· 制剂与质量 ·

Studies on raising yield of effective constituent in Trigonella foenum-graecum by cellulase

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Abstract: **Object** To improve the yield of the effective constituent in *Trigonella foenum-graecum* L. by cellulase. **Methods** To take the yield of saponin as index to confirm the optimal enzyme and the best enzymolysis condition for improving the yield of saponin. **Results** The yield of the effective constituent in experimental group is about 3.6 times of that in control group; the best experimental condition for enzymolysis is: temperature 50 ℃, pH 4.4, time 72 h. **Conclusion** By using cellulase, the yield of effective constituent of *T. foenum-graecum* — saponin could be increased.

Key words: *Trigonella foenum-graecum* L.; cellulase; saponin

纤维素酶提高胡芦巴有效成分收率的研究

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摘 要: **目的** 利用纤维素酶提高胡芦巴有效成分的收率。**方法** 以甾体皂苷收率为指标, 确定提高胡芦巴有效成分收率的最佳酶及最佳酶解条件。**结果** 实验组有效成分的收率约是对照组的 3.6 倍; 酶解的最佳实验条件为: 温度 50 ℃, pH 4.4, 反应 72 h。**结论** 利用纤维素酶可以提高胡芦巴有效成分——甾体皂苷的收率。

关键词: 胡芦巴; 纤维素酶; 甾体皂苷

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The research of extracting the effective constituent from the natural materials depending on enzyme preparation or enzymatic engineering is a new technology just arising after the middle of 1990's. Domestic and overseas researches indicate that the biological enzyme can be utilized to realize the extraction and isolation of the effective consti- tuent from the Chinese medicinal materials[1~3]. In order to improve the yield of the effective consti- tuent from the medicinal materials, some scholars have ever made some research using this technology[4,5]. At present, the domestic and overseas scholars mainly concentrate on the research of the cellulose as for the application of enzyme method to extraction of traditional Chinese medicine. Because the cell wall of most Chinese medicinal materials is composed of cellulose, the effective constituents are always wrapped within the cell wall. Cellulase can be used to break the β-D-glucose chain, which is in favor of the extraction of effective constituents.

Trigonella foenum-graecum L. (TFL) is the seed of *Trigonella* Linn., one Leguminosae plant, which is considered as the traditional use for warming the kidney to invigorate *yang* for a long history. The application of the dried mature seed of TFL has already been recorded in *Chinese Pharmacopoeia* as the typical Chinese medicine. The study of modern pharmacology shows that TFL has the functions of reducing the sugar and fat in the blood

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as well as fighting against tumor, in which more attention of domestic and overseas scholars has been attracted. The main chemical constituents of the plant are saponin, steroidenin, flavone and its glycoside, triterpene, alkaloid, coumarin, organic acid, lipa, and others, among which the content of diosgenin and yamogenin is about 0.6%—1% but only 0.15%—0.32% in other plants from same genus[6].

Four different enzymes (including cellulase, glucoamylase, β-glucanase NS44053, cellobiase NS37040) were used to carry out the enzymolysis on TFL and also confim the best enzyme and best enzymolysis condition of improving the yield of saponin from TFL.

1 Materials and apparatus

1.1 Materials: TFL is provided by the Research Center of New Medicine of Liaoning College of Traditional Chinese Medicine. The sample is kept with constant weight by fire dry and kept in the drying apparatus after grinding and screening. The standard saponin is purchased from the Chinese Biological Products Authentication Station; Cellulase comes from Shanghai Lizhu-Dongfeng Biological Technology Co., Ltd.; Glucoamylas, β-glucanase, and cellobiase are all provided by Beijing Novey Company. The other reagents are purely analyzed.

1.2 Apparatus: JC101—A Digital Electrothermic Blasting Dry Box from Shanghai Jiacheng Apparatus & Equipments Factory; 721—Type Spectrophotometer from Shanghai Analytical Instrument Factory; DK—97—Ⅰ Electric Constant Temperature Water Bath from Tianjin City Taist Apparatus Co., Ltd.

2 Methods and results

2.1 Preparation of experimental reagent: Citric acid buffer solution is prepared according to the file[7]: Enzyme solution: Cellulase solution, to weigh 50 mg cellulase in accuracy and dissolve it in 10 mL measuring flask, keep the concentration at 5 mg/mL. The concentration of other enzymic preparation solution is 5%.

Standard solution: To accurately weigh 1.0 mg saponin standard and dissolve it in 5 mL methyl alcohol with constant volume, then preserve it in the refrigerator at 4 ℃ (the concentration of the saponin is 0.2 mg/mL).

