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Production of Bromelain Enzyme from Pineapple Waste by Membrane Process

Thang X. Nguyen, Tan M. Le, Yen H.P. Duong, Phung K. Le, Viet T. Tran*

Faculty of Chemical Engineering, Ho Chi Minh City University of Technology (HCMUT), VNU-HCM, Ho Chi Minh City, Vietnam
trantanviet@hcmut.edu.vn

Bromelain, a proteolytic enzyme derived from pineapple waste, which accounts for more than two-thirds of the total volume of pineapples utilized, has gained significant attention in recent years. The employment of membrane technology has surfaced as a promising approach for the production of enzymes. A complete comprehension regarding the purification and preservation of bromelain enzymes is yet to be achieved. To address this gap, the present study aimed to extract and purify bromelain from pineapple waste to enhance the value of these by-products. The continuous microfiltration (MF), ultrafiltration (UF), nanofiltration (NF), and lyophilization system were optimized to enhance enzyme recovery and purity. The filtration process increased bromelain purity by 1.37-fold and achieved a 96.5 % enzyme recovery rate, indicating its effectiveness in separating bromelain from the pineapple crude extract. The highest activity of 3279.02 GDU/g was achieved through lyophilization, combined with 10 % of skim milk as a cryoprotectant. The product was detected and quantified using Liquid Chromatography with Refractive Index Detector (LC-RID). The combination of membrane and lyophilization processes could potentially separate bromelain from pineapple waste, with promising applications in biotechnology and the food industry.

1. Introduction

The pineapple (*Ananas comosus* L. Merr.) significantly impacts the worldwide commercial market, with a global production of 28.65 million t in 2021 (Shahbandeh, 2023). Pineapple contains nutrients that benefit human health, including sugars, vitamins, and several highly functional proteolytic enzymes, including ananain, comosain, and bromelain. Pineapple production generates a significant amount of waste, such as bud (Avila et al., 2022), core, crown, and peel (Ketnawa et al., 2012). Nowadays, utilizing waste to produce high-value products following the circular economy concept (Le et al., 2023) is one of the appropriate solutions for managing waste from pineapple.

Bromelain is a proteolytic enzyme found in the pineapple stem, fruit, eye, peel, and crown (Chia et al., 2019). Many studies have shown that pineapple wastes have great potential for bromelain production (Misran et al., 2023). Bromelain finds various commercial applications in the food industry (Nanda et al., 2020), pharmaceuticals (Sorokin et al., 2023), and skin care (Abbas et al., 2021). Despite its high applicability and potential advantages, the high efficiency purification of bromelain enzyme from pineapple by-products on a pilot scale have not been extensively studied.

Membrane technology enhances bromelain purity and offers advantages in cost and environmental impact (Banerjee et al., 2022). The membrane processes can act as an interface between feed and product while separating particles from dissolved ions to bacteria (Singh, 2015), making it widely used to purify food and pharmaceutical products. Misran et al. (2023) found promising results in bromelain purification using a single-stage nanofiltration membrane, but purity needs improvement. Membrane fouling can reduce efficiency and lifespan, making it unsuitable for industrial applications. This study used microfiltration, ultrafiltration, and nanofiltration membrane to purify bromelain from pineapple by-product extract. MF is a separation process with a 0.1-5 μm pore to remove colloids, fat, and bacteria while allowing proteins and low molecular weight molecules (Ghenghesh et al., 2005). UF has a molecular weight cut-off (MWCO) range of 3 to 100 kDa with pore sizes of 0.001 - 0.1 μm. NF membranes exhibit high rejection for multivalent ions and larger organic molecules but low

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selectivity for monovalent and nonionized organic molecules (Mw < 150 Da, pore size < 0.001 µm). Bromelain can pass through the filtration system with a molecular weight of approximately 30 kDa (Park and Snyder, 2020). Freeze-drying (lyophilization) is an efficient approach to maintaining the physical structure and extending the shelf-life of materials. Under vacuum pressure, this process involves the direct transition from ice to vapor without an intermediate liquid phase (Mensink et al., 2017). The ice crystal formation during freezing can alter the properties of the product, leading to reduced bromelain activity. Cryoprotectants are used as protectors by binding to the functional sites of bromelain to prevent protein denaturation, lipid oxidation, and ice crystal formation (Joshi, 2016). To integrate bromelain into food contexts, the cryoprotectant must meet criteria such as edibility, safety for human consumption, and stability during freeze-drying. Skim milk is a cryoprotectant that protects biological samples from freezing damage due to its ability to form a coating layer and provide calcium ions (Abadias et al., 2001).

