Enhancing extraction of proanthocyanidins from Chinese quince fruit by ball-milling and enzyme hydrolysis

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Abstract

Chinese quince fruit is rich in proanthocyanidins (PAs), which have antioxidant properties. This work describes a method to isolate PAs from Chinese quince fruits by a combination of ball-milling pretreatment and enzyme-assisted extraction. Untreated PA fractions (UMP), ball mill pretreated PA fractions (BMP) and enzymatically hydrolysed PA fractions (BEP-1, BEP-2, BEP-3, BEP-4) were obtained. The total PA and total phenolic contents, antioxidant activity and α-amylase inhibitory activity of the six PA samples were determined, and their structural characteristics were also analyzed. BEP and BMP fractions were structurally similar with UMP, but had more total phenolics and PAs, and had higher antioxidant activities, lower thermal stability, and lower average molecular weight. Among them, BEP-4 was the most outstanding. Its total phenolics and PAs were 750.98 ± 6.95 and 948.72 ± 3.50 mg/g extract per gram dry weight, respectively. Its DPPH, ABTS, hydroxyl radical scavenging ability, and α-amylase inhibitory capacity had maximum values of 90.38%, 98.99%, 97.89%, and 92.98%, respectively. These investigations provide a new, efficient way to isolate proanthocyanidins from Chinese quince fruits, and reveal that Chinese quince fruits may be a good source of antioxidants, worthy of further development.

Introduction

The fruits of Chaenomeles sinensis (Thouin) Koehne, commonly known as Chinese quince, have traditionally been used as a source of medicine and food (Sawai-Kuroda et al., 2013). In terms of Chinese medicine, it has the effects of benefiting the stomach, promoting digestion, relaxing tendons, reducing cholesterol, dispelling wind and cold, regulating immunity and relieving pain (Fang et al., 2021). Recently, the yield of Chinese quince has increased rapidly; however, the overall utilization rate of Chinese quince in industry is not high (Hamauzu et al., 2010). Previous papers have shown that Chinese quince fruits are rich in bioactive compounds, such as phenolics (Jiao et al., 2020). Chinese quince polyphenols include procyanidin (PC) polymers, flavan-3-ols, epicatechins and various phenolic acids. These polyphenols could be useful in the food industry because they have powerful antioxidant, anti-influenza, anti-virus and anti-inflammatory effects (Qin et al., 2020). Thus, finding and developing ways to isolate and extract the polyphenols from Chinese quince would benefit industry and increase the value and utilization rate of Chinese quince fruits.

Proanthocyanidins (PAs), are a group of phenolic compounds derived from flavan-3-ols monomers (catechin or epicatechin) (Lv et al., 2021). PAs have been widely studied for their high biological activities. They play a positive role in human health, in particular protecting against cardiovascular diseases (Wojdylo et al., 2013; Szychowskia et al., 2018). Based on where they are found in the Chinese quince fruits and their chemical structure, PAs are categorized as either free or insoluble-bound. Free PAs have low molecular weight, and are composed of simple flavane-3-alcohol monomers. Because they are not bound to other cell major components, they can be readily extracted by organic solvents (Fernandes et al., 2020); Insoluble-bound PAs have high molecular weight, and are mainly composed of polymers. They have non-covalent bonds with cell wall components such as protein and pectin, and therefore typically need pretreatment before they can be extracted (Zeller, 2019). Compared with other fruits containing PAs, Chinese quince fruits have higher contents of bound PAs (up to 40% of total PAs) (Hamauzu & Mizuno, 2011). In addition, some free PAs undergo enzymatic reaction with cell wall components to convert into bound PAs during the separation process (Waterelot et al., 2014). However, while PA content is high in Chinese quince fruit, extraction is difficult. Due to the strong interactions between PAs and cell wall components, conventional separation methods cannot effectively extract bound PAs. Therefore, a significant amount of bound PAs remain in the extraction residue, which leads to low overall separation efficiency and low purity of the extracted PAs.

Pretreatment can promote the release of bound PAs during extraction (Domínguez-Rodríguez et al., 2017), improving the overall separation and extraction efficiency of PAs from Chinese quince. Acid and alkali hydrolysis are the most common pretreatment methods. However, temperature and pH must be controlled in order to prevent the rupture of covalent bonds in the structure of the PAs. Enzymatic hydrolysis could be another option for promoting the differential release of PAs, as it is more selective than other hydrolyses (Shashidi & Yeo, 2016). Enzyme-assisted extraction is based on the degradation of cell wall components, thereby liberating the bound PAs (Cascaes-Teles et al., 2021; Osete-Alcaraz et al., 2019). In addition, ball-milling technology has been reported to be an efficient and environmentally friendly method of ultrafine grinding, which can help to break the bonds between cell wall components and the PAs, thereby facilitating the action of solvents. Using a lower temperature in the ball-milling treatment process can help avoid oxidation or transformation of PAs and ensure the stability of PA structures (Liu et al., 2017). Enzymatic extraction of PAs has been studied (Bautista-Ortíñ et al., 2013); however, the combination of ball-milling treatment and enzyme hydrolysis for the extraction of PAs has not been reported.

