

## Original Research Article

# Characteristics of Coagulants from Moringa Seed Extract (*Moringa oleifera*) with Microwave-Assisted Extraction (MAE) Method

### ABSTRACT

Moringa seed is an alternative coagulant that can be used in various industrial fields, generally used for water purification and cheese making. In addition, to obtain the required chemical compounds, Moringa seed can be extracted by conventional or modern methods. This research was conducted to obtain the coagulant characteristics of Moringa oleifera extracts using the Microwave-Assisted Extraction (MAE) method with different extraction times. This research was conducted at the Animal Products Laboratory, Faculty of Animal Science, and Biochemical Laboratory, Faculty of Mathematics and Natural Sciences, Brawijaya University, Malang, between September 2021 and November 2022. The research material used was Moringa seed powder with different extraction times, designed by 4 treatments and 4 replications consisting of P0: maceration for 48 hours (control), P1: extraction for 6 minutes, P2: extraction for 9 minutes, and P3 extractions for 12 minutes with the MAE method. The variables tested were protein content, protein profile (SDS PAGE), milk clotting activity (MCA), and caseinolytic activity (CA), also the ratio of MCA to CA. The research method was a laboratory experiment analyzed by Analysis of Variance (ANOVA) using Duncan's Multiple Range Test (DMRT) if there were differences. The result showed a highly significant difference ( $P < 0.01$ ) in protein content, milk clotting activity (MCA), and caseinolytic activity (CA). However, there was no significant difference ( $P > 0.05$ ) in the ratio of MCA to CA. The characteristics of Moringa seed extract in this study were protein content ranging from 0.979-1.022 mg/ml, milk clotting activity (MCA) 3.837-3.999 SU/ml, caseinolytic activity (CA) 2.308-2.455 U/ml, and the ratio of MCA to CA 1.627-1.663. In conclusion, the best treatment for the extraction time of Moringa seed extract is 6 minutes with the MAE method, which can be used as a coagulant in the manufacture of mozzarella cheese.

**Keywords:** Caseinolytic activity; coagulant; Microwave-Assisted Extraction; milk clotting activity; Moringa seed extract

### 1. INTRODUCTION

Milk is a functional food that contains various nutrients such as protein, carbohydrates, fat, minerals, and vitamins needed by humans. This food material is easily damaged and needs to be preserved to extend its shelf life by processing it into dairy products such as cheese [1]. Cheese can be classified into three types based on its texture, namely hard cheese, semi-hard cheese, and soft cheese [2]. Mozzarella cheese is a soft

cheese whose manufacturing process does not go through a ripening or fermentation process and includes fresh cheese. This cheese is used as a topping on food because of its soft texture, meltable and delicious taste [3]. Cheese is made by coagulation using bacteria or the rennin enzyme to separate casein from whey [4]. Generally, the rennin enzyme used is derived from calf abomasum, which is limited in availability and relatively expensive, so alternative

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coagulants are needed as a substitute for rennin.

Moringa seed is an alternative coagulant that has been used in water purification and cheese making [5]. The Moringa seed coat contains protein at a level of 15,680 ppm/gram lower than the inner seed/cotyledon of Moringa, which is 147,280 ppm/gram, so the use of Moringa seed as a coagulant should be peeled first [6]. According to [7], moringa is also a source of milk clotting enzymes (MCE). Partial purification of Moringa seed extract resulted in the highest ratio of milk clotting activity to proteolytic activity compared to Moringa leaf extract and was able to produce a white and hard curd. Milk clotting activity was highest at pH 5, the temperature of 65°C, while the concentration of partial purification of 10% Moringa seed extract and Moringa leaf extract yielded 35.36 U/ml and 12.88 U/ml [8]. The Rennin enzyme derived from plants has been in great demand for research in the cheese-making industry because of its abundant availability and use of a simple purification process [9].