This study aims to extract bromelain from the pineapple by-products through membrane filtration and freeze-drying. The obtained bromelain was identified and quantified by HPLC analysis. The effects of parameters for purified bromelain in different processes were also investigated using the specific activity, enzyme recovery, volume reduction factor, and purification factor. During lyophilization, the impact of skim milk with different concentrations and ratios on bromelain was studied. The microbial loading of the bromelain was evaluated with various microorganisms. The results of this study demonstrate the potential for developing an effective process for producing bromelain from pineapple by-products.

2. Materials and Methods

2.1 Materials and chemicals

The queen pineapples (*Ananas comosus* L. Merr) wastes, including crown, peel, and eye, were collected from Tien Giang province, Vietnam, and were rinsed and cut into small pieces. All the samples were stored at -5 °C for further use. All reagents were obtained from commercial suppliers with the pure grade, including bovine serum albumin, copper (ii) sulfate pentahydrate ($CuSO_4.5H_2O$), trisodium citrate dihydrate ($C_6H_5O_7Na_3.2H_2O$), Folin-Ciocalteu's phenol reagent, 3,5-dinitro salicylic acid ($C_7H_4N_2O_7$), sodium potassium tartrate ($KNaC_4H_4O_6.4H_2O$) was purchased from Sigma Aldrich. Sodium hydroxide (NaOH), formaldehyde (HCHO), phenolphthalein ($C_{20}H_{14}O_4$), glucose ($C_6H_{12}O_6$), sodium carbonate (Na_2CO_3), disodium hydrogen phosphate dodecahydrate ($Na_2HPO_4.12H_2O$), hydrogen peroxide (H_2O_2), potassium dihydrogen phosphate (KH_2PO_4) methanol (MeOH), hydrochloric acid (HCl), were purchased from Xilong. Gelatin Bloom A250 was purchased from Rousselot. Skim milk was purchased from Himedia.

2.2 Bromelain extraction from pineapple waste

The raw pineapple wastes (40 % crown, 40 % peel, and 20 % core) were extracted using phosphate buffer (pH 7.0) in the ultrasonic bath at a solid-to-liquid ratio of 1:2 (w/v), 25 °C for 10 min. The mixture was then filtered through a cloth filter (Nylon Monofilament Mesh (NMO), 35 µm) to remove residue and gain the crude extract.

2.3 Bromelain purification process

This study's purification process included 4 stages using a membrane system at the pilot scale at room temperature and pressurized by a Japanese electric water pump. At stage 1, the crude extract was fed into a membrane module with a 1-micron filter (Taiwan) to remove impurities. For stages 2, 3, and 4, the extract was gone the membranes modules in turn 0.2 Micron Polysulfone Membrane Filters (Taiwan) at 0.058 mPa from 40 L HDPE tank, Taiwan nano-silver activated carbon filter, 100 kDa ceramic ultra-filtration membrane, a 10 kDa NanoCeram Filter to obtain purified bromelain (30 kDa). Bromelain was concentrated in the retentate, and low molecular weight compounds were separated in the permeate.

Enzyme recovery, which represents the percentage of feed extract filtered as permeate in the ultrafiltration stage, was calculated as Eq(1).

$$\text{Enzyme Recovery} = \frac{C_P.V_P}{C_F.V_F} \times 100\ \% \quad (1)$$

where C_p is the bromelain concentration of permeate, V_p is the volume of the permeate. C_F is the bromelain concentration of the feed, V_F is the volume of the feed.

