Process Optimization of Tannase Hydrolysis of Pomegranate Peel Polyphenols and Identification of Enzymatic Hydrolysis Products Post-Print

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Date: 2017-11-08T00:00:00+00:00

Abstract

Polyphenols extracted from pomegranate peel exhibit various bioactivities such as anti-inflammatory, antioxidant, and anticancer effects; however, large molecular weight ellagitannins such as punicalagin (PG) cannot be directly absorbed by the animal gastrointestinal tract and must first be hydrolyzed into small molecular substances such as ellagic acid (EA) to be absorbed into the bloodstream and exert their biological functions. This study utilized tannase to hydrolyze large molecular weight tannins (relative molecular weight $>$ 500) in pomegranate peel polyphenols into small molecular phenolic acids, aiming to optimize the enzymatic hydrolysis process and product purification protocol, elucidate the compositional changes in pomegranate peel polyphenols before and after hydrolysis, and provide fundamental data for the development and utilization of pomegranate peel polyphenols as functional feed additives to promote animal health. By comparing the high-performance liquid chromatography (HPLC) fingerprint profiles of pomegranate peel polyphenols before and after tannase hydrolysis, and establishing PG degradation rate and EA generation rate as indicators for hydrolysis extent, the enzymatic hydrolysis conditions were optimized using single-factor experiments and orthogonal experiments. Macroporous resin purification conditions were determined through static and dynamic adsorption experiments, and liquid chromatography-mass spectrometry (LC-MS) was employed to identify compositional changes in pomegranate peel polyphenols before and after enzymatic hydrolysis. The results showed that the optimal enzymatic hydrolysis conditions were: pH 5.2, temperature 45 °C, enzyme dosage 30 mL/g substrate, substrate concentration 4 g/L, and time 16 h. The optimal purification conditions for the hydrolysis products were: D101 macroporous resin, 95% ethanol as eluent, sample loading volume of 300 mL, and elution volume of 120 mL. HPLC analysis revealed that original pomegranate peel polyphenols contained 43.64% PG (relative molecular weight 1,084) and 4.85% EA (relative molecular weight 302), while the hydrolysis products contained 0.00% PG and

45.73% EA. Seven major polyphenol compounds were identified in the original pomegranate peel polyphenols, with mass-to-charge ratios (m/z) of 1,083, 1,083, 783, 757, 633, 401, and 300, and relative molecular weights ranging from 302 to 1,084; four major polyphenol compounds were identified in the hydrolysis products, with m/z values of 600, 300, 291, and 247, and relative molecular weights ranging from 248 to 602. This study utilized tannase to hydrolyze pomegranate peel polyphenols containing abundant ellagitannins predominantly composed of PG into hydrolysis products rich in small molecular polyphenol EA, and established optimal conditions for both enzymatic hydrolysis and product purification, which can provide fundamental data for the development of pomegranate peel polyphenols and their application in feed production.

Full Text

Preamble

In livestock production, high-density housing, weaning stress, heat stress, bacterial and viral infections, moldy feed, and oxidized feed can trigger stress responses in animals, leading to disease or physiological abnormalities. Research indicates that oxidative stress is closely related to the vast majority of stress responses and diseases in both humans and animals. Oxidative stress not only induces conditions such as sepsis, mastitis, enteritis, pneumonia, and arthritis in animals, but also increases lipid peroxidation and reduces polyunsaturated fatty acid content in muscle, resulting in decreased meat quality [**?**]. Intake of exogenous antioxidants can help maintain redox balance, alleviate oxidative stress, and improve animal health and meat quality.

