International Journal of
Dairy Science

ISSN 1811-9743

Academic
Journals Inc.

www.academicjournals.com

OPEN ACCESS International Journal of Dairy Science

ISSN 1811-9743
DOI: 10.3923/ijds.2017.254.265

Research Article
Purification and Characterization of Milk Clotting Enzyme from Artichoke (*Cynara cardunculus* L.) Flowers as Coagulant on White Soft Cheese

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Abstract

Background and Objective: Global increase in cheese production with decreased supply of calf rennet is leading to search for coagulant alternatives from available resources such as plant extracts. Therefore, the main objective of this study was to extract, purify and characterize Milk Clotting Enzyme (MCE) from artichoke (*Cynara cardunculus* L. ssp. *scolymus*) violet flowers and the effect of artichoke crude and purified MCE as a coagulant on chemical, texture profile and sensory properties of white soft cheese compared to calf and microbial rennets during cheese storage were studied to determine the technological suitability of vegetable coagulant for cheese manufacturing. **Materials and Methods:** The MCE was extracted and purified from artichoke violet flowers. White soft cheese coagulated with artichoke crude extract (CMCE) and Purified Milk Clotting Enzyme (PMCE) compared to Liquid Calf (LC) and Powdered Microbial (PM) rennets were analyzed for chemical, textural and sensorial properties during storage, also the results were analyzed by SAS software using the one way ANOVA procedure for analysis of variance. **Results:** The CMCE had 425.082 U milk clotting activity per milliliter, yield of partial purified MCE precipitated with 20-40% of ammonium sulfate was 22.58% with 1.32 purification fold, while final recovery yield of PMCE was 17.65% with increase of purification fold up to 1.43. The optimum activity of CMCE and PMCE was at (pH 6.5 and 5.5), respectively and 60°C for both. Moreover, optimal milk clotting activity was observed at 40 and 160 mM of $CaCl_2$ for CMCE and PMCE, respectively. Cheeses made with artichoke MCE exhibited higher levels of acidity and soluble nitrogen content with lower values of cheese yield than those coagulated by LC or PM. Texture profile of CMCE cheese was softer with lower hardness and cohesiveness than those of LC or PMCE. As regards sensorial properties, the flavor and body and texture scores of CMCE cheese were significantly ($p \leq 0.05$) higher than those with PMCE, LC and PM cheeses, while PMCE cheese had flavor scores close to those produced with LC or PM during storage period. **Conclusion:** The MCE from artichoke waste could be used as calf rennet alternative in white soft cheese making.

Key words: Milk clotting enzyme, artichoke flowers, alternative calf rennet, white soft cheese, coagulant

Received: March 16, 2017 Accepted: May 03, 2017 Published: June 15, 2017

Citation: Baraka Abo El-Yazeed Abd El-Salam, Osama Abd El-Hamid Ibrahim and Howida Abd El-Razek El-Sayed, 2017. Purification and characterization of milk clotting enzyme from artichoke (*Cynara cardunculus* L.) flowers as coagulant on white soft cheese. Int. J. Dairy Sci., 12: 254-265.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Coagulating enzymes have been used in cheese making from thousands of years and they seem to be the oldest known application of enzymes[1]. Calf rennet, the conventional milk clotting enzyme obtained from the fourth stomach of suckling calves, which consists of chymosin (EC 3.4.23.4) as the major component and another proteolytic enzyme, pepsin (EC 3.4.23.1); when rennet is extracted from adult animals this proportion is inverted and there is predominance of pepsin[2]. Chymosin is known for its high specificity for cleaving the caseinomacropeptide from k-casein which triggers the destabilization of the casein micelles and, therefore, induces milk clotting[3], whereas pepsin is much less specific and hydrolyses bonds with Phe, Tyr, Leu or Val residues[4]. However, the global increase in cheese production, along with the decreased supply of calf rennet leading to search for coagulants substitutes from easily and locally available resources such as plant extracts, thus, not only feasible but also essential in order to meet the demand for milk coagulants for cheese making[5]. Moreover, use of calf rennet may be limited for religious reasons (e.g., Judaism and Islam), diet (vegetarianism) or consumer concern regarding genetically engineered foods (e.g., Germany, Netherlands and France forbid the use of recombinant calf rennet)[6].

Much research interest has been directed towards discovering milk clotting enzyme which would satisfactorily replace calf rennet produced by genetically engineered bacteria have proven suitable substitutes for animal rennet but increasing attention has been directed towards natural rennet extract of plant origin[7].

There are so many rennet substitutes obtained from the plants such as fruits (e.g., *Actinidia chinensis* and *Cucumis melo*), latex (e.g., *Ficus carica* and *Calotropis procera*), flowers (e.g., *Cynara cardunculus* and *Centaurea calcitrapa*), seeds (e.g., *Albizia lebbeck* and *Solanum dubium*), leaves (e.g., *Lactuca sativa*) and roots (e.g., *Zingiber officinale*) but some of these plant coagulants had excessive proteolytic activity which reduced cheese yield and increased the perception of bitter tastes, making their use more difficult for cheese production[8,9].

Nevertheless, the aqueous extract obtained from the cardoon (*Cynara cardunculus* L.) flowers, which has been used for years in artisanal cheese making such as Serra da Estrela, Manchego, La Serena or Serpa[10], especially in Mediterranean countries, Southern Europe and Western Africa, provides the desired effect[11]. Furthermore extracts of artichoke flowers have been insufficiently investigation as a source of natural enzymes to be used in cheese manufacture as a calf rennet alternative.

Therefore, the main objectives of the present study were to extract, purify and characterize of milk clotting enzyme from artichoke (*Cynara cardunculus* L. ssp. *scolymus*) flowers and study the effect of artichoke crude and purified milk clotting enzyme as a plant coagulant on chemical, texture profile and sensory properties of white soft cheese compared to calf rennet and microbial rennet during cold storage.

