

# Characterization of *Ficus benjamina* and *Artocarpus heterophyllus* Proteases as Potential Rennet Alternatives

Andreas Binar Aji Sukmana\*, Indy Widyaningrum, Rischa Karmila Lani, Sri Kasmiyati

Faculty of Biology, Universitas Kristen Satya Wacana, Indonesia

\*Email: andreas.sukmana@uksw.edu

Submitted: 21 February 2020. Revised: 17 June 2020. Accepted: 1 July 2020

**Abstract.** Rennet, a milk coagulant exhibiting proteolytic activity, is a crucial component in cheese industries. Its price and availability have discouraged the growth of some small scale cheese industries. Therefore, an alternative for rennet will be beneficial for the industries. Among other sources, plant proteases offer some advantages as rennet alternatives. This study aimed to investigate the potential of plant proteases obtained from the latex as potential rennet alternatives. A total of six plants from the genus *Ficus* and *Artocarpus* were screened for their proteolytic activity and milk coagulating ability. The screening indicated that all six tested plants displayed proteolytic activity at various levels, but only *Ficus benjamina* and *Artocarpus heterophyllus* produced a firm milk curd. Hence, both *F. benjamina* and *A. heterophyllus* were determined to be the most potential. Further characterizations suggested that *F. benjamina* and *A. heterophyllus* protease were optimum at pH 7.0 also at 50°C and 40°C, respectively. At their optimum conditions, both proteases exhibited a lower MCA/PA ratio than that of the rennet. This study contributed to scientific knowledge development by becoming the first to characterize the optimum conditions of *F. benjamina* and *A. heterophyllus*' proteases, investigate their MCA/PA ratio, and compare their activity against commercial rennet. The examination of their potentials as rennet alternatives could benefit small cheese industries and the communities.

**Key words:** *A. heterophyllus*; *F. benjamina*; Milk-Clotting; Proteolytic; Rennet

**How to Cite:** Sukmana, A. B. A., Widyaningrum, I., Lani, R. K., & Kasmiyati, S. (2020). Characterization of *Ficus benjamina* and *Artocarpus heterophyllus* Proteases as Potential Rennet Alternatives. *Biosaintifika: Journal of Biology & Biology Education*, 12 (2), 213-219

**DOI:** <http://dx.doi.org/10.15294/biosaintifika.v12i2.23516>

## INTRODUCTION

The trend of cheese consumption in Indonesia is increasing every year. The cheese was originally not a typical food in Indonesia, but it gains popularity in recent decades, increasing the demand for cheese steadily (Mansur, 2018). In 2020, the cheese consumption in Indonesia is predicted to grow by 4.43%, with the projected industrial revenue to increase by 8.7% (*Cheese—Indonesia | Statista Market Forecast*, 2020). The growths are reflected in an increase in the number of cheese industry in Indonesia, including small scale industries, which is expected to grow annually by 5.2% (*Cheese—Indonesia | Statista Market Forecast*, 2020).

One of the critical components in the cheese-making process is rennet. Rennet is added to the milk and transforms the milk into solid curd and liquid whey, in which the curd will be processed further into cheese. The main constituent of the rennet is rennin, an aspartic protease that cleaves kappa-casein protein. Kappa-casein possesses both hydrophobic and hydrophilic regions. It protects and stabilizes the hydrophobic alpha and beta-casein against the water component in the milk allowing casein complex to be soluble (Kethireddipalli & Hill, 2015). In the presence of rennin, kappa-casein is hydrolyzed at the Phe105-

Met106 bond, removing the hydrophilic portion as well as exposing both hydrophobic alpha and beta-casein to the aqueous environment. This exposure further destabilizes the casein complex, which leads to milk coagulation (Amira et al., 2017).

Rennet was initially extracted from the calf abomasum, and nowadays, many alternatives produced by microorganisms are available (Benlounissi et al., 2014). Lengthy and intricate processes in that rennet production drive the selling price to be relatively high. The high cost of rennet is unfavorable for small scale cheese industries to grow (Bouma et al., 2014). Other than the price, rennet availability also becomes an issue. The small industries usually do not have a direct access to rennet. Therefore, a cheaper and more readily available rennet alternatives will be beneficial for those small industries.

