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Pectinase-Ultrasound Synergistic Extraction of Chlorogenic Acid from Flos Lonicera Japonicae

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Abstract

A pectinase-ultrasound synergistic extraction (PUSE) method was developed to extract chlorogenic acid (CGA) from Flos Lonicerae Japonicae (FLJ). The screening and optimization experiments revealed that 20% (v/v) ethanol concentration in conjunction with an ultrasonic power 90 W was the optimal condition. Moreover, other parameters including pectinase concentrations, liquid-solid ratio, ultrasonic time and pH were systematically optimized. The scanning electronic micrographs revealed that the treatments with pectinase and ultrasound distinctly facilitated the extraction process, in which the plant cell wall was destructed in large extent. Compared with other previously reported methods, PUSE was more efficient with higher CGA extraction yield and CGA yield can reach 5.13%.

Keywords: Ultrasound; Pectinase; Extraction; Chlorogenic acid; Flos Lonicerae Japonicae

Introduction

FLJ was known as golden-and-silver honeysuckle with sweetly vanilla scented [1,2]. As both food and medicinal plants, it has been reported to have multiple biological activities and it is applied in traditional Chinese medicine to treat fever, headache, thirst and carbuncles [3,4]. As main bioactive component of FLJ, CGA has been reported to have multiple pharmacological and biological activities. CGA slightly reduces blood pressure and it has been investigated for anti-inflammatory effects taken as a dietary supplement or in coffee [5,6]. A series of methods for CGA extraction including ultrasonic assisted extraction (UAE) [7-9], enzyme extraction (EE) [10,11], water extraction (WE) [12,13], refluent ethanol extraction (REE) [14-16] and microwave-assisted extraction (MAE) [17,18] had been reported. Nonetheless, these methods usually have limitations, such as relative long extraction time and unsatisfactory CGA yield. Therefore, the article developed a new method called pectinase-ultrasound synergistic extraction (PUSE) for the extraction of CGA from FLJ.

Experimental

Reagents and materials

FLJ was acquired from Xinyi Honeysuckle Agricultural Development Co., Ltd. (Xinyi, Jiangsu). FLJ with diameters of 1.5-3.0 mm was dried in hot air oven for extraction. CGA standard was obtained from Aladdin (Shanghai, China). Pectinase was obtained from Shanghai Yuan ye Biotechnology Co., Ltd. (Jiangsu China). Sample solutions were prepared with deionized water. All other reagents are of analytical grade.

Analysis method

An Amethyst C_{18}-H column, (4.6 mm × 250 mm, 5 μm) (Sepax Technologies, Inc.) was used to detected CGA content. The elution was performed in gradient mode using methanol-water as the mobile phase and monitored at a wavelength of 327 nm. Standard curve for CGA was linear at a range of 0.01-1 mg/ml. The CGA yield was calculated using a standard formula: $y=0.999x+1.296$ ($r^2=0.999$; x, concentration in g/ml; y, peak area).

Extraction process optimization

Studies have shown that the activity of pectinase is greatly affected by ultrasound power and ethanol concentration, so a series experiments were conducted to investigate these two factors.

Screening of ethanol concentration: Ethanol with concentrations of 0, 10, 20, 30, 40, 50, 60 and 70% (v/v) and were mixed with 80 mg of pectinase in a flat-bottomed flask to obtain ethanol-pectinase solution with a concentration of 1 mg/ml. After ethanol-pectinase solution pH was adjusted to 3, the flasks were placed in a 40°C ultrasonic bath for 100 min. The resulting pectinase solution was subsequently used for analysis by hypoiodite method. Similarly, 2.0 g FLJ powder was mixed with eight different ethanol concentrations from 0-70% (v/v) and the final extract were filtered through a 0.45 μm filter prior for subsequent analysis.

