

Isolate and Extract for Milk Clotting Enzymes from the leaves of *Moringa Oleifera*, *Carica Papaya* and *Mangifera Indica* and Use in Cheese Making: The Case of Western Hararage Region, Ethiopia

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Abstract Milk-clotting activity was screened from leaves of *Carica papaya*, *Mangifera indica* and *Moringa oleifera* in order to use the leaf with the highest milk-clotting activity as a source of the enzyme. The results of the present study showed that milk-clotting activity was only detected in the leaf extracts of *Carica papaya* and *Moringa oleifera* and the leaf extract from the leaf of *Mangifera indica* showed no activity. Ammonium sulfate precipitation was used in this study and the results showed that the highest milk-clotting activity was obtained with 40 % ammonium sulfate saturation. Maximum temperature for the crude extract of *Carica papaya* and *Moringa oleifera* showed milk clotting activity at 70°C and 65°C respectively and also showed the highest clotting activity at a pH of 7.5 and 7 respectively. There was more than 80% retention of the milk-clotting activity of the crude extract of *Carica papaya* and *Moringa oleifera* after 1h incubation at 60°C and 55°C respectively and the result also indicated that the crude extract retained more than 80% of its activity between pH 5 to pH 7.5 and pH 5 to pH 6 respectively. Finally the crude extract of *Carica papaya* and *Moringa oleifera* has a highest enzymatic activity at a concentration of 70 and 90 grams respectively.

Keywords: activity, milk clotting enzyme, *Carica papaya* and *Moringa oleifera*

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1. Introduction

Milk coagulation is the main step for producing cheese, and coagulating enzymes, which are preparations of proteolytic enzymes, have been used in cheese making for thousands of years, and they seem to be the oldest known application of enzymes. Historically, most enzyme preparations used for cheese have been extracts from the stomachs of ruminants, but coagulants from microbes and plants were also used at very early dates [1].

The transformation of raw milk to cheese is very important for developing countries such as Ethiopia. Milk and milk products play a very important role in feeding the rural and urban population of Ethiopia and have a high nutritional value and are daily produced, sold for cash or readily processed. Cheese-making starts with coagulation of milk, which is widely achieved by rennin, extracted from calf's abomasums before weaning. The worldwide increase in cheese production has reduced the availability

of rennin, which became short in supply and expensive for local farmers. The reduced supply of rennin has led to the search for coagulant substitutes. Numbers of proteolytic enzymes from various sources have been used: bovine, porcine and chicken pepsins, fish chymotrypsins as well as proteases of fungi and transgenic microorganisms [2].

The use of these coagulants gave rise to unwanted final products, and led to ethic (been against genetically engineered foods), religious (Judaism and Islam), diet (vegetarianism) and public health problems (bird flu, bovine spongiform encephalopathy, H1N1 virus and microbial toxins) [3]. Recent publications on new proteases from vegetable origin for milk coagulation indicated that they are subject with growing interest for dairy technology [4,5,6]. Plant coagulants have been used in cheese-making since fifty centuries before our era. Since the renewal of interest in 1960, vegetable coagulants have been used the more in dairy technology; especially at the artisanal scale [7].

Cheese production with plant coagulants contributes significantly to the socioeconomic development of a

locality, region and hence whole country where it is produced. Protein input is improved for those populations to whom restrictions are imposed by the use of animal and microbial coagulants. The technology led by farmers is easy and straightforward [3]. Such know-how can be interesting to farmers. Several plant preparations are known for cheese making. Species from *Cynara* genus are used successfully as source of milk-clotting enzymes in the Iberian Peninsula and Latin America. Tropical plants such as: *Carica papaya*, *Ananas comocus*, *Ficus glabra*, *Calotropis procera* and *Lactuca sativa* are also sources of milk-clotting enzymes [8,9,10]. The fruits and leaves of these plants have not yet been experimented as a source of milk-clotting enzymes, which can substitute rennin.

West Hararghe zone is characterized by mixed farming system where crop production is adjacent to livestock rearing. Though there is problem of adequate range land, farmers adapt to feed crop residues their livestock. In such a way milk is produced by smallholder farmers in which its quality and quantity affected by community location variation, lack of improved technology, availability of feed and water. Rural areas of West Hararghe zone are the sole sources of milk for the nearby town users of the zone where irregularity in demand and supply for fresh milk is affected. Moreover, by its product nature milk is perishable that can be consumed in shorter period. But many of the products are transported from far rural areas that vulnerable to loss in quality of the milk which tends to reduce the market price. It is observed that smallholder farmers of West Hararghe zone produces fermented milk such as cheese through traditional methods. Cheese is one of the numerous products from the processing of milk. Cheese is used as a form of preserving essential nutrients in milk and is an excellent source of nutrients such as protein, fat, minerals and vitamins. Cheese manufacture is essentially a dehydration process in which the fat and casein of milk are concentrated 6-10-fold [11]. Since long

the animal rennin (or rennet) is employed in making cheese. The enzyme rennet is obtained on a commercial scale from the fourth or true stomach of the un-weaned calves which are specifically slaughtered for this purpose. The objective of the present work is to find alternative sources of milk-clotting enzymes from the *Carica papaya*, *Mangifera indica* and *Moringa oleifera* found in West Hararghe zone; with the aim to select the best among them for the promotion of cheese making. This preliminary study describes the milk-clotting activity of each leave extract from each species, the efficiency of enzyme extraction from fresh and dried leave with distilled water and 5% sodium chloride as extracting medium. Besides, enzymatic activity of each leave extract was determined.

2. Materials and Methods

2.1. Study Area

The study was conducted in Chiro Woreda, West Hararghe Zone, Oromia region. The site was selected based on preliminary survey and due to the fact that these areas are highly known for the growth of *Carica papaya*, *Mangifera indica*, and *Moringa oleifera*, huge dependence of the people in the area those plants due to recent development of basic infrastructure; mainly roads and the proximity to market centers. The researcher has contacted with the woreda agricultural officers to identify which woreda is famous in those plants. Based on responses from the officials Chiro Woreda was identified due to its proximity and more famous in the production of *Carica papaya*, *Mangifera indica*, and *Moringa oleifera*. This Woreda is 325 kilometer away from Addis Ababa. It is geographically located between 34°18'43" to 43°04" E Latitude and 10°09'24" to 30°18'43" N longitude.