In this present study, an enhanced method for isolating PAs from Chinese quince fruits was developed; the method is a combination of ball-milling and enzyme-assisted extraction. The yield of the method was calculated, and the structure, α-amylase inhibitory activity and antioxidant activity of PAs isolated were analyzed. This study provides a new extraction method for obtaining PAs from Chinese quince fruits, thereby laying the foundation for the potential applications of Chinese quince PAs as natural antioxidants in foods.
Materials And Methods

Plant material and chemicals

Fresh Chinese quince fruits were obtained from a local plantation base (Zhengzhou, China). Chemicals and reagents were of analytical grade and purchased from Zhiyuan (Tianjin, China). Celluclast 1.5 L (700 EGU/g, endoglucanase units per gram), Pectinex XXL (10000 PECTU/mL, pectin transeliminase units per gram), and xylanase Shearzyme 500L (500 FXU-S/g, endo-1,4-β-D-xylan hydrolases units per gram) were all obtained from Novozymes (Beijing, China).

Sample preparation

Chinese quinces were washed, seeded, sliced, then freeze-dried for 48 h, ground and sieved by an 80-mesh sieve. The powder was defatted for 8 h at 50°C with petroleum ether. The defatted dried Chinese quince powder was sealed and stored in a desiccator for later use. This powder, which had not been subjected to ball-milling, was defined as UMS.

Isolation and extraction of PAs

Conventional organic solvent extraction

Firstly, 10 g of UMS was soaked in a 400 mL solvent mixture of acetone: water: acetic acid (70: 29.5: 0.5, v/v/v). The mixture was magnetically stirred for 3 h, and centrifuged for 10 min at 4800 g to remove insoluble solids. The supernatant was recovered and concentrated under vacuum at 30°C until the organic solvent was completely removed (Li et al., 2015). The remaining fraction was freeze-dried and purified by Macroporous resin column AB-8 and Sephadex LH-20, using a method described in a published paper (Qi et al., 2016); the resulting powder was named UMP.

Organic solvent extraction after ball-milling

The ball-milling process was run for 48 cycles by a Fritsh planetary ball mill (CTPT Co., Ltd., China). A single cycle comprised of milling for 10 min and pausing for 20 min. Briefly, two grinding tanks (volume of 500 ml) were each filled with 100 g UMS, and grinding balls (material: zirconia; size: 60 mm) were added in a 6:1 ratio of agate ball to sample (Yang et al., 2019). The resulting powder was named BMS. BMS was extracted with organic solvent and purified as described in Conventional organic solvent extraction Section. The PA fraction obtained was named BMP.

Organic solvent extraction after ball-milling and enzyme-assisted pretreatments

Based on our previous study (Qin et al., 2018) with minor modifications, briefly, sample buffer was obtained by adding 20 g of BMS to 250 ml of 0.2 M HAc-NaAc buffer (pH 4.7). Four sample buffers were prepared in this way. Then 2 mL cellulase (700 EGU/g) was added to the first one, 2 mL xylanase (500 U/g) was added to the second, 2 mL pectinase (10000 PECTU/mL) was added to the third, and multi-enzyme (2 mL cellulase, 2 mL xylanase, and 2 mL pectinase) was added to the fourth. The mixtures were reacted at 37°C for 24 h, then centrifuged for 5 min at 4800 g. The insoluble residues after cellulase, xylanase, pectinase, and multi-enzyme hydrolysis pretreatments were labeled as BES-1, BES-2, BES-3, and BES-4, respectively. Then PAs were isolated from these four, using the conventional organic solvent extraction and purification method described in in Conventional organic solvent extraction Section. The four PA fractions obtained from BES-1, BES-2, BES-3, and BES-4 were named BEP-1, BEP-2, BEP-3, and BEP-4, respectively.

Evaluation of bioactive compounds

Total phenolic content (TPC)

The TPCs of the six purified samples were determined according to the previous study (Zhang et al., 2016). The TPC of each sample was recorded as milligrams of epicatechin (EC) per gram of extracts.

Total PA content (TPA)

The TPAs of the six purified samples were measured according to a previous report (Lv et al., 2015). The TPA of each sample was recorded as mg proanthocyanidin B1 equivalents (PB1)/g extracts.

X-ray diffraction (XRD) analysis

UMS, BMS, BES-1, BES-2, BES-3, and BES-4 were evaluated by XRD analysis according to a published study (Udeh et al., 2017). The crystallinity index (CrI) was determined using the XRD spectra. Samples were scanned at a speed of 2° min⁻¹ from 10° to 60°. The step size
was set at 0.013°.

**Scanning electron microscope (SEM) examination**

UMS, BMS, BES-1, BES-2, BES-3, and BES-4 were examined by SEM. Samples were placed on stubs, and sputter coated. Samples were observed at 3.0 kV. The images were obtained at 1000× magnifications.

**Fourier transform infrared spectroscopy (FT-IR) analysis**

FT-IR spectra of the six purified samples were obtained on a Frontier FT-IR 115089 spectrometer to further verify their structures, using a KBr disk containing 1% finely ground samples. Thirty-two scans from 4000 to 400 cm⁻¹ in transmission mode were performed (Ji et al., 2020).

