Australian Journal of Basic and Applied Sciences, 7(1): 482-493, 2013
ISSN 1991-8178

Purification and Characterization of a Novel Milk-Clotting Enzyme from *Brassica napus* Seeds

[1]Sanaa T. El-Sayed, [2]Mohamed M.A. Elmazar, [1]Rehab A. Al-Azzouny

[1]Bio-chemistry, National Research Center, Egypt. [2]Ahram Canadian University.

Abstract: *Brassica napus* (rape) seeds were chosen as source for milk-clotting activity (MCA). The extract shows the most potent clotting activity (164 U/g dry seeds) with firm clotting with the minimum proteolytic activity at pH 4.5. The purification scheme for milk-clotting enzyme (MCE) included precipitation with $(NH_4)_2SO_4$ (20-60%- saturation) followed by chromatography columns on Sephadex G-100, and two successive Sephadex G-200 columns to give a pure enzyme with final specific activity 144 (U/mg), 22.2 purification fold. The purity of the purified enzyme was confirmed by Sodium dodecyl sulphate polyacrylamide gel electrophoresis showed a single band of 26 kDa. While the molecular masses of the purified enzyme was found to be 30 KDa by gel filtration technique. The purified enzyme has optimum activities in 0.1 M acetate or citrate buffer at pH range from 3.5 to 4.5 at 60 C˚. The milk-clotting activity increased slightly by preincubation of the enzyme with different buffers. The MCE should high heat stability up to 40 ˚C for 120 minute without loss in activity. The enzyme is dependent on calcium ions to perform its activity up to 0.44% and almost loss of activity with sodium chloride concentration above 3% w/v. Linear relationship (inversely proportional) was observed between milk substrate concentration and enzyme concentration.

Key words: *Brassica napus* (rape), milk-clotting enzyme, purification, physicochemical characterization, seeds.

INTRODUCTION

Calf rennet, which contains chymosin (EC 3.4.23.4) as the main enzyme component, has been widely used as a milk-clotting enzyme (Ahmed *et al.,* 2009). The reduced supply of calf rennet, calf diseases like bovine spongiform encephalopathy (BSE) has led to an increase in the demand for alternatives sources of milk coagulants (Roseiro *et al.,* 2003 and Cavalcanti *et al.,* 2004). Milk-clotting enzymes from different sources have been suggested. Fish and aquatic invertebrates as a source has been studied (Shahidi and Kamil, 2001).Various microbial alternatives are used for chymosin production *Rhizomucor pusillus* and *Cryphonectria parasitica* (Egitoa *et al.,* 2007), *Thermomucor indicae-seudaticae* N31 (Merheb-Dini *et al.,* 2009)], *Nocardiopsis* sp (Cavalcanti *et al.,* 2004), *Rhizomucor miehei* (Silveira and Contiero, 2001), *Penicillium oxalicum* (Hashem, 2000). Most of the companies produce recombinant rennet of cattle calf origin in different microbial hosts (Seker *et al.,* 1999 and Neelakantan *et al.,* 1999). Microbial rennet produced by genetically engineered bacteria has proven suitable substitutes for animal rennet, but increasing attention has been directed toward natural rennet extracted from plants (Ahmed *et al.,* 2009). The consumer constraints on the use of animal rennet for religious reasons (e.g., Judaism and Islam) as well as diet (vegetarianism), or consumer concern regarding genetically engineered foods (e.g., Germany, Netherlands and France forbid the use of recombinant calf rennet) have led to a growing interest in vegetable coagulants (Egitoa *et al.,* 2007). Plant coagulants includes enzymes from *Cynara cardunculus* (Louro Martins *et al.,* 1996 and Sousa and Malcata, 2002), seven *papilionoideae* species (*Eriosema shirense*, *E. ellipticum*, *E. pauciflorum*, *E. gossweilleri*, *E. psoraleoides*, *Adenolichos anchietae* and *Droogmansia megalantha* (Lopes *et al.,* 1998), *Solanum dobium fresen* (Ahmed *et al.,* 2009) and *Centaurea calcitrapa* (Reis *et al.,* 2000; Pintado *et al.,* 2001), *Cynara scolymus* (Sidrach *et al.,* 2005), *Calotropis procera* (Sanni, *et al.,* 1999), *Helianthus annuus* (Park *et al.,* 2000), *Lactuca sativa* (Lo Piero *et al.,* 2002), fig (*Ficus carica*), paw paw (*Carica papaya*), pineapple (*Ananas sativa*) and castor oil seeds (*Ricinus communis*) coagulate milk (Garg and Johri, 1994). In a previous study (Elmazar *et al.,* 2012), the extract of *Brassica napus* (rape) seeds show the most potent milk-clotting activity among the twenty eight plant seed members tested. In the present study, the purification and characterization of the milk-clotting enzyme from rape seeds was performed. The purification scheme to be adopted was simple, efficient and easy to scale up techniques. The purified milk-clotting enzyme shows some unique physicochemical characteristics that made the enzyme promising in cheese industry application.

Corresponding Author: Sanaa T. El-Sayed, National Research Center, Egypt,
E-mail: santsayed@yahoo.com

MATERIALS AND METHODS

Materials:

The *Brassica napus* (rape) dry seeds were brought from local markets. The dry skim milk powder was obtained from local market in Cairo. Soluble casein and L-Tyrosine were obtained from BDH, England.

Chemicals:

Sephadex G-100, Sephadex G-200, Diethyl aminoethyl cellulose (DEAE-cellulose), molecular weight markers and all resins and reagent for electrophoresis were obtained from Sigma Chemicals Co., St. Louis, USA and from Pharmacia Fine Chemicals, Sweden. Other chemicals were of analytical grade.

Methods:

***Preparation and Extraction of The Crude Enzyme*:**

The rape dry seeds were extracted as previously mentioned (Elmazar *et al.*, 2012). Briefly; seeds were crushed and mixed with dist. water at 9 °C with continuous shaking over a period of 12 hours. The resulted extract was then centrifuged at 3000 r.p.m for 15 minutes, and the supernatant was collected, dialyzed against dist. water and then used as the crude enzyme preparation.

