



# Cheese-making Ability of *Plumeria alba* and *Plumeria rubra* Latex: Milk Clotting and Proteolytic Activities

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**Abstract:** In cheese making industry, plant enzymes are used to produce cheese. In Owest Africa, they produced the cheese “waragashi” with the mix milk and the plant *Calotropis procera*. The objective of our study is to assess ability to coagulate milk of the latex from news plants *Plumeria alba* and *Plumeria rubra*. If the two plants can supplied *Calotropis procera* in cheese making. For the results, we observed that the latex of *Plumeria alba* and *Plumeria rubra* are richer in proteins and mineral elements than the latex of *Calotropis procera*. It appears that the milk clotting and proteolytic activities of the latex of *Plumeria alba* and *Plumeria rubra* reached their optimum at 75°C while those of *Calotropis procera* reached the optimum at 55°C but remained active above 60 and 70°C. The Proteolytic Activity of the latex of *C. procera* and the two species of *Plumeria* was maximal at concentrations of 10% and 20% in the reaction medium, respectively. Polyacrylamide gel electrophoresis revealed that *Plumeria alba* latex had proteins with molecular weights of 10 and 25 kDa while the molecular weights *Plumeria rubra* latex proteins were 10, 25, 45kDa. The MCA/ PA ratio of *Calotropis procera*, *Plumeria alba* and *Plumeria rubra* was 1142.86; 810.81 and 576.92 respectively. These two *Plumeria* latex could validly replace *C. procera* latex in the production of cheese.

**Keywords:** Plumeria, Latex, Enzymatic Activity, Wagashi

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## 1. Introduction

Proteases are one of the largest groups of industrial enzymes. They are found in animals and microorganisms as well as in plants [1, 2]. The oldest protease used in the production of cheese is rennet. Nowadays, proteases of plant origin are increasingly in demand for several reasons, including: vegetarian diet, religious affiliation and the high cost of animal rennet. West African countries, notably Benin, have for several decades used *Calotropis procera* as only source of protease in the production of a soft cheese commonly known as wagashi [3, 4]. In addition, several authors have tested extracts from papaya leaves, pineapple juice and lemon juice in the production of soft cheeses [5]. However, *Plumeria* has rarely been used as a source of coagulant.

*Plumeria alba* and *Plumeria rubra* are two species of ornamental plants cultivated in the tropics and subtropics. Their latex, less documented, would nevertheless exhibit significant enzymatic activities. However, they could validly replace *Calotropis procera* in the production of cheese.

## 2. Materials

The latex of *Calotropis procera*, *Plumeria alba* and *Plumeria rubra* was collected from the Abomey-Calavi campus in the Atlantic region of the Republic of Benin. The latex sample was taken according to the work of Macalood [6]. the analysis have in Unity of Research in Enzymatic Food (URGEA) to Laboratory of Study and research in chemical Apply (LERCA) University Abomey-Calavi.



Figure 1. Different latex plants collected.

## 3. Methods

### 3.1. Physico-Chemical Analysis

The water and ash contents were determined according to official methods of analysis (AOAC) [7]. The total lipid and protein contents were determined according to the methods of Darné and Madero-Tamargo [8] and BIURET, respectively.

#### 3.1.1. Extraction of the Enzymatic Extract

The extraction of the enzyme extract was done according to the modified method of Oséni [9]. The latex from each plant was filtered and centrifuged at 4000 rpm for 15 min. The supernatant was collected and stored at 4°C to serve as an enzyme for analyzes.

#### 3.1.2. Determination of the Coagulant Activity of Milk

The milk clotting activity was measured according to the method used by Arima and Yu [10], with some modifications. The substrate was skimmed milk (1% fat) containing 0.02% calcium chloride ( $\text{CaCl}_2$ ). To 10 ml of milk (substrate), was added 1 ml of each enzymatic extract. Incubation was done at a temperature ranging from 40°C to 80°C for 15 minutes. The time taken for the milk to coagulate is used to determine the milk clotting activity (MCA).

$$\text{MCA (US)} = 2400/t \times S/E$$

With US: Soxhlet Unit; t: clotting time; S: volume of milk withdrawn (ml); E: volume of coagulant (ml).

