Article

Enhanced Enzymatic Production of Antioxidant Peptides from *Carya cathayensis* Cake Using an Enzymatic Membrane-Coupled Reactor

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Abstract: This study establishes an enzymatic membrane-coupled production process for antioxidant peptides from *Carya cathayensis* cake by comparing the effects of not feeding, water feeding, substrate feeding, and gradient dilution feeding supplementary material modes, to assess their impact on production efficiency. The optimal operational conditions were determined as follows: pH 10.5, temperature 50 °C, and enzyme-to-substrate ratio of 10% (*w*/*w*). The continuous production using the gradient dilution supplementary material mode resulted in Chinese pecan antioxidant peptides with 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging rate of 0.044 mg/mL, 2, 2-azinobis-(3-ethylbenzthiazoline-6-sulfonate) (ABTS) radical scavenging rate of 0.518 mg/mL, and ferrous ion chelating ability (IC_{50}) of 0.252 mg/mL. Compared with traditional enzymatic hydrolysis processes, the gradient dilution supplementary material enzymatic membrane-coupled production process increased peptide yield, peptide production, and unit enzyme-peptide production by 14.36%, 11.35%, and 235.63%, respectively. This continuous production method facilitates scalability, enabling the production of high-yield and high-activity *Carya cathayensis* cake peptides, making better use of byproducts after oil extraction, and laying a solid foundation for the comprehensive development and deep processing of Chinese pecan, thus enhancing its economic value.

Keywords: enzyme membrane reactor; *Carya cathayensis* cake; enzyme membrane coupling process; antioxidant peptides

Citation: Wang, Q.; Chang, Y.; Yuan, Q.; Wu, F.; Hu, H.; Zhang, J.; He, Z. Enhanced Enzymatic Production of Antioxidant Peptides from *Carya cathayensis* Cake Using an Enzymatic Membrane-Coupled Reactor. *Processes* **2024**, *12*, 2. https://doi.org/10.3390/pr12010002

Academic Editor: Yonghui Li

Received: 30 October 2023
Revised: 7 December 2023
Accepted: 15 December 2023
Published: 19 December 2023

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1. Introduction

The enzymatic membrane-coupled reactor, an innovative apparatus integrating enzymatic catalysis and membrane separation technologies, integrates the high efficiency and selectivity of conventional enzyme-catalyzed reactions with the proficient mass transfer characteristics of membrane separation techniques. It finds extensive applications in biotechnology, food engineering, pharmaceuticals, and beyond. The core principle of the enzyme membrane coupling technology lies in the selective separation of products and unreacted substrates through the membrane's separation function, facilitating uninterrupted substrate reactions and continuous product separation, thus elevating production efficiency. Compared to traditional enzyme hydrolysis techniques, the enzyme membrane coupling technology actualizes enzyme recycling, resulting in improved enzyme utilization efficiency. Furthermore, it promptly separates products, alleviating product inhibition and enhancing product yield and purity. By employing membrane components with varying pore sizes for product separation, uniformity, and stability of the final product are ensured [1]. In the food industry, the enzyme membrane reactor is predominantly employed for protein hydrolysis, yielding peptides and amino acids [2], the reutilization of whey in milk [3], juice clarification [4], and the synthesis of bioactive substances [5]. Currently, the

enzyme membrane coupling technology has been applied to the preparation of diverse bioactive peptides, such as antioxidant peptides from sunflower seed cake [6], collagen peptides [7], corn germ ACE inhibitory peptides [8], whey-derived DPP-IV inhibitory peptides [9]. However, its application in nut peptides is scarcely documented.

Carya cathayensis Sarg., also known as Chinese pecan or small Chinese pecan, predominantly thrives in the border regions of Zhejiang and Anhui provinces in China, exhibiting a rich composition of proteins, oils, and other essential components [10]. Chinese pecan cake, a byproduct generated during the oil extraction process, is frequently employed as feed or disposed of directly, leading to environmental pollution. Within these byproducts, proteins account for approximately 20%, encompassing 17 types of amino acids, rendering them an excellent raw material to produce bioactive peptides [11]. Our previous research has also elucidated that peptides isolated from Chinese pecan protein hydrolysates demonstrate exceptional antioxidant activity [12–14].

In this study, we conduct a comparative analysis with traditional enzymatic processes to explore the influence of pH, temperature, and enzyme-to-substrate ratio on the yield of antioxidant peptides from *Carya cathayensis* cake, ultimately determining the optimal conditions. Additionally, we assess the production efficiency and antioxidant activity of the enzyme membrane coupling production process under various supplementary material modes, thus laying the foundation for the development of an efficient method for producing Chinese pecan antioxidant peptides through enzyme membrane coupling, thereby providing a theoretical basis for the continuous production of nut peptides.

2. Materials and Methods

2.1. Materials

Chinese pecan was purchased from Changhua County, Hangzhou City, Zhejiang Province. Alkaline protease (200 U/mg) and flavourzyme (60 U/mg) were purchased from Beijing Solarbio Biotechnology Co., Ltd., Beijing, China. Trypsin (bovine pancreas, 250 U/mg), protamex (120 U/mg), aprotinin (6000 U/mg), cytochrome c, and 2, 2-azinobis-(3-ethylbenzthiazoline-6-sulfonate) (ABTS) were purchased from Shanghai Yuanye Biotechnology Co., Ltd. (Shanghai, China). 1,1-diphenyl-2-picrylhydrazyl (DPPH) was purchased from Shanghai Aladdin Biochemical Technology Co., Ltd., Shanghai, China. Insulin, glycine-glycine-tyrosine-arginine, glycine-glycine-glycine, and 17 amino acid standards were purchased from Shanghai Anpu Experimental Technology Co., Ltd., Shanghai, China. High-performance liquid chromatography (HPLC) grade methanol and acetonitrile were purchased from American Tiandi Co., Ltd. (Shanghai, China). Other chemicals used in this study were analytical grade, purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

