Pineapple Peel Bromelain Extract Improves Poultry *In Vitro* Protein Digestibility

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Abstract

This study aimed to assess the proteolytic activity of pineapple peel crude bromelain extracts using different extractants and its impact on *in vitro* protein digestibility, simulating poultry digestion. This study compared the proteolytic activity of bromelain extracted using deionized water alone and in combination with 0.02 M dithiothreitol (DTT), 0.02% sodium azide, and 0.02 M ethylenediaminetetraacetic acid (EDTA). Subsequently, the effects of bromelain extracts on protein solubilization and digestibility using a poultry gut simulation model were evaluated. The findings indicated that bromelain extractions using deionized water (82.22 U/mg) and a combination of deionized water + sodium azide + EDTA (87.84 U/mg) exhibited the highest proteolytic activities compared to other extractants ($P<0.05$). Furthermore, pineapple peel bromelain extracts resulted in up to 24% increase in protein solubility and up to 6% increase in protein digestibility ($P<0.05$, respectively). Notably, bromelain extracted using deionized water (60.69 U/mg) proved to be the most effective in digesting protein *in vitro*.

Keywords: protease, pineapple waste, proteolytic activity, chicken.

Introduction

Bromelain is a mixture of enzymes, mainly cysteine proteases, present in the pulp, core, stem, peel, and crown of pineapple (*Ananas comosus*). The pineapple stem (EC 3.4.22.32) and fruit (EC 3.4.22.3) bromelains are commercially available and widely used in industries such as medicine, cosmetics, and food. During the processing of canned pineapple, approximately half of the total weight of pineapple consisting of peel, core, crown, and leaves is disposed of in landfills. The pineapple peel itself contributes to almost 30% of total pineapple waste, and the peel is the second largest source of bromelain after the crown of the fruit (Misran et al., 2019; Vieira et al., 2022). However, this most significant waste material usually goes to landfills or is used for animal feedstock that has low economic value. Various research studies have indicated that

supplementing the diet of broiler chickens with microbial protease can significantly enhance protein digestion (Freitas et al., 2011; Stefanello et al., 2024; Peñuela-Sierra et al., 2024) Meanwhile, the inclusion of fruit bromelain in the broiler diet has been shown to improve protein digestibility (Akit et al., 2019), and dietary stem bromelain has been found to enhance broiler feed efficiency (Mahfudhoh et al., 2023). Recent research has also demonstrated that bromelain can mitigate the negative effects of necrotic enteritis in broilers, prompting further exploration into its impacts on nutrient digestion and intestinal health (Gharib-Naseri et al., 2024).

Crude bromelain extracts from fruit and peel portions had the highest proteolytic activity, and the stem was reported to have the lowest proteolytic activity (Misran et al., 2019). Bromelain extract from the pineapple peel portion showed the second-highest proteolytic activity after the crown and the lowest activity was reported in the stem portion (Ketnawa et al., 2012). It was postulated that the differences in proteolytic activity may be due to different enzymes present in different parts of pineapple (Misran et al., 2019). The stem bromelain contains other minor proteases, including ananain (EC3.4.22.31), comosain, and acidic stem bromelain. Differences in clinical results using pineapple stem and fruit bromelains were likely due to the presence of different thiol-endopeptidases in bromelain preparations (Fissore et al., 2023).

Various methods of pineapple peel crude bromelain extract preparation have been studied with the general aim of obtaining high bromelain activity intended for various purification methods (Silvestre et al., 2012; Bresolin et al., 2013; Wan et al., 2016). The costs of solvents and additives have to be justified for the scaling-up process of the desired use of bromelain. It is crucial to extract bromelain using extractants that promote the maximum activity of bromelain as well as being cost-effective and eco-friendly. Pineapple peel crude bromelain prepared using the extractant sodium phosphate buffer of pH 6.0 containing 5 mM EDTA yielded 3,404 CDU/mg specific activity (Nor et al., 2015). Purified water has also been used as an extractant of pineapple peel crude bromelain, yielding high proteolytic activity (Ketnawa et al., 2012).

