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Research Article

A NOVEL MILK-CLOTTING ENZYME FROM CUMIN SEEDS (*CUMINUM CYMINUM* L.) AND ITS IMPACTS ON THE CHARACTERISTICS OF BRINED SOFT CHEESE

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ABSTRACT

As cheese production increased globally and bovine rennet became rare and costly, other milk-clotting enzymes (MCE) were required. The objective of this study was to extract MCE from cumin seeds (Cuminum cyminum L), evaluate its characteristics, and consider its potential application in the production of brined soft cheese (BSC). Purified MCE maintained more than 80% of its initial activity across a broad temperature range (20–60°C) and pH range (4–6), with the optimal temperature and pH being 50°C and 6, respectively. When purified MCE was used to make soft cheese, there were no major differences in cheese yield and protein recovery compared to calf and microbial rennet. Also, there were no noticeable differences in the composition and texture profile of BSC with purified MCE from cumin seeds (BSC-CE) compared to those made with calf (BSC-CR) and microbial (BSC-MR) rennet until week 5 when stored at 5±2°C. At week 8, the moisture and proteolysis contents of BSC-CE were the highest, while its hardness and gumminess were the lowest. The antioxidant activity of BSC-CE against DPPH and ABTS radicals was the highest and increased with time of storage, followed by BSC-MR and BSC-CR. BSC-CE was also more palatable up until week 5, but by week 8, the texture became very soft.

1.Introduction

Cheese making is the process of separating curd from whey and kneading it until it takes on a mouldable consistency. Cheese is a nutritional milk product made from the milk of cows, buffaloes, goats, sheep, and camels. Cheese makers have historically used chymosins extracted from the abomasal tissues of young ruminants, due to their milk-coagulating properties (Nájera-Domínguez *et al.,* 2022). Calf rennet can include up to 95% chymosin (EC 3.4.23.4) and a small amount of pepsin (EC 3.4.23.1), depending on the age of animals (Jacob *et al*., 2011). Nowadays, cheese is mostly made using microbial and recombinant chymosin proteases. Yeast (*Kluveryomyces lactis*), molds (*Aspergillus niger* var. *awamori*), and bacteria (*E. coli*) frequently express recombinant chymosins (Kumar *et al.,* 2010; Fguiri *et al.,* 2021). Microbial and Recombinant chymosins are rapidly becoming more and more popular among cheese producers because of their enhanced milk-clotting properties, vegetarian acceptability, and protection of animal rights. However, a number of factors, including vegetarianism, religious prohibitions, high rennet costs, increased cheese production and animal disease incidence, consumer concerns or negative opinions about genetically modified organisms (GMOs), and dietary changes that limit the use of animal and microbial rennet, encourage the search for alternative milk-clotting sources (Pagthinathan *et al.,* 2020; Nicosia *et al.,* 2022). According to Huppertz *et al.* (2018), alternative coagulants must have biochemical properties that are comparable to those of calf rennet, including high milk clotting power, high specificity for κ-casein, proteolytic activity at the pH and temperature required for cheese making, and adequate thermolability for keeping whey products free of coagulant residue.

Plant-based natural rennet has becoming more and more popular in the cheese industry since it is readily available and requires little extraction or purification (Silva *et al.,* 2021). As a result, the search for new possible plant-based milk-clotting enzymes continues in order to make them commercially feasible and meet the growing global demand for diverse, high-quality cheese manufacturing. The utilization of plant extracts as alternatives to rennet has been the subject of numerous investigations (Ben Amira

et al., 2017; Rincon *et al.,* 2017; Sorin *et al.,* 2021**).** Proteases, such as ficin from *Ficus* sp. *latex*, papain from *Carica papaya*, and cardosins from *Cynara* sp., are most commonly used as milk coagulants. Additionally, Silvestre *et al*. (2012) found that the optimal temperature for the clotting and proteolytic activities of plant extracts is determined by a variety of parameters, including plant source, tissue, concentration, and protease type. Since ancient times, the Mediterranean, West African, and southern European nations have utilized vegetable extracts as coagulants in the production of cheese. However, most plant proteases hydrolyze milk proteins extensively, which results in weak milk gels or no gels at all. Additionally, a large number of plant proteases have not been fully identified or studied (Ben Amira *et al.*, 2017; Nájera-Domínguez *et al.,* 2022). Cumin (*Cuminum cyminum* L.), Belonging to the *Apiaceae* family, is an important commercial spice with a variety of medical, nutraceutical, and pharmacological uses as well as a broad use as a flavoring agent, either whole or powdered powder. It is indigenous to several nations and is extensively cultivated there, mostly in arid and semi-arid areas such as China, Egypt, Saudi Arabia, the Mediterranean, India, and Iran (Srinivasan, 2018). Traditionally used as an astringent, carminative, and stimulant, cumin seed also possesses far greater functional activity, including antioxidant, antibacterial, anticancer and anti-inflammatory actions, in addition to enhancing food taste and flavor. It has also been discovered that cumin seeds have milk-clotting properties (Siow *et al*., 2017; Allaq *et al*., 2020). Proteolytic enzymes, found in cumin, break down proteins. Pepsin proteolytic activity has been shown to be enhanced by cumin seed peptides, which are produced from cumin. Therefore, the main objectives of this study were to extract, purify, and characterize the milk-clotting protease from cumin (*Cuminum cyminum* L.) seeds and compare it with microbial and animal rennet in the production of brined soft cheese. Cumin may be a new milk-clotting enzyme (MCE) and provide the dairy industry with alternatives for traditional rennet.

2. Materials and methods

2.1.Materials

Cumin seeds (*Cuminum cyminum* L.) were purchased from a local market in Cairo, Egypt, and then powdered using an electric grinder. The average composition of cumin seed powder was 17.81, 22.27, 44.24, and 10.5% for protein, oil, carbs, and dietary fiber, respectively. Buffalo's milk was obtained from the Animal Production Institute, Agricultural Research Centre, Giza, Egypt. Animal rennet was provided by El-Serw Station, Egypt. Microbial rennet powder was obtained from Chr. Hansen's Lab. (A/S Copenhagen, Denmark). Cheese starter cultures, Lactococcus lactis ssp. lactis and Lactococcus lactis ssp. cremoris, were obtained from stock cultures of the Dairy Microbiology Lab (National Research Centre, Egypt). Skim milk powder, made in Ukraine, was purchased from a local market in Cairo, Egypt. Sodium chloride (NaCl) and calcium chloride ($CaCl_2$) were obtained from El-Nasser Company, Alexandria, Egypt. The 2,2-diphenyl-1-(2,4,6-trinitrophenyl)-hydrazinyl (DPPH) and 2,2-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Acetate, phthalate-NaOH, and citrate salts were obtained from Fluka AG (Chemische Fabrik, Germany). All other chemicals used in the present study were of analytical grade.

2.2. Methods

2.2.1. Preparation of crude enzyme extract

Three buffer solutions (acetate, phthalate-sodium hydroxide, and citrate) with concentrations of 0.1 *M* and pH 5, as well as distilled water (pH 6.8), were used to select the optimal solution for extracting MCE from cumin seeds. All buffers were prepared using the procedure of Gomori (1955). Using 100 ml of buffer solution, 40 g of cumin seeds powder were soaked in a conical flask for 24 h at 5±1ºC. For the first 3 h, the flask was shaken frequently. The mixture was centrifuged under cooling (4ºC) at 6000 xg for 20 min and then filtered through filter paper, Whatman No. 1. The aqueous filtrate was used to test for milk-clotting activity (MCA) according to Arima and Iwasaki (1970). Briefly, the milk substrate was prepared (10% skim milk powder in 0.01 M $CaCl_2$) and the pH was adjusted to 6.5. After the substrate (2.0 ml) was pre-incubated for 5 min at 37°C, adding 0.2 ml of crude MCE while periodically rotating the test tube by hand, the formation of curd was observed. When the split particles were visible, the termination point was recorded. According to Gutiérrez et al. (2012), one milk-clotting unit is the amount of enzyme required to clot 10 ml of the substrate in 40 min. Protein content was determined calorimetrically at 595 nm using Coomassie brilliant blue G-250 and bovine serum albumin according to Bradford (1976). Specific activity was calculated by dividing the determined MCA by the protein content.

$$MCA = \frac{2400}{t_c} \times \frac{Vs}{Vc} \quad (1)$$

Where: MCA, the milk-clotting activity units (U/ml); Vs, the volume of milk (ml); Vc, the volume of clotting enzyme added into the milk (ml); t_c, the time span to coagulation (sec).