2.2. Preparation of Pecan Cake Protein

The pecan cake protein was extracted by the aqueous enzymatic method, and some modifications were made according to the method of Liu et al. [15]. A total of 100 g crushed and sieved pecan cake powder was dispersed in deionized water with a solid–liquid ratio of 1:16 (*w*/*v*). The pH of the mixture was adjusted to 10.0 using 1 M NaOH solution, and then 500 mg alkaline protease was added. The mixture was stirred in a 50 °C water bath for 1.5 h. After cooling, the solution was centrifuged at 7000 r/min for 15 min (5804R, Eppendorf, Hamburg, Germany) and the supernatant of the intermediate layer was collected. The extract was adjusted to pH 3.7 with 1 M HCl solution and allowed to stand for 2 h. The protein was obtained by centrifugation at 7000 r/min for 15 min and washed with deionized water for neutralization. After freeze-drying, the pecan cake protein powder was obtained and stored at −20 °C for use.

2.3. Batch Enzymatic Hydrolysis Method of Enzymatic Membrane Reactor

Batch operation refers to the simultaneous operation of an enzymatic hydrolysis reactor and a membrane separation device. During the enzymatic hydrolysis process, no

water or substrate was added, and only the initial volume of the substrate was used for the reaction. The specific operation process was as follows: 5 g protein was dissolved in 1 L deionized water and denatured in a 95 °C water bath for 20 min. The protein solution was adjusted to set pH and temperature, and protease was added for enzymatic hydrolysis. The pH and temperature in the reactor were kept constant during the enzymatic hydrolysis process. After 10 min of enzymatic hydrolysis, the circulating pump and valve were opened, and the valve was adjusted to make the initial transmembrane pressure 0.2 MPa. The liquid in the reactor flowed out from the bottom and was separated into two parts by a 10 kDa ultrafiltration membrane. The pecan cake peptide with a molecular weight of less than 10 kDa was collected. The macromolecular protein and protease were intercepted by an ultrafiltration membrane and circulated to the reactor to continue the reaction. When the volume of intercepted liquid was 200 mL, the circulation system was stopped, and the reaction was ended. The collected permeate was dried in a vacuum and stored at −20 °C for later use.

2.4. Optimization of Batch Enzymatic Hydrolysis Conditions in an Enzymatic Membrane Reactor

The optimum reaction pH of alkaline protease was 9–12, and the optimum reaction temperature was 40–55 °C. The previous experiments showed that the color of the protein solution was dark brown when the pH was higher than 11, which affected the accuracy of the experimental results. Therefore, the upper limit of the reaction pH was set to 11, and the upper limit of the reaction temperature was set to 55 °C. Based on the substrate concentration of 5 g/L, the operating conditions of the enzyme membrane-coupled reactor in the batch enzymatic hydrolysis process were optimized with the peptide yield (%) as the index, including enzymatic hydrolysis pH (9, 9.5, 10, 10.5, 11), enzymatic hydrolysis temperature (35, 40, 45, 50, 55 °C), and enzyme-to-substrate ratio (2.5%, 5%, 7.5%, 10%, 12.5%).

2.5. Constant Feeding Enzymatic Membrane Coupling Test

The operating conditions of the constant feeding enzyme membrane coupling process were based on the optimal conditions of the batch enzymatic membrane process. In the process of enzymatic hydrolysis, water, protein, or gradient diluted protein solution was continuously supplemented, and the flow rate of the feeding solution was consistent with the flow rate of the penetrating solution. After continuous enzymatic hydrolysis for 4 h, the feeding pump was closed, and the solution in the reactor continued to be separated by ultrafiltration until the volume of the retained liquid was 200 mL. The collected permeate was concentrated, vacuum freeze-dried, and stored at −20 °C for later use. The protein concentration of the gradient dilution feeding solution was first high and then low (3.33 g/L pecan protein solution at 0–80 min; 2.5 g/L hickory protein solution at 81–160 min; 1.67 g/L pecan cake protein solution at 161–240 min).

2.6. Yield of Peptide

Peptide yields were measured using Wu et al.'s method [12] with a slight modification. The same volume of 10%(*w*/*v*) of trichloroacetic acid was mixed with the permeate solution and stood for 10 min to precipitate the insoluble peptides and proteins in the solution. The mixture was centrifuged at 7000 r/min, 4 °C for 10 min, and diluted with 5% trichloroacetic acid to a suitable multiple. The soluble peptide content in the supernatant was measured by spectrophotometer (Model T6, General Instrument for General Analysis, Beijing, China) at 540 nm using the biuret method. The peptide yield can be calculated by Formula (1):

$$\text{Peptide yield}(\%) = \frac{C \times V \times N \times 100}{2.5 \times 1000 \times M} \times 100\% \tag{1}$$

where C (mg/mL) is the peptide concentration calculated according to the standard curve, N is the dilution multiple, V (mL) is the volume of the enzymatic hydrolysate, and M (g) is the quantity of the Chinese pecan cake protein powder.