There have been few studies on the use of stem and fruit bromelains to enhance the growth performance of broiler chickens. Despite the high proteolytic activity of peel bromelain, there is currently no research on its use for determining feed protein digestibility. The objectives of this study were to determine the proteolytic activity of pineapple peel bromelain extracts and to investigate the effect of bromelain on *in vitro* protein digestibility of corn and soybean meal-based feed. The results from this study were intended to provide initial insights into a simple method of crude bromelain extraction and its effect on protein digestibility, serving as a starting point for further investigations on bromelain

stability for feed processing and animal experiments.

Materials and Methods

Preparation of Pineapple Peel Crude Bromelain Extract

The pineapple variety of MD2 was obtained from Ayer Hitam, Johor, Malaysia. The pineapple peels were washed with tap water, cut into small pieces and weighed. The crude bromelain extract from pineapple peel was prepared by blending the pineapple peel with deionized water in a 1:1 ratio (v/w) using a blender, with or without the presence of either one or combinations of antimicrobial agents, mineral chelator and reducing agent, namely, 0.02% sodium azide (Sigma Aldrich, USA), 0.02 M EDTA or 0.02 M DTT (Merck, Germany) (Table 1). The fibrous part of the peels was then removed and the liquid containing crude bromelain was centrifuged at 10,000 x g, 4 °C for 20 minutes (Hitachi, Japan) (Nor et al. 2015). The estimated amount of liquid obtained was between 60 to 70% of the total volume. The supernatant obtained was filtered using Whatmann No. 1 filter paper. The filtrate obtained was the crude pineapple peel bromelain extract.

Determination of Bromelain Extract Specific Proteolytic Activity

Proteolytic activity was determined using an azo-casein assay as described by Toe *et al.* (Toe et al., 2019). Briefly, 250 µl of pineapple peel bromelain extract was added to 400 µL of 0.1 M sodium acetate buffer solution (pH 5) containing 2.5% (w/v) azo-casein (Sigma Aldrich, USA) dissolved in 0.1 M sodium hydroxide (Merck, Germany).

Table 1. Preparation of pineapple crude bromelain extracts using deionized water with or without the combination of sodium azide, dithiothreitol (DTT), and ethylenediaminetetraacetic acid (EDTA).

Bromelain extractants
Deionized water
Deionized water + 0.02% sodium azide
Deionized water + 0.02 M DTT
Deionized water + 0.02 M EDTA
Deionized water + 0.02% sodium azide + 0.02 M DTT
Deionized water + 0.02% sodium azide + 0.02 M EDTA
Deionized water + 0.02% sodium azide + 0.02 M DTT + 0.02 M EDTA
Deionized water +0.02 M DTT + 0.02 M EDTA

The solution mixture was incubated at 37 °C for 30 minutes and subsequently centrifuged at 12,000 x g, 4 °C for 10 minutes. Then, 600 µL of supernatant was removed from the solution mixture and added to 600 µL of 0.1 M sodium hydroxide. The absorbance of the mixture was read at 450 nm using a spectrophotometer (Varian Cary® 50 UV-Vis, Agilent, USA). One unit of specific proteolytic activity (U/mg) was defined as the amount of enzyme capable of hydrolyzing sulfanilamide azo-casein to produce 0.001 changes in absorbance per minute per amount of protein (mg) under the assay condition.

In vitro Protein Digestibility

Bromelain extracts were prepared using deionized water according to the method described above. The specific proteolytic activities of 0.01% (w/v) commercial

fruit bromelain (Nutra Choice, Malaysia), 1:3 bromelain extract (pineapple peel to deionized water ratio) and 1:1 bromelain extract (pineapple peel to deionized water ratio) were determined according to the method described above. The specific proteolytic activity of the commercial bromelain yielded 69.10 U/mg, 1:3 ratio (pineapple peel to deionized water) yielded 60.69 U/mg and 1:1 ratio (pineapple peel to deionized water) yielded 52.39 U/mg. The inclusion rate of bromelain for the *in vitro* experiment was then determined using the following equation:

Bromelain inclusion rate (%) = 1/(a x b) x 100, where

a = specific proteolytic activity of bromelain

b = amount of degraded substrate of 2.5% (w/v) azo-casein in 0.01 changes in absorbance. The value of the constant b is 615.7 g.