One strategy of finding rennet alternatives is by screening proteases for their ability to coagulate milk similar to that of rennet. Proteases are prevalent enzymes, and they can be found in all organisms (Mótyán et al., 2013). Compared to microorganisms and animals, plants are simpler to process and less sensitive to many ethical issues. Also, locally available and abundant ones are potentially easier to collect. Hence, plants can be considered as potential candidates for protease sources (Amira et al., 2017).

Some studies have been conducted in studying plant proteases as a milk coagulating agent to replace rennet. Bromelain, ficin, zingibain, cucumisin, and papain; proteases from pineapple, cluster fig, ginger, melon, lettuce, and papaya respectively have been studied for their milk coagulating ability (Faccia et al., 2012; Huang et al., 2011; Silvestre et al., 2012). However, their application in cheese production is still limited. In South Sulawesi, Indonesia, the latex from unripe papaya containing papain had been traditionally utilized to coagulate milk during the process of making a cheese-like food known as *dangke* (Sulmiyati, 2017).

To increase the probability of obtaining potential proteases, the screening in this study targeted plants that produce latex. A similar approach has been applied to screening potential plant protease for a milk-coagulating enzyme in the genus *Euphorbiaceae* (Fibriana & Upaichit, 2015). The reasoning of targeting the plants which produce latex is because the latex is a form of defense mechanism against pathogens and herbivores, and it may contain proteases (Siti-Balqis & Rosma, 2020). For this study, the examined plants were selected from the Moraceae family. This plant family is known for its ability to produce latex (Somashkhar et al., 2013).

This study aimed to investigate the potential of plant latex-containing protease from *Ficus* and *Artocarpus* genera, members of the Moraceae family, as rennet alternatives. Some studies have been conducted to examine the protease from *Ficus* spp. and *Artocarpus* spp., however, the information on their proteolytic characteristics related to milk-coagulating properties are still limited. The novel data obtained from this study are not only crucial in enriching scientific knowledge but also essential in unraveling the potential of local plants to support small industries and the communities.

## METHODS

This study focused on *Ficus* and *Artocarpus* genera. Three members from each genus were selected based on their local availability and abundance. The three selected members from the genus *Ficus* are *F. benjamina*, *F. lyrata*, and *F. septica*. Three members from the genus *Artocarpus* are *A. heterophyllus*, *A. altilis*, and *A. camansi*. The exploration was conducted by examining their protease activity and screening their milk-coagulating ability. For the two most prospective plants (*F. benjamina* and *A. heterophyllus*), their optimum conditions for protease activity and their MCA/PA ratio were studied to examine their potential as rennet alternatives further.

## Latex collection and crude enzyme extract preparation

Latex from each plant was collected by incising the young shoots. The latexes were mixed with 50 mM cold phosphate buffer pH 7.0 to obtain a final concentration of 0.1 g latex.ml<sup>-1</sup> buffer. The latex-buffer mixtures were thoroughly mixed by vortexing followed by centrifugation at 10,000 rpm for 5 minutes. Soluble protease in the supernatant fraction was collected and was kept cold until subsequent analyses. The supernatants will be referred to as crude enzyme extracts from now on.

## Proteolytic activity determination

The proteolytic activity of the latex proteases was determined using the Folin-Ciocalteu method (Cuppenyard, 2008). The activity was calculated from the enzyme's ability to hydrolyzed substrate casein and produce free tyrosine. Dilutions of the crude enzyme extract were performed when necessary. Rennet 1 mg.ml<sup>-1</sup> was included as the control. Briefly, 500 µl crude enzyme extract from each plant was mixed with 500 µl pre-warmed 1% casein (Merck) at 37°C in a test tube. The mixtures were then incubated at 37°C in a water bath for 10 minutes. The reaction was stopped by the addition of 1.25 ml TCA 5%. The precipitated proteins from the mixtures were separated by cold centrifugation at 10,000 rpm for 10 minutes, and then the supernatants were recovered and kept cold on ice. As much as 500 µl of the supernatants were each mixed with 1.25 ml Na<sub>2</sub>CO<sub>3</sub> 500 mM and 250 µl Folin-Ciocalteu reagent (Merck). The mixtures were incubated at 37°C for 10 minutes and were brought to room temperature before measurement. The absorbances were read at λ 660 nm (Shimadzu UVmini-1240). The concentration of released tyrosine was calculated by comparing the absorbance with the tyrosine standard curve. The proteolytic activity was expressed as Unit per mg protein. One unit of proteolytic activity is described as the amount of tyrosine being produced in µmol per minute. This measurement was conducted in triplicate.