Enzyme Assay:

Preparation of Milk-Clotting Substrate:

Skim milk was used as a substrate for the assay of milk-clotting activity. It was prepared according to Kawai and Mukai (1970) with slight modification. Twelve gm of skimmed milk powder were dissolved in 100 ml distilled water or in 0.1M acetate buffer, pH 4.5 containing 0.11 gm $CaCl_2$ (0.01M final concentration) and used as substrate for assaying milk-clotting activities.

Determination of Milk-Clotting Activity (MCA):

The prepared enzyme solutions were assayed for their ability to produce extracellular milk-clotting activity using the standard assay procedure as described by Berridge (1955) with slight modification. The reaction mixture contained 0.5 ml of enzyme solution was added to 2.0 ml of milk-clotting substrate solution already incubated at 37 °C. The time necessary for the formation of curd fragment was measured. One unit of enzyme activity was taken to be that which clotted 10ml. milk in 10 min. at 37^{o}C. The activity of milk-clotting enzyme was expressed in term of Otani units.

Calculated as follows: MCA units = (2400/T) x S/E

Where:

T = time (in sec) necessary for the curd fragment formation.

S = volume (in ml) of substrate (milk).

E = volume (in ml) of enzyme.

Estimation of Protein:

The protein concentration was determined by Lowry *et al.* (1951) method using bovine serum albumin as a standard.

Purification of the Crude MCE:

Fractional precipitation with acetone for MCE revealed unsuitability as precipitating agent due to the poor yield obtained relative to the crude enzyme (data not show). The crude enzyme was precipitated by using ammonium sulfate with different concentrations (0-20, 20-40, 40-60 and 60-80% saturation) of saturation according to the method of Green and Hughes (1955). Each fraction was obtained by centrifugation at 13,000g and 4°C for 15 min. The resulting precipitates were dissolved in appropriate amount of distilled water and dialyzed exhaustively against distilled water for 2 days at 4°C to get rid of the excess of ammonium sulfate. Undissolved protein was removed by centrifugation before enzyme assay. Enzyme activity and protein content were determined in each fraction.

Gel Filtration by Using Sephadex G-100:

The dialyzed ammonium sulfate fraction with high MCA was concentrated by lyophilization and then applied to Sephadex G-100 column (3.2 x 35 cm) previously equilibrated with 0.01M phosphate buffer pH 7.0 The protein was eluted with the same buffer at a flow rate of 15 ml/hr. The most active fractions were collected and concentrated by lyophilization.

Ion Exchange Using DEAE-Cellulose:

The concentrated active fraction was applied directly on the top of the column (2 x 18 cm) of pre-activated DEAE-cellulose equilibrated with 0.01M phosphate buffer pH 7.0. Elution was carried out using the same buffer at a flow rate of 30ml/h, with a linear gradient of NaCl (0.1-0.5M). Fractions of 5ml were collected at the elution rate 30 ml/hr. The eluted fractions were dialyzed against water for 48 h at 4 °C and monitored at 280 nm for protein and assayed for enzyme activity and protein content. Poor yield of activity was obtained and this step was ignored (data not show).

Gel Filtration by Using Sephadex G-200:

The concentrated dialyzed active fractions of enzyme from Sephadex G-100 column were loaded to the top of two Sephadex G-200 column successively (1.6 x 55 cm) previously equilibrated with 0.01 M phosphate buffer pH 7.0. The protein was eluted with the same buffer at a flow rate of 15 ml/hr.

Physicochemical Properties of The Purified MCE:

Molecular Weight Determination by Gel Filtration:

The molecular weights of the enzyme were determined by gel filtration on Sephadex G-200 column (1x 37 cm) as described by Andrew (1970). The column was packed with Sephadex G-200 and pre-equilibrated with 0.01 M sodium borate buffer, pH 8.5. Bovine serum albumin (66,000), trypsin from porcine (33,000), carbonic anhydrase from bovine erythrocytes (29,000), trypsin from soybean inhibitor (20,000) and lysozyme (14,200) beside the purified MCE were applied to the top of the column at rate 16 ml/hr. The protein concentration in each fraction was determined. Then, the curve was constructed between elution volume and log molecular weights.

Molecular Weight Determination by Soduim Dodecyl Sulphate (SDS) Polyacrylamide Gel Electrophoresis (PAGE):

The molecular weight for homogeneous preparation was determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (Laemmli, 1970).

Effect of pH Value on The Activity and Stability of The Purified MCE:

Small aliquots of the purified enzyme were assayed with three buffering systems, namely acetate (0.1 M, pH 3.5-5.5), citrate-phosphate (0.1 M, pH 2.5-6.5) and phosphate buffer (0.1 M, pH 4.5-8.0). In case of the effect of pH on stability of the enzyme, small aliquots of the purified enzyme were stored with the three previous buffering systems at 5 °C for 30 min before testing the enzymatic activities, then relative activities were calculated.

Effect of Temperature on The Activity and Stability of The Purified MCE:

The maximum activity of the tested enzyme was determined at different temperatures ranged from 30 to 65 °C. Small aliquots of the purified enzyme were preheated at different temperatures (30-80 °C) for time intervals from 15 to 120 min. The remaining enzymatic activities were then assayed using the standard assay conditions.

Results:

Purification of The Milk-Clotting Enzyme (MCE) From Brassica napus Seeds:

Partial purification of the crude enzyme was carried out by fractional precipitation using ammonium sulfate fractionation as an initial step of purification; about 89.6% of the total enzyme activity was recovered in the fraction of 20-60% saturation (1.34 fold purification). The ammonium sulfate fraction (20-60% saturation) was applied on Sephadex G-100 column (Fig. 1). Three protein peaks were obtained only one have high MCA was obtained. It has 30.1 U/mg proteins with a yield of about 36.6%. Further purification of the pooled active fractions was concentrated by lyophilization and applied on two successive Sephadex G–200 column (Fig. 2&3). The data in figure (3) reveal that a single peak having high MCA which also indicates its purity and homogeneity in the final preparation. Data of purification was summarized in table (1). The purified MCE has purification folds of 22.2 times, with 19 % recovery and high specific activities of 144 U/mg.