Pomegranate is a fruit rich in antioxidant compounds, with particularly high concentrations found in its peel [**?**]. Studies have shown that pomegranate peel extract ranks first in antioxidant activity among over 1,000 plant extracts tested in vitro [**?**], with polyphenols being the primary bioactive components responsible for this effect. Beyond antioxidant activity, pomegranate peel polyphenols exhibit anti-inflammatory, antibacterial, antiviral, and wound-healing properties [**?**]. China is a major pomegranate producer, with the world' s largest cultivation area and an annual output exceeding 1.2 million tons [**?**]. As a byproduct of juice processing, pomegranate peel is generated in massive quantities, representing a substantial resource of polyphenols. Consequently, pomegranate peel polyphenols show excellent potential for development as feed additives to regulate animal health. However, pomegranate peel polyphenols contain substantial amounts of tannins, primarily ellagitannins such as punicalagin (PG), which are polyphenolic compounds with relative molecular masses between 500 and 3,000 [**?**]. According to Lipinski' s rules [**?**], these compounds exhibit poor absorption and permeability due to having more than 5 hydrogen bond donors, molecular weights exceeding 500, and more than 10 hydrogen bond acceptors. Numerous studies have confirmed that these high-molecular-weight tannins cannot be completely absorbed through the digestive tract [**?**, **?**], limiting their in vivo

antioxidant activity. Additionally, high-molecular-weight tannins have antinutritional effects, readily forming precipitates with proteins and metal ions that impair nutrient digestion and absorption [**?**].

Tannase is a tannin acyl hydrolase that hydrolyzes ester bonds and depside bonds, widely distributed in microorganisms such as bacteria and fungi. It has been recognized as a safe food additive by both the U.S. Food and Drug Administration (FDA) and China' s Ministry of Health [**?**]. Tannase can hydrolyze ellagitannins like PG into smaller phenolic acids such as ellagic acid (EA) [**?**]. Following oral administration, EA is primarily absorbed through the stomach [**?**] and can also be further metabolized by intestinal microorganisms in the lower gut before absorption [**?**], thereby exerting antioxidant and other physiological activities in vivo.

In view of these considerations, this study utilized tannase to degrade high-molecular-weight tannins in pomegranate peel polyphenols. We investigated the optimal conditions for tannase hydrolysis and the purification process for enzymatic hydrolysates, analyzed compositional differences in pomegranate peel polyphenols before and after enzymatic treatment, and aimed to provide insights and guidance for the development and application of pomegranate peel polyphenols in feed production.

1.1 Materials, Reagents, and Instruments

Materials and Reagents: Tannase enzyme (powder) was purchased from Hubei Jianuoxin Biochemical Co., Ltd.; pomegranate peel polyphenols (60% purity, powder) from Shaanxi Ciyuan Biotechnology Co., Ltd.; D101 macroporous resin from Anhui Samsung Resin Technology Co., Ltd.; ADS-17 macroporous resin from Tianjin Nankai Hecheng Technology Co., Ltd.; SP-700 macroporous resin from Mitsubishi Chemical Corporation (Japan); PG and EA standards from Sigma-Aldrich (USA); HPLC-grade methanol, acetonitrile, and trifluoroacetic acid (TFA), along with other analytical-grade reagents, from Hangzhou Changqing Chemical Co., Ltd.

Instruments: Agilent 1200 reversed-phase high-performance liquid chromatography (HPLC) system (Life Technologies, USA); UPLC-Triple TOF 5600+ time-of-flight mass spectrometer (Sciex, Canada); SpectraMax M5 multi-mode microplate reader (Molecular Devices, USA); DSK-24 electric water bath (Hangzhou Lantian Instrument Co.); DGG-9140BD constant temperature drying oven (Shanghai Senxin Experimental Instrument Co.); DL-720D ultrasonic cleaner (Shanghai Zhixin Instrument Co.).

1.2 Preparation of Pomegranate Peel Polyphenol Solution and Tannase Solution

Pomegranate peel polyphenol solution: To prepare solutions of specific concentrations, accurately weighed pomegranate peel polyphenol powder was

added to phosphate-buffered saline (PBS) of varying pH values. After ultrasonic treatment for 30 minutes, the mixture was filtered, and the filtrate served as the pomegranate peel polyphenol solution, with its polyphenol concentration subsequently measured.

Tannase solution: Tannase powder was weighed and mixed with water at a mass-to-volume ratio of 1:2. The mixture was magnetically stirred for 1 hour, filtered through a stainless-steel filter, and then passed through a 0.45 μm microporous membrane. The filtrate' s tannase activity was measured at 41 U/L, where 1 U represents the degradation of 1 μmol of tannic acid per minute.