Moringa seeds can be extracted using one of the currently developed methods, namely Microwave-Assisted Extraction (MAE), which utilizes microwaves with a frequency of 300 MHz–300 GHz [10]. The use of the MAE method with a power level and extraction time has been investigated to extract oil from Moringa seeds, which yields 91-94% and a shorter time of 7 minutes with a power of 300 W compared to conventional methods [11]. This research used the MAE method to obtain Moringa seed extract with a power of 300 W and different extraction times treatments, namely 6, 9, and 12 minutes. Based on the description above, Moringa seed extract was then analyzed to obtain its characteristics as a coagulant in terms of protein content, protein profile, milk clotting activity (MCA), and caseinolytic activity (CA), also the ratio of MCA to CA.

## 2. MATERIAL AND METHODS

### 2.1 Materials

The materials used in this research were Moringa seed powder, Reagent A (2 g Na<sub>2</sub>CO<sub>3</sub> in 100 ml NaOH), Reagent B (5 ml CuSO<sub>4</sub>.5H<sub>2</sub>O 1% in 3 ml Na/K tartrate 1%), Reagent D (folin ciocalteu), standard solution of BSA (*Bovine Serum Albumin*), ethanol 96%, trichloroacetic acid (TCA), phosphate buffer pH 7, tritone (X-100) at 0.1%, skimmed milk, EDTA, distilled water, sodium phosphate pH 7.5, sodium caseinate, reducing sample buffer (RSB), coomassie brilliant blue (CBB) R-250, destaining solution, sodium dodecyl sulfate (SDS), ammonium persulfate (APS), tetramethylethylenediamine (TEMED), acrylamide:bis-acrylamide solution, tris HCl 1.5 M (pH 8.8), tris HCl 1.5 M (pH 6.8), and protein marker (11-245 kDa). The equipment used in this research is a shaker, electrophoresis tank, power supply from Bio-Rad electrophoresis, Eppendorf micropipette, gelling glass plate, pH meter, spatula, freezer, hot plate, magnetic stirrer, centrifugator, paper filter (Whatman No.1), microwave (Sharp), beaker glass (Pyrex-50), stainless steel sieve (40 mesh), micropipette, digital scale, UV-Vis spectrophotometer, rotary vacuum evaporator, incubator, and blender.

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### 2.2 Methods

The research method was a laboratory experiment with 4 treatments and 4 replications. Extraction of Moringa seed powder with different extraction times consisting of P0: maceration for 48 hours (control), P1: extraction for 6 minutes, P2: extraction for 9 minutes, and P3: extraction for 12 minutes with the MAE method.

#### 2.2.1 The Preparation of Moringa Seed Powder

The preparation of Moringa seed powder is based on [12], which has been slightly modified. Dried Moringa seeds in the sun

for 2 days. The Moringa seeds were peeled from the skin and mashed with a blender. The seeds were then sieved using a 40-mesh sieve and stored in a closed container at room temperature.

### 2.2.2 Extraction of Moringa Seed by The Maceration Method

Moringa seed extraction by the maceration method according to [13] with slight modification. Moringa seed powder was macerated with 96% ethanol (1:10, w/v) at room temperature for 48 hours. Stirring is done every 6 hours for 5 minutes [14]. The maceration extract was filtered using Whatman filter paper No. 1, and the filtrate obtained was concentrated with a rotary vacuum evaporator at a temperature of 45°C to about 10% of the original volume.

### 2.2.3 Preparation and Extraction of Moringa Seed by The MAE (Microwave-Assisted Extraction) Method

Moringa seed extraction preparation according to [15] with modification. Weighed 100 grams of Moringa seed powder, then added 300 ml of sodium phosphate pH 7 buffer solution. After that, 10 ml of EDTA solution was added and homogenized with a magnetic stirrer at 6 rpm for 30 minutes.

