PARTIAL PURIFICATION OF MILK-CLOTTING ENZYME FROM THE SEEDS OF *MORINGA OLEIFERA*

Amna E. Tajalsir[1], Ali S. A. Ebraheem[1], Abdelmoez M. Abdallah[1], Fatima J. Khider[1], Mohamed O. Elsamani[2], and Isam A. Mohamed Ahmed[1,3]*

Address(es):
[1] Department of Food Science and Technology, Faculty of Agriculture, University of Khartoum, Shambat 13314, Khartoum North, Sudan.
[2] Department of Food Science and Technology, Faculty of Agriculture, Omdurman Islamic University, Omdurman, Sudan.
[3] Arid Land Research Center, Tottori University, Tottori 680-0001, Japan.

*Corresponding author: isamnawa@yahoo.com

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ABSTRACT

The aim of the present study was to search for milk clotting substitute from different parts (flowers, seeds, stem, leaves, ripe and unripe fruits) of *Moringa oleifera*. The samples were blended and extracted using different types of extracting solutions. The most reliable, quick and efficient enzyme extracting solution was found to be 5% NaCl in 100 mM sodium acetate buffer, pH 5.0, which was used throughout the study. The milk clotting activity was only observed in the seeds extract while the other parts were either deficient or has very low milk clotting activity. Thus, the moringa seeds were used as source of milk clotting enzyme. The extracted proteins were fractionated with ammonium sulfate at concentration of 20, 30, 40, 50 and 60 %. Highest milk clotting activity was observed in the 20 % fraction. This fraction was assumed to contain the clotting enzymes and characterized for its heating stability (30 – 90°C) and optimum temperature (30 – 90°C). The results demonstrated that moringa seeds milk clotting enzyme is stable up to 50°C with an optimum milk clotting activity of 70°C. The high ratio of milk-clotting to proteolytic activity of the partially purified enzyme indicates the potential of this enzyme as suitable rennet substitute in dairy industry. However, further study is needed to completely purify and characterize this promising milk clotting enzyme from moringa seeds.

Keywords: Ammonium sulfate fractionation, Milk-clotting activity, Moringa seeds, Rennet substitutes

INTRODUCTION

Cheese is the generic name for a group of fermented milk-based food products, with more than 1000 types, produced in a wide range of flavours and forms throughout the world that has played a key role in human nutrition for more than 8000 years (**Fox & McSweeney 2004**). The main objective has always been and still is to convert milk, which is perishable, into a product with a longer shelf life whilst preserving its nutrients (**Mohamed Ahmed et al., 2010**). Milk coagulation is the basic step in the manufacture of most types of cheeses and calf rennet has been and still the most widely used milk-clotting enzyme preparation (**Mohamed Ahmed et al., 2009a**). However, the last decades have witnessed by the population explosion that lead to an increased demand for cheese production and consumption. Together with that the price of calf rennet's was greatly increased, along with a reduced supply of natural calf rennet (**Mohamed Ahmed et al., 2010**). In addition to that, the use of animal rennet is limited for religious reasons (e.g. Judaism and Islam), safety reasons (bovine spongiform encephalopathy), diet (vegetarianism), or being against genetically engineered foods (**Roseiro et al., 2003**). All these reasons have necessitated the search for new protease with high specific milk-clotting activity and low general proteolytic activity to be used as a rennet substitute. Accordingly, much research interest has been directed towards discovering a milk-clotting enzyme which would satisfactory replace calf rennet in cheese manufacture, and numerous enzyme preparations of animal, microbial, and plant origin have been studied (**Jacob et al., 2011**). Microbial rennets produced by genetically engineered bacteria have proven to be suitable substitutes for animal rennet, but increasing attention has been directed toward natural rennet extracted from plants such as *Ananas comosus*, *Carica papaya*, *Calotropis porcera*, *Ficus carica*, *Calm viscera*, *Cynara cardunculus* (**Roseiro et al., 2003**), *Cynara scolymus* (**Sidrach et al., 2005**), and *Solanum dubium* (**Mohamed Ahmed et al., 2009 a, b**) among others. Unfortunately, most of these plant rennets were found inappropriate because they possess high general proteolytic activity, which leads to the production of short peptides that are responsible defect in flavor and texture of cheese (**Anusha et al., 2014**). An exception to this general rule is represented by the aqueous extract of *Cynara cardunculus* flowers containing two aspartic acids-type proteases, named

cardosin A and B (**Verissimo et al., 1995**), which has been used for years for the manufacture of sheep milk cheese in several areas of Portugal and Spain. However, the flowers of *Cynara cardunculus* are not used for the production of cow-milk cheeses as they tend to taste bitter because of the formation of several peptides identifiable in the digests of isolated bovine β- and α-casein (**Macedo et al., 1996**). Thus, the search for a rennet substitute from plant sources having high ration of milk clotting to general proteolytic activity is highly needed to overcome the above mentioned problems.