#### 3.1.3. Determination of the Proteolytic activity of Milk

Proteolytic activity was determined by the method of Ladd and Butter [11] which uses bovine serum albumin (BSA) or casein as protein substrate. The protein substrate used in this case is casein. It was prepared in a phosphate citrate buffer (0.05 M) at pH = 7.5 and incubated in a water bath at 100°C for 15 minutes. To 1 ml of the 1% protein substrate solution (0.05M of

phosphate buffer, at pH = 7.5), was added 1 ml of latex. The preparation was incubated at room temperature (37°C) for one hour. On the other hand, 1 ml of the protein substrate mixed with 1 ml of buffer was used for the control. After 1 hour of incubation, the reaction was stopped by adding 3 ml of 10% trichloroacetic acid (TCA). The mixture is vortexed and left in ice for one hour and centrifuged at 3000 rpm for 30 minutes. After centrifugation, the supernatant is recovered and its absorbance measured with a spectrophotometer.

Optical Density (OD) was read at 280 nm wavelength. The proteolytic activity (PA) was expressed in g of rennet per g of latex.

#### 3.1.4. Effect of Temperature on Coagulant Activity and Proteolytic Activity

To assess the effect of temperature, the proteolytic and coagulant activities of the milk were determined after an incubation time of one hour, at different temperatures (40, 50, 60, 70 and 80°C), under the same conditions.

#### 3.1.5. Effect of Cofactors on Proteolytic Activity

Effect of the cofactors ( $\text{Ca}^{2+}$ ,  $\text{K}^+$ ,  $\text{Na}^+$ ) on the activity of the different enzymes was assessed. The solution of the protein substrate was prepared in a phosphate citrate buffer (0.05 M) at pH = 7.5 by adding the different salts at different concentrations (0-10-15mM), followed by incubation in a water bath at 100°C for 15 minutes. The other steps of the reaction were carried out as described in section 3.1.3.

#### 3.1.6. SDS-Polyacrylamide Gel Electrophoresis

Protein electrophoresis was performed using 10% (w / v) sodium dodecyl sulfate (SDS) according to the method described by Laemmli [12]. The apparent molecular masses of the proteins were estimated by co-electrophoresis of protein markers with molecular weight ranging from 10 to 102 kDa. The gel used for the separation consisted of H<sub>2</sub>O (6 ml), 40% acrylamide (3.1 ml), 1.5 M tris pH 8.8 (3.1 ml), 10% SDS (125 µL), 10% APS (62.5 µL) and TEMED (6.25 µL). The samples were loaded in a gel consisting of H<sub>2</sub>O (4.35 ml), 40% acrylamide (750 µL), 1.5 M tris pH 6.8 (750 µL), 10% SDS (60 µL), 10% PSA (60 µL) and TEMED (6 µL). Two types of dyes were used, one with Coomassie blue and the other with silver nitrate. The proteins separated on SDS-Polyacrylamide gel at 120 V for two hours and were stained with Coomassie Brilliant Blue R-250. Regarding the silver nitrate staining, the gel was incubated for one minute in a sensitizing solution (0.02% sodium thiosulfate) and then rinsed twice for 1 minute in distilled water. The gel was then stained by shaking at 4°C in a solution of silver nitrate (0.1%) for 20 minutes and then rinsed for 1 min with distilled water before being developed by incubation with vigorous shaking in an aqueous solution of formalin (0.04%) and sodium carbonate (2%). The reaction was stopped by replacing the development solution with an aqueous solution of acetic acid (5%).

## 3.2. Data Analysis

Data were analyzed using descriptive statistics. Thus, means and standard deviations were calculated using Excel 2016.

SPSS 17.0 software was used to process the data. The test was considered statistically significant for values less than 5%.

## 4. Results and Discussion

### 4.1. Analyzes Physicochemical

The latex of *Calotropis procera*, *Plumeria alba* and

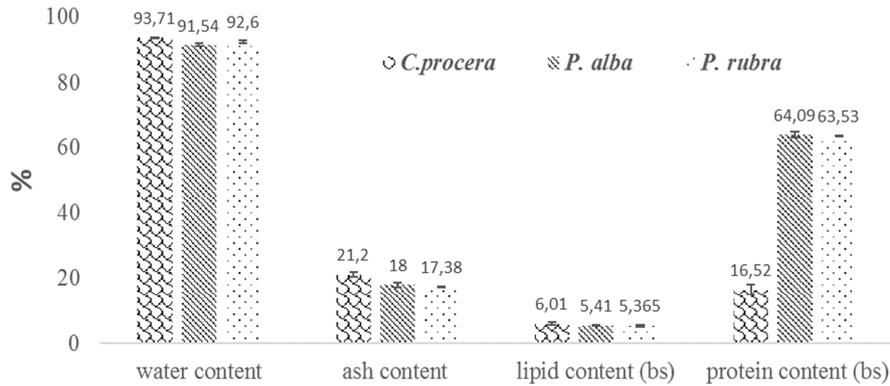


Figure 2. Physico-chemical characteristics of latex.