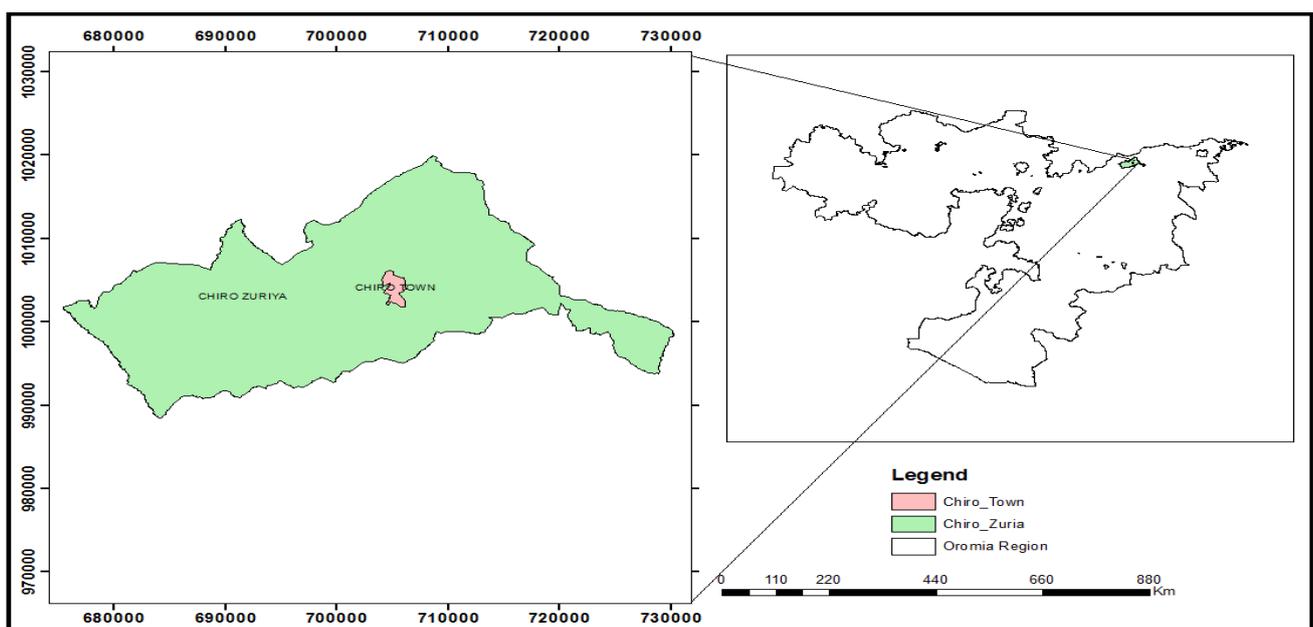


Figure 1. Map of Study Area

2.2. Methodology

2.2.1. Materials and Equipment's

The following chemicals were used in all the analysis of the experiment: Iron Sulphate hydrated, Magnesium Sulphate hydrated, Ammonium Sulphate, Di potassium Hydrogen Sulphate, Copper Sulphate Calcium carbonate Calcium chloride, Sodium chloride, potassium chloride, Zinc Sulphate hydrated buffer, Hydroxide-Chloride buffer, Acetate buffer, Sodium hydroxide.

The equipment's which were used in the process include: sample collection units, Centrifuge, Portable pH meter and centrifuge.

2.3. Experimental Procedures

2.3.1. Sample Collection

The leaves of the following plants *Carica papaya*, *Mangifera indica*, and *Moringa oleifera* were collected early in the morning. The leaves were carefully cleaned, and then coarsely grounded using an electric grinder and kept in polyethylene bags at 4°C until used for enzyme extraction.

2.3.2. Enzyme Extraction

The crude extract enzyme of *Carica papaya*, *Mangifera indica*, and *Moringa oleifera* leaves were prepared using different extracting buffers as described previously (Mohamed Ahmed et al. 2009a). Briefly, duplicate samples of 10 g of *Carica papaya*, *Mangifera indica*, and *Moringa oleifera* powder was immersed in 100 mL of sodium acetate buffer. The extraction procedure was continued for 24 h at 4°C. The extracts were filtrated through cheese cloth and centrifuged at 5000 × g for 20 min. The supernatant was used for enzyme purification by ammonium sulfate fractionation method.

2.3.3. Partial Purification of the Enzyme

Initially, solid ammonium sulfate was slowly added with stirring to the crude extract (600 mL) preparations to 30% saturation. The pH of the enzyme/ammonium sulfate solution was maintained at pH 5.0 by a drop wise addition of either 0.1 mol/L H₂SO₄ or 7% NH₄OH, and the mixture was then maintained on ice for 30 min. Thereafter, the precipitates were separated from the supernatant by centrifugation at 8000 ×g (Centurion Scientific Co., Ltd, west Sussex, UK) for 20 min at 4°C. Solid ammonium sulfate was further slowly added to the supernatant to 20-100% saturation, and the solution was preserved on ice for another 30 min before being centrifuged at 8000 ×g for 20 min at 4°C.

The precipitates collected was dissolved in a small volume (about 2 mL) of 50 mmol/L Na acetate buffer, pH 5.0, and then dialyzed overnight at 4°C against the same buffer (10 L).

The extract was then centrifuged, and the supernatants were examined for milk-clotting and protease activities. The partially purified enzyme was either directly used for enzyme characterization and cheese making or freeze-dried at -50°C. The latter step was repeated until the quantity of the freeze-dried partially purified enzyme reached 2 g and stored at -20°C until used for cheese making and enzyme characterization

2.4. Analytical Methods

2.4.1. Determination of Milk-clotting Activity

Milk clotting activity was determined according to the method of Arima [12] and expressed in terms of Soxhlet units (SU). One SU is defined as the amount of enzyme which clots 1ml of a solution containing 0.1 g skim milk powder and 0.00111 g calcium chlorides in 40 min at 35°C. In brief, 0.5 ml of tested materials was added to a test-tube containing 5ml of reconstituted skim milk solution (10g dry skim milk/ 100ml, 0.01 M CaCl₂) pre-incubated at 35°C for 5 min. The mixture was mixed well and the clotting time T (sec), the time period starting from the addition of test material to the first appearance of clots of milk solution, was recorded and the clotting activity was calculated using the following formula:

$$SU = 2400 \times 5 / T \times 0.5; T = \text{clotting time (sec)}.$$

The test materials include liquid solution of crude enzyme from extracts of fruit and leaves.

