Purification and Characterization of Bromelain from Waste Parts of *Ananas comosus* for its Application in Detergent Industry

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A B S T R A C T

Bromelain is a thiol-containing cysteine protease isolated from the stem, peel, and leaf parts of *Ananas comosus*. Protease accounts for 60% of the total enzyme market. Being a plant-based cysteine protease, it has various pharmaceutical and biotechnological applications. The isolation and purification cost of the enzyme is highest, so there is a need to develop cost-effective purification methods. This research work aims to purify BRM by using magnetic nanoparticles. After purification, BRM enzyme was characterized and utilized in the detergent industry due to its stability at a wide range of pH and temperature; results showed that stem BRM has the highest enzyme activity at 23.5 U/ml as compared to crown leaf (11.7 U/ml) and peel (19.5 U/ml). The specific activity of leaf, peel, and stem BRM was determined 0.8, 1.2, and 1.4 U/mg, respectively. Total protein content was 660 mg, 810 mg, and 865 mg from leaf, peel, and stem samples. The percentage yield of BRM was determined in the range of 60 to 93 % from these waste parts of the plant. This study achieved 4, 6, and 7-fold purification for leaf, peel, and stem BRM, respectively. This study showed positive results for utilizing affinity-purified BRM enzyme in the laundry and detergent industry due to its stability in different detergents and destaining properties.

INTRODUCTION

Around the globe, plant proteins are being used as a food source to accomplish human physiological needs. The plant proteins are mostly proteases that break the peptide bond leading to the degradation of protein into amino acids and peptides (Nehete *et al*., 2013; Muntari *et al*., 2012; Manzoor *et al*., 2016). Bromelain is extracted from the stem, fruit, crown, leaves, and peel of the pineapple plant (*Ananas comosus*). It has great commercial value worldwide (Pavan *et al*., 2012; Chobotova *et al*., 2010; Ramli *et al*., 2017). Crude extract of BRM has a complex mixture of cysteine proteases, including other components such as phosphatases, peroxidases, carbohydrates, and organic calcium. BRM works at a pH range of 5.5-8.0, where it is stable for longer period of time. The nature of stem BRM is alkaline, but fruit BRM is slightly acidic (Agrawal *et al*., 2022; Ramalingam *et al*., 2012; De Lencastre Novaes *et al*., 2016; Arshad *et al*., 2014). Pineapple is 3rd most important fruit, and 18.7 million tons of production was estimated by 2014 (Bresolin *et al*., 2013; Hossain, 2016). Pineapple fruit represents 23% of the plant part rest is considered agro-industrial residue, which contains a huge amount of BRM. Many industrial and pharmaceutical applications increase the market value of BRM (Martins *et al*., 2014).

BRM is considered a promising drug in the pharmaceutical industry and has many health benefits, e.g., reduce pain and swelling, decrease formation of clots by preventing platelet aggregation (Lopez and Bond, 2008; Souza *et al*., 2015; Bala, 2015; Rathnavelu *et al*., 2016). BRM has gained attention in different industries due to its eco-friendly nature and stability, e.g., food, detergent, baking, textile, wine-making, cosmetics, meat tenderization, and leather. Proteases account for 60% of all commercial enzymes worldwide (Zambare *et al*.,

2011; Shah *et al.*, 2014; Watanabe *et al.*, 2000; Kaur and Chakraborty, 2015; Levy and Emer, 2012). In the detergent industry, proteases are used to enhance the effectiveness of detergent. The use of enzyme in detergents is environment friendly because less amount of detergents or chemicals are used during washing. The major hindrance to protease utilization in the detergent industry is its stability in commercial detergents at broad pH and temperature ranges.

Commercially BRM is produced and purified by using chromatographic techniques. Purification and downstream processing of BRM increases the production cost. The present study aims to develop a new cost-effective procedure for purification of BRM by using interactions between arginine-conjugated magnetic nanoparticles and BRM enzyme. The purified BRM was characterized and used to evaluate its stability in different detergents. The destaining properties of the enzyme were also determined under designed experimental conditions.

MATERIALS AND METHODS

Materials

The pineapple variety *Kona sugarloaf*, a type of smooth cayenne, was purchased from the local market. Casein (Sigma-Aldrich), 1-Ethyl-3-(3-dimethylaminopropyl) *carbodiimide* (EDAC) (Thermo Fisher Scientific), Bovine serum albumin (Thermo Fisher), Arginine (Thermo Scientific) were utilized. Highly purified and analytical-grade chemicals were used in this study. Commercially available detergents were purchased from the local market.