Moringa oleifera is native to the sub-Himalayan tracts of India, Pakistan, Bangladesh and Afghanistan and it is an important crop in India, Ethiopia, the Philippines and the Sudan. All parts of this rapidly-growing tree are edible and have long been consumed by the ancient Romans, Greeks and Egyptians (**Fahey 2005**). It is the most nutrient-rich plant yet discovered as it contains all the essential nutritional elements that are essential for livestock and human beings (**Fahey 2005**). Many parts of this miracle tree have been used in various applications such as food for overcoming malnutrion, feed for weight gain and milk production, medicine for the treatment and/or prevention of many diseases, and environment for water clarification (**Fahey 2005**). Despite the wide spread and huge uses of *Moringa oleifera*, it uses as a source of milk clotting enzymes is scarce. Only one report has recently been published indicating that the presences of caseinolytic and milk-clotting activities from flowers of *Moringa oleifera* (**Pontual et al., 2012**). This activity has only been detected after the precipitation of the protein with 60% ammonium sulfate (**Pontual et al., 2012**) indicating low concentration of such enzyme in the flowers. Nevertheless, extracts from other parts of this important tree have not been tested for their milk-clotting ability. Moreover, isolation and characterization of milk-clotting enzymes from any part of this tree has not yet been reported. Therefore, in the current study crude extracts from different parts of *Moringa oleifera* has been screened for their milk-clotting activity using skimmed milk as substrate. Milk-clotting activity was only detected in the seeds extract of this tree. This enzyme was further partially purified by using ammonium sulfate precipitation techniques, and then milk-clotting properties and temperature profile of the partially purified enzyme were evaluated.

MATERIAL AND METHODS

Material

Moringa oleifera samples were supplied by engineer Salah Abdoun at Moringa Research and Development Group Farm, Khartoum North, (Al-Samrab). These samples include leaves, stems, flowers, unripe fruits, ripe fruits and seeds. All fresh samples are dried for two days at room temperature. Thereafter, samples were ground using house grinder and store in closed containers in the freezer at -20°C until use. All chemical used were of analytical grade.

Enzyme extraction

Different extracting buffers were tried for the extraction of milk-clotting enzyme from different parts of *Moringa oleifera* (flowers, seeds, stem, leaves, ripe and unripe fruits), which were distilled water, 5 % NaCl in distilled water, sodium acetate buffer (pH 5.0), and 5% NaCl in sodium acetate buffer (pH 5.0). *Moringa oleifera* samples (5.0 g each) were finely ground by grinder and extracted with 50 ml of each extractant for 4h with stirring. The extracts were filtrated through cheesecloth and centrifuged at $12,000 \times g$ for 20 min. The supernatants were dialyzed overnight against 0.1 M sodium acetate buffer, pH 5.0. Milk-clotting and protease activities in addition to protein concentration in each extract were determined.

Ammonium sulfate fractionation

Ammonium sulfate fractionation was carried out following the method described elsewhere (**Mohamed Ahmed *et al.*, 2009a**). Solid ammonium sulfate was slowly added with stirring to the crude extract (50 ml) preparations to 20% saturation. During the addition of solid ammonium sulfate, the pH of the enzyme solution was kept at pH 5.0 by a drop wise addition of either 7% NH_4OH or 0.1 M H_2SO_4, and the mixture was kept on ice for 30 min. The precipitates were separated from the supernatant by centrifugation at $12,000 \times g$ for 20 min at room temperature. Solid ammonium sulfate was further slowly added to the supernatant to 30% saturation and the solution was kept on ice for another 30 min before being centrifuged at $12,000 \times g$ for 20 min at 4°C. This process was repeated for each concentration of ammonium sulfate until reach 60 % saturation. The precipitates collected were dissolved in a small volume (about 10 ml) of 50 mM sodium acetate buffer, pH 5.0, and then dialyzed overnight at 4°C against the same buffer (1.0 liter) with several change of the dialysis buffer. The dialyzed solutions were further centrifuged to remove any solid particles, and their protein concentration, milk-clotting and proteolytic activities were measured. The most active fraction with high yield was pooled and used to characterize the enzyme.

Determination of milk-clotting activity

Milk-clotting activity was determined according to the methods described elsewhere (**Arima *et al.*, 1970**). The substrate (10% skimmed milk in 0.01 M $CaCl_2$) was prepared and the pH was adjusted to 6.5. Then, ten millimeters of the substrate was pre-incubated for 5 min at 37°C, and 1.0 ml of enzyme extract was then added, and the curd formation was observed at 37°C while manually rotating the test tube from time to time. The end point was recorded when discrete particles were discernible. One milk-clotting unit is defined as the amount of enzyme that clots 10 mL of the substrate in 40 min.

MCA (U/ml) = (2400∕clotting time (sec))×Dilution factor

Protease activity assay

In this method (**Sarath 1989**), 1% casein solution was prepared in 50 mM potassium phosphate buffer, pH 6.5. To start the assay, 0.5 ml of temperature-equilibrated substrate was added to 0.1 ml of the enzyme solution (in centrifugal tube) and mixed gently. The reaction mixture was incubated at 30°C for 30 min, and then the reaction was terminated by the addition of 1.0 ml of 5% trichloroacetic acid (TCA). The blank was prepared in the same way by adding TCA prior to the addition of the substrate. After standing for 30 min at room temperature, the reaction mixtures were centrifuged at $12,000 \times g$ for 20 min to separate the TCA-soluble products. The absorbance was read at 280 nm with reference to the blank. One unit of the enzyme activity was defined as the amount of the enzyme required to cause a unit increase in absorbance at 280 nm across a 1 cm path length, under the assay conditions.