2.2.2. Purification of milk-collecting enzyme (MCE)

Crude MCE extract was precipitated by different concentrations of ammonium sulfate from 10 to 100% saturation (Colowick and Kaplan, 1955). A suitable quantity of ammonium sulfate was added to the supernatant and then centrifuged undercooling at 8000 xg for 20 min. The precipitate was collected with a minimum quantity of 0.1 *M* citrate buffer at pH 5 as the best extraction solution. The precipitate fraction was dialyzed in the same buffer using a dialysis bag and kept in the refrigerator for 48 h. A Sephadex G-100 column (2.5 x 40 cm) was then used to purify the crude MCE extract using the gel filtration procedure described by Dioxn and Webb (1968). The same buffer was used to elute the extract at a flow rate of 0.7 ml/min. Five ml fractions were collected and assayed for enzyme activity and protein (mg/ml) at 280 nm.

2.3 Characterization of purified MCE

2.3.1. Optimum pH

The optimum pH of the purified MCE was determined according to the method described by Gomori (1955). 0.1 *M* citrate buffer pH 5 in the clotting assay was substituted with 0.1 *M* citrate buffer (pH 4-6), 0.1 M phosphate buffer (pH 6-7), 0.1 *M* Tris-HCl buffer (pH 8-9), and Glysine NaOH buffer (pH 10). The milk clotting test technique was used to conduct the reaction.

2.3.2. Optimum temperature

The reaction mixture of the clotting assay was incubated at different temperatures ranging from 20 to 100°C for 10 min in order to determine the optimum temperature of the pure enzyme. The optimum temperature for MCE was then determined by measuring the enzyme activity at each temperature.

2.3.3. Effect of $CaCl_2$ and NaCl concentrations

The effect of different $CaCl_2$ and NaCl concentrations, which ranged from 0–50 m*M* and 0–10%, respectively on the relative activity of purified MCE, was determined according to Ahmed and Helmy (2012).The relative activity was determined by measuring the activity under standard assay conditions and calculating the percentage of activity that remained following incubation with different concentrations of $CaCl_2$ and NaCl.

2.3.4. Effect of metal ions

The effects of $MnSO_4.H_2O$, $BaCl_2.2H_2O$, EDTA, $MgCl_2.6H_2O$, $FeCl_2.6H_2O$, $ZnSO_4.7H_2O$, $CuSO_4.5H_2O$, $MgSO_4.7H_2O$, and $NiSO_4.H_2O$ at concentrations of 1 and 5 mM on the enzyme activity were determined according to the method of Cui et al. (2007).The percentage of activity that remained after incubation with different chemicals was used to calculate the relative activity.

2.3.5. Thermal stability

Purified MCE extract aliquot was heated for 15, 30, 45, and 60 minutes in a water bath set at various temperatures between 30 and 100°C. It was then quickly cooled to 37 °C and examined rapidly for any remaining enzyme activity according to El-Bendary *et al.* (2007)

2.4. Cheese making

Standardized buffalo's milk (~4.13% total protein and ~4.2% fat, 1:1) was heated to 75°C for 30 sec and then cooled to 38°C. It was inoculated with starter culture at a rate of 1% (w/w) and then held for 30 min. Cheese milk was divided into three equal portions, and each portion received one of the following three types of rennet to achieve coagulation within a period of 40 min: 1.9, 1.7, and 2.8 ml/L of calf, microbial, and cumin MCE, respectively. After coagulation, the curd was cut and transferred to a mold, where it was left to rest overnight at room temperature. Cheese blocks were cut and weighed, and cheese yield was calculated as the weight of finished cheese divided by the weight of milk used. The resultant soft cheeses were individually covered in plastic wrap in brine with 13% salt (1:2) and analyzed during storage at 5±2°C for 1, 4, and 8 weeks.

2.4.1. Chemical analysis

Total solids, total nitrogen (TN), and fat content of brined soft cheese were determined according to AOAC methods (2012). The protein content was calculated by multiplying the percentage of TN by 6.38. The concentration of sodium chloride (salt) in the soft cheese was measured using the Volhard method (Michael Wehr and Frank, 2004). Cheese samples were measured for pH using a pH-meter 646 with glass electrodes (Ingold, Knick, Germany). Water-soluble nitrogen (WSN/TN ratio) was determined according to a method described by Hassan *et al*. (2020). A mixer (Heidolph No. 50 111, Type RZRI, Germany) was used to mix 20 g of cheese samples with 100 ml of distilled water at 40°C for 5 min on speed setting 10. The extract was filtered through filter paper Whatman No. 40, and the filtrate was used for the determination of WSN using the Kjeldahl method. The WSN/TN ratio was considered an indicator of cheese proteolysis.

2.4.2. Antioxidant activities

Antioxidant activity of cheese samples was estimated in cheese supernatant, prepared by the method of Hassan *et al.* (2020), using stable DPPH- and ABTS radical assays developed by Brand-Williams *et al.* (1995) and Re *et al.* (1999), respectively. Briefly, 10 g of cheese sample was mixed with 10 ml of distilled water, and the cheese mixtures were then incubated at 40 °C for 1 h. The extracts were then separated by centrifugation at 4000 xg for 5 min. 100 µL of diluted extract was added to 3.9 ml of either the ABTS working solution (7 mM ABTS solution with 2.45 mM $K_2S_2O_8$) or the DPPH working solution (25 mg DPPH/L methanol). After 30 sec of vortexing and 30 min of dark incubation at room temperature, the degree of decolorization was measured using a Shimadzu

spectrophotometer (UV-Vis. 1201, Japan) at 517 nm for the DPPH and 734 nm for the ABTS radical-scavenging activity assays. In the same manner as the assay mixture, control solutions—DPPH and ABTS without cheese extract—were prepared. For both ABTS and DPPH scavenging activities, the formula shown below was used:

$$RSA\ (\%) = \frac{A_0 - A_1}{A_0} \times 100 \quad (2)$$

A_0 is the absorbance of the control (DPPH or ABTS solution), and A_1 is the absorbance of the sample.

2.4.4. Texture profile analysis

The brined soft cheese samples were subjected to texture profile analysis (TPA) using a texture analyzer (TA-XT2 Texture Analyzer, Texture Technologies Crop, Scarsdale, NY) that was linked to a PC running texture analysis software. An artificial plastic cylinder (45 Perspex Cone, 432-081) was installed on the moving crosshead. The plastic cylinder was inserted 20 mm below the surface of the cheese sample, which was placed on a flat holding plate at 5± 2°C, and the penetration speed was set at 70 mm/min. The hardness (N), cohesiveness, adhesiveness, and gumminess (N) were calculated from the obtained TPA according to the definition given by the IDF (1991).

2.4.5. Sensory evaluation

The cheese samples were evaluated by a panel of staff members from the Dairy Department, National Research Center, Egypt. A nine-point hedonic scale, ranging from extremely like (9), through like or dislike (5), to extremely dislike (1), was used to score the cheese samples as described by Wadhwani and McMahon (2012). Cheese samples were cut into 1.5 x 1.5 x 1.5 cm cubes and covered in plastic to prevent dehydration. Three-digit random numbers were utilized to code the cubes. Cheese samples were kept at 20°C for a least of an hour to provide for equilibration. Three cheese cubes were provided for each judge's sample. Between tastings, water and unsalted crackers were given to keep their palates fresh.

2.5. Statistical analysis

For statistical analysis (mean of three replicates), statistical analyses system user's guide (SAS Institute, Version 2008, Cary, North Carolina, U.S.A.) was used. Duncan's multiple comparison procedure was used to compare the means. A probability of ≤ 0.05 was used to establish statistical significance.