## Protein concentration determination

The protein concentration of the crude enzyme extracts was determined using the Bradford assay. Briefly, 25 µl of the crude enzyme extract was mixed with 1 ml Bradford reagent followed by a 5 minutes dark incubation at room temperature. The developed blue color was measured at λ 595 nm, and the absorbance was compared to Bovine Serum Albumin (Merck) standard curve. This measurement was conducted in triplicate.

### Milk coagulation screening assay

Milk coagulation potential for the screening was conducted by mixing 20 µl crude enzyme extract with 5 ml 10% skim milk containing 10 mM CaCl<sub>2</sub> in phosphate buffer pH 7.0 (Pontual et al., 2012). Rennet 1 mg.ml<sup>-1</sup> and phosphate buffer were included as a positive and negative control, respectively. The mixtures were then incubated in a 37°C water bath for 15 minutes. The tubes were tilted to examine the sign of coagulation. This assay was conducted in triplicate.

### Optimum pH and temperature determination

Crude enzyme extract of *F. benjamina* and *A. heterophyllus* were characterized for their optimum pH and temperature by measuring their proteolytic activity at varying pH and temperature, respectively. The pH and temperature yielding the highest proteolytic activity were determined to be the optimum condition. Overall, the assay was conducted similarly as in the proteolytic activity determination. For optimum pH determination, the pH of the reactions was adjusted to 3.0, 4.0, 5.0, 6.0, 7.0, and 8.0 using 50 mM phosphate buffer while the temperature was set at 37°C. For optimum temperature determination, the reaction mixtures were incubated at 4, 25, 30, 40, 50, 60, and 70°C while the pH was kept at 7.0. The measurement was conducted in triplicate.

### Milk-coagulating activity (MCA), proteolytic activity (PA), and MCA/PA ratio determination

The milk-coagulating activity of *F. benjamina*, *A. heterophyllus*, and commercial rennet were determined by a similar procedure as the milk coagulating screening assay. The pH and incubation temperature for each sample was set to their optimum conditions. The time needed for each sample to show a sign of coagulations was recorded. The MCA was calculated by the following equation (Wu et al., 2013):

$$MCA = \frac{2400 \times V}{t \times v}$$

Note,

MCA = Milk-coagulating activity

t = coagulating time (in seconds)

V = skim milk volume (in ml)

v = volume of crude enzyme extract (in ml)

One unit of MCA is expressed as the amount of milk (in ml) that can be coagulated by 1 ml crude enzyme extract in 40 minutes.

Proteolytic activity of *F. benjamina*, *A. heterophyllus*, and commercial rennet were determined as previously described at each of their respective optimum condition. Here, the proteolytic activity is expressed as Unit per ml crude enzyme extract. One unit

of proteolytic activity is described as the amount of tyrosine being produced in µmol per minute.

MCA/PA ratio was calculated by dividing MCA with PA. The ratio of MCA/PA is unitless. The MCA and PA determination were conducted in triplicate.

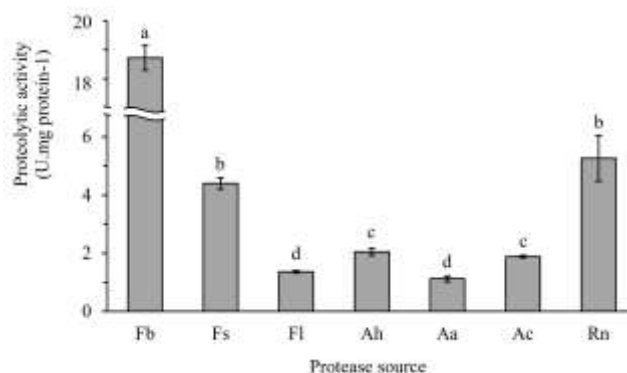
### Data analysis

The data were analyzed statistically using SPSS ver.22. The mean differences among samples or treatments were examined using one-way ANOVA, and the significant differences were analyzed by Tukey's test with  $p < 0.05$ .