Determination of the protein content

The protein content of the fractions obtained before and after ammonium sulfate fractionation was estimated by measuring the absorbance at 280 nm.

Effect of temperature on the enzyme activity and stability

The effect of temperature on the activity of the partially purified enzyme was studied using skimmed milk as substrate. The substrate at a final concentration of 10% skimmed milk in 0.01 M calcium chloride, pH 6.5, was pre-incubated at specified temperatures in a range of 30 - 90°C for 10 min. Then the enzyme was added, and the reaction was proceeded at the same temperature until the discrete particles were discernible. The control tube was incubated without the enzyme at each temperature.

The thermal stability of the enzyme was tested by incubating the partially purified enzyme in 50 sodium acetate buffer, pH 5.0, at different temperatures (30 - 90°C) for 30 min. Then, the remaining activity was measured at 37°C using 10% skimmed milk as substrate and expressed as percentage of the control.

Storage stability

Partially purified enzyme of moringa seeds was stored at 4 and 25°C for 7 days. The milk-clotting activity of the stored enzyme was assayed on every third day using skimmed milk as substrate as described above.

Digestion of food protein

Food protein digestion experiment was performed following the method described by **Singh and Dubey (2011)**. Briefly, three cubical pieces of boiled egg albumen, 1 g each, were placed in 16 ml test tubes. Then, 5 ml of either moringa seed extract or rennet solution were added and the tubes were incubated at 37 °C for overnight. A control was used for comparison in which enzymes were replaced with distilled water.

Statistical analysis

Each determination was carried out and analyzed in triplicate and the figures were then averaged. The variation was assessed by the analysis of variance (ANOVA).

RESULTS AND DISCUSSION

Screening for milk-clotting activity from different parts of *Moringa oleifera*

Milk-clotting activity was screened from various parts (leaves, stems, flowers, unripe fruits, ripe fruits and seeds) of *Moringa oleifera* in order to use the part with the highest milk-clotting activity as a source of the enzyme. The results showed that the milk-clotting activity was only detected in the seeds extract and the other parts showed no activity for more than 5 hours incubation at 37°C (Table 1). However, when the reaction is continued for overnight at 37°C very mild curds were observed in all reaction mixtures. The overnight curds formation could be attributed to either the action of very weak and/or low concentration milk-clotting enzyme in these extracts or to the acidification effect of lactic acid that released during the fermentation of milk lactose by indigenous lactic acid bacteria. This has been supported by the findings of **Pontual et al. (2012)** who reported that moringa flower extract (2,940 mg of protein) was not able to hydrolyze azocasein, and it did not show milk-clotting activity using milk supplemented or not with $CaCl_2$. High milk clotting activity in the moringa seeds extract was expected since most of the proteolytic enzymes in the plants are concentrated in the seeds where it degrade the storage protein during seed germination (**Antao & Malcata 2005**). Similar to our findings, many previous works had been reported on the isolation of milk-clotting enzymes for the seeds of numerous plants (**Elmazar *et al*., 2012; Egito *et al*., 2007; Mohamed Ahmed *et al*., 2009a,b; Naz *et al*., 2009; Nestor *et al*., 2012**). However, various milk-clotting enzymes has also been extracted from other parts of the plants (**Anusha *et al*., 2014; Beka *et al*., 2014; Hashim *et al*., 2011; Guiama *et al*., 2010; Mazorra-Manzano *et al*., 2013; Pontual *et al*., 2012**).

Table 1 Screening for milk clotting activity from various parts of *Moringa oliefera*

Moringa parts	Extraction solution			
	Distilled Water (DW)	5% NaCl in DW	Sodium acetate buffer	5% NaCl in Sodium acetate buffer
Leaves	nd	nd	nd	nd
Stems	nd	nd	nd	nd
Flowers	nd	nd	nd	nd
Ripe fruits	nd	nd	nd	nd
Un ripe fruits	nd	nd	nd	nd
Seeds	19.62 U/mL	230.84 U/mL	220.01 U/mL	3814.80 U/mL

Results are average of triplicate samples.
nd: not detected.

Effect of different extractant on the milk-clotting activity

In order to obtain the highest milk-clotting activity from moringa samples various extracting solution were tested. The results showed that moringa seeds extracted with 5% NaCl in 50 mM sodium acetate buffer, pH 5.0, had higher milk-clotting