Screening of ultrasonic power: Eighty milligrams pectinase and 20% (v/v) ethanol were mixed in a flat-bottomed flask and ethanol-pectinase solution mixture with a concentration of 1 mg/ml was obtained. The flask was placed in an ultrasonic bath and operated at different ultrasonic powers including 60, 75, 90, 105, 120, 135, 150 and 200 W for 100 min after the ethanol-pectinase solution pH was adjusted to 3. The resulting pectinase solution was used for subsequent analysis by hypoiodite method. Similarly, 2.0 g FLJ was mixed with eight different ethanol concentrations and the extract was filtered through a 0.45 μm filter for subsequent analysis.

Determination of pectinase activity: Important factors affecting pectinase activity including ethanol concentrations and ultrasonic power were optimized. Pectinase activity was determined by hypoiodite method. Pectinase activity is correlated with the quantity of galacturonic acid and the pectinase activity was calculated by Eq. (1):

$$\text{Pectinase activity} = \frac{(B-A)\times N\times 0.51\times S}{E\times t\times M} \quad \text{.....(1)}$$

In the equation, B is the consumption of $Na_2S_2O_3$ in control, A is the consumption of $Na_2S_2O_3$ in sample, N is $Na_2S_2O_3$ equivalent concentration, 0.51 is a constant (1 mg of equivalent $Na_2S_2O_3$ equals to 0.51 mg of equivalent free galacturonic acid), S is the total reaction liquid volume, E is the volume of enzyme used, t is the holding time, and M is quantity of absorbing reaction. The amount of enzyme produced in the enzymatic reaction that is equivalent to 1 μg of galacturonic acid per minute is defined as 1 enzyme activity unit (U).

Pectinase Ultrasound Synergistic Extraction (PUSE): In addition to the two sensitive factors of ultrasound power and ethanol concentration, other important factors affecting the extraction CGA yield were examined. Certain amount of pectinase was accurately weighed and various concentrations of pectinase aqueous solution (0-0.7 mg/ml) were prepared. The solutions pH was then adjusted from 2.0 to 6.0, 2.0 g dried sample was mixed with ethanol-pectinase aqueous solvent and then placed in a KQ-5200DE ultrasonic bath at 40°C for 30 min to 4 h. The extract was subsequently filtered and the residue was discarded. The filtrate was concentrated to 15 ml for subsequently diluted by 625 times using a 1 ml aliquot. The final extract was filtered through a 0.45 μm filter for subsequent analysis. All experiments were performed in duplicate.

Scanning electron micrographs of different samples: The structural change of the FLJ powder after the treatment was examined using a TM3000 tabletop scanning electron microscope (Hitachi, Japan) under high vacuum of 15.0 kV.

Results and Discussion

Pectinase activity had an impact on CGA extraction yield on certain extent. Two important factors impacting pectinase activity including ethanol concentration and ultrasonic power were investigated. Additionally, other factors including pectinase concentration, liquid-solid ratio, ultrasonic time and pH value were also studied.

Screening of ethanol concentration and ultrasonic power

CGA is a polar substance and ethanol was used as the extraction solvent (accordingly to the compatibility principle). A serious of experiment on optimizing ethanol concentration was conducted because ethanol concentration had a great influence on enzyme activity [19]. The influences of different ethanol concentrations on enzyme activity were determined under the following conditions: ultrasonic temperature 40, ultrasonic power 75 W, pectinase concentration 1 mg/ml, liquid ratio 40 ml/g, ultrasonic time 100 min and pH 3. The results showed that the effect of ethanol concentration (0-45%) on the activity of pectinase gradually increased (Figure 1) and the effect of ethanol concentration in this range on the extraction CGA of CGA also gradually increased (Figure 2). In addition, the pectinase activity was steeply declined when ethanol concentration was over 45% (v/v) (Figure 1a), whereby CGA yield was slightly increased (Figure 2a) and it is likely that the dissolution of ethanol may play a major role in this phase. When ethanol concentration exceeded 80% (v/v), pectinase was completely inactivated.

