

Extraction of Crude Bromelain from Pineapple (*Ananas comosus* L.) Fruit Waste and its *in vitro* Protein Digestibility

J. Wiboonsirikul^{1*}, P. Khuwijitjaru², and R. Klahan³

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ABSTRACT

Purpose: Crude bromelain is abundant in pineapple fruit waste and is possible to use in shrimp farming. The objectives of this present study were to investigate extraction conditions and to evaluate *in vitro* protein digestibility of the shrimp feed supplemented with the crude bromelain extract.

Research Method: Crude bromelain was extracted from pineapple fruit waste with either distilled water or tap water and the pH was adjusted, ranging from 5 to 9. The different ripeness stages and weight ratios of waste to water were further investigated. The highest proteolytic and specific proteolytic activities of the extract were selected for suitable extraction conditions. The *in vitro* protein digestibility of soybean meal and shrimp feed pretreated with crude bromelain extract was evaluated to determine their feasibility for use in shrimp farming.

Findings: The crude bromelain derived from the pH 7 preboiled tap water extract exhibited the highest values of both proteolytic activities. The weight ratio of fruit waste to the water of 1:0.5 and a ripeness stage of more than 50% yellow peel was the conditions suitable to obtain the highest values of both activities. The *in vitro* protein digestibility of shrimp feed pretreated with crude bromelain was 68.68 ± 2.70 %, which indicated the promotion capability for protein digestion in shrimp during shrimp farming.

Research Limitations: A suitable crushing tool for pineapple peel was not available. Therefore, the fragments after crushing the peel were not consistent.

Originality/value: The crude bromelain can be extracted with tap water and used to supplement shrimp feed for the promotion of digestibility.

Keywords: Crude bromelain, Pineapple fruit waste, Proteolytic activity, Ripeness stage, Water

INTRODUCTION

Thailand is one of the top exporters of canned pineapple fruit in Asia, along with Indonesia and the Philippines. Around 400,000 tons a year of canned pineapples and other products such as juice, concentrates, and preserves are dispatched all over the world (Wattanakul *et al.*, 2020). The pineapple fruits used in food industries are separated into edible parts containing pulp and inedible so-called wastes, including peels, crowns and cores. The 'Smooth Cayenne' cultivar of the pineapples with yellow peel, about 40–90% of whole fruit, is mostly used for the production of

pineapple products in the industries in Thailand, and consequently, a great amount of peels and

^{1*} Division of Food Science and Technology, Faculty of Agricultural Technology, Phetchaburi Rajabhat University, Phetchaburi 76000, Thailand.

wiboonsirikul@yahoo.com

² Department of Food Technology, Faculty of Engineering and Industrial Technology, Silpakorn University, NaKhon Pathom 73000, Thailand.

³ Division of Aquaculture, Faculty of Agricultural Technology, Phetchaburi Rajabhat University, Phetchaburi 76000, Thailand.

 <https://orcid.org/0000-0002-9519-5597>

crowns of the fruits, representing more than 40% of the waste, is generated after processing. Most waste is sold for animal feed with low value (Ketnawa *et al.*, 2012). At present, a zero waste policy is normally applied by many entrepreneurs and industries for the reduction of cost and waste. Many pineapple industries have made an effort toward the production of high-value products from pineapple fruit waste. Whole pineapple fruit, including pulp, peel, core, and crown, contains a significant quantity of bromelain, which is a well-known digestive enzyme used in many food and feed industries. Several studies have shown a few times higher total protease activity in the extract from pineapple peel and crown than from the pulp and core parts (Ketnawa *et al.*, 2012; Aravind and Gokulakrishnan, 2015; Sirijariyawat and Nontaloon, 2020). Optimization conditions for extraction of the bromelain from pineapple fruit waste have been tested in many studies. The main types of extractants used for crude bromelain extraction are purified water, distilled water (Nadzirah *et al.*, 2012; Mohan *et al.*, 2016) and various types of buffers (Ketnawa *et al.*, 2012; Aravind and Gokulakrishnan, 2015). However, those aqueous solutions are not practical in use for shrimp farmers and community entrepreneurs because they are expensive and need a downstream process to remove additional salt. The use of tap water obtained from the local provincial water work authority is convenient and more practical in use for aquatic farmers. However, there is no published report available for using tap water as an extractant for crude bromelain in comparison with the other extractants. Besides the types of extractants, other factors of the crude bromelain extraction from pineapple fruit affect proteolytic activity, such as the pH of the extractant and the ripeness stage of the fruit (Mohan *et al.*, 2016; Poba *et al.*, 2019). Moreover, many studies involving solid-liquid extraction report that an increase in the ratios of waste material to extractant tends to increase extraction efficiencies (Perva-Uzunalić *et al.*, 2006; Wiboonsirikul *et al.*, 2007).