activity compared to that extracted with the other extractants (Table.1, Fig. 1). 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 *et al.* (1996)** and **Mohamed Ahmed *et al.* (2009a,b)** 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 *et al.* (2010)** obtained the maximum milk-clotting activity by soaking dried berries of nine Solanum species in 5% NaCl solution. An increase in the ionic strength of the extracting solution apparently increases the solubility of the milk-clotting enzyme, and thus allows for increased extraction.
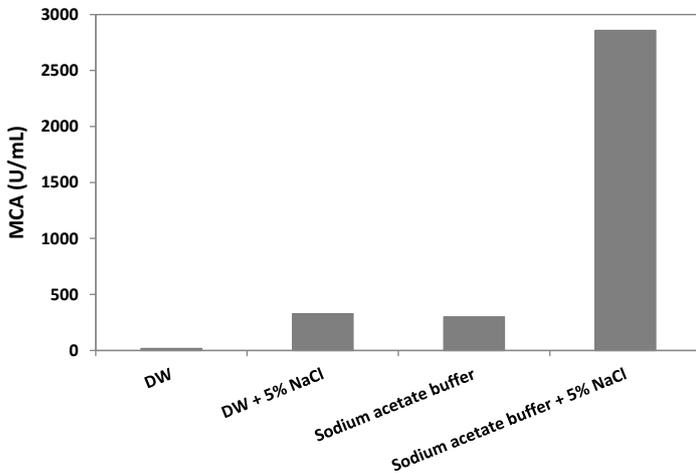

Figure 1 Ability of different extracting solutions to extract milk-clotting enzyme from the seeds of *Moringa oleifera*.

Partial purification of the enzyme

Ammonium sulfate precipitation was performed as a single purification step of milk-clotting enzyme from the seeds of *Moringa oleifera*. The crude extract (50 ml in 50 mM sodium acetate buffer, pH 5.0) was precipitated with 0-60% ammonium sulfate with 20 % intervals as described in Material and Methods. The results showed that with increasing the ammonium sulfate concentration the milk clotting activity was gradually decreased ($P \leq 0.05$) until completely vanished at 60% saturation (Fig. 2). Exceptionally, the highest milk-clotting activity was obtained with 20 % ammonium sulfate saturation, which was 7 times higher compared to that of the crude extract. This result was comparable to those of **Nestor *et al.* (2012)** who reported that 20% ammonium sulfate gave the highest milk-clotting activity of *Solanum elaeagnifolium* seeds extract. Previously, a milk-clotting serine protease was partially purified from the seeds of *Solanum dubium* by using ammonium sulfate at saturation of 35-55% that lead to the salting out of more than 86% of the total protease in the crude extract (**Mohamed Ahmed *et al.*, 2009a**). The application of ammonium sulfate at low saturation (20%) in the current study is superior to high concentrations that usually used for the purification of milk-clotting enzymes from various sources (**Mohamed Ahmed *et al.*, 2009a; Pontual *et al.*, 2012**). The results obtained in the current study indicated that the degree of saturation of ammonium sulfate greatly affected the enzyme activity, yield and total protein as well as the enzyme purity. This procedure not only facilitates the effective removal of the brown-colored materials in the crude seeds extract, but also concentrated the enzyme to 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 *Moringa oleifera* seeds. Such an economic purification procedure combined with the easy availability of the plant seeds makes large scale preparation of the enzyme is possible, allowing a broad study of its various aspects and hence probable applications.
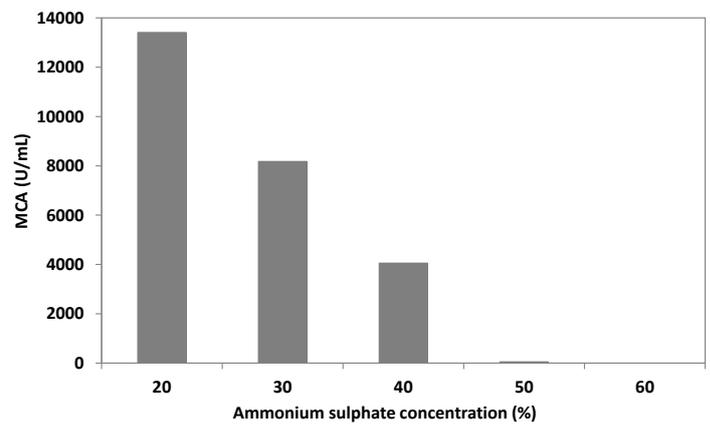

Figure 2 Ammonium sulfate fractionation of a milk-clotting enzyme from the seeds of *Moringa oleifera*.

Milk-clotting and proteolytic activity of the enzyme

The ratio of milk-clotting activity to proteolytic activity of the enzyme is a useful indicator for its suitability as a rennet substitute for cheese making (**Arima *et al.*, 1970**). An enzyme with a higher ratio of milk-clotting to proteolyic activity is better able to form curd, obtain higher yield and with less bitterness development during cheese processing, while that with low ratio may developed lower curd recovery, weak curd firmness and the release of bitter peptides that affect the sensory properties of the final product (**Mazorra-Manzano *et al.*, 2013**). Thus in the current study, the ratio of milk-clotting activity to proteolytic activity of the partially purified enzyme was evaluated and compared with those of some commercial milk coagulating enzymes (Table 2). Strikingly, partially purified enzyme from moringa seeds showed higher ratio of milk clotting activity to proteolytic activity compared to that of calf rennet, dubiumin and papain. The results obtained indicated that the partially purified enzyme of moringa seeds was highly active compared to calf rennet. This result is also supported by the coagulation behavior of the enzyme (Fig. 3a) which indicated that the partially purified enzyme greatly coagulated the skim milk compared to calf rennet and the control sample. Additionally, the partially purified enzyme from moringa seeds efficiently clotted the skimmed milk and formed a white and firm curd (Fig. 3b). It is well known that Chymosin, the main enzyme in commercial rennet's has a higher ratio of milk-clotting activity to protolytic activity than most coagulant substitutes from microbial and plants sources (**Jacob *et al.*, 2011**). It has been previously reported that the ratio of milk-clotting activity/proteolytic activity of vegetable rennet was lower, resulting in cheese that was bitter during ripening time (**Sousa *et al.*, 2001**). For example, papain, ficin, and bromelain have a higher proteolytic activity and produce a soft and bitter cheese. It is worth to note that in the current study the partially purified milk-clotting enzyme from moringa seeds demonstrated the highest ratio of milk-clotting to proteolytic activity among all milk clotting enzymes including calf rennet that is widely used in cheese making industry. The capability of the partially purified enzyme from moringa seeds to coagulate milk and form a firm curd combined with its high ratio of milk clotting to proteolytic activity could make it a useful rennet substitute in the dairy industry. Though, more studies about quality of both milk curds and the cheese formed should be carried out in the future to confirm usefulness of this new and cheap coagulant in the dairy industry.