2.5. Cheese Making

Cheese was produced according to the procedure of Talib et al. (2006, 2009) with slight modifications. Briefly, 25 L of fresh cows' milk was heated at 72°C for 15 sec and then cooled to 45°C, and CaCl₂ was added at the rate of 0.02%. Then, a starter culture of lactic acid (*Lactobacillus bulgaricus* and *Lactobacillus thermophilus*) was added at the rate of 2.0% w/v and left for 30 min to develop acidity. The partially purified freeze-dried enzymes of the leaves were added to the milk at the rate of 2 g/50 L of milk. The milk was mixed and left until coagulation completed.

2.6. Characterization of Partially Purified Enzyme

2.6.1. Effect of Temperature on Activity of the Extract

To study the effect of temperature on milk-clotting activity assay the reaction mixture containing 10% skim milk was incubated at a temperature range of 35-65°C. The enzyme thermo stability was also determined by pre incubating the enzyme in the temperature range of 35-70°C. The incubation time of samples varied from 10 to 60 min. After incubation, the samples were submitted for determination of residual activity

2.6.2. Effect of pH on Activity of the Extract

To study the effect of pH on milk-clotting activity assay, the reaction mixture containing 10% skim milk was adjusted to different pH (3.5-8.5). The buffers used were: 0.1M citrate-phosphate (pH 3.5-6.0). For pH stability, the enzyme will be dispersed (1:1) in the following 0.1M buffer solutions: HCl- KCl (pH 1.0-2.0), citrate-phosphate (pH 3.5-7.0), sodium-phosphate (pH 6.0-7.5) and maintained at room temperature for 60 min, and afterwards MCA was determined.

2.6.3. Effect of Concentration of the Crude Extract

To study the effect of concentration of the crude extract on milk-clotting activity assay, the reaction mixture

containing 10% skim milk was adjusted to concentration of 1-100 mg of the crude extract, and afterwards MCA was determined.

2.7. Experimental Design and Data Analysis

All statistical analysis of different parameters was computed using appropriate software statistical package (EXCELL, SPSS). Enzymes assay was carried out in triplicates with analytical grade reagents and the average values and standard errors were calculated. Relationships among studied factors were presented using appropriate curves, tables and where necessary pictures.

3. Result and Discussion

3.1. Screening of Milk-clotting Enzyme from Leaves of *Carica Papaya*, *Mangifera Indica* and *Moringa Oleifera*

Milk-clotting activity was screened from leaves of *Carica papaya*, *Mangifera indica* and *Moringa oleifera* in order to use the leaf with the highest milk-clotting activity as a source of the enzyme. The results of the present study showed that milk-clotting activity was only detected in the leaf extracts of *Carica papaya* and *Moringa oleifera* and the leaf extract from the leaf of *Mangifera indica* showed no activity. *Carica papaya* and *Moringa oleifera* leaf

extract used for coagulation or clotting of milk for cheese preparation throughout this study and milk clotting activity of the extracted leaves presented in Table 1.

Similar to our findings, many previous works had been reported on the isolation of milk-clotting enzymes for the leaves and seeds of numerous plants [5,13,14,15,16]. However, various milk clotting enzymes has also been extracted from other parts of the plants [16,17].

3.2. Effect of Different Extracting Solution on the Milk-clotting Activity

In order to obtain the highest milk-clotting activity from the leaves of *Carica papaya* and *Moringa oleifera* samples various extracting solution were tested (Figure 2). The results showed that of *Carica papaya* and *Moringa oleifera* leaf extracted with 5% NaCl in sodium acetate buffer, pH 5.0 had higher milk-clotting activity compared to that extracted with the other extracting solution (Figure 2). Therefore, 5% NaCl in sodium acetate buffer, pH 5.0, was used as an extracting buffer throughout the study.

This result is in a good agreement with the observation of Yousif [19] and Mohamed Ahmed [14,15] who stated that water extract of *Solanum dubium* berries and seeds had less milk clotting activity compared to that extracted with 5% NaCl in acetate buffer. Moreover, Guiama [18] obtained the maximum milk-clotting activity by soaking dried berries of nine *Solanum* species in 5% NaCl solution.

Table 1. Extracting for milk clotting activity from the leaves of *Carica papaya*, *Mangifera indica* and *Moringa oleifera* in different extracting solutions

Leaves of plants	Distilled Water (DW)	5% NaCl in DW	Sodium acetate buffer	5% NaCl in Sodium acetate buffer
<i>Carica papaya</i>	19.35	34.16	82	195
<i>Moringa oleifera</i>	16.18	30.12	68.9	135
<i>Mangifera indica</i>	ND	ND	ND	ND

Results are average of triplicate samples. nd: not detected. MCA in Su/ml.