**Gel permeation chromatography (GPC) analysis**

GPC was performed using a Waters 1525 instrument with an RI Detector Waters 2414, with a flow rate of 1 mL/min at 25 °C for 15min. A standard calibration was created using standard polystyrene in a molecular weight range of 200-100000 Da. Before the determination, the six purified PA samples were subjected to acetylation according to a previous report (Roy et al., 2021).

**Thermal analysis**

The TGA spectra were acquired on a Q5000 TGA analyzer (TA Instruments, USA) in a nitrogen atmosphere (60 mL·min⁻¹), and TGA analysis was done according to the published report (Zhang et al., 2017). 5 mg of each of the six purified PA samples was transferred to a platinum pan, and the data was recorded from 25 to 700 °C at a heating rate of 10°C·min⁻¹.

**MALDI-TOF-MS analysis**

The MALDI-TOF-MS spectra of the purified PA samples were acquired with a MALDI-TOF-MS instrument (Bruker Reflex III, Germany). Samples were irradiated at 337 nm with a pulsed nitrogen laser, and the duration of the laser pulse was 3 ns. The analytical parameters were done according to a previous report (Esquivel-Alvaradoa et al., 2021).

**LC-MS/MS analysis**

The monomers in the oligomers and homopolymers in the purified PA extracts were detected using an LC-MS/MS method as previously reported (Karl et al., 2016).

**Nuclear magnetic resonance (NMR) analysis**

The heteronuclear single quantum coherence (HSQC) spectra of the purified PA samples were recorded on a Bruker Avance III HD 500 MHz NMR spectrometer (Bruker Instruments, Inc., Switzerland). 50 mg of sample was dissolved in 0.5 mL mL of CD₃OD and put in a 5 mm NMR tube. The 2D-HSQC NMR experiment was carried out according to the previous report (Huang et al., 2015).

**Assays of antioxidant and α-amylase inhibitory activities**

**DPPH assay**

DPPH radical scavenging activity was measured according to the previous report (Plaza et al., 2013). Methanol and vitamin c (Vc) were used as blank and positive control, respectively.

**ABTS assay**

The ABTS scavenging potency was assayed according to the method described by Li et al (Li et al., 2016). Methanol and Vc were used as the blank and positive control, respectively.

**FRAP assay**

The FRAP assay was carried out according to the procedure described by Li et al (Li et al., 2016). The results were expressed as absorbance; the higher the absorbance, the stronger the reduction ability of the sample.

**Capacity to inhibit the formation of hydroxyl radical assay**

The assay was performed according to the published paper (Hernández-Corroto et al., 2018). Vc was used to replace the sample as the positive control.

**α-Amylase inhibitory activity**
The α-amylase inhibitory activity was measured according to the method proposed by Fu et al. (Fu et al., 2015), and the absorbance was recorded at 540 nm.

**Statistical analysis**

All assays were performed in triplicate. The results were expressed as mean ± SD (standard deviation). Values were expressed as mean ± standard deviation of at least three independent experiments. The analysis of variance (ANOVA) was carried out using a significant level of 0.05. Other statistical analyses were performed using Microsoft Excel 2019. The standard curves were plotted using Origin Pro 2021 software (Version 9.8, OriginLab Corporation, Northampton, MA, USA).

**Results And Discussion**

**Total phenolic and total PA content**

The data of TPC and TPA are shown in Fig. 1 (a) and Fig. 1 (b), respectively. The total phenolic contents of UMP, BMP, BEP-1, BEP-2, BEP-3, and BEP-4 were 467.15 ± 6.26 mg/g, 516.05 ± 4.55 mg/g, 711.31 ± 6.56 mg/g, 581.13 ± 7.26 mg/g, 718.88 ± 6.05 mg/g, and 750.98 ± 6.95 mg/g of per gram extracts dry weight (DW), respectively. The total PA contents of UMP, BMP, BEP-1, BEP-2, BEP-3, and BEP-4 were 808.62 ± 3.45 mg/g, 837.07 ± 4.11 mg/g, 859.65 ± 3.72 mg/g, 849.36 ± 4.75 mg/g, 909.54 ± 3.96 mg/g, and 948.72 ± 3.50 mg/g of per gram extracts (DW), respectively. Thus, pretreatments by ball-milling and enzymatic hydrolysis increased the total phenolic and PA contents significantly (P < 0.05). In comparison with other PA samples, BEP-4 had the highest TPC and TPA. The results showed that the combination of multi-enzyme assisted extraction and ball milling extracted more phenolic compounds and PAs than the other five methods.

**XRD analysis**

According to XRD patterns (Fig. 2), the CrI of UMS was 15.8%, which was lower than those of the pretreated samples. The increase in crystallinity after ball-milling was due to the removal of amorphous materials such as pectin and hemicellulose. Results showed that comparing BES-1, BES-2, BES-3, and BES-4 to BMS, the CrI was decreased from 22.5–19.6%, 16.6%, 19.7%, and 15.1%, respectively. The decrease in crystallinity after enzymatic hydrolysis pretreatment was due to the disruption of cellulose by various enzymes.