Physicochemical Properties of The Purified MCE:

The purified MCE was subjected to study its physicochemical properties. As a general rule, most experiments were carried out in triplicate.

Table 1: Purification steps and yields recovery of MCE from *Brassica napus* seeds (10 gm dry seeds).

Purification steps	Total MCA (Otani units)	Total protein (mg/ml)	Specific activity (U/mg)	Yield recovery (%)	Purification fold
Crude	1462 ± 2	224.9 ± 0.03	6.5	100	1
Ammonium sulphate fraction (20-60%)	1312 ± 3.8	151.4 ± 3.2	8.7	89.6	1.34
Sephadex G-100 column	536 ± 3.05	17.8 ± 0.15	30.1	36.6	4.63
Sephadex G-200 column (First column)	299 ± 0.57	4.5 ± 0.08	66.0	20.5	10.2
Sephadex G-200 column (Second column)	278± 0.5	1.93± 0.08	144	19.0	22.2

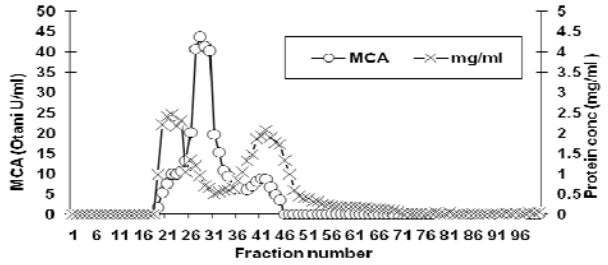

Fig. 1: Typical elution profile of MCE on Sephadex G-100.

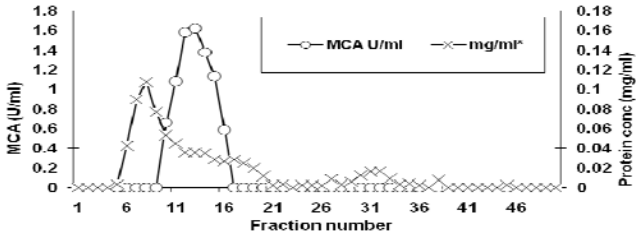

Fig. 2: Typical elution profile of MCE on Sephadex G-200 (First column).

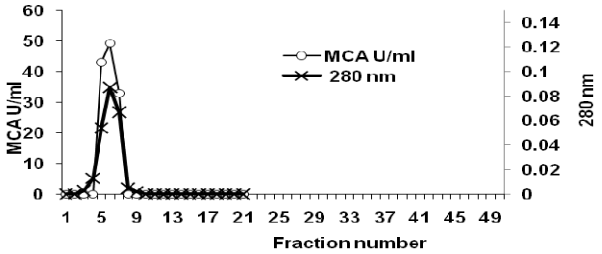

Fig. 3: Typical elution profile of MCE on Sephadex G-200 (Second column).

Determination of The Molecular Weight of The Purified MCE by Gel Filtration on Sephadex G-200 Column:

The molecular weight of purified MCE was determined using the gel filtration technique using different standard proteins with known molecular weights. The molecular weight of MCE was 30 KDa (Figure 4).

Determination of The Molecular Weight of The Purified MCE by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE):

The electrophoretic behaviour of MCE on polyacrylamide gel electrophoresis (PAGE-SDS) is shown in figure (5). Only one single band was detected which confirmed their homogeneity. The molecular weight of the purified enzyme was estimated to be 26 KDa (Figure 5).

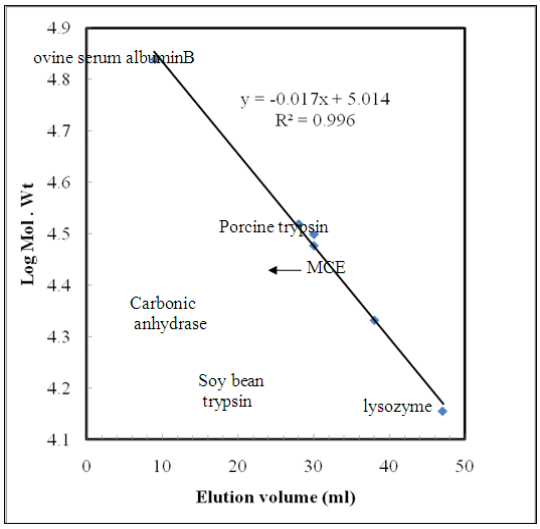

Fig. 4: Determination of molecular weight of MCE by gel filteration on Sephadex G-200 (1× 37cm), using the five denoted protein markers.

Effect of pH Value on The Activity and Stability of The Purified MCE:

Figure (6 & 7) shows both the effect of different pH on MCA and MCE stability. At pH value higher than 6.0, the MCA was completely inactivated and the enzyme failed to clot the milk in the reaction mixture. Maximum MCA was found at pH from 3.5 to 4.5. The MCA was increased slightly by preincubation of the enzyme with different buffers.