Soybean meal is an alternative protein source in shrimp feed. Shrimp farmers in Thailand usually use soybean meal to partially substitute for fish

meal due to its economical price, relatively good digestibility, and well-balanced amino acid composition (Sookying *et al.*, 2013). Currently, shrimp feed contains about 20–30% soybean meal. However, the soybean meal contains some anti-nutritional components including lectins, phytic acid and protease inhibitors, especially soybean trypsin inhibitor (SBTI), which lead to a lower capability to utilize nutrients and subsequently lower growth rates of shrimp during cultivation in comparison with other animal protein sources. Utilization of natural protease obtained from agricultural waste, such as bromelain, locally available in pineapple fruit waste from pineapple fruit processing industries, is an alternative way to overwhelm the hindrance in the utilization of soybean meal in the shrimp feed without significantly increasing the cultivation cost (Francis *et al.*, 2001; Bae *et al.*, 2020). Feed cost is a major concern for shrimp farmers, and it represents about 50–60% of the total variable cost of cultivation. Although there is commercial powdered bromelain available in the market, the feed cost of shrimp farming would be increased if it was used. In addition, bromelain from pineapple fruits is not commercially available (Larocca *et al.*, 2010), but the pineapple fruit waste is more easily available for feed production industries and aquatic farming as a result of many pineapple fruit processing industries in Thailand. Therefore, many shrimp farmers prefer to use the fruit waste from pineapple industries for crude bromelain extract and use it directly in shrimp feed instead of commercial bromelain powder. Our previous research reported the possibility of utilizing crude bromelain extract from pineapple fruit waste in shrimp feed to contribute to better digestion by Pacific white shrimp during cultivation. The results showed that the growth rate and feed utilization of the shrimp were not significantly increased at the volume of 1–4% per feed weight concentration of the pineapple fruit waste extract. In addition, the survival and molt rates were not significantly increased at the 2% volume extract concentration per feed weight (Klahan *et al.*, 2021b). Furthermore, Yuangsoi *et al.* (2018) reported that the use of the pineapple fruit waste extract to supplement the feed for Nile tilapia significantly improved pepsin digestibility

but not protein digestibility. However, there is rarely an investigation of extraction conditions for crude bromelain involving water types as extractants besides distilled water and buffer, in addition to ripeness stages, and the ratios of pineapple fruit waste to water. Based on the standpoints mentioned above, the objectives of this study were to identify the extraction conditions of crude bromelain from pineapple fruit waste and its effect on *in vitro* protein digestibility.

MATERIALS AND METHODS

Materials and Chemicals

Pineapple fruits (*Ananas comosus* L.) from the 'Smooth Cayenne' cultivar, shrimp feed, and soybean meal were purchased from a local market at the Banlard District in Phetchaburi province, Thailand a day before extraction. Shrimp feed (Integc®, Samutsakorn, Thailand) contained 38% crude protein and was subjected to crude fat removal by using petroleum ether extraction. All chemicals and reagents used in the experiments were analytical grade.

Preparation of Pineapple Fruit Waste

Pineapple waste was prepared from the peel and crown of the fruits, which were washed thoroughly with water and 0.1% H₂O₂ solution. The fruits were air-dried at room temperature before manually peeling using a pineapple borer (80 mm diameter). The waste was cut into small pieces before grinding in a grinder (SKG PN-2562, Thailand). The weight of whole pineapple fruit and waste was recorded separately to evaluate the weight proportion.

Preparation of Crude Bromelain Extract from Pineapple Fruit Waste

First, 200 g of pineapple fruit waste, including peel and crown, was crushed together with 200 g of cold preboiled tap water or distilled water. The resulting mixture was filtered through a cotton cloth and then the filtrate was adjusted to studied pHs of 5, 6, 7, 8, and 9 (as called pH5, pH6, pH7, pH8, and pH9) using sodium hydroxide (5 M) and phosphoric acid (5 M). The filtrate without pH adjustment was used as a control. All filtrates at different pH values were stored at -18 °C until further analysis. On the day for analyses, the frozen filtrate was thawed and centrifuged at 6000 rpm at 4°C for 10 min by using a refrigerated centrifuge (MPW 260R, Korea). The supernatant after centrifugation was the crude bromelain extract used for evaluation of the physical and chemical properties resulting from the type of water extractant (preboiled tap water or distilled water) and adjusted pH.

The various weight ratios of the pineapple fruit waste to selected water were studied at 1:0.25, 1:0.50, 1:0.75, and 1:1 (w/w). The different ripeness stages based on the percentage of yellow-orange and green peel, including fully green peel (FGP), less than 50% yellow peel (LYP), more than 50% yellow peel (MYP), and fully yellow peel (FYP), were also studied. The waste and water were crushed, filtered, stored in the freezer, and centrifuged according to the above procedures to obtain the crude bromelain extracts at the different weight ratios of fruit waste and water, as well as the different ripeness stages.