MATERIALS AND METHODS

The study was conducted at Dairy Research Department, Agricultural Research Centre and Dairy Science Department, National Research Centre, Egypt from March to August 2016.

Raw materials: Artichoke violet flowers (*Cynara cardunculus* L. ssp., *scolymus*) were obtained from Egyptian local market as a waste produced from cleaning and preparing artichoke. The flowers were dried for about 3 weeks at 25°C and shielded from light. Citric acid was added to eliminate the flowers color changing.

Fresh buffaloe's milk was obtained from Faculty of Agriculture, Cairo University, Egypt. Liquid calf rennet 1 N was purchased from Mifad, Misr food additives, Egypt. Microbial rennet powder from *M. miehei* (Reniplus 2000 IMCU g^{-1}) was purchased from Caglio star Espana Pol. Inds, Spain.

Extraction of milk clotting enzyme from artichoke violet flowers: Extraction of milk clotting enzyme was performed according to Nouani *et al.*[12]. The recovered fractions were pooled and considered as crude enzymatic extract and then the milk clotting enzyme activity, proteolytic activity and protein content were determined.

Purification of milk clotting enzyme

Ammonium sulfate precipitation: The crude enzymatic extract was partial purified by adding of solid ammonium sulphate to 80% saturation at 4°C according to Colowick and Kaplan[13]. The sedimentary protein was collected by centrifugation at 5000 rpm for 15 min at 4°C. The supernatant is discarded while the precipitate is re-dissolved in 5 mL of 0.1 M sodium acetate buffer (pH 5.0). The highly active fraction was dialyzed against a large volume of the same buffer overnight.

Size exclusion column chromatography on sephadex G-100: The dialyzed fractions were further purified by applied on sephadex G-100 column (2.5×37 cm) (Phamacia, Uppsala, Sweden), equilibrated with 0.1 M sodium acetate buffer pH 5.0

and the sample eluted with the same buffer at a flow rate of 1.0 mL min^{-1}. The recovered fractions were assayed for milk clotting enzyme activity and protein detection at OD 280 nm. The rich fractions of milk clotting enzyme activity were pooled and considered as purified milk clotting enzyme.

Milk clotting enzyme activity (MCA): The milk clotting enzyme activity (MCA) of crude, partial and purified enzymatic extract were measured according to the method of IDF[14]. Milk clotting activity is expressed in term of Soxhlet unit. One Soxhlet Unit (SU) of milk clotting activity was defined as the amount of enzyme required to clot 1 mL of substrate within 40 min at 35°C. Soxhlet units were calculated using the following equation according to IDF[14]:

$$\text{Soxhlet (U mL}^{-1}) = \frac{M \times 2400}{E \times t}$$

where, M is the volume of substrate (mL), E is the amount of enzyme extract (mL) and t is the clotting time (sec).

Proteolytic activity: The proteolytic activity of the enzymatic extracts was measured by the method described by Chopra and Mathur[15]. One unit of proteolytic activity is defined as the amount of enzyme required to release TCA-soluble fragment giving a blue color equivalent to 1 μg of tyrosine under the standard assay condition.

Protein content determination: Protein content in all enzymatic extracts was carried out according to Bradford[16] procedure using coomassie brilliant blue G-250 dye. The concentration is measured from a standard curve of bovine serum albumin.

Specific activity calculation: The specific activity is calculated by divided the enzyme activity (U mL^{-1}) to the protein content (mg mL^{-1}).

Biochemical characterization of milk clotting enzyme: Some of biochemical characteristics of milk clotting enzyme were determined for the crude and purified enzyme extracts as follow:

- **Optimum reaction pH:** The milk clotting enzyme activity was measured at different pH values ranging from 5-8 using 0.2 M acetate buffer (pH 5.0), 0.2 M phosphate buffer (pH 6.0-7.0) and 0.2 M tris-HCl buffer (pH 8.0)
- **Optimum reaction temperature:** The milk clotting enzyme activity was determined after the reaction mixture was incubated at different temperatures ranging from 30-80°C to define the enzyme optimal temperature
- **Effect of $CaCl_2$ concentration on MCA:** The milk clotting enzyme activity was measured using casein substrate dissolved in different concentrations of $CaCl_2$ ranging from 2.5-1280 mM

Tallaga cheese manufacture: Four batches of Tallaga cheese were manufactured according to Mehanna and Rashed[17] except, the milk wasn't treated with CO_2. Tallage cheese was made from buffalo's milk coagulated with liquid calf rennet, microbial rennet powder compared to crude and purified plant coagulant extracted from artichoke violet flowers (*Cynara cardunculus* L. ssp. *scolymus*).

Fresh buffaloe's milk was standardized to 5.5% fat, calcium chloride and sodium chloride were added at the rate of 0.02 and 4.0% (w/w), respectively. Milk was pasteurized at 72°C for15 sec and after cooled to 37°C it was divided to four equal portions as follow: The first portion was coagulated with liquid calf rennet (T1); the second portion was coagulated microbial rennet powder (T2) while the third and fourth portions were coagulated with crude (T3) and purified (T4) milk clotting enzyme from artichoke, respectively. The resultant Tallaga cheese was packed in plastic cups filled with pasteurized whey from the respective treatment and stored in refrigerator (7°C±1) for 3 weeks. The whole experiment was repeated in triplicates.

Tallaga cheese chemical composition: Samples of different cheese batches were analyzed chemically at 0,14, 15 and 21 days of cold storage. Moisture, titratable acidity, fat, ash, protein and soluble nitrogen contents were determined by methods of AOAC[18]. The pH on warm water macerates was determined using a digital pH-meter HANNA 213 (Italy). Salt content of cheese treatments was estimated using Volhard method according to Richardson[19]. The yield of fresh cheese was calculated as a percentage of cheese weight divided by weight of cheese milk. The total amount of curd protein was divided by the total amount of milk protein to obtain the protein recovery percentage in the cheese by Madadlou *et al.*[20].