Extraction of crude BRM from pineapple

Peel, crown leaf, and stem samples (200 g each) were cut into pieces, washed with distilled water, homogenized separately in chilled phosphate buffer (25 mM, pH: 7), filtered on a muslin cloth, and centrifuged at 10,000 rpm for 20 min at 4 °C. The supernatant was subjected to ammonium sulfate precipitation at 40-60% saturation and dialyzed.

Enzyme assay

For enzyme assay, BRM extract (1 ml) was added to a 15 ml falcon tube and mixed with casein (15% w/v solution in 50 mM potassium phosphate buffer) and incubated for 10 min in a water bath at 37 °C. Trichloroacetic acid solution (3 ml) was added and kept at room temperature for 5-10 min, then centrifugation was done at 10,000 rpm for about 5 min. The supernatant was taken in a 15 ml falcon tube and combined with 5 ml of Na_2CO_3 (500 mM). After mixing the contents, Folin-Ciocalteu (FC) reagent (2 ml) was added. The optical density was evaluated at 660 nm

(Folin and Ciocalteu, 1927). One unit of enzyme activity is defined as the amount of BRM required to release 1 µM of L-tyrosine per minute under standard experimental conditions.

Determination of protein concentration

Bradford assay was used to estimate protein concentration using bovine serum albumin as standard (Bradford, 1976).

Conjugation of magnetic nanoparticles (MNPs) with arginine (ARG)

MNPs were prepared as described by Lodhi and Samra (2019). Arginine (0.1 g) was added to a 50 ml falcon tube and mixed with 25 ml of distilled water. The pH 8 of the arginine solution was adjusted by NaOH (1 M). Magnetic nanoparticles (0.5 g) were added to the solution and sonicated at 4 °C for 10 min on ice. The solution was placed at 37 °C in a water bath for 15 min. After cooling the mixture on ice, 0.4 g of 1-Ethyl-3-(3-dimethylaminopropyl) *carbodiimide (EDAC)* was mixed, and the pH was adjusted at 6 with HCl (1 M). The mixture was kept in the dark for 6 h at 25 °C with constant stirring. Again 0.4 g of EDAC was added and kept in the dark for 15 h with continuous stirring. The magnetic field was applied to separate arginine-conjugated magnetic nanoparticles (ARG-MNPs) and washed three times with acetone. Particles were air-dried and stored in an airtight container. The conjugated particles were processed for Fourier transform infrared (FTIR) analysis to confirm the conjugation of arginine with MNPs.

Purification of BRM by ARG-MNPs conjugates

MNPs (0.1 g) were taken in a 50 ml falcon tube and combined with 5 ml of dialyzed ammonium sulfate precipitated crude bromalin. The tube was placed in a shaker for 5 h at 25 °C. The conjugated complex (BRM-ARG-MNPs) was separated by an external magnetic field and washed with 5 ml of 10 mM Tris-Cl, pH 7.2. Then 2 ml of elution buffer (5 mM Tris-Cl, pH 7.4) was added for the elution of the enzyme. The magnetic field was applied again to separate ARG-MNPs, and the enzyme was eluted and dialyzed against 500 ml of 5 mM Tris-Cl, pH 7.4. The sample was run on 12 % SDS PAGE. The protein concentration was determined by Bradford assay (Bradford, 1976).

Characterization of bromelain (BRM)

For determination of molecular weight affinity-purified BRM was run on 12% SDS PAGE, stained with coomassie brilliant blue, and then destained. The relative molecular mass of BRM was assessed using a standard

protein marker (Pageruler prestained protein ladder 10-180 kDa).

For determination of optimum pH the effect of pH on BRM activity was determined at different pH ranges (3, 5, 6, 7, and 8) under standard experimental conditions in 0.05 M buffer (glycine-HCl buffer pH: 2-3, Na-citrate buffer pH: 4-5, Na-phosphate buffer pH: 6-7, Tris-Cl buffer pH 8-10).

For determination of optimum temperature aliquots of affinity-purified BRM were kept at different temperatures (20, 30, 40, 50, 60, 70, and 80 $^{\circ}$C) under standard experimental conditions. BRM activity was estimated by using enzyme assay as described above.

Thermal stability profile was also determined by incubating the enzyme for 30 min at different temperatures (20, 30, 40, 50, 60, 70, and 80 $^{\circ}$C) under standard assay conditions.