The effect of bromelain on *in vitro* protein digestion was simulated in the chicken's crop, proventriculus, and duodenum. The simulation was carried out following the protocol described by (Suresh et al., 2019) with modifications. All incubations were carried out in duplicates at 42°C, with a constant stirring of 70 rpm in an incubator shaker (Innova®, Eppendorf, USA). The feed was composed of a mixture of 60% corn and 40% soybean meal. The control group consisted of 5 g of feed without any added bromelain enzyme. The commercial bromelain treatment consisted of 5 g of feed, combined with the required amount of commercial bromelain to achieve a final concentration of 69.10 U/mg. The low bromelain treatment included 5 g of feed mixed with the required amount of bromelain extract to achieve a final concentration of 52.39 U/mg. Finally, the high bromelain treatment contained 5 g of feed mixed with the required amount of bromelain extract to achieve a final concentration of 60.69 U/mg. To simulate crop digestion, the sample was pre-incubated with 7 ml of 0.1 M hydrochloric acid for 30 minutes (pH 5.2) in a 50-ml Falcon tube. Subsequently, proventriculus digestion was simulated by adding 1.5 ml of 1.5 M hydrochloric acid (pH 2) containing 3000 U pepsin/g (Sigma Aldrich, USA) for a 45-minute incubation period. After that, duodenal digestion was simulated by adding 3 ml of 1 M sodium bicarbonate (pH 6.8) containing 8.4 mg/ml pancreatin (Sigma Aldrich, USA) for a 120-minute incubation period. At the end of each digestion phase, the samples were centrifuged at 10,000 x g for 15 minutes (4°C). The supernatant was collected and analyzed for soluble protein concentration and protein digestibility.

The soluble protein concentration of the supernatant collected at the end of the crop, proventriculus and duodenal digestion phases were determined using the Bradford method. Bovine serum albumin (Sigma Aldrich, USA) was used as a standard. A volume of 500 µL of collected supernatant was added with 500 µL of Bradford reagent (Sigma Aldrich, USA). The mixture was then incubated at room temperature for 5 minutes before reading the absorbance at 595 nm using

a spectrophotometer (Varian Cary® 50 UV-Vis, Agilent, USA).

In vitro protein digestibility was calculated using the following equation (Koyum et al., 2023):

Digestibility (%) = (A-B)/A x 100, where

A = total soluble protein concentration after digestion

B = total soluble protein concentration before digestion

Statistical Analysis

The data was analyzed using a one-way analysis of variance and differences between means using the Duncan Multiple Range test. Data was considered significant when $P<0.05$ and trends when $0.05<P<0.10$. The statistical test was carried out using Statistical Analysis Software 9.4 (SAS).

Results and discussion

The specific proteolytic activity of the pineapple peel bromelain extracts prepared using deionized water, with or without the combinations of sodium azide, EDTA, and DTT, is shown in Figure 1. In the present study, the highest proteolytic activities were observed in bromelain extracts prepared using deionized water (82.22 U/mg) and a combination of deionized water + sodium azide + EDTA (87.84 U/mg) ($P<0.05$). A previous study found that the proteolytic activity of *Phu Lae* pineapple peel extracted using distilled water was higher at 13.26 U/mg compared to the extract with a combination of cysteine and EDTA, which measured 11.79 U/mg (Ketnawa et al., 2011). The second highest proteolytic activity was obtained in bromelain extracts prepared using deionized water + EDTA (68.84 U/mg) and deionized water + DTT (69.1 U/mg). The decrease in the specific proteolytic activity of bromelain in the presence of EDTA can be attributed to the chelation of metal ions on the active site of bromelain by EDTA. This chelation causes a structural shift in bromelain, leading to a reduction in its proteolytic activity (Hidayani et al., 2020). Bromelain extracts prepared using deionized water + sodium azide + DTT (58.45 U/mg), deionized water + sodium azide + DTT + EDTA (55.4 U/mg), and deionized water + sodium azide (35.5 U/mg) showed lower proteolytic activities. To produce a significant quantity of pineapple peel extract for use as a feed supplement, it is important to consider the cost of additives. Therefore, we chose to use deionized water without sodium azide and EDTA as the extractant for extracting pineapple peel bromelain in the subsequent *in vitro* experiment.