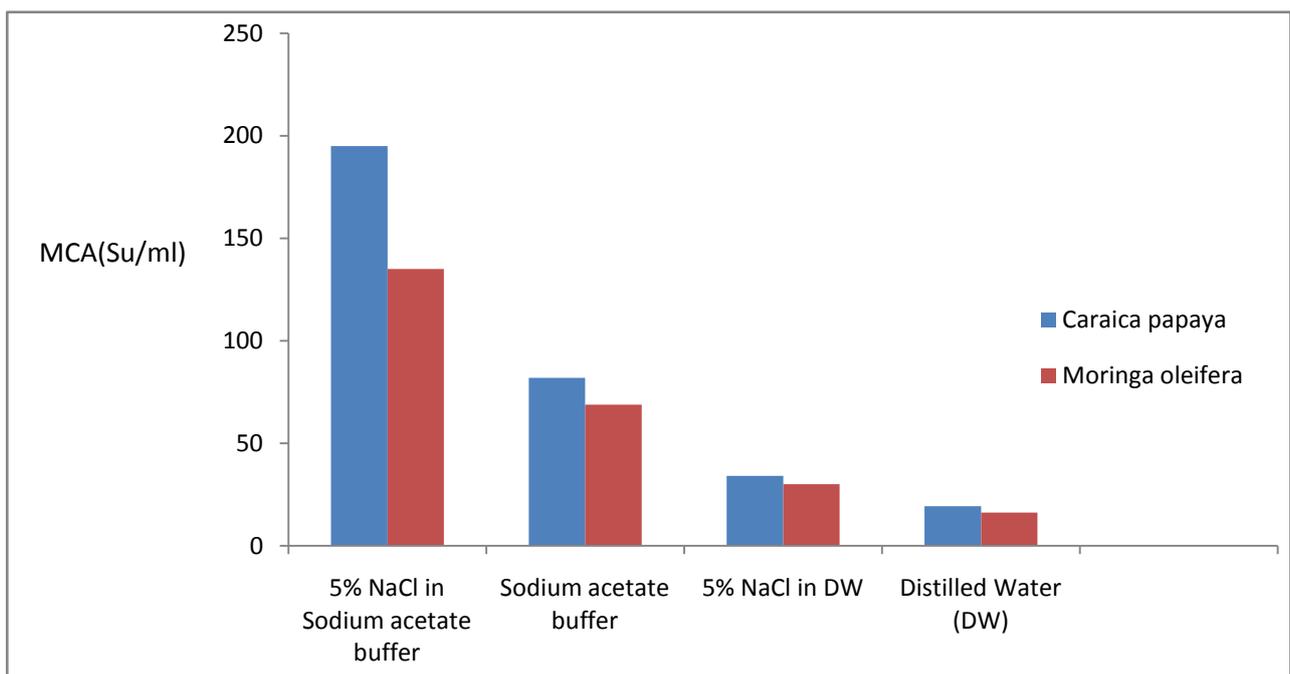


Figure 2. Ability of different extracting solutions to extract milk-clotting enzyme from the leaves of *Carica papaya* and *Moringa oleifera*

3.3. Partial Purification of the Enzyme

Ammonium sulfate precipitation was used in this study, a single purification step of milk-clotting enzymes from the leaves of *Carica papaya* and *Moringa oleifera*. The crude extract was precipitated with 0-100% ammonium sulfate with 20 % intervals as described in Material and Methods.

The results showed that the highest milk-clotting activity was obtained with 40 % ammonium sulfate saturation, which was 5 times higher compared to that of the crude extract. With increasing the ammonium sulfate concentration the milk clotting activity was gradually decreased until completely disappeared above 100 % saturation (Figure 3). Accordingly 40 % ammonium sulfate saturation was selected for potential purification of the milk clotting enzyme for the leaves of *Carica papaya*

and *Moringa oleifera*. This result disagree to those of Nestor [16] who reported that 20% ammonium sulfate gave the highest milk-clotting activity of *Solanum Elaeagnifolium* seeds extract. The results obtained in the current study indicated that the degree of saturation of ammonium sulfate greatly affected the enzyme activity, yield and as well as the enzyme purity. This procedure causes the enzyme to concentrate a workable volume that could efficiently be used for milk coagulation in cheese making industry. Overall, a one-step and cheap purification procedure has been developed to partially purify milk-clotting enzyme from *Carica papaya* and *Moringa oleifera* leaves. Such an economic purification procedure combined with the easy availability of the plant leaves make large scale preparation of the enzyme possible, allowing a wide study of its various aspects and hence probable applications.

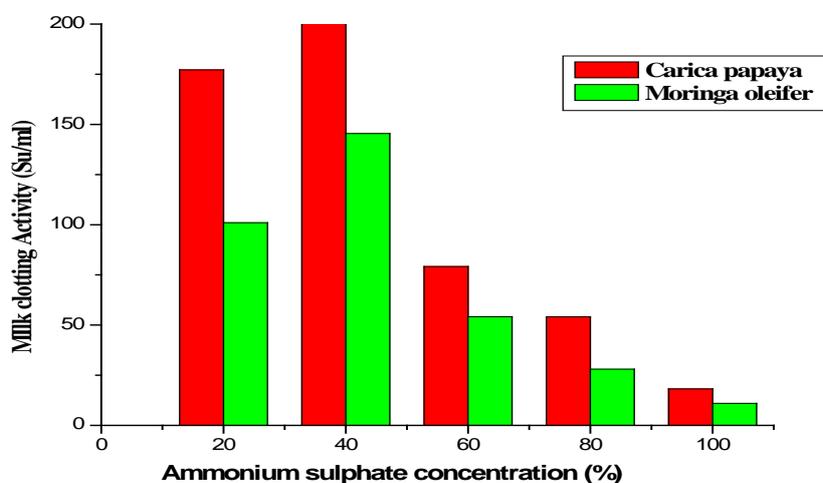


Figure 3. Ammonium sulfate fractionation of a milk-clotting enzyme from the leaves of *Carica papaya* and *Moringa oleifera*

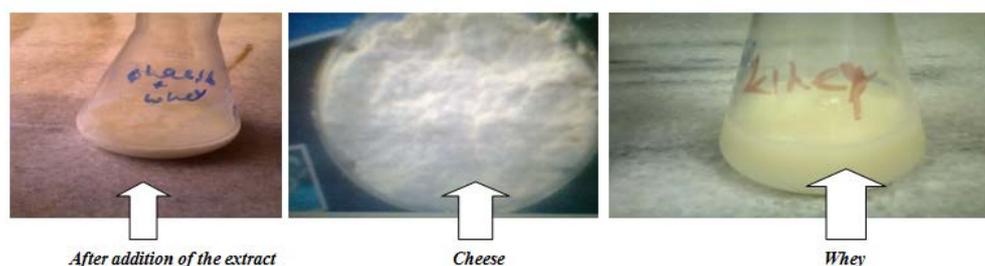
3.4. Preparation of Cheese from Cow Milk Using the Enzyme

Picture A and B below has shown cheese curd produced by the partially purified milk clotting enzyme from the leaves of *Moringa oleifera* and *Carica papaya* respectively.

Picture A Making of cheese from leaves of *Carica papaya*



Picture B Making of cheese from leaves of *Moringa oleifera*



3.4. Characterization of the Crude Enzyme

3.4.1. Effect of Temperature on the Enzyme Activity and Stability

The temperature profile of the milk-clotting enzyme from plant extracts depends on several factors such as the plant source, tissue, concentration and type of protease [20]. The milk-clotting activities of the partially purified enzymes were examined using skim milk as a substrate at different temperatures ranging from 30 to 90°C for 30 min. Optimum temperature range were between 30 to 90°C for both leaves of *Carica papaya* and *Moringa olifera* and with the maximum activity at 70°C and 65°C respectively.