2.7. Peptide Production

The production of pecan peptides was calculated as the ratio of pecan crude peptide mass (mass after liquid freeze-drying) to pecan cake protein mass.

$$\text{Peptide production}(\%) = \frac{\text{Weight of freeze} - \text{dried pecan cake peptide}}{\text{Quality of pecan cake protein}} \times 100\% \quad (2)$$

2.8. Unit Enzyme Peptide Production

The production of peptide per unit enzyme is expressed as the ratio of pecan crude peptide mass to the mass of the protease used.

$$\text{Unit enzyme peptide production } (g/g) = \frac{\text{Weight of freeze} - \text{dried pecan cake peptide}}{\text{Protease weight}} \times 100\% \quad (3)$$

2.9. Membrane Flux

Membrane flux (or permeation rate) is an important process operating parameter in the membrane separation process, which refers to the amount of fluid passing through the unit membrane area per unit time:

$$J = \frac{V}{A \times t} \times 100\% \quad (4)$$

where J ($mL/(min \cdot m^2)$) is the membrane flux, V (mL) is the permeate volume, A (m^2) is the effective area of the ultrafiltration membrane, and t (min) is the ultrafiltration time.

2.10. Antioxidant Activity

2.10.1. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) Free Radical Scavenging Activity

The DPPH free radical scavenging activity of the samples was determined according to the method reported by Moaveni et al. [16]. The 0.5 mL samples with different concentrations were mixed with 0.5 mL 0.1 mM DPPH-methanol solution, and the mixture reacted at room temperature in the dark for 30 min. The reduction of DPPH free radical was determined by the measurement of absorbance at 517 nm. The equal volume of mixed methanol and DPPH was used as the blank control of the sample, the sample was mixed with methanol as the reagent blank group, and ascorbic acid was used as the positive control. The DPPH free radical scavenging rate of the sample was calculated using Formula (2):

$$\text{Scavenging activity } (\%) = \frac{A_0 - (A_i - A_j)}{A_0} \times 100\% \quad (5)$$

where A_i, A_j, and A_0 represent the absorbance of the sample group, reagent blank group, and sample blank group, respectively.

2.10.2. 2,2-Azinobis (3-ethylbenzthiazoline-6-sulfonate) (ABTS) Free Radical Scavenging Activity

The ABTS free radical scavenging activity of the samples was determined according to the method reported by Hu et al. [17]. The ABTS working solution was obtained by mixing ABTS solution (7 mM) and $K_2S_2O_8$ solution (2.45 mM) in equal volume and reacting under dark conditions at room temperature for 14 h. The ABTS working solution was diluted with deionized water before use until the absorbance of the solution at 734 nm was 0.70 ± 0.02. A total of 0.2 mL of the sample was mixed with 4 mL of diluted ABTS working solution by vibration, and the absorbance was measured at 734 nm after reacting for 6 min at room temperature and protected from light. After reacting for 6 min away from light, the absorbance was measured at 734 nm. The reagent blank group and the sample blank group used deionized water instead of ABTS working solution and sample, respectively. The ABTS radical scavenging rate of the samples was calculated using Equation (5).

2.10.3. Ferrous Ion Chelating Ability

The chelating activity of ferrous ions was determined according to Abeynayake et al.'s method with a slight modification [18]. After mixing 1 mL sample with 0.1 mL 2 mM $FeSO_4$ solution, the sample was diluted with 3.7 mL deionized water. Then, 0.2 mL of 5 mM phenazine was added and mixed vigorously. The mixture was allowed to stand at room temperature for 10 min, and the absorbance was measured at 562 nm. The reagent blank group and the sample blank group were treated with deionized water instead of phenazine solution and sample, respectively. EDTA was used as a positive control. The ferrous ion chelation rate of the sample was calculated using Formula (5).

2.11. Analysis of Amino Acid Composition via High-Performance Liquid Chromatography

Amino acid composition analysis was determined by PITC pre-column derivatization HPLC. A total of 50 mg of the sample was accurately weighed and placed in a hydrolysis tube containing 10 mL 6 M HCl and hydrolyzed in an oven at 110 ± 1 °C for 20 h. An amount of 1 mL of the cooled digestive juice was placed in an oven at 80 ± 1 °C to evaporate the solvent. The dried samples were re-dissolved with 6 mL of deionized water and then derivatized. The samples were filtered by 0.45 µm organic filter membrane and analyzed.

Amino acid analysis was performed by a high-performance liquid chromatograph (LC-20AT, Shimadzu Corporation, Kyoto, Japan). The mobile phase was 0.02 mol/L sodium acetate buffer and 80% acetonitrile–water solution. Exactly 10 µL of sample solution was directly injected into the C18 Inertsil ODS-SP column (4.6 mm × 250 mm, 5 µm) at a flow rate of 1.0 mL/min, the detection wavelength was 254 nm, and the column temperature was 40 °C. The amino acid content in the sample was calculated by comparing the relative retention time of the peak of the sample with that of the 17 standard samples.

2.12. Statistical Analysis

The data were analyzed by IBM SPSS Statistics 27 (IBM SPSS Inc., Chicago, IL, USA). All samples were replicated three times, and the results were expressed as mean ± standard deviation. Duncan's model was used to analyze the significant differences between different samples, and $p < 0.05$ indicated that the difference was significant. Origin 2021 software (OriginLab Co., Northampton, MA, USA) was used for graphing.