Texture profile analysis of Tallaga cheese: The Texture Profile Analysis (TPA) was carried out using at least three samples for each cheese treatments with Universal Testing Machine (Cometech, B type, Taiwan), provided with the

software. Back extrusion cell with 35 mm diameter compression disc was used. Two cycles were applied at a constant crosshead velocity of 1 mm sec^{-1} G1 to 35% of sample depth then returned. From the resulting force-time curve the values for texture attributes, i.e., hardiness, chewiness, cohesiveness, gumminess and springiness were calculated TPA graphic.

Sensory evaluation of Tallaga cheese: Sensory properties of the resulted cheese samples at zero, 7, 14 and 21 days of cold storage were evaluated according to the method of Pappas *et al.*[21]. Cheese were assessed by means of 15 panelists from the staff of the Dairy Department at Food Technology Research Institute, with maximum score points of 50 points for flavor, body and texture (40 points) and 10 points for the cheese appearance.

Statistical analysis: Statistical analysis of the average data values were analyzed by the Statistical Analysis System (SAS) using the one way ANOVA procedure for analysis of variance, using the General Linear Model (GLM) procedure of SAS software (version 6.04), SAS Institute[22]. The results were expressed as Mean±Standard Error and the difference between means were tested for significance using Duncan's multiple range at ($p \leq 0.05$).

RESULTS AND DISCUSSION

Artichoke (*Cynara cardunculus* L. ssp. *scolymus*) violet flowers was used as a plant by-product resulted during preparation of artichoke for producing milk clotting enzyme as a calf rennet substitute in order to meet the demand of milk coagulants for cheese production. Milk clotting enzyme was extracted, purified and characterized from artichoke violet flowers (*Cynara cardunculus* L. ssp. *scolymus*) with successive purification processes such as, ammonium sulfate precipitation and size exclusion column chromatography.

Purification of milk clotting enzyme from artichoke flowers: Milk clotting enzyme was extracted and purified from artichoke violet flowers (*Cynara cardunculus* L. ssp. *scolymus*) but the excessive oxidation of crude extract leads to browning therefore the crude extract was subjected on Sephadex G-50 column in order to eliminate most of these pigments[12]. The recovered fractions were pooled and considered as crude enzymatic extract with 425.082 SU mL^{-1} and their specific activity was 727.88 SU mg^{-1} protein. Moreover, the proteolytic activity of crude milk clotting enzyme was 4.12 U mL^{-1} with 103.27 as Milk Clotting Activity/Proteolytic Activity (MCA/PA) ratio as shown in Table 1. Milk clotting activity observed in artichoke violet flowers (*Cynara cardunculus* L. ssp. *scolymus*) which is in agreement with Heimgartner *et al.*[23] and Lorente *et al.*[24], who demonstrated that the artichoke extracts from the upper part of mature (violet) flowers had milk clotting activity. Also, Lorente *et al.*[24] observed that no milk clotting activity could be detected in roots, young leaves, midribs, receptacles and inflorescence stems and leaves within the assay time (300 min). Moreover, more extensive literature confirmed that the artichoke extracts contain aspartic proteases with similar activities as chymosin (Cardosin A) and pepsin (Cardosin B) of animal origin and they are accumulated within the flowers (flosculi), in the vacuoles of stigmatic papilla and inside the epidermal cells of the stylum. Cardosins A catalyzing the k-casein hydrolysis in correspondence to the sensitive Phe^{105}-Met^{106} peptide bond, while cardosins B with their highly proteolytic action[25-31].

Ammonium sulfate salts was used to partial purify of milk clotting enzyme from artichoke crude extract at the level of 20-40% saturation with 240 SU mL^{-1}. The proteolytic activity of partial purified milk clotting enzyme was 2.67 U mL^{-1} with 89.72 as MCA/PA ratio as shown in Table 1. Ammonium sulfate saturation was similar to that found for milk clotting enzyme extracted from artichoke (*Cynara scolymus*) leaves[32].

The dialyzed partial purified milk clotting enzyme was purified by size exclusion column chromatography using sephadex G-100 gel. The highly active fractions in MCA from a single peak were pooled and labeled as purified milk clotting enzyme as shown in Fig. 1, which was similar to that found for a cynarase extracted from artichoke (*Cynara scolymus*) flowers[12] while unlike with Verissimo *et al.*[25] and Sidrach *et al.*[33], who observed 3 forms of cynarase, also Lorente *et al.*[24] isolated 2 forms of cynarase. The MCA of the purified milk clotting enzyme from artichoke violet flowers (*Cynara cardunculus* L. ssp. *scolymus*) was 15.635 while their proteolytic activity was 0.25 U mL^{-1} with 63.81 as MCA/PA ratio as shown in Table 1. The artichoke purified fractions showed milk clotting activity lower than crude extract due to dilution but which are clear from inactive proteins which similar to that found for a Cynarase extracted from artichoke (*Cynara scolymus*) flowers[12].

Milk clotting enzyme purification steps and their effect on the enzyme yield and purification fold are summarized in Table 2. Yield of partial purified milk clotting enzyme precipitated with ammonium sulfate was 22.58% with 1.32 purification fold and their specific activity was 960 SU mg^{-1} protein while the final recovery yield of purified milk clotting enzyme was 17.65% with increasing