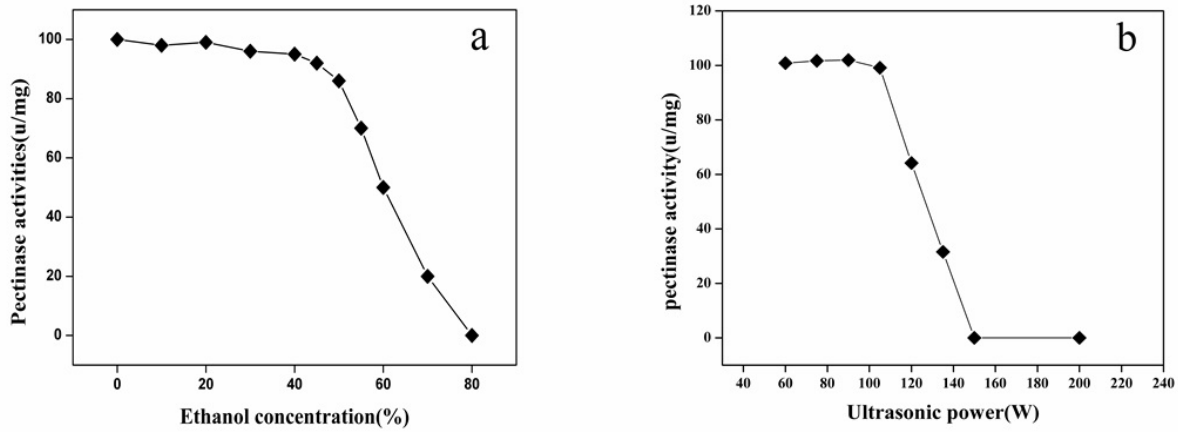

Figure 1: (a) Effects of ethanol concentration on pectinase activity (b) and ultrasonic power on pectinase activity.

It was reported that the ultrasound power had a significant effect on the pectinase activity and it could accelerate the isomerization of unsaturated double bonds and ester bonds of CGA [20,21]. Thus, it is necessary and meaningful to measure pectinase activity and CGA yield under different ultrasonic power. The impacts of different ultrasonic powers on enzyme activity were investigated under conditions as follows of ultrasonic temperature 40°C, 20% (v/v) ethanol concentration, 1 mg/ml pectinase concentration, liquid ratio 40 ml/g, ultrasonic time 100 min, and pH 3. Although the CGA yield was slightly increased (Figure 2b), the ultrasonic power from 60 to 100 W had no impact on the pectinase activity (Figure 1b).

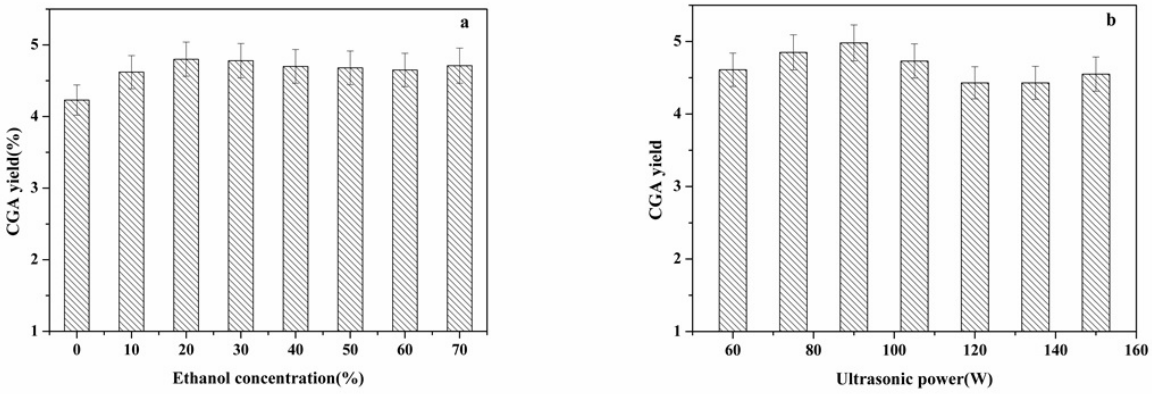

Figure 2: (a) Effect of ethanol concentrations on the extraction yields (b) and ultrasonic power on the extraction yields of CGA from FLJ.

However, the pectinase activity was rapidly decreased when the ultrasonic power was higher than 100 W, while the CGA yield was slightly increased when that was higher than 140 W. This indicated that ultrasonic power played a role in the deactivation of pectinase. The activity of pectinase completely diminished when the ultrasonic power was higher than 150 W.