Moringa seed extraction using the MAE method refers to [11] that has been modified. The homogeneous mixture of Moringa seed was added to the Erlenmeyer as much as 100 ml and then extracted in the microwave for 6, 9, and 12 minutes at 100% power (one-minute radiation, two minutes off). Then, the extraction results were filtered using filter paper and allowed to stand at 4°C for 30 minutes. The filtrate was centrifuged for 30 minutes at a speed of 4,000 rpm, after that the results of the centrifugation were filtered using filter paper and then 60% (w/v) ammonium sulfate was added. Centrifugation was carried out again for 30 minutes at a speed of 4,000 rpm and

the precipitate was taken and the sample was stored in a freezer at a temperature of -20°C.

### 2.2.4 Protein Content

Protein content was analyzed according to [16] using the Lowry method. 1 ml of the extracted filtrate was taken and then added with distilled water until the volume became 4 ml. It was mixed with 5 ml of reagent C, and the mixture was stirred well and then allowed to stand for 15 minutes at room temperature. 0.5 ml of reagent D was added rapidly and stirred and then allowed to stand for 30 minutes at room temperature. Absorption was measured using a UV-vis spectrophotometer at a wavelength of 650 nm. The standard curve of BSA (Bovine Serum Albumin) was used with a concentration of 0; 20; 40; 60; 80; 100; 120; 140; 160; 180; 200; 220; 240; 260; 280; 300 ppm. The determination of protein content in samples of Moringa seed protein extract was performed using linear regression equations obtained from graphs in standard solutions.

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### 2.2.5 Protein Profile (SDS PAGE)

The protein profile was determined using SDS PAGE with a 15% (w/v) polyacrylamide gel according to [17,18].

#### 1) Creating of separating gel:

The concentration of the separating gel used is 15%. The composition of the separating gel solution is 3.95 ml of distilled water, 30% acrylamide, bis-acrylamide 3.35 ml, tris HCl 1.5 M (pH 8.8) 2.5 ml, 10% SDS 0.2 ml, 10 % APS 75 µl, and TEMED 10 µl. The separating gel solution is inserted into the space between 2 glass plates as high as 5 cm, then distilled water is added until it is full and the surface of the gel is flat.

#### 2) Preparation of the stacking gel:

The concentration of the stacking gel used was 2.75 ml of distilled water, 30% acrylamide, bis-acrylamide 700 µl, tris HCl 1.5 M (pH 6.8) 750 l, 10% SDS 0.2 ml, 10 % APS 75 µl and TEMED 10 µl. Discard the distilled water solution that was above the separating gel then a stacking gel solution was prepared when the separating gel began to solidify and allowed to polymerize for 30 minutes. Dispose of the distilled water that was above the separating gel after which the stacking gel solution was inserted between the two glass plates. A well-forming comb is installed on the surface of the gel stacking, then the glass plate containing the solidified gel is transferred into the electrophoresis tank.

### 3) Injection and Sample Running

The sample is taken with a pipette and put into an Eppendorf. Protein is used as a marker with a molecular weight of 11-245 kDa [19]. The marker and sample were added by loading or reducing sample buffer (RSB) (1:1) and heated at 100°C for 5 minutes. The sample is cooled at room temperature, then the sample is inserted with a volume of 10–20 µl using a micropipette into a well that has been soaked by running buffer solution and printed by a polyacrylamide gel comb, then connected to an electrophoresis device with a power supply for the running process with a current of 40 mA at a voltage of 120 volts for about 1 hour.

### 4) Staining

The gel was removed from the electrophoresis and then the gel was immersed in 100 ml of CBB R-250 dye solution for approximately 30 minutes on a shaker.

### 5) Color removal (destaining)

Polyacrylamide gel was put in the destaining solution and rinsed with distilled water for 1 hour. After that, it was washed with distilled water and placed in a container and then analyzed for the

presence of protein bands and finally scanned with a digital camera.