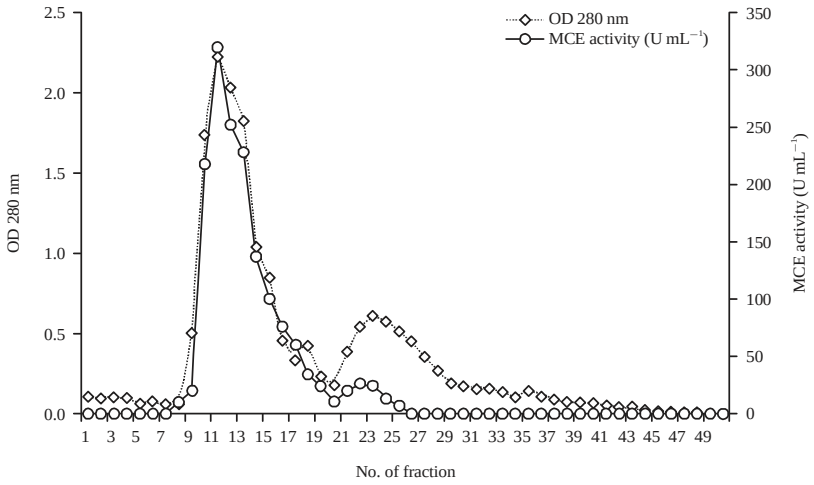

Fig. 1: Size exclusion chromatography pattern of MCE from artichoke (*Cynara cardunculus* L. ssp. *scolymus*) flowers on Sephadex G-100 column

Table 1: Milk clotting activity (MCA) and proteolytic activity (PA) of artichoke (*Cynara cardunculus* L. ssp. *scolymus*) violet flowers

Milk clotting enzyme fractions	MCA* (SU mL^{-1})	PA* (U mL^{-1})	MCA:PA
Crude enzyme	425.082	4.12	103.27
ASP (20-40%)	240.000	2.67	89.72
SEC on Sephadex G-100	15.635	0.25	63.81

*Calculation based on 0.1 g mL^{-1} of dried artichoke violet flowers in 0.1 M sodium acetate buffer pH 5.0, ASP: Ammonium sulfate precipitation, SEC: Size exclusion column chromatography

Table 2: Purification results of MCE from artichoke (*Cynara cardunculus* L. ssp. *scolymus*) violet flowers

Purification steps	Total activity* (U)	Total protein* (mg)	Specific activity (U mg^{-1} protein)	Yield (%)	Purification fold
Crude enzyme	10627.05	14.60	727.88	100.00	1.00
ASP (20-40%)	2400.00	2.50	960.00	22.58	1.32
SEC on Sephadex G-100	1876.20	1.80	1042.33	17.65	1.43

*Calculation based on 0.1 g mL^{-1} of dried artichoke violet flowers in 0.1 M sodium acetate buffer pH 5.0, Specific activity: Enzyme activity/Protein content, Total activity: Enzyme activity×fraction volume, Total protein: Protein content×fraction volume, Yield: Total activity of purified enzyme/total activity of crude enzyme×100, Purification fold: Specific activity of purified enzyme/specific activity of crude enzyme, ASP: Ammonium sulfate precipitation, SEC: Size exclusion column chromatography

of purification fold up to 1.43 with specific activity of 1042-33 SU mg^{-1} protein. Chazarra *et al.*[34] and Esposito[32] also found that MCA had the lowest coagulant activity compared to artichoke crude extract, while MCA showed the highest values. Nevertheless, the use of pure extracts of aspartic proteases is of less interest for the cheese industry due to the cost of this type of extract[24]. This is why it is important to determine the coagulant activity of vegetable crude extracts, which may be of greater interest for the food industry.

Biochemical characterization of milk clotting enzyme: Artichoke (*Cynara cardunculus* L. ssp. *scolymus*) milk clotting enzyme exhibited the optimum activity at 60°C for the crude and purified extracts as shown in Fig. 2. The optimal reaction temperature of artichoke milk clotting enzyme obtained was similar to that found for a milk clotting proteinase extracted from artichoke (*Cynara scolymus* L.)[33] while it was quite lower than that observed in cynarase extracted from artichoke (*Cynara scolymus*) flowers[12].

Figure 3 shows the optimum reaction pH for crude milk clotting enzyme was 6.5 while the purified enzyme it was observed at pH 5.5 with a broad pH range of pH 5.0-6.5 where artichoke milk clotting enzyme was sharply lost their activity at alkaline pH. The maximum activity of milk clotting proteinase isolated from the upper part (violet) of mature flowers normally lies in acidic pH between (3.5 and 5.0)[24], in the case of artichoke (*Cynara cardunculus* L. ssp. *scolymus*) milk clotting enzyme optimal pH was similar to that found for a milk clotting proteinase obtained from flowers of *Cynara cardunculus*[23,35] and artichoke (*Cynara scolymus* L.)[12, 33].

The optimal $CaCl_2$ concentration for the crude and purified milk clotting enzyme extracted from artichoke flowers were found at the level of 40 and 160 mM, respectively as

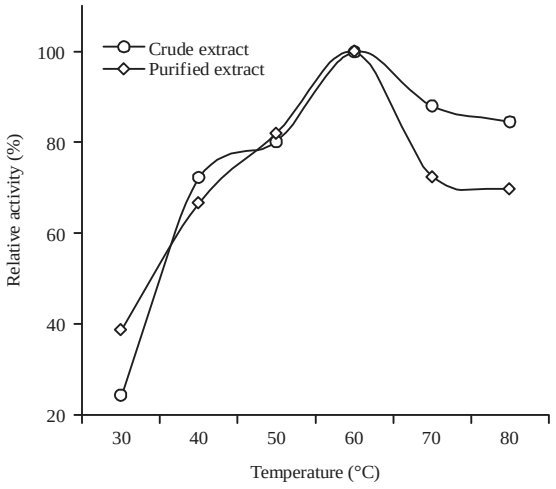

Fig. 2: Optimum reaction temperature of MCE from artichoke (*Cynara cardunculus* L. ssp. *scolymus*) flowers