Table 2 Ratio of milk-clotting activity to proteolytic activity of *Moringa oleifera* seeds enzyme compared to that of other coagulants

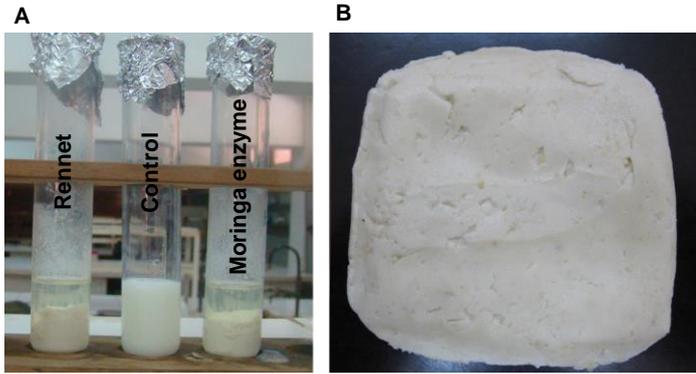| Enzyme	Clotting Activity (Unit/ml)	Proteolytic Activity (Unit/ ml)	Ratio
M. oleifera seed extract	13407.8	2.50	5279.0
Rennet	249.6	0.05	4992.0
Dubiumin	880.0	0.35	2490.0
papain	216.0	0.59	367.0

Figure 3 Milk-clotting properties of partially purified milk-clotting enzyme from the seeds of *Moringa oleifera*. Panel A: coagulation of skimmed milk by moringa seeds enzyme and commercial rennet. Panel B: cheese curd formed by the partially purified enzyme from moringa seeds.

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 (**Mazorra-Manzano** *et al.*, **2013**). The milk-clotting activity of the partially purified enzyme was examined using skim milk as a substrate at different temperatures ranging from 20 to 90°C for 30 min. The results (Fig. 4) showed that the enzyme activity increased as the (reaction) temperature increased from 20 to 70°C. The activity at 70°C was 10-fold higher than that of the activity at 20°C. The activity sharply decreased as the temperature of the reaction raised over 80°C. However, at 80°C and 90°C the milk-clotting activity of the enzyme was still higher compared to that at temperatures below 60°C. The effect of temperature on the catalytic activity of partially purified milk clotting enzyme of moringa seeds 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 (**Najera** *et al.*, **2003**). 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 (**Mohamed Ahmed** *et al.*, **2009a,b; Mazorra-Manzano** *et al.*, **2013; Pontual** *et al.*, **2012**). Thermostability of the partially purified enzyme was examined by measuring the residual activity after incubating the enzyme at different temperatures. Since the enzyme had a high optimum temperature, its stability at temperature between 20 to 90 °C was studied. There was more than 80% retention of the milk-clotting activity of the enzyme after 1h incubation at 50 °C (Fig. 4). However, the residual activity was quickly diminished when the enzyme was incubated for 1 h at 60°C or higher temperatures. With regards to the stability of the partially purified enzyme from moringa seeds 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 (**Anusha** *et al.*, **2014; Nestor** *et al.*, **2012**). However, the partially purified milk-clotting enzyme in the current study was characterized by its 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 (**Shehata** *et al.*, **1996**). The storage stability of proteins and enzymes is usually a factor that limits their usefulness in many applications (**Mohamed Ahmed** *et al.*, **2009b**). The storage stability of the partially purified enzyme was determined by incubating the enzyme at either 4°C or 25°C for one week (Fig. 5). The results showed that the enzyme is quite sensitive to the storage temperature and retain about 50 and 30% of its milk-clotting activity when incubated for 1 week at 4°C and 25°C, respectively. The reduction of the enzyme activity during the storage could be attributed to the either the autolysis or the degradation by other contaminating proteolytic enzymes since the enzyme is not fully purified yet. Most of the isolated plant proteases studied undergo autodigestion at low protein concentrations such as procerain (**Dubey & Jagannadham 2003**). However, some of the isolated milk-clotting plant serine proteases were found to resist autodigestion such as Religiosin B (**Kumari** *et al.*, **2012**) and dubiumin (**Mohamed Ahmed** *et al.*, **2009b**). The low storage stability of the partially purified enzyme from moringa seeds could be overcome by either complete purification or by the freeze drying of the enzyme. Complete purification will remove contaminating proteases while freeze drying will terminate autodigestion.
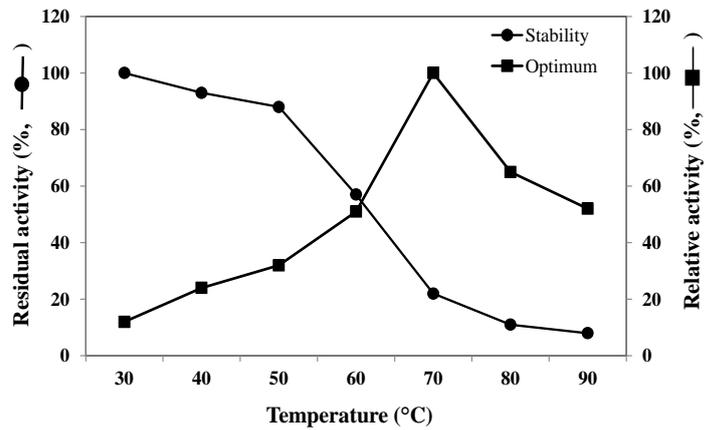