### 6) Protein band BM (Molecular Weight) calculation

The molecular weight of the protein bands that appear on the gel is calculated based on the standard (marker). The results of the calculation of retardation factor (Rf) using the formula:

$$Rf = \frac{JP}{JW} \quad (20)$$

Description:

Rf = Molecular mass  
 JP = Protein band movement distance from the starting point  
 JW = Tracking color movement distance from starting point

The Rf value in the linear regression equation refers to [21] using the formula:

$$Y = a + bX$$

Description:

Y = molecular weight  
 X = sample Rf value

### 2.2.6 Milk Clotting Activity (MCA)

The MCA was determined using [22] 10% skimmed milk (w/v). Skimmed milk heated at 50°C, dissolved skimmed milk in distilled water and 10 mM CaCl<sub>2</sub> at pH 6.5. The curd formation was observed at 37°C, after 10 ml of milk was incubated with 1 ml of Moringa seed extract. One unit of milk coagulation is defined as the amount of enzyme that can coagulate 10 ml of milk in 180 seconds and can be calculated using the formula:

$$MCA (U/ml) = \frac{S \times 100}{CT \times E} \quad (12)$$

Description:

S : milk volume;  
 CT : coagulation time;  
 E : enzyme;  
 100 : dilution factor.

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### 2.2.7 Caseinolytic Activity (CA)

CA was analyzed according to [23] using azocasein as a substrate. The dissolved extract from the Moringa seed was added to 300 µl of sodium phosphate (0.1 M) at pH 7.5 containing 0.6% azocasein (w/v). The mixture was supplemented with 100 µl of tritone (X-100) at 0.1% (v/v). The mixture was incubated at 37°C for 1 hour. 200 µl trichloroacetic acid (TCA) 10% (w/v) was added to stop the reaction. Incubated at 4°C for 30 minutes, then centrifuged at 9,000 rpm for 10 minutes, and the absorbance of the supernatant was measured at 270 nm.

$$CA \text{ (U/ml)} = \frac{\lambda_{270 \text{ nm}} \times 10 \times \text{dilution factor}}{E \times t} \quad (8)$$

Description:

270 nm : maximum wavelength in the experiment;

E : enzyme volume;

t : reaction time.

### 2.2.8 The Ratio of MCA to CA

The ratio of MCA to CA or milk clotting index (MCI) was described by [24] and calculated using the formula:

$$MCI = \frac{MCA}{CA} \quad (24)$$

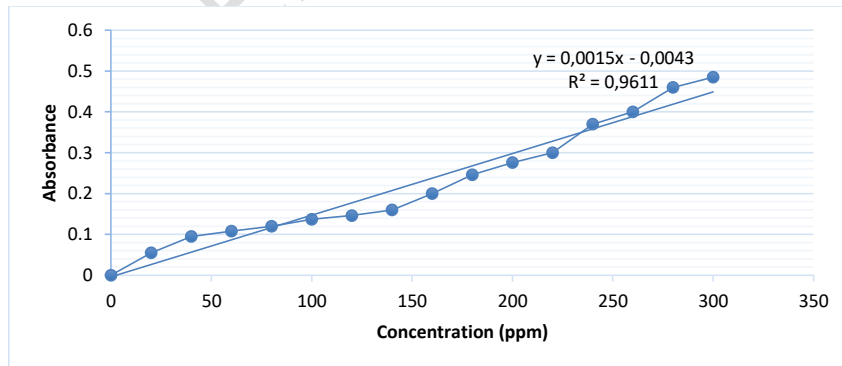


Fig.1 The BSA Standard Curve and Linear Regression Equation

## 2.3 Data Analysis

The data for protein content, protein profile, milk clotting activity (MCA), caseinolytic activity (CA), and the ratio of MCA to CA were analyzed by analysis of variance (ANOVA) carried out using the Microsoft Excel Software. The level of significance was determined by the Duncan's Multiple Range Test (DMRT) method.