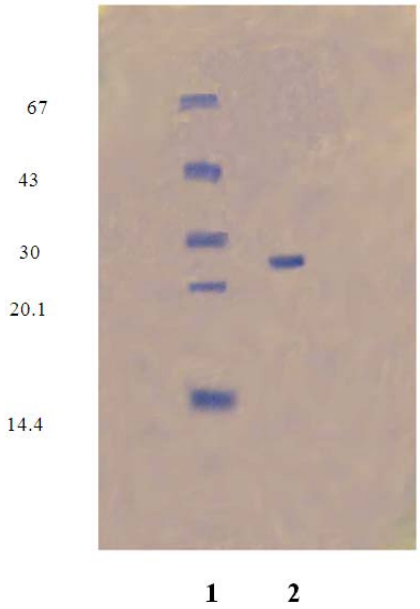

Fig. 5: SDS-PAGE of the purified MCE from Brassica napus seeds.
Lane 1: Standard proteins, bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and a lactalbumin (14.4 kDa).
Lane 2: Purified MCE

Effect of Temperature on The Activity and on the Stability of the Purified MCE:

The activity of the purified milk-clotting enzyme was determined at different reaction temperatures between 30 to 65 ºC under standard reaction conditions (Figure 8). The MCA increased progressively with the incubation temperature reaching the highest MCA value at 60 ºC. At higher incubation temperatures, total loss of MCA was found. Figure (9) shows the MCA of the purified enzyme after heating in absence of substrate for different

times from 20 to 120 minute in a water bath set at different temperatures (30-80 ºC). It is clear that the purified enzyme could stand heating up to 40 ºC for 120 minute without apparent loss of activity (only 5% loss of its activity). However, heating the enzyme at 60 ºC reduced the activity to only 8%, whereas total denaturation of the enzyme was evident at higher temperatures (70 ºC). It is of interest to note that in the presence of substrate the enzyme activity increased up to 60 ºC due to the protective effect of the substrate (skimmed-milk) on enzyme activity. When compared to the thermal liability of the purified enzyme in absence of substrate when heated alone at 60 ºC for 20 minutes.

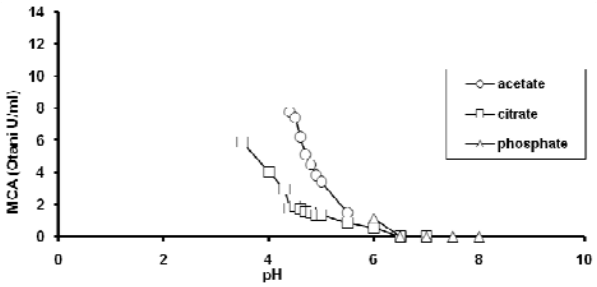

Fig. 6: Effect of pH value on the activity of the purified MCE.

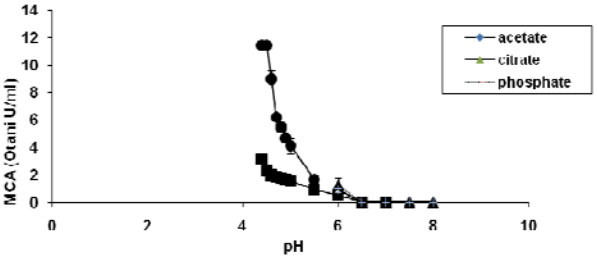

Fig. 7: Effect of pH value on the stability of the purified MCE.

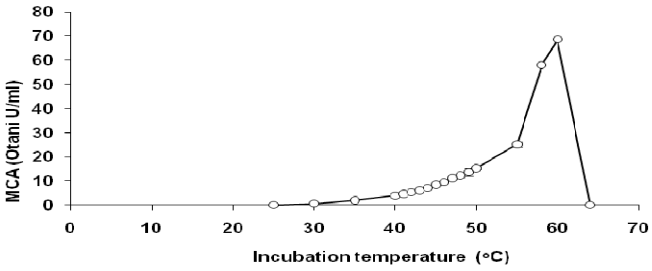

Fig. 8: Effect of incubation temperature on the activity of the purified MCE.

Effect of Enzyme Concentration on The MCA:

The concentration of the purified enzyme ranging from 0.0153-0.153 µg/reaction mixture was used in standard reaction mixtures and the MCA was determined (Figure 10). The MCA was increased proportionally and in strict linear relation with enzyme concentration up to 0.137 µ/ml.

Effect of Skim Milk Concentration on MCA of The Purified Enzyme:

The effect of increasing the concentration of skim dry milk from 12 to 30% (w/v) on MCA of the purified enzyme is shown in figure (11). Milk at 12% (w/v) concentration and 50 ºC is stable and less than 12% (w/v) produced no clotting activity.

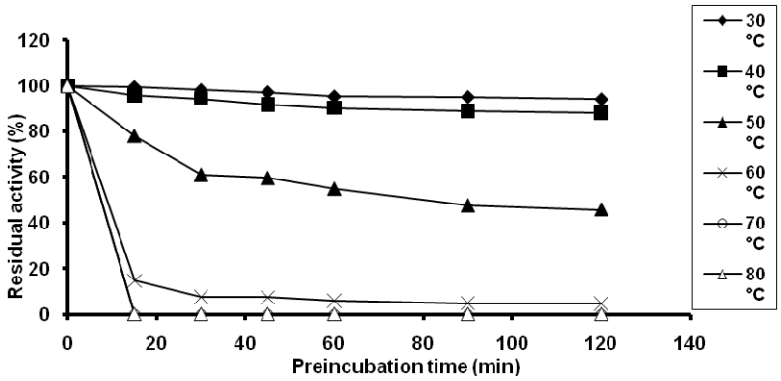

Fig. 9: Thermal stability of the puified MCE. The enzyme was heated at the indicated temperatures for different times and rapidly cooled under running water before enzyme assay was carried ou under standard conditions.

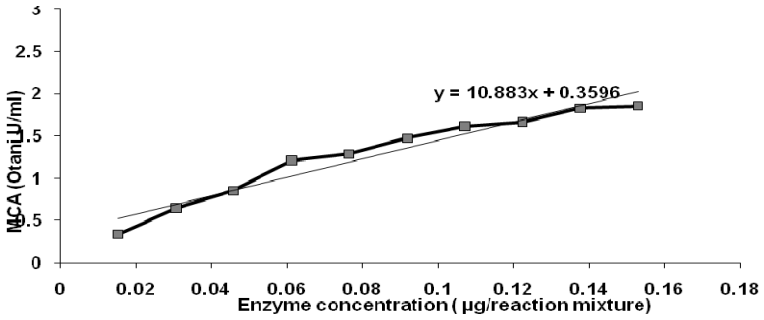

Fig. 10: Effcet of enzyme concentration on the activity of the purified MCE.