All experiments were carried out in triplicates.

Stain-removing property of purified BRM

The stain-removing property of BRM was evaluated by dissolving affinity-purified BRM in phosphate buffer (pH 7). Cotton fabric (8 cm x 8 cm) was stained with 1 ml of goat blood, cut into pieces, and placed in labeled glass plates. Stained cotton cloths were treated with detergent and BRM enzyme. Glass plates were incubated at 37 $^{\circ}$C for 30 min. Pieces were taken out after 30 min, washed with water, and visualized to check the stain-removing property of BRM. An untreated, stained piece of cloth was taken as control. After that, all cotton pieces were washed, air dried, cut into smaller pieces separately, and put in 50 ml falcon tubes individually (labeled as A, B, C, and D) having 30 ml normal saline. Centrifugation was carried out at 10,000 rpm for 15 min. The optical density of the supernatant was measured at 660 nm after removing the pellet.

Stability of purified BRM in different detergents

Affinity-purified BRM was used to check its stability in different local detergents (Surf-Excel, Brite, and Ariel). BRM solution (2 mg/ml) was mixed with the above detergent (20 mg/ml) and incubated for 60 min at 40 $^{\circ}$C. BRM activity was determined by using enzyme assay as described above. Without the detergent, the activity of BRM was taken as 100 %.

Experimental design for destaining procedure

Three glass plates with blood stained cotton were treated as follows.

A: 15ml BRM (2 mg/ml) + 30ml phosohate buffer

B: 30 ml detergent (20 mg/ml)

C: 30 ml detergent + 15ml BRM

RESULTS AND DISCUSSION

Crude extract of BRM is a mixture of proteases that contain sulfhydryl group and hydrolyze peptide bonds. In this study, BRM was purified from waste parts of *Ananas comosus* by using a novel magnetic affinity purification technique.

The related peaks of magnetic nanoparticles (MNPs) were analyzed by the FTIR spectrum (Fig. 1A), and Figure 1B shows the FTIR spectrum of ARG-MNPs. The change in spectrum functional group peaks confirmed the successful conjugation of arginine on MNPs.
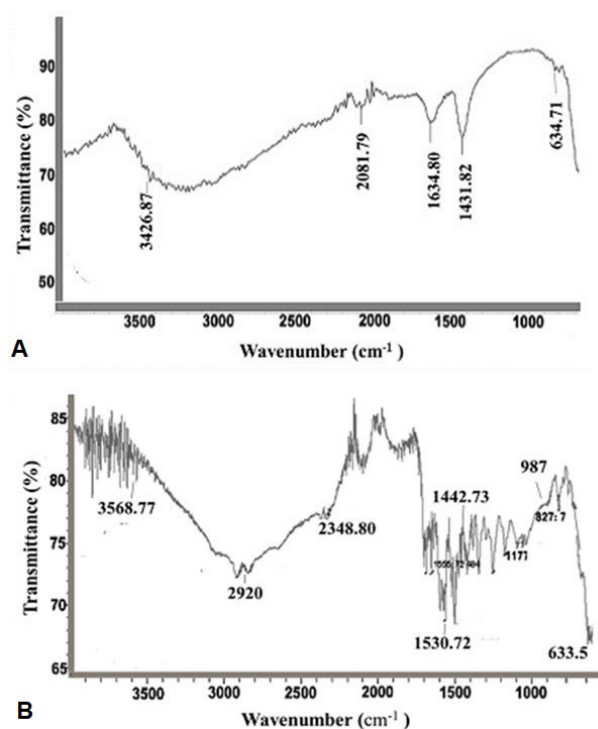

Fig. 1. A, FTIR spectrum of magnetic nanoparticles (MNPs). A peak at 634.71 cm^{-1} is due to Fe-O bond vibrations confirming MNPs synthesis. For OH groups, a peak is observed at 1634.80 cm^{-1}. B, FTIR spectrum of conjugated ARG-MNPs. FeO peak (633.5 cm^{-1}), N=C peak (2348.8 cm^{-1}), and the overlapping peak of OH-NH (3568.7 cm^{-1}) confirm successful conjugation of arginine with MNPs.