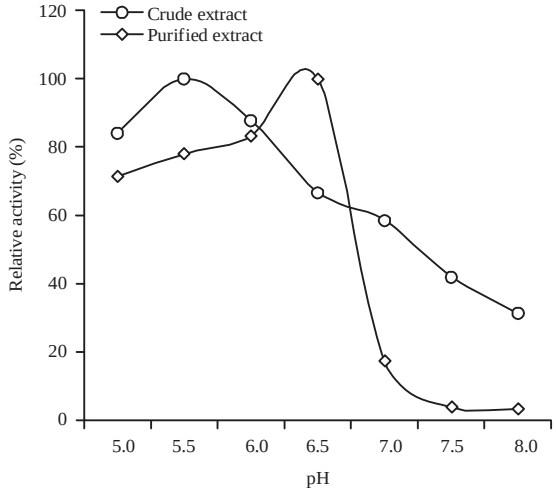

Fig. 3: Optimum reaction pH of MCE from artichoke (*Cynara cardunculus* L. ssp. *scolymus*) flowers

presented in Fig. 4. It is well known that the non-enzymatic secondary phase, in which the aggregation of para-k-casein and other casein components occurs in association with Ca^{+2} ions and eventually results in the formation of a gel[36]. Therefore, clot formation is Ca^{+2} dependent[33], it was confirmed with milk clotting enzyme extracted from artichoke flowers which required suitable $CaCl_2$ concentration for it is optimal activity.

White soft cheese making: Tallaga cheese as a type of white soft cheese was manufactured from buffalo's milk coagulated with the crude and purified milk clotting enzyme (denominated as CMCE and PMCE, respectively) extracted

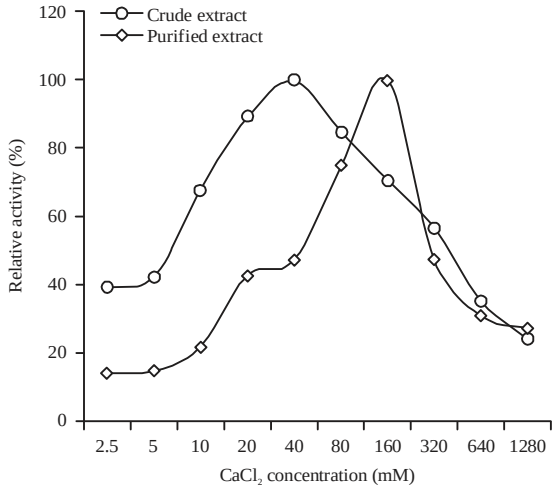

Fig. 4: Effect of $CaCl_2$ concentration on MCE activity from artichoke (*Cynara cardunculus* L. ssp. *scolymus*) flowers

from Artichoke (*Cynara cardunculus* L. ssp. *scolymus*) violet flowers compared to Liquid Calf rennet (LC) and microbial rennet powder (PM). The resultant cheeses were analyzed for chemical, rheological and sensorial properties.

Chemical composition of Tallaga cheeses during cold storage: Table 3 shows the chemical composition of Tallaga cheese coagulated using CMCE or PMCE extracted from artichoke (*Cynara cardunculus* L. ssp. *scolymus*) violet flowers compared to LC and PM commercial rennet. It could be seen from the results that, the moisture levels of Tallaga cheese coagulated with CMCE were significantly ($p \leq 0.05$) higher than those produced with LC rennet or PMCE extracted from artichoke flowers. No significance difference between moisture levels of Tallaga cheese coagulated with CMCE extracted from artichoke flowers and PM rennet. Moisture levels of all cheese treatments decreased significantly ($p \leq 0.05$) with progressing storage period. Similar results were obtained by El-Kholy[37], who found that, Tallaga cheese made with extracts of artichoke *(Cynara scolymus* L.) flowers had a significant higher ($p \leq 0.05$) moisture content compared to cheese coagulated with calf rennet while Galán *et al.*[27] found that the moisture levels of cheeses produced with vegetable coagulant obtained from *Cynara cardunculus* were lower than those produced with calf rennet.

The fat levels of Tallaga cheese coagulated with CMCE extracted from artichoke flowers or PM rennet were significantly ($p \leq 0.05$) lower than those coagulated with PMCE extracted from artichoke flowers or LC rennet. The fat levels significantly ($p \leq 0.05$) increased in all cheese treatments

Table 3: Chemical composition protein recovery and yield of Tallaga cheese made with artichoke (*Cynara cardunculus* L. ssp. *scolymus*) coagulant compared to commercial coagulants during cold storage at 7°C

