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Analytical Methods

Enzyme assisted extraction of luteolin and apigenin from pigeonpea [*Cajanus cajan* (L.) Millsp.] leaves

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ABSTRACT

Luteolin and apigenin are naturally occurring flavones with a wide spectrum of pharmacological properties. In the present study, enzyme assisted extraction of luteolin and apigenin from pigeonpea leaves using commercial plant cell wall degrading enzyme preparations including cellulase, beta-glucosidase and pectinase were examined. We found that pectinase offered a better performance in enhancement of the extraction yields of luteolin and apigenin than cellulase and beta-glucosidase. The pectinase assisted extraction process was further optimized by varying different parameters such as pectinase concentration, time of incubation, pH of pectinase solution, and incubation temperature. The optimum parameters were obtained as follows: 0.4 mg/ml pectinase, incubation for 18 h at 30–35 °C, pH of pectinase solution 3.5–4. Under the optimum conditions, the extraction yields of luteolin and apigenin achieved 0.268 and 0.132 mg/g in pectinase treated sample, which increased 248% and 239%, respectively, compared with the untreated ones.

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1. Introduction

Flavonoids are natural polyphenolic compounds widely present in medical and edible plants, the relatively strong dietary representation of flavonoids renders them particularly relevant to human health (Hollman & Katan, 1999a, 1999b). Epidemiological studies have indicated a consistent protective effect of fruits and vegetable intake on cardiovascular diseases (Formica & Regelson, 1995; Ness & Powles, 1997; Verbeek, Plomp, van Tol, & van Noort, 2004). Although there is still uncertainty about the relationship between specific dietary components and cardiovascular disease risk, the current literature suggests that the cardioprotective action of fruits and vegetable consumption is at least partly attributable to the antioxidant activity of flavonoids (Hertog et al., 1995; Knekt, Järvinen, Reunanen, & Maatela, 1996; Witztum, 1994). Similarly positive effects of flavonoids have been reported on coronary disease (Hertog, Feskens, Hollman, Katan, & Kromhout, 1993) and cancer (Knekt et al., 1997; Wang, 2000; Wenzel, Kuntz, Brendel, & Daniel, 2000).

Pigeonpea [*Cajanus cajan* (L.) Millsp.] is one of the major grain legume crops in the tropics and subtropics. Endowed with several unique characteristics, it takes an important place in the farming systems adopted by smallholder farmers in a large number of developing countries. Although pigeonpea ranks sixth in area and production in comparison with other grain legumes, it is used in more diverse ways than others (Fu et al., 2006, 2007). Besides its main usage as dhal, its tender, green seeds are used as a vegetable, its crushed dry seeds as animal feed, and the medical applications of its leaves have provoked much interest. Pigeonpea leaves have been used to treat trauma, burn infections, bedsores, arrest blood, for pain relief and to kill worms, and have been brought to market as a product of traditional Chinese medicine (TCM) for the therapy of ischemic necrosis of the femoral head (Fu et al., 2006, 2007; Zu, Fu, Liu, Hou, & Kong, 2006). Chemical constituent investigations indicated pigeonpea leaves are rich in polyphenolic compounds, especially flavonoids, and flavonoids have been considered as being responsible for the beneficial efficacies of pigeonpea leaves on human health (Duker-Eshun, Jaroszewski, Asomaning, Oppong-Boachie, & Christensen, 2004; Lin, Xie, & Cheng, 1999; Zu et al., 2006). Among the flavonoids in pigeonpea leaves, luteolin and apigenin (Fig. 1) are the main flavones with better pharmacological activities. They were found to possess anti-inflammatory, anti-allergic, anti-proliferative, antioxidant and free radical scavenging activities (Wang & Huang, 2004). Therefore, the extraction and separation of flavones in pigeonpea leaves represents a hot spot in pigeonpea research.

Plant cell walls consist of cellulose, hemi-cellulose and pectin, which is the barrier for the release of intracellular substances.