The latex of *Calotropis procera*, *Plumeria rubra* and *Plumeria alba* have a water content greater than 50% (i.e. 93.71%; 92.6% and 91.54%, respectively). The protein content of the various latex is 16.52%, 63.53% and 64.09% for *Calotropis procera*, *Plumeria rubra* and *Plumeria alba*, respectively. The protein content of *C. procera* latex ( $16.52 \pm 0.21\%$ ) is statistically lower than the content obtained by Jeana [15] for dried latex of *C. procera* (57.24%). Furthermore, the work of Milaiti [16] on *C. procera* from Ouagadougou made it possible to note an average protein content of 16.30% (a value statistically identical to that obtained during this work). The observed differences in total latex protein content may be due to the high variability of agro-ecological conditions [17]. It should be noted that the protein content of the latex of *C. procera*, both for the experimental value and that of the literature, is lower than the average protein content of the latex of *Plumeria alba* and *Plumeria rubra* (64%). Furthermore, the latex of *C. procera* has a lipid content ( $6.01 \pm 0.20\%$ ) statistically higher than that of the latex of *Plumeria alba* and *Plumeria rubra* (moderately, 5.51%). These results are very close to those obtained by Jeana and Milaiti [15, 16] on the latex of *C. procera* (6.17%) and *C. papaya* (5.21%). The latex of *C. procera* is however richer in mineral elements ( $21.2 \pm 0.04\%$ ) than the latex of *Plumeria alba* (18%) and *Plumeria rubra* (17.38%). This observation was also made by Mazou [18].

### 4.2. Biochemical Characteristics of Latex

The coagulant and proteolytic activities of latex depend on several factors, including the type, nature of the tissue, the concentration of the enzyme or the type of protease [19].

#### 4.2.1. Effect of Temperature on the Enzymatic Activity of Latex

Temperature is a very important factor in cheese making,

*Plumeria rubra* constitute a large and diverse group of plants characterized by the presence of a milky white latex, an aqueous emulsion present in the vacuole of specialized secretory cells, called lactifers. The latex is rich in water, it contains rubbers, resins, sugars and many proteins and enzymes [13, 14]. Figure 1 shows the physico-chemical characteristics of the latex of each plant.

especially in the coagulation stage [2]. Mastery of cheese technology requires knowledge of the renneting temperature, which must correspond to the optimum temperature of the proteases used. Figure 2 shows the variation in the relative activity of the latex of *Calotropis procera*, *Plumeria alba* and *Plumeria rubra* as a function of the temperature of the reaction medium.

Initially, casein was used as a substrate, then milk. The use of casein as a substrate made it possible to determine the proteolytic activity and the use of milk help to evaluate the coagulant activity of different latex.

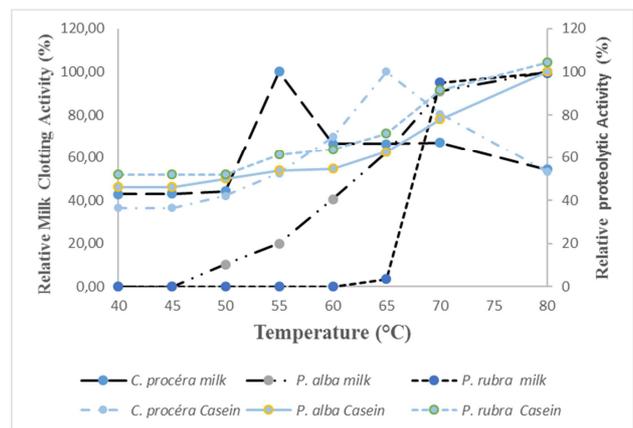


Figure 3. Relative Coagulant and Proteolytic Activity Variation as a Function of Temperature.

In the presence of latex from *Plumeria alba* and *Plumeria rubra*, the coagulation activity of the milk increases as the temperature rises. This activity is optimal at 70°C. Indeed, the proteolytic activity of the latex of *Plumeria alba* and *Plumeria rubra* as a function of temperature is 75°C and is almost stable above 75°C. The high temperatures are very favorable to the enzyme present in these latex during the milk coagulation.