Figure 4 Effect of temperature on the activity and stability of partially purified milk-clotting enzyme from seeds of *Moringa oleifera*.
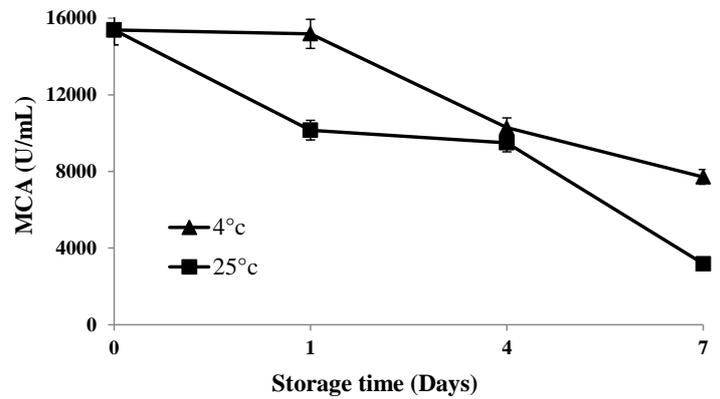

Figure 5 Storage stability of the partially purified milk-clotting enzyme from seeds of *Moringa oleifera*.

Digestion of egg white protein

Enzymatic hydrolysis of food proteins can release peptides able to exert different biological activities. Egg white proteins are used extensively in food systems due to their unique functional (foaming, gelling, emulsifying) and nutritional properties (**Mine & Zhang 2013**). In order to assess the digestive ability of the partially purified enzyme from moringa seeds on food proteins others than those of milk, the enzyme was incubated with egg white proteins for 24 h at 37°C. Commercial calf rennet and distilled water were used as positive and negative controls, respectively. Unexpectedly, the results showed that the partially purified enzyme of moringa seeds was incapable to digest egg white proteins, whereas, the commercial calf rennet digested egg white protein at lower rate (Fig. 6). The digestibility of egg white proteins by both enzymes (moringa seeds enzyme and calf rennet) was not visible in the test tubes incubated for 24 h. However, when removing the cubes of egg white proteins from the test tubes, the protein cubes that treated with calf rennet was fragile with many creaks and wholes, whereas, those incubated with either the partially purified enzyme or distilled water were still compact with no changes. Contradictory to this results, a milk-clotting cysteine protease from *Calotropis procera* (Procerain B) was found to digest cubes of egg white proteins and reducing their weight from 500 to 195.5 mg (**Singh & Dubey 2011**). The results of the current study indicated the inability of the partially purified enzyme from moringa seeds to digest egg white proteins. Although this phenomena narrowed the application of the new enzyme in food industry, it is however, increased the suitability of the enzyme to be used in cheese making as a new and specific rennet substitute. The specificity of this enzyme toward milk proteins was supported by its high ratio of milk-clotting to proteolytic activity (Table 2). The specificity of milk-clotting enzyme toward milk caseins and especially k-casein is a great determinant of the enzyme to be used in cheese making.
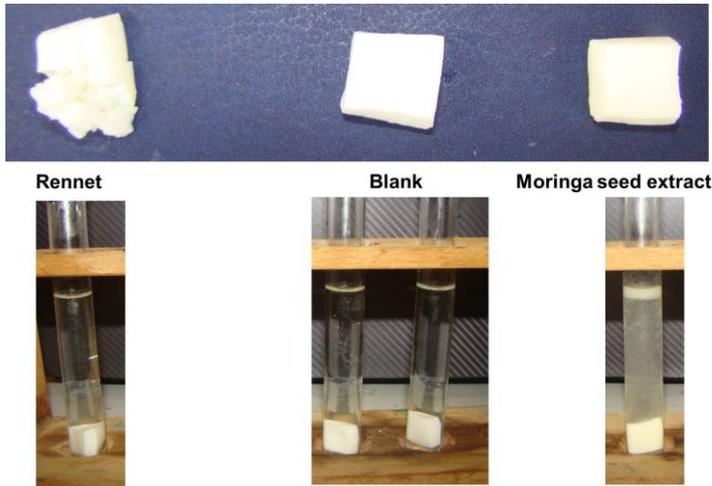
Figure 6 Digestion of egg white protein by partially purified milk-clotting enzyme from seeds of *Moringa oleifera* in comparison with calf rennet and the blank (with no enzyme).