BRM enzyme activity was 8.5 U/ml (crown leaf), 11.2 U/ml (peel), and 14.5 U/ml (stem) after ammonium sulfate precipitation, as given in Table I. At this saturation level, most of the BRM enzyme was precipitated, which is consistent with the findings of Ramalingam *et al*. (2012). After affinity purification, enzyme activity was measured as 11.7 U/ml (crown leaf), 19.5 U/ml (peel), and 23.5 U/ml

(stem). Stem BRM showed maximum activity, as was also observed in Gautam *et al*. (2010). They reported that stem BRM is more active and superior than BRM isolated from other parts of the plant. Bala (2015) reported 1.19 U/ml activity of stem BRM by using N α-carbobenzoxy-lysine p-nitrophenyl ester (LNPE) substrate with a purification fold of 4.64 after ion exchange chromatography. Bresolin *et al*. (2013) studied that after ion exchange chromatography, the specific activity of peel BRM was increased up to 1.13 U/mg, which is 1.2 U/mg after magnetic affinity purification. Silvestre *et al*. (2012) reported a higher value of specific activity (1.6 U/mg) for peel BRM by using hemoglobin as a substrate. In our study, specific activity for peel and stem BRM was achieved up to 1.2 and 1.4 U/mg, respectively, using casein as a substrate. Susanti *et al*. (2022) also reported partial characterization of crown BRM isolated from the Queen variety of pineapple plant by employing 60 % ammonium sulfate precipitation. The specific activity was achieved as 0.33 U/mg at 40 ºC. The protein content and activity of BRM were calculated as 4.41 mg/ml and 1.36 U/ml, respectively. In our findings, the specific activity was 0.4 U/mg after 60% ammonium sulfate precipitation, and it was increased up to 0.8 U/mg after the affinity purification process. Variation in specific activity and protein content of BRM depends on various factors, i.e., isolation from different varieties and different plant parts, the maturity level of the plant, purification strategy, cultivation conditions, the composition of BRM extract, etc. BRM can be purified using various techniques, but our method is simple, cost-effective, gives a good yield with minimum loss in enzyme activity, and is reliable.

Further, compared to the traditional method, protein elution increases the volume of purified protein which requires an additional lyophilization step but unfortunately, lyophilization produces freezing and drying stress that can denature protein to various extents (Cerdan-Leal *et al*., 2020). Here, the magnetic field application prevents time by reducing the number of purification steps and purifying the protein in concentrated form. Hence, there is no requirement for lyophilization.

A band of 23 kDa was observed for affinity-purified BRM isolated from the leaf, peel, and stem of the *Ananas comosus* (Fig. 2). Molecular weight of stem BRM was reported as 24 kDa (Ferreira *et al*., 2011). Silveira *et al*. (2009) reported a band of 31 kDa for stem and fruit BRM. The molecular weight of BRM was reported as 26 kDa by Umesh *et al*. (2008). Maurer (2001) reported a band of 23 kDa for stem BRM. This variation arises due to the isolation of BRM from different sources, variety of *Ananas comosus*, age, cultivation time, conditions, and area that can alter enzyme content and activity (Bartholomew *et al*., 2003; Wu *et al*., 2017). The single band indicated complete

integrity and purity of BRM, as shown in Figure 2. Our results indicated that BRM could be purified more by magnetic affinity purification.
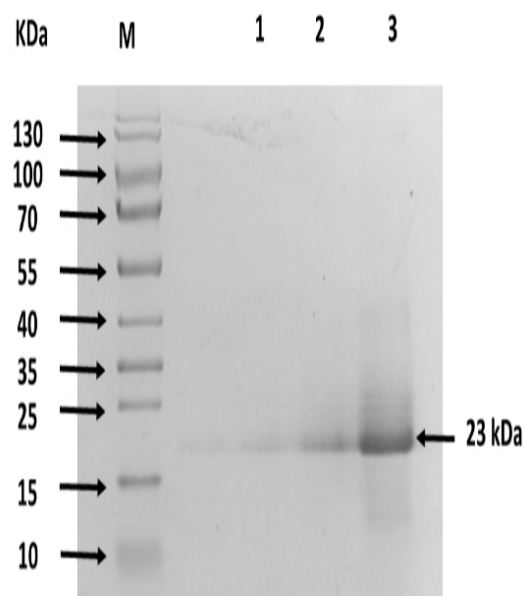

Fig. 2. 12% SDS PAGE indicating 23 kDa band of affinity purified BRM enzyme. Lane M; Prestained protein ladder (Pageruler 10-180 kDa). Lane 1; Leaf BRM. Lane 2; Peel BRM, Lane 3; Stem BRM.

