 8 mg/g), G group (8 mg/g:0 mg/g). The effect of two different enzymes on the extraction rate of total flavonoids from Ginkgo biloba leaves was investigated.

(2) The effect of pH on the extraction rate of total flavonoids from ginkgo leaves

Accurately weigh 7 parts of 1g ginkgo leaf powder with 4mg of cellulase and 4mg of pectinase. The enzymatic hydrolysis temperature is 50℃ for 100min, and the pH is 3.0, 3.5, 4.0, 4.5, 5.0, 5.5 and 6.0, to investigate the effect of different enzymatic hydrolysis pH on the extraction effect of total flavonoids in ginkgo.

(3) The effect of enzymatic hydrolysis temperature on the extraction rate of flavonoids in ginkgo leaves

Accurately weigh 7 parts of 1g ginkgo leaf powder with 4mg of cellulase and 4mg of pectinase. The pH of enzymatic hydrolysis is 4.5, the enzymatic hydrolysis time is 100 min and the enzymatic hydrolysis temperature is 35℃, 40℃, 45℃, 50 ℃, 55℃, 60℃, 65℃, to investigate the effect of different enzymatic hydrolysis temperature on the extraction effect of ginkgo biloba total flavonoids.

(4) The effect of enzymolysis time on the extraction rate of flavonoids

Accurately weigh 7 parts of 1g ginkgo leaf powder with 4mg of cellulase and 4mg of pectinase. Under the conditions of 50℃ for enzymatic hydrolysis and 4.5 for enzymatic hydrolysis, the enzymatic hydrolysis time is 25min, 50min, 75min, 100min, 125min, 150min, 175min, to investigate the effect of different enzymolysis time on the extraction effect of Ginkgo biloba total flavonoids.

3. RESULTS AND ANALYSIS

3.1. Single Factor Test of Double Enzyme Extraction of Total Flavonoids in Ginkgo

3.1.1. The effect of enzyme addition on the extraction rate of total flavonoids from ginkgo leaves

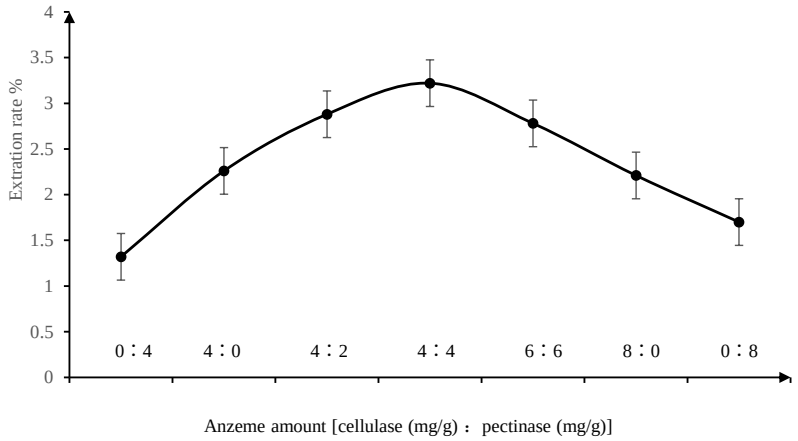

Figure1. *The effect of enzyme addition on the extraction rate of total flavonoids from Ginkgo biloba leaves*

The different types and dosages of enzymes will lead to different extraction rates of total flavonoids in Ginkgo biloba leaves. The extraction rate of only adding pectinase or cellulase is not as high as the extraction rate of adding both enzymes. When adding 4mg/g cellulose With enzyme and 4mg/g pectinase, the extraction rate of total flavonoids from ginkgo leaves reached the maximum 3.12% (Figure 1).