3. Results and Discussion

3.1. Establishment of Enzymatic Hydrolysis Method for Chinese Pecan Antioxidant Peptides Enzyme Selection

Based on the data presented in Figure 1, it was found that alkaline protease exhibited the highest yield in producing Chinese pecan cake peptides, with a notable percentage of 18.90 ± 0.64%. This was followed by flavourzyme (15.33 ± 1.24%), protamex (12.68 ± 1.07%), and trypsin (11.12 ± 1.64%). Concurrently, alkaline protease showcased the most substantial DPPH free radical scavenging activity at 57.28 ± 0.71%. The amino acid composition of Chinese pecan protein was analyzed, revealing the presence of aromatic residues (Phe, Trp, and Tyr), acidic residues (Glu), sulfur-containing residues (Met), aliphatic residues (Leu and Ala), hydroxyl residues (Ser), and basic residues (Lys), accounting for 52.06% of the total composition. Hydrophobic amino acids accounted for 34.94% of the overall content. Alkaline protease demonstrated exceptional specificity towards these amino acid residues, effectively hydrolyzing Chinese pecan cake protein into smaller peptides. The release of hydrophobic amino acids significantly contributed to the remarkable antioxidant activity of the resulting peptides [14,19]. Therefore, alkaline protease was chosen as the ideal candidate for producing Chinese pecan antioxidant peptides.

3.2. Impact of Enzymatic Hydrolysis pH on the Yield of Chinese Pecan Cake Peptides in a Batch Enzymatic Process

As depicted in Figure 2a, the yield of peptides demonstrates a rising tendency across the range of enzymatic pH values from 9.0 to 10.5. The apex of this yield is attained at

a pH of 10.5, culminating at 27.47 ± 0.76%. The alkaline environment induced a state of heightened disorder in the Chinese pecan protein structure, leading to the exposure of active sites and rendering the protein more susceptible to enzymatic hydrolysis, which facilitated the formation of peptides [20]. Additionally, as the pH rose from 9.0 to 10.5, the enzymatic activity of the protease underwent enhancement, thereby promoting more efficient hydrolysis of the Chinese pecan cake protein and ultimately resulting in an increase in peptide yield. As a result, enzymatic hydrolysis at pH 10.5 was identified as the optimal condition.

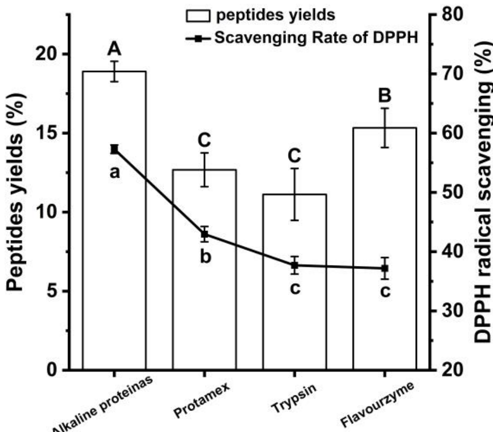

Figure 1. Effects of different proteins on peptides yield and scavenging rate of DPPH of pecan cake in the batch enzymatic hydrolysis process. Different capital letters (A, B, C) indicate significant differences in peptide yields ($p < 0.05$). Different lowercase letters (a, b, c) indicate significant differences in DPPH free radical scavenging rates ($p < 0.05$).

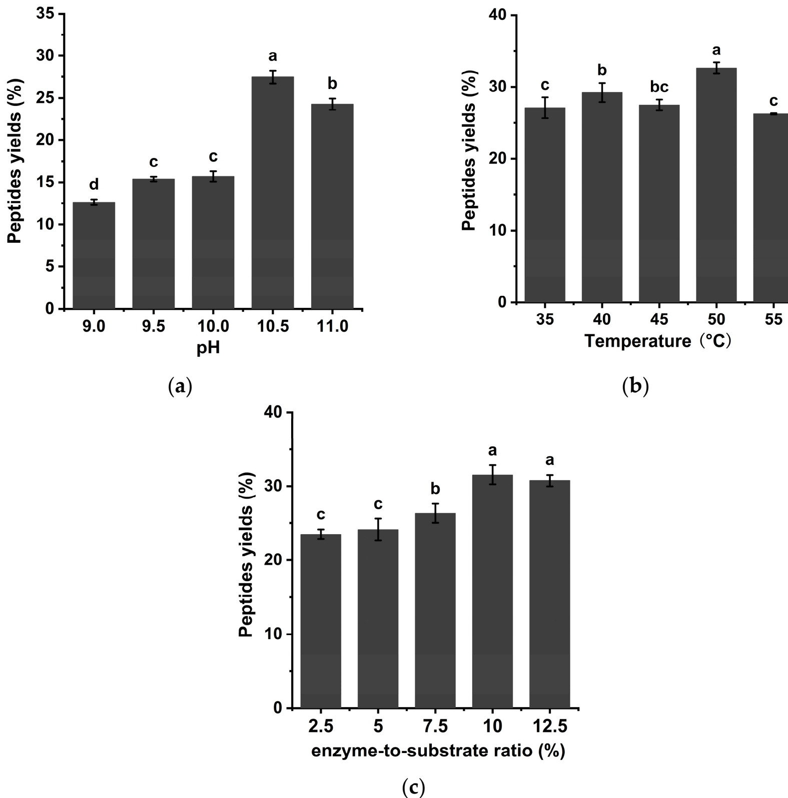

Figure 2. Effects of pH, temperature, and enzyme-to-substrate ratio on peptides yields of *Carya cathayensis* cake in batch enzymatic hydrolysis. (**a**) pH, (**b**) Temperature, (**c**) enzyme-to-substrate ratio. The bars with different letters indicate the significant differences ($p < 0.05$).