Unlike Chanda [20] who showed that the *Plumeria rubra* latex has a maximum proteolytic activity at 55°C. Few studies have been made on these two new latex about their coagulant activity. Furthermore, the coagulant activity of *C. procera* latex as a function of temperature peaks at 55°C. However, residual activity is observed between 60 and 70°C. Similar temperature profiles have been reported by Beka R, Lo peiro and Asakura, respectively on latex of *Balanites aegyptiaca*, *Lactuca sativa* and *Oriza sativa* [2, 21, 22]. They showed that the optimum temperature for proteases in these plants is around 50°C. This behavior of plant proteases has also been reported by Rapaso and Domingos for *Centaruea calcitrapa* [23]. This result is also similar to that of Dele Raheem who demonstrated that the proteolytic activity of *C. procera* reaches its optimum at 70°C [24]. Finally, the high activity temperatures exhibited by these different latex (temperature

above 50°C) testify to the thermostability of their enzymes.

4.2.2. Effect of Latex Concentration

Figure 3 shows the relative activity of the different latex as a function of their concentration in the reaction medium. An increase in proteolytic activity is noted when increasing the amount of latex from 2 to 20% (m / m) for the latex of *C. procera*, *P. alba* and *P. rubra*. The maximum activity was obtained at 20% for the *Plumeria* latex. These results are similar to those obtained by Mazou [18]. On the other hand, the latex activity of *C. procera* reached its optimum for a concentration of 10%. Beyond each optimum, any increase in latex does not result in an increase in proteolytic activity. In fact, previous work has shown that enzyme activity is highly dependent on the concentration of the enzyme in the reaction medium, up to a given threshold [25].

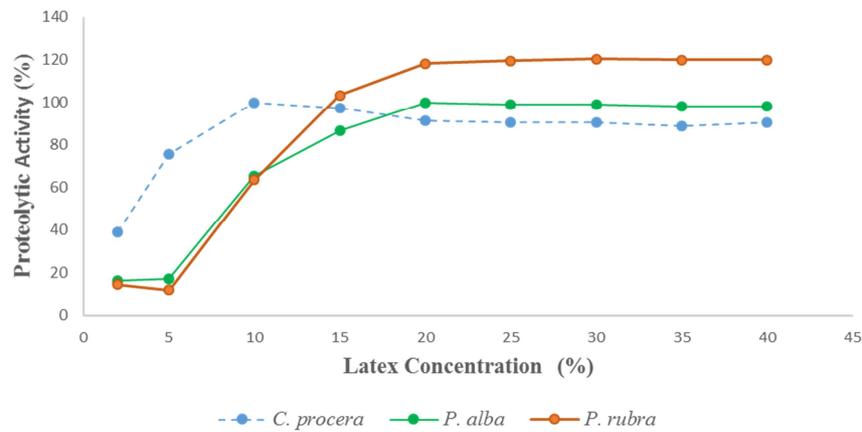


Figure 4. Relative activity as a function of the latex concentration.

4.2.3. Effect of Calcium Chloride, Sodium Chloride and Potassium Chloride

Table 1 shows the effect of different ions on the performance of the three latex. There is no significant difference between the proteolytic activity of the latex of *Plumeria alba* and *Plumeria rubra* in the presence of the different salts used. However, Ca<sup>2+</sup> and Na<sup>+</sup> increase the proteolytic activity of the *Calotropis procera* latex.

The effect of ions on enzyme activity has been reported in previous work. Chazarra found that calcium ion promotes increased protease activity in *Cynara scolymus* extracts at a concentration of 50mM [26]. Corrons also observed an increase in the protease activity of *Maclura pomifera*, after the addition of 25 Mm of calcium ion [27]. For Mukundan, chloride ion has no effect on proteases [28].

Table 1. Effects of the different ions on the proteolytic activity of latex.

g/ml	<i>Calotropis procera</i>	<i>Plumeria alba</i>	<i>Plumeria rubra</i>
CaCl <sub>2</sub>	0,534±0,19b	0,326±0,02a	0,31±0,02a
NaCl	0,525±0,03b	0,318±0,10a	0,35±0,03a
KCl	0,31±0,04a	0,324±0,05a	0,32±0,04a
Witness	0,31±0,12a	0,320±0,08a	0,32±0,04a

Values with the same letters in the same column are not significantly different from 5%.

4.2.4. Electrophoresis of Latex on Polyacrylamide Gel

The tissues of plants contain a set of proteins which differ in their properties and functions. To get an idea of protein size, molecular weights are determined by electrophoresis under denaturing conditions (SDS medium).