## 3. RESULTS AND DISCUSSION

### 3.1 Protein Content

Measurement of protein content in Moringa seed extract used the Lowry method to determine soluble protein quantitatively. There was the reduction of Cu<sup>2+</sup> from Lowry B reagent (CuSO<sub>4</sub>) to Cu<sup>+</sup> by Trp, Tyr, and Cys contained in the protein. Then folin ciocalteu reagent containing phosphomolybdate and phosphotungstate will form a blue color [25]. The standard curve used in protein measurement is BSA (Bovine Serum Albumin) at λ 650 nm. The results of the BSA standard curve obtained a linear regression equation  $y = 0.0015x - 0.0043$  where x is the concentration and y is the absorbance, with a correlation coefficient (r<sup>2</sup>) of 0.9611. BSA Standard Curve and Linear Regression Equation can be seen in Fig. 1.

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**Comment [D15]:** The Duncan's Multiple Range Test (DMRT) method determined the significance level.

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The result of different extraction times of Moringa seed showed a highly significant difference ( $P < 0.01$ ) in Moringa seed extract protein content (Table 1). The lowest average value was found in P3, which was  $0.979 \pm 0.01$  (mg/ml) with an extraction time of 12 minutes, and the highest average protein content value was found in P1, which was  $1.022 \pm 0.01$  (mg/ml) with an extraction time of 6 minutes. The mean value of P1 and P0 as the control treatment was not much different, whereas the P0 treatment was the extraction of Moringa seed using the maceration method. Extraction of Moringa seeds for 6 minutes using the MAE method resulted in higher protein content than the extraction times of 9 and 12 minutes. The MAE method is one of the methods used for the extraction of compounds from plants by utilizing microwaves and requires less time than conventional methods such as maceration. Research that has been done by [11] extraction using the MAE method is more efficient in producing 91-94% Moringa seed oil extract for 7 minutes compared to the conventional method of 90%, which takes 50 minutes.

The decrease in the average value of protein content from Moringa seed extract was caused by the fact that it took too long a time to damage the metabolite compounds contained in the plant during the extraction process. Heating treatment can cause the protein to be denatured rapidly, affecting the change in protein structure and reducing its solubility. The statement [26] stated that the factors that affect the solubility of proteins, such as heat treatment, pH, solvent, and ionic strength. The main parameters that need to be considered to increase the extraction yield of the MAE method are sample solubility, solid-liquid ratio, time, power, and microwave temperature [27].

The nature of the protein can change to become less soluble and more viscous due to denaturation at high temperatures so that it changes the secondary, tertiary, and quaternary protein structures [28]. The use of high power and extraction time for too long causes the cells to burst suddenly, and an increase in the number of extracts becomes a complex mixture, making it difficult to isolate the desired single compound [28].

### 3.2 Protein Profile (SDS PAGE)

The determination of the protein profile in Moringa seed extract was carried out using the SDS PAGE electrophoresis method (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) to separate proteins based on their molecular size and shape. The use of markers in electrophoresis aims to determine the type of protein and molecular weight with gel dye using a Coomassie brilliant blue (CBB) solution. According to [29], protein molecules will migrate from the negative pole to the positive pole and form a band of the same length. Staining on the gel using Coomassie brilliant blue (CBB) solution and rinsed with a destaining solution, the protein band will turn red under acidic conditions, otherwise, the protein in the bound sample after being reacted with SDS anionic detergent will form a blue color. The results of the SDS PAGE electrogram of Moringa seed extract can be seen in Fig. 2. The estimation curve of the molecular weight of the Moringa seed extract contained in Fig. 3. obtained the equation  $y = -2.1775x + 2.3918$  with  $R^2 = 0.9628$ . The molecular weight of the sample is obtained from the linear formula.

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**Comment [D17]:** The MAE method's extraction of Moringa seeds for 6 minutes resulted in higher protein content than the extraction times of 9 and 12 minutes.

