



The Physicochemical Characteristics and Protein Profile of Cottage Cheese Produced by Using Crude Bromelain Enzyme Extracted from *Ananas Comosus*

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Abstract

The utilization of an alternative enzyme for rennet substitution in cheese production offers promising potential for product diversification. In this research, the physicochemical characteristics and protein profile of cottage cheese made by using the crude extract of bromelain enzyme from *Ananas comosus* were evaluated. The observed variables include protein, fat, water, and ash content, as well as the cottage cheese yields, texture, meltability and protein profile. The research was conducted in a completely randomized design with 3 different bromelain concentrations at 1.5% (K1); 3% (K2); and 4.5% (K3) and each was replicated for 4 times. The obtained data were analyzed with analysis of variance (ANOVA) and followed with the Least Significant Difference (LSD) test to measure significant differences, while the protein profile of the cottage cheese was observed by using SDS-PAGE and analyzed descriptively. The different enzyme concentrations showed that the different bromelain concentrations had significant differences ($P < 0.01$) to the overall physicochemical properties of the cottage cheese, with the protein content at 11.50 to 12.64%; fat content at 2.13 to 5.21%; water content at 56.01 to 57.65%; and ash content at 0.59 to 1.68%. The produced cottage cheese had yields at 8.46 to 10.62%; texture at 3.67 to 3.81 N; and meltability at 1.89 to 2.82%, while the protein profile observation showed different bands at 4.58 to 160.47 kDa. The research concludes that 3% bromelain concentration produces cottage cheese with the best physicochemical properties in this research, that contained $12.64 \pm 0.43\%$ protein, $2.13 \pm 0.90\%$ fat, $56.06 \pm 0.22\%$ water, $0.59 \pm 0.38\%$ ash, with cheese texture of 3.81 ± 0.11 N and $282 \pm 0.13\%$ meltability from a total yield of $10.62 \pm 0.06\%$.



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Introduction

The continuous increase of cheese products consumption in Indonesia expand a promising market for local dairy industries as well as offers a new market for local cheese manufacturers. On its production, the cheese curds are commonly produced by adding acetic acid and rennet enzyme to the milk and then further processed according to need. The rennet enzyme is added to coagulate and separate milk into curds and liquid whey. The total curd yields then indicate the effectivity of the used acetic acid and rennet.¹

Aside from the rennet, which could only extracted from the stomachs of ruminants, other coagulants from microbes and plants has also been used even though relatively less popular.^{2, 3} The utilization of ruminant stomach extracted enzyme is known to be expensive and limited due to the availability of ruminant stomach.³ In addition, various factors such as religious concerns, diet limitation and ban on calf rennet in several countries have encouraged for the alternative sources.⁴ Research then directed towards cheese production by utilizing alternative milk-clotting enzymes, including plant-based enzymes to tackle the problem.³

Cottage cheese is one of the cheese products that highly valued and demanded by the consumers. Chandan *et al.*⁵ defined that cottage cheese is a soft cheese with fat content less than 4% and water content less than 80%, with the common consumption of the cheese is by mixing with cream. Those characteristics differentiate cottage cheese with other cheese products such as cheddar which has less than 50% fat and less than 39% water, or cream cheese which has less than 33% fat and less than 55% water. Moreover, Chandan *et al.*⁵ also added that the production of cottage cheese does not require curing.

An enzyme is a group of protein which responsible to catalyze various chemical changes such as hydrolysis, oxidation and reduction, isomerase, bond cleavages and also radical transfers.⁶ Chang⁷ showed that enzyme catalysis could provide a thousand times more efficient chemical reaction, and commonly work on neutral pH and low temperature, had a specific reaction and selective to a certain substrate. Along with the development of cheese processing technology, an approach to the utilization

of natural enzymes, especially extracted from plants began to emerge. One of the promising enzymes to be used in cheese production is bromelain, with the enzyme can be extracted from all parts of pineapple (*Ananas comosus*) whether the fruit, stem or the knobs. The biochemical properties of properties of bromelain extracted from *Ananas comosus* fruit is provided in Table 1.