The results (Figure 4) showed that the enzyme activity of *Carica Papaya* increased as the (reaction) temperature increased from 30 to 70°C. The activity at 70°C was 7-fold higher than that of the activity at 30°C. The activity sharply decreased as the temperature of the reaction raised over 80°C. However, at 80°C the milk-clotting activity of the enzyme was still higher compared to that at temperatures below 45°C.

The results (Figure 5) showed that the enzyme activity of *Moringa olifera* increased as the (reaction) temperature increased from 30 to 65°C. The activity at 65°C was 5-fold higher than that of the activity at 30°C. The activity sharply decreased as the temperature of the reaction raised over 75°C. However, at 80°C the milk-clotting activity of the enzyme was still higher compared to that at temperatures below 35°C. The effect of temperature on the catalytic activity of partially purified milk clotting enzymes of *Carica papaya* and *Moringa olifera* leaves exhibited a typical activity-temperature relationship of enzymes. The increase of the milk-clotting activity at higher temperature could be attributed to the protein aggregation and molecular rearrangement in the protein structure [21]. Different enzymes have different optimum temperatures; mainly depending on the enzyme structure. The conformational changes in the protein structure under high temperature could make it vulnerable to proteolysis because, protein unfolding can expose new cleavage sites to enzymatic hydrolysis. In agreement with our findings, high optimal temperature (70°C) of milk-clotting enzymes from various plants has been reported [14,15,20].

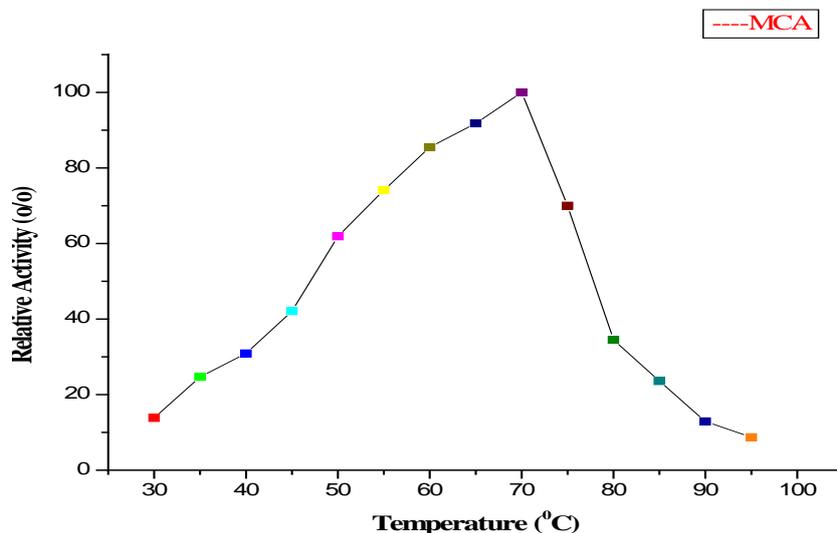


Figure 4. Optimal temperature of milk clotting enzyme from leaves of *Carica papaya*

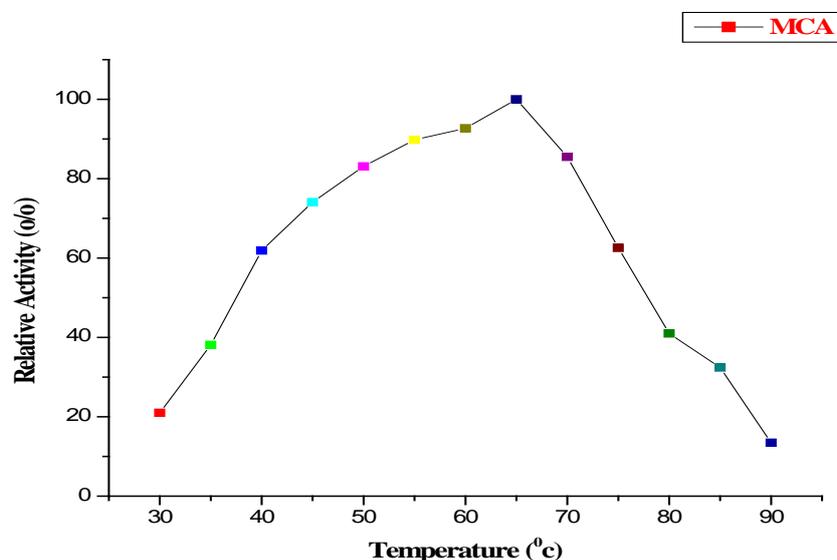


Figure 5. Optimal temperature of milk clotting enzyme from leaves of *Moringa olifera*

Thermo-stability of the partially purified enzymes was examined by measuring the residual activity after incubating the enzymes at different temperatures. Since the enzyme had a high optimum temperature, its stability at temperature between 20 to 75 °C was studied.

The results from leaves of *Carica Papaya* (Figure 6), there were more than 80% retention of the milk-clotting activity of the enzyme from after 1h incubation at 60 °C and also there was 70% retention of the milk-clotting activity of the enzyme after 1h and 30 minutes incubation at 60°C. There was more than 60% retention of the milk-clotting activity of the enzyme after 2h incubation at 60°C. However, the residual activity was quickly diminished when the enzyme was incubated for 1 h at 70°C or higher temperatures.

The results from leaves of *Moringa olifera* (Figure 7), there were more than 80% retention of the milk-clotting activity of the enzyme from after 1h incubation at 55 °C

and also there was 70% retention of the milk-clotting activity of the enzyme after 1h and 30 minutes incubation at 55 °C. There was more than 50% retention of the milk-clotting activity of the enzyme after 2h incubation at 60°C. However, the residual activity was quickly diminished when the enzyme was incubated for 1h at 65°C or higher temperatures.

With regards to the stability of the partially purified enzymes from both *Carica Papaya* and *Moringa olifera* leaves at high temperature, it behaved like milk-clotting enzymes extracted from other vegetable and microbial sources. Thermal stability in the range of 30-60°C for milk clotting enzymes from different plant sources has been reported [16,17]. However, the partially purified milk-clotting enzymes in the current study were characterized by their high thermal resistance as compared to calf rennet, since calf rennet reached its maximum activity at 45°C, followed by a sharp decline when the temperature exceeded 50°C [22].