3.Results and Discussion

3.1.Cumin enzyme extract

3.1.1.Activity of crude enzyme extract

Three 0.1 *M* buffer solutions were used: acetate pH 5.0, phthalate-sodium hydroxide pH 5.0, and citrate pH 5.0, in addition to distilled water pH 6.8, to select the most suitable solution for extracting milk-clotting enzyme (MCE) from cumin seeds (*Cuminum cyminum* L.). The milk clotting activity (MCA), specific MCA, protein content, proteolytic activity, and specific MCA/PA ratio of crude MCE extracts are presented in Table 1.

Table 1. Milk clotting activity (MCA) characteristics of crude cumin milk-clotting enzyme (MCE) extracted with different buffering solutions.

Items	Type of buffer solutions			
	Water	Acetate	Phthalate-NaOH	Citrate
pH	6.80	5.00	5.00	5.00
MCA (U/ml)	94.7	112.6	107.1	158.1
PA (U/ml)	1.13	2.04	1.35	1.48
PC (mg/ml)	0.019	0.022	0.017	0.014
Specific MCA (U/mg)	4992	5117	6299	11295
Specific PA (U/mg)	59.47	92.72	79.41	105.70
Specific MCA/PA ratio	83.94	55.19	79.32	106.80

MCA, milk clotting activity; PA, proteolytic activity; PC, protein content

The MCE extracted with 0.1 *M* citrate pH 5.0 exhibited the highest MCA, specific MCA, and specific MCE/PA ratio, which are equivalent to 158.13 U/ml, 11295, and 106.8, respectively. The acetate buffer solution displayed a high MCA (112.6 U/ml) and specific MCA (5117), and the phthalate-sodium hydroxide buffer solution, which also showed a high MCA (107.1) and specific MCA (6299), while the least of them was the enzyme extraction by distilled water. These findings were contradicted by Guiama *et al.* (2010), who found that extraction using 5 % NaCl in 50 m*M* acetate buffer (pH 5.0) yielded more MCA than distilled water. In a different study, Kholif and Hamed (2022) found that MCE extracted from the *Solanum elaeagnifolium* plant using sodium phosphate buffer pH 5.9 showed the maximum MCA. Additionally, Kholif *et al.* (2024) noted that, in comparison to other buffers, phosphate buffer solution had the highest activity and the maximum extraction of MCE from purslane. This suggests that the type of plant and the characteristics of plant proteins—such as differences in protease structure and their

location in plant tissues—determine the most suitable extraction solution.

3.2.Purification steps of MCE

A simple purification method used to obtain a highly stable and active MCE from cumin seed extract. In the initial stage of enzyme purification, 50 ml of crude cumin extract (extracted in 0.1 *M* citrate buffer pH 5.0) was purified using different ammonium sulfate concentrations (0–90% saturation). MCA (282.3 U/ml), specific activity (12272), total activity (2822.6 U/ ml), yield (35.69 %), and rate purification (1.09) for MCE were all maximum at 50% saturation, according to the data presented in Table 2. These results are in line with those of Kholif *et al*. (2016), who found that the highest MCA and specific activity occurred at ammonium sulfate concentrations ranging from 30 to 50%. Nasr *et al*. (2016) found that sunflower (*Helianthus annus*) seeds extracts had the highest MCA levels, between 30 and 50% ammonium sulfate concentrations. According to Arbita *et al.* (2024), the *Gracilaria edulis* algal extract exhibited optimum saturation with ammonium sulfate at a 50% concentration. However, after the MCE precipitated by ammonium sulfate, a dialysis bag was used to reduce proteolytic activity (PA), which causes curd patronization and off-flavor, particularly a bitter taste in cheese making. The MCA, yield, and purification rate were 262.67 U/ml, 26.57%, and 1.16, respectively. Finally, a Sephadex G-100 column equilibrated with 0.1 *M* citrate buffer pH 5 was loaded with the dialyzed pooled fraction. The purification results, as displayed in Table 2 and Figure 1, showed only one peak with the greatest MCE concentration (at fraction 29), with a rate purification of 5.63, a yield of 8.044 %, and a specific MCA of 63605 U/mg. The results are consistent with those of Kholif *et al.* (2024), who discovered that a purslane extract contained one diffuse protein band and just one peak. Nevertheless, Abdalla *et al.* (2010) found that two peaks containing PA were eluted from the purification of papaya and *Jacaratia corumbensis*. The primary causes of the peak number fluctuation are different protein contents or changing separation conditions.

Table 2. Milk clotting enzyme from Cumin during purification steps extract using ammonium sulfate, dialysis bag and gel filtration sephadex G-100

Items	Purification steps			
	Crude extract	Ammonium sulfate (50%)	Dialysis bag	Sephadex G-100
Volume (ml)	50.0	10.0	8.0	5.0
MCA (U/ml)	158.1	282.3	262.7	127.2
PC (mg/ml)	0.014	0.023	0.02	0.002
Specific MCA (U/mg)	11295	12272	13133	63605
Total activity	7906.5	2822.6	2101.4	636.1
Total protein	0.70	0.23	0.16	0.01
Yield (%)	100.0	35.69	26.57	8.04
Rate purification	1.00	1.09	1.16	5.63

MCA, milk clotting activity; PC, Protein content

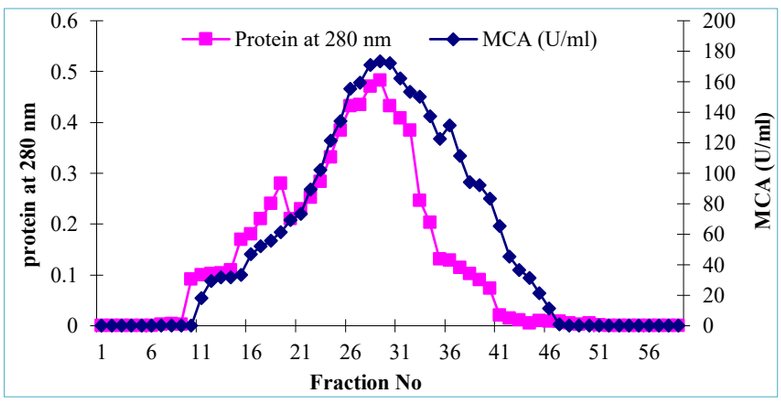

Figure 1. Gel filtration for the chromatography of cumin MCE extract on a Sephadex G-100 column (40 x 2.5 cm). The column was equilibrated with 0.1 *M* acetate buffer (pH 5.0) at a flow rate of 0.7 ml/min and 5 ml fractions.

3.3.Characterization of purified MCE

3.3.1.Optimum pH and temperature

The pH and temperature have a major effect on activity of the enzymes. The extracted enzyme maintained its activity between pH 4.0 and pH 7.0 and then gradually dropped at higher pH values, as shown in Figure 2. At pH 10, the enzyme exhibited no activity. The highest MCA

was obtained at pH 6.0 and these agreements with Amer *et al.* (2022) and Zhang *et al*. (2023) mention that the optimal pH is 6 for MCE from *A. lebbeck* seeds and *Bacillus velezensis*, respectively. The decrease in enzyme activity is caused by changing the acidic to basal medium, which affects the enzyme activity. On the other hand, the activity of enzyme increased with temperature up to 50°C, then began to slowly decrease until it reached 60°C. After that, it sharply declined above 60°C, and heating it to 90°C rendered it completely inactive because the protein became thermally denaturated, which prevented the enzyme from binding to the substrate. Similar behavior of optimum pH and temperature was found by Kholif *et al.* (2016) and Narwal *et al.* (2016) for MCE obtained from fruit seeds of *Solanum elaeagnifolium* and *Bacillus subtilis* MTCC 10422. Mamo *et al.* (2022) reported that MCE extracted from *Aspergillus oryzae* DRDFS13 exhibited optimal activity at pH 5 and stability at 35–45°C. El-Sayed *et al.* (2013a) states that extracted MCE from *Streptomyces pseudogrisiolus* NRC 15 also has an ideal pH of 6.5 and a temperature of 45°C. On the contrary, the coagulant enzyme from noni seeds (*Morinda citrifolia* L.) preferred a temperature of 60°C, according to de Oliveira and Junio (2022). Furthermore, Wang (2009) discovered that the MCE generated by Nocardiopsis sp. was most active at 55°C. In this respect, this enzyme is better than a large number of others that have been documented in the literature, many of which are most active at high temperatures.

