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High-Yield Production of Apigenin from Celery with Ultrasonic-Assisted β-Glucosidase Hydrolysis

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Apigenin, being an important dietary compound, is a natural product mainly contained source of celery, is negatively associated with various diseases. However, high-efficient extraction of apigenin, with low cost from complex-component plant vegetable tissue, is still a problem. The aim of the study was to evaluate the influence of different operational conditions during ultrasonic-assisted β-glucosidase hydrolysis on apigenin extraction from celery leaves. Enzymatic hydrolysis showed high apigenin extraction capacity at 0.1 mg/mL concentration, after 15 h, at pH 6. Ultrasonic-assisted enzymatic hydrolysis also achieved high extraction activity towards apigenin with these optimal parameters: an exposure period of 40 min at 60 °C, power source 60 W and with 70 % methanol concentration.

Keywords: Apigenin extraction, β-Glucosidase hydrolysis, Ultrasonic-assisted, Operational conditions, Celery.

INTRODUCTION

The compound 4',5,7-trihydroxyflavone, commonly referred to as apigenin, is a natural product mainly contained in food sources such as thyme, cherries, tea and celery. As a biologically active flavonoid that is plentiful in a variety of dietary constituents, numerous studies provide evidence that apigenin may exert some influence over the transition from normal to carcinogenic and has value as a chemopreventive agent that underlies the benefit of a healthy diet[1]. However, high-efficient extraction of apigenin, with low cost from complex-component plant vegetable tissue, is still a problem. It is well known that the plant cell wall consists of cellulose, hemicellulose and pectin, which constitute the barrier for the release of intracellular substances.

The 3 constituents can be hydrolyzed using cellulase, β-glucosidase and pectinase[2]. A previous study in our lab optimized the experimental conditions for pectinase extraction on the yield of apigenin from celery and compared the performance of pectinase and cellulase on the extraction[3]. However, besides pectinase and cellulase, which could improve the release of intracellular contents, there is the third enzyme, β-glucosidase, which cleaves the β-1,4 linkage in cellulose and which can also break down the plant cell wall by damaging the hydrogen-bond interactions to obtain more free flavonoids. Due to the fact that β-glucosidase has substrate specificity for the hydrolysis of short-chain cello-oligosaccharides and cellobiose into glucose[4], the efficient conversion of ligocellulose to fermentable sugars has been recognized as the main bottleneck in the economical production of natural compounds from plant tissues.

Therefore, in the present study we focused on the optimal conditions both in ultrasonic treatment using methanol extraction and enzymatic hydrolysis with β-glucosidase treatment.

EXPERIMENTAL

Apigenin (4',5,7-trihydroxyflavone), β-glucosidase and acetonitrile of HPLC grade were purchased from Sigma-Aldrich (Steinheim, Germany). Other chemicals were of analytical grade. Deionized water was purified using a Milli-Q Water Purification system (Millipore Corp., Bedford, Mass., USA.).

Fresh celery (*Apium graveolens var. dulce*) was obtained from local green grocers in Nantong city of China. After being dried, celery leaves were divided into several groups (5 g each).

General procedure: A group of celery leaves (5 g) was ground with β-glucosidase solution using a Thermomix (Wuppertal, Vorwerk, Germany) laboratory mill for 20 s. Methanol and distilled water with specific ratio were added to obtain stock solutions at the concentration of 0.5 mg/mL. The stock solutions were further diluted with methanol solvent solution to obtain serial solutions with desired concentrations and pH. The mixture was incubated in an automatic incubator at 30-75 °C for 0-27 h with 0.05-0.2 mg/mL enzyme concentration at pH 5-7. The temperature, pH, enzyme concentration and duration time were 30 °C, 7, 0.1 mg/mL and 24 h except for the tests of their effects. In ultrasonic-assisted extraction

procedure, we investigated the effect of methanol concentration on the yield of apigenin using different concentrations (30, 50, 70, 80, 90 and 100 %) and following ultrasonic parameters were investigated *i.e.*, an exposure period, which varied from 0 to 50 min; the temperature of the bath, which varied from 50 to 70 °C and the power which varied from 40 to 99 W. The prior ultrasonic conditions of each single-variable treatment were set as follows. An extraction temperature of 60 °C, an extraction time of 0.5 h, an ultrasonic intensity of 80 W. Three replicates of apigenin preparations were carried out. Apigenin production was analyzed directly after processing.