Table 1: Bromelain enzyme characteristics extracted from *Ananas comosus* fruit

Characteristics	Descriptions
Structural chemistry ⁸	Single-chain protein
Molecular weight ⁸	25 kDa
Isoelectric point ⁹	4.6
Specific activity ¹⁰	4.5 units/mg protein
Total activity ¹⁰	128 units x 10 ⁻²

The research on cheese production by using protease enzyme extracted from *Ananas comosus* plant is still limited, even though the enzyme is expected to be able to produce soft cheese with good meltability and stretchability, and distinctive fibers suitable for pizza or spread cheese.^{11,12} Bromelain enzyme is one of the proteolytic enzymes that can be used to coagulate milk compounds. Jaya and Hadikusuma¹³ described that the utilization of bromelain enzyme extracted from pineapple knobs on the skim milk produced cottage cheese with protein content at 10.34 – 12.77%; water content at 70.5 – 85.78%; pH at 4.36 – 4.75 and total yields at 11.85 – 15.77%, while the utilization of other parts of pineapple has yet to be available.

The North Sulawesi, Indonesia, is the national producer of pineapples (*Ananas comosus*). Data¹⁴ has shown the positive growth of *Ananas comosus* in North Sulawesi, which reached¹⁵ tonnes in 2017. In this research, researchers further determine the potential of *Ananas comosus* as a crude enzyme extract to be used for cottage cheese production. The observed variables include physicochemical characteristics (protein, fat, water, and ash content, as well as the cottage cheese yields, texture and meltability) of the produced cottage cheese. The results of this research are expected to provide information on the local cottage cheese production by using crude enzyme extracted from *Ananas comosus*.

Materials and Methods

The research was conducted from April to May 2018 in the Laboratory of Animal Products Technology, Faculty of Animal Science, the University of Brawijaya Malang for the sample preparation, curd production and meltability measurement. The chemical content of the cottage cheese is measured in the Laboratory of Food Science, Faculty of Agricultural Technology, University of Brawijaya Malang.

Materials

The materials used in this study include bromelain enzyme extracted from pineapple fruit, fresh dairy milk obtained from the Laboratory of Animal Product Technology, University of Brawijaya Malang, plastic container, whey container, heater, thermometer, filter cloth, mixer, and cheese container.

Sample Preparation

The research was conducted as a laboratory experiment with a completely randomized design, consisted of 3 treatments and 4 replications. The research treatments were different bromelain enzyme concentration levels (K1 = 1.5%; K2 = 3%; K3 = 4.5%), with all treatments were incubated at 40°C for 35 minutes. The crude bromelain extraction was done by peeling and cut the *Ananas comosus* as much as 500 g into small pieces and grinded in mortar and pestle to extract the filtrate. The filtrate was then added with 0.1 M phosphate buffer and centrifuged at 3500 rpm for 15 minutes. The solution was then incubated at 4°C and filtered by using What man filter paper (150 mm) to obtain the crude bromelain enzyme.¹⁵ The cottage cheese was made without culture starters and the processing was solely made by using crude bromelain extract. The fresh dairy milk was firstly pasteurized at 70°C for 30 minutes, and then cooled down to 40°C before added with crude bromelain enzyme. The milk was then incubated at room temperature for 35 minutes and filtered to obtain the cottage cheese curd.

Protein Content

The protein content of the produced cottage cheese was measured by the Kjeldahl method.¹³ Three grams of cottage cheese were grounded and put into 30 mL Kjeldahl flask, and then added with 5 gr Na₂SO₄, 20 mL H₂SO₄, and 0.2 gr CuSO₄. The solution was then heated for 3 to 4 hours and until the solution turned clear. The clear solution

was put into distillation flask, added with 100 mL aquadest and 60 mL NaOH 45%. The solution was then distilled with HCL 0.1 N and 1% phenolphalen solution until the solution color turned into pink. The protein content was then determined by the following formula:

$$\text{Protein (\%)} = \frac{\text{Total NaOH (mL)} - \text{Total sample (mL)} \times 14 \times 6.25}{\text{Sample weight} \times 100}$$