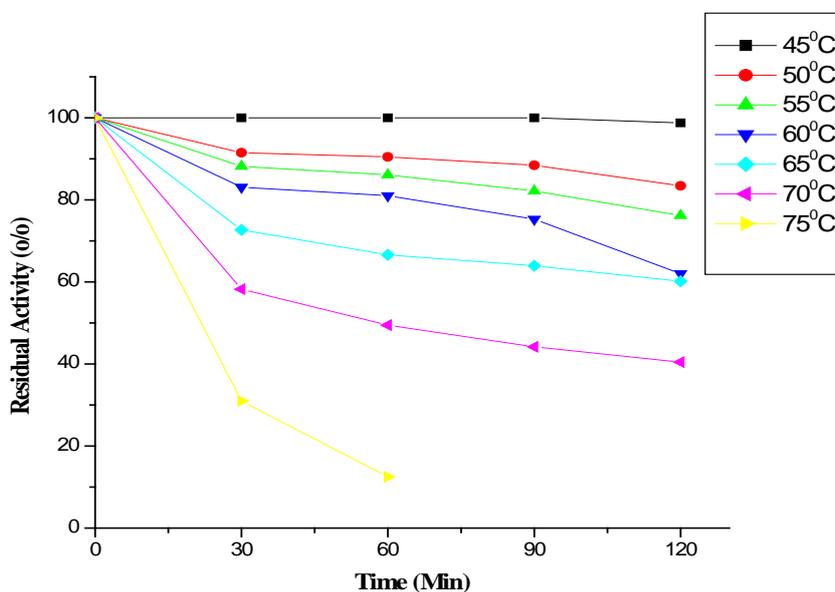


Figure 6. Thermal Stability of milk clotting enzyme from leaves of *Carica Papaya*

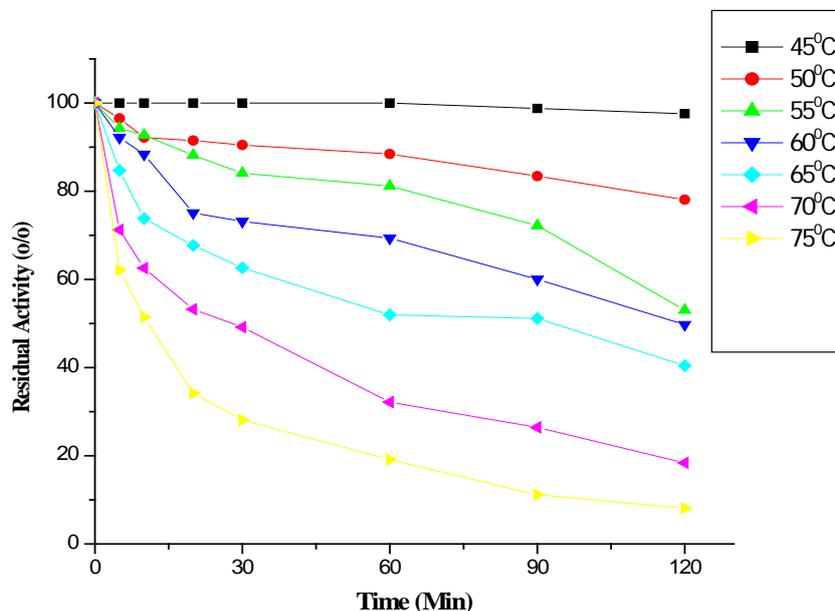


Figure 7. Thermal Stability of milk clotting enzyme from leaves of *Moringa olifera*

3.4.2. Effect of pH on Enzyme Activity and Stability

Results (Figure 8) showed from leaves of *Carica Papaya* at different pH values were tested, the enzyme showed the highest protease activity at a pH of 7.5 and the lowest activity at a pH of 3. This higher activity at alkaline pH suggests that the enzyme belongs to a group of proteases that are often used commercially [23]. Similar results were observed for the protease in the latex of *Streblus asper*, which had an optimum pH of 9.0, keeping it in the alkaline range [24]. The optimum pH of calf rennet about 6–6.3 as reported by Okigbo *et al.*, 1985.

Results (Figure 9) showed from leaves of *Moringa olifera* at different pH values were tested, the enzyme showed the highest protease activity at a pH of 7 and the lowest activity at a pH of 3. Similar results were observed for a protease from *Jacaratia corumbensis* O. Kuntze showed greater activity in a nearly neutral pH range— pH 6.5 for the crude extract of the enzyme and pH 7.0 for the purified enzyme (Duarte *et al.*, 2009). However, a protease from *Ficus racemosa* showed higher activity at an acidic pH, with 80% of its activity occurring at a pH of 4.0 [25].

According to Demir [26], high pH enzymes are advantageous in food production because at lower milk pH, it formed bad and non-firmed clots therefore, and their results were excluded. Coagulants should not be sensitive to variations in the pH of milk because this sensitivity can decrease the yield of cheese or cause defective cheese formation owing to clots too soft for cutting [27].

The stability of the purified enzyme from *Carica Papaya* at different pH values was shown in Figure 10. pH has an effects on the activity of MCE produced by *Carica Papaya*, held for 60 min and at different pH levels (pH 5-10), as shown in Figure 10. The results indicated that the crude enzyme retained more than 80% of its activity between pH 5 and pH 7.5 and more than 70% of its activity at pH 6.5 for more than 120 min. According to Demir [26], pH stable enzymes are advantageous in food production. Papain and bromelain are traditional plant proteases reported to be stable between pH 5.0 and 9.0 [28]. Coagulants should not be sensitive to variations in the pH of milk because this sensitivity can decrease the yield of cheese or cause defective cheese formation owing to clots too soft for cutting [27].