The Volume Reduction Factor (VRF) was the ratio between the feed and retentate volumes (as Eq(2)), representing the concentration of the concentrated enzyme (Castro-Muñoz and Yáñez-Fernández, 2015). The determination of the purification – fold was calculated according to the enzyme's specific activity following Eq(3).

$$\text{VRF} = \frac{V_F}{V_R} \times 100\ \% \quad (2)$$

where V_F is the volume of the feed (mL), V_R is the volume of the retentate (mL).

$$\text{Purification} - \text{fold} = \frac{\text{SA}_\text{R}}{\text{SA}_\text{CE}} \quad (3)$$

where SA_R is the specific activity of the enzyme in the retentate (GDU/g), SA_{CE} is the specific activity of the enzyme in the crude extract (GDU/g).

2.4 Effect of skim milk on the stability of bromelain in the lyophilization process

Skim milk is diluted in phosphate buffer pH 7.0 following the suitable concentration. The purified bromelain is mixed with cryoprotectant solution, freezing for 24 h at – 60 °C before evacuation. The effects of skim milk on bromelain during the free-drying process were estimated with different concentrations of skim milk and the ratios of the extract and cryoprotectant.

2.5 Enzyme-specific activity of purified bromelain

Proteolytic activity was assessed using the Gelatin Digestion Unit method (Krishnan and Gokulakrishnan, 2015). The protein content was estimated according to (Lowry et al., 1951). High-performance liquid chromatography (HPLC) of 250 nm with an ultraviolet (UV) detector was used to quantify the vitamin C content in the product. The HPLC analysis used a C18 250 mm column maintained at 30 °C and a 1 mL/min dynamic flow rate. The mobile phase comprised Formic acid (0.1 %) and MeOH for HPLC.

2.6 Liquid chromatography with refractive index detector

The extract was subjected to analytical HPLC with RID set at 35 °C, Ultrahydrogel 250 Å 6 µm column to verify the bromelain. The mobile phase comprised 10 % acetonitrile, 0.2 % trifluoroacetic acid, and deionized water. The bromelain standard of 182.9 Units per milliliter (U/mL) was prepared by mixing 29.5 mg of bromelain with mobile phase to 5 mL solution and storage at 4 °C. The column temperature was maintained at 50 °C with a dynamic flow rate of 1 mL/min (Encarnação et al., 2020).

2.7 Microbiological loading of bromelain

The microbiological loading of final product was performed by The Center for Analytical and Experimental Services of Ho Chi Minh City, including *Coliform*, *Escherichia coli (E. coli)*, total yeast, molds, and total plate counts. *Coliform* bacteria form in the (ISO 4832:2006) Colony-count technique. *E. coli* were identified based on (ISO 16649-3:2015). Yeast and molds were evaluated on mycological agar medium described in (ISO 21527-1:2008). The total plate counts method uses selective culture media based on (ISO 4833-1:2013).

3. Result and Discussion

3.1 Effect of pre-filtration and micro-filtration on crude extract

The protein content of crude extract varied from around 1250 - 1350 (mg/mL) after pre-filtration. Figure 1a shows the specific activity and protein content through the microfiltration process. The highest specific activity was achieved at 15 min (599.593 GDU/g) and decreased for the remaining process due to insufficient filtration time to eliminate large molecular proteins. Longer filtration time causes high friction between extract and pipeline, which decreases the specific activity (Martins et al., 2014). The protein content showed a high value in low operating pressure and peaked at 25 min (1284.20 mg/mL).

3.2 Effect of ultra and nanofiltration on filtered extract

Figure 1b shows the relationship between the enzyme recovery and the permeate flow rate during the UF stage. The enzyme recovery percentage was used to determine the efficiency of the purification process, with 96.5 % at 37 mL/min. After that, the membrane was almost fouled, due to near surface effect or blockage and constriction of the membrane pores, causing a decrease in flow rate (Polyakov and Zydney, 2013). The highest value of bromelain content was 22.87 mg/mL in permeate and retained in the retentate stream, even when the enzyme recovery was 96.5 %. At NF stage, the association between the flow rate behavior and VRF was shown in Figure 1c. Increased VRF is associated with a lower permeate flow rate and improved purification fold. The membrane pore fouling and/or constriction in the NF stage may lead to the permeate flow rate declines observed (Polyakov and Zydney, 2013). The results suggest that the enzyme purification can be changed according to different VRF to increase the applicability of bromelain. The optimized purification factor is 1.38 fold which is higher than the result of Babu (1.2 fold), who also purified pineapple core and peel extract with ultrafiltration membrane (Babu, 2008).