## RESULTS AND DISCUSSION

### Proteolytic activity

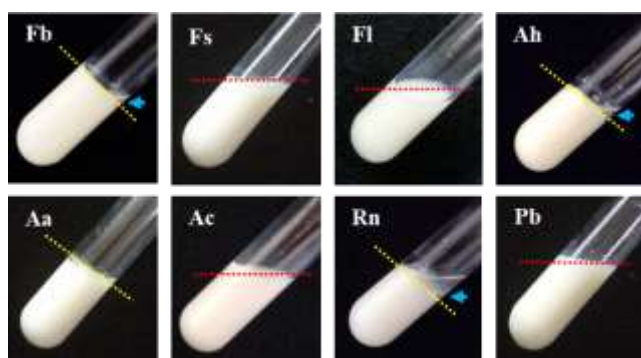
The first step of screening was conducted by examining the level of proteolytic activity exhibited by the latex of each tested plants. Rennet was used in this experiment to serve as a positive control. From the six tested plants, the proteolytic activity from the highest to the lowest were *F. benjamina*, *F. septica*, *A. heterophyllus*, *A. camansi*, *F. lyrata*, and *A. altilis* at 18.72, 4.40, 2.04, 1.88, 1.36 and 1.12 U.mg protein<sup>-1</sup> respectively while the rennet exhibited a proteolytic activity at 5.27 U.mg protein<sup>-1</sup> (Figure 1).



**Figure 1.** A proteolytic assay using crude latex extract from the tested plants. Fb, Fs, Fl, Ah, Aa, Ac, and Rn represent *F. benjamina*, *F. septica*, *F. lyrata*, *A. heterophyllus*, *A. altilis*, *A. camansi*, and commercial rennet, respectively. Error bars represent standard deviations from three replicates. Different letter on top of each bar indicates a significant difference (Tukey's test  $p < 0.05$ ). The Y-axis is split to display the high activity of Fb better.

The different levels of proteolytic activity from the tested plants might be an early indication for their potential. However, proteolytic activity is not the single determining factor in milk coagulation. A high proteolytic activity does not directly translate to an excellent milk-coagulating property (Amira et al., 2017). Therefore, to further evaluate their potential, the ability of the crude enzyme extracts in coagulating the milk was examined.

The milk coagulation screening assay presented in Figure 2 suggested that not all six tested plants had the ability to coagulate milk. Crude enzyme extract of *F. septica*, *F. lyrata*, and *A. camansi* failed to coagulate the milk (Figure 2-Fs, Fl, and Ac). The milk stayed liquid even after 30 minutes of incubation. Meanwhile, the extract of *F. benjamina*, *A. heterophyllus*, and *A. altilis* successfully coagulated the milk (Fig 2-Fb, Ah, and Aa). Rennet and phosphate buffer were included as a positive and a negative control, respectively. Rennet addition coagulated the milk resulted in a clear separation between the solid white curd and the clear liquid whey when the tube was tilted (Figure 2-Rn). Phosphate buffer addition did not change the state of the milk, the milk stayed liquid, and it did not display any separation (Fig 2-Pb).

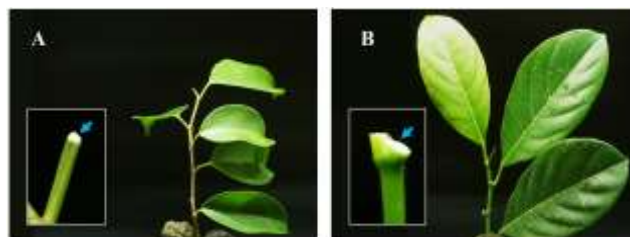


**Figure 2.** Milk coagulation screening assay on crude latex extract from the tested plants. The assay was conducted in triplicate, and one tube from each triplicated was documented above as a representative. Fb, Fs, Fl, Ah, Aa, Ac, Rn, and Pb represent *F. benjamina*, *F. septica*, *F. lyrata*, *A. heterophyllus*, *A. altilis*, *A. camansi*, commercial rennet, and buffer respectively. The tubes were tilted to examine milk clot formation. Red-dashed lines, which are parallel to the earth's surface, depict the surface of liquid milk. Yellow-dashed lines depict the surface of solid curd, which no longer parallel to the earth's surface, and the blue arrows indicate the liquid whey. Both solid curd and liquid whey are the results of a successful milk clotting.