Parameters	Storage period (weeks)	Treatments T1	T2	T3	T4
Moisture (%)	0	61.61^{b,A}	62.30^{a,A}	62.18^{a,A}	59.66^{c,A}
	1	60.11^{b,B}	61.69^{a,B}	60.66^{a,B}	59.36^{c,B}
	2	58.08^{b,C}	58.47^{a,C}	58.50^{a,C}	58.04^{cb,C}
	3	56.96^{b,D}	58.38^{a,D}	58.44^{a,D}	56.80^{c,D}
Fat (%)	0	22.00^{a,D}	21.80^{c,D}	21.70^{d,D}	21.90^{b,D}
	1	22.50^{a,C}	21.90^{c,C}	21.80^{d,C}	22.00^{b,C}
	2	22.80^{b,B}	22.60^{c,B}	22.60^{dc,B}	23.00^{a,B}
	3	23.00^{b,A}	22.90^{c,A}	22.80^{d,A}	23.20^{a,A}
Protein (%)	0	16.48^{a,A}	16.50^{a,A}	16.51^{a,A}	16.52^{a,A}
	1	16.41^{a,B}	16.41^{a,B}	16.43^{a,B}	16.45^{a,B}
	2	16.28^{a,C}	16.29^{a,C}	16.32^{a,C}	16.33^{a,C}
	3	16.03^{a,D}	16.05^{a,D}	16.12^{a,D}	16. 15^{a,D}
Soluble nitrogen (%)	0	0.11^{d,D}	0.14^{c,D}	0.21^{a,D}	0.19^{b,D}
	1	0.16^{d,C}	0.17^{c,C}	0.46^{a,C}	0.40^{b,C}
	2	0.25^{d,B}	0.32^{c,B}	0.73^{a,B}	0.58^{b,B}
	3	0.46^{d,A}	0.49^{c,A}	0.77^{a,A}	0.63^{b,A}
Ash (%)	0	3.41^{a,A}	3.40^{a,A}	3.44^{a,A}	3.46^{a,A}
	1	3.45^{a,A}	3.45^{a,A}	3.48^{a,A}	3.47^{a,A}
	2	3.47^{a,A}	3.48^{a,A}	3.49^{a,A}	3.50^{a,A}
	3	3.49^{a,A}	3.50^{a,A}	3.50^{a,A}	3.51^{a,A}
Salt (%)	0	2.25^{a,D}	2.20^{a,D}	2.21^{a,D}	2.22^{a,D}
	1	2.28^{a,C}	2.25^{a,C}	2.24^{a,C}	2.28^{a,C}
	2	2.33^{a,B}	2.31^{a,B}	2.35^{a,B}	2.32^{a,B}
	3	2.37^{a,A}	2.34^{a,A}	2.37^{a,A}	2.36^{a,A}
pH	0	6.45^{a,A}	6.40^{b,A}	6.36^{d,A}	6.38^{c,A}
	1	6.39^{a,B}	6.19^{b,B}	6.10^{d,B}	6.15^{c,B}
	2	6.00^{a,C}	5.67^{b,C}	5.32^{d,C}	5.37^{c,C}
	3	5.77^{a,D}	5.34^{a,D}	5.00^{d,D}	5.22^{c,D}
Acidity (%)	0	0.29^{d,D}	0.30^{c,D}	0.33^{a,D}	0.32^{b,D}
	1	0.32^{d,C}	0.33^{c,C}	0.37^{a,C}	0.35^{b,C}
	2	0.35^{d,B}	0.37^{c,B}	0.43^{a,B}	0.40^{b,B}
	3	0.41^{d,A}	0.42^{c,A}	0.54^{a,A}	0.49^{b,A}
Yield (%)	0	26.11^{b}	26.65^{a}	24.45^{d}	25.13^{c}
Protein recovery (%)	0	4.99^{a}	5.00^{a}	5.00^{a}	5.01^{a}

T1: Liquid calf rennet, T2: Microbial rennet powder, T3: Crude MCE form artichoke flowers, T4: Purified MCE form artichoke flowers. Different small letters on the same row are differ significantly at $p \leq 0.05$ between treatments. Different capital letters on the same column are differ significantly at $p \leq 0.05$ during storage period

during storage period which might be due to the moisture content reduction during storage period[37].

It could be noticed from the results that, the protein content of all cheese treatments was not affected significantly by used coagulant type. Pino *et al.*[38] found no significant differences between cheeses made with calf rennet and plant coagulant concerning protein values. The protein content of all cheese treatments significantly decreased ($p \leq 0.05$) with increasing storage period which might be due to the degradation of proteins into soluble nitrogen and hence, loss of some water soluble nitrogen from the degraded proteins[37,39].

Soluble Nitrogen (SN) values in cheeses made with PMCE or CMCE extracted from artichoke flowers were higher than those coagulated with LC or PMrennet which due to the intense proteolytic activity of plant coagulant[40], also it could be observed that the SN of CMCE (T3) was higher than PMCE (T4) which due to the proteolytic activity of CMCE was higher than PMCE at the level of 4.12 and 0.25 U mL^{-1}, respectively. Moreover, SN values of all cheese treatments significantly increased ($p \leq 0.05$) with storage period progressing. Similar results were observed by Pino *et al.*[38], who found statistically higher levels of SN in cheese made with plant coagulant than in cheese made with calf rennet. In cheese, the water soluble nitrogen is primarily formed by coagulating enzymes (e.g., chymosin), plasmin or cell-wall envelope proteases at the early stage of proteolysis. It is well known that protein breakdown is an important factor for both flavors and texture development during ripening[2].

The ash content and sodium chloride of all cheese treatments was not affected significantly ($p \leq 0.05$) by coagulant type were used. Similar observations were

Table 4: Sensory properties of Tallaga cheese manufactured with artichoke (*Cynara cardunculus* L. ssp. *scolymus*) coagulant compared to commercial coagulants during cold storage at 7°C

Parameters	Storage period (weeks)	Treatments T1	T2	T3	T4
Flavour (50)	0	$46^{cb,A}$	$46^{b,A}$	$48^{a,A}$	$45^{db,B}$
	1	$45^{db,B}$	$46^{b,A}$	$48^{a,A}$	$46^{cb,A}$
	2	$44^{cb,C}$	$45^{b,B}$	$47^{a,B}$	$44^{db,C}$
	3	$44^{a,C}$	$45^{a,B}$	$45^{b,C}$	$44^{a,C}$
Body and texture (40)	0	$36^{d,A}$	$37^{c,A}$	$39^{a,A}$	$38^{b,A}$
	1	$36^{dc,A}$	$36^{c,B}$	$38^{a,B}$	$37^{b,B}$
	2	$34^{d,B}$	$35^{c,C}$	$37^{a,C}$	$36^{b,C}$
	3	$33^{c,C}$	$34^{b,D}$	$35^{d,D}$	$35^{a,D}$
Appearance (10)	0	$9.0^{a,A}$	$9.0^{a,A}$	$9.0^{a,A}$	$9.0^{a,A}$
	1	$8.7^{a,A}$	$9.0^{a,A}$	$8.8^{a,A}$	$8.8^{a,A}$
	2	$8.5^{a,A}$	$8.8^{a,A}$	$8.7^{a,A}$	$8.7^{a,A}$
	3	$8.5^{a,A}$	$8.8^{a,A}$	$8.6^{a,A}$	$8.7^{a,A}$

T1: Liquid calf rennet, T2: Microbial rennet powder, T3: Crude MCE form artichoke flowers, T4: Purified MCE form artichoke flowers. Different small letters on the same row are differ significantly at $p \leq 0.05$,between treatments. Different Capital letters on the same column are differ significantly at $p \leq 0.05$ during storage period

obtained by Tejada *et al.*[26]. Moreover, it could be noticed that sodium chloride content was significantly increased ($p \leq 0.05$) in all cheese treatments with storage period progressing which might be due to the moisture content reduction during storage period[41].