In the XRD profile of UMS, the peak at approximately 2θ = 22.5° (200) indicated the cellulose had a highly ordered crystalline region. Conversely, the diffraction peak at approximately 2θ = 18.0° (110) indicated a less organized amorphous region. After pretreatment, the peak of the pretreated samples had shifted a little, indicating changes in the structure. The intensity of the two peaks corresponding to the (110) and (200) lattice planes of the crystalline cellulose polymorph was increased for the samples that had been ball-milled or treated with enzymes compared to UMS.

These findings show that combination of enzymatic hydrolysis and ball-milling pretreatments enhance the degradation of the crystalline cellulose and other chemical components in the cell wall of the Chinese quince.

**SEM analysis**

The SEM graphics are shown in Fig. 3. The results indicate that UMS had many spherical and uneven lumps, suggesting that the structures of the crystalline cellulose and other chemical components in the cell wall of the Chinese quince were not destroyed. In comparison with UMS, BMS had many smaller lumps and thick lamellas. This difference indicated that the cell wall components of the samples pretreated by ball milling had been slightly damaged. BES-1 had the thinnest sheets with irregularly folded edges and the smallest lumps in all areas, indicating that the cell wall components of the sample after enzymatic hydrolysis of cellulose were damaged the most seriously, and that the cellulolytic effect was the strongest. In comparison with UMS and BMS, BES-2, BES-3, and BES-4 had more and larger pores on the surface, as well as smaller spherical and uneven lumps, and smaller lamellar shapes. BES-4 had the most porous surface and largest pores. Results showed that the cell walls of the samples were disrupted in varying degrees according to the different treatments; the effects of multi-enzyme and cellulose enzymatic hydrolysis were particularly remarkable.

**FT-IR analysis**
The FT-IR spectra of the six PA fractions are shown in Fig. 4. All six samples exhibited an intense peak at around 3410 cm−1, which probably corresponds to -OH stretching in the phenolic structure of the PAs. The weaker absorption peaks at approximately 2915 cm−1 were due to the C-H stretching vibrations assigned to the -CH and -CH2 groups of the aliphatic hydrocarbons. Distortion of aromatic and non-aromatic C-H bonds occurred around 3000 cm−1. The absorption peak at 1654 cm−1 was due to the stretching vibration of the carboxylic acid C = O, suggesting the presence of a galloyl group on the epicatechin gallate. The most striking feature of the infrared spectrums was the dense and relatively broad band around 1600 cm−1. The bands between 1606 cm−1 and 1441 cm−1 were attributed to aromatic ring stretching, indicating that the core structure of the PAs was not changed significantly by ball-milling and enzymatic hydrolysis. The single peak at 1521 cm−1 indicated that the six samples predominantly consisted of PAs, and the high intensity of the C-C stretching peak at 1606 cm−1 indicated the presence of many C4-C8 interflavonoid linkages. The peaks near 1441 cm−1 were due to the C-H stretching vibration of benzene rings. The bands at approximately 1380 cm−1 were due to the deformation and vibration of -C=O, and the bands observed from 1281 to 1058 cm−1 were the characteristic bands of C-O-C, including aromatic C-O and aliphatic C-O. The bands at 830 cm−1 were associated with C-H of benzene rings, suggesting the presence of three hydrogens on the benzene ring. The peaks at 764 cm−1 were attributed to bending vibration of -CH out-of-plane conformations of procyanidin (PC), indicating its significant presence. Bands at about 674 cm−1 were associated with C-H of benzene rings and O-H of alcohol. Collectively, this information indicates that the PAs contained a large number of benzene rings and hydroxyl groups. Specifically, it seems that Chinese quince fruit PAs contain polyhydroxy-substituted benzene rings. This conclusion is consistent with the results of FT-IR spectra for known PAs (Zhang et al., 2017).

Insert Fig. 4 here

**GPC analysis**

The molecular characteristics and GPC chromatograms of the six PA extracts isolated by different pretreatments are listed in Fig. 5. Each of the six samples exhibited bimodal peaks, with the main peak at 8.5–9.5 min and a shoulder peak at 10.5–11.5 min, and a low polydispersity index (PDI), which demonstrated that they were homogeneous (Jiao et al., 2020). The average Mws of the six fractions were in the order: BEP-3 > BMP > BEP-2 > UMP > BEP-1 > BEP-4. In contrast, the Mw (weight-average molecular weight), Mn (number-average molecular weight), and PDI of BEP-1 and BEP-4 were significantly lower than the other four samples. This data suggests that BEP-1 and BEP-4 mainly comprise low Mw PAs. In other words, the samples pretreated with cellulase and multiple enzymes could better release a large amount of oligomeric PAs. Meanwhile, BMP showed a higher Mw than UMP probably because more polymeric PAs were released after ball-milling. BEP-3 has the highest Mw among the six samples. This is presumably because the pectinase pretreatment enabled solvents to infiltrate the cell wall more easily, resulting in the release of large amounts of high molar mass PA components (Yang et al., 2019). A previous report (Dorenkott et al., 2014) has shown that PAs with lower DP and Mw have stronger antioxidant capacity and biological activities. Therefore, the results obtained from GPC analysis were consistent with those of the antioxidant capacity and biological activity analysis, to be described in Antioxidant capacity and biological activity analysis Section.