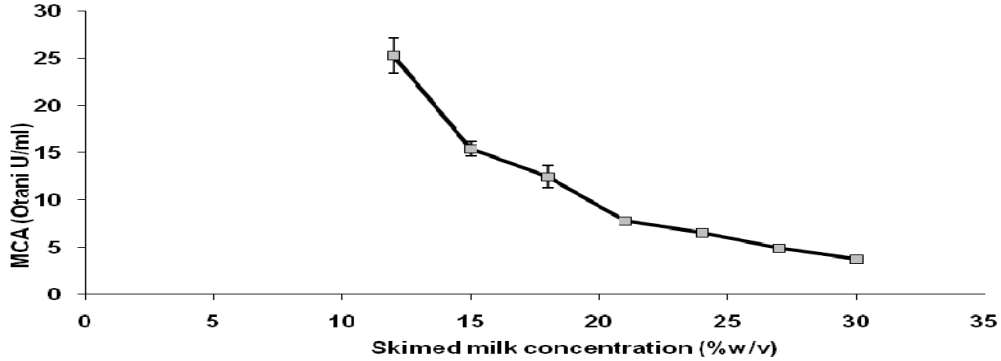

Fig. 11: Effect of substrate concentration on the activity of the purified MCE.
Note: Milk at 12% concentration is stable at assay temperature (50^{o}C), less than 12% blank quicky disturbed.

Effect of Calcium Chloride Concentration on MCA of the Purified Enzyme:

In some processes during the manufacture of some types of cheese, appropriate concentrations of $CaCl_2$ may be added. To study this effect, $CaCl_2$ was added in different concentrations from 0.03 to 0.44%. The clotting activity of the enzyme was greatly stimulated from 7 to 25 µ/ml with addition of $CaCl_2$ in a linear relationship (Figure 12).

Effect of Addition NaCl on MCA of the Purified Enzyme:

Sodium chloride is usually used during the processes of Domiati cheese manufactured in Egypt. Figure (13) results indicate an apparent progressive inhibition of the MCA from 11.0 to near inactivity with the increase of the concentration of sodium chloride.

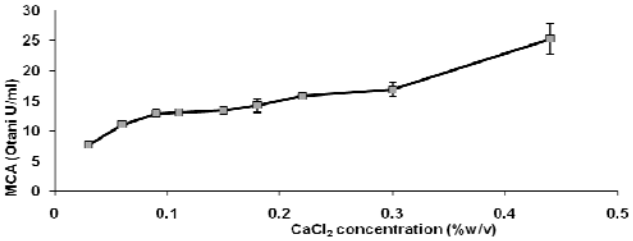

Fig. 12: Effect of calcium chloride concentration on the activity of the purified MCE.

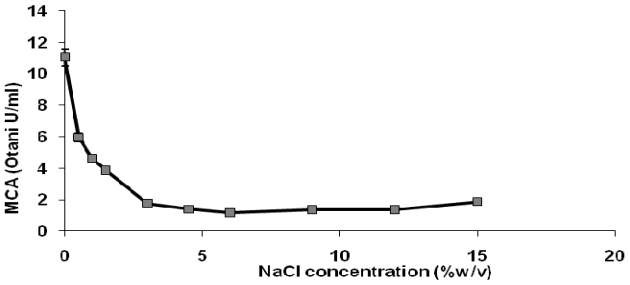

Fig. 13: Effect of sodium chloride concentration on the activity of the purified MCE.

UV Spectrum:

The ultraviolet absorbency profile and the maximum absorbency of the purified enzyme was determined by using wave lengths range from 240-350nm in 0.01 M phosphate buffer, pH 6.8 using CECIL instrument CE595 U.V. spectrophotometer. The purified enzyme exhibited a maximum absorbance at 279 nm. No significant absorbance could be traced in the visible region indicating the absence of chromophore. The ratio of absorbance at 280 to 260 nm was 1.3.

Effect of Activators and Inhibitors on The activity of MCE:

This effect was done by preincubation of some cations; Co2+, Pb2+, Mg2+, Cu2+, Mn2+ and K+ with the purified enzyme at concentration of 1.0 mM for 30 min and 12 hours at 9 °C before determination of the residual enzyme activity. It was clear that great activation of the clotting process was obtained with Pb^{2+} after 30 minutes preincubation and with Co^{2+}, Mg^{2+}, Cu^{2+}, K^{+} and Mn^{2+} after 12 hours at 9 ºC preincubation with the purified enzyme.

Discussion:

Previously, it was reported that *Brassica napus* seeds extract shows a potent milk-clotting activity in comparison with 28 plant seeds extract (Elmazar *et al.,* 2012). It is a member of the family *Cruciferae*. With increasing reports on extraction of milk-clotting enzymes from plants, it was noted to be family related (Lopes *et al*., 1998 and Llorente *et al*., 2004). The seeds were chosen as the target plant tissue to be studied as they contain high amounts of storage protein and hence a high percentage of proteolytic activity (Muntz, 1996). In this study, purification of the milk-clotting enzyme was adopted using a simple and efficient 3 steps. Precipitation with ammonium sulphate is an effective way to produce substantial amounts of active proteinases (Raposo and Domingos, 2008). The step of precipitation with ammonium sulphate helps removal of several non-protein components including the polyphenols that have absorbance at 280 nm (Devaraj *et al.,* 2008). The MCE from *Brassica napus* appears to be labile to organic solvents where a total loss of the activity occurred when the acetone concentration increased to 60%. Some enzymes reported a great loss upon acetone precipitation (Krishnapillai, *et al.,* 1999). A loss of protease activity and non adsorption on DEAE-cellulose have been previously reported (Wang and Ng, 2001). Most probably because of different pH or ionic strength that alters the net charge of the enzyme (Illanes, 2008). Size exclusion chromatography using Sephadex G-100 followed by Sephadex G-200 was used because it is non-detrimental and safe for enzymatic purification as no use of organic solvent or high ionic strength that might interfere with the clotting-activity and hence mask the elution of this targeted protease. Also gel filtration chromatography is cost effective with minimum chemical residues, therefore safer for food or drug application [(Aehle, 2007). Proteins eluted from Sephadex G-100 were resolved into three fractions. Fraction two that exhibited maximum milk-clotting activity was pooled separately with 5 fold purity than the applied ammonium sulphate fraction. The eluted peak was then applied on the first Sephadex G-200 column showing a single peak of milk-clotting and proteolytic activity with a final purification