The titratable acidity (%) with opposite trends in pH values of Tallaga cheese coagulated with CMCE or PMCE extracted from artichoke flowers was higher than those coagulated with LC or PM rennet, probably due to the proteolytic activity of artichoke flower extracts. The acidity of all cheese treatments significantly ($p \leq 0.05$) increased with storage period progressing. These findings were also noted by Abdel-Kader[42] for Domiati cheese made with microbial and recombinant rennet. Al-Jasser and Al-Dogan[43] also observed higher acidity for white soft cheese produced using rennet substitute extracted from *Solanum* seeds compared to control cheese. It well known that pH variation during ripening depends on the buffering capacity of the cheese, due to the amount of proteins and minerals present, the formation of ammonium and/or catabolism of lactic acid[44].

It could be seen that a higher yield of cheese was obtained using liquid calf rennet rather than plant coagulant, it could be due to proteolytic activity of vegetable coagulants which responsible for increased N losses in whey and reduced cheese yield[45]. No significance difference between protein recovery percentage were observed for Tallaga cheese treatments.

Texture profile analysis of cheese: Texture profile of all cheese treatments is presented in Fig. 5, cheese texture was significantly ($p \leq 0.05$) influenced by coagulant type where Tallaga cheese coagulated with CMCE extracted from artichoke (*Cynara cardunculus* L. ssp. *scolymus*) violet flowers was softer and had lower hardness and cohesiveness of than those produced with LC rennet or PMCE extracted from artichoke flowers. No significance difference between hardness and cohesiveness of Tallaga cheese coagulated with CMCE and (PM) rennet. The hardness and cohesiveness of all cheese treatments increased with progressing storage period which may be due to relationship between moisture and cheese hardness. Calvo *et al.*[46] found that hardness significantly increased ($p \leq 0.05$) as a consequence of the low moisture content. Negative correlation was found between hardness values and the respective amounts of SN. The higher proteolytic activity in the breakdown of caseins and the first degradations products in cheeses made with vegetable coagulant led to a softer and creamier texture than in those obtained with calf rennet[27]. The springiness showed no significant differences between the different cheeses, while differences were found in chewiness and gumminess. The gumminess and chewiness followed the same trend of hardness and cohesiveness and had the same differences of means between the cheese types. The significant differences ($p \leq 0.05$) observed in cheese gumminess and chewiness may be related to differences in the moisture content as described by Pinto *et al.*[38].

Sensory evaluation of cheese: Sensorial characteristics of Tallaga cheese coagulated with CMCE and PMCE extracted from artichoke (*Cynara cardunculus* L. ssp. *scolymus*) violet flowers compared to commercial LC and PM rennet during cold storage were presented in Table 4. The flavor scores of cheese coagulated with CMCE was significantly ($p \leq 0.05$) higher than those with PMCE, LC and PM rennet until the 2nd week of storage period, while the cheese coagulated with