Fat Content

The fat content was measured by following AOAC.¹⁶ The boiling flask was firstly heated on the oven at 105°C and then weighed after cooled down in the desiccator. As much as 5 grams of dry sample was placed into the filter paper and then into the Soxhlet extractor. A condenser was then placed above the boiling flask. Hexane solvent was added to the boiling flask accordingly. Sixteen hours reflux was then performed until the sample solution turned clear color. The extracted sample was then dried in the oven for 105°C to evaporate any solvent residue and reached a constant weight. The sample was then cooled in the desiccator and weighed. The fat content is measured by the following formula:

$$\text{Fat (\%)} = \frac{C - B}{A} \times 100\%$$

A	=	Sample weight
B	=	Initial boiling flask weight
C	=	Final boiling flask weight

Water Content

The water content was measured by using an oven as described by AOAC.¹⁶ The sample was dried in the oven at 100 – 105°C for 3 – 5 hours. The sample was then cooled down on the desiccator for 30 minutes. The initial weight and final weight of the sample was then recorded to be calculated by the following formula:

$$\text{Water (\%)} = \frac{\text{Initial weight} - \text{Final weight}}{\text{Initial weight}} \times 100\%$$

Ash Content

The petri dish was heated at 400°C for 1 hour, and then weighed after cooled down on the desiccator for 1 hour (A). The cheese sample for 5 grams was heated for 400°C along with the petri dish for 1 hour, the sample along with the petri dish was then

weighed after cooled down on the desiccator (B). The ash content is calculated as follow¹³

$$\text{Ash (\%)} = \frac{B - A}{\text{Sample weight}} \times 100\%$$

Cheese yield

The yield of cottage cheese was determined by calculating the produced cheese weight and the total weight of the ingredients before processing. The formula is as follow.¹³

$$\text{Yield (\%)} = \frac{\text{Final product weight}}{\text{total Ingredients weight}} \times 100\%$$

Cheese Texture

The cottage cheese texture was measured by using Penetrometer.¹⁷ The texture was then calculated by the following formula:

$$\text{Texture} = \frac{\text{Measured value (mm)}}{\text{load weight (g)} / \text{time (s)}}$$

Cheese Meltability

The meltability of cottage cheese was measured by following Tunick *et al.*¹⁸ The cheese was placed on a tube and heated at 232°C for 5 minutes. After cooled down, the melting length was measured by

using calipers in a horizontal position. The length was measured in millimeters (mm) and scored 1 if there is no increase in length; scored 2 if the length is increased until 5 mm, and the score increased by 1 for every 5 mm increase in length.

Protein Profile

The observation of cottage cheese protein profile was done by using SDS-PAGE electrophoresis.²⁰ The protein molecules are separated by its molecular masses in an electric field. A comassie brilliant blue staining was used as marker to determine the molecular weight of the protein. Sample was added with buffer solution at the ratio of 1:1, and heated at 100°C for 5 minutes and cooled down to the room temperature before placed in the electrophoresis chamber. As much as 10-20 µL was placed in the gel column and then electrophoresis was done at 100 V for 2 hours. The produced bands were then compared to the standard to determine the molecular weight of each band.

Data Analysis

All of the data was analyzed by using analysis of variance (ANOVA) and followed with the Least Significant Difference (LSD) test to determine any significant differences by using SPSS version 16.0.

Table 2: Physicochemical characteristics of cottage cheese

Physicochemical characteristics	Bromelain enzyme concentration		
	K1	K2	K3
Protein (%)	11.93 ± 0.29 ^a	12.64 ± 0.43 ^b	11.50 ± 0.22 ^a
Fat (%)	3.25 ± 0.99 ^{a,b}	2.13 ± 0.90 ^a	5.21 ± 2.01 ^b
Water (%)	57.96 ± 0.12 ^b	56.06 ± 0.22 ^a	57.46 ± 0.51 ^b
Ash (%)	1.68 ± 0.46 ^b	0.59 ± 0.38 ^a	2.06 ± 0.57 ^b
Yield (%)	9.53 ± 0.35 ^b	10.62 ± 0.06 ^c	8.46 ± 0.60 ^a
Texture (N)	3.67 ± 0.11 ^b	3.81 ± 0.11 ^b	3.38 ± 0.81 ^a
Meltability (%)	2.28 ± 0.20 ^{ab}	2.82 ± 0.13 ^b	1.89 ± 0.59 ^a