of 10.2 fold. It is noted that the final pure enzyme shows a proteolytic activity at acidic pH with an R value = 14.8 (the ratio between milk-clotting to proteolytic activity) that was probably masked in the crude extract by the protein or non protein contaminant present. Upon application on the second Sephadex G200 column a final single pure peak with 22.2 purification fold was finally collected for the homogeneity studies. Although, the extraction of milk clotting proteinases from the intact plant requires labor –intensive procedures (Raposo and domingos, 2008) , the 3 steps adopted in this study was able to purify the enzyme using a simple purification steps in an extra pure form at the final step. The rape seed extract maintained a potent milk-clotting activity during the steps of purification; this allows the possibility of enzyme application in an excellent cost benefit as in food industry with minimal required purity or in the analytical application in pharmaceutical industry with higher purity recommendation. The purity of the extracted enzyme was confirmed by having a single peak of protein eluted from Sephadex G-200 which coincides with the activity peak of the enzyme. SDS-polyacrylamide gel electrophoresis of the extracted enzyme showed a single band of 26KDa.

The purified protease shows a limited pH range for activity and stability. The activity was mostly in pH range from 3.5 to 5 where it retains about 25% of the activity at pH 5 while a total loss was found after pH 6. The molecular weight determined by both size exclusion and SDS-gel electrophoresis shows, in addition to the results of the pH optimum, that the enzyme belongs to the class acidic protease. Sumantha, *et al*., (2006) reported that the acidic proteases molecular weight usually lies between 30-40 KDa with an acidic pH optimum at 2-5.

In agreement with previous findings, reducing the milk pH results in a significant decrease in clotting time (Awad, 2007). Several authors have reported a similar decline in activity near neutrality (Castillo, *et al*., 2000 and Chazara *et al*., 2007). A newly isolated milk-clotting enzyme from *Thermomucor indicae-seudaticae* (Merheb-Dini, *et al*., 2009) found the same steep decrease in activity upon raising the pH till a total loss at pH 7. Reports for milk clotting enzymes isolated from plants as *cyanara scolymus* L. flower showed a loss of activity of 87% at pH 7 (Chazara *et al*., 2007). Chymosin shows maximum activity at pH 5.5 and loss of activity at pH 7 (Foltmann, 1970 and Mohanty, *et al*., 2003).

This is of great advantage for industrial application as the enzyme shows a high specificity of action in only the acidic range with an activity that could be easily controlled in the industrial application as designed. Its stability that is also lost after pH 6 is of special interest for food application especially in cheese industry as this controls the unwanted proteolytic activity during ripening process in certain cheese varieties (Rao et al., 1998). For an application of a milk-clotting enzyme as an efficient rennet substitutes, the enzyme must not be more heat stable than animal rennet (Merheb-Dini et al., 2009). This is because the residual activity remaining after heating the curd is responsible for the bitterness and the uncontrolled proteolytic activity upon curd storage (Silva and Malcata, 2005). Cattle chymosin is stable up to 50 ºC and lost its activity at 60 ºC. Buffalo chymosin is stable up to 55 ºC and at 60ºC the relative milk clotting activity was 50% and calf chymosin became inactive at temperature above 56 ºC (Mohanty *et al. ,* 2003 ; Kumar *et al.,* 2006 and Merheb-Dini *et al.,* 2009). The milk-clotting protease from the flower of Cyanara cradunculus is among the famous and traditional plant source used for cheese production in Portugal, exhibit similar pattern of the purified protease from Brassica napus where optimum temperature was 52 ºC and total enzyme loss of activity was above 60 ºC (Raposo and Domingos, 2008). This predicts a possible efficiency for the purified protease as an alternative to the animal chymosin in its different application in food or industry. For an application of a milk-clotting enzyme as an efficient rennet substitutes, the enzyme must not be more heat stable than animal rennet. Milk-clotting activity is dependent on the enzyme concentration. Different models were reported to relate this relation. The purified protease although shows a linear relationships between 1/enzyme concentration2 and clotting time in Hyslop *et al.,* model (1979), it shows a best fit with linear regression of 0.99 in Van Hooydonk and walstra model (1987) (data not shown).

It is noteworthy that the model that relates clotting time with the reciprocal enzyme concentration was also the best fit for animal chymosin (Verissimo et al., 1995). As the skim milk concentration increases, the clotting time and hence clotting activity decreases. This would be attributed to the less available water for enzyme to perform its action on casein. The more solutes are dissolved the less water activity or free water available and hence less enzymatic action. The milk clotting time decreases with increasing enzyme concentration (Chitipinityol and Crabbe, 1998). The increase in enzyme concentration increases the rate of κ-casein proteolysis (Lopes *et al.,* 1998). The higher the κ-casein hydrolysis (more enzymatic specificity) the firmness the gel produced which is in agreement to the results of the present study. Calcium ion in the reaction mixture was decrease the rennet clotting time (Montilla *et al.,* 1995; Balcones *et al.,* 1996 and Kumar *et al.,* 2006). Addition of calcium improved curd firmness, gel strength, aggregation rate and adhesiveness (Patel and Reuter, 1986). The purified enzyme shows a steep increase in activity with increasing calcium concentration this means the enzyme is dependent on calcium ions to perform its activity. Also this is in favour for rennet-gelled micro-encapsulation since the addition of calcium chloride could be of usefulness for the tailor control of the aggregate size (Karlsson et al., 2007). Addition of sodium chloride was reported to decrease the rate of the enzymatic reaction and also the coagulation of renneted micells (Zoon et al., 1989). The purified enzyme shows a strong inhibition and steep decrease to 4% w/v sodium chloride addition. The enzymatic reaction shows a plateau at

higher concentration, this could be of benefit for certain types of cheese during their ripening as no full inactivation is performed but a slight proteolytic activity can be still maintained for required targeted organoleptic characteristics. The purified enzymes appeared unaffected by different cations which favor the use in pharmaceutical or industrial application for proteolytic activity in presence of heavy metal impurities or in sewage disposal.