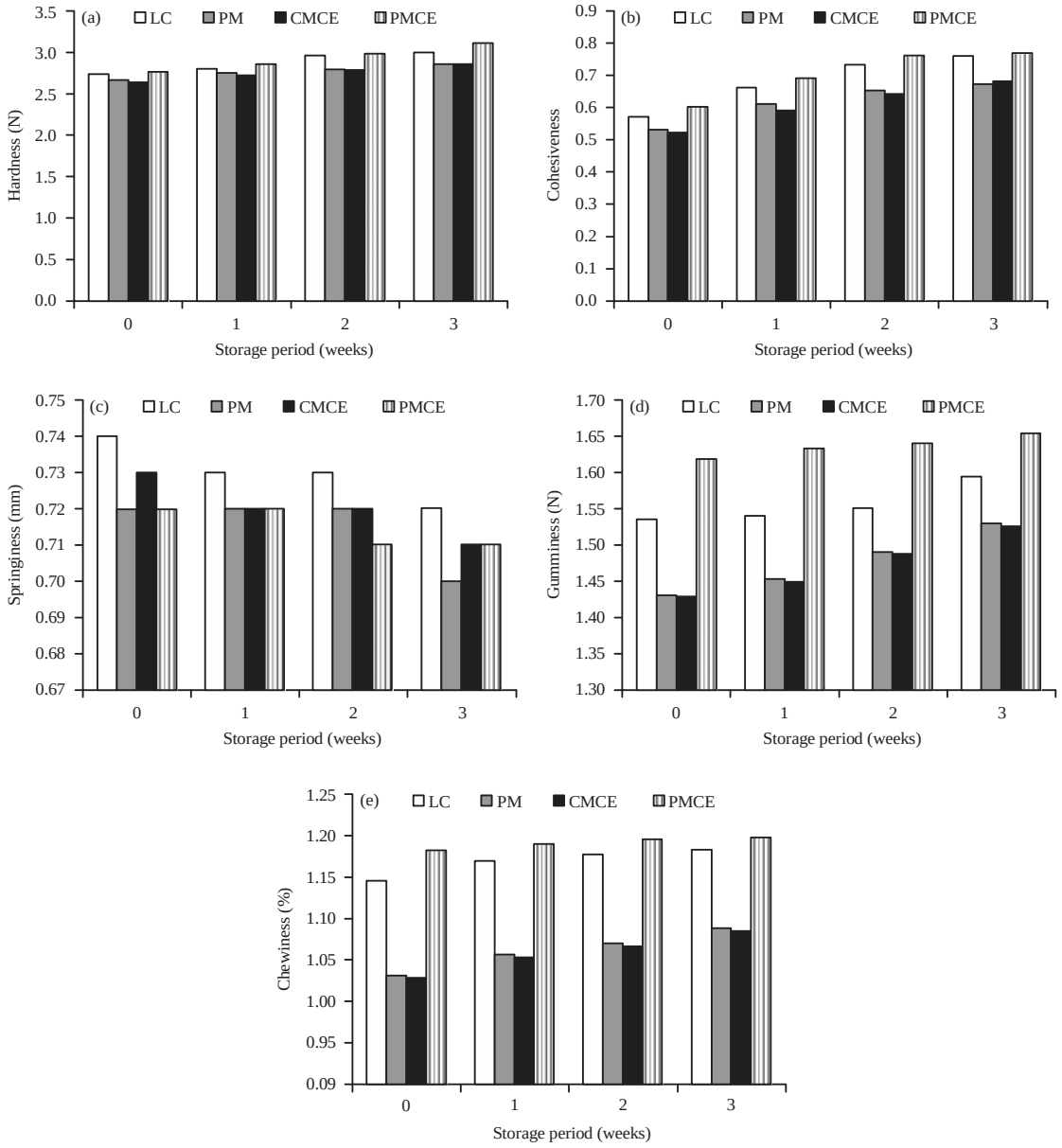

Fig. 5: Texture profile analysis of Tallaga cheese made with artichoke (*Cynara cardunculus* L. ssp. *scolymus*) coagulant compared to commercial coagulants during cold storage at 7°C (a) Hardness, (b) Cohesiveness, (c) Springiness, (d) Gumminess, (e) Chewiness

LC: Liquid calf rennet, MP: Microbial rennet powder, CMCE: Crude extract of MCE form artichoke flowers, PMCE: Purified MCE form artichoke flowers

PMCE extracted from artichoke had scores close to those with LC or PM rennet for flavor during studied storage period. The body and texture scores of cheese coagulated with CMCE extracted from artichoke were significantly ($p \leq 0.05$) higher than those with PMCE, LC or PM coagulant during studied storage period followed by Tallaga cheese coagulated with PMCE extracted from artichoke flowers. These results are in agreement with those obtained by Nazni *et al.*[47], who indicated that the cardoon rennet cheese exhibited softer texture and higher creaminess scores as compared with the calf and microbial rennet cheeses. This may be contributed to the higher artichoke cheese contents of SN which serve as precursors of certain flavor components[48]. No significance differences for appearance were observed among all cheese treatments during storage period, it could be due to eliminate the artichoke extract color which this discoloration prevents the coagulant extract from transmitting an undesirable color and odor to cheese milk and their

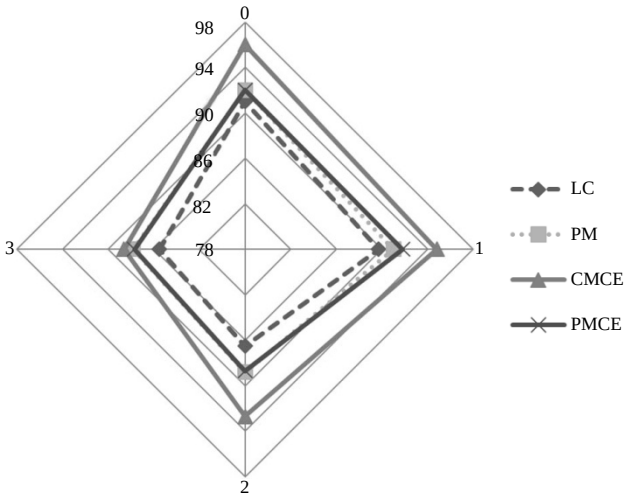

Fig. 6: Sensory properties of Tallaga cheese made with artichoke flowers (*Cynara cardunculus* L. ssp. *scolymus*) coagulant compared to commercial coagulants during cold storage at 7°C

LC: Liquid calf rennet, MP: Microbial rennet powder, CMCE: Crude extract of MCE form artichoke flowers, PMCE: Purified MCE form artichoke flowers. (Each point describes total score for each sample per week including flavor, body, texture and appearance)

appearance[12]. Overall, cheese coagulated with CMCE (T3) extracted from artichoke flowers characterized with soft body and acceptable texture compared with other cheese treatments as shown in Fig. 6 during the storage period while PMCE (T4) was close to other commercial rennet during the storage period.

This study has significant implications in the milk coagulants field, where the artichoke violet flowers (waste) was used to produce of milk clotting enzyme as a calf rennet alternative and thus, resulting in protect the environment from pollution. This enzyme has been applied for production of soft cheese compared to calf and microbial rennet and the results indicated that it caused a good flavor. It could be recommended to use the milk clotting enzyme extracted from artichoke flowers as calf rennet substitute in soft cheese manufacture. Many studies were needed to identify the structure and other characteristics of this enzyme.

CONCLUSION AND FUTURE RECOMMENDATION

Milk Clotting Enzyme (MCE) was isolated and purified from artichoke (*Cynara cardunculus* L. ssp. *scolymus*) flowers, which produced during cleaning and preparing artichokeas a waste resulting in protect the environment from pollution, to use it as a plant coagulant and substitute of calf rennet. The crude MCE exhibited optimum activity at pH 6.5 while the at pH 5.5 and 60°C for both crude and purified artichoke MCE. Artichoke MCE as a vegetable coagulant caused greater casein hydrolysis with resultant softer cheese texture and good flavor especially cheese coagulated with crude MCE while purified MCE was close to other commercial rennet during cheese storage period. It could be recommended that MCE extracted from artichoke flowers could be applied as a food additive with beneficial role in cheese production and improvement of their sensorial properties without any defects of the resultant cheese. Many studies are needed to identify the structure and other characteristics of artichoke MCE and their effect on different milk and cheese types.

SIGNIFICANCE STATEMENTS

This study discovers the calf rennet alternatives from artichoke (*Cynara cardunculus* L. ssp. *scolymus*) violet flowers that can be beneficial for cheese production and improvement of their sensorial properties. This study will help the researcher to uncover t he critical areas of artichoke milk clotting enzyme kinetics that many researchers were not able to explore. Thus a new theory on kinetics of artichoke milk c lotting enzyme and their effect on white soft cheese properties would be known.

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