Measurement of Physical and Chemical Properties of Water Types as Extractants

The preboiled, cold tap water and distilled water used for the extraction of the crude bromelain were evaluated for their physical and chemical properties, including pH, total dissolved solids and salt concentration (ppm), specific gravity, electrical conductivity ($\mu\text{S}/\text{cm}$), and redox potential (mV) by using a water quality tester (Juanjuan, China).

Measurement of Peel Color of Pineapple Fruits

The different ripeness stages (FGP, LYP, MYP, and FYP) were measured by the evaluation of chroma values of peels after the preparation of pineapple fruit waste by using a colorimeter (WR 18 FRU, China). Ten pieces of peels from each ripeness stage were randomly selected to measure the color values of L , a , and b on the surface of the peels. The chroma value was calculated using equation (1).

$$\text{Chroma} = (a^2 + b^2)^{1/2} \dots\dots\dots(1)$$

Measurement of Proteolytic Activity in the Extracts

The proteolytic activity of the crude bromelain extracts was evaluated according to the method proposed by Ketnawa *et al.*, (2012) with slight modification. Casein was used as a substrate. Briefly, 0.5-1.0 mL of extract was mixed with 1 mL of a reaction solution containing 1% (w/v) of casein, 0.03 mol/L cysteine, 0.006 mol/L EDTA in 0.05 mol/L phosphate buffer at pH 7.0, which was freshly prepared for analysis. The enzymatic reaction was conducted at 37 °C for 10 min and was then stopped by the addition of 3 mL of 5% (w/v) trichloroacetic acid. A blank was prepared by mixing the same volume of the extract with 5% (w/v) trichloroacetic acid before the addition of casein. The reaction mixture and blank were centrifuged at 10,000 rpm for 10 min, and the supernatant was used for recording the absorbance value at 275 nm as the soluble

tyrosine and non-precipitated peptides obtained from enzymatic digestion of casein. A serial dilution of L-tyrosine was evaluated at the same absorbance as a standard. The proteolytic activity (unit/mL) was calculated by equation (2) (Mohan *et al.*, 2016):

$$\text{Proteolytic activity} = (TE \times DF) / (V \times t) \dots\dots\dots(2)$$

Where,

TE = tyrosine equivalent released ($\mu\text{g}/\text{mL}$),

DF = dilution factor,

V = volume of crude bromelain extract used (mL),

t = reaction time of assay (min).

The proteolytic-specific activity (unit/min.mg) of the crude bromelain extract was calculated by equation (3).

$$\text{Specific proteolytic activity} = \text{proteolytic activity/protein content} \dots\dots\dots(3)$$

Measurement of Total Protein Content

The protein content in the crude bromelain extract was evaluated according to the Bradford protein method (Bradford, 1976). Bovine serum albumin (BSA) was used as a standard for serial dilution.

Measurement of Total Carbohydrate Content

Total carbohydrate content in the crude bromelain extract was measured according to the phenol-sulfuric method (Dubois *et al.*, 2002). Glucose was used as a standard for serial dilution.

Measurement of pH and Total Soluble Solid

The apparent and adjusted pH and total soluble solids ($^{\circ}\text{Brix}$) were measured using a pH meter

(Ohaus ST20, USA) and a hand refractometer (Atago N1, Japan), respectively.

Measurement of In vitro Protein Digestibility in Soybean Meal and Shrimp Feed Pretreated with Crude Bromelain Extract

Commercial defatted soybean meal (SBM) and shrimp feed with a particle size of 1.5 mm pretreated with crude bromelain extract (PTSF) were evaluated for *in vitro* protein digestibility. The PTSF was prepared by soaking the shrimp feed with the crude bromelain extract based on the protein contents of the extract to obtain 0.8 g/g of protein to feed and then drying in the desiccator for a few days before measurement of *in vitro* protein digestibility. The SBM and PTSF were subjected to protein extraction according to Fageer and El-Tinay (2004), with slight modification. Briefly, a 2 g of SBM and PTSF was mixed with 20 mL of 0.1 mol/L phosphate buffer at different pH values of 6.0, 7.0, 8.0, and 9.0 in several Erlenmeyer flasks and then incubated at 30 °C in a temperature-controlled shaker (WiseCube, Korea) at 90 rpm/min for 16 h. After incubation, the supernatant of each flask, which contained soluble protein, was withdrawn into two plastic tubes. The first tube was evaluated for protein content before digestion by following the Bradford method (Bradford, 1976). The second one was subjected to digestion by thoroughly mixing 1 mL of the supernatant, 2 mL of crude bromelain extract, and 20 mL of the phosphate buffer at the same pH value of the protein extraction as above mentioned. After incubation at different temperatures of 25, 30, or 40 °C for 24 h, the supernatant of each tube was evaluated for soluble protein content. The *in vitro* protein digestibility of crude bromelain extract was calculated by equation (4).

$$\text{Protein digestibility (\%)} = [(SP_1 + EP) - SP_2] / SP_1 \times 100 \dots \dots \dots (4)$$

Where,

SP_1 = protein content in soluble protein before

the addition of crude bromelain extract,

SP_2 = protein content in soluble protein after the addition of crude bromelain extract,

EP = protein content in crude bromelain extract.