Description: Different superscripts on each row indicates highly significant differences (P<0.01)

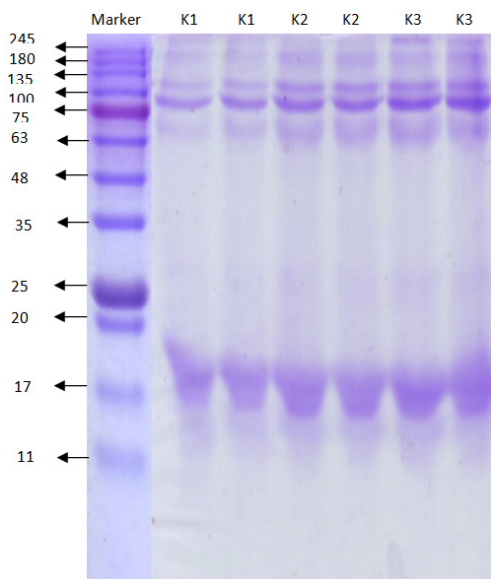
Results and Discussions

The physicochemical characteristics of cottage cheese which include protein, fat, water, and ash content, as well as cheese yield texture

and meltability in this research are presented in Table 2. The molecular weight of cottage cheese as well as SDS-PAGE observation of each treatment is presented in Table 3 and Figure 1, respectively.

Table 3: Molecular weight of the cottage cheese (kDa)

No	K1	K2	K3
1	160.47	133.72	160.47
2	133.72	122.07	133.72
3	101.72	101.72	122.07
4	84.76	84.76	101.72
5	64.48	64.48	84.76
6	7.23	7.23	64.48
7	4.58	4.58	17.99
8			7.23
9			4.58

**Fig 1: Protein profile of the cottage cheese as observed with SDS-PAGE**

Protein

In this research, the protein content of cottage cheese added with different bromelain enzyme concentrations showed significant differences, with the highest protein content was shown in K2 (3% bromelain concentration) which contained $12.64 \pm 0.43\%$ protein. The different protein concentration was due to the proteolytic characteristics of bromelain enzyme. Yamada *et al.*¹⁰ showed that bromelain enzyme from *Ananas comosus* had specific activity at 4.5 units/mg protein with a total of $128 \text{ units} \times 10^{-2}$. This research showed that 3% bromelain concentration produced cottage cheese with higher protein content, which noting that

bromelain enzyme by itself is a protein, thus higher concentration would yield higher protein content, but also showed the 3% bromelain had more efficient protease activity which affect the protein content of the product. The bromelain enzyme would initiate destabilisation of the casein micelles in milk, along with a cooperative aggregation and thus resulting in formation of a three-dimensional protein matrix.^{20,21} The finding by Rusnakova and Jaroslav,²² and Metzger *et al.*²³ showed that the initial acidification on cheese processing could affect the protein content in cheese. The activity of plant-based enzyme is mainly associated with the proteolytic activity of the enzyme with serine and cysteine residues,²⁴ thus different plant-based enzyme would show different proteolytic activity. Macedo *et al.*²⁵ added that different types of plant protease in cheese technology affect the degradation of protein matrix in milk, resulting in different sensory properties of the cheese.

The protein content of cottage cheese in this research is lower compared with the finding of Kariyawasam *et al.*,²⁶ which used different microbial starters in cheese processing. However, the lower protein content is expected as it has been widely known that the addition of microbial starters in a food product would increase the protein content of the food, while the addition of plant-based enzyme would increase the protein degradation of milk.