Protein content was higher in the stem than peel and crown leaf. The former exhibited high protease activity. Ramalingam *et al*. (2012) reported that the pulp part has a higher protein concentration than the leaf parts. In our study, after affinity purification, protein content was 660 mg, 810 mg, and 865 mg from leaf, peel, and stem parts, respectively. Results showed that BRM content was less in the leaf and highest in the stem comparatively. Ketnawa *et al*. (2012) reported the leaf part's highest protein content (141 to 220 mg) and protease activity. This study achieved a 2.5-fold increase in specific activity after ammonium sulfate precipitation and dialysis. Bresolin *et al*. (2013) reported a 2.2-fold increase in specific activity for peel BRM. Devakate *et al*. (2009) and Singh *et al*. (2004) also described almost the same purification fold of crude BRM from pineapple waste parts. Chaurasiya and Hebbar (2013) reported 3.96-fold purification for fruit BRM by employing reverse micelles extraction system. Arumugam and Ponnusami (2013) reported a 6.2-fold purification of fruit BRM using functionalized mesoporous silica. Our results indicated a 4, 6, and 7-fold increase in specific activity of leaf, peel, and stem BRM, respectively (Table I), which is highest than other purification methods.

Table I. Comparison of specific activity, Purification fold and Yield for peel, crown leaf, and stem BRM.

| Purification strategy	Total volume ml	Enzyme activity U/ml	Enzyme concentration mg/ml	Total protein mg	Total activity units	Specific activity U/mg	Yield %	Purification fold
Crown leaf BRM								
Bromelain extract	250	5.61	25.3	6325	1402.5	0.2	100	1
40-60 % $(NH_4)_2SO_4$ and dialysis	100	8.5	18.5	1850	850	0.4	60	2
Magnetic affinity purification	50	11.7	13.2	660	585	0.8	41.7	4
Peel BRM								
Bromelain extract	250	4.2	30.1	7525	1050	0.2	100	1
40-60 % $(NH_4)_2SO_4$ & dialysis	100	11.2	23.5	2350	1120	0.5	107	2.5
Magnetic affinity purification	50	19.5	16.2	810	975	1.2	92.8	6
Stem BRM								
Bromelain extract	250	7.3	37.5	9375	1825	0.2	100	1
40-60 % $(NH_4)_2SO_4$ & dialysis	100	14.5	29.2	2920	1450	0.5	79.4	2.5
Magnetic affinity purification	50	23.5	17.3	865	1175	1.4	64	7
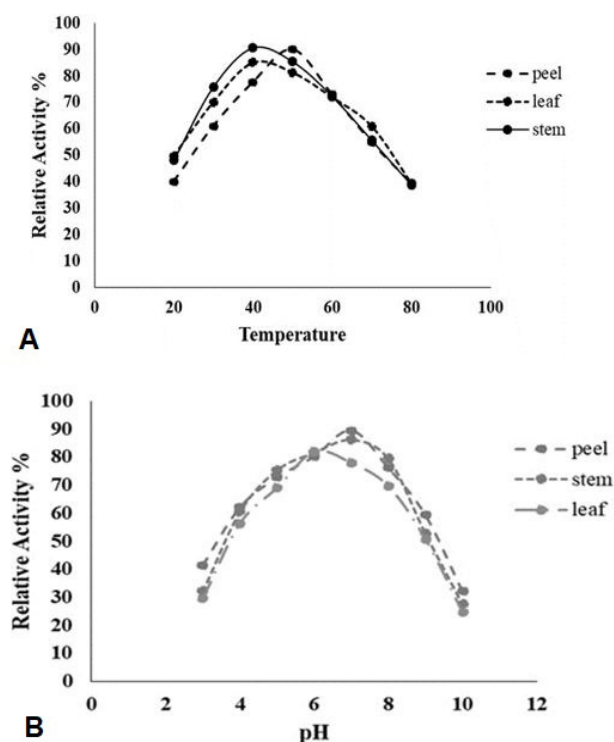
Fig. 3. Optimum temperature (A) and pH (B) for peel, crown leaf, and stem BRM. Stem and peel BRM showed maximum activity 40 °C and at pH 7. Peel BRM exhibited maximum activity at 50 °C. Leaf BRM showed the highest activity at pH 6.