In this report, an industrially promising milk-clotting enzyme from plant origin was purified and its physicochemical characteristics were determined.

Conclusion:

The present basic study indicates that rape seeds are considered an economic and available source of milk-clotting enzyme that could be possibly utilized industrially. The use of seed part of the plant allows the continuous availability for industry. The purification procedures described in this study were simple, inexpensive and gave a final homogenous enzyme with high specific activity. The purified milk-clotting enzyme shows a unique physicochemical characteristics that could be promising for different industrial applications. The enzyme shows a high specificity of action in only the acidic range, the optimum temperature was 60 ºC which favor its use as rennet-substitute where a successful one must not be more heat stable than animal rennet, the enzyme is dependent on calcium ions to perform its activity. It is highly recommended to apply the described procedure extraction and purification of milk-clotting enzyme on industrial scale to be used in the food and pharmaceutical fields.

REFERENCES

Aehle, W., 2007. Enzymes in Industry; Production and Applications. WILEY-VCH, Weinheim.

Ahmed, I.A.M., I. Morishima, E.E. Babiker and N. Mori, 2009. Characterisation of partially purified milk-clotting enzyme from *Solanum dubium* Fresen seeds. Food Chem., 116: 395-400.

Andrews, P., 1970. Estimation of molecular size and molecular weights of biological compounds by gel filtration. Method Biochem Anal., 18: 1-53.

Awad, S., 2007. Effect of sodium chloride and pH on the rennet coagulation and gel firmness. LWT- Food Science and Technology, 40: 220-224.

Balcones, E., A. Olano and M.M. Calvo, 1996. Factors affecting the rennet clotting properties of ewe's milk. J Agr. Food Chem., 44: 1993-1996.

Berridge, N.J., 1955. Purification and assay of rennin. Method Enzymol., 2: 69-77.

Castillo, M., F.A. Payne, C.I. Hicks and M.B. Lopez, 2000. Predicting cutting and clotting time of coagulating goat's milk using diffuse reflectance: effect of pH, temperature and enzyme concentration. International Dairy Journal, 10: 551-562.

Cavalcanti, M.T.H., M.F.S. Teixeira, J.L. Lima Filho and A.L.F. Porto, 2004. Partial purification of new milk-clotting enzyme produced by *Nocardiopsis* sp. Bioresource Technol., 93: 29-35.

Chazara, S., L. Sidrach, D. Lo´pez-Molina and J.N. Rodrı´guez-Lo´pez, 2007. Characterization of the milk-clotting properties of extracts from artichoke (Cynara scolymus, L.) flowers. International Dairy Journal, 17: 1393-1400.

Chitipinityol, S. and M.J.C. Crabbe, 1998. Chymosin and aspartic proteinases. Food Chemistry, 61: 395-418.

Devaraj, K.B., L.R. Gowda and V. Prakash, 2008. An unusual thermostable aspartic protease from the latex of Ficus racemosa (L.). Phytochemistry, 69: 647-655.

Egitoa, A.S., J.M. Girardetc, L.E. Lagunaa, C. Poirsonc, D. Molle´b, L. Micloc, G. Humbertc and J.L. Gaillardc, 2007. Milk-clotting activity of enzyme extracts from sunflower and albizia seeds and specific hydrolysis of bovine k-casein. Int Dairy J., 17: 816-825.

Elmazar, M.M., S.T. El-sayed and R.A. Al-Azzouny, 2012. Screening some local Egyptian seeds extract for milk-clotting activity and physicochemical characterization of *Brassica napus* seed extract. J. Agric. Food. Tech., 2: 28-34.

Foltmann, B., 1970. Prochymosin and chymosin (Prorennin and rennin). Methods in Enzymlogy, 19: 421-436.

Garg, S.K. and B.N. Johri, 1994. Rennet: current trends and future research. Food Rev Int., 10: 313-355.

Green, A.A. and W.L. Hughes, 1955. Protein fractionation on the basis of solubility in aqueous solutions of salts and organic solvents. Meth. Enzymol., 1: 67-90.

Hashem, A.M., 2000. Purification and properties of a milk-clotting enzyme produced by *Penicillium oxalicum*. Bioresource Technol., 75: 219-222.

Hyslop, D.B., T. Richardson and D.S. Ryan, 1979. Kinetics of pepsin-initiated coagulation of kappa casein. Biochim Biophys Acta, 9: 390-396.

Illanes, A., 2008. Enzyme Biocatalysis: Principles and Applications, third edition. Springer Science.

Morris, K.D.A. and P.C. Quantick, 1999. Extraction and purification of hyaluronoglucosidase (EC 3.2.1.35) from Norway lobster (Nephrops norvegicus). Food chemistry, 65: 359-365.

Karlsson, A.O., R. Ipsen and Y. Ardö, 2007. Rheological properties and microstructure during rennet induced coagulation of UF concentrated skim milk. Int Dairy J., 17: 674-682.

Kawai, M. and N. Mukai, 1970. Studies on milk clotting enzymes, produced by Basidiomycetes. Agr Biol Chem Tokyo, 34: 159-163.

Kumar, A., J. Sharma, A.K. Mohanty, S. Grover and V.K. Batish, 2006. Purification and characterization of milk clotting enzyme from goat (*Capra hircus*). Comp Biochem Phys., B.145: 108-113.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227: 680-685.

Llorente, B.E., C.B. Brutti and B.O. Caffini, 2004. Purification and characterization of a milk-clotting aspartic proteinase from Globe Artichoke (Cynara scolymus L.). Journal of Agricultural and Food Chemistry, 52: 8182-8189.