Fat

The highest fat content of cottage cheese in this research is found on K3 ($5.21 \pm 2.01\%$), while the lowest was found on K2 ($2.13 \pm 0.90\%$). According to Schoumacker *et al.*,²⁷ the common fat content of commercial cottage cheese is 0 to 7.8%. This showed that the overall fat content of produced cottage cheese in this research is suitable for the market. In this research, no starter culture and sodium chloride were used to produce the cottage cheese. Research by Kariyawasam *et al.*²⁶ showed that the addition of starter culture would reduce the fat content in cottage cheese. A combination of bromelain enzyme and starter cultures thus provides a possibility to produce low-fat cottage cheese. Aside from the addition of starter culture, other studies mentioned that the addition the addition of NaCl may inhibit lipolysis during cheese production,²⁷ thus affect the fat content of the cheese, even though with the NaCl addition at 0.5 to 3.0% (w/w) showed no effect on lipolysis.²⁸

Water

Water or moisture is an essential component of food products as it would affect the texture, flavor, and overall acceptability and shelf-life of the products. The water content in food products is accumulated from the water content in food added with another external ingredient addition and/or processing.³⁰ In cheese processing, the loss of moisture would significantly affect the weight loss during the ripening period.³¹ The addition of salt is also known to affect the loss of moisture in cheese.^{32,29} In this research, cottage cheese with the highest water content was found with the addition of 1.5% bromelain enzyme (K1), which was at $57.96 \pm 0.12\%$. The moisture has then resulted in soft cheese. Heller *et al.*³³ described that cheese products can be grouped based on its water content, with soft cheese is a category for cheese products with more than 55% water content. McSweeney³⁴ further explained that high water content would disrupt casein bonds and resulted in a weak texture of the cheese. According to the USDA,³⁵ the water content of cottage cheese in this research has met the standard.

Ash

The ash content in food indicates the inorganic compounds or minerals, which can be found after a certain heating method. In cheese processing, ash content represents the minerals in final product and depends upon the brining used. In this research, K3 (4.5% bromelain concentration) showed the highest ash content, which reached $2.06 \pm 0.57\%$; while the lowest was found in K2 ($0.59 \pm 0.38\%$). The highest ash content of cottage cheese in this research (K3) is higher compared to the finding of Kariyawasam *et al.*²⁶ which used different starters to make the cottage cheese. The ash content of cottage cheese by using *Lactobacillus rhamnosus* was $1.84 \pm 0.02\%$; while by using *Weissella cibaria* D30 $1.80 \pm 0.02\%$. The higher ash content in this research could be affected by several factors, such as the milk origin as well as the absence of starters which could utilize mineral for its metabolism. Research by Mijan *et al.*³⁶ showed higher ash content (2.7%) in the cheese prepared from dairy milk. In this research, NaCl was not added during cheese making, thus lower ash content is to be expected, though Khan and Masud³⁷ showed that higher ash content in cottage cheese was recorded in plant-based enzyme compared to rennet.

Yield

One of the most important parameters in assessing the enzyme alternatives for cheese making is the yield by weight of the produced cheese. Optimal cheese yield is important for cheese production,³⁷ as it would determine the cost and benefit. The total cheese yield during processing is affected by several factors, such as enzyme concentration, temperature, and pH during processing.³⁸ In this research, the total cheese yield was significantly different between each treatment, which is $9.53 \pm 0.35\%$ (K1); $10.62 \pm 0.06\%$ (K2); and $8.46 \pm 0.60\%$ (K3), with the pH during processing were varied from 4.5 to 5.5. It can be seen that the usage of bromelain enzyme as much as 4.5% produced lower cheese yield, while 3% bromelain had higher yield compared to the 4.5% and 1.5% bromelain enzyme concentration. This showed that 3% of bromelain enzyme concentration would produce more optimum cheese yield compared to other concentrations. Purwadi³⁹ explained that an optimum pH and enzyme concentration could initiate efficient milk coagulation, resulting in a compact curd and higher cheese yield. Previous studies on the plant-based enzymes for cheese processing showed that most of them are not suitable for industrial scale, noting that the cheese undergo protein loss as a result of proteolytic activities.³⁷

The effective enzyme activity would be affected by temperature, pH and enzyme concentration. On a higher temperature, milk coagulation will be reduced. In this research, the incubation was performed at 40°C for 35 minutes and showed a good enzyme activity. The milk coagulation was occurred due to the specific bromelain activity toward casein until reached its isoelectric condition. Cavalli *et al.*⁴⁰ further describe that at acidic pH, around 75 to 95% of total extracted enzyme activity correspond to cardosin A. This enzyme was shown to be similar to chymosin in terms of kinetic parameters and specificity by cleaving similar peptide bond of κ -casein.