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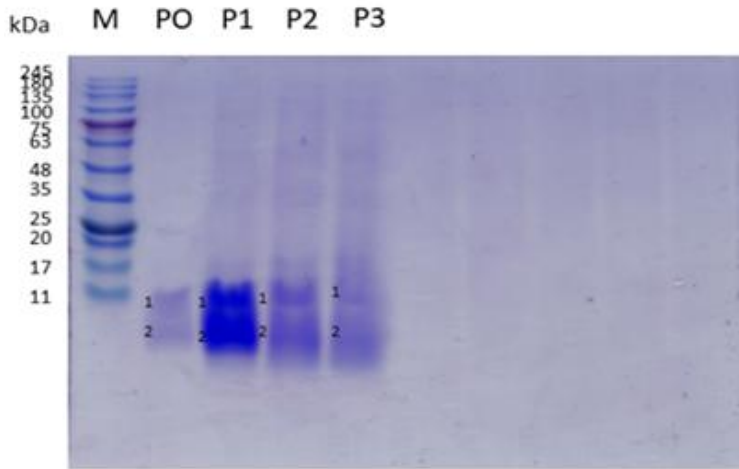


Fig. 2. An Electrogram protein profile of Moringa seed extract

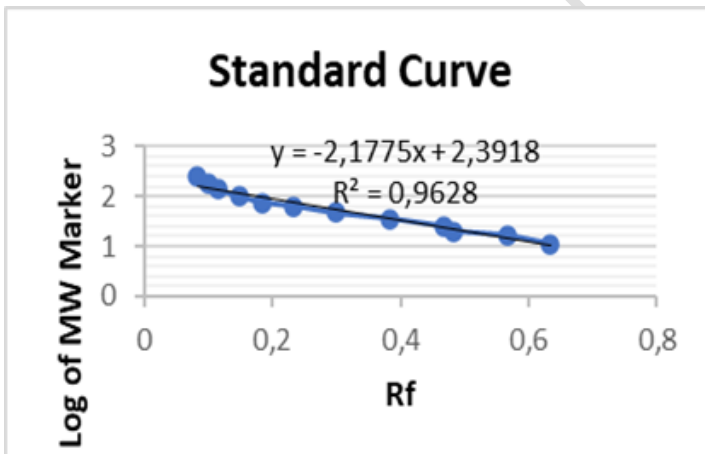


Fig. 3. Protein molecular weight calculation using a linear equation curve

Based on the results of protein profile analysis in Moringa seed extract, each MAE treatment showed 2 main protein bands, namely P0 having a molecular weight of 8.0 kDa and 5.3 kDa, P1 9.5 kDa and 4.9 kDa, P2 9, 5 kDa and 4.1 kDa, and P3 9.5 kDa and 4.1 kDa (Figure 2). Previous researchers demonstrated Moringa seed protein with different molecular weights (6.5-26.5 kDa) [30, 31, 32, 33]. The protein solubility of Moringa

seed depends on the material (salt, alkali, and water) [34] and various parameters such as pH, temperature, ionic strength, type of salt or solvent, extraction time, solid-solvent ratio, and components causing intermolecular bonds [35].

The thickness of the protein band indicates a higher protein content. The protein band in P1 was thicker than in the other treatments. This protein profile was

higher in line with the higher P1 protein content data in Table 1 compared to other treatments. Extraction using the MAE method for 6 minutes resulted in a thicker protein band intensity. The addition of 9 and 12 minutes of extraction time could reduce the intensity of the protein band thickness. According to [36], the different thicknesses of protein bands in the electrophoresis results indicate the protein content contained. The thicker the band formed, the higher the protein concentration. Peptides that have a molecular weight of < 10 kDa are antibacterial, and < 3 kDa indicates a fairly high proteolytic activity [37]. [38] reported that hydrolysis of goat's milk using bromelain enzymes showed the presence of peptides at molecular weights between 12-35 kDa. The number of protein subunits can be determined by the number of protein bands. If it is at the top position, it is included in the largest subunit and vice versa. The number or type of constituent amino acids determines the size of the protein subunit [39]. Several factors that affect electrophoresis are the electric field, sample, and sample solution. The rate of movement of molecules in the sample is influenced by the charge, size, and shape of the molecule. [40] adds that the molecular displacement will decrease and the transfer energy required is quite large if the molecular size is larger than different molecular shapes of the same size. The electric field also acts as a source of electric current with a constant voltage. The conductivity of the buffer solution affects the electrophoresis results because it has OH<sup>-</sup> ions so that it can quickly attract negatively charged molecules to the positive pole [41].