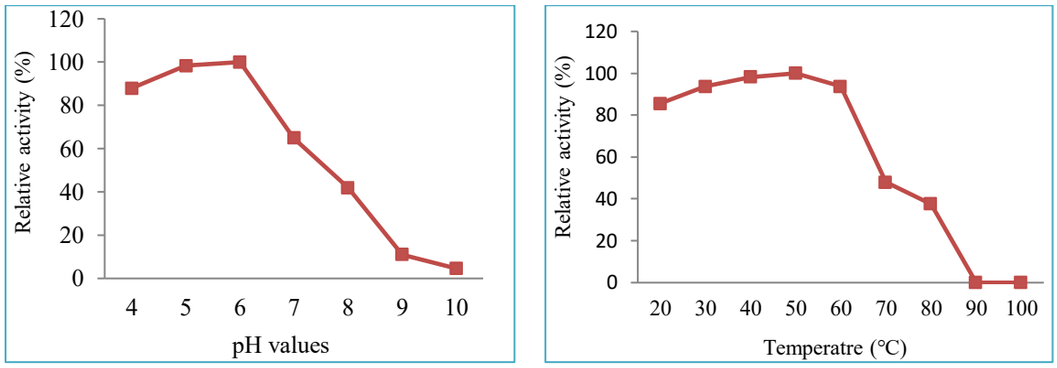

Figure 2. Effect of pH value and temperature degree on the MCA of purified cumin MCE extract.

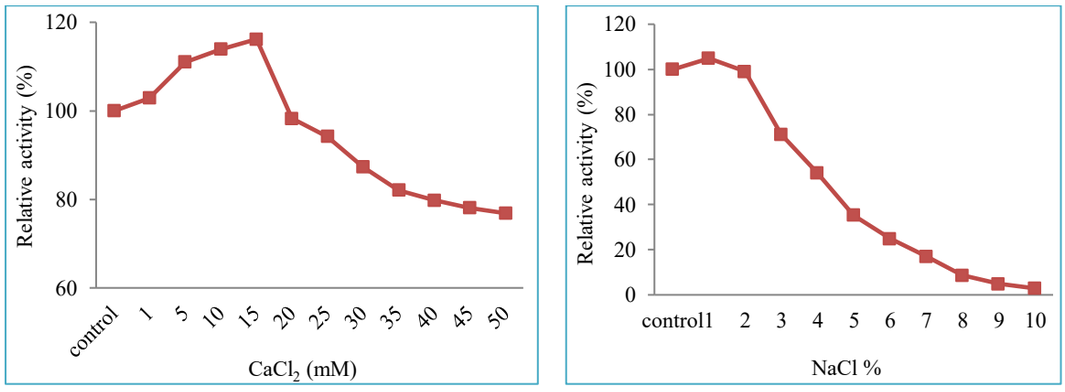

Figure 3. Effect $CaCl_2$ and NaCl concentrations on the MCA of purified cumin MCE extract.

3.3.2. Effect of $CaCl_2$ and NaCl concentrations

The addition of calcium chloride ($CaCl_2$) to milk prior to curdling was found to favor not only the rate of reaction but also the extraction of clear whey; appropriate concentrations of $CaCl_2$ may be added. Also, sodium chloride (NaCl) is usually used during the process of cheese manufacture because it protects milk against spoilage by various microorganisms. As shown in Figure 3, the activity of the purified MCE increased on addition of $CaCl_2$, up to 15 mM, or NaCI, up to 1 %, and thereafter decreased on further addition of either. The purified MCE lost around 45 % of its activity at a concentration of 4 % NaCl and became inactive at 10 % NaCl. However, the enzyme MCA extracted from *A. lebbeck* seeds and *Bacillus velezensis* was activated by the addition of $CaCl_2$ up to 30 m*M* (Amer *et al*., 2022 and Zhang *et al.* (2023). Calcium not only creates iso-electric conditions but also forms ion bridges between the phosphate moieties of casein micelles (Sun *et al*., 2014). However, MCA decreased at concentrations ≥ 20 m*M*, probably due to the increase in ionic force or the saturation of negative residues in micelles at increasing Ca^{+2} concentrations (Vairo-Cavalli *et al*., 2005). Regarding NaCl, coagulation time increased and MCA reduced as the concentration of NaCl increased (Abdalla *et al*., 2010).

3.3.3. Effect of some metal ions

The enzyme converts k-casein in milk to para-casein in the first stage, and in the second

stage, para-casein in the presence of calcium ions gives a firm clot. Other divalent cations, like magnesium, are also known to cause coagulation. The effect of various monovalent and divalent ions on the activity of MCE in the presence of chlorides and sulfates at concentrations of 1 and 5 mM is shown in Figure 4. In general, different metal ions have different effects on the activity of the pure MCE extract. There was an increase in the activity of the purified MCE extract when certain mineral ions, such as Ba^{2+}, Mg^{2+}, and Fe^{2+}, were added at concentrations of 1 and 5 mM. However, Mn^{2+}, EDTA, Zn^{2+}, Cu^{2+}, Mg^{2+}, and Ni^{2+} were shown to inhibit the MCE extract; the rate of inhibition increased as the ion concentration increased. The enzyme was most inhibited by $Mg^{2+}$and Ni^{2+}at 5 mM, which caused the MCA to less than double. Similarly, Amer *et al*. (2022) reported that the Fe^{2+} and Ba^{2+} led to the activation of MCE from the *A. lebbeck* seeds, and Ni^{2+} and Cu^{2+} led to the inhibitor MCE. Our findings are in contrast to those of El-Bendary *et al*. (2007), who found that Fe^{2+} ions had no effect on the enzyme's activity. Zhang *et al*. (2023) showed that Mn^{2+} significantly increased the *Bacillus velezensis* MCA to about 128 % at 0.25 M, and Mg^{2+} had no influence on *Bacillus velezensis* MCE.

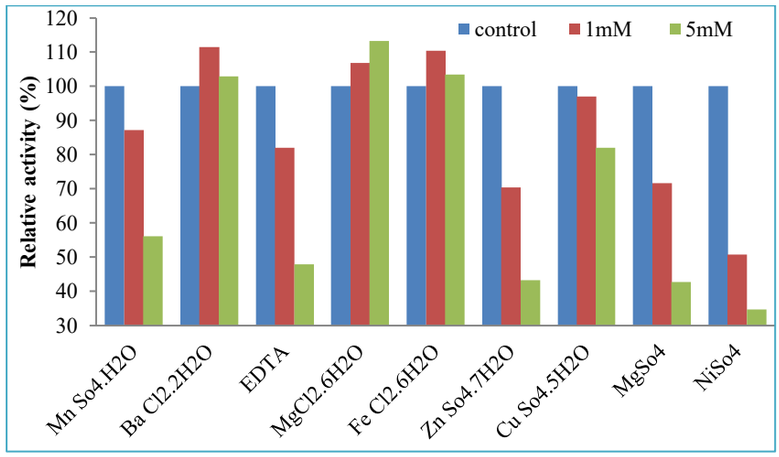

Figure 4. Effect of different metal ions on the MCA of purified cumin MCE extract.