3.1.2. The effect of enzymatic hydrolysis pH on the extraction rate of total flavonoids from ginkgo leaves

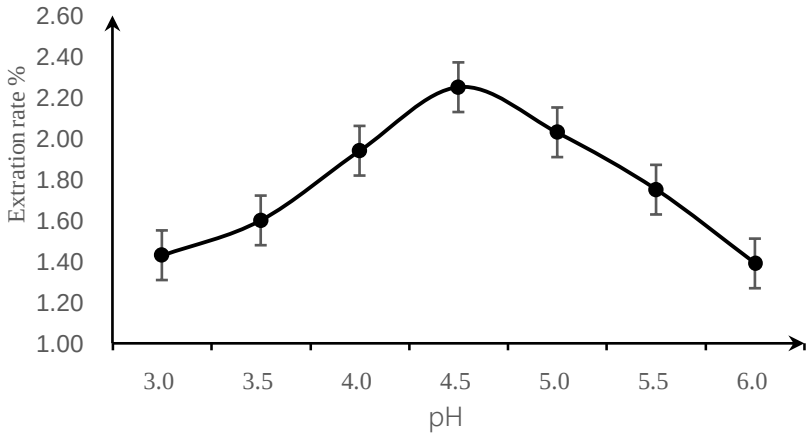

Figure2. *The effect of pH of enzymatic hydrolysis on the extraction rate of total flavonoids from Ginkgo biloba leaves*

The PH is between 3.0 and 4.5. With the increase of PH, the extraction rate of ginkgo biloba total flavonoids shows an upward trend; when PH4.5, the extraction rate of ginkgo biloba total flavonoids

reaches the maximum 2.25%; when the PH value is greater than After 5.0, the extraction rate of total flavonoids from Ginkgo biloba showed a downward trend (Figure 2). Therefore, the extraction rate of total flavonoids from Ginkgo biloba leaves is best when the pH is 4.5.

3.1.3. The effect of enzymatic hydrolysis temperature on the extraction rate of flavonoids in ginkgo leaves

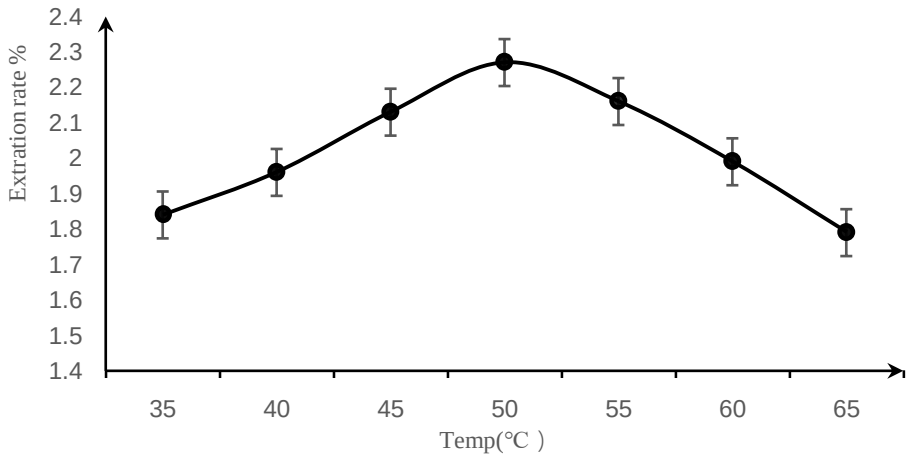

Figure3. *The effect of enzymatic hydrolysis temperature on the extraction rate of flavonoids in ginkgo leaves*

When the enzymolysis temperature is between 35°C and 50°C, the extraction rate of total flavonoids continues to increase, indicating that as the enzymolysis temperature rises, the extraction rate is also increasing, and the extraction rate reaches 2.27% when the temperature reaches 50°C. After 50°C, it began to drop continuously, indicating that the optimal temperature for enzymatic hydrolysis is 50°C (Figure 3).