3.3. Impact of Enzymatic Hydrolysis Temperature on the Yield of Chinese Pecan Cake Peptides in Batch Enzymatic Process

As evidenced by Figure 2b, the peptide yield exhibits a gradual increment with the elevation of enzymatic hydrolysis temperature, culminating at 50 °C with an impressive yield of 32.59 ± 0.79%. This is similar to the results of Qu et al., whose study found that hydrolysis of purslane protein using alkaline protease resulted in the highest degree of protein conversion in the batch CEH-MS system at 50 °C [21]. This phenomenon can be attributed to the heightened enzymatic activity and augmented collision frequency between the enzyme and substrate as the temperature rises. Consequently, the enzyme's catalytic efficiency is accelerated, leading to an enhancement in the peptide yield. However, it is imperative to exercise caution, as excessively high enzymatic hydrolysis temperatures may trigger enzyme denaturation and deactivation, consequently impeding its capacity to cleave peptide chains [22]. Consequently, a prudent choice is to set the enzymatic hydrolysis temperature at 50 °C as the optimal condition.

3.4. Impact of Enzyme-to-Substrate Ratio on the Yield of Chinese Pecan Cake Peptides in Batch Enzymatic Process

As illustrated in Figure 2c, the peptide yield exhibits a significant increase within the enzyme-to-substrate ratio range from 2.5% to 12.5%, followed by a slight decline. Remarkably, at an enzyme-to-substrate ratio of 10%, the highest peptide yield of 31.55 ± 1.30% is achieved, demonstrating the efficacy of this specific ratio. Zhang et al. also found that the extraction rate of chrysin increased with increasing xylanase concentration, but when the enzyme concentration was too high, a decrease in the extraction rate was observed [23]. This phenomenon can be primarily attributed to the dynamic interplay between the enzyme and substrate at lower enzyme-to-substrate ratios, leading to enhanced contact and binding, thus accelerating the enzyme-catalyzed reaction. However, as the enzyme-to-substrate ratio further increases, saturation of enzyme–substrate binding sites occurs, coupled with competitive inhibition at higher enzyme concentrations, which ultimately impedes the reaction efficiency and results in a minor reduction in peptide yield [24]. Consequently, the optimal enzyme-to-substrate ratio of 10% is selected for the production of Chinese pecan cake peptides.

3.5. Comparative Analysis of Enzyme Membrane Coupling Production Processes for Chinese Pecan Cake Peptides Employing Different Supplementary Feeding Modes

3.5.1. Operational Performance Comparison in Different Supplementary Feeding Modes for Enzyme Membrane Coupling Process

As illustrated in Figure 3, under the specified conditions of substrate concentration (5.0 g/L), pH level (10.5), temperature (50 °C), and enzyme-to-substrate ratio (10%), the utilization of diverse constant feeding modes results in varying degrees of enhancement in the yield of Chinese pecan cake peptides. The gradient dilution feeding approach, involving continuous replenishment for a duration of 4 h while maintaining the solution in the ultrafiltration reactor, progressively elevated the yield of Chinese pecan cake peptides. Eventually, the peptide yield reached an impressive pinnacle at 48.33 ± 1.19%. Throughout this process, the membrane flux underwent a gradual decline of 645.16 mL/(min·m^2), and the transmembrane pressure experienced a slow increase of 0.020 MPa. In the case of the constant water supplementation mode, the peptide yield attained 34.61 ± 0.06%, accompanied by a reduction in membrane flux of 583.72 mL/(min·m^2), and an increase in transmembrane pressure by 0.016 MPa. Conversely, for the constant substrate supplementation mode, the peptide yield reached 43.83 ± 0.98%, but there was a rapid decline in membrane flux, a 0.030 MPa rise in transmembrane pressure, and a significant extension in ultrafiltration time, spanning 550 min. These observed distinctions arose from the application of the gradient dilution feeding mode, which effectively supplemented both water and substrate, maintaining an optimal concentration range of reactant proteins. This enabled efficient utilization of substrates and enzymes, while mitigating the degree of membrane

fouling compared to the substrate supplementation mode, thereby effectively bolstering production efficiency.