3.3 HPLC-RID results of bromelain

Figure 1d indicates the purity of bromelain extract in different stages. The peak of impurities for the crude extract is substantial, with a retention time of 9.59 min. The MF stage had lower impurities with a peak at 9.51 min. At the UF stage, the impurities smaller than 100 kDa have not been removed and show in a small peak next to the bromelain peak. The bromelain content increased up to 35.5 % compared to the crude extract. In NF stage, the sharp peak of bromelain at 9.50 min indicates a high purity of the solution. The enzyme content dramatically increased from 17.05 mg/mL to 28.50 mg/mL after 4 stages of purification.

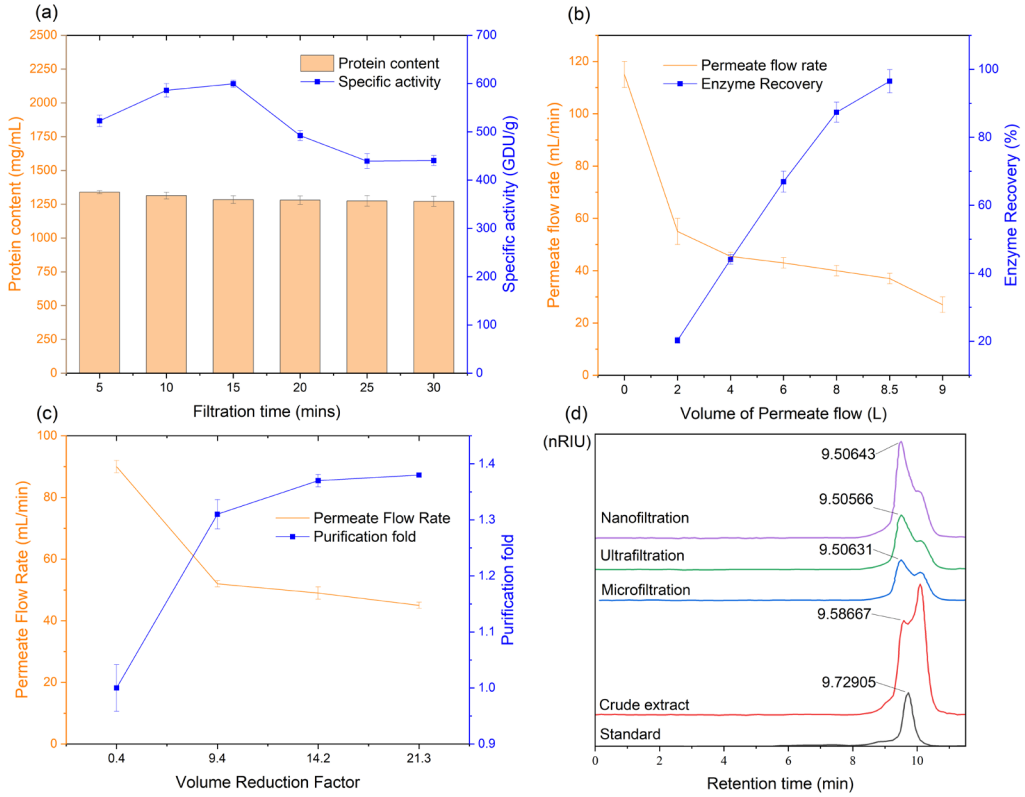

Figure 1: Specific activity and protein content of the enzyme through the (a) MF stage, (b) Permeate flow rate - enzyme recovery according to the volume of permeate flow in UF stage, (c) permeate flow rate and purification fold at different VRF of NF stage, and (d) HPLC chromatogram of the extract at different filtration stages

3.4 Effect of cryoprotectant on bromelain preservation

Figure 2a shows that the specific activity of the product decreased gradually when the concentration of skim milk increased. The enzymatic activity was highest at 10 % skim milk concentration with 3279.022 GDU/g. The high concentration of cryoprotectant leads to the waste of materials and decreases the proteolytic activity during drying.