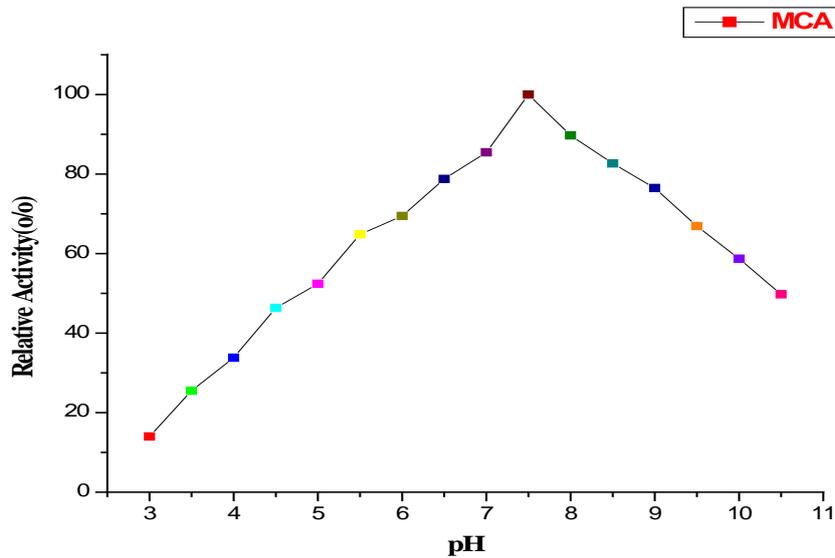


Figure 8. Optimal pH for milk clotting enzyme from leaves of *Carica Papaya*

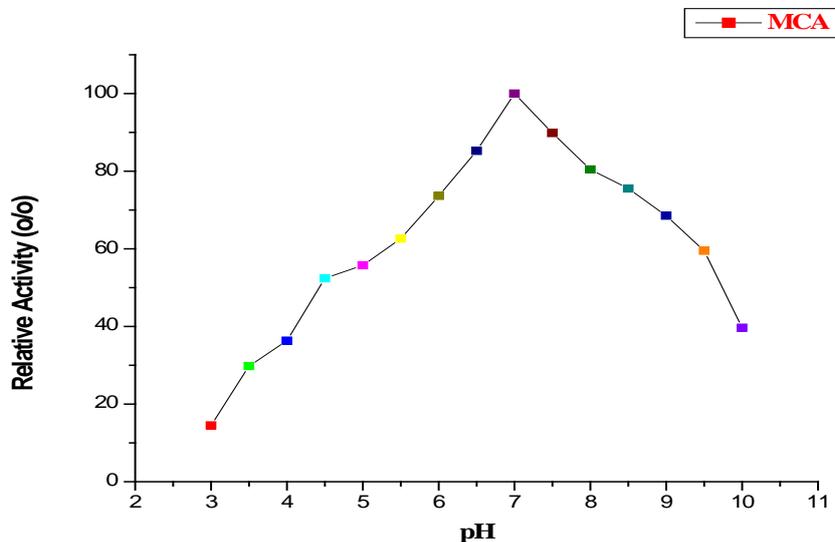


Figure 9. Optimal pH for milk clotting enzyme from leaves of *Moringa*

The stability of the purified enzyme from *Moringa oleifera* at different pH values was shown in Figure 11. pH has an effect on the activity of MCE produced from *Moringa oleifera*, held for 60 min and at different pH levels (pH 5-9), as shown in Figure 11. The results indicated that the crude enzyme retained more than 80% of its activity between pH 5 and pH 6.5 and more than 70% of its activity at pH 6 for more than 120 min.

3.4.3. Effect of Concentration on the Activity of the Crude Extract

The effect of concentration of the crude extract on milk clotting activity was checked between 1-100 grams of the crude extracts of *Carica Papaya* and *Moringa oleifera* as shown below in Figure 12 and Figure 13 respectively. The isolated protein fraction has a highest enzymatic activity at a concentration of 70 and 90 mg respectively, of the crude extracts of *Carica Papaya* and *Moringa oleifera* leaves.