For HPLC analysis, apigenin samples were performed on the Agilent 1200 Chromatographic system, consisting of G1311A isocratic pump, a thermostatted column compartment, a variable-wavelength UV detector (VWD) and Agilent Chemstation software. Chromatographic separation and collection of apigenin were achieved on the Agilent Eclips Plus C18 reverse-phase column (250 mm × 4.6 mm, 5 mm), with a pre-column (Agilent Eclips Plus C18, 10 mm × 4.6 mm. 5 mm) and an Agilent ZORBAX stablebond semi-preparation column, respectively. Determination of apigenin was carried out with mobile phase composed of acetonitrile and 0.2 % phosphoric acid aqueous solution (30:70, v/v) at a flow-rate of 1 mL/min. The optimum separation of HPLC was achieved at 30 °C and monitored at 347 nm.

RESULTS AND DISCUSSION

Chromatographic selectivity: The procedure afforded efficient separation and quantification of apigenin in celery leaves, without interference of peaks of endogenous constituents from celery. Fig. 1 shows the typical chromatograms of stock solution spiked with standard substances. Apigenin was eluted in approximately at 9.5 min (Fig. 1).

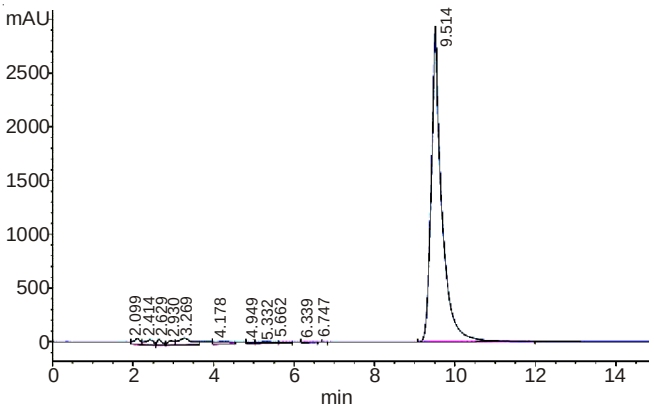

Fig. 1. High performance of liquid chromatography analysis of apigenin standard solution

Evaluation of changes in the content of apigenin with different ultrasonic conditions: Evaluation of changes in the content of apigenin in celery juices allowed to demonstrate the variation in ultrasonic conditions on methanol extraction, after enzymatic hydrolysis.

Addition of methanol solution with different concentrations (30, 50, 70, 80, 90 and 100 %) to the extraction pool, during ultrasonic-assisted process, has a significant impact on the content of apigenin produced from celery juices (Fig. 2A). Among apigenin produced with methanol extraction, the highest average content of this component was determined in the pool of 70 % methanol solution. With the average value of 2.62 mg/g in apigenin produced with 30 % methanol treatment, 70 % methanol solution increased the mean value of apigenin up to 10.43 mg/g of celery juice. This demonstrates the susceptibility of different concentrations of methanol on the ultrasonic extraction occurring after enzymatic hydrolysis.

It is well known that substance could be dissolved in some organic solvents with similar structure due to the principle of similarity of polarity[5]. In current study, the extraction yield of apigenin has been shown as improving trends with the increase of methanol concentrations from 50 to 70 % and achieved to the highest yield of apigenin among the different concentration treatments. Excessive methanol (80 %) might not dissolve apigenin thoroughly since the structure of apigenin does not totally agree with that of methanol. Hence, with the methanol concentration continuously increasing, the active effect is substituted by the inhibitory effect (Fig. 2A). To optimize corresponding ultrasonic treatment conditions for apigenin extraction, following procedures were operated in 70 % methanol solution during the ultrasonic-assisted procedure.