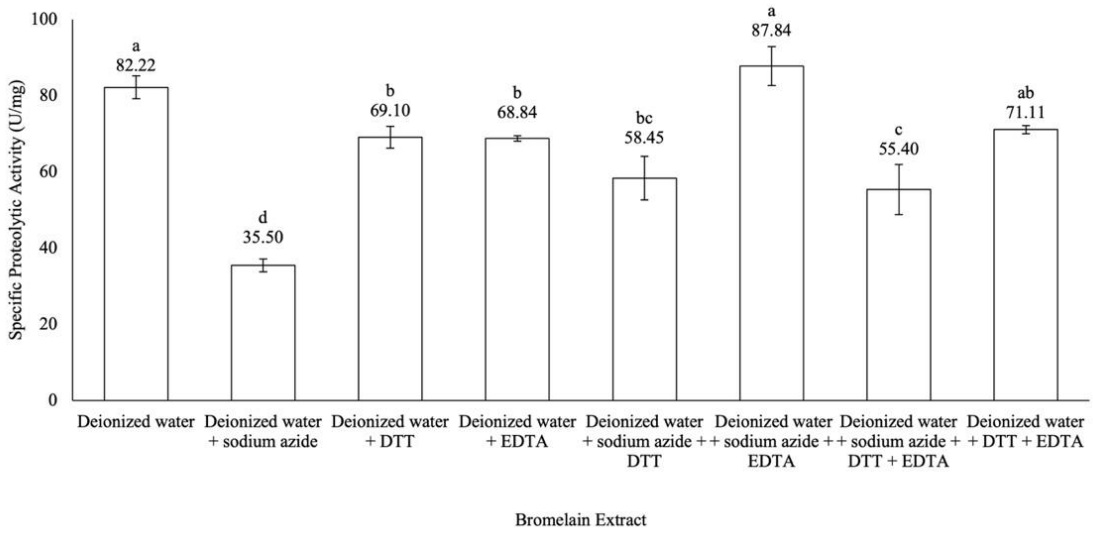

Figure 1: The specific proteolytic activity (U/mg) of pineapple peel bromelain extracts prepared using deionized water with or without combinations of sodium azide, dithiothreitol (DTT), and ethylenediaminetetraacetic acid (EDTA).

Figure 2 illustrates the concentration of soluble proteins in the crop, proventriculus, and duodenal digestion phases. Bromelain is a proteolytic enzyme that breaks down peptide links in protein chains. The proteolytic enzyme consists of soluble proteins such as thiol-endopeptidases, phosphatases, glucosidase, peroxidases, cellulases, and protease inhibitors (Bhattacharya & Bhattacharyya, 2009). Although not significant, high bromelain extract showed a trend of increased soluble protein content compared to the control in the crop ($P=0.08$) and proventriculus ($P=0.06$) digestion phases. The increase of soluble protein (up to a 24% increase) was observed more prominently in the duodenal digestion phase. The high bromelain extract showed the highest soluble protein followed by the low bromelain extract, commercial bromelain, and the control ($P<0.05$). The higher soluble protein in the bromelain treatments was due to a large number of soluble peptides initially present in bromelain compared to the control. Throughout the digestive process from the crop to the duodenal phase, there was a noticeable increase in soluble protein. Notably, the duodenal phase exhibited the highest concentration of soluble protein, attributed to the increased production of soluble peptides resulting from the action of digestive enzymes.

Similarly, it was reported that the soluble protein increased as protease levels rose in an *in vitro* digestion study using serine protease (Fru-Nji et al., 2011). In agreement, an *in vitro* study simulating human digestion showed that bromelain increased the soluble protein of pork samples measured after gastric and intestinal digestion phases (Gallego et al., 2023). The authors observed a consistent pattern of rising levels of soluble peptides and free amino groups, suggesting enhanced protein digestibility facilitated by bromelain.