Texture

In this research, different bromelain enzyme concentrations resulted in a highly significant difference in cheese texture ($P < 0.01$). It is caused by the acidic condition of the bromelain enzyme which increases the proteolytic activity and initiates the breakage of peptide bonds in cheese. Aggraini

*et al.*⁴¹ explained that the protein breakdown into simpler compounds would increase whey solubility and resulted in soft cheese texture. The different cheese texture in this research indicates that different bromelain enzyme concentrations resulted in different curd formations, thus affect the texture. Moreover, research by Sousa *et al.*⁴² showed that plant-based enzyme affects the cheese texture due to the primary proteolysis of milk caseins, while Al-Otaibi⁴³ added that the cheese hardness will be decreased due to the hydrolysis of α 1 casein.

Aside from the curd formation, the water content in cheese could also affect its texture. Gunasekaran and Mehmet⁴⁴ showed that the texture in cheese correlates with water content, with higher water content resulted in lower cheese texture and affect consumers' acceptability to the cheese product. Susanto⁴⁵ also described that water content in cheese has a negative correlation to the elasticity, with lower water content that would result in harder cheese texture.

Meltability

The cheese meltability is mainly affected by fat content as well as protein and water interaction,³⁴ with the preferred cheese meltability is at 2 – 4%. In this research, the addition of 4.5% bromelain enzyme did not meet the preferred cheese meltability, while both 1.5% and 3% bromelain enzyme concentration had more than 2% cheese meltability, which were $2.28 \pm 0.20\%$ and $2.82 \pm 0.13\%$, thus had a more preferred meltability. Lucey *et al.*⁴⁶ described that attractive and repulsive forces between casein control the melting of the cheese. The different cheese meltability in this research indicates that the bromelain enzyme affects the casein compounds which determine the cheese texture.

Protein Profile

The protein profile observation showed that the visible bands were at 4.58 to 160.47 kDa. The protein profile of each treatment in this research were observed two times and showed that on K1 and K2, seven protein bands, while the K3 showed nine protein bands (Table 3 and Fig. 1). The results showed that the casein protein was seen on all

treatments with molecular weight at 64.48 kDa. Similar results⁴⁷ showed that the casein proteins, which include α -casein, β -casein and κ -casein would be seen at around 60 kDa. The results also showed that higher crude enzyme concentrations showed more bands (9 bands) in the SDS-PAGE. The condition is expected as higher enzyme concentration could initiate more proteolysis reactions and resulted in more lysed protein products. This also indicates that the addition crude bromelain enzyme concentrations at more than 4.5% would possibly produce more bands as well. Aside from the enzymatic properties, other factors known to affect the protein degradation of the cheese are storage time⁴⁸ and ripening temperature.⁴⁹

Conclusion

The research concludes that 3% bromelain concentration produce the best physicochemical properties of cottage cheese, which shown the highest protein content as well as cheese yield, texture and meltability with low water, fat and ash content with significant difference at $P < 0.01$. The treatment produced cheese that contained $12.64 \pm 0.43\%$ protein, $2.13 \pm 0.90\%$ fat, $56.06 \pm 0.22\%$ water, $0.59 \pm 0.38\%$ ash, with cheese texture of 3.81 ± 0.11 N and $282 \pm 0.13\%$ meltability from a total yield of $10.62 \pm 0.06\%$. This showed that crude bromelain enzyme could be used as a cheap rennet substitution for cheese making, especially in area rich of *Ananas comosus* production.

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Conflict of Interest

There was no conflict of interest associated with this study by any of the authors.

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