Ultrasonic extraction time had a significant impact on the average content of apigenin produced in celery juice (Fig. 2B). In the group of apigenin produced with different ultrasonic time treatments, the highest content of apigenin have samples obtained in 40 min (an average of 29.5 mg/L), while the lowest content in the celery juices produced in 20 min (an average of 14.2 mg/L).

The result demonstrated that after the diffusing of methanol extracts arrived at extraction equilibrium at 40 min, a decreasing apigenin yield might occur with prolonged heating time. According to the investigation, the cavitation effect of ultrasound, including macro-turbulence created by the implosion of cavitation micro-bubbles and microjets generated by the cavitation on the surface of plant matrix[6], facilitated the interior apigenin release to extraction solvent. Additionally, most of the apigenin in plant cells diffused at the early stage of extraction with a sharp increase of extraction yield in the first 40 min (Fig. 2B). However, prolonged time for more than 40 min might induce the degradation of apigenin and lead to the decrease of its yield. The chemical decomposition of some natural active constituents might be occurred on account of the negative effect of long-time ultrasonic irradiation, resulting in a decrease of extraction yield[7]. When the cavitation micro-bubbles collapsed asymmetrically onto the surfaces of target extracts, the generated microjets scoured the surfaces and caused damage to plant matrix in the solution[8]. The continuous collapse of cavitation bubbles[9] could be responsible for the decomposition of apigenin and the decline of its stability. Moreover, it was also potential that the high reactive hydroxyl radicals generated by cavitation effect in the matrix with the existence of small quantities of water could cause the decomposition of the extract[10]. Hence, ultrasonic time presented a significant impact on the apigenin yield, but additional extraction time was not necessary for the yield enhancement.

Variation of ultrasonic temperature, during ultrasonic extraction process, has also a significant impact on the level of apigenin extraction. With the average value of 20 mg/g in