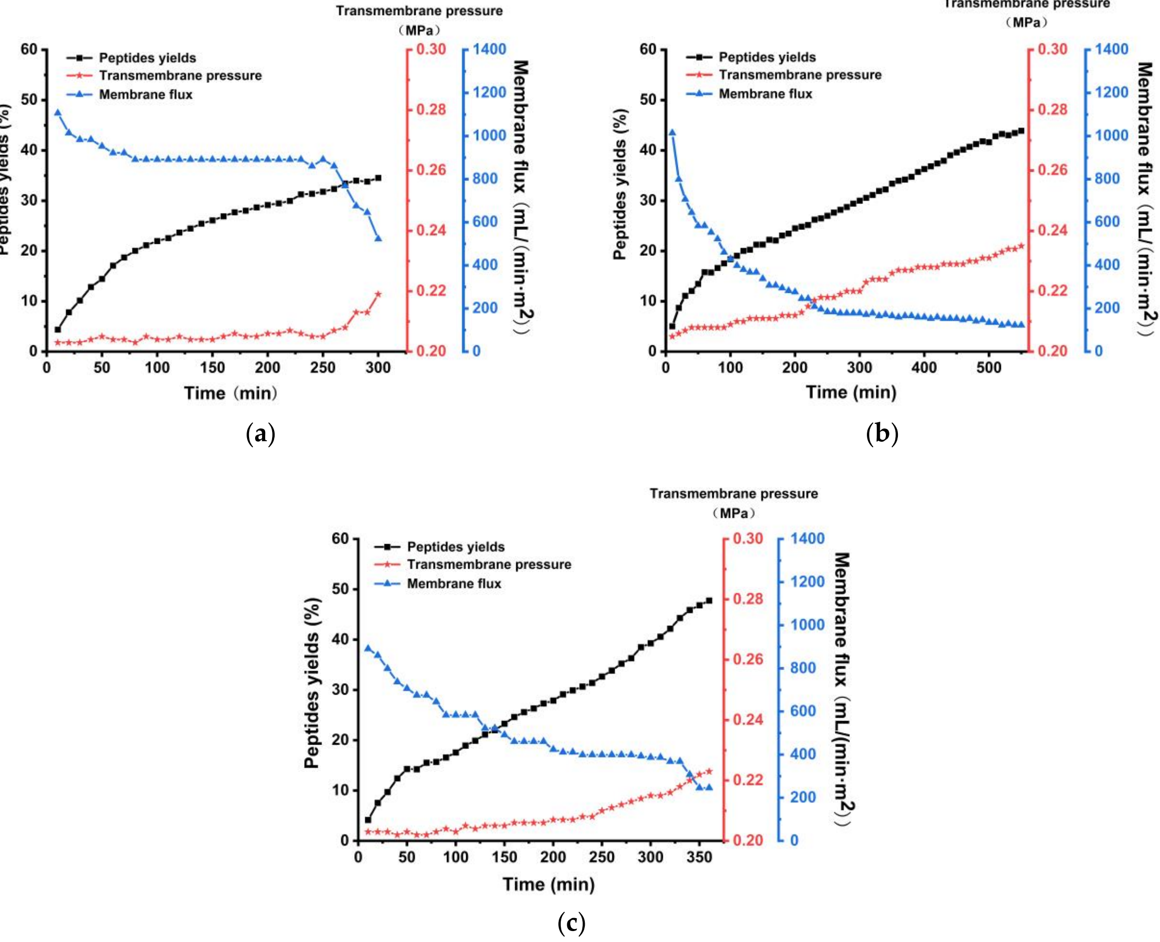

Figure 3. Effects of EMR on protein transformation degree, osmotic flow rate, and transmembrane pressure through constant water feeding, substrate feeding, and gradient dilution feeding. (**a**) Water feeding. (**b**) Substrate feeding. (**c**) Gradient dilution feeding.

3.5.2. Comparative Analysis of Production Efficiency in Enzyme Membrane Coupling Process with Various Supplementary Feeding Modes

As presented in Table 1, the gradient dilution feeding mode manifests substantial enhancements in peptide yield, peptide production, and enzyme-to-peptide production by 14.36%, 11.35%, and 235.63%, respectively, when compared to the conventional enzyme hydrolysis method. In contrast to the continuous feeding mode, the gradient dilution feeding mode demonstrates significant improvements of 4.50%, 7.42%, and 13.32% in the aforementioned parameters. These remarkable advancements can be attributed to the continuous separation of small molecular products from the reaction system facilitated by the ultrafiltration membrane device, thus mitigating product inhibition due to product accumulation. Moreover, the gradient dilution feeding effectively mitigated the efficiency decline resulting from excessively high protein concentrations during the hydrolysis process. This result was also higher than the reported yields of certain peptides, which were 34.1% ± 0.2% from hickory cake meal protein using complex proteases and 17.41% from walnut isolate protein using trypsin [12,25]. Collectively, these results underscore the efficacy of the gradient dilution feeding enzyme membrane coupling process, showcasing its ability to substantially boost the production efficiency of Chinese pecan cake peptides. It serves as an efficient and continuous method for the preparation of Chinese pecan antioxidant peptides.

Table 1. Peptides yield, peptides production, and unit enzyme peptides production of different enzymatic hydrolysis processes.

Method	Traditional Enzymatic Hydrolysis	Enzyme Membrane Coupling			
		Batch Enzymatic Hydrolysis	Water Feeding	Substrate Feeding	Gradient Dilution Feeding
Peptides yield (%)	33.97 ± 0.60 c	31.12 ± 1.70 d	34.61 ± 0.06 c	43.83 ± 0.98 b	48.33 ± 1.19 a
Peptides production (%)	21.27 ± 0.95 c	20.38 ± 0.63 c	22.12 ± 1.90 c	25.20 ± 0.96 b	32.63 ± 0.31 a
Unit enzyme peptides production (g/g)	2.13 ± 0.10 c	2.04 ± 0.06 c	2.21 ± 0.19 c	6.30 ± 0.24 b	7.14 ± 0.07 a

Note: Different lowercase letters (a, b, c, d) in the same column indicate significant differences ($p < 0.05$).

3.6. Comparison of Antioxidant Activity

As illustrated in Figure 4, the Chinese pecan cake peptides produced through the gradient dilution feeding enzyme membrane coupling process manifested optimal DPPH free radical scavenging, ABTS free radical scavenging, and ferrous ion chelating capacities, achieving half-maximal inhibition rates at concentrations of 0.044, 0.518, and 0.250 mg/mL, respectively, which were lower than the reported IC_{50} values of some peptides. For example, the DPPH radical scavenging rate was 0.3 mg/mL for peptides extracted from hickory cake meal protein with complex proteases, and the ABTS radical scavenging rate was 1.8 mg/mL for peptides extracted from watermelon seed protein with alkaline proteases [12,26]. The Chinese pecan cake meal peptides produced by the gradient dilution feeding mode had a ferric iron chelating rate of 71.94% at a concentration of 0.375 mg/mL, which was comparable to that of mulberry leaf protein hydrolysate (73.2%, 0.4 mg/mL), and higher than alfalfa leaf peptides (65%, 0.5 mg/mL) [27,28]. This superiority can be ascribed to the copious presence of hydrophobic amino acids in Chinese pecan cake protein. The gradient dilution feeding mode meticulously regulated the protein concentration within a suitable range within the reactor, ensuring the complete binding of proteinases to substrates. Consequently, this induced the abundant release of specific functional groups, which actively engaged with free radicals and metal ions, leading to remarkable antioxidant effects [17].