Lo Piero, A.R., I. Puglisi and G. Petrone, 2002. Characterization of ''Lettucine'', a serine-like protease from *Lactuca sativa* leaves, as a novel enzyme for milk clotting. J Agr Food Chem., 50: 2439-2443.

Lopes, A., G. Teixerira, M.C. Liberato, M.S. Pais and A. Clemente, 1998. New vegetal sources for milk clotting enzymes. J Mol Catal B-Enzym., 5: 63-68.

Louro Martins, A.P., M.M. Pestana de Vasconcelos and R.B. De Sousa, 1996. Thistle (*Cynara cardunculus* L) flower as a coagulant agent for cheese making. Short characterization. Lait., 76: 473-477.

Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall, 1951. Protein measurement with Folin Phenol reagent. J Biol Chem., 193: 265-275.

Merheb-Dini, C., E. Gomes, M. Boscolo and R. da Silva, 2009. Production and characterization of a milk-clotting protease in the crude enzymatic extract from the newly isolated Thermomucor indicae-seudaticae N31. Food Chem., 120(1): 87-93.

Mohanty, A.K., U.K. Mukhopadhyay, J.K.K. Aushik, S. Grover and V.K. Batish, 2003. Isolation, purification and characterization of chymosin from riverine buffalo (Bubalos bubalis). J Dairy Res., 70: 37-43.

Montilla, A., E. Balcones, A. Olano and M.M. Calvo, 1995. Influence of heat treatments on whey protein denaturation and rennet clotting properties of cow's and goat's milk. J Agr Food Chem., 43: 1908-1911.

Muntz, K., 1996. Proteases and proteolytic cleavage of storage proteins in developing and germinating dicotyledonous seeds. Journal of Experimental Botany, 47: 605-622.

Neelakantan, S., A.K. Mohanty and J.K. Kaushik, 1999. Production and use of microbial enzymes for dairy processing: a review. Curr Sci India, 77: 143-8.

Park, H., N. Yamanaka, A. Mikkonen, I. Kusakabe and H. Kobayashi, 2000. Purification and characterization of aspartic proteinase from sunflower seeds. Biosci Biotech Bioch., 64: 931-939.

Patel, R.S. and H. Reuter, 1986. Effect of sodium, calcium and phosphate on properties of rennet coagulated milk. Lebensm Wiss Technol., 19: 288-291.

Pintado, A.I., A.C. Macedo, G. Teixeira, M.S. Pais, A. Clemente and F.X. Malcata, 2001. Caseinolytic activity of fruit extract from Opuntia ficus-indica on bovine, caprine and ovine sodium caseinates. Biotechnol Progr., 17: 643-646.

Rao, M.B., A.M. Tanksale, M.S. Ghatge and V.V. Deshpande, 1998. Molecular and biotechnological aspects of microbial proteases. Microbiol Mol Biol R., 62: 597-635.

Raposo, S. and A. Domingos, 2008. Purification and characterization milk-clotting aspartic proteinases from Centaurea calcitrapa cell suspension cultures. Process Biochem., 43: 139-144.

Reis, P.M., P.L. Lourenco, A. Domingos, A.F. Clemente, M.S. Pais and F.X. Malcata, 2000. Applicability of extracts from *Centaureae calcitrapa* in ripening of bovine cheese. Int Dairy J., 10: 775-780.

Roseiro, L.B., M.M. Barbosa, J. Ames and R. Wilbey, 2003. Cheese making with vegetable coagulants; the use of Cynara L. for the production of ovine milk cheeses. Int J Dairy Technol., 56: 76-85.

Sanni, A.I., A.A. Onilude and M.O. Momoh, 1999. Selection of starters and a starter-mediated novel procedure for production of wara, aWest African soft cheese. Int J Food Sci Tech., 34: 325-333.

Seker, S., H. Beyenal and A. Tanyolac, 1999. Modeling milk clotting activity in the continuous production of microbial rennet from Mucor miehei. J Food Sci., 64: 525-529.

Shahidi, F. and Y.V.A. Janak Kamil, 2001. Enzymes from fish and aquatic invertebrates and their application in the food industry. Trends Food Sci Tech., 12: 435-464.

Sidrach, L., F. Garcıa-Canovas, J. Tudela and J. N. Rodrıguez-Lopez, 2005. Purification of cynarases from artichoke (*Cynara scolymus* L.): Enzymatic properties of cynarase A. Phytochemistry, 66: 41-49.

Silva, S.V. and F.X. Malcata, 2005. Partial identification of water soluble peptides released at early stages of proteolysis in sterilized ovine cheese-like systems: Influence of type of coagulant and starter. J Dairy Sci., 88: 1947-1954.

Silveira, G.G. and J. Contiero, 2001. Production of microbial rennin from Mucor miehei in batch fermentation. Eur J Pharm Sc., 13: 38.

Sousa, M.J. and F.X. Malcata, 2002. Advances in the role of a plant coagulant (*Cynara cardunculus*) in vitro and during ripening of cheeses from several milk species. Lait, 82(2): 151-170.

Sumantha, A., C. Larroche and A. Pandey, 2006. Microbiology and industrial biotechnology of food-grade proteases: A perspective. Food Technology and Biotechnology, 44: 211-220.

Van Hooydonk, A.C.M. and P. Walstra, 1987. Interpretation of the kinetics of the renneting reaction in milk. Neth Milk Dairy J., 41: 19-47.

Verissimo, P., C. Esteves, C. Faro and E. Pires, 1995. The vegetable rennet of *Cynara cardunculus* L. contains two proteinases with chymosin and pepsin-like specificities. Biotechnol Lett., 17: 621-626.

Wang, H. and T.B. Ng, 2001. Pleureryn, a novel protease from fresh fruiting bodies of the edible mushroom pleurotus eryngii. Biochemical and biophysical research communications, 289: 750-755.

Zoon, P., T. Van Vliet and P. Walstra.1989. Rheological properties of rennet-induced skim milk gels. Neth Milk Dairy J., 43: 17-34.