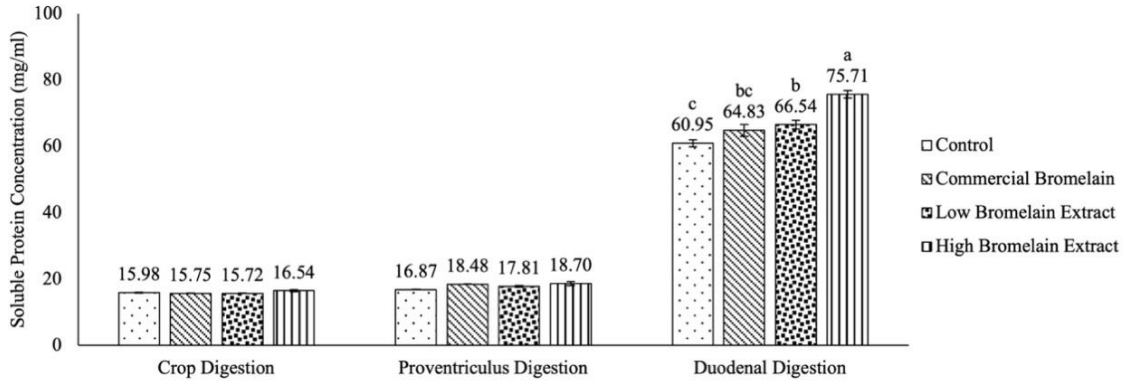

Figure 2: The concentration of soluble protein (mg/ml) in the crop, proventriculus, and duodenal digestion phases *in vitro*. Control = Feed without Bromelain; Commercial Bromelain = Feed + 69.10 U/mg commercial bromelain; Low Bromelain Extract = Feed + 52.39 U/mg bromelain extract; High Bromelain Extract = Feed + 60.69 U/mg bromelain extract. Different alphabetical letters indicate significant differences ($P<0.05$) among treatments.

Figure 3 illustrates the impact of bromelain on protein digestibility in the proventriculus and duodenal digestion phases *in vitro*. In the proventriculus phase, the bromelain treatment group had a higher protein digestibility than the control ($P<0.05$). In the duodenal phase, bromelain increased protein digestibility by up to 6%. The high bromelain extract had the highest protein digestibility followed by the low bromelain extract, commercial bromelain, and the control ($P=0.03$). The wide range of pH in different parts of the poultry digestive tract can influence the optimal activity of proteases.

The pH values of the poultry proventriculus content ranged between 0.3 and 4.1, and the intestinal content ranged between 5.5 and 7.7 (Ao et al., 2008). The crude bromelain found in pineapple peel exhibited consistent proteolytic activity within the pH range of 3.0 to 5.0, reaching its peak between pH 6.0 and 7.0 (Silvestre et al., 2012). The same authors observed a decline in specific proteolytic activity of bromelain at pH 8.0, followed by stability at pH 9.0. In the present study, the proventriculus phase was simulated by exposing the feed mixture to pepsin under pH 2, followed by a duodenal phase simulated by increasing the pH to 6.8 with the addition of pancreatin. In the proventriculus phase, bromelain enhanced protein digestibility. However, there was no significant difference in protein digestibility between the bromelain extracts and the commercial bromelain.

The bromelain remained active at pH 2, indicating its stability under acidic conditions. Previous works have also proven that bromelain was resistant to inactivation and retained its proteolytic activity at pH 2 (Corzo et al., 2012; Fru-Nji et al., 2011). Protein digestibility improvement was more evident in the duodenal phase, with the highest digestibility observed in high bromelain extract, followed by low bromelain extract, commercial bromelain, and the lowest in the control. Concurrent with the current *in vitro* study, dietary supplementation of fruit bromelain improved protein digestibility in broilers

(Akit et al., 2019). Bromelain also enhanced the *in vitro* gastrointestinal digestion of whey protein, as indicated by an increased degree of hydrolysis (Jadhav et al., 2021). It was evident that bromelain remained active during the duodenal phase (pH 6.8). Likewise, studies indicated that bromelain from pineapple peel exhibited its optimal proteolytic activity between pH 6.8 and 9.0 (Ketnawa et al., 2011, 2010). In the current study, the peel bromelain extract (60.69 U/mg) was found to be the most effective in digesting the protein from the mixture of corn and soybean meal.