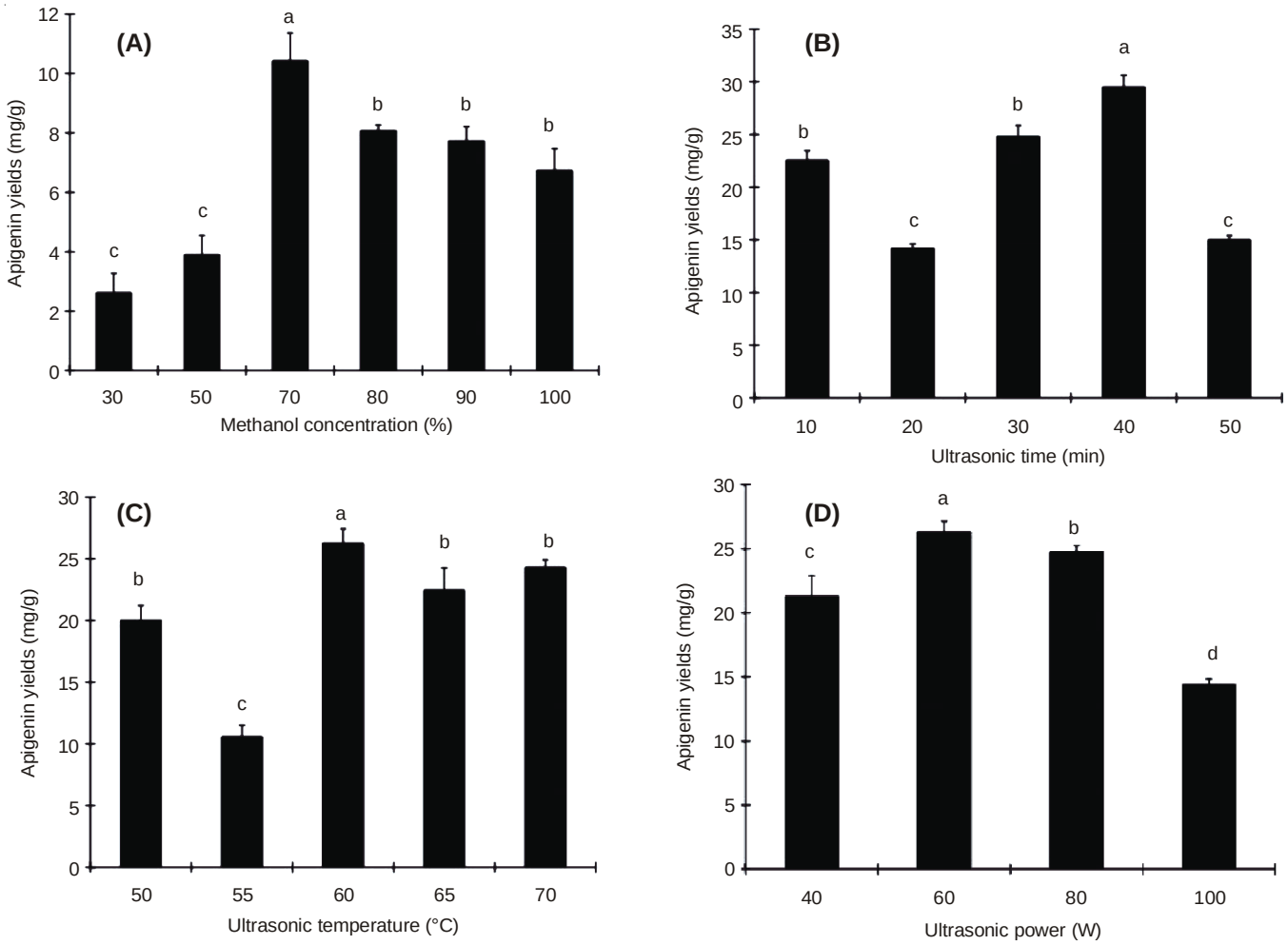

Fig. 2. Effect of ultrasonic parameters with methanol treatments (A) Methanol concentration; (B) Ultrasonic time; (C) Ultrasonic temperature; (D) Ultrasonic power on the yield of apigenin

apigenin produced at 50 °C, the mean value of apigenin was increased to 26.27 mg/g, after temperature increased to 60 °C (Fig. 2C). The lower yield of apigenin produced with immoderately high temperature (65 °C, 70 °C) was probably caused by deduced cavitation intensity and methanol volatilization.

The effect of ultrasonic temperature on the yield was probably due to the acceleration of molecular thermal motion and the increase of solubility of apigenin in methanol as temperature raising from 50 to 70 °C. Combined acoustic cavitation and thermal effect[11] resulted in the variation of apigenin extraction yield. However, immoderately high temperature (65 °C, 70 °C) might also deduce the cavitation intensity[12] and result in methanol volatilization. The physical characteristics of organic solvent such as surface tension, vapor pressure and viscosity might become primary factors affecting cavitation intensity and the vapor pressure might be the crucial factor among these properties[13]. The increasing of the ultrasonic temperature generated a positive effect on vapour pressure, therefore, the more high temperature, the more the increase of vapor pressure of solvent molecules within cavitation microbubbles, which caused the damping of the collapse and the decrease of cavitation intensity[14,15]. Thus, temperature was a sensitive variable for methanol ultrasonic extraction of apigenin and the optimum temperature was 60 °C, which gave the highest extraction yield with cavitation effect and thermal effect arrived at an equilibrium state with the variation of temperature[11].