TFL solution for experiment: To weigh 5 g TFL and separately place it in the 250 mL measuring flask (20 mg/mL), then add citric acid to balance solution, make a constant volume after 10 minutes supersonic oscillations, then store it in the refrigerator at 4 ℃ for standby.

2.2 The drawing of standard curve of saponin: According to the method in the literature[8], to take the content of saponin (C, μg) to the corresponding absorbency A in order to process linear regression; saponin regression linear equation: $C = 79.93A - 0.5201$; $r = 0.9975$; and the corresponding absorbency A within (0—60 μg) possess good linear dependence.

2.3 The optimum enzyme for hydrolyzed TFL: In control group to weigh 20 mg/mL TFL preparation TFL solution for experiment 2.5 mL, and 2.5 mL distilled water, pH 4.4, 50 ℃, water bath 48 h; and 1 mL water saturated normal butyl alcohol into the 1 mL liquid, then after shaking, standing, demixing, centrifugation, put supernatant liquid in evaporating dish; repeat the above operation four times, totally collect 5 mL water saturated normal butyl alcohol in evaporating dish, after 100 ℃ volatilizing on the bain-marie use formaldehyde to dissolve the saponin sticking to the 25 mL evaporating dish, wash it several times and volatilize the water bath at 60 ℃, take 1 mL saponin solution in the test tube with 1 mL marks, according to the method of testing the transparency at the wavelength of 560 nm, then account yield of enzymatic saponin through the regression equation and the following formula.

$$\text{Saponin yield} = (25CV \times 10^{-3}/W) \times 100\%$$

Four pieces of 2.5 mL TFL preparation: TFL solution for experiment with 20 mg/mL concentration were measured in control group by separately adding 2.5 mL and 5 mg/mL cellulase; pH 4.4, 50 ℃, water bath 48 h; 5% β-glucanase, pH 5.8, 50 ℃ water bath 48 h; 5% β-glucanase Ⅱ;

pH 5.8, 50 ℃, 48 h; and 5% glucoamylase, pH 4.4, 25 ℃, water bath 48 h, according to the method of testing the transparency at the wavelength of 560 nm and account the rate of enzymatic saponin using formula (Ⅰ). The comparison of different results of enzyme hydrolyzed saponin yield can be seen in Table 1. The result is cellulase > glucoamylase > cellobiase > β-glucanase Ⅰ > control group. The best is the group of celluase, the high yield of saponin is about 3.6 times than that in the control group.

Table 1 Saponin yield comparison of different enzyme hydrolyzed TFL

No.	Methods	Saponin yield / %
1	control group	0.564
2	cellulase	2.041
3	glucoamylase	1.245
4	β-glucanase	0.699
5	cellobiase	0.915

2.4 Choose for optimum condition for cellulase hydrolyzed TFL: Take TFL, and 5 mg/mL the cellulase into triangular bottle, in different time (4, 24, 48, 72, 96, 108 h), different temperature (20 ℃, 37 ℃, 45 ℃, 50 ℃, 55 ℃, 65 ℃), different pH (4.0, 4.2, 4.4, 4.6, 4.8) and different TFL concentrations. Account the yield of saponin according to the method in 2.3 after the reaction termination.

2.4.1 The determination of the best reaction time of cellulase hydrolyzed TFL: Under the condition of 50 ℃ hot water with pH 4.4, heating at different times, the relation curve of reaction time of cellulase hydrolyzed TFL and the yield of cellulase hydrolyzed saponin is indicated in Fig. 1. With the time running, the cellulase hydrolyzed TFL function was increased by and by, the function was improved step by step in 4—72 h as well, until 72 h later it reached the peak, then lowered down. It suggests that the most adaptive time for cellulase hydrolyzed TFL be 72 hours. This sounds that cellulase can develop the largest function under the most suitable conditions.

2.4.2 The determination of the best reaction temperature of cellulase hydrolyzed TFL: Under the condition of pH 4.4, hot water with heating at

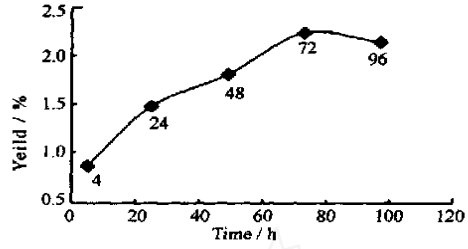

Fig. 1 Effect on saponin yield in different enzyme hydrolyzing time

72 h, in different temperatures, the relation curve of reaction temperature of cellulase hydrolyzed TFL and the yield of cellulase hydrolyzed saponin is indicated in Fig. 2. The cellulase hydrolyzed TFL function began from 40 ℃, it nonoccurrenced in 20 ℃—40 ℃. The function was improved step by step from 45 ℃ as well, until 50 ℃ it reached the peak, then lowered down. It suggests that the most adaptive temperature for cellulase hydrolyzed TFL be 50 ℃. This sounds that cellulase can develop the largest function under the most suitable conditions.