CONCLUSION

To the best of our knowledge, this is the first report the partial purification of a milk-clotting enzyme from the seeds of *Moringa oleifera*. The simple and cheap procedure for partial purification of the enzyme applied in the current study, combined with the availability of the plant seeds, could possibly be used for large-scale production of the enzyme, allowing a broad study of its various aspects and hence probable applications. Moreover, the high specificity in terms of its high ratio of milk-clotting to proteolytic activity could pave the way for its uses in the cheese industry as alternative to calf rennet. Additional studies on the complete purification and characterization of this promising enzymes together with the intense evaluation of the quality of cheese curd produced by its action will shed more light into its commercial suitability.

REFERENCES

ANTAO, C.M. & MALCATA, F. X. 2005. Plant serine proteases: biochemical, physiological and molecular features. *Plant Physiology and Biochemistry*, 43, 637–650. http://dx.doi.org/10.1016/j.plaphy.2005.05.001

ANUSHA, R., SINGH, M. K. & BINDHU O.S. 2014. Characterization of potential milk coagulants from *Calotropis gigantean* plant parts and their hydrolytic pattern of bovine casein. *European Food Research and Technology*, 238, 997-1006. http://dx.doi.org/10.1007/s00217-014-2177-0

ARIMA, K., YA, J. & IWASAKI, S. 1970. Milk-clotting enzyme from *Mucor pusillus* var. Lindt. In: Methods in enzymology, (eds. Pearlman, E. G., and Lorand, L.). Academic Press, New York, pp. 446-459. ISBN: 978-0-12-181881-4

BEKA, R.G., KRIER, F., BOTQUIN, M., GUIAMA, V.D., DONN, P., LIBOUGA, D.G., MBOFUNG, C.M., DIMITROV, K., SLOMIANNY, M.-C., GUILLOCHON, D. & VERCAIGNE-MARKO, D. 2014. Characterisation of a milk-clotting extract from *Balanites aegyptiaca* fruit pulp. *International Dairy Journal*, 34, 25–31. http://dx.doi.org/10.1016/j.idairyj.2013.06.013

DUBEY, K.V. & JAGANNADHAM, V.M., 2003. Procerain, a stable cysteine protease from the latex of *Calotropis procera*. *Phytochemistry*, 62, 1057–1071. http://dx.doi.org/10.1016/S0031-9422(02)00676-3

EGITO A.S., GIRARDET, J.-M., LAGUNA, L. E., POIRSON, C., MOLLE, D., MICLO, L., HUMBERT, G. & GAILLARD, J. –L. 2006. Milk-clotting activity of enzyme extracts from sunflower and albizia seeds and specific hydrolysis of bovine k- casein. *International Dairy Journal*, 17, 816–825. http://dx.doi.org/10.1016/j.idairyj.2006.09.012

ELMAZAR M. M. E., EL-SAYED S. T. & AL-AZZOUNY R. A. 2012. Screening Some Local Egyptian Seeds Extract for Milk-Clotting Activity and Physicochemical Characterization of *Brassica Napus* Seed Extract. *Journal of Agriculture and Food Technology*, 2(2), 28-34.

Fahey, J. W. 2005. *Moringa oleifera*: A Review of the Medical Evidence for Its Nutritional, Therapeutic, and Prophylactic Properties. Part 1. Trees for Life.

FOX, P.F. & MCSWEENEY, P. L. H. 2004. Cheese: An Overview in Cheese: Chemistry, Physics and Microbiology, 3rd Edn. Chapman and Hall London. ISBN: 0-1226-3651-1

GUIAMA V. D., LIBOUGA D. G., NGAH E. & MBOFUNG C. M. 2010. Milk-clotting activity of berries extracts from nine *Solanum* plants. *African Journal of Biotechnology*, 9(25), 3911-3918.

HASHIM M. M., MINGSHENG D., IQBAL M. F. & XIAOHONG C. 2011. Ginger rhizome as a potential source of milk coagulating cysteine protease. *Phytochemistry*, 72, 458–464. http://dx.doi.org/10.1016/j.phytochem.2010.12.002

JACOB, M., JAROS, D. & ROHM, H. 2011. Recent advances in milk clotting enzymes. *International Journal of Dairy Technology*, 64(1), 14–33. http://dx.doi.org/10.1111/j.1471-0307.2010.00633.x

KUMARI, M., SHARMA, A. & JAGANNADHAM, M. V. 2012. Religiosin B, a milk-clotting serine protease from *Ficus religiosa*. *Food Chemistry*, 131, 1295-1303. http://dx.doi.org/10.1016/j.foodchem.2011.09.122