Statistical Analysis

Triplicate analyses were conducted for each measurement, and data were expressed as mean ± standard error. The mean difference among treatments was evaluated by analysis of variance (ANOVA) followed by Duncan’s new multiple range test at a significant level of $p < 0.05$ using R version 4.1.2 (R Core Team, 2021).

RESULTS AND DISCUSSION

Weight Proportion of Pineapple Fruit Waste

The average weight proportion of the waste, including peel and crown to whole pineapple fruit was about 30.31–40.62% (w/w), which was close to that obtained from the report of the other researchers for ‘Smooth Cayenne’ cultivar (Mulyono *et al.*, 2013; Sirijariyawat and Nontaloon, 2020). The rest of the pineapple fruits, containing the pulp and core, were not used for extraction of the crude bromelain because the pulp is used in pineapple processing for various products, and the core part contains very high fiber content and several times lower proteolytic activity than the peel and crown (Prakongpan *et al.*, 2002; Ketnawa *et.al*, 2012; Sirijariyawat and Nontaloon, 2020; Abbas *et al.*, 2021). The weight proportion of each part in the pineapple fruits varied according to their sizes and varieties. The bigger the fruit size was, the larger the edible parts were (Ketnawa *et al.*, 2012; Sirijariyawat and Nontaloon, 2020).

Physical and Chemical Properties of Distilled Water and Preboiled Tap Water

Two water types as the extractants, including distilled water and preboiled tap water, were used to extract the crude bromelain from the pineapple fruit waste. The physical and chemical properties of the distilled water and preboiled tap water are shown in Table 01.

The preboiled tap water exhibited higher salt concentration, and electrical conductivity but lower pH and redox potential values than the distilled water. The total dissolved solid and specific gravity of the preboiled tap water were equivalent to those of the distilled water. According to the salt concentration and electrical conductivity, the preboiled tap water contained a higher concentration of inorganic ions that could conduct electricity than the distilled water. Common ions in water that conduct electrical current include sodium, chloride, carbonate, calcium, and magnesium (Damo and Icka, 2013; Provincial Waterworks Authority, 2022). The negative redox potential of the preboiled tap water indicated a lower concentration of dissolved oxygen and carbon dioxide than distilled water due to not boiling the water before use (Račys *et al.*, 2010). In addition, the pH of both waters indicated that the distilled water was slightly basic and the preboiled tap water was slightly acidic.

Effect of Water Types as Extractants and Adjusted pH on Chemical Properties of Crude Bromelain Extract

After extraction with both water types and filtration, the pH of the filtrate was adjusted from 5 to 9 as well as a control without pH adjustment before storage in a freezer. All crude extract solutions were exposed to centrifugation, and the supernatant, as crude bromelain extract, was evaluated for chemical properties, including apparent pH, total soluble solid, protein and total carbohydrate contents, and general and specific proteolytic activities. The results are presented in Table 02 and Table 03.

The apparent pH was close to the adjusted pH of the same crude bromelain extract obtained from both distilled water and tap water, but the crude bromelain extract adjusted pH of 9 showed a slight decrease in the apparent pH after freezing and thawing, which could be attributed to the acidity of crude fruit bromelain (Ramli *et al.*, 2018). The pH of crude bromelain extract without adjustment (control) obtained from extraction with both water types was 4.4–4.5, which was slightly higher than the pH of the extract reported by the other researchers. Bartolome *et al.*, (1995) reported that the pH of the pineapple peel extract from the ‘Smooth Cayenne’ cultivar was around 3.54, while Ketnawa *et al.* (2012) reported that the pH was around 4.0 for the peel extract and around 4.8–5.19 for the crown extract.

Table 01: Physical and chemical properties of distilled water and preboiled tap water.

Physical and chemical properties	Distilled water	Preboiled tap water
pH	8.62±0.10	6.60±0.05
Total dissolved solid (mg/L)	0	0
Salt concentration (mg/L)	0	133±3.51
Specific gravity	1.001	1.001
Electrical conductivity (µS/cm)	(1.00±0.05)×10 ⁻⁴	(271±4.04) ×10 ⁻⁴
Redox potential (mV)	30.7±2.52	-95.7±2.08

Table 02: Chemical properties of crude bromelain extract obtained from extraction of pineapple fruit waste crushed together with distilled water at various pH values.