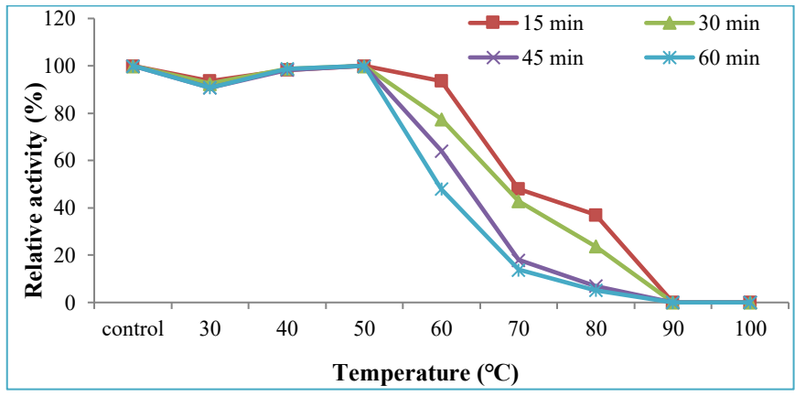

Figure 5. Thermal stability of the purified cumin MCE extract on different heat temperatures.

3.3.4.Effect of thermal stability

Thermal stability is one of the most important properties of the coagulate enzyme, due to its importance in cheese making. In Figure 5 shows the thermal stability of purified MCE after 15–60 min of incubation at temperatures between 30 and 100°C. The MCA was more stable at all incubation times (15, 30, 45, and 60 min) up to 50°C, and it slightly decreased at 60°C when incubated for 15 min. Over 60°C, the MCA sharply decreased, and at 90°C, it was completely inhibited. Moreover, the incubation period was proportionate to the rate of inhibition. Kholif *et al.* (2024) found that the coagulate enzyme activity from the *Portulaca oleracea plant* is stable at temperatures from 30 to 60 °C on all incubation times. Inversely, Bakr *et al*. (2022) mentioned that the activity of MCE from edible mushrooms decreased with increasing temperature and time. The calf rennet is stable up to 50°C and loses its activity above 60 °C (Kumar *et al*., 2005). El-Sayed *et al.* (2013a) showed that heating isolated MCE from *Streptomyces pseudogrisiolus NRC* 15 to 45 °C for 20 min reduced its activity by 20 %, while heating it to 60°C for 20 min completely deactivated it. According to El-Sayed *et al.* (2013b), the purified MCE from *Brassica napus* seeds could withstand heating to 40°C for 120 min without showing any signs of activity loss. But when the enzyme was heated to 60 °C, its activity dropped to just 8 %, while at higher temperatures (70 °C), complete denaturation of the enzyme was observed.

3.5.Application of purified MCE in brined soft cheese making

3.5.1.Cheese yield and protein recovery

Table 3 shows the effects of using MCE extracted from cumin seeds on the soft cheese yield (%) and protein recovery (%) in comparison to calf and microbial rennet. The yield and protein recovery of soft cheese made with purified cumin extract (SC-CE) did not differ significantly (P≤0.05) from that made with microbial (SC-MR) and calf rennet (SC-CR).The numerical difference in cheese yield may be related positively with the moisture content (Table 4). However, a decrease in protein recovery was slightly less in BSC-CE than in BSC-MR and BSC-CR. These findings were incompatible with those of Khan *et al.* (2013), who reported that cheese made with MCE from plant sources had a significant loss of protein due to excessive hydrolytic activities. Mazorra-Manzano *et al.* (2013) found the curd yield obtained using extracts of kiwi (17.8%), melon (15.1%), and ginger (15.4%) was less than that obtained with commercial rennet (20.2%). Protein recovery ranged between 84.43±1.63 and 85.92±2.34 % when using MCE extract from cumin plant and calf rennet, respectively.

Table 3. Yield and protein recovery of soft cheese made with purified cumin MCE compared to calf and microbial rennet.

Items	Soft cheese mad with different MCE		
	SC-CR	SC-MR	SC-CE
Yield (%)	25.15^{A}±0.71	24.74^{A}±0.63	25.86^{A}±0.37
Protein recovery (%)	85.92^{A}±2.34	85.20^{A}±1.89	84.43^{A}±1.62

Means (n=3 ±SE) with the same capital letters within the same row are not different significantly at P ≤ 0.05; MCE, milk-clotting enzyme; SC-CR, soft cheese made with calf rennet; SC-MR, soft cheese made with microbial rennet; SC-CE, soft cheese made with purified cumin extract

Table 4. Composition of brined soft cheese made with purified cumin MCE compared to calf and microbial rennet during storage at 5±2°C for 8 weeks.

Items	Storage period (week)	Brined soft cheese mad with different MCE		
		BSC-CR	BSC-CR	BSC-CR
pH	1	5.81^{Aa}±0.22	5.91^{Aa}±0.12	5.76^{Aa}±0.21
	5	5.64^{Aa}±0.11	5.66^{Aa}±0.09	5.63^{Aa}±0.11
	8	5.16^{Ab}±0.20	5.08^{Ab}±0.17	4.96^{Ab}±0.19
Moisture (%)	1	61.57^{Aa}±0.78	60.46^{Aa}±0.66	62.79^{Aa}±1.01
	4	63.94^{Aab}± 0.73	61.98^{Aba}±0.51	64.12^{Aab}±0.59
	8	64.45^{Bb}±0.95	63.64^{Bb}±0.67	67.73^{Ab}±0.88
Proteins (%)	1	13.94^{Aa}±0.77	14.05^{Aa}±0.16	13.42^{Aa}±0.22
	5	13.77^{Aa}±0.23	13.99^{Aa}±0.41	13.02^{Aa}±0.44
	8	12.83^{Aa}±0.35	12.98^{Aa}±0.27	11.84^{Ab}±0.18
WSN/TN ratio (%)	1	6.88^{Aa} ±0.41	7.72^{Aa}±0.46	8.21^{Aa}±0.56
	5	12.43^{Ab}±0.88	13.65^{Ab}±1.04	14.61^{Ab}±0.62
	8	19.81^{Bc}±0.42	19.15^{Bc}±1.01	24.59^{Ac}±0.94
Fat (%)	1	15.95^{Aa}±0.11	16.25^{Aa}±0.10	15.25^{Aa}±0.31
	5	15.50^{Aa}±0.08	15.75^{Ab}±0.17	14.65^{Bb}±0.13
	8	14.90^{Bb}±0.12	15.50^{Ab}±0.28	13.75^{Cc}±0.19
Salt (%)	1	3.44^{Aa}±0.18	3.43^{Aa}±0.13	3.56^{Aa}±0.14
	5	3.51^{Aa}±0.10	3.47^{Aa}±0.15	3.59^{Aa}±0.12
	8	3.59^{Aa}±0.16	3.51^{Aa}±0.14	3.73^{Aa}±0.09

Means (n=3 ±SE) with the same capital letters within the same row and the same small letters within the same column are not different significantly at P ≤ 0.05; BSC-CR, brined soft cheese made with calf rennet; BSC-MR, brined soft cheese made with microbial rennet; BSC-CE, brined soft cheese made with cumin extract; WSN/TN, water-soluble nitrogen/total nitrogen

3.5.2.Changes in cheese composition

The chemical composition of brined soft cheese made with purified cumin extract (BSC-CE) compared to that made with calf rennet (BSC-CR) or microbial rennet (BSC-MR) during 8 weeks of storage at 5±2°C is presented in Table 4. The type of MCE used may have an impact on the composition of brined soft cheese during cold storage. In particular, BSC-CE had higher moisture content and a higher water-

soluble nitrogen/total nitrogen ratio (WSN/TN ratio) than BSC-CR and BSC-MR; the difference was significant only after 8 weeks of cold storage ($P < 0.05$). Protein, fat, and salt contents, as well as pH values, did not differ significantly between the brined soft cheese treatments ($P > 0.05$). During cold storage, the pH steadily decreased while the WSN/TN ratio and moisture content steadily increased. The pH reduction was statistically significant only at week 8 of cold storage ($P = 0.021$). The increase in the WSN/TN ratio was significant at weeks 4 and 8 ($P < 0.001$) in all cheese treatments but was more noticeable in BSC-CE. Similarly, in ginger extract-fortified cheese, Abd El-Aziz *et al.* (2012) found that the effect of ginger extract on protein proteolysis was more pronounced at week 6. The proteolytic activity of the starter bacteria, non-starter bacteria, and residual coagulant may be responsible for the increase in the WSN/TN ratio (Hassan *et al.*, 2020). Soltani *et al.* (2019) reported that the residual coagulant activity was influenced by the type and concentration of the coagulant as well. The high WSN/TN ratio in BSC-CE might be caused by the nonspecific activity of plant proteases against caseins, which produce peptides that are readily hydrolyzed by proteases of starter culture into free amino acids or low molecular weight peptides (Nicosia *et al.*, 2022). Additionally, only the BSC-CE showed a statistically significant increase in moisture content during cold storage at week 8 ($P = 0.047$). As moisture content increased, salt increased, but protein and fat content decreased; the changes were not statistically significant. A similar trend was found by Abd El-Aziz *et al.* (2015) when UF-white soft cheese was stored in a brine solution containing ginger extract. A decrease in protein-protein interactions at low temperatures could be the reason for the variations in moisture during storage. McMahon *et al.* (2009) observed an increase in cheese weight after brining at 3, 6, or 10°C, while it decreased at 22°C. The cheese matrix contracted and lost 13 to 18 g/100 g of weight at 22°C, while it expanded by 20 to 30% at lower temperatures. Thus, the cheese matrix may have expanded more with cumin MCE extract compared with either calf or microbial rennet at the 8th week of storage at 5±2°C.