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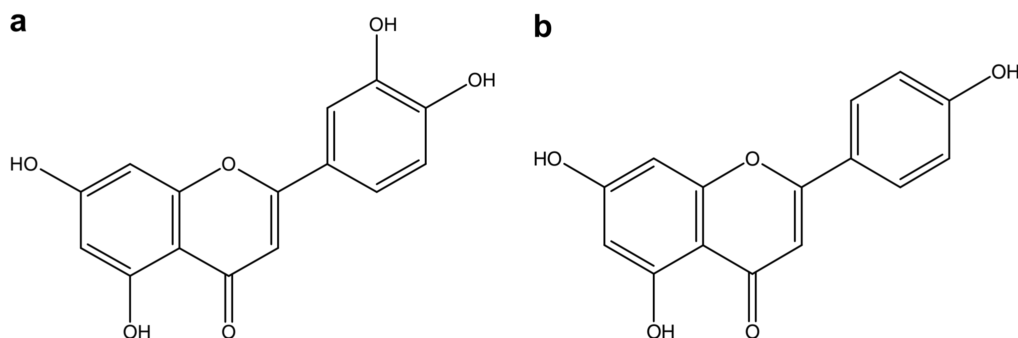


Fig. 1. Chemical structures of (a) luteolin and (b) apigenin.

Hydrolytic enzyme preparations can hydrolyze and degrade the plant cell wall constituents, and improve the release of intracellular contents. Cellulose, hemi-cellulose and pectin can be hydrolyzed using cellulase, beta-glucosidase, and pectinase enzymes (Wilkins, Widmer, Grohmann, & Cameron, 2007). Cellulase randomly breaks cellulose chains into glucose, beta-glucosidase cleaves beta-1,4 linkages in cellulose, and commercial pectinase preparations from *Aspergillus niger* have pectinesterase (PE), polygalacturonase (PG) and pectin lyase (PL) activity (Çinar, 2005; Grohman & Baldwin, 1992). In addition, there are many phenolic hydroxyl groups existing in flavonoids, that combine with cellulose, hemi-cellulose and pectin as complexes due to hydrogen bonding interactions. The hydrogen bonding interactions can be broken by cellulase, beta-glucosidase, and pectinase enzymes, then more free flavonoids were obtained. Moreover, flavonoids are always occurring in combination with glucoses as glucosides with glucosidic linkages, when the glucosidic linkages were broken, free flavonoids were released. Beta-glucosidase possesses the ability to break the beta-1,4 glucosidic linkages in glucosides. Nowadays, enzyme assisted extraction has been reported for the extraction of various kinds of compounds. Cellulase and pectinase have been employed for the extraction of lycopene from tomato tissues (Choudhari & Ananthanarayan, 2007), extraction of capsaicinoids and carotenoids from chilli (*Capsicum annuum* L.) (Barzana et al., 2002; Santamaría et al., 2000). Carotenoids were enzymically extracted from orange peel, sweet potato and carrot using different concentrations of cellulase and pectinase combinations (Çinar, 2005). Beta-glucosidase was used in the vapor phase extraction of rose oil (Yang, Yao, & Qian, 2006). In another report, enzyme assisted extraction of antioxidative phenols from black currant juice press residues with pectinase and protease was studied (Landbo & Meyer, 2001). To our knowledge, enzyme assisted extraction of luteolin and apigenin in plant materials has not been reported.

The objective of the present study was to investigate the performance of cellulase, beta-glucosidase and pectinase in enzyme assisted extraction of luteolin and apigenin from pigeonpea leaves. Various parameters such as enzyme type, pectinase concentration, time of incubation, pH of pectinase solution, and incubation temperature were optimized to improve the yields of luteolin and apigenin in the extraction process.