Adjusted pH of extract	Apparent pH	Total soluble solid (°Brix)	Proteolytic activity (Unit/mL)	Specific proteolytic activity (Unit/mg)	Total protein content (mg/mL)	Total carbohydrate content (mg/mL)
control	4.40±0.50	4.2±0.10 ^b	4.11±0.10 ^b	13.09±0.31 ^a	0.31±0.01 ^e	22.54±0.23 ^c
pH5	4.96±0.10	4.4±0.05 ^a	4.92±0.26 ^a	13.52±0.73 ^a	0.36±0.01 ^{de}	24.85±0.10 ^b
pH6	6.05±0.05	4.3±0.05 ^{ab}	5.01±0.12 ^a	10.48±0.26 ^b	0.48±0.03 ^c	25.49±0.20 ^a
pH7	6.88±0.05	4.4±0.05 ^a	5.17±0.24 ^a	3.76±0.18 ^c	1.38±0.09 ^b	24.61±0.36 ^b
pH8	8.01±0.10	4.5±0.10 ^a	5.08±0.15 ^a	3.14±0.09 ^c	1.61±0.10 ^{ab}	24.56±0.13 ^b
pH9	8.65±0.10	4.5±0.10 ^a	5.17±0.12 ^a	12.91±0.31 ^a	0.46±0.10 ^{cd}	25.67±0.41 ^a

Data presented in Table 02 represent the mean ± standard error. Different superscripts in a column indicate significant differences among adjusted pH of extracts by Duncan's new multiple range test at $p < 0.05$.

Table 03: Chemical of crude bromelain extract obtained from the extraction of pineapple fruit waste crushed with the preboiled tap water at various pH values.

Adjusted pH of extract	Apparent pH	Total soluble solid (°Brix)	proteolytic activity (unit/mL)	Specific proteolytic activity (unit/mg)	Total protein content (mg/mL)	Total carbohydrate content (mg/mL)
control	4.58±0.15	4.2±0.10	7.16±0.02 ^b	22.04±0.55 ^b	0.32±0.01 ^b	27.53±0.27 ^c
pH5	5.10±0.10	4.4±0.10	8.32±0.08 ^a	20.92±0.20 ^c	0.40±0.01 ^a	27.91±0.21 ^c
pH6	6.07±0.08	4.5±0.10	8.11±0.05 ^a	21.61±0.13 ^{bc}	0.37±0.00 ^a	31.63±0.96 ^a
pH7	7.05±0.10	4.5±0.10	8.07±0.03 ^a	24.22±0.80 ^a	0.33±0.02 ^b	29.83±1.11 ^b
pH8	7.92±0.08	4.3±0.10	4.35±0.02 ^d	15.44±0.78 ^c	0.28±0.03 ^c	27.83±0.24 ^c
pH9	8.75±0.15	4.0±0.10	4.69±0.01 ^c	18.08±0.57 ^d	0.26±0.03 ^c	27.46±0.33 ^c

Data presented in Table 03 represents the mean ± standard error. Different superscripts in a column indicate significant differences among the pH of extracts by Duncan's new multiple range test at $p < 0.05$.

The difference in pH of the extract depended on several parameters, such as ripeness stages, cultivar, extracted parts of pineapple, and the number of organic acids in the pineapple waste (Ketnawa *et.al*, 2012). The total soluble solids of the crude bromelain extract at all adjusted pH values, including the control obtained from extraction with both water types, was 4.0–4.5 °Brix. The slight difference in the total soluble solid content of each extract mainly depended on the dissolved carbohydrate content of the pineapple waste extract and the other minor contents of soluble proteins, acids, and minerals

according to the refractometer. The total protein and carbohydrate contents of all extracts obtained from the distilled water extraction were 0.31–1.61 and 22.54–25.67 mg/mL, respectively, and those obtained from the preboiled tap water extraction were 0.26–0.40 and 27.46–31.63 mg/mL, respectively.

Water types affected the availability of proteolytic activity, specific activity and protein contents of the extract. The extraction with tap water exhibited higher proteolytic and specific proteolytic activities, total protein and