3.5.3.Changes in cheese texture profile

The effects of MCE type on the textural profile attributes of brined soft cheese (hardness, cohesiveness, adhesiveness, and gumminess) during the period of 8 weeks of cold storage are displayed in Table 5. BSC-CE showed less hardness, cohesiveness, and gumminess when compared to BSC-CR and BSC-MR. Significant differences were found for hardness at week 8 ($P = 0.007$) and gumminess at weeks 1 and 8 ($P = 0.0013$), but not for cohesiveness ($P > 0.05$). Inversely, the adhesiveness values were the highest in BSC-CE; however, the variations were not significant ($P \leq 0.05$). Similar findings were obtained by Nájera-Domínguez *et al.* (2022) in milk gels made with *Solanum elaeagnifolium* proteases and Abd El-Aziz *et al*. (2015) in UF-white soft cheese stored in a brine solution containing ginger extract. Darwish (2016) also found that the hardness, gumminess, and adhesiveness of Domiati cheese made with plant proteases derived from *Abizia lebbeck* and *Helianthus annuus* seeds were lower than those made with chymosin. Conversely, other research has shown that the soft cheese made with plant extract had a firmer texture profile than the control sample (García *et al*., 2012; Abebe and Emire, 2020). During cold storage, cheese hardness, cohesiveness, and gumminess in all soft cheeses decreased, but adhesiveness decreased as the time increased. Statistical analysis showed the decrease in cheese hardness and gumminess was significant after 4 weeks of cold storage, but the increase in adhesiveness was significant at week 8. The cheese hardness was found to be negatively correlated with moisture content ($r^2 = -0.48$) and WSN/TN ratio ($r^2 = -0.75$). According to Martínez-Ruiz *et al.* (2013), Asadero cheese, in northern Mexico, made with MCE isolated from *Solanum elaeagnifolium* berries and stored at 4–6°C for 28 days, was softer than that made with chymosin due to its higher moisture content and proteolysis. Fathollahi *et al.* (2010) found that proteolysis can contribute to textural softening during ripening of Iranian UF white cheese. However, some other works reported Ca solubilization as a main factor of some cheese softening.

3.6.Antioxidant activity

Figure 6 shows the antioxidant activity of brined soft cheese against DPPH and ABTS radicals as affected by the type of MCE. Antioxidant activity against DPPH radicals in brined soft cheese was not significantly affected by the type of MCE at weeks 1 and 4. In comparison to BSC-MR and BSC-CR, BSC-CE showed greater antioxidant activity after week 8; the difference was only significant between BSC-CE and BSC-CR ($P < 0.001$). However, BSC-CE showed stronger antioxidant activity against ABTS radicals at weeks 1, 4, and 8 compared to BSC-CR ($P < 0.001$), but it also showed stronger antioxidant activity at weeks 1 and 8 compared to BSC-MR. A number of factors, such as the kind of protease, the degree of hydrolysis, and the amino acid contents and sequences, influence the antioxidant activity of cheese (Wu *et al.,* 2012). Over the cold storage period, there was a gradual increase in antioxidant activity of all brined soft cheese against DPPH and ABTS free radicals ($P < 0.001$); the increase was more pronounced in BSC-CE. These results were consistent with those of Yasar and Kose (2024) in Malatya cheese that was ripened in dry salted polyethylene bags or in brine using plastic drums

at 7 and 20°C for 120 days. Hassan *et al.* (2020) found that when cheese proteolysis increased, brined soft cheese's antioxidant activity increased significantly ($p < 0.05$). According to Yasar and Kose (2024), ripening causes an increase in the forming of water-soluble peptides, which in turn increases the antioxidant activity of cheese.

Table 5. Texture profile of brined soft cheese made with purified cumin MCE compared to calf and microbial rennet during storage at 5±2°C for 8 weeks.

Items	Storage period (week)	Brined soft cheese mad with different MCE		
		BSC-CR	BSC-CR	BSC-CR
Hardness (N)	1	14.10^{Aa}±0.98	14.22^{Aa}±1.21	12.80^{Aa}±0.67
	5	10.08^{Ab}±0.45	10.77^{Ab}±0.73	9.11^{Ab}±0.59
	8	8.73^{ABb}±0.82	9.75^{Ab}±1.25	7.15^{Bb}±0.75
Cohesiveness	1	0.68^{Aa}±0.06	0.66^{Aa}±0.09	0.65^{Aa}±0.09
	5	0.70^{Aa}±0.05	0.72^{Aa}±0.05	0.67^{Aa}±0.08
	8	0.63^{Aa}±0.14	0.65^{Aa}±0.13	0.62^{Aa}±0.13
Adhesiveness	1	0.23^{Ab}±0.06	0.19^{Ab}±0.03	0.29^{Ab}±0.02
	5	0.29^{Ab}±0.02	0.27^{Ab}±0.03	0.33^{Ab}±0.04
	8	0.51^{Aa}±0.07	0.52^{Aa}±0.08	0.62^{Aa}±0.08
Gumminess (N)	1	9.83^{Aa}±0.24	9.34^{Aa}±0.15	7.61^{Ba}±0.39
	5	7.92^{Ab}±0.41	7.35^{Ab}±0.75	6.63^{Aab}±0.47
	8	7.42^{Ab}±0.38	7.22^{Ab}±0.57	5.97^{Bb}±0.31

Means (n=3 ±SE) with the same capital letters within the same row and the same small letters within the same column are not different significantly at $P \leq 0.05$; BSC-CR, brined soft cheese made with calf rennet; BSC-MR, brined soft cheese made with microbial rennet; BSC-CE, brined soft cheese made with cumin extract.

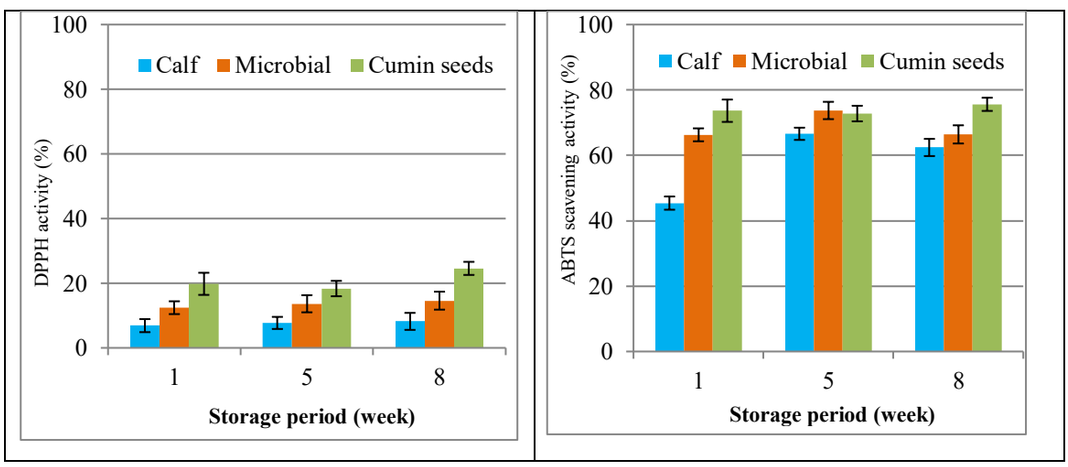

Figure 6. Antioxidant activity of brined soft cheese made with purified cumin MCE compared to calf and microbial rennet during storage at 5±2°C for 8 weeks.