Upon further examination, the curd on *A. altilis* treatment was looser than that of the *F. benjamina* and *A. heterophyllus*. *F. benjamina* and *A. heterophyllus* produced a firm curd with notable whey formation, comparable to that of rennet (Figure 2). Both the results in Figure 1 and Figure 2 suggest that *F. benjamina* and *A. heterophyllus* may be good candidates for rennet alternatives. Therefore, it is crucial to investigate their biochemical characteristics to determine the best condition for the cheese-making application.

### *F. benjamina* and *A. heterophyllus* proteases

A further examination of *F. benjamina* and *A. heterophyllus* was conducted using the latex-containing protease to examine their biochemical characteristics. *F. benjamina* and *A. heterophyllus* young shoots and their latex were displayed in Figure 3. Upon further examination, *F. benjamina* and *A. heterophyllus*' proteases exhibited similar optimum conditions (Figure 4 and Figure 5). The optimum condition for *F. benjamina* protease in the crude enzyme extract was determined to be at pH 7.0 and 50°C (Figure 4A and 4B). Similar to that of *F. benjamina*, *A. heterophyllus* protease worked optimally at pH 7.0 and 40°C (Figure 5A and 5B).



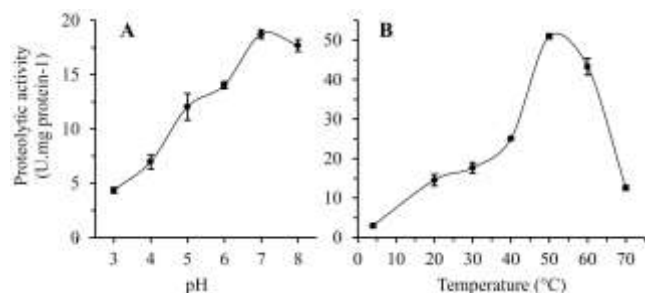
**Figure 3.** The young shoots of the two most potential plants which were used in this study, *F. benjamina* (panel A) and *A. heterophyllus* (panel B). The latex (blue arrows) was collected from the incised young shoots (insets on both panels).

*F. benjamina*'s activity at pH 7.0 in Figure 4A was comparable with the result presented in Figure 1, which was conducted in the same condition. Its activities at pH 6.0 and below were significantly lower than at pH 7.0. Its activity at pH 8.0, although it was lower than at pH 7.0, was not significantly different from pH 7.0. This result indicated that *F. benjamina*'s protease was more active at neutral pH as well as at slightly alkaline conditions.

*F. benjamina*'s proteolytic activity was increasing as the temperature increased. Its optimum activity was observed at 50°C (Figure 4B). *F. benjamina* activity at 50°C was 50.99 U.mg<sup>-1</sup>; this value was about 2.5 times higher than the previously observed activity at 37°C (Figure 1). The activity measured at 37°C (Figure 1) falls between the activities measured at 30°C and 40°C (Figure 3B) indicated the validity of the assay. At higher temperatures, 60°C and 70°C, the activities decreased significantly. This decrease was possibly due to the partial enzyme denaturation at higher temperatures (Huang et al., 2011).

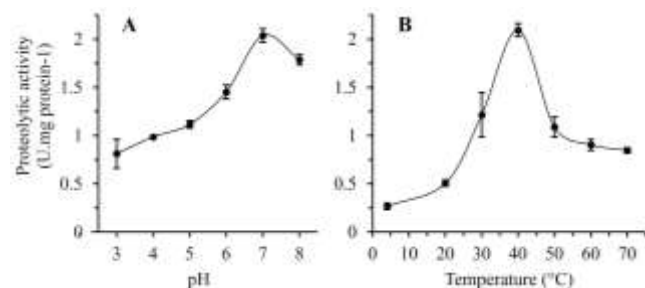
This finding was consistent with the previous finding in which a purified cysteine protease of *F. benjamina* worked optimum between pH 6 to 8 at 60°C (Wanderley et al., 2018). The result in this study was slightly different on its optimum temperature, possibly due to the crude enzyme extract being used instead of the purified enzyme. Also, the biochemical

characteristics of *F. benjamina*'s protease are comparable to that of the other *Ficus* spp. They were most active at pH 6.0 to 8.0 and at 50°C to 70°C (Afsharnezhad et al., 2019; Sharma et al., 2012; Wahyuni et al., 2015).