carbohydrate contents than that with the distilled water. The proteolytic activity of the extract derived from the extraction with the distilled water and preboiled tap water were 4.11–5.17 and 4.35–8.32 unit/mL, respectively, which were in accordance with the report from the other researchers (Mohan *et al.*, 2016). However, some researchers reported higher proteolytic activity of the extract from pineapple fruit waste than the present study (Ketnawa *et al.*, 2012; Mulyono *et al.* 2013). The difference in the proteolytic activity may be due to the cultivar, ripeness stages, and fruit sizes of pineapples used for extraction (Mulyono *et al.*, 2013). For the distilled water as the extractant, the extracts at adjusted pH of 5 and 9 exhibited the highest proteolytic and specific proteolytic activities, while for the preboiled tap water as the extractant, the extract at adjusted pH of 7 showed the highest activities ($p < 0.05$). It should be noted that the extract obtained from the preboiled tap water at adjusted pH of 7 showed higher proteolytic and specific proteolytic activities than that from using distilled water ($p < 0.05$). In addition, considering the relationship among the proteolytic and specific proteolytic activities as well as protein content, the higher the proteolytic activity and the lower the protein content, the higher the specific activity that was obtained. The inverse proportion between specific proteolytic activity and protein content in the crude bromelain extract was from the other peptidases and proteins besides bromelain, such as phosphatases, glucosidases, peroxidases, cellulases, and glycoproteins (Bhattacharyya, 2008). Moreover, fruit bromelain exhibited a broad aptitude for protein cleavage and stability in a broad pH range from 3 to 8 (Mohapatra *et al.*, 2013; Manzoor *et al.*, 2016). Changes in specific proteolytic activity and protein content according to pH and water types may be attributed to the availability of ions, such as calcium and magnesium, in the preboiled tap water used for extraction. The preboiled tap water contained calcium and magnesium, along with chloride of about 300 and 250 mg/L, respectively (Provincial Waterworks Authority, 2022). The presence of calcium, magnesium, or calcium chloride as the

activator or stimulatory agent at 0.1 mmol/L increased the proteolytic activity of the bromelain about two times higher than with their absence (Fadhilah *et al.*, 2018; Chakraborty *et al.*, 2021).

Effect of Weight Ratios of Pineapple Fruit Waste to Water on Chemical Properties of Crude Bromelain Extract

The crude bromelain extract obtained from extraction with the preboiled tap water adjusted pH to 7 was selected to further investigate the effect of various weight ratios of pineapple fruit waste to water from 1:0.25 to 1:1 on the chemical properties of the crude bromelain extracts. The results are shown in Table 04.

The apparent pH of the extracts after centrifugation at all weight ratios of waste to water were not significantly different ($p > 0.05$) from each other. An increase in the ratios of fruit waste to water increased the total soluble solid, proteolytic activity, protein, and carbohydrate contents in the extracts ($p < 0.05$). However, the specific proteolytic activity, in the extract obtained from the different ratios of the fruit waste to water was not significantly different ($p > 0.05$). It is noticeable that the increase in the total carbohydrate and protein contents was related to the proportional increase in total soluble solid and proteolytic activity. Considering the significantly higher general proteolytic and insignificant different specific proteolytic activities, the extract obtained from the ratio of waste to water at 1:0.5 was chosen to continue the investigation of the effect of ripeness stages on the chemical properties of the crude bromelain extracts. In addition, the limited availability of water at the weight ratio of the waste to water at 1:0.25 caused inconsistency of fragment sizes and crude bromelain from the large fragments of the fruit waste after crushing with water was not extracted.

Table 04: Characteristics of crude bromelain extract obtained from extraction at various ratios of pineapple fruit waste and the preboiled tap water.

The ratio of waste to water (w/w)	Apparent pH	Total soluble solid (°Brix)	proteolytic activity (unit/mL)	Specific proteolytic activity (unit/mg)	Total protein content (mg/mL)	Total carbohydrate content (mg/mL)
1:0.25	6.88±0.07 ^a	5.83±0.06 ^a	12.01±0.39 ^a	24.24±0.79 ^a	0.50±0.02 ^a	32.25±0.46 ^a
1:0.50	7.03±0.08 ^a	5.40±0.00 ^b	11.42±0.43 ^a	23.96±0.90 ^a	0.48±0.01 ^{ab}	31.73±0.16 ^a
1:0.75	6.85±0.05 ^a	4.60±0.05 ^c	10.74±0.19 ^b	23.59±0.42 ^a	0.45±0.02 ^b	25.22±0.84 ^b
1:1	6.99±0.08 ^a	3.63±0.06 ^d	10.26±0.16 ^b	24.98±0.39 ^a	0.41±0.01 ^c	19.69±0.10 ^c

Data presented in Table 04 represent the mean ± standard error. Different superscripts in a column indicate significant differences in the ratio of waste to water by Duncan's new multiple range test at $p < 0.05$.

Table 05: Characteristics of crude bromelain extract obtained from extraction of pineapple wastes at different ripeness stages.