The ultrasonic power with methanol treatment had significant effects on the apigenin production (Fig. 2D). The incidence of apigenin yield enhancement occurred with the increase in ultrasonic power within the experimental range from 40 to 100 W, which reached a maximum at 60 W. This was most likely because of the effect of ultrasonic treatment on damaging cell walls and allowing easier penetration of biocides[16]. Any further increase in ultrasonic power did not increase the extraction efficiency (Fig. 2D). The yield declined as the methanol treatment, reaching the lowest value at 100 W, partly because excessive ultrasonic power might result in methanol volatilization and the degradation of apigenin.

Evaluation of changes in the content of apigenin with different enzymatic conditions: The variations of different conditions during enzymatic reaction helped to demonstrate the differences in susceptibility of β-glucosidase activities on the apigenin extraction.

The effect of the variation of enzyme concentrations on apigenin extraction is shown in Fig. 3A. The results indicated that with an increase in the concentration of β-glucosidase from 0.05 to 0.1 mg/mL, the percentage hydrolysis also

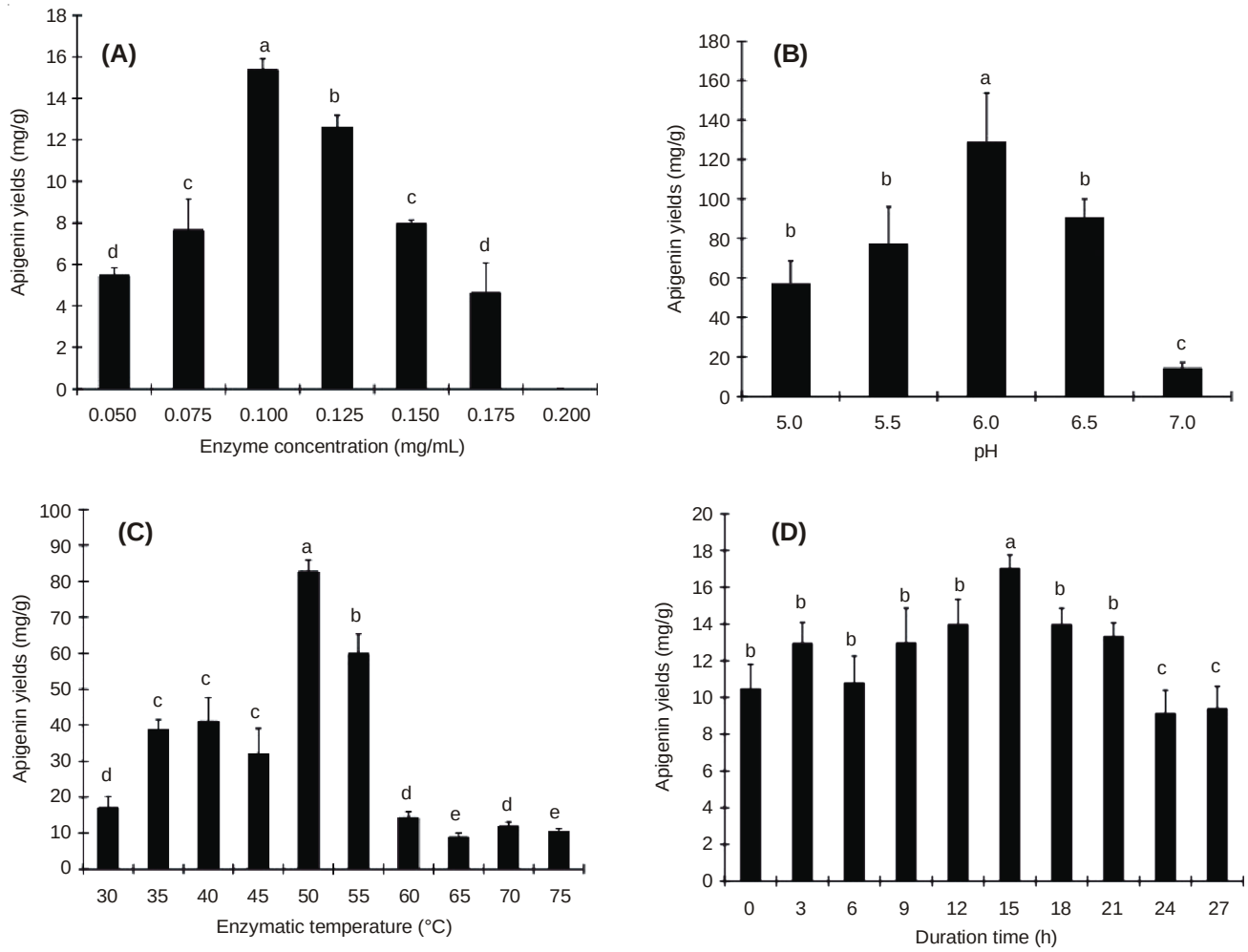

Fig. 3. Effect of different conditions during enzymatic hydrolysis (A) Enzyme concentration; (B) pH value; (C) Enzymatic temperature; (D) Duration time on the yield of apigenin

increased, while with continuous enzyme content increasing, the yield of apigenin was demonstrated as decreasing trend, indicating that the increase of enzyme concentration in the hydrolysis was not directly proportional to the apigenin yield. When enzyme concentration was doubled, the yield of apigenin was deduced, instead. As the reaction took place at the interface, an increase in the bulk enzyme concentration led to an enhancement in the enzyme concentration at the interface[17], and hence, the yield of apigenin has been increased. Nevertheless, for given hydrodynamic parameters, the extent of hydrolysis was likely to increase with an increase in the enzyme concentration till the interface was saturated with the enzyme[18]. Once the enzyme saturated into the reaction interface, any further increase in the enzyme concentration might not necessarily lead to a further increase in the hydrolysis rate[18]. Similarly, it was also reported that the enzyme had tendency to form aggregates at high concentration[18], and this could be another reason for no appreciable change in the yield of apigenin with an increase in the enzyme solution concentration.