Effect of pectinase concentration on CGA yield: The pectinase concentration considerably affected the CGA yield. Owing to its ability to accelerate the dissolution of cell contents, pectinase had been widely used to digest plant cell wall [12]. The influence of pectinase concentration on CGA yield was investigated under the following conditions of ultrasonic temperature 40°C, 20% (v/v) ethanol concentration, ultrasonic power 90 W, liquid-solid ratio 40 ml/g, ultrasonic time 40 min and pH 3. From Figure 3a, the yield reached a maximum value when pectinase concentration was increased to 0.25 mg/ml and remained essentially unchanged at pectinase concentration higher than 0.25 mg/ml. 0.25 mg/ml was considered as the optimal concentration for PUSE taking the cost into account.

Effect of liquid-solid ratio on CGA yield: A series of experiments were carried out for finding a proper liquid-solid ratio. The influence of liquid-solid ratio on CGA extraction yield was investigated under the following conditions of ultrasonic temperature 40°C, 20% (v/v) ethanol concentration, ultrasonic power 90 W, 0.25 mg/ml pectinase concentration, ultrasonic time 40 min and pH 3. From Figure 3b, the CGA yield was greatly increased with increasing liquid-solid ratio from 20 to 50 ml/g. It is likely that the interactions between FLJ and solvent increase with increasing amount of solvent [22].

It appeared that the interactions between sample and solvent reached saturated state when the liquid-solid ratio exceeded 50 ml/g. Considering the cost, 50 ml/g was chosen as the optimum liquid-solid ratio.

Effect of ultrasonic time on CGA yield: From Figure 3c, the influence of ultrasonic times on CGA yields was evaluated under the following conditions of ultrasonic temperature 40˚C, 20% (v/v) ethanol concentration, ultrasonic power 90 W, pectinase concentration 0.25 mg/ml, and pH value 3. The results in Figure 3c illustrated that the CGA yield increased with increasing extraction time of up to 50 min, whereas that decreased with increasing extraction time higher than 50 min.

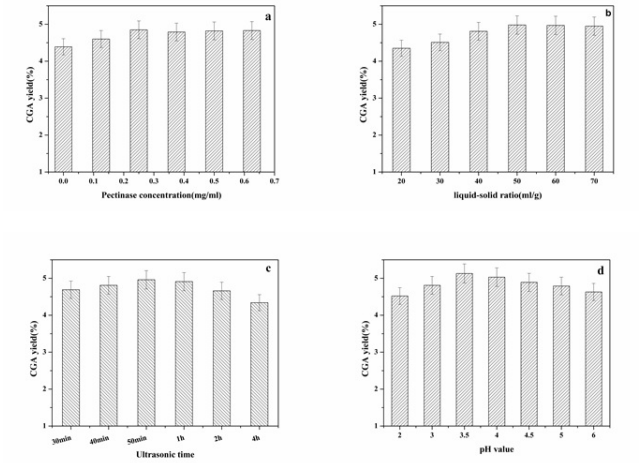

Figure 3 Effects of pectinase concentration (a) liquid-liquid ratio; (b) ultrasonic time; (c) and pH; (d) on the extraction yields of CGA from FLJ.

The results indicated that extended ultrasonic time provided more thorough interactions between the solvent and FLJ, however, extended time may destruct the chemical structure of the extract. Thus, 50 min was selected as the optimal time used in subsequent experiments.

Effect of pH value on CGA yield: The effects of pH range of 2.0-6.0 were investigated considering that CGA is an acidic compound and pH had been found to have important effect on pectinase. The experimental conditions were as follows of ultrasonic temperature 40°C, 20% (v/v) ethanol concentration, 0.25 mg/ml pectinase concentration, liquid-solid ratio 50 ml/g, ultrasonic time 50 min and ultrasonic power 90 W. The results in Figure 3d showed that the extraction efficiency of CGA initially increased with the increase of pH and reached maximum CGA yield at pH 3.5, while the efficiency decreased with pH beyond 3.5. It appears that the increase of pH is beneficial to the ionization of CGA; thereby the H-bond between

solvent and CGA is weakened [23-25]. Thus, pH 3.5 was considered most suitable for the extraction experiments.