PBS preparation: A sodium hydrogen phosphate solution (28.4 g/L, i.e., 0.2 mol/L) and a citric acid solution (21.01 g/L, i.e., 0.1 mol/L) were prepared. These two solutions were mixed in different volumes and pH-calibrated to prepare buffers of various pH values.

1.3 Single-Factor Experimental Design for Optimal Enzymatic Hydrolysis Conditions

Single-factor experiments were conducted to examine temperature, pH, reaction time, enzyme amount, and substrate concentration (i.e., polyphenol concentration), investigating their effects on PG degradation rate and EA production rate in the reaction mixture. The enzymatic hydrolysis reaction was initiated by mixing the reaction components according to the conditions listed in Table 1 . After completion, the reaction was terminated by placing the mixture in a 95 °C water bath for 8 minutes. After cooling to room temperature, the mixture was filtered through a 0.45 μm membrane, and PG and EA contents in the supernatant were measured by HPLC. Each reaction condition was performed in triplicate.

PG degradation rate (%) = (1 - PG concentration after reaction / PG concentration before reaction) × 100
EA production rate (%) = (EA concentration after reaction / EA concentration before reaction - 1) × 100

1.5.2 Determination of Eluent Concentration

Static desorption methods were used to measure desorption rates at ethanol concentrations of 50%, 70%, 90%, and 95%. To a conical flask containing 1 g of saturated adsorption resin, 50 mL of ethanol solution at different concentrations was added. After sealing, the flask was shaken at 120 r/min at room temperature for 24 hours. The polyphenol concentration in the supernatant was measured, and the desorption rate was calculated.

1.5.3 Determination of Loading Volume

Using wet packing, macroporous resin was loaded into a glass chromatography column ($ $2.0 cm × 30.0 cm). Pomegranate peel polyphenol enzymatic hydrolysate was loaded at a flow rate of 1.5 mL/min. The eluate was collected in 50 mL fractions, and the polyphenol concentration in each fraction was measured to plot the dynamic curve of polyphenol concentration.

1.5.4 Determination of Elution Volume

After loading, the column was washed with deionized water until the eluate became colorless. The eluent was then applied at a flow rate of 1.5 mL/min. The eluate was collected in 10 mL fractions, and the polyphenol concentration in each fraction was measured.

1.6 HPLC Detection of PG and EA Content

Chromatographic conditions: ZORBAX SB-C18 column (4.6 mm × 250 mm, 5 μm); column temperature 30 °C; UV detector at 280 nm; injection volume 20 μL; mobile phase consisting of methanol and 0.1% TFA aqueous solution at a flow rate of 1.0 mL/min. Linear gradient elution program: 0–10 min, 3%–14% methanol; 10–20 min, 14%–40% methanol; 20–40 min, 40%–100% methanol.

1.7 Polyphenol Content Detection

The Folin-Ciocalteu method described by Sun Yuqing [**?**] was referenced. Gallic acid was used as the standard to prepare a calibration curve and fit a regression equation. The same method was applied to measure the absorbance of test samples, and polyphenol content was calculated based on the regression equation.

1.8 Collection of Enzymatic Hydrolysis Products

Under optimal enzymatic hydrolysis conditions, pomegranate peel polyphenols were reacted with tannase. After reaction completion, the mixture was filtered through a stainless-steel filter, and the precipitate was collected and dried. The supernatant was heated in a 95 °C water bath for 8 minutes, and protein precipitate was removed by stainless-steel filtration. The supernatant was then purified under optimal purification conditions, and the resulting polyphenol solution was evaporated to dryness in an 80 °C water bath. The dried precipitate and purified supernatant were combined, thoroughly ground, and mixed to obtain the final powdered enzymatic hydrolysis product.

1.9 Liquid Chromatography-Mass Spectrometry Identification of Components

Accurately weighed 10 mg of purified pomegranate peel polyphenols or its enzymatic hydrolysis product powder was dissolved in 10 mL methanol, filtered through a 0.45 μm microporous membrane, and subjected to LC-MS analysis.