2. Materials and methods

2.1. Chemicals and reagents

Luteolin (3′,4′,5,7-tetrahydroxyflavone) and apigenin (4′,5,7-trihydroxyflavone) were bought from Sigma–Aldrich (Steinheim, Germany). Cellulase (Celluclast 1.5 l, ⩾1000 U/mg) and beta-glucosidase (Novozym 188, ⩾200 U/mg) were obtained from Novozymes A/S (Bagsvaerd, Denmark), pectinase from *A. niger* (1.41 U/mg) was purchased from Sigma–Aldrich (Steinheim, Germany). Acetonitrile of HPLC grade was purchased from Sigma–Aldrich (Steinheim, Germany). All other reagents were of analytical grade. Deionized water was purified by a Milli-Q water-purification system from Millipore (Bedford, MA, USA). Appropriate amount of reference compounds were dissolved in methanol to obtain the stock solutions at concentration of 0.5 mg/ml for luteolin and apigenin, respectively. All solutions prepared for HPLC were filtered through 0.45 μm nylon membranes prior to use.

2.2. Plant material

Fresh leaves of *Cajanus cajan* (L.) Millsp. were collected from the arboretum of the Key Laboratory of Forest Plant Ecology, Ministry of Education, Northeast Forestry University, Harbin, PR China, and authenticated by Prof. Nie Shaoquan from the same Key Laboratory. Voucher specimens were deposited in the herbarium of this Key Laboratory.

2.3. Luteolin and apigenin determination

The determination of luteolin and apigenin was carried out on a Waters liquid chromatographic system (Waters Company, USA) consisted of Millennium32 system software, Model Waters Delta 600 pump, and Model Waters 2996 Photodiode Array Detector (PAD). Chromatographic separation was carried out by HIQ sil C18V reversed-phase column (4.6 mm Ø × 250 mm, KYA TECH Corporation, Japan) packed with 5 μm diameter particles, the mobile phase was acetonitrile–water–acetic acid (30:69.3:0.7, v/v/v). The mobile phase was filtered through a 0.45 μm membrane filter (Millipore, USA), and then deaerated ultrasonically prior to use.

Luteolin and apigenin were quantified by a PAD at 347 nm following RP-HPLC separation. The flow rate was 1 ml/min, the injection volume was 10 μl, the column temperature was maintained at 30 °C, and the retention time for luteolin and apigenin were 14.1 and 24.5 min, respectively. Chromatographic peaks of luteolin and apigenin were confirmed by comparing their retention time and UV spectrum with those of the reference compounds. The working calibration curves based on reference compounds of luteolin and apigenin showed good linearity over the range of 0.5–100, and 0.45–90 μg/ml, respectively. The regression lines were $Y = 117890X - 18301$ ($R^2 = 0.9997$, $n = 8$), and $Y = 112680X - 15539$ ($R^2 = 0.9998$, $n = 8$), where Y is the peak area of analyte, and X is the concentration of reference compound (μg/ml).

2.4. Enzyme assisted extraction

Enzyme assisted extraction of luteolin and apigenin from pigeonpea leaves was carried out as detailed below. Calculated

amounts of cellulase, beta-glucosidase and pectinase were weighed, and distilled water was added to obtain stock solutions at the concentration of 0.5 mg/ml, respectively. The stock solutions were further diluted with distilled water and adjusted with acetate buffer to obtain serial solutions with desired concentration and pH. A batch of 20 g of fresh pigeonpea leaves was homogenized for 2 min to increase the surface area for efficient enzyme treatment, with a domestic blender in 150 ml of the solution obtained above, and then the well-homogenized mixture was transferred into an Erlenmeyer flask covered with aluminum foil. The mixture was incubated in an automatic incubator at 25–50 °C for 0–24 h, the temperature and pH were 25 °C and 7 except for the tests of temperature and pH effects. After the enzyme treatment, the mixture was vacuum filtered, filtrate and the residue were separated to achieve efficient and complete extraction.

The obtained residue was subjected to solvent extraction using aqueous-ethanol (20:80, v/v) solution in an ultrasonic bath (Kunshan Ultrasonic Instrument, China) for 15 min, repeated three times. The extracts were isolated by membrane filtration and combined with the filtrate, and then transferred to a rotary evaporator device (RE-52AA, Shanghai Huxi Instrument, China) and concentrated under vacuum until the residual volume was 100 ml. The residue was washed by petroleum ether and extracted by ethyl acetate successively, followed by filtering and drying. Mobile phase were added to yield a sample solution for HPLC determination. Control samples not treated with enzyme preparations were extracted, purified, processed as described above, and then analyzed by HPLC to calculate the contents of luteolin and apigenin in pigeonpea leaves.