### 3.3 Milk Clotting Activity (MCA)

The result of different extraction times of Moringa seed showed a highly significant

difference ( $P < 0.01$ ) in Moringa seed extract milk clotting activity (MCA) Table 1. The average value in Table 1 of the results of the Milk Clotting Activity (MCA) from Moringa seed extract decreased with the extraction time using the MAE method. The highest MCA value was found in P1 which was  $3.999 \pm 0.02$  (SU/ml) with an extraction time of 6 minutes and the lowest value was found in P3 which was  $3.837 \pm 0.01$  (SU/ml) with an extraction time of 12 minutes. Moringa seed extract using the MAE method for 6 minutes resulted in higher MCA, the addition of 9 and 12 minutes of extraction time could reduce MCA. MCA data along with protein content and protein profile of Moringa seed extract decreased if the extraction time was more than 6 minutes.

Coagulation activity is an important parameter to determine the ability of an enzyme to hydrolyze casein in milk. Coagulation protein can be affected by factors such as temperature, pH, and chemicals added during the enzyme extraction process. The decrease in MCA in Table 1 is thought to be caused by a decrease in the protein content of Moringa seed which decreases with the extraction time using MAE and conventional methods such as maceration. This is consistent with the opinion of [42], who states that the percentage of protein will decrease if the heating time is too long due to protein denaturation at a temperature of 70°C, and affects its coagulation activity. [43] added that protein aggregation is related to gelation properties that occur due to protein denaturation, causing proteins to coalesce and form a network structure or protein matrix. The ability of coagulant materials and protein content are also closely related to forming a curd, so the higher the protein content, the denser the curd produced.

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**Comment [D22]:** The number of protein bands can determine the number of protein subunits.

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**Table 1. The characteristics analysis of Moringa seed extract with different extraction times using maceration and the MAE method**

Treatment	Analysis
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	Protein content (mg/ml)	Milk Clotting Activity (MCA) (SU/ml)	Caseinolytic Activity (CA) (U/ml)	Ratio of MCA to CA
P0	1,019 <sup>b</sup> ± 0,01	3,994 <sup>c</sup> ± 0,03	2,442 <sup>b</sup> ± 0,02	1,636 ± 0,03
P1	1,022 <sup>c</sup> ± 0,01	3,999 <sup>c</sup> ± 0,02	2,455 <sup>b</sup> ± 0,05	1,627 ± 0,04
P2	0,985 <sup>a</sup> ± 0,01	3,919 <sup>b</sup> ± 0,01	2,354 <sup>a</sup> ± 0,06	1,665 ± 0,04
P3	0,979 <sup>a</sup> ± 0,01	3,837 <sup>a</sup> ± 0,01	2,308 <sup>a</sup> ± 0,02	1,663 ± 0,01

### 3.4 Caseinolytic Activity (CA)

The result of different extraction times of Moringa seed showed a highly significant difference ( $P < 0.01$ ) in Moringa seed extract caseinolytic activity (CA). **Table 1.** The lowest mean CA value is found in P3, which is  $2,308 \pm 0.02$  (U/ml) with an extraction time of 12 minutes, and the highest average value is found in P1, which is  $2.455 \pm 0.05$  (U/ml) with an extraction time of 6 minutes. The extraction of Moringa seed extract using the MAE method for 6 minutes resulted in a higher CA, while the addition of 9 and 12 minutes of extraction time reduced CA. CA data along with protein content, protein profile, and MCA decreased if the extraction was carried out for more than 6 minutes.