Liquid chromatography conditions: Agilent Zorbax SB-C18 column (250 mm × 4.6 mm, 5 μm); mobile phase A: 0.1% formic acid solution; mobile phase B: 0.1% formic acid in acetonitrile; linear gradient elution: 0–10 min, 3%–14% B; 10–20 min, 14%–40% B; 20–43 min, 40%–100% B; flow rate 1 mL/min; column temperature 20 °C; UV variable wavelength detector at 280 nm; injection volume 10 μL.

Mass spectrometry conditions: Negative ion mode; mass-to-charge ratio (m/z) scan range 100–1,500; nebulizer gas pressure 1 (GS1) 50 psi, nebulizer gas pressure 2 (GS2) 50 psi, curtain gas pressure (CUR) 30 psi; ion source temperature (TEM) 550 °C, ion source voltage (IS) 4,500 V; declustering potential for primary scan 100 V; focusing potential 10 V; secondary scan mode: information-dependent acquisition (IDA); collision-induced dissociation (CID) energies 20, 40, and 60 V.

1.10 Statistical Analysis

Data from single-factor and orthogonal experiments are expressed as mean ± standard deviation. Orthogonal experimental design and result analysis were performed using Orthogonal Design Assistant V 3.1 software. Mass spectrometry data were analyzed by comparison with the NIST database.

2.1 Effect of Enzymatic Hydrolysis on Major Polyphenol Compounds

As shown by the peak areas in the HPLC fingerprint (Figure 1 [Figure 1: see original paper]), raw pomegranate peel polyphenols contained a high proportion of PG components [including two isomers, punicalagin a (PGa) and punicalagin b (PGb)], while the proportion of EA was relatively low. After reaction between tannase and pomegranate peel polyphenols under specific conditions, the PG peak area decreased significantly while the EA peak area increased substantially, indicating that PG underwent hydrolysis under the action of tannase, generating EA in large quantities. Therefore, this study selected PG degradation rate and EA production rate as the two indicators to evaluate reaction efficiency and extent.

2.2.1 Effect of Temperature on Enzymatic Hydrolysis of Pomegranate Peel Polyphenols

As shown in Figure 2 [Figure 2: see original paper], both PG degradation rate and EA production rate exhibited an increasing trend between 30–45 °C, reaching their peak at 45 °C. Upon further temperature increase to 50 °C, both rates declined sharply, demonstrating that the enzymatic hydrolysis reaction achieved maximum efficiency at 45 °C.

2.2.2 Effect of pH on Enzymatic Hydrolysis of Pomegranate Peel Polyphenols

As shown in Figure 3 [Figure 3: see original paper], PG degradation rate remained relatively high at pH values below 5.0, but decreased sharply when pH exceeded 5.0. EA production rate fluctuated with pH changes, reaching its maximum at pH 5.0, with significant reductions observed both below and above this value. Therefore, pH 5.0 was selected as the optimal reaction pH.

2.2.3 Effect of Time on Enzymatic Hydrolysis of Pomegranate Peel Polyphenols

As shown in Figure 4 [Figure 4: see original paper], both PG degradation rate and EA production rate increased with prolonged reaction time. For PG degradation rate, a rapid increase was observed between 4–10 h, after which the rate of increase slowed, and the curve became smoother after 16 h. For EA production rate, a slow increase occurred between 4–8 h, followed by a rapid increase from 8–16 h, after which the curve plateaued. Considering both reaction extent and efficiency, 16 h was selected as the optimal reaction time.

2.2.4 Effect of Enzyme Amount on Enzymatic Hydrolysis of Pomegranate Peel Polyphenols

As shown in Figure 5 [Figure 5: see original paper], PG degradation rate increased rapidly when enzyme amount was 10–30 mL/g substrate, plateauing thereafter. For EA production rate, a rapid increase occurred at 10–20 mL/g substrate, with a slower increase beyond 20 mL/g substrate, and a slight decrease observed above 40 mL/g substrate. Considering both reaction efficiency and enzyme conservation, 30 mL/g substrate was selected as the optimal enzyme amount.