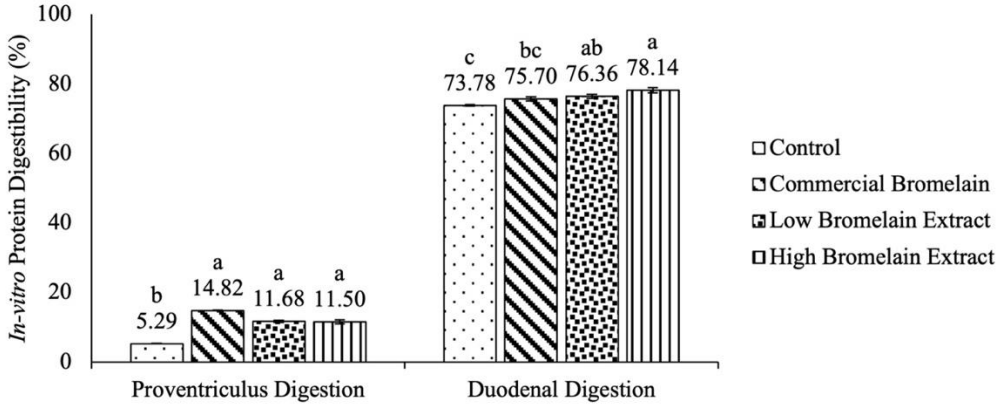

Figure 3: The impact of bromelain on protein digestibility (%) in the proventriculus and duodenal digestion phases *in vitro*. Control = Feed without Bromelain; Commercial Bromelain = Feed + 69.10 U/mg commercial bromelain; Low Bromelain Extract = Feed + 52.39 U/mg bromelain extract; High Bromelain Extract = Feed + 60.69 U/mg bromelain extract. Different alphabetical letters indicate significant differences ($P<0.05$) among treatments.

Exogenous enzymes, produced industrially, are utilized in various commercial applications, including incorporation into animal feed. Proteolytic enzymes, among the exogenous enzymes added to animal diets, are employed to enhance the breakdown and utilization of protein. Most of the protein digestion occurs in the intestine, with some taking place in the proventriculus. Pepsin, an endogenous protease, functions to break down dietary proteins into smaller peptides in the proventriculus. The digesta then transits through the gizzard for grinding and mixing, after which endogenous pancreatin hydrolyzes and solubilizes the protein in the duodenum. Studies demonstrating increased protein digestibility using commercial and extracted bromelains indicate that bromelain can complement endogenous pepsin and pancreatin, thereby improving protein digestion. Stem bromelain was shown to complement the natural endogenous enzymes produced by broiler chickens (Yu et al., 2006). In addition, bromelain supplementation did not affect pepsin activity in the gizzard of broilers (Yu et al., 2002). However, it was reported that there was still no conclusive evidence as to whether exogenous proteases would have a positive or negative impact on endogenous enzymes (Romero et al., 2013). Since soybean meal and corn had a certain degree of resistance to endogenous proteases (Zheng et al., 2023), it was possible that peel bromelain further assists in improving

protein digestibility. Moreover, it was shown that microbial protease degraded anti-nutrient factors of soybean meal, specifically glycinin, β-conglycinin and trypsin inhibitor, resulting in improved *in vitro* nutrient digestibility (Zheng et al., 2017). Bromelain enhanced the performance of broilers affected by necrotic enteritis (Gharib-Naseri et al., 2024), which may be partly due to improved nutrient digestibility and alteration of microbiome homeostasis.

However, *in vitro* protein digestibility data in the present study is limited to a controlled environment, excluding the complex physiological processes involving endogenous protein secretions, anti-nutritional factors, and activity of other gut enzymes and microbiomes. Hence, it is imperative to conduct an *in vivo* study to validate the impact of peel bromelain on protein digestibility. Additionally, it's important to consider that the storage and processing of feed pellets in the feed mills may lead to a reduction in bromelain activity, thereby limiting its efficacy in enhancing nutrient digestion. Therefore, it is essential to explore methods for preserving bromelain activity for potential utilization in poultry feed and conduct further research in this area.

Conclusion

The bromelain extracted from pineapple peel using deionized water (60.69 U/mg) was found to be effective in breaking down protein *in vitro*. Deionized water was identified as a practical extractant for crude bromelain preparation, facilitating scalability. Further investigation is needed to explore the preservation of bromelain's proteolytic activity for potential applications in poultry feed.

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Conflict of Interest

The authors declare that there are no conflicts of interest.

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