3.7.Sensory attributes

Sensory attributes of brined soft cheese, which were evaluated with a nine-point hedonic scale during storage at 5±2ºC for 8 weeks, are shown in Table 6. After, weeks 1 and 4 of cold storage, statistical analysis showed no significant variations in appearance, flavor and body & texture among BSC-CE, BSC-MR, and BSC-CR. However, BSC-CE had numerically higher sensory scores than BSC-MR and BSC-CR ($P < 0.05$). BSC-CR had a softer body, smoother texture, and a more palatable flavor without being bitter. Similar results were obtained by Kholif and Hamed (2022) in white soft cheeses made with purified *Solanum elaeagnifolium* seed extract against animal and microbial rennet, and Bruno *et al*. (2010) in the production of cheese using *Bromelia hieronymi* fruit extract that did not exhibit a bitter taste. Afsharnezhad *et al.* (2019) also found that the cheese produced using the pure MCE extracted from *Ficus johannis* had textural characteristics that were comparable to those of the cheese made with commercial calf rennet. After 8 weeks, BSC-CE had the lowest body & texture scores (very soft); the difference was only significant when compared to BSC-CR. However, the appearance and flavor scores of BSC-CE only started to decline at week 8, and the difference was not statistically significant.

Table 6. Sensory attributes of soft cheese made with purified cumin MCE compared to calf and microbial rennet during storage at 5±2°C for 8 weeks.

Items	Storage period (week)	Brined soft cheese mad with different MCE		
		BSC-CR	BSC-CR	BSC-CR
Appearance	1	8.36^{Aa}±0.15	8.27^{Aa}±0.14	8.45^{Aa}±0.21
	5	8.45^{Aa}±0.16	8.36^{Aa}±0.20	8.36^{Aa}±0.24
	8	8.10^{Aa}±0.21	8.00^{Aa}±0.27	7.63^{Ab}±0.15
Flavor	1	8.09^{Aa}±0.23	7.91^{Aa}±0.25	8.27^{Aa}±0.19
	5	8.36^{Aa}±0.15	8.27^{Aa}±0.14	8.45^{Aa}±0.16
	8	8.10^{Aa}±0.18	8.00^{Aa}±0.23	7.63^{Ab}±0.15
Body & texture	1	7.71^{Aa}±0.22	7.81^{Aa}±0.22	8.18^{Aa}±0.18
	5	8.18^{Aa}±0.20	7.18^{Aa}±0.18	8.45^{Aa}±0.16
	8	8.18^{Aa}±0.18	7.91^{ABa}±0.21	7.45^{Bb}±0.16

Means (n=3 ±SE) with the same capital letters within the same row and the same small letters within the same column are not different significantly at $P \leq 0.05$; BSC-CR, brined soft cheese made with calf rennet; BSC-MR, brined soft cheese made with microbial rennet; BSC-CE, brined soft cheese made with cumin extract.

4.Conclusion

It can be concluded that cumin seeds contain a type of protease (MCE) that can coagulate milk and proteolytic activity in a way that is similar to calf and microbial rennet; there is no clear difference between cheese yield and protein recovery. Purified cumin MCE also showed the highest activity when the pH was 6 and the temperature was between 40 and 50 °C, which are the ideal temperatures for the production of most types of cheese. Soft cheese made with cumin MCE (BSC-CE) was softer, smoother, and more palatable than the calf and microbial rennet-induced cheese when stored in brine solution for 5 weeks at 5±2 °C. However, high proteolysis and partial BSC-CE dissolution were observed when the brine storage duration was prolonged beyond 5 weeks at 5±2 °C. Consequently, the cumin MCE can be effectively substituted for traditional rennet in the production of fresh soft cheese or soft cheese that has been brined for a maximum of 5 weeks at 5±2 °C. Finally, further studies are required to identify the type of protease and confirm the properties of cumin MCE, particularly if soft cheese is stored in brine at temperatures over 5±2 °C. It can also be used to accelerate the maturation of semi-hard and hard cheese.

5.References

Abd El-Aziz, M., Mohamed, S.H.S. and Seleet, F.L. (2012). Production and evaluation of soft cheese fortified with ginger extract as a functional dairy food. *Polish Journal of Food and Nutrition Sciences,* 62 (2), 77-83. https://doi.org/10.2478/v10222-011-0046-0

Abd El-Aziz, M., Mohamed, S.H.S., Seleet, F.L. and Abd El-Gawad, M.A.M. (2015). Effect of brine solution containing ginger extract on the properties of Egyptian white cheese. *American Journal of Food Technology*, 10 (1), 37-47. https://doi.org/10.3923/ajft.2015.37.47

Abdalla, M.O.M., A.A.D. Ali and E.B. Mohamed, 2010. Extraction, milk-clotting activity measurements and purification of Solanum dubium Fresen (Gubbain) for cheesemaking. *World J. Dairy Food Sci.,* 5(2): 152-159. https://www.idosi.org/wjdfs

Abebe, B. and Emire, S. (2020). Manufacture of fresh cheese using east African Calotropis procera leaves extract crude enzyme as milk coagulant, Food Science & Nutrition, 8, 4831-4842. https://doi.org/10.1002/fsn3.1765

Afsharnezhad, M., Shahangian, S.S., Sariri, RA. (2019). A novel milk-clotting cysteine protease from *Ficus johannis*: Purification and characterization. *International Journal of Biological Macromolecules,* 121, 173–182. https://doi.org/10.1016/j.ijbiomac.2018.10.006

Ahmed, S.A. and Helmy, W.A. (2012). Comparative evaluation of *Bacillus licheniformis* 5A5 and *Aloe variegata* Milk-clotting enzyme. *Brazilian Journal of Chemical Engineering,* 29, 1, 69–76. https://doi.org/10.1590/s0104-66322012000100008

Allaq, A.A., Sidik, N.J., Abdul-Aziz, A. and Ahmed, I.A. (2020). Cumin (*Cuminum cyminum* L.*):* A review of its ethnopharmacology, phytochemistry. *Biomedical Research and Therapy,* 7(9), 4016-4021. https://doi.org/10.15419/bmrat.v7i9.634

Amer, A.G., Omar, M.A and Abdrabou, N.S (2022). Screening of some Egyptian plants for milk clotting activity. *Al-Azhar Journal of Agricultural Research*, 47 (1), 241-252. https://doi.org/10.21608/ajar.2022.266506

AOAC (2012). Official methods of analysis, 19th ed. Method 947.05. Association of Official Analytical Chemists, Washington.