2.2.5 Effect of Substrate Concentration on Enzymatic Hydrolysis of Pomegranate Peel Polyphenols

As shown in Figure 6 [Figure 6: see original paper], PG degradation rate increased rapidly at substrate concentrations below 1 g/L, indicating that tannase was not saturated relative to the polyphenol substrate and its activity was

not fully expressed. Beyond 1 g/L, PG degradation rate remained relatively stable at approximately 95%. EA production rate became essentially constant when substrate concentration exceeded 2 g/L, indicating high EA production efficiency at this concentration. From the perspective of reaction efficiency, higher substrate concentrations within the 0–4 g/L range were preferable.

2.3 Orthogonal Analysis of Enzymatic Hydrolysis Conditions

As shown in Table 3 , the order of factors influencing PG degradation rate was pH > temperature > enzyme amount > time. However, these factors had minimal impact on PG degradation rate, with PG degradation exceeding 95% under all conditions, indicating relatively complete degradation. Therefore, primary consideration was given to the effect on EA production rate. The order of factors influencing EA production rate was pH > time > temperature > enzyme amount. The optimal reaction conditions were pH at level 3, temperature at level 2, enzyme amount at level 1, and time at level 3, corresponding to pH = 5.2, temperature = 45 °C, enzyme amount = 30 mL/g substrate, and time = 16 h. These conditions corresponded to trial number 8 in the orthogonal array (Table 3), which yielded the highest EA production rate among all groups. Therefore, these conditions were selected as the optimal enzymatic hydrolysis conditions.

2.4 Optimization Results for Pomegranate Peel Polyphenol Purification Process

2.4.1 Comparison of Adsorption and Desorption Characteristics of Three Macroporous Resin Types

As shown in Table 4 , the adsorption rates of D101, ADS-17, and SP-700 macroporous resins for pomegranate peel polyphenols were all approximately 90%, with desorption rates above 95%. D101 exhibited the highest desorption rate at 99.8%, and was therefore selected as the final resin type.

2.4.2 Effect of Eluent Concentration on Desorption Rate of Pomegranate Peel Polyphenols

As shown in Figure 7 [Figure 7: see original paper], the desorption rate of pomegranate peel polyphenols increased gradually with increasing ethanol concentration. The highest desorption rate was achieved with 95% ethanol, which was selected as the final eluent concentration.

2.4.3 Effect of Loading Volume on Adsorption Efficiency

As shown in Figure 8 [Figure 8: see original paper], when loading volume was less than 300 mL, polyphenol concentration in the effluent increased rapidly

with increasing loading volume. When loading volume exceeded 300 mL, effluent polyphenol concentration remained essentially constant, indicating that the macroporous resin had reached saturation for pomegranate peel polyphenol adsorption. Therefore, 300 mL was selected as the final loading volume.

2.4.4 Effect of Elution Volume on Elution Efficiency

As shown in Figure 9 [Figure 9: see original paper], effluent polyphenol concentration exhibited a peak-shaped curve with increasing elution volume, first increasing then decreasing. When elution volume exceeded 120 mL, effluent polyphenol concentration approached zero. Therefore, 120 mL was selected as the final elution volume.

2.5 Polyphenol, PG, and EA Contents in Pomegranate Peel Polyphenols and Their Enzymatic Hydrolysis Products

Analysis revealed that pomegranate peel polyphenols contained 43.64% PG and 4.85% EA. Under optimal enzymatic hydrolysis and macroporous resin purification conditions, the enzymatic hydrolysis product of pomegranate peel polyphenols was obtained with a polyphenol content of 80.40%. PG was not detected (0.00%), while EA content reached 45.73%.

2.6 Mass Spectrometric Analysis of Components in Pomegranate Peel Polyphenols and Their Enzymatic Hydrolysis Products

The UV absorption spectra of pomegranate peel polyphenols and their enzymatic hydrolysis products are shown in Figure 10 [Figure 10: see original paper], and the total ion current chromatograms are presented in Figure 11 [Figure 11: see original paper].