**Figure 4.** The optimum condition for *F. benjamina*'s proteolytic activity. Panel A and B display the examination for optimum pH and optimum temperature, respectively. The Y-axis in both panels represents proteolytic activity. Each point represents the average of triplicates, and each error bar represents standard deviations.

*A. heterophyllum* demonstrate a pH-related activity profile similar to that of *F. benjamina* (Figure 5A). Its activity was increasing as the pH increased, and it reached optimum at pH 7.0 before it decreased at pH 8.0. The activity at pH 7.0 was 2.03 U.mg<sup>-1</sup>, and it was consistent with the previous measurement conducted at pH 7.0 in Figure 1 at 2.04 U.mg<sup>-1</sup>. Slightly different from *F. benjamina*, *A. heterophyllum* protease exhibits optimum activity at 40°C, as seen in Fig 5B. At 2.09 U/mg, this activity is comparable with the activity at a slightly lower temperature (37°C in Figure 1). As the temperature increased, at 50°C and above, the activities were significantly lower. The information on the biochemical characteristics of *Artocarpus* spp. proteases are still limited. *A. altilis* and *A. integer* exhibited a proteolytic activity which most active at pH 10 and 40°C (Siti Balqis & Rosma, 2011; Siti-Balqis & Rosma, 2020). Those conditions were similar to that of the *A. heterophyllum* except that the pH was different.



**Figure 5.** The optimum condition for *A. heterophyllum*'s proteolytic activity. Panel A and Panel B display the examination for optimum pH and optimum temperature, respectively. The Y-axis in both panels represents proteolytic activity. Each point represents the average of triplicates, and each error bar represents standard deviations.

resents proteolytic activity. Each point represents the average of triplicates, and each error bar represents the standard deviations.

Both *F. benjamina* and *A. heterophyllum* optimum pH was 7.0 and less active at lower pH. This pH requirement suggested that the mode of action of *F. benjamina* and *A. heterophyllum* proteases might be different from that of chymosin in the rennet, which is active in an acidic environment (Kethireddipalli & Hill, 2015). Therefore, their milk coagulating properties need to be examined.

**The MCA/PA ratio of both *F. benjamina* and *A. heterophyllum***

MCA/PA ratio is considered as one of the standards to determine the quality of rennet alternatives (Mazorra-Manzano et al., 2013). A good rennet alternative displays a similar or higher MCA/PA ratio than that of rennet, indicating decent milk coagulating activity without an excessive proteolytic activity, which results in bitter taste formation. The examination of MCA, PA, and MCA/PA ratio of *F. benjamina* and *A. heterophyllum* with their comparison to a commercial rennet are shown in Table 1. *F. benjamina* displayed an exceedingly high MCA and PA, resulted in an overall low MCA/PA ratio at 32% than that of the rennet. *A. heterophyllum* showed the opposite trend of *F. benjamina*; its MCA and PA were lower than that of rennet. Its MCA/PA ratio is 58% than that of the rennet.

**Table 1.** The comparison of milk coagulating potential-related properties

Protease Source	MCA (U)	PA (U/ml)	MCA/PA
<i>F. benjamina</i>	8301.58 ± 287.03 <sup>a</sup>	23.26 ± 0.20 <sup>a</sup>	357.66 ± 12.09 <sup>a</sup>
<i>A. heterophyllum</i>	663.26 ± 16.67 <sup>c</sup>	1.02 ± 0.03 <sup>c</sup>	649.48 ± 32.22 <sup>b</sup>
Rennet	2433.97 ± 74.67 <sup>b</sup>	2.20 ± 0.32 <sup>b</sup>	1121.67 ± 172.77 <sup>c</sup>

Note: MCA and PA represent Milk Coagulating Activity and Proteolytic Activity, respectively. Each value represents the average of triplicates ± its standard deviation. The following letters indicate significant differences for each parameter (Tukey's test p < 0.05).

Based on the MCA/PA ratio, it was suggested that *A. heterophyllum* was more potential than *F. benjamina* as a rennet alternative. Its low PA can potentially minimize the bitter taste formation. Its relatively low MCA activity can theoretically be compensated by using a more concentrated crude enzyme extract. It is predicted that this will not be a significant problem

because *A. heterophyllus* is known as a latex producing plant (Siritapetawee et al., 2012). Therefore, the availability of its latex might be sufficient and could satisfy the needs of a small-scale cheese industry.