The protease activity, also known as caseinolytic activity (CA), of an enzyme is expressed in one unit of activity (U), which is the number of proteases that cause an increase in one unit of absorbance at 270 nm. The enzyme activity is related to the protein content produced by Moringa seed extract. The higher the protein content, the greater the coagulation activity followed by enzyme activity to hydrolyze

k-casein. [44] also stated that proteolytic activity (PA) also plays an important role in evaluating the suitability of milk coagulation enzymes that affect casein degradation, yield, and sensory properties in cheese.

P1 treatment had high protein content and high casein activity, while P2 and P3 treatments decreased, presumably due to the high temperature generated during

extraction. This is in line with the opinion of [45], who states that the factors that affect the activity of enzymes are temperature and pH. [15] also added that the enzyme has an active site that matches the casein substrate and causes the formation of an enzyme complex with a maximum substrate at the optimum pH. The resulting product is maximized when the proton donating and accepting groups on the catalytic site of the enzyme are at the desired ionization level. In addition, the enzyme activity decreased after passing the optimum temperature caused by damage to the active group (denaturation) so that the active site of the enzyme changed conformation and reduced its catalytic activity.

### 3.5 The Ratio of MCA to CA

The result of different extraction times of Moringa seed had no effect ( $P > 0.05$ ) on the ratio of MCA to CA of Moringa seed extract (Table 1). The average value in Table 1 can be seen that the results of the average value of the MCA to CA ratio increased from P1 to P2, then P3 began to decrease. The highest average value is found in P2, which is  $1.665 \pm 0.04$  with an extraction time of 9 minutes, and the lowest average value is found in P1, which is  $1.627 \pm 0.04$  with an extraction time of 6 minutes.

The extraction time of Moringa seed using the MAE method tends to increase the ratio of MCA to CA. The high ratio in the P2 treatment was thought to be due to the higher coagulation activity obtained than in P3, in contrast to the P1 treatment, which had higher coagulation activity than

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P2 but the ratio obtained was lower. This proves that although the coagulation activity and proteolytic activity of the P1 treatment were higher, the ratio of MCA to CA was lower. This is in line with [46], who stated that the enzyme is derived from *C. gigantealatex* showed the highest caseinolytic activity, followed by *P. rubra*, *E. antiquorum*, *A. cathartica*, as well as *J. curcus* but the highest milk clotting index (MCI) was found in *A. cathartica* then *C. gigantea*. The ratio of milk coagulation to proteolytic activity is an important standard used as a reference to replace the enzyme rennin, although *A. cathartica*

has lower coagulation activity than *C. gigantea*, the resulting ratio is higher. [47] also added that the milk clotting index (MCI) is an indicator to determine the ratio of milk clotting activity (MCA) to caseinolytic activity (CA). A higher milk clotting index (MCI) ratio indicates that the enzyme is more capable of forming curd. The resulting yield is also higher and the taste is less bitter during cheese making. On the other hand, the enzyme with a lower ratio produces a more bitter taste and is less able to form curd, thus affecting the sensory of the final product.

**Table 2. The best treatment of Moringa seed extraction times using maceration and the MAE method**

No.	Treatment	Total Value of Each Parameter
1.	P0	0.817
2.	P1	1.095
3.	P2	0.514
4.	P3	0.230

### 3.6 Best Treatment

The best Treatment of Moringa seed extraction times using the maceration and MAE methods was carried out using the effective index method according to [48]. The value of the product can be seen in **Table 2**. Based on the description above, the best treatment was obtained, namely the extraction time of Moringa seed for 6 minutes (P1) with a product value of 1.095.

### 4. CONCLUSION

Moringa seed extraction time using the MAE method for 6 minutes is the best treatment and can be used as a coagulant, especially in the manufacture of mozzarella cheese. Based on this research, which resulted from characteristics of Moringa seed protein content at 1.022 mg/ml, Milk Clotting

Activity (MCA) at 3.999 SU/ml, Caseinolytic Activity (CA) at 2.455 u/ml, the ratio of MCA to CA at 1.627, and has a molecular weight of 4.9-9.5 kDa.

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**Comment [D28]:** Not mentioned in the article??

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