3. Results and discussion

3.1. Selection of enzyme type

Plant cell wall consists of a rigid skeleton of cellulose embedded in a gel-like matrix composed of pectic compounds, hemi-cellulose and glycoprotein. Cellulase catalyzes the breakdown of cellulose into glucose, cellobiose and higher glucose polymers, beta-glucosidase breaks the beta-1,4 glucosidic linkages in cellulose and glucosides. Pectinase has the ability to disintegrate pectic compounds and pectin. The latter is a polymer of 100–200-galacturonic acids, found in the middle lamella and primary walls.

Enzyme assisted extraction of luteolin and apigenin were conducted with two enzyme concentrations and two incubation times for cellulase, beta-glucosidase, and pectinase, respectively. The contents of luteolin and apigenin in pigeonpea leaves were 0.077 and 0.039 mg/g, respectively. As shown in Fig. 2a and b, the extraction yields of luteolin and apigenin in treated samples increased notably with treatment by the three enzyme preparations. Cellulase and pectinase hydrolyzed cellulose and pectin, beta-glucosidase hydrolyzed cellulose and glucosides in pigeonpea leaves, released luteolin and apigenin from the cell walls, and increased the extraction efficiency. In addition, the amounts of luteolin and apigenin extracted with pectinase were much higher than those with cellulase and beta-glucosidase by using different concentrations and incubation times. The possible reason is that pectinase comprises the activities of pectinesterase (PE), polygalacturonase (PG) and pectin lyase (PL). The cooperative effects of these activities may result in an increased degradation of cell walls of pigeonpea leaves, resulting in higher extraction yields of luteolin and apigenin. Hence, pectinase was considered as more efficient for the extraction of luteolin and apigenin and selected further optimizing the extraction process in the following tests.

3.2. Effect of pectinase concentration

The effect of using different concentrations of pectinase on the extraction of luteolin and apigenin at an incubation time of 24 h was shown in Fig. 3. With an increase of pectinase concentration up to 0.4 mg/ml, the extraction yields of luteolin and apigenin increased accordingly. Concentration of 0.4 mg/ml gave almost the same extraction yields as those of 0.5 mg/ml, indicating that a concentration of 0.4 mg/ml provided sufficient amounts of pectinase for the hydrolysis of cell wall. Thus, 0.4 mg/ml was selected as suitable concentration for the consumption of pectinase.

3.3. Effect of incubation time

Next, the effect of incubation time on the extraction yields of luteolin and apigenin was examined. An increase in the extraction yields was observed with the extending of time (Fig. 4). During the initial 18 h, the extraction of luteolin and apigenin was considerably enhanced. After 18 h, the extraction yields almost kept at a steady level indicating that 18 h is long enough for pectinase to degrade the cell wall.

3.4. Effect of pH of pectinase solution

Enzyme activities are easily influenced by pH. Keeping the pectinase concentration at 0.4 mg/ml and incubation time at 18 h, we