*F. benjamina*, with its low MCA/PA ratio, might require more optimization. *F. benjamina* latex coagulated milk effectively, but its high proteolytic activity increased the risk of bitter taste development (Mazorra-Manzano et al., 2013). Theoretically, the risk can be minimized by limiting its proteolytic activity through incubation condition manipulations (e.g., temperature and pH). Lowering the incubation temperature to 40°C from its optimum temperature at 50°C decreased its proteolytic activity by half (Figure 4B). The other option is by adjusting the amount of latex extract for the coagulating process. The lower the enzyme concentration, the lower its enzyme activity will be (Robinson, 2015).

The novel biochemical characteristics of *F. benjamina* and *A. heterophyllus* proteases, together with their MCA/PA ratios, were described in this study. From the pure science perspective, this new information enhances the scientific understanding of local plants and their characteristics. From the applied science perspective, this study has the potential to benefit the community. The data on biochemical characteristics of those proteases are essential to optimize the milk clotting process during cheese production as rennet alternatives. The availability of rennet alternatives will be beneficial in supporting small cheese industries economically as well as the communities.

## CONCLUSION

The investigation on six tested plants indicated different levels of proteolytic and milk coagulating potentials. *F. benjamina* and *A. heterophyllus* proteases were determined to have better potentials as rennet alternatives. Their proteases were the most active at pH 7. *F. benjamina*'s protease was optimum at 50°C, while *A. heterophyllus*' was at 40°C. At their optimum conditions, both proteases were not perfect rennet alternatives because their MCA/PA ratios were lower than those of the rennet. Therefore, further adjustments are required to optimize their potential. Comprehensively, the results demonstrated in this study contributed to the development of both the pure and applicative scientific.

## ACKNOWLEDGMENT

The authors are grateful to the Faculty of Biology Universitas Kristen Satya Wacana for providing the facilities and financial support. The authors also thank the staff at Biochemistry and Biology Molecular Lab as well as Microbiology Lab for assistance.

## REFERENCES

- Afsharnezhad, M., Shahangian, S. S., & Sariri, R. (2019). A novel milk-clotting cysteine protease from *Ficus johannis*: Purification and characterization. *International Journal of Biological Macromolecules*, *121*, 173–182.
- Amira, A. B., Besbes, S., Attia, H., & Blecker, C. (2017). Milk-clotting properties of plant rennets and their enzymatic, rheological, and sensory role in cheese making: A review. *International Journal of Food Properties*, *20*(sup1), S76–S93.
- Benlounissi, A., Mechakra-Maza, A., Blum, L. J., & Marquette, C. A. (2014). Identification and characterization of milk-clotting proteases produced by two species of mold. *African Journal of Biotechnology*, *13*(11).
- Bouma, A., Durham, C. A., & Meunier-Goddik, L. (2014). Start-up and operating costs for artisan cheese companies. *Journal of Dairy Science*, *97*(6), 3964–3972.
- Cheese—Indonesia | Statista Market Forecast*. (2020). Statista. <https://www.statista.com/outlook/40010400/120/cheese/indonesia>
- Cupp-Enyard, C. (2008). Sigma's Non-specific Protease Activity Assay—Casein as a Substrate. *Journal of Visualized Experiments : JoVE*, *19*, 899.
- Faccia, M., Picariello, G., Trani, A., Loizzo, P., Gambacorta, G., Lamacchia, C., & Di Luccia, A. (2012). Proteolysis of Caciocotta cheese made from goat milk coagulated with caprifig (*Ficus carica sylvestris*) or calf rennet. *European Food Research and Technology*, *234*(3), 527–533.
- Fibriana, F., & Upaichit, A. (2015). Proteases from Latex of *Euphorbia* spp. And Its Application on Milk Clot Formation. *Biosaintifika: Journal of Biology & Biology Education*, *7*.
- Huang, X. W., Chen, L. J., Luo, Y. B., Guo, H. Y., & Ren, F. Z. (2011). Purification, characterization, and milk coagulating properties of ginger proteases. *Journal of Dairy Science*, *94*(5), 2259–2269.
- Kethireddipalli, P., & Hill, A. R. (2015). Rennet Coagulation and Cheesemaking Properties of Thermally Processed Milk: Overview and Recent Developments. *Journal of Agricultural and Food Chemistry*, *63*(43), 9389–9403.
- Mansur, A. (2018). *Menakar Peluang dan Potensi Usaha Keju*. <http://www.neraca.co.id/article/99191/menakar-peluang-dan-potensi-usaha-keju>
- Mazorra-Manzano, M. A., Perea-Gutiérrez, T. C., Lugo-Sánchez, M. E., Ramirez-Suarez, J. C., Torres-Llanez, M. J., González-Córdova, A. F., & Vallejo-Cordoba, B. (2013). Comparison of the milk-clotting properties of three plant extracts. *Food Chemistry*, *141*(3), 1902–1907.