Ripeness stage	Apparent pH	Total soluble solid (°Brix)	Proteolytic activity (unit/mL)	Specific proteolytic activity (unit/mg)	Total protein content (mg/mL)	Total carbohydrate content (mg/mL)
FGP	6.88±0.04 ^a	5.08±0.03 ^a	7.76±0.24 ^a	17.59±0.54 ^b	0.44±0.01 ^a	32.72±0.05 ^c
LYP	6.95±0.05 ^a	5.42±0.03 ^b	7.13±0.24 ^b	20.97±0.71 ^a	0.34±0.02 ^c	36.91±0.10 ^b
MYP	6.85±0.04 ^a	6.18±0.03 ^c	7.95±0.15 ^a	19.73±0.37 ^a	0.40±0.01 ^b	42.17±0.47 ^a
FYP	6.93±0.03 ^a	6.40±0.05 ^d	3.93±0.51 ^c	10.25±1.34 ^c	0.38±0.01 ^c	41.95±0.62 ^a

Data presented in Table 05 represents the mean ± standard error. Different superscripts in a column indicate significant differences among ripeness stages by Duncan's new multiple range test at $p < 0.05$. Abbreviation: FGP, fully green peel extract; LYP, less than 50% yellow peel extract; MYP, more than 50% yellow peel extract; FYP, fully yellow peel extract.

Effect of Ripeness Stages on the Chemical Properties of the Crude Bromelain Extracts

The chemical properties of the extracts obtained from the extraction of pineapple fruit waste at different ripeness stages with the preboiled tap water adjusted to pH 7 are shown in Table 05.

The difference in yellowish skin color of pineapple fruit peel based on human eyes is one of the parameters to differentiate ripeness stages (Wang and Chai, 2022). Fully green peel designates unripeness but full maturity of the pineapple fruits. On the other hand, 100% yellow peel shows full ripeness. An increase in the percentage of yellow peel starting from the bottom to the top of the fruits implies the development of ripeness toward senescence or over-ripeness (Assumi *et al.*, 2021). The different

ripeness stages of the selected pineapple fruits were based on the percentage of yellow peel. The crude bromelain extracts obtained from the fruit waste with fully green peel, less than 50% yellow peel, more than 50% yellow peel, and fully yellow peel are represented with FGP, LYP, MYP, and FYP, respectively. The chroma values of FGP, LYP, MYP, and FYP were 28.69±2.68, 70.43±15.70, 99.37±5.46, and 124.69±14.02, respectively. An increase in the chroma values of the pineapple peel is one of the indices for the difference in the ripeness stages of the pineapple fruits (Ullah, *et al.*, 2018). The apparent pH of each extract obtained from different ripeness stages of the pineapple fruit waste was not significantly different, but the total soluble solid and carbohydrate content of the extract significantly increased with the percentage of yellow peel ($p < 0.05$), which was in accordance

with the report of Kumara and Hettigh (2020), where the increase in total soluble solids in the extract from all parts of pineapple fruits, except the core part, was a function of an increase in the percentage of yellow peel. On the other hand, the protein content of the extract did not depend on an increase in the percentage of yellow peel. The highest and lowest proteolytic and specific proteolytic activities were obtained from MYP and FYP, respectively ($p < 0.05$). However, those activities of the extracts of FGP, LYP, and MYP were only slightly different from one another. The lowest proteolytic and specific proteolytic activities, as well as the protein content of FYP, were attributed to fruit ripening and senescence involving in proteolysis and protein catabolism (Koia *et al.*, 2012; Pang *et al.*, 2020). Koia *et al.* (2012) reported that the fruit bromelain activity decreased as pineapple ripens from green peel to yellow peel fruit. In addition, the pineapple fruit waste with less than 50% yellow peel and more than 50% yellow peel is abundant as a byproduct from pineapple processing industries. For the above-mentioned reasons and proteolytic and specific proteolytic activities in Table 05, the pineapple fruits with a ripeness stage of less than 50% yellow or more than 50% yellow peel were used for extraction of the crude bromelain to evaluate the *in vitro* digestibility of soybean meal and shrimp feed.

Effect of Incubation Temperatures and pHs Values of Shrimp Feed Supplemented with Crude Bromelain on *in Vitro* Protein Digestibility

The *in vitro* protein digestibility of soybean meal (SBM) and pretreated shrimp feed (PTSF) with crude bromelain extract are shown in Table 06.

The PTSF was composed of SBM and shrimp feed, which was pretreated with crude bromelain extract before analysis of the *in vitro* protein digestibility. The shrimp feed used for pretreatment contained fishmeal and hydrolysates, liver paste, wheat flour, grains or crops, fish oil, and various feed additives, such as binders, synbiotics, and antioxidants (Prachom,

2022). The results indicate that the *in vitro* protein digestibility of SBM and PTSF varied from 28.56 ± 0.17 to 89.31 ± 1.19 % and from 33.64 ± 0.97 to 68.68 ± 2.70 %, respectively. While the *in vitro* protein digestibility of SBM increased with increasing pH, that of PTSF decreased with increasing pHs ($p < 0.05$). It should be noted that at the same incubation temperature, the *in vitro* protein digestibility of SBM and PTSF showed the highest values at different pH of 9 and 6, respectively. The incubation temperature of 25–40°C had little effect on the *in vitro* protein digestibility.