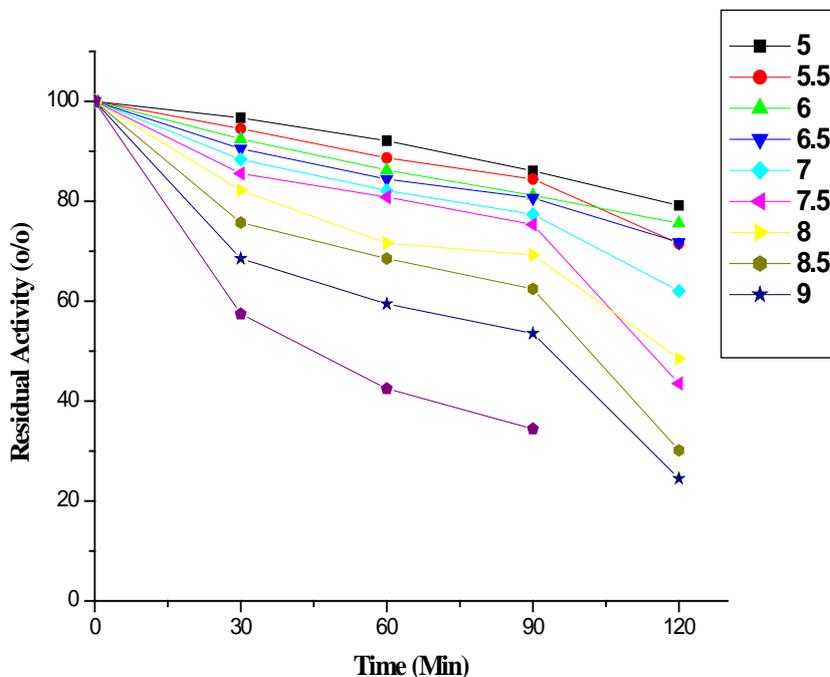


Figure 10. PH stability of milk clotting enzyme from leaves of *Carica Papaya*

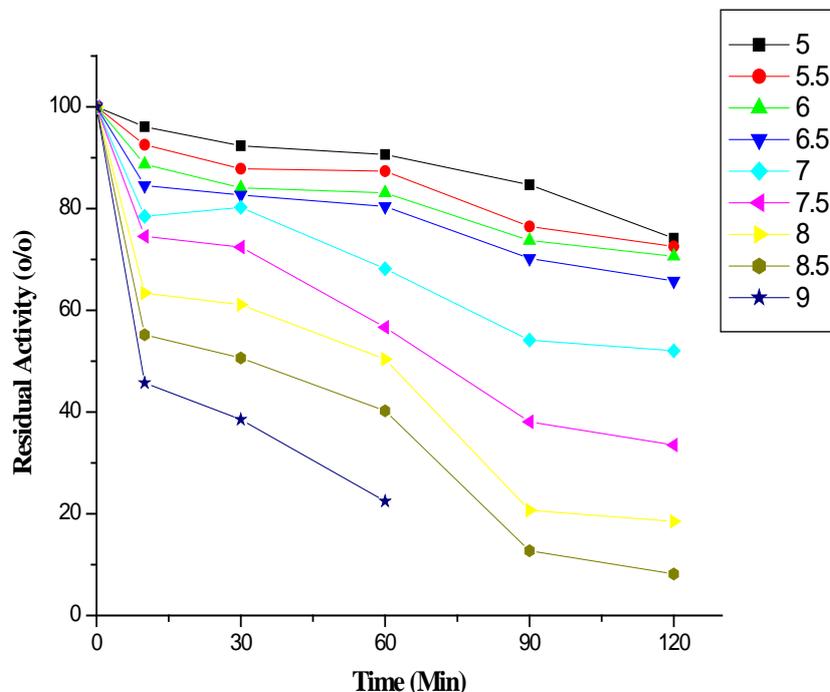
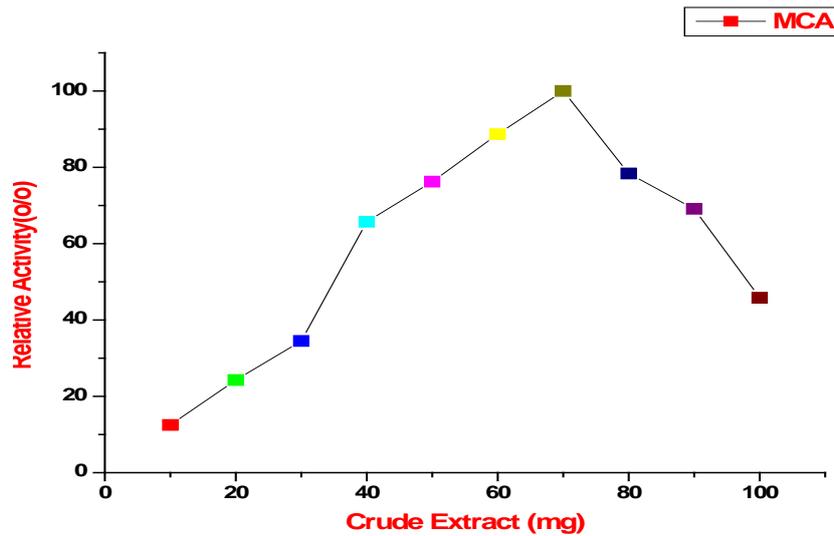
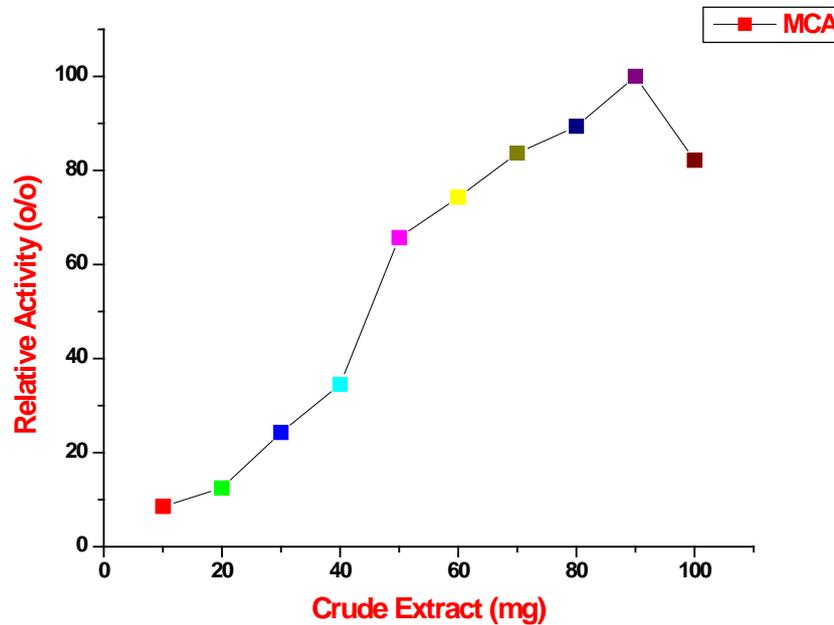


Figure 11. PH stability of milk clotting enzyme from leaves of *Moringa oleifera*

Figure 12. MCA of *Carica papaya* crude extractFigure 13. MCA of *Moringa oleifera* crude extract

4. Conclusion

This study could prove to the commercial production of milk clotting enzyme leaves of *Carica papaya* and *Moringa oleifera*. We develop a simple purification procedure for the production of substantial amounts of active milk-clotting enzymes from leaves of *Carica papaya* and *Moringa oleifera* as a cheap milk-clotting preparation for cheese making. In addition, the partially purified enzyme demonstrated great milk-coagulation and curd formation ability indicating its potentiality in cheese making as rennet substitute.

This paper describes the characterization and partial purification of the enzyme using ammonium sulphate. As a result milk-clotting activity increased with increasing incubation temperature and reached its maximum activity at 70°C and 65°C for leave of *Carica papaya* and *Moringa oleifera* respectively, while the activity also reached its maximum at pH 7.5 and 7 respectively, it had showed excellent pH and thermal stability. Moreover, the

high stability of the purified enzyme under various conditions, in accordance with the availability of raw materials, in addition to its high milk-clotting ability, could therefore pave the way for its use in the cheese industry.

5. Recommendations

- Extract the crude enzymes for large-scale production for industrially purpose and try it with trace amounts (milliliters);
- Experiment with other plants to make effective choice to replace animal rennet coagulant by a cheap plant in traditional cheese making in rural areas.
- Further on purification procedures to increase the activity of enzymes at lower concentration
- Further studies should be conducted on the texture, taste, chewiness, gumminess and color of the produce cheese

- Study should be made on toxicological analysis of the produced cheese
- Study on shelf life estimation of the extract using different packaging materials.
- Study on the assessment of the MCA and proteolytic activity of extract enzyme of different species grown in Ethiopia at different maturation stages.
- Research should also be done on the evaluation of the suitability of the extract enzyme for cheese making from different milk sources such as cow, ewe, goat milk and soy milk

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