- Mótyán, J. A., Tóth, F., & Tózsér, J. (2013). Research Applications of Proteolytic Enzymes in Molecular Biology. *Biomolecules*, 3(4), 923–942.
- Pontual, E. V., Carvalho, B. E. A., Bezerra, R. S., Coelho, L. C. B. B., Napoleão, T. H., & Paiva, P. M. G. (2012). Caseinolytic and milk-clotting activities from *Moringa oleifera* flowers. *Food Chemistry*, 135(3), 1848–1854.
- Robinson, P. K. (2015). Enzymes: Principles and biotechnological applications. *Essays in Biochemistry*, 59, 1–41.
- Sharma, A., Kumari, M., & Jagannadham, M. V. (2012). Religiosin C, a cucumisin-like serine protease from *Ficus religiosa*. *Process Biochemistry*, 47(6), 914–921.
- Silvestre, M. P. C., Carreira, R. L., Silva, M. R., Corcosinho, F. C., Monteiro, M. R. P., & Morais, H. A. (2012). Effect of pH and Temperature on the Activity of Enzymatic Extracts from Pineapple Peel. *Food and Bioprocess Technology*, 5(5), 1824–1831.
- Siritapetawee, J., Thammasirirak, S., & Samosornsuk, W. (2012). Antimicrobial activity of a 48-kDa protease (AMP48) from *Artocarpus heterophyllus* latex. *European Review for Medical and Pharmacological Sciences*, 16(1), 132–137.
- Siti Balqis, Z., & Rosma, A. (2011). *Artocarpus integer* leaf protease: Purification and characterisation. *Food Chemistry*, 129(4), 1523–1529.
- Siti-Balqis, Z., & Rosma, A. (2020). Serine protease from *Artocarpus altilis* (breadfruit) latex. *IOP Conference Series: Earth and Environmental Science*, 411, 012014.
- Somashekhar, M., Nayeem, N., & Sonnad, B. (2013). A review on family Moraceae (mulberry) with a focus on *Artocarpus* species. *World Journal of Pharmacy and Pharmaceutical Sciences (WJPPS)*, 2(5), 2614–2626.
- Sulmiyati, S. (2017). Karakteristik Fisik dan Kimia Air Dadih (Whey) Dangke dengan Level Enzim Papain yang Berbeda (Chemical and Physical Properties of Dangke Whey Using Different Levels of Papain Enzyme). *Jurnal Ilmu dan Teknologi Peternakan*, 5(2), 102–106.
- Wahyuni, S., Susanti, R., & Iswari, R. S. (2015). Isolation and characterization of ficin enzyme from *Ficus septica* Burm F stem latex. *Indonesian Journal of Biotechnology*, 20(2), 161–166.
- Wanderley, L. F., Soares, A. M. dos S., Silva, C. R. e, Figueiredo, I. M. de, Ferreira, A. T. da S., Perales, J., Mota, H. R. de O., Oliveira, J. T. A., Costa Junior, L. M., Wanderley, L. F., Soares, A. M. dos S., Silva, C. R. e, Figueiredo, I. M. de, Ferreira, A. T. da S., Perales, J., Mota, H. R. de O., Oliveira, J. T. A., & Costa Junior, L. M. (2018). A cysteine protease from the latex of *Ficus benjamina* has in vitro anthelmintic activity against *Haemonchus contortus*. *Revista Brasileira de Parasitologia Veterinária*, 27(4), 473–480.
- Wu, F.-C., Chang, C.-W., & Shih, I.-L. (2013). Optimization of the production and characterization of milk clotting enzymes by *Bacillus subtilis* natto. *SpringerPlus*, 2.