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Modification of cellulase and its application to extraction of diosgenin from *Dioscorea zingiberensis* C.H. Wright

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ABSTRACT

In order to enhance the thermostability and efficiency of cellulase in the extraction of diosgenin from *Dioscorea zingiberensis* C.H. Wright, we applied polyethylene glycol (PEG) (400, 1000, 2000, and 4000) to modify cellulase. The modified cellulase, α-amylase and β-glycosidase were used to hydrolyze the material. The results show that the thermostability of modified cellulase is better than that of natural cellulase, the optimum pH value and temperature of modified cellulase are wider than that of natural cellulase, the activity of cellulase modified by activated PEG2000 is higher than that of cellulase modified by other modifiers, and its remaining activity is 58% of its initial value. With this technique, the purity of the product reaches 96%, the melting point is 201–204 °C, the yield rate and the extraction rate of the diosgenin reaches 2.80% and 96.6%, respectively. IR spectra and ^{1}H NMR spectroscopy were used to confirm the structure of the product.

1. Introduction

Diosgenin (25R-Spriost-5-en-3β-ol) is a hydrolysate of dioscin contained in the rootstock of yam, and it exists widely in the natural plant such as glucoside [1].

Diosgenin is a source of helpful biologically active components found in plant foods, such as phytochemicals and it is a functional food. Published literature reports many health benefits of products associated with the diosgenin, for example, prevention against cardiovascular disease, contraception, prevention against cancer and coronary disease [2,3].

Diosgenin is an important steroidal metabolite used as a starting material for the synthesis of steroidal drugs. It has an estrogenic effect on the mammary gland [4], and plays an important role in the control of cholesterol metabolism [5,6]. It is mainly used as the initial material for partial synthesis of oral contraceptives, sex hormones and other steroids.

Methods of extraction of diosgenin from *Dioscorea zingiberensis* C.H. Wright include direct acid hydrolysis, spontaneous fermentation, supercritical CO_2 extraction and so on. Recently, some researchers used a single enzyme (such as cellulase [7–9], theamylase [10] and so on) combined with acid hydrolysis to treat Chinese yam (*Dioscorea opposita* Thunb) material, demonstrating that about 70% diosgenin can be extracted from the material. However, the activity of enzyme gradually reduces owing to the change of catalysis environment, so that the catalysis efficiency of enzyme will also reduce.

Therefore it is urgent to find efficient methods to enhance the stability of natural cellulase. Current methods that enhance the stability of natural cellulase include selection, protein engineering, enzyme immobilization, enzyme chemical modification, and adding a cosolvent agent [11]. The method of enzyme chemical modification uses a chemical dressing agent to modify the side chain of amino acid (such as lysine) on the surface of the enzyme covalently, thus the stability of the enzyme is enhanced [12,13]. Compared to other methods, enzyme chemical modification is simple and effective. Ying [14] used mono-methoxy polyethylene glycol (MPEG) to modify lumbrulcinase, so that the thermostability of the modified enzyme was better than that of natural cellulase. Park [15] used hydrophilic and hydrophobic copolymers to modify cellulase, the introduction of a hydrophilic copolymer enhanced the hydrolysis rate and the conversion rate of cellulase saccharying cellulose and also changed the adsorption parameter of cellulase. Polyethylene glycol (PEG) was used to modify cellulase in this paper. The

Abbreviations: CC, cyanuric chloride; CC-PEG, the activated cyanuric chloride PEG; CMC, carboxymethylcellulose; FT-IR, Fourier transform infrared spectroscopy; HPLC, high-pressure liquid chromatography; MPEG, mono-methoxy polyethylene glycol; MWCO, molecular weight cut off; NMR, nuclear magnetic resonance; PEG, polyethylene glycol; TNBS, 2,4,6-trinitrobenzenesulfonic acid.

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results show that the stability of the modified cellulase is improved and the deactivation rate is reduced. Thus it is feasible to apply the modified cellulase to the extraction of diosgenin from *D. zingiberensis*.

2. Materials and methods

2.1. Materials and reagents

Cellulase (20,000 U/g), β-glycosidase (4000 U/g) and α-amylase (100,000 U/g) were obtained from Novozymes in China. *D. zingiberensis* was supplied by Xunyang yam Corp. of Shanxi province, China. The content of diosgenin in the raw material was 2.87%. Polyethylene glycol (PEG400, 1000, 2000 and 4000), cyanuric chloride (CC), concentrated sulfuric acid and anhydrous sodium carbonate (analytical grade) were purchased from Tianjin Kewei Chemical Reagent Company. 120# gasoline was supplied by Petroleum Corp. of Tianjin, China. All other reagents were of analytical grade.