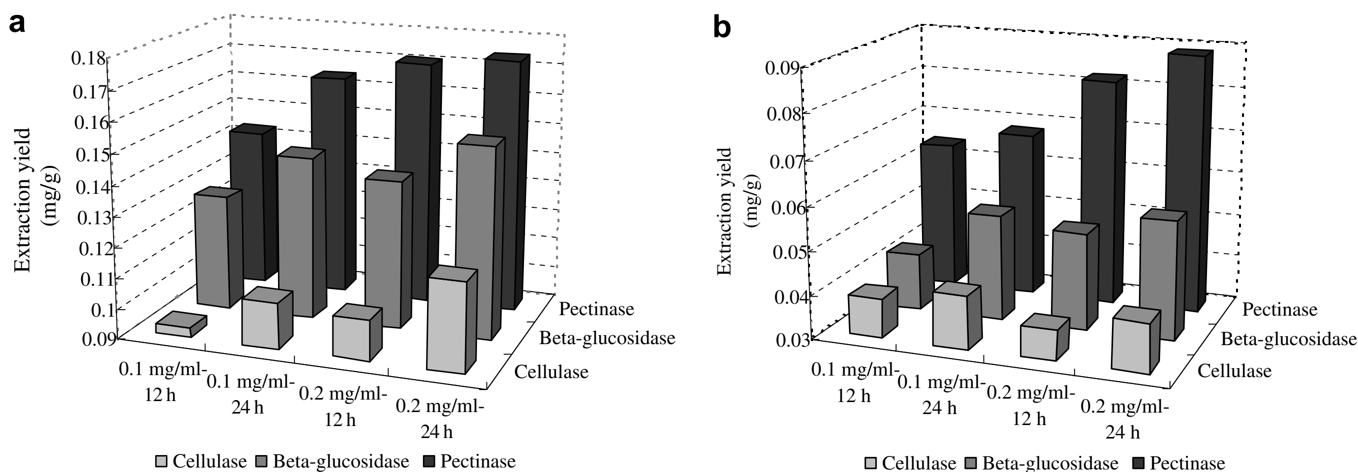


Fig. 2. Effect of different enzyme preparations on the extraction yields of (a) luteolin and (b) apigenin.

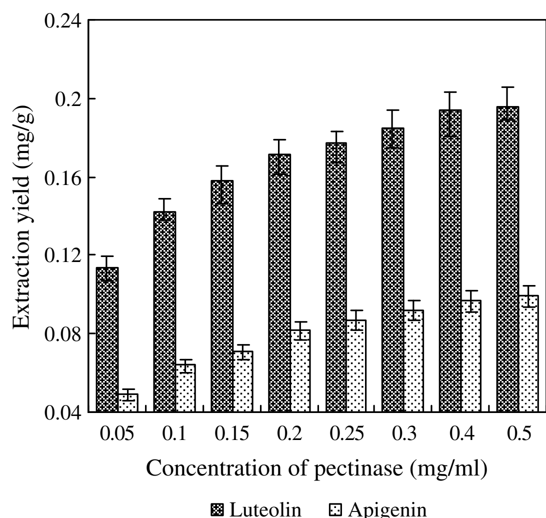


Fig. 3. Effect of pectinase concentrations on the extraction yields of luteolin and apigenin.

varied pH values of pectinase solution in a range from 3 to 7 to investigate its effect on extraction yields of luteolin and apigenin (Fig. 5a and b). The highest amount of luteolin (0.237 mg/g) was obtained at pH 4. For apigenin, the highest extraction yield (0.117 mg/g) was achieved at pH 3.5. Thus, pH 3.5–4 was chosen as optimum pH for simultaneously obtaining optimal extraction yields of luteolin and apigenin.

3.5. Effect of incubation temperature

Likewise, temperature also affects enzyme activity. Reaction rates of enzymes are accelerated with the increase of temperature up to an optimum, above which enzymes are denaturalized. The effect of incubation temperature on the extraction yields of luteolin and apigenin was studied with the other conditions set above. As it can be seen from Fig. 6, the highest extraction yields of luteolin and apigenin appeared at temperatures of 30 and 35 °C, respectively. Therefore, 30–35 °C was considered as the optimum incubation temperature.

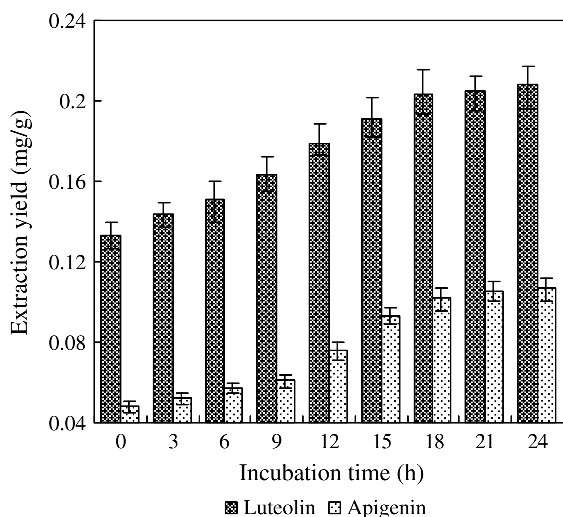


Fig. 4. Effect of incubation time on the extraction yields of luteolin and apigenin.