3.7. Comparison of Amino Acid Composition

As presented in Table 2, all five variants of Chinese pecan cake peptides demonstrate a predominant abundance of hydrophobic amino acids, comprising proportions of 40.17%, 39.45%, 39.32%, 39.25%, and 39.50%, respectively. Additionally, they exhibit significant richness in acidic amino acids and arginine. Higher levels of hydrophobic amino acids, and negatively charged acidic amino acids (aspartic acid and glutamic acid) have been reported to promote the free radical scavenging activity of peptides [29]. In contrast to the conventional enzymatic hydrolysis method, the Chinese pecan cake peptides obtained through the gradient dilution feeding enzyme membrane coupling process exhibited a remarkable rise in the proportion of acidic and basic amino acids. Scientific literature has documented the ability of these amino acids to effectively quench free radicals through electron donation, consequently amplifying the overall antioxidant activity [30]. Furthermore, the cumulative proportion of aspartic acid, glutamic acid, histidine, and lysine, found in the Chinese pecan cake peptides produced via the gradient dilution feeding mode, amounted to 34.49%. This constituted one of the contributing factors to the peptides' robust metal ion chelating capability. This is in line with the study of Phongthai et al. who found that rice bran protein hydrolysate containing the above-mentioned amino acids exhibited good metal chelating ability [31].

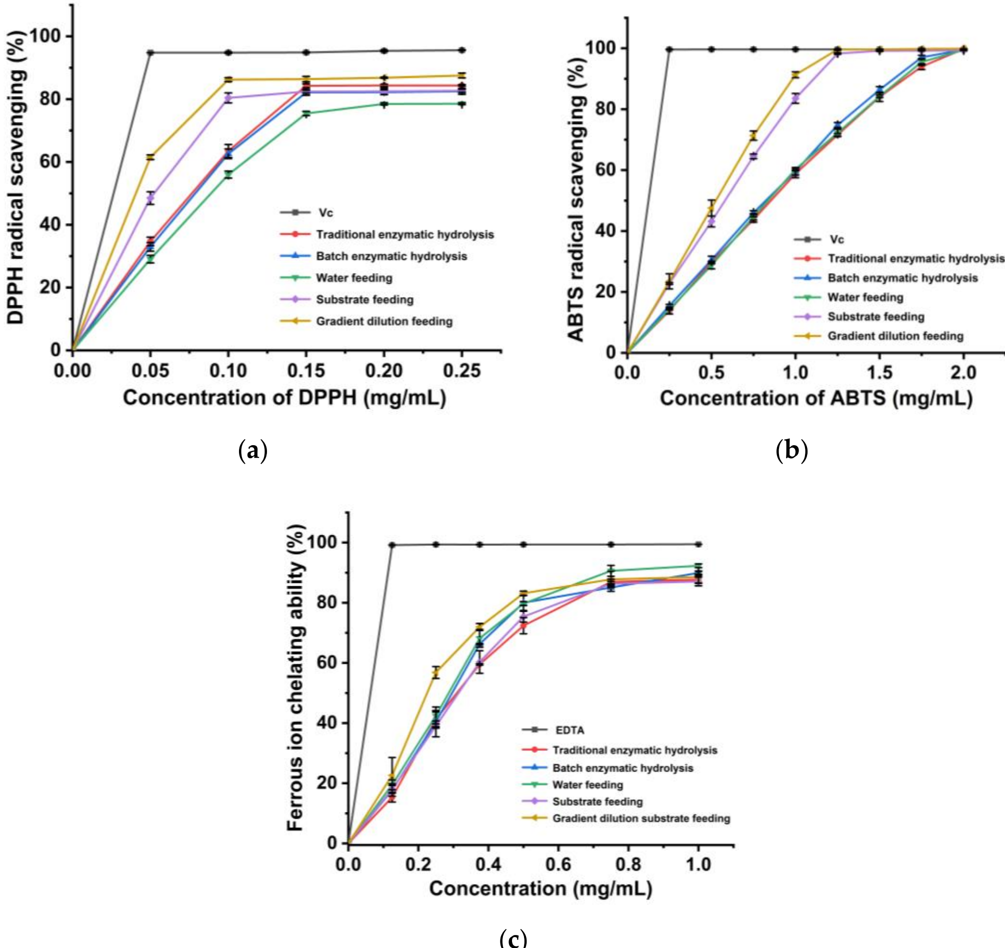

Figure 4. Antioxidant activity of polypeptides from hickory cake produced by different enzyme membrane processes. (**a**) DPPH radical scavenging. (**b**) ABTS radical scavenging. (**c**) Ferrous ion chelating ability.

Table 2. Analysis of amino acid composition of protein and peptide in *Carya cathayensis* cake.