3.1.4. The effect of enzymolysis time on the extraction rate of flavonoids

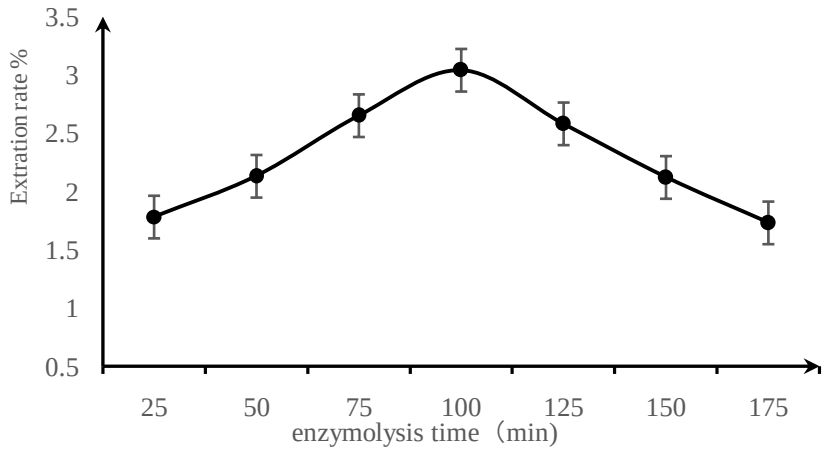

Figure4. *The effect of enzymolysis time on the extraction rate of flavonoids*

When the enzymolysis time is 25 min to 90 min, the extraction rate of ginkgo biloba total flavonoids is continuously increasing, indicating that the enzymolysis time continues to increase, and the extraction rate of total flavonoids is also increasing; at 100 min, the extraction rate of ginkgo biloba total flavonoids reaches 3.04 %, it began to decrease within 100 minutes, and the extraction rate of ginkgo biloba flavonoids began to decrease, indicating that the best time for enzymatic hydrolysis was 100 Minutes (**Figure** 4).

3.2. Orthogonal Test for Extracting Total Flavonoids from Ginkgo Biloba Leaves by Double Enzyme Hydrolysis

The results of the orthogonal test for the extraction of total flavonoids from Ginkgo biloba leaves by double enzyme hydrolysis are shown in Table 2

Table2. *Orthogonal test table for extracting total flavonoids from Ginkgo biloba leaves by double enzyme hydrolysis*

No.	Factor				Extraction rate（%）
	A AMOUNT	B pH	CTEMP	DTIME	
1	1	1	1	1	3.03
2	1	2	2	2	3.56
3	1	3	3	3	4.13
4	2	1	2	3	4.56
5	2	2	3	1	5.08
6	2	3	1	2	2.93
7	3	1	3	2	2.84
8	3	2	1	3	3.96
9	3	3	2	1	3.09
K1	3.573	3.443	3.207	3.733	
K2	4.157	3.627	3.703	3.110	
K3	3.197	3.383	4.017	4.083	
R1	0.960	0.243	0.810	0.973	

From the orthogonal test table in Table 2, it can be concluded that the primary and secondary factors affecting the extraction rate are as follows: the amount of enzyme added> the concentration of enzymatic hydrolysis> the temperature of enzymatic hydrolysis> the pH value of enzymatic hydrolysis. The comparison of the three levels of the same factor shows that: A2> A1>A3, B2>B1>B3, C3>C2>C1, D3>D1>D2, so the best process conditions are A2B2C3D3.

3.3. Verification Test

Take the optimal combination of orthogonal experiment (A2B2C3D3) and the combination 1 (A2B2C3D1) with the highest extraction rate in Table 3 for verification test, repeat 3 times respectively, calculate the average, the optimal combination extraction rate is 5.48%, the extraction rate of combination 1 Compared with 5.08%, the optimal combination of the two has a higher extraction rate than the combination 1, so the optimal combination determined by the orthogonal experiment is the optimal extraction condition.

4. Conclusions and Prospects

4.1. Conclusion

(1) The single factor test results show that the addition amount of dual enzymes 4mg of cellulase and 4mg of pectinase is the best addition amount for the extraction of total flavonoids from Ginkgo biloba leaves. The pH value of enzymatic hydrolysis is 4.5 is the optimal pH value for extracting the total flavonoids of Ginkgobiloba leaves. The temperature of enzymolysis at 50℃ is the optimum temperature for extracting total flavonoids from Ginkgo biloba leaves. Enzymatic hydrolysis time is 100 minutes when it is the best time to extract the total flavonoids of Ginkgo biloba.

(2) The results of the orthogonal test showed that the optimal conditions for the extraction of total flavonoids from Ginkgo biloba by the two enzymes were A2B2C3D3, that is, 4mg of cellulase 4mg of pectinase was added to 20ml of buffer solution, and the enzymatic hydrolysis temperature was 50 ℃ and pH was 4.5. Act for 125min under the conditions. The extraction rate of total flavonoids from Ginkgo biloba leaves was 5.48%, which was 24.9% higher than that of cellulase alone in the preliminary test.

4.2. Outlook

This experiment explores the process of extracting total flavonoids from ginkgo biloba by double-enzyme hydrolysis. The results of the experiment show that using cellulase and pectinase to extract total flavonoids from ginkgo biloba has a higher extraction rate than cellulase alone. The total flavonoids provide new ideas and lay a good foundation for the later separation and purification.

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