Comparison with reference methods: The GCA yield obtained in the present study was compared with the previous reported methods. The comparison listed in Table 1 indicated that our method has superior extraction efficiency with higher yields than other reported methods.

Method	Optimized extraction parameters	CGA yield(%)
WE	Liquid ratio 15 ml/g, extraction temperature 70°C, extraction time 1 h	3.8 [24]
REE	Ethanol concentration 80% (v/v), liquid- solid ratio 30 ml/g, extraction time 70 min, extraction temperature 90°C.	3.63 [25]
MAE	Extraction time 30 min, ethanol concentration 75%, liquid- solid ratio 30 ml/g, extraction temperature 65°C, pH 7	5.07 [18]
EAE	Dosage of cellulase 3.0%, extraction temperature 46°C, extraction time 4 h, leaching at 56°C for 1 h.	3.57 [25]
	Dosage of pectinase 0.5%, operating temperature 45°C, operating time 120 min, pH 5.0.	4.15
UAE	Ultrasonic temperature 40°C, ultrasonic power 280 W, ultrasonic time 80 min, ethanol concentration 70%, liquid-solid ratio 30 ml/g.	4.37
PUSE	Ultrasonic temperature 40°C, ethanol concentration 20% (v/v), ultrasonic power 90 W, ultrasonic time 50 min, pectinase concentration 0.25 mg/ml, liquid- solid ratio 50 ml/g, pH 3.5.	5.13

Table 1: Comparison of PUSE with the reference and conventional methods.

Scanning electron micrographs (SEM) of FLJ samples: As a result of PUSE process, the SEM showed the structural change of FLJ and revealed the extraction mechanism. Figure 4 showed the SEM of untreated sample, sample treated with ultrasound, sample treated with pectinase, sample treated with PUSE. Figure 4a illustrated the leaves of the untreated samples had clear and smooth surface. The ultrasound treatment caused the surface become rough with apparent fractures in some locations from Figure 4b.

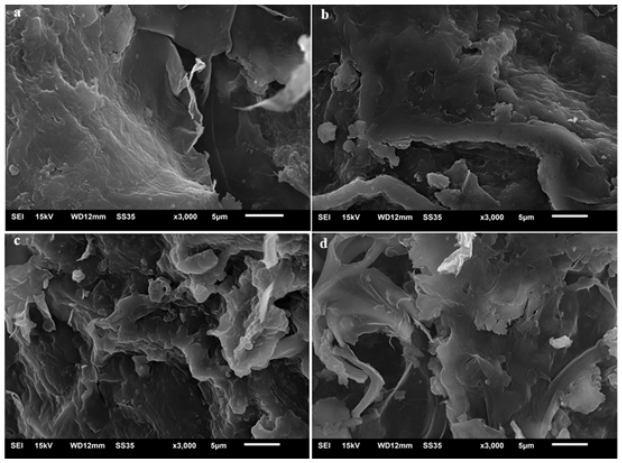

Figure 4: The scanning electron micrographs of FLJ, Untreated sample (a) treated only with ultrasound; (b) treated only with pectinase; (c) and treated with PUSE.

The SEM of the sample treated with pectinase showed that the surface was largely uneven with some wrinkles, which may be the result of the exposure to extraction solution from Figure 4c. The sample treated with PUSE had slightly rough sheet-like surface that appeared to be thinner compared with other samples and most of the bulges were disappeared. The breakage of plant cell and the degree of protrusions of extraction solvent were linked to the dissolution and extraction effectiveness. These findings indicated that the PUSE method caused the destruction of FLJ cell wall to a large extent, which led increasing of CGA extraction yield.

Conclusions

In the present study, a novel pectinase-ultrasound synergistic extraction (PUSE) was developed for the first time to extract CGA from FLJ. Compared to other reported extraction methods, the PUSE method showed higher extraction efficiency and yield. The finding indicates PUSE can be a highly potential method for extraction of biologically active compounds from natural resources.

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