Klahan *et al.* (2021a) reported that the *in vitro* protein digestibility at higher values than 50% could promote protein digestion during shrimp farming. Regardless of incubation temperature and pH, the *in vitro* protein digestibility of PTSF (28.56 ± 0.17 to 89.31 ± 1.19 %) was higher than that of shrimp feed (4.27 ± 0.29 to 63.22 ± 1.58 %) at the same particle size used ($p < 0.05$). In addition, the *in vitro* protein digestibility of PTSF and shrimp feed showed the same highest value at the same pH of 6. This may be because the PTSF was predigested before measurement of the *in vitro* protein digestibility, as swelling of feed particles was observed during soaking, which indicated protein digestion in comparison with feed particles not swollen during soaking in distilled water, as reported by Jintanawit *et al.*, (2004). Moreover, PTSF contained other compositions, such as protein hydrolysate, oil, starch, and some additives other than SBM, which may influence the proteolytic activity of the crude bromelain extract. Corzo *et al.* (2012) reported that the proteolytic activity of the fruit bromelain depended on types of substrate, including azocasein, casein, azoalbumin, and haemoglobin. Each individual substrate has a different optimal pH varying from 2.9 to 7.7 and optimal temperatures varying from 37 to 59 °C for proteolytic activity. The shrimp feeds supplemented with crude bromelain extract are beneficial to not only protein digestibility, but also to growth performance, feed utilization, and molting stimulation of aquatic animals, as reported by other researchers (Choi *et al.*, 2016; Klahan *et al.*, 2021b).

Table 06: The *in vitro* protein digestibility (%) of soybean meal and pretreated shrimp feed with crude bromelain extract at different incubation temperatures (T) and pH values.

T [°C]	pH	<i>In vitro</i> protein digestibility (%)	
		SBM	PTSF
25	6	43.47±1.20 ^{figh}	68.68±2.70 ^c
	7	55.91±1.35 ^c	58.52±1.41 ^{de}
	8	46.70±3.55 ^{ef}	58.87±1.74 ^{de}
	9	89.31±1.19 ^a	47.74±2.11 ^g
30	6	28.56±0.17 ^j	54.28±3.51 ^{ef}
	7	48.65±2.44 ^e	32.92±2.83 ^{hi}
	8	48.14±1.55 ^e	30.16±1.29 ^{hij}
	9	87.84±3.04 ^a	33.64±0.97 ^{hi}
40	6	34.00±2.01 ⁱ	48.11±2.07 ^g
	7	40.60±1.01 ^h	50.13±1.77 ^{fg}
	8	42.08±3.01 ^{gh}	61.41±1.01 ^d
	9	78.74±1.95 ^b	35.41±0.29 ^h

Data presented in Table 06 represents the mean ± standard error. Different superscripts in a column indicate significant differences among incubation temperature and pH by Duncan's new multiple range test at $p < 0.05$.

In addition, water in shrimp ponds fed with feed containing bromelain shows better quality by decreasing the ammonia nitrogen (Klahan *et al.*, 2020). Moreover, the particle size of the shrimp feed seemed to affect the protein digestibility. The bigger the particle size, the lower the *in vitro* protein digestibility of the shrimp feed treated with the crude bromelain extract was. (Klahan *et al.*, 2021a).

CONCLUSIONS

The suitable extraction of crude bromelain from pineapple fruit waste was investigated. The overall findings suggest that either preboiled tap water or distilled water could be used to obtain the crude bromelain extract at a pH adjusted to 7 with slightly different values of proteolytic and specific proteolytic activities. The suitable ratio of pineapple fruit waste to water was 1 to 0.50 by weight and the optimal ripeness stage was more than 50% yellow peel to acquire the highest

values of both activities. The crude bromelain extract was applied to soybean meal and shrimp feed for their *in vitro* protein digestibility. The shrimp feed pretreated with the crude bromelain extract showed the highest *in vitro* protein digestibility at pH 6 and a 25 °C incubation temperature, and the soybean meal showed the highest *in vitro* protein digestibility at pH 9 and a 25 °C incubation temperature. Consequently, it is possible to use tap water to extract the crude bromelain from the pineapple fruit waste, at the suitable weight ratio of the waste to water and ripeness stage, and apply it to shrimp feed for shrimp farming to reduce feed cost.

Data Availability Statement

The dataset of *in vitro* protein digestibility of shrimp feed for analysis of mean comparison between that of shrimp feed and PTSF is available from co-author (Klahan, R.) on reasonable request.

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Conflict of Interest

The authors declare that there is no conflict of interest for this research.

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