The stability of pH is an important parameter affecting enzyme activities[19]. The bioconversion of both cellulose and hemicellulose contents of cell walls in celery leaves into sugars during the β-glucosidase hydrolysis was studied under different pH control conditions[19]. As shown in Fig. 3B, pH had an impact on yield by affecting the hydrolytic activities of β-glucosidase. The highest apigenin production was achieved at pH 6.

Temperature also had a significant effect on the average value of the apigenin yield (Fig. 3C). The highest yield, in the group of apigenin produced with different temperature treatments, was determined in 50 °C (an average of 82.71 mg/g), while the lowest in 65 °C (an average of 8.88 mg/g). Further increase in hydrolysis temperature to 75 °C reduced the apigenin yield even much lower as compared with that in low temperature reactions (30-45 °C). Appropriate temperature control for the reaction is thus required to avoid thermal deactivation of the β-glucosidase. Extreme temperature reaction causes modification of the active site enzyme and reduced available sites for the reaction process. The optimal temperature of 50 °C for β-glucosidase was also found to be optimum for enzymatic hydrolysis of different lignocellulosic biomass such as empty fruit bunches fibre[20], soybean straw[21], spruce[22], and wheat straw[23,24].

The duration time also significantly affected the average yield of apigenin (Fig. 3D). During the β-glucosidase hydrolysis with different duration time treatments (0, 3, 6, 9, 12, 15, 18, 21, 24 and 27 h), the highest apigenin yield was determined in 15 h. The average value of the yield of apigenin was 17.05 mg/g. The lowest value of apigenin yields was determined in

24 h, in which the average apigenin yield stood at 9.17 mg/g. Suitable performance time is required for the enzyme to maintain high activities of β-glucosidase. This may be due to end production inhibition occurred with the increase of the hydrolysis time that contributed to the activities of the enzyme.

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