Insert Fig. 5 here

**Thermogravimetric analysis (TGA)**

The thermogravimetric curves are presented in Fig. 6. The weight loss of the six samples occurred in three stages (Fig. 6A). In the first stage, ranging from 25°C to 150°C, there was an initial weight loss, presumably due to loss of adsorbed water and evaporation of small molecular substances such as low molecular weight phenolic acids (Zhang et al., 2017). The second stage ranged from 150°C to 500°C. The rapid mass loss at this stage was associated with the breakdown of PAs (Phitsuwan et al., 2016). The third stage of weight loss was in the range of 500°C-700°C. During this stage there was a slow mass loss due to the breakdown of residual carbohydrates; it was basically a carbonization process. The stage at which a PA decomposes depends on its structure, the substitution pattern of substituents, the degree of polymerization (DP), and the type of chemical bonds (Wen et al., 2019). As shown, UMP and BMP had higher final solid residue yields compared to the four BEP fractions. At 50% weight loss, thermal decomposition occurred at temperatures ranging from 670 °C to 680 °C for the BEP-1, BEP-3, and BEP-4 fractions, while decomposition occurred at temperatures above 700 °C for the UMP and BMP fractions. The higher the temperature at which PAs reaches 50% weight loss, the better its thermal stability. Therefore, BEP-1 had the lowest temperature at 50% weight loss, indicating BEP-1 was the most unstable of the six samples. UMP and BMP had higher thermal stabilities than the four BEP fractions.

The DTG curves are shown in Fig. 6 (B). Three notable inflection points can be seen. The inflection point occurring below 100°C was related to the loss of water and substances such as low molecular weight phenolic acids. The second inflection point, which generally occurred at temperatures ranging from 150 °C to 200 °C, was caused by the degradation of oligomeric PAs. The third inflection point occurred at temperatures ranging from 250 °C to 300 °C, and may be associated with the degradation of polymeric PAs. As can be seen from Fig. 6B, during the second stage, the order of the maximum degradation temperatures was as follows: BEP-2 > BEP-3 > BEP-1 > BEP-4 > UMP > BMP,
indicating that more low molecular weight PAs were isolated after pretreatment with multi-enzyme hydrolysis. In the third period, the order of the maximum degradation temperatures was as follows: BMP > UMP > BEP-3 > BEP-2 > BEP-4 > BEP-1, suggesting that BMP and UMP contained more polymeric and higher molecular weight PAs than the BEP fractions. Generally, the higher the molecular weight of samples, the stronger the thermal stability. These results are consistent with those of the GPC analysis.

MALDI-TOF-MS analysis

The MALDI-TOF-MS spectra of the six PAs samples are shown in Fig. 7. All samples exhibited mass spectra with a primary set of peaks with differences of 288 Da (Li et al., 2016). The masses were calculated based on the following equation (Chai et al., 2014) : 290 + 288a + 152b + 16c − 2d + 133, where 290 is the molecular weight of the terminal epicatechin or catechin unit, 288 is the molecular weight of a catechin unit, 152 is the relative molecular weight difference between a catechin gallate and a catechin monomer, 16 is the mass of an oxygen atom, and 133 is the molecular weight of cesium; a is the number of extended catechin/epicatechin units, b is the number of gallate groups, c is the number of additional hydroxyl groups in the preproenzyme peptide (PD), and d is the number of A-type interflavan bonds. Substituting the mass-charge ratio shown in Fig. 7 into the formula according to the above method shows that the peaks at m/z 863, 999, 1151, 1287, 1575, 1863, 2151, 2439, 2727, 3015, 3303, 3591, 3879, and 4167 can be considered to be dimeric, trimeric, tetramer, pentamer, hexamer, heptamer, octamer, nonamer, decamer, undedimeric, dodecamer, tridimeric, and tetramer PCs with B-type linkages, respectively. In addition, the signal of the molecular ion peak, which was 16 Da away from the main molecular ion peak, could be detected, the signal was produced by B-type prodelphinidin of PD structures. These results show that the PAs in the six samples were mainly B-type PC and PD. In addition, B-type PCs were the main components of all six samples. A few molecular ion peak signals differing from the main molecular ion peak by 2 Da were also detected, indicating that A-type linkages also existed in the six samples. MALDI-TOF-MS analysis showed that the degree of polymerization (DP) of the six samples ranged from DP 3 to DP 15. The results demonstrate that the six samples were mainly dimers to octamers consisting of the same flavonoid-3-ol subunits. This conclusion is consistent with the results of a previous study (Wang et al., 2021).