Seven major polyphenol compounds were identified in pomegranate peel polyphenols: (1) retention time = 15.31 min, m/z = 1,083.0618, PGa; (2) retention time = 15.88 min, m/z = 783.0700, pedunculagin; (3) retention time = 17.38 min, m/z = 1,083.0614, PGb; (4) retention time = 20.92 min, m/z = 757.0900, tellimagrandin; (5) retention time = 22.27 min, m/z = 633.0737, corilagin; (6) retention time = 30.41 min, m/z = 300.9996, EA; (7) retention time = 36.60 min, m/z = 401.0879, cetraric acid. The relative molecular masses of compounds in pomegranate peel polyphenols ranged from 302 to 1,084.

Four major polyphenol compounds were identified in the enzymatic hydrolysis products: (1) retention time = 23.14 min, m/z = 291.0152, brevifolin carboxylic acid; (2) retention time = 25.68 min, m/z = 600.9895, gallagic acid; (3) retention time = 26.20 min, m/z = 247.0258, brevifolin; (4) retention time = 30.33 min, m/z = 300.9995, EA. The relative molecular masses of compounds in the enzymatic hydrolysis products ranged from 248 to 602. Compared with

pomegranate peel polyphenols, the enzymatic products showed increased diversity of small-molecular compounds and disappearance of high-molecular-weight compounds.

Tannase is widely distributed in Aspergillus fungi and is extensively used in the food industry, commonly employed to degrade tannins in beer to prevent precipitation with proteins and clarify solutions. It is also used in instant tea production to prevent turbidity caused by aggregation of high-molecular-weight tannins [**?**]. International reports have described the production of EA through solid-state fermentation of pomegranate peel using tannase-producing *Aspergillus niger* [**?**, **?**]. Cheng Yan [**?**] used pomegranate peel tannins as an inducer to stimulate *A. niger* tannase production, then reacted the extracted enzyme with pomegranate peel tannins to obtain EA through acid-base dissolution purification. Unlike previous studies focused solely on EA production, this study optimized tannase hydrolysis conditions and purification processes for pomegranate peel polyphenols using PG degradation rate and EA production rate as targets. This approach maintains product diversity rather than complete conversion to EA alone, aiming to obtain more bioactive compounds from PG hydrolysis without compromising the comprehensive activity of the enzymatic hydrolysates.

During enzymatic hydrolysis, tannase altered the composition and proportions of compounds in pomegranate peel polyphenols. HPLC fingerprints revealed that raw pomegranate peel polyphenols showed numerous peaks with large areas at early retention times, while enzymatically treated polyphenols exhibited increased peak numbers and areas at later retention times. Mass spectrometric identification demonstrated that tannase hydrolysis significantly reduced the variety of compounds and decreased their relative molecular masses, with the representative high-molecular-weight compound PG being completely hydrolyzed. Structural comparison indicated that tannase hydrolysis primarily caused cleavage of acyl and glycosidic bonds, generating small-molecular polyphenols dominated by EA. Based on the liquid chromatography elution conditions and the principle of "like dissolves like," early-eluting peaks represent highly polar water-soluble compounds, while later-eluting peaks indicate less polar, more lipophilic substances. These results demonstrate that enzymatic hydrolysis reduced polar compounds and increased lipophilic compounds in pomegranate peel polyphenols. Increased lipophilicity facilitates transport across the lipid bilayer of cell membranes to exert intracellular effects. Our previous research investigated three pomegranate peel polyphenols with similar structural units but different molecular weights—PG (relative molecular mass 1,084), punicalin (relative molecular mass 783), and EA (relative molecular mass 302)—and found that EA exhibited the strongest antioxidant activity against oxidized fish oil-induced oxidative damage in mice, as evidenced by alleviated weight loss, increased antioxidant enzyme activities in liver and blood, and reduced malondialdehyde (MDA) content [**?**]. Therefore, hydrolyzing high-molecular-weight ellagitannins like PG into small-molecular phenolic acids such as EA enhances the antioxidant activity of pomegranate peel polyphenols in animals, representing a practical approach

for developing pomegranate peel as a functional feed additive.