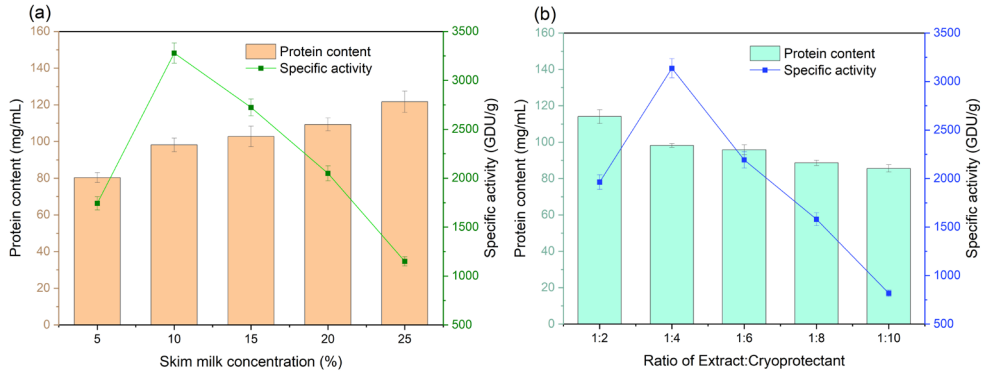

Figure 2: Effect of (a) concentration of cryoprotectant and (b) the ratio between cryoprotectant and crude extract in protein content and proteolytic activity of purified bromelain

With the investigation of the appropriate ratio between cryoprotectant and crude extract in Figure 2b, as the skim milk content was increased, the protein content and specific activity of bromelain decreased. The specific activity increased when the extract/cryoprotectant ratio was approximately 1:4.

3.5 Product qualification

Table 1 presents compelling evidence of the exceptional efficacy of bromelain in controlling microbial growth and ensuring the safety of the product, surpassing the performance exhibited by commercial products. These findings underscore the impressive capacity of bromelain to fulfill the stringent criteria mandated for its safe utilization within the food industry. The recorded colony count in the product demonstrates an exceptionally low magnitude, registering levels below 01 CFU/mL, further confirming its suitability for consumption. Bromelain exhibits broad-spectrum activity against various microorganisms, indicating its potential superiority over existing alternatives (Mamo and Assefa, 2019). The presence of Vitamin C in pineapple extract is a noteworthy nutritional attribute. With a molecular weight of 176.12 Da (Dawood and Koshio, 2018). The concentrated extracts maintain a consistent vitamin C content of 36.54 µg/mL, reflecting its stability and resistance to degradation due to its small size and inherent instability.

Table 1: Product qualification

Microbial Profile	Bromelain	Bromelain (commercial)	Method
Coliform	< 01 (CFU/mL)	-	ISO 4832:2006
Escherichia coli	Negative	Negative	ISO 16649-3:2015
Total plate counts (TPC)	< 01 (CFU/mL)	≤ 1000 (CFU/g)	ISO 4833-1:2013
Total yeast mold	< 01 (CFU/mL)	≤ 100 (CFU/g)	ISO 21527-1:2008
Vitamin C	36.54 (µg/mL)		

4. Conclusion

The membrane technology employed in this study effectively separated bromelain from other components, increasing bromelain purity and achieving a 96.5 % enzyme recovery rate. The study demonstrated that 10 % of skim milk as a cryoprotectant achieved the highest activity of 3,279.02 GDU/g. The sequential filtration steps can result in a highly purified bromelain extract with improved activity and purity, which is beneficial for its commercial applications in various industries. Freeze-drying maintains the stability and activity of bromelain during storage. These processes are cost-effective, efficient, and environmentally friendly, but they require specialized equipment and skilled operators, which can increase production costs.

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