2D-HSQC NMR analysis

The 2D-HSQC NMR spectra are displayed in Fig. 8. The attribution of signals is based on previous publications (Saive et al., 2020; Reeves et al., 2020; Crestini et al., 2016). We selected the most representative BEP-4 from the four samples treated by enzymatic hydrolysis and performed NMR analysis on them, together with UMP and BMP. In the NMR spectra, UMP, BMP and BEP-4 showed strong and similar signals, with only slight differences, indicating that the different pretreatments had not significantly changed the "core" of the PC structure. More specifically, the signals at δC/δH 112–120/6.4–7.4 ppm were present in the spectra of UMP, BMP and BEP-4 HSQC, which correspond to the C2'-H2', C5'-H5', C6'-H6' correlations peak for B-ring substitution pattern of PC structures. The signals at δC/δH 92–96/5.8–6.2 ppm were appeared in both UMP and BEP-4 HSQC spectra, but not in the HSQC spectrum of BMP, which correspond to the C6-H6, C8-H8 correlations peaks for phloroglucinol units of PD structures. The C2-H2 (trans), C2-H2 (cis) correlation peaks for the A-ring substitution pattern occurring at δC/δH 74–78/4.8–5.6 ppm was present in all UMP, BMP and BEP-4 HSQC spectra. In addition, the presence of the C4-H4 of A-ring was confirmed by a corresponding small but distinguishable cross-peak at δC/δH 26–29/2.7-3.0 ppm. At δC/δH 36–38/4.2–4.8, B-rings for (epi) catechin and (epi) catechin flavan-3-ol subunits are represented by C4-H4 (trans) and C4-H4 (cis) correlations. Furthermore, the signals at δC/δH 68–72/3.4–4.2 ppm presumably represent non-aromatic hydroxylated C-H.

Thus, the HSQC analysis nicely confirmed the presence of PC and PD structures in the PA samples, and provided information about the composition, structural subtypes, and the linkage mode of flavane-3-ol monomers. To further clarify the structural differences among these three samples (UMP, BMP, and BEP-4), quantitative NMR analysis was performed, and the results are presented in Table 1. As shown, UMP contained a higher proportion of PD subunits, PC content, cis-conformation, and flavan-3-ol subunits. By comparison, BEP-4 contained more A-ring substitution patterns. In addition, the absence of PD structures in BMP was observed. Results of HSQC analysis suggest that the PAs in the three samples were mainly B-type PC, which is consistent with results of a previous study (Lv et al., 2021).
Table 1
Structural features of A- and B-rings, PC and PD structure, and flavan-3-ol content in UMP, BMP, and BEP-4 as evaluated by HSQC spectroscopy

| sample | % A-type | % B-type | % PC | % PD | % cis | % flavan-3-ol subunits |
|--------|----------|----------|------|------|-------|------------------------|
| UMP    | 24.38    | 75.63    | 60.98| 4.67 | 31.58 | 14.64                  |
| BMP    | 31.69    | 68.31    | 56.93| 0    | 24.47 | 11.38                  |
| BEP-4  | 43.13    | 56.87    | 46.35| 0.78 | 21.87 | 10.52                  |

LC-MS/MS analysis

The constituents of the six PA extracts were analyzed by LC-MS/MS, and the results are shown in Table 2. A total of nine PA oligomers were detected. There were five flavan-3-ol basic units, namely, catechin, (+)-epicatechin, (-)-epicatechin, epicatechin gallate, and epigallocatechin-3-gallate, three PA dimers (PB1, PB2, PC1), and one PA tetramer. The highest content of PA dimer was found among these oligomers. This result was consistent with our previous study (Wang et al., 2021).

Table 2
The content of compounds (ug/mg extracts) in various proanthocyanidins extracts and percentage content (%) of each component based on total oligomeric procyanidins

|                  | UMP    | BMP    | BEP-1 | BEP-2 | BEP-3 | BEP-4 |
|------------------|--------|--------|-------|-------|-------|-------|
| Epicatechin      | 1.32   | 4.67   | 32.64 | 0.30  | 9.20  | 0.35  |
| catechin         | 1.32   | 4.67   | 32.64 | 0.30  | 9.20  | 0.35  |
| epicatechin      | 0.27   | 0.11   | 0.78  | 0.24  | 7.51  | 0.19  |
| epicatechin      | 0.09   | 0.27   | 0.22  | 0.11  | 7.51  | 0.19  |
| Epigallocatechin-3-gallate | 0.11  | 0.11  | 0.79  | 0.17  | 5.30  | 0.09  |
| Procyanidin B1   | 0.67   | 0.52   | 3.63  | 0.11  | 3.43  | 0.44  |
| Procyanidin B2   | 0.67   | 0.52   | 3.63  | 0.11  | 3.43  | 0.44  |
| Procyanidin      | 3.50   | 1.67   | 11.64 | 0.15  | 4.66  | 2.83  |
| Procyanidin      | 2.83   | 1.67   | 11.64 | 0.15  | 4.66  | 2.83  |
| Procyanidin      | 0.46   | 3.91   | 0.37  | 2.60  | 4.08  | 0.41  |
| Procyanidin      | 0.46   | 3.91   | 0.37  | 2.60  | 4.08  | 0.41  |
| Procyanidin      | 3.50   | 1.67   | 11.64 | 1.73  | 53.18 | 2.83  |
| Procyanidin      | 3.50   | 1.67   | 11.64 | 1.73  | 53.18 | 2.83  |
| Procyanidin      | 11.83  | -      | 14.32 | -     | 7.94  | 8.07  |

As shown in Table 2, UMP, BMP, and BEP-1 contained relatively high proportions of catechins and epicatechins—namely, 11.13%, 32.64%, and 9.20%, respectively. Epigallocatechin gallate (7.51%) in BEP-1 were higher than in the other five fractions. Epigallocatechin-3-gallate (7.44%) in BEP-3 were higher than in the other five fractions. PA trimers in BEP-2 and BEP-3 were also higher, up to 35.65% and 30.12%, respectively. PC1 in BEP-4 were higher than in the other fractions, up to 65.71% of oligomers.