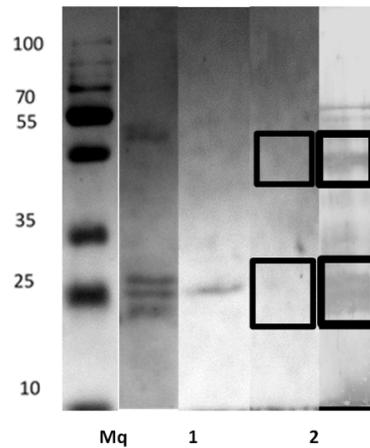


Figure 5. Electrophoresis of plant extracts used on polyacrylamide gel in SDS medium. (Mq: size marker for proteins 1: *Calotropis procera*; 2: *Plumeria alba*; 3: *Plumeria rubra*; 4: *Plumeria rubra* treated with Silver Nitrate).

Analysis of Figure 4 shows that qualitatively, the protein profiles of *Calotropis procera*, *Plumeria alba* and *Plumeria rubra* are almost equivalent as bands appear at similar molar mass levels. More particularly, a cluster of three protein bands with molar masses of 25 kDa and 55 kDa is identified within these latex. Moreover, electrophoretic analysis shows the presence of several protein bands between approximately 10 and 100 kDa, the number of which depends on the nature of the latex. The bands corresponding to the proteins of *Calotropis procera* latex are more intense than those corresponding to the latex of *Plumeria alba* and *Plumeria rubra*. *Calotropis procera* latex shows four distinct bands of 15, 20, 25 and 55 kDa. As for the sample of *Plumeria alba*, it is observed the presence of two protein bands of 10 and 25 kDa. On the other hand, with regard to the latex of *Plumeria rubra*, the bands could not be identified well. Then a nitrate staining was carried out and then made it possible to better see the bands of 10, 25 and 45kDa. Cambon has also reported the identification of three protein bands with molar masses close to 10 kDa, 25 kDa, 45 kDa within *Plumeria rubra* latex [29].

The proteins contained in the latex of *Plumeria alba* and *Plumeria rubra* would behave in the same way as those contained in the latex of *C. procera* (25kDa and 45 kDa). These proteins of similar sizes present in the latex studied would therefore be responsible for the coagulation of milk and could certainly be of technological interest in the manufacture of cheese.

#### 4.2.5. Relationship Between Proteolytic Activity and Coagulant Activity

The coagulant and proteolytic activities of latex are different from each other (Table 2). This difference can be attributed to the different proteases contained in the latex [30, 31].

**Table 2.** Milk coagulation activity (MCA) and latex proteolytic activity (PA).

Latex	MCA (US /ml)	PA (U/ml of latex)	MCA/PA
<i>C. procera</i>	400	0,35	1142,86
<i>P. alba</i>	300	0,37	810,81
<i>P. rubra</i>	225	0,39	576,92

The type of specificity of the protease is of great importance for its use in food. A protease with a high MCA / PA ratio is more likely to clot and gives a higher yield in cheese making. A low ratio leads to a low yield and a low organoleptic quality [31]. It appears from this work that *C. procera* has the highest ratio (1142.86), followed by *P. alba* (810.81) and *P. rubra* (576.92). The two latex of *Plumeria* have a ratio close to that of *C. procera*, they can validly be used as substitutes in the manufacture of cheese.

## 5. Conclusion

This study allowed us to perform an enzymatic characterization and to determine the influence of temperature and salts on the latex of *Plumeria alba* and *Plumeria rubra* in comparison to *Calotropis procera* latex. The optimum points for the different latex are respectively around 55°C for *Calotropis procera* and 75°C for *Plumeria*

*alba* and *Plumeria rubra*. The size of the *Plumeria alba* latex proteins is 10 and 25 kDa and that of the *Plumeria rubra* latex proteins is 10, 25, 45 kDa. These latex have the privilege of starting from thermostable biocatalysts and are therefore predisposed to be useful in many food industries. They would represent an asset for the dairy industry.

## Authors Contributions

This work was supervised by Fidèle Paul TCHOBO and carried out by Ibidjokè Rachidatou BANKOLE. The conceptualization of this subject was made by Ibidjokè Rachidatou BANKOLE and Mouaïmine MAZOU. Mouaïmine MAZOU then set up the methodology used. A methodology validated by Ali MAHAMAT SEID and Fidèle Paul TCHOBO. The data analysis was however carried out by Sènan Christa Marie Josephine LOKOSSOU

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