2.2. Modification of cellulase

PEG (average molecular masses: 400, 1000, 2000 and 4000) and cyanuric chloride were added into a reactor which contained 300 mL benzene solution, 6.5 g anhydrous sodium carbonate and 5.0 g 4A molecular sieves. The mole ratio of PEG to cyanuric chloride was 2:1. It reacted for 4 h under a controlled temperature 40–45 °C. After the reaction, the solution was concentrated, crystallized, filtered and vacuum dried at low temperature, in this way, the activated cyanuric chloride PEG (CC-PEG) was obtained.

The natural cellulase was mixed with CC-PEG in a 100 mL boric acid buffer solution at 8 °C and pH 8.5. The mass ratio of natural cellulase to CC-PEG was 1:20. During the reaction, 10% NaOH solution was added dropwise in the reaction solution to ensure the alkalescent circumstance. After the modification reaction, modified cellulase was separated by a polysulfone ultrafiltration membrane, which was 10,000 molecular weight cut off (MWCO). Modified cellulase was freeze-dried and kept at 4 °C.

The reaction principle [16] is shown in Fig. 1.

2.3. Determination of modification degree of modified cellulase

2,4,6-Trinitrobenzenesulfonic acid (TNBS) was used to determine the modification degree of modified cellulase [17]. The basic principle is as follows: TNBS reacts with the free amino-group on the protein molecule and produces a coloured chemical compound. Thus the modification degree can be calculated by detecting the changes of absorbance before and after enzyme modification.

The following steps were carried out:

(1) 1.0 mL TNBS (0.01 (w/v) aqueous solution) was added into 1.5 mL Tris–HCl buffer solution (pH 8.2), and the solution mixed evenly to be used as reference.
(2) 1.0 mL TNBS (0.01 (w/v) aqueous solution) was added into 1.5 mL natural cellulase sample solution (concentration 0.25 mg/mL), mixed evenly and left to stand for 35 min at room temperature. Its absorbency value was detected at 420 nm.
(3) 1.0 mL TNBS (0.01 (w/v) aqueous solution) was added into a 1.5 mL modified cellulase sample solution (concentration 0.25 mg/mL), mixed evenly and left to stand for 40 min at room temperature. Its absorbency value was detected at 420 nm.
(4) Comparing the absorbency value of (2) with (3), the proportion of modified amino-group was calculated, and then the modification degree was determined according to Eq. (1) as follows:

$$\text{Modification degree} = 1 - \text{the ratio of residual amino-group} = \frac{1 - \text{the absorbency value after modification}}{\text{the absorbency value before modification}} \quad (1)$$

2.4. Thermostability of modified cellulase

Natural cellulase and different modified cellulase were both prepared to be citric acid buffer solution. The concentration of the citric acid buffer solutions was identical in both the natural and modified cellulase and the pH was 4.5. The citric acid buffer solu-

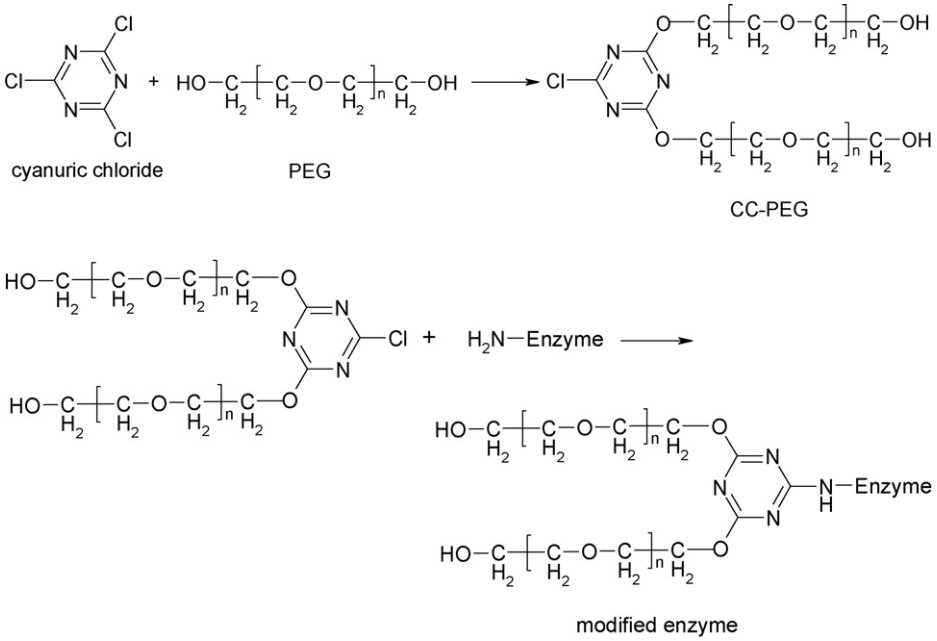

Fig. 1. Chemical modification of cellulase. The activated cyanuric chloride PEG (CC-PEG) was obtained first, then the modified cellulase was synthesized through modification reaction between the CC-PEG and natural cellulase.