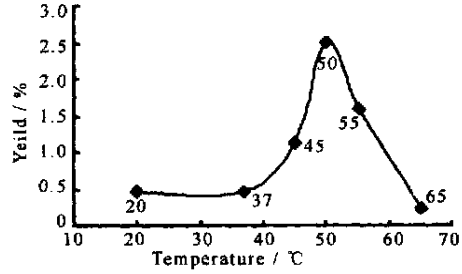

Fig. 2 Effect on saponin yield in different enzyme hydrolyzing temperature

2.4.3 The determination of the best reaction pH of cellulase hydrolyzed TFL: Under the condition of 50 ℃, hot water heated for 72 h, at different pH values, the relation curve of reaction pH of cellulase hydrolyzed TFL and the yield of cellulase hydrolyzed saponin is indicated in Fig. 3.

It showed in Fig. 3 that, cellulase hydrolyzed TFL function was increased by and by with pH running. When pH = 4.4, it reached the peak, then lowered down. So the optimum adaptive pH for cellulase hydrolyzed TFL is 4.4, which inosculates with the most adaptive pH of cellulase. This sounds like that cellulase can develop the strongest function under the most suitable pH conditions.

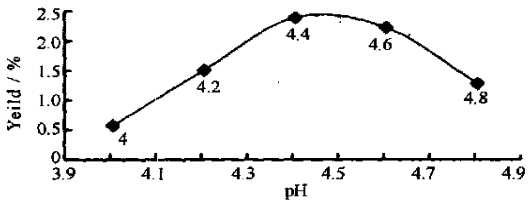

Fig 3 Effect on saponin yield in different pH

2.4.4 Influence of enzymatic reaction caused by the change of sample concentration, and the determination of Michaelis-Menten equation: After 72 h under the condition of 50 ℃, pH 4.4, and the hydrolytic action by 5 mg/mL cellulase, the result for the reaction velocity of various sample with different concentrations is shown in Table 2.

Table 2 Change of reaction speed of different sample after enzyme hydrolyzing

Sample /mg	$[S]$/ (mg·mL^{-1})	$\frac{1}{[S]}$	V/ (mg·d^{-1})	V^{-1}/ (d·mg^{-1})
5	5	0.20	0.011 1	90.09
100	10	0.10	0.015 1	66.22
150	15	0.07	0.042 0	23.81
200	20	0.05	0.045 4	22.03
250	25	0.04	0.044 4	22.52

Under the condition of constant reaction temperature, pH and enzymatic concentration, the primary speed of enzymatic reaction increased following the increase of samples' concentration $[S]$. While as sample concentrations reached a certain limit, the reaction speed became constant, considering as the largest reaction speed. The relation between the speed and concentration of substratum can be shown by Michaelis-Menten equation.

$$V = \frac{V_{max} \cdot [S]}{K_m + [S]}$$

K_m: M-constant, V_{max}: the most reaction speed, $[S]$: sample concentration. when $V = V_{max}/2$, $K_m = [S]$

K_m is one characteristic-constant of enzyme. It is one method to research enzyme by measuring K_m. If the Michaelis-Menten equation changes into double reciprocal equation, one picture is made by $1/V$ to $1/[S]$, each point is linked together and make the nodal increment is made to be $-1/K_m$ on the lateral axis in order to calculate K_m value

$$\frac{1}{V} = \frac{K_m}{V_{max}} \times \frac{1}{[S]} + \frac{1}{V_{max}}$$

The Michaelis-Menten constant of hydrolysis TFL by cellulase is $K_m = 200$ mg/mL; $1/V_{max} = 2.5$, $V_{max} = 0.04$ mg/d. So the relationship equation between the reaction speed of hydrolysis TFL by cellulase and concentration of sample as below:

$$V = \frac{0.04 \cdot [S]}{200 + [S]}$$

3 Discussion

The result of such research has indicated that the important function of cellulase hydrolytic TFL is enzyme's function of breaking wall of plant cell, because comparing with the control group, the high yield of saponin is about 3.6 times than that in the control group. The result of the research is the same as the predecessor's result, and the argument is also identified. So the research points out: during the period of extracting effective medi- cine element from the plant, firstly it can be hydrolyzed by cellulase accordingly the release of the effective constituent has been increased, the yield of the effective constituent has been improv- ed, for improving the technology of extracting the effective constituent, raising the yield of the traditional Chinese medicine the above method has possessed a very important applied cost.

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