The optimum temperature for the crown leaf and stem was 40 °C, and for the peel, it was 50 °C (Fig. 3A). BRM extracted from all parts was stable up to 60 °C and retained 60-70% activity at 70 °C after that, the enzyme activity was lost due to denaturation of enzyme's active site. Ketnawa et al. (2010) also reported that the optimum temperature was 55 °C for BRM isolated from pineapple peel. BRM is stable at 40 to 60 °C for a more extended period, where most enzymes show denaturation. This property of BRM makes it useful for its application in biotechnology, e.g., detergent, textile, and pharmaceutical industries. A large quantity of fiber is left behind after BRM isolation from all these parts, which can be utilized in the paper industry. The optimum pH for the leaf was 6.00, and for the stem and peel, it was 7.00 (Fig. 3B). Ketnama et al. (2012) reported a pH 7.00 for BRM from the peel and stem parts of the pineapple in the Nang Lae variety. Our results are similar to previous reports. Different types of BRM isolated from other sources, e.g., peel, fruit, stem, and leaf, show optimum temperature and pH variation.

Stem BRM activity was highest, so it was used to check its stability and activity in detergent. BRM solution was mixed in three different detergents and incubated at optimum temperature for 1 h. Stability was determined using casein hydrolysis assay after incubation of enzyme in detergent for 1 h in the form of % relative activity. Maximum activity of the enzyme was observed in 81 to 90 % of all three detergents. This data indicates that stem BRM is stable in detergent and alkaline conditions.

Stain-removing properties were visualized, as shown in Figure 4. Results indicate that BRM is more active alone and removes stains effectively. It was observed that stains were obliterated when treated with BRM in combination with detergent (Table II). This activity difference is due

to the presence of various components in detergents. Protease helps to get rid of proteinaceous stains rapidly with minimal utilization of detergent. But detergent alone cannot remove these stains easily; an enormous amount of detergent will be used to remove these stains. Similar results were reported by Jaouadi *et al.* (2008) and Rao *et al.* (2009) for alkaline and cysteine proteases, respectively. Adding BRM in detergent minimizes the quantity of detergent used, which is eco-friendly because higher concentrations of chemicals used in detergents cause water pollution. Using stable and more active enzymes in detergent can boost its effects and reduce pollution. High concentration of detergent in water leads to drinking water contamination, reducing crop production, etc.
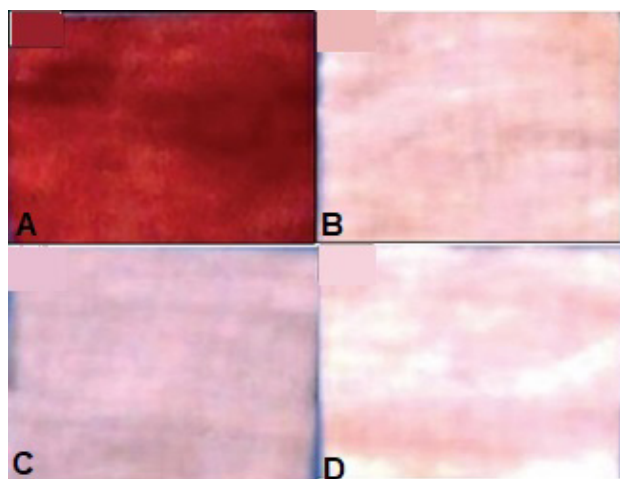

Fig. 4. Blood stains removed by affinity-purified stem BRM and detergent. Control showing cotton fabric stained with goat blood (A). Treated with BRM enzyme only (B). Treated with detergent only (C). Treated with BRM and detergent (D).

Table II. Destaining profile of blood-stained fabric.

Treatment given to stained cotton fabric	Destaining property measured as % relative activity	Observations
Bromelain	80	More active + effectively destained
Detergent	69.5	Moderate destaining
Bromelain + Detergent	75	Less active + completely destained

CONCLUSION

This work describes that BRM purification using arginine-bound magnetic nanoparticle complexes is a cost-effective, less time-consuming method to avoid laborious procedures of purification that increase downstream processing time and cost. BRM recovery is highest compared to crude extract, and bands show structural coherence and stability of the enzyme. This process achieved high protein content and protease activity, comparable to chromatographic techniques. Impurities can reduce enzyme activity, so it is essential to enhance purification, increasing enzyme activity. This technique is simple and has the potential to purify the enzyme with a high specific activity. BRM's stability at broad pH and temperature ranges and stain-removing properties make this plant-based enzyme beneficial for its use in the detergent industry.

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