Antioxidant capacity and biological activity analysis

DPPH radical scavenging activity
As shown in the Fig. 9 (a), the six PA samples showed a wide range in antioxidant activity, from 1.77–90.38%. The scavenging abilities increased with increasing concentration and reached maximum values of 84.84% (UMP), 84.44% (BMP), 86.29% (BEP-1), 84.20% (BEP-2), 86.93% (BEP-3), 90.38% (BEP-4) and 82.00% (Vc). In comparison with other PA samples, BEP-4 exhibited higher antioxidant capacity, and its DPPH radical scavenging ability was significantly higher than Vc. The radical scavenging activity could also be indicated as the antioxidant concentration required for a 50% radical reduction (IC$_{50}$) (Li et al., 2016). The IC$_{50}$ values of these samples are shown in Table 3. BEP-4, with the highest PA content, showed the lowest IC$_{50}$ values and the highest antioxidant capacity. All six samples showed higher radical scavenging capacities than Vc (P < 0.05). The DPPH radical scavenging abilities of BEP-1, BEP-2, BEP-3, and BMP, were between the scavenging ability of UMP (lowest) and BEP-4 (highest). The results show that the chemical antioxidant capacity increased with the increase of TPC value and PA content and indicated a moderately strong relationship between antioxidant capacity and the PA content (Dorta et al., 2013).

| sample | DPPH IC$_{50}$ (µg/ml) | ABTS IC$_{50}$ (µg/ml) | OH IC$_{50}$ (µg/ml) | α-amylase IC$_{50}$ (µg/ml) |
|--------|------------------------|-------------------------|----------------------|----------------------------|
| UMP    | 253.87 ± 5.06$^b$      | 122.67 ± 1.47$^b$       | 215.47 ± 0.51$^b$    | 276.58 ± 0.52$^a$          |
| BMP    | 250.57 ± 4.88$^b$      | 94.55 ± 0.54$^c$        | 203.75 ± 1.57$^c$    | 248.20 ± 0.76$^h$          |
| BEP-1  | 244.80 ± 1.82$^c$      | 42.15 ± 0.68$^e$        | 170.56 ± 0.66$^c$    | 215.23 ± 0.67$^c$          |
| BEP-2  | 232.57 ± 1.44$^d$      | 41.91 ± 0.45$^e$        | 156.25 ± 0.77$^e$    | 215.97 ± 0.88$^c$          |
| BEP-3  | 216.71 ± 0.89$^e$      | 68.64 ± 0.56$^d$        | 151.18 ± 0.39$^f$    | 205.36 ± 0.31$^d$          |
| BEP-4  | 131.45 ± 0.97$^f$      | 22.66 ± 0.32$^f$        | 134.05 ± 0.71$^g$    | 182.07 ± 0.28$^e$          |
| Vc     | 276.23 ± 1.31$^a$      | 185.45 ± 0.26$^a$       | 217.54 ± 0.90$^a$    | –                          |

Values are expressed as mean ± standard deviation (n = 3). Different superscript letters in every column represent significantly different mean values (p < 0.05)

Insert Fig. 9 (a) here

ABTS scavenging potency

As shown in Fig. 9 (b), the ABTS scavenging activities of the six samples increased with increasing concentration and reached maximum values of 90.52% (UMP), 93.71% (BMP), 97.40% (BEP-1), 96.76% (BEP-2), 94.78% (BEP-3), 98.99% (BEP-4) and 88.79% (Vc). The ABTS scavenging activity of BEP-4 was the highest (P < 0.05). The IC$_{50}$ values of these samples are shown in Table 3; all are lower than the IC$_{50}$ values of DPPH (Lv et al., 2016). Obviously, the ABTS radical scavenging activities of BEP-1, BEP-2, BEP-3, BMP are higher than that of UMP, but lower than BEP-4 (P < 0.05). We interpret this as demonstrating that the PA and phenol content determine antioxidant ability (Jiang et al., 2016). These results indicate that Chinese quince fruit PAs have relatively strong antioxidant properties.

Insert Fig. 9 (b) here

Ferric ion reducing antioxidant power

The reducing powers of the six PA samples and reference sample Vc are shown in Fig. 9 (c). The absorbance values increased with concentrations, reaching maximum values of 2.47 (UMP), 2.53 (BMP), 2.63 (BEP-1), 2.56 (BEP-2), 2.57 (BEP-3), 3.27 (BEP-4) and 2.14 (Vc). Our data revealed that all samples have a strong reducing power (Erdogan-Orhan et al., 2019). As can be seen in Fig. 9 (c), the effective reducing capacities of the six samples were significantly better than Vc. BEP-4, which had the highest absorbance values in the ferric ion reducing ability test, also exhibited significantly higher reducing power than the five other samples and Vc. Overall, the reducing ability of PA samples pretreated by ball milling and enzymatic hydrolysis were higher than that of the untreated sample. The results were similar to ABTS and DPPH assays. Therefore, the higher the content and purity of PAs isolated from Chinese quince fruits, the stronger the antioxidant and reducing ability.