MACEDO, I. Q., FARO, C. J. & PIRES, E. M. 1996. Caseinolytic Specificity of Cardosin, an Aspartic Protease from the Cardoon *Cynara cardunculus* L.: Action on Bovine β- and α -Casein and Comparison with Chymosin. *Journal of Agricultural and Food Chemistry*, 44, 42-47. http://dx.doi.org/10.1021/jf9406929

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, 1902–1907. http://dx.doi.org/10.1016/j.foodchem.2013.05.042

MINE, Y. & ZHANG, H. 2012. Egg components in food systems. Biochemistry of Foods (3rd ed.), Academic Press, San Diego, pp. 215–241. ISBN: 978-0-12-242352-9

MOHAMED AHMED, I. A., MORISHIMA, I., BABIKER, E. E. & MORI, N. 2009a. Characterization of partially purified milk-clotting enzyme from *Solanum dubium* Fresen seeds. *Food Chemistry*, 116 (2), 395–400. http://dx.doi.org/10.1016/j.foodchem.2008.11.072

MOHAMED AHMED, I. A., MORISHIMA, I., BABIKER, E. E. & MORI, N. 2009b. Dubiumin, a chymotrypsin-like serine protease from the seeds of *Solanum dubium* Fresen. *Phytochemistry*, 70 (4), 483-491. http://dx.doi.org/10.1016/j.phytochem.2009.01.016

MOHAMED AHMED, I.A., BABIKER, E.E. & MORI N. 2010. pH stability and influence of salts on activity of a milk-clotting enzyme from *Solanum dubium* seeds and its enzymatic action on bovine caseins. *LWT of Food Science and Technology*, 43, 759-764. http://dx.doi.org/10.1016/j.lwt.2009.12.011

NAJERA, A. I., DE RENOBALES, M. & BARRON, L. J. R. 2003. Effects of pH, temperature, CaCl$_2$ and enzyme concentrations on the rennet-clotting properties of milk: a multifactorial study. *Food Chemistry*, 80, 345-352. http://dx.doi.org/10.1016/S0308-8146(02)00270-4

NAZ, S., MASUD, T. & NAWAZ, M. A. 2009. Characterization of milk coagulating properties from the extract of *Whithania coagulans*. *International Journal of Dairy Technology*, 62, 315–320. http://dx.doi.org/10.1111/j.1471-0307.2009.00492.x

NESTOR, G.-M., RUBI, C.-G. D. & HECTOR, J.-C. 2012. Exploring the Milk-Clotting Properties of a Plant Coagulant from the Berries of *S. elaeagnifolium* var. *Cavanilles*. *Journal of Food Science*, 71(1), C89-C94. http://dx.doi.org/10.1111/j.1750-3841.2011.02468.x

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, 1848–1854. http://dx.doi.org/10.1016/j.foodchem.2012.06.087

ROSEIRO, L. B., BARBOSA, M. M., AMES, J. & WILBEY, R., 2003. Cheese making with vegetable coagulants; the use of *Cynara cardunculus* L.for the production of ovine milk cheeses. *International Journal of Dairy Technology*, 56, 76 - 85. http://dx.doi.org/10.1046/j.1471-0307.2003.00080.x

SARATH, G., MOTTE, R. S. & WANGER, F. M. 1989. Protease assay methods. In: Beynon, R. J., Bond, J. S. (Eds), *Proteolytic enzyme- A practical approach*. IRL Press, Oxford, Pp. 25-55. ISBN: 0-19-963058-5

SHEHATA, A.E., FAYET, E. A., ISAMIL, A. A. & SALEM, M. M. 1996. Production and characterization of bacterial coagulants as calf rennet replace for Egyptian cheese making. *Egyptian Journal of Food Science*, 24 (3), 417-449.

SIDRACH, L., GARCIA-CANOVAS, F., TUDELA J. & RODRIGUEZ-LOPEZ, J. N. 2005. Purification of cynarases from artichoke (*Cynara scolymus* L.): enzymatic properties of cynarase A. *Phytochemistry*, 66, 41–49. http://dx.doi.org/10.1016/j.phytochem.2004.10.005

SINGH, A. N. & DUBEY, V. K. 2011. Exploring application of Procerain B, a novel protease from Calotropis procera, and characterization by N-terminal sequencing as well as peptide mass fingerprinting. *Applied Biochemistry and Biotechnology*, 164, 573-580. http://dx.doi.org/10.1007/s12010-011-9158-6

SOUSA, M. J., ARD, Y. & MCSWEENEY, P. L. H. 2001. Advances in the study of proteolysis during cheese ripening. *International Dairy Journal*, 11, 327-345. http://dx.doi.org/10.1016/S0958-6946(01)00062-0

VERISSIMO, P., ESTEVES, C., FARO, C. & PIRES, E. 1995. The vegetable rennet of *Cynara cardunculus* L. contains two proteinases with chymosin and pepsin-like specificities. *Biotechnology Letters*, 17, 614–645. http://dx.doi.org/10.1007/BF00129389

YOUSIF, B. H., MCMAHON, J. D. & SHAMMET, M. K., 1996. Milk-clotting Enzyme from *Solanum dobium* Plant. *International Dairy Journal*, 6, 637-644. http://dx.doi.org/10.1016/0958-6946(95)00025-9