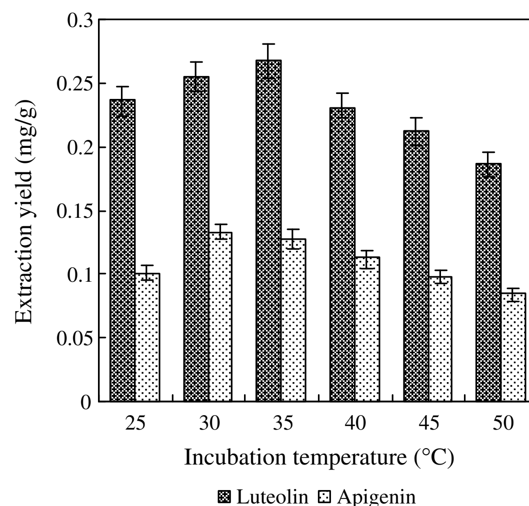
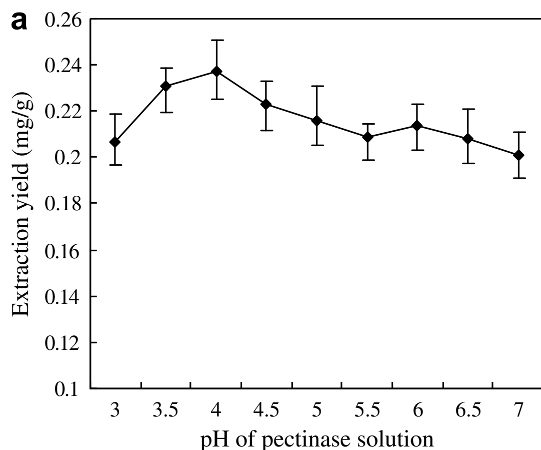


Fig. 6. Effect of incubation temperature on the extraction yields of luteolin and apigenin.



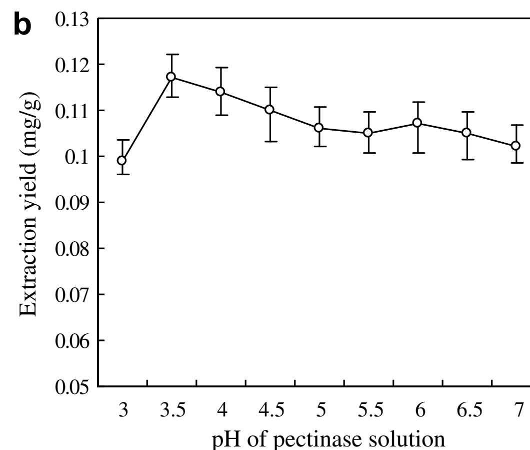


Fig. 5. Effect of pH of pectinase solution on the extraction yield of (a) luteolin and (b) apigenin.

4. Conclusions

In the present study, enzyme assisted extraction of luteolin and apigenin from pigeonpea leaves was investigated with cellulase, beta-glucosidase and pectinase. All three enzyme preparations proved to be effective in extracting luteolin and apigenin from pigeonpea leaves. At same enzyme concentrations and incubation times, pectinase was found to be more efficient than the other two enzyme preparations. Therefore, this enzyme was used for further investigations. Several parameters affecting pectinase assisted extraction of luteolin and apigenin were optimized, e.g., pectinase concentration, time of incubation, pH of pectinase solution, and incubation temperature. At optimized conditions, the extraction yields of luteolin and apigenin were 0.268 and 0.132 mg/g, represents an increase of 248% and 239%, respectively, compared to untreated controls. The results in this study can serve as a reference for the enzyme assisted extraction of other compounds from herbal plants.

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