Insert Fig. 9 (c) here
Hydroxyl radical formation inhibition capacity

The ability of PAs to scavenge hydroxyl radicals is presented in Fig. 9 (d). Extracts UMP, BMP, BEP-1, BEP-2, BEP-3, BEP-4 and Vc showed scavenging capacity up to 83.52%, 85.03%, 90.36%, 93.58%, 94.96%, 97.89% and 82.97%, respectively. The IC$_{50}$ values of the six samples are shown in Table 3. Among them, the scavenging activity of BEP-4 was the highest, nearly 100%. The IC$_{50}$ values of BEP-1, BEP-2, BEP-3, and BEP-4 were significantly lower than UMP and BMP, and all samples exhibited IC$_{50}$ values lower than Vc (P < 0.05). As stronger scavenging activity is reflected by lower IC$_{50}$ values, the results showed that PAs isolated from Chinese quince fruits have powerful antioxidant activity, and they are more effective at scavenging free radicals than ascorbic acid. The higher the content of phenolic compounds and PAs in these samples, the stronger the antioxidant activity (Aladdedunye et al., 2014), which is consistent with the results of the DPPH, ABTS, and FRAP assays.

α-amylase inhibitory capacity

As shown in Fig. 9 (e), UMP, BMP, BEP-1, BEP-2, BEP-3, and BEP-4 showed inhibitions up to 77.00%, 81.66%, 84.14%, 83.59%, 86.82%, and 92.98%, respectively. IC$_{50}$ values of six samples are shown in Table 3. Inhibitory effects of the six samples were in the following order: BEP-4 > BEP-3 > BEP-1 > BEP-2 > BMP > UMP. BEP-4 showed the highest inhibitory effect with the lowest IC$_{50}$ value (P < 0.05) (Sun et al., 2016). Thus, the results of these experiments indicate that enzyme assistance and ball-milling as pretreatments of Chinese quince fruits improve the α-amylase inhibitory ability of extracted PAs (Nguyen et al., 2019).

Conclusion

In the present investigation, ball milling and enzymatic hydrolysis after ball milling were tested as pretreatments for Chinese quince fruits before PA extraction by organic solvent. The sample that was treated with ball-milling and multi-enzyme hydrolysis (BEP-4) had the highest antioxidant activity of all. Its DPPH, ABTS, hydroxyl radical scavenging abilities, and α-amylase inhibitory capacity were 90.38%, 98.99%, 97.89%, and 92.98%, respectively. It also had the highest total phenolic and PA contents, namely 750.98 ± 6.95 and 948.72 ± 3.50 mg/g of dry weight extract, respectively. However, the thermal stability and molecular weight of BEP-4 were lower than other fractions. The six PA samples exhibited different surface morphology. The combination of enzymatic hydrolysis and ball-milling pretreatments enhanced the degradation of chemical components in the cell walls, thereby promoting the release of PAs. All six PA fractions were mainly composed of catechin/epicatechin oligomers. Overall, the combination of ball-milling and enzyme assistance improved the extraction of PAs from Chinese quince fruits, giving higher yields with higher purity and greater antioxidant activity. The results of this investigation provided experimental support for developing Chinese quince fruit proanthocyanidins as natural antioxidants.

Declarations

Author’s contribution statement

Wan-Qing Kong: Methodology, Investigation, Data curation, Software, Writing-original draft.

Ming-Wei Liu: Investigation, Resources, Software.

Hui-Hui Gao: Investigation, Resources, Software.

Shou-Tao Wang: Resources, Investigation.

Zhao Qin: Project administration, Funding acquisition, Supervision, Writing-review & editing.

Hua-Min Liu: Project administration, Funding acquisition, Supervision.

Xue-De Wang: Project administration, Supervision.

Jing-Ren He: Investigation, Data curation.

Data Availability
The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declaration of Competing Interest

The authors declare that there is no conflict of interests regarding the publication of this article.

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Figures

**Fig. 1. (a)**

**Fig. 1. (b)**

**Figure 1**

Total phenolic content (a) and total proanthocyanidin content (b) of six proanthocyanidins extracts of Chinese quince fruits.
Figure 2

XRD patterns of untreated, ball-milled and enzymatic hydrolysed pretreated samples.

Figure 3

SEM micrographs of UMS, BMS, BES-1, BES-2, BES-3, BES-4 (1000-fold)
Figure 4

FT-IR spectra of UMP, BMP, BEP-1, BEP-2, BEP-3, and BEP-4
Figure 5

GPC chromatograms of various proanthocyanidin extracts isolated from the Chinese quinces by different pretreatments
Figure 6

TG curves (A) and DTG curves (B) of various proanthocyanidin extracts isolated by different pretreatments
Figure 7

MALDI-TOF-MS spectra of various proanthocyanidin extracts isolated from the Chinese quinces by different pretreatments
Figure 8

HSQC NMR spectrum of three proanthocyanidin samples isolated from Chinese quince fruits after different pretreatments
Antioxidant activity against DPPH (a), ABTS (b), ferric ion reducing antioxidant power (c), hydroxyl radical formation inhibition capacity (d), α-Amylase inhibitory capacity (e) of UMP, BMP, BEP-1, BEP-2, BEP-3, BEP-4, and Vc.