During optimization of enzymatic hydrolysis conditions, temperature and pH exhibited the greatest influence. With increasing temperature, both EA production rate and PG degradation rate initially increased then decreased, with EA production rate declining more rapidly than PG degradation rate after 45 °C. This phenomenon can be attributed to two factors: first, tannase specifically produces EA; second, when temperature exceeds the optimum, tannase gradually loses activity, causing a sharp decline in EA production rate, while PG can undergo spontaneous hydrolysis at elevated temperatures independent of enzymatic action, producing non-EA products. The effects of pH differed between EA production and PG degradation rates. Under acidic conditions ($pH < 5.0$), PG degradation rate remained high, but decreased sharply at $pH \geq 5.0$. This occurs because PG can spontaneously hydrolyze in acidic environments to produce EA; as pH increases, spontaneous hydrolysis efficiency and extent decrease, potentially leading to incomplete hydrolysis and formation of EA derivatives conjugated with one glucose molecule. At pH 4.5, incomplete PG hydrolysis was evident, resulting in lower EA production rate. As pH continued to increase toward the optimum for enzymatic reaction, tannase-mediated EA production peaked; beyond this point, reduced spontaneous hydrolysis and enzymatic activity caused rapid decline in EA production rate. These findings demonstrate that tannase hydrolysis of pomegranate peel polyphenols is not solely dependent on enzymatic processes, requiring comprehensive consideration of various reaction conditions. This study ultimately determined optimal enzymatic hydrolysis conditions through orthogonal experimental design.

To maintain stable pH during enzymatic hydrolysis, substantial amounts of PBS were added, resulting in high inorganic salt content in the hydrolysates. Additionally, glucose moieties were released during hydrolysis of PG and other compounds. To remove these inorganic salts and glucose, D101 macroporous resin was selected through static adsorption and desorption experiments using polyphenol content as the evaluation index, and purification process parameters were optimized. D101 macroporous resin is a styrene-based nonpolar copolymer with strong adsorption capacity for nonpolar or weakly polar compounds and broad applicability. Although pomegranate peel polyphenols are dominated by ellagitannins containing multiple phenolic hydroxyl groups, the acyl and ellagoyl structures possess certain hydrophobic properties, making them suitable for adsorption by nonpolar resins. Zhang Lihua et al. [**?**] compared the adsorption of polyphenols from pomegranate peel extracts by D101, AB-8, and DA-201 macroporous resins, finding that D101 exhibited the best adsorption and desorption performance, consistent with our experimental results.

China is a major pomegranate producer with enormous annual output. In modern juice processing, whole pomegranate fruits are pressed for juice, generating abundant peel waste that is inexpensive and readily available. The primary cost of pomegranate peel enzymatic hydrolysis technology stems from tannase. To reduce costs for future feed applications, crude enzyme preparations from mi-

croorganisms such as *A. niger* could be used for pomegranate peel polyphenol hydrolysis, or tannase-producing microorganisms could be employed for direct fermentation of pomegranate peel. From the perspective of improving enzyme utilization efficiency, research on tannase immobilization is also warranted.

Conclusions

1. The optimal enzymatic hydrolysis conditions for tannase hydrolysis of pomegranate peel polyphenols were established as: pH 5.2, temperature 45 °C, enzyme amount 30 mL/g substrate, reaction time 16 h, and substrate concentration 4 g/L.
2. D101 macroporous resin was selected for purification of enzymatic hydrolysates, with 95% ethanol as the optimal eluent. Using a glass chromatography column ($ $2.0 cm × 30.0 cm) for dynamic adsorption, the optimal loading volume was 300 mL and optimal elution volume was 120 mL.
3. Under these conditions, the enzymatic hydrolysis product of pomegranate peel polyphenols contained 80.40% polyphenols. The high-molecular-weight polyphenol PG content decreased from 43.64% before hydrolysis to 0.00%, while the small-molecular polyphenol EA content increased from 4.85% before hydrolysis to 45.73%. The relative molecular mass distribution of compounds in pomegranate peel polyphenols decreased from 302–1,084 before hydrolysis to 248–602 after hydrolysis.

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Note: Figure translations are in progress. See original paper for figures.