Amino Acid	Proportion (%)				
	Traditional Enzymatic Hydrolysis	Batch Enzymatic Hydrolysis	Water Feeding	Substrate Feeding	Gradient Dilution Feeding
Asp	7.45 ± 0.51 b	7.37 ± 0.66 b	8.64 ± 0.30 a	8.00 ± 0.15 ab	7.81 ± 0.40 b
Glu	20.08 ± 0.20 a	20.47 ± 0.56 a	20.63 ± 0.39 a	20.15 ± 0.13 a	20.7 ± 0.37 a
Ser	5.29 ± 0.08 a	5.51 ± 0.02 a	5.11 ± 0.17 ab	4.91 ± 0.01 bc	4.66 ± 0.03 c
Gly	4.66 ± 0.06 b	4.51 ± 0.02 c	4.66 ± 0.08 b	4.89 ± 0.05 a	4.29 ± 0.13 d
His	1.97 ± 0.03 a	1.82 ± 0.01 b	1.72 ± 0.06 c	1.74 ± 0.08 c	1.94 ± 0.01 a
The	2.89 ± 0.01 a	3.06 ± 0.01 a	2.61 ± 0.44 a	2.96 ± 0.05 a	2.89 ± 0.02 a
Ala	4.77 ± 0.15 a	4.51 ± 0.01 b	4.28 ± 0.07 c	4.16 ± 0.01 d	4.06 ± 0.05 d
Arg	11.54 ± 0.22 b	11.43 ± 0.03 ab	11.46 ± 0.01 b	11.52 ± 0.03 b	11.98 ± 0.02 a
Pro	8.35 ± 0.07 c	8.72 ± 0.06 b	8.99 ± 0.11 a	8.68 ± 0.01 b	9.06 ± 0.01 a
Tyr	2.73 ± 0.08 c	2.75 ± 0.02 bc	2.79 ± 0.01 b	2.85 ± 0.01 a	2.72 ± 0.01 c
Val	5.99 ± 0.01 a	5.76 ± 0.01 b	5.92 ± 0.05 ab	6.06 ± 0.01 a	6.00 ± 0.02 a
Met	1.59 ± 0.01 bc	1.84 ± 0.02 a	1.51 ± 0.02 cd	1.69 ± 0.04 ab	1.41 ± 0.01 d
Cys	0.79 ± 0.01 b	0.99 ± 0.01 a	0.77 ± 0.01 d	0.79 ± 0.01 b	0.78 ± 0.01 c
Ile	4.39 ± 0.03 d	4.49 ± 0.03 ab	4.50 ± 0.04 a	4.45 ± 0.01 bc	4.41 ± 0.01 cd
Leu	8.56 ± 0.13 a	7.93 ± 0.06 b	7.56 ± 0.04 d	7.74 ± 0.01 c	7.79 ± 0.04 c
Phe	5.37 ± 0.06 ab	5.29 ± 0.04 b	5.28 ± 0.02 ab	5.32 ± 0.01 ab	5.46 ± 0.02 a
Lys	3.59 ± 0.04 b	3.55 ± 0.03 b	3.57 ± 0.02 b	4.10 ± 0.01 a	4.04 ± 0.05 a
Aci AAs	27.53 ± 0.31 e	27.84 ± 0.33 d	29.27 ± 0.23 a	28.15 ± 0.02 c	28.51 ± 0.03 b
BAAs	17.10 ± 0.16 c	16.8 ± 0.02 d	16.76 ± 0.08 d	17.36 ± 0.05 b	17.96 ± 0.03 a
EAAs	34.34 ± 0.01 a	33.74 ± 0.15 a	32.66 ± 0.20 a	34.06 ± 0.04 a	33.94 ± 0.11 a
HAAs	40.17 ± 0.32 a	39.45 ± 0.14 b	39.32 ± 0.06 b	39.25 ± 0.01 b	39.50 ± 0.14 b
Aro AAs	8.10 ± 0.07 b	8.04 ± 0.05 c	8.07 ± 0.03 bc	8.17 ± 0.01 a	8.18 ± 0.03 a

Note: Acidic amino acids (Aci AAs), essential amino acids (EAAs), basic amino acids (BAAs), hydrophobic amino acids (HAAs), aromatic amino acids (Aro AAs); different lowercase letters (a, b, c, d, e) in the same column indicate significant differences ($p < 0.05$).

4. Conclusions

Within the context of this investigation, we have successfully devised an enzymatic membrane-coupled production protocol for the synthesis of antioxidant peptides derived from Chinese pecan cake. This accomplishment was achieved through meticulous comparisons of the outcomes yielded by different supplementary feeding modes. Drawing insights from preliminary groundwork and pre-experimental trials, we thoughtfully determined the operative parameters, encompassing a pH of 10.5, a temperature of 50 °C, and an enzyme-to-substrate ratio of 10% (w/w). By employing the gradient dilution feeding mode for continuous production, we attained remarkable results, obtaining Chinese pecan antioxidant peptides showcasing exceptional DPPH free radical scavenging, ABTS free radical scavenging, and ferrous ion chelating capabilities (IC_{50}) quantified at 0.044, 0.518, and 0.252 mg/mL, respectively. Compared to the conventional enzymatic hydrolysis method, the gradient dilution feeding enzyme membrane coupling process has demonstrated substantial augmentations in peptide yield, peptide production, and enzyme-to-peptide production, attaining improvements of 14.36%, 11.35%, and 235.63%, respectively. This continuous production approach significantly simplifies scalability, facilitating the generation of ample quantities of highly potent Chinese pecan cake peptides. Furthermore, it enables more efficient utilization of byproducts arising from the oil extraction process, thereby establishing a sturdy basis for the comprehensive development and sophisticated processing of Chinese pecans, ultimately elevating their economic value.

Author Contributions: Methodology, Q.W., F.W. and H.H.; Validation, Q.W.; Data curation, Q.W., Y.C. and Q.Y.; Writing-original draft preparation, Q.W.; Visualization, Y.C. and Q.Y.; Supervision, Q.Y., F.W., H.H. and J.Z.; Writing-review and editing, J.Z. and Z.H.; Project administration, Z.H.; Funding acquisition, Z.H. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Natural Science Foundation of Xinjiang Uygur Autonomous Region (2021D01A71) and the Key Research and Development Program of Zhejiang Province (2020C02040).

Data Availability Statement: Data are contained within the article.

Conflicts of Interest: The authors declare no conflict of interest.

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