Arbita, A.A., Paul, N.A., Cox, J. and ·Zhao, J. (2024). Screening and isolation of milk clotting enzymes from seaweed. *Journal of Applied Phycology*, 36:887–896. https://doi.org/10.1007/s10811-023-02975-4

Arima, K., Ya, J. and Iwasaki, S. (1970). Milk-clotting enzyme from *Mucor pusillus* var. *Lindt*. In: E. G. Pearlman & L. Lorand (Ed.), Methods in Enzymology (pp. 446–459). New York: Academic Press. https://doi.org/10.1016/0076-6879(70)19033-1

Bakr, A., Ibrahim, O., El-Ghandour, A and El-Deeb, N. (2022). Purification and characterization of milk-clotting enzyme from edible mushroom (*Pleurotus florida*). *Letters in Applied NanoBioScience* 11, 2, 3362–3373. https://doi.org/10.33263/lianbs112.33623373

Ben Amira, A., Besbes, C. S., Attia, H. and Blecker, C. (2017). Milk-clotting properties of plant rennet's and their enzymatic, rheological, and sensory role in cheese making: A review. *International Journal of Food Properties*, 20 (Supplement 1), S76–S93. https://doi.org/10.1080/10942912.2017.1289959

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Annual Biochemistry,* 72, 248-254. https://doi.org/10.1006/abio.1976.9999

Brand-Williams, W., Cuvelier, M.E. and Berset, C. (1995). Use of a free radical method to evaluate antioxidant activity. *LWT - Food Science and Technology*, 28(1), 25-30. https://doi.org/10.1016/s0023-6438(95)80008-5

Bruno, M.A., Lazza, C.M., Errasti, M.E., López, L.M.I., Caffini, N.O. and Pardo, M.F. (2010). Milk clotting and proteolytic activity of an enzyme preparation from *Bromelia hieronymi* fruits. *LWT - Journal of Food Science and Technology*, 43, 695–701.https://doi.org/10.1016/j.lwt.2009.12.003

Colowick, S.P. and Kablan, N.O. (1955). Methods in Enzymology, Vol. 1. Academic press, Inc. Elsevier publishers, USA, 835 pp.https://doi.org/10.1016/0076-6879(55)01001-x

Cui, L., Guocheng D., Dongxu, Z., He L. and Jian C. (2007). Purification and characterization of transglutaminase from a newly isolated Streptomyces hygroscopicus. *Food Chem*. 105, 612-618. https://doi.org/10.1016/j.foodchem.2007.04.020

Darwish, M. (2016). Influence of plant based coagulant (enzyme extracts from Albizia and Sunflower Seeds) on quality of Domiati cheese. *Journal of Food and Dairy Sciences*, 7, 501–506. https://doi.org/10.21608/jfds.2016.46072

de Oliveira, J.D and Junior, C.L.S (2022). A new milk-clotting enzyme from noni seeds (*Morinda citrifolia* L). *Hoehnea,* 49, e562021. https://doi.org/10.1590/2236-8906-56-2021

Dioxn, M. and Webb, E. (1968) Enzymes (2nd Ed.) Longman Green Co. Ltd London and Academic Press, New York, pp. 380

El-Bendary, A., Magda, M.G., Maysa, E.A., Thanaa, H. (2007). Purification and characterization of milk-clotting enzyme produced by *Bacillus sphaericus*. *The Journal of Applied Sciences Research,* 3, (8), 695-699. https://www.researchgate.net/publication/248380258

El-Sayed, E.M., Saad, M.M., Selim, M.H. and El-Hadedy, D.E. (2013a). Isolation of clt genes encoding milk-clotting enzyme and characterization of milk-clotting enzyme from new isolate *Streptomyces pseudogriseolus* NRC-15. *American Journal of Biotechnology and Molecular Sciences,* 3(1), 8-23. https://doi.org/10.5251/ajbms.2013.3.1.8.23

El-Sayed, S.T. and Elmazar, M.M.A. and Al-Azzouny, R A. (2013b). Purification and characterization of a novel milk-clotting enzyme from *Brassica napus* seeds *Australian Journal of Basic and Applied Sciences*, 7, 482-493 https://www.researchgate.net/publication/236017342

Fathollahi, I., Hesari, J., Azadmard, S., Oustan S. (2010). Influence of proteolysis and soluble calcium levels on textural changes in the interior and exterior of Iranian UF white cheese during. *World Academy of Science, Engineering and Technology*, 66, 844–849. https://www.researchgate.net/publication/279895887

Fguiri, I., Atigui, M., Sboui, A., Samira, A., Marzougui, C., Dbara M. and Khorchani, T. (2021). Camel milk-clotting using plant extracts as a substitute to commercial rennet. *Journal of Chemistry*, 4, 1-8. https://doi.org/10.1155/2021/6680246

García, V., Rovira, S., Teruel, R., Boutoial, K., Rodríguez, J., Roa, I. and López, M. B. (2012). Effect of vegetable coagulant, microbial coagulant and calf rennet on physicochemical, proteolysis, sensory and texture profiles of fresh goats' cheese. *Dairy Science & Technology*, 92(6), 691–707. https://doi.org/10.1007/s13594-012-0086-1

Gomori, G. (1955). Preparation of Buffers for Use in Enzyme Studies, Handbook of Biochemistry and Molecular Biology. https://doi.org/10.1201/b10501-77

Guiama, V. D. Libuoga, G., Ngah, E., Baka, R.G., Ndi, K.C., Maloga, B., Bindzi, J.M.. Donn, P. and Mbofung, C.M. (2010). Milk-

clotting potential of fruit extract from *Solanum esculentum*, *Solanum macrocarpon* L. and *Solanum melongena*. *African Journal of Biotechnology,* 9 (12), 1797-1802. https://doi.org/10.5897/ajb10.1079

Gutiérrez, M.N., Chavez-Garay. D.R. and Jimenez-Campos H. (2012). Exploring the milk clotting properties of a plant coagulant from the Berries of *S. elaeagnifolium* var. *Cavanilles*. *Journal of Food Science,* 77, (1), c89-c94. https://doi.org/10.1111/j.1750-3841.2011.02468.x

Hassan, L.K., Shazly, A.B., Kholif, A.M., Sayed, A.F. and Abd El-Aziz, M. (2020). Effect of flaxseed (*Linum usitatissimum*) and soybean (*Glycine max*) oils in Egyptian lactating buffalo and cow diets on the milk and soft cheese quality. *Acta Scientiarum Animal Sciences*, 42, e47200. https://doi.org/10.4025/actascianimsci.v42i1.47200

Huppertz, T., Fox, P.F. and Kelly, A.L. (2018). The caseins: Structure, stability, and functionality. In Proteins in Food Processing, 2nd ed.; Yada, R.Y., Ed.; Wood head Publishing: Sawston, UK, pp. 49–92. https://doi.org/10.1016/b978-0-08-100722-8.00004-8

IDF (1991). Rheological and fracture properties of cheese. Bulletin of the International Dairy Federation No. 368/1991, Brussels, Belgium, pp.1-67.

Jacob, M., Jaros, D. and Rohm, H. (2011). Recent advances in milk clotting enzymes, *International Journal of Dairy Technology*, 64, 1, 14–33. https://doi.org/10.1111/j.1471-0307.2010.00633.x

Khan, R.S. and Masud, T. (2013). Comparison of buffalo cottage cheese made from aqueous extract of Withania coagulans with commercial calf rennet. *International Journal of Dairy Technology,* 66(3), 396–401. https://doi.org/10.1111/1471-0307.12048

Kholif, A.M.M. and Hamed, A.M. (2022). Evaluating milk coagulants from seeds of *Solanum elaeagnifolium* plant against animal and microbial rennet in manufacturing white soft cheese. *Egyptian Journal of Food Science*, 50, 2, 191-202. https://doi.org/10.21608/ejfs.2022.151430.1136

Kholif, A.M.M., Mahran, G.A., Khorshid, M.A., El-Nawawy, M. A., El-Tanboly, E. E and Abd-Rabou, N.S. (2016). Isolation, purification and characterization of milk-clotting enzyme from fruit seeds of *Solanum Elaeagnifolium* plant. *Egyptian Journal of Food Science*, 44, 1 –27.

Kholif, A.M.M., Mohamed M. El-Metwally, M.M and Mohamed, M.M (2024). Isolation, purification and characterization of milk-clotting enzyme from leaves of *Portulaca Oleracea* (purslane) plant against animal and microbial rennet in manufacturing white soft cheese. *The Arab Journal of Agricultural Sciences*, 7 (24), 231 -270. https://doi.org/10.21608/asajs.2024.386889

Kumar, A., Grover, S., Sharma, J. and Batish, V.K. (2010). Chymosin and other milk coagulants: sources and biotechnological interventions. *Critical Reviews in Biotechnology*, 30, 4, 243–258. https://doi.org/10.3109/07388551.2010.483459

Mamo, J., Kangwa, M., Orellana, J.F.S., Yelemane, V., Fernandez-Lahore, H.M and Assefa, F (2022). Purification and characterization of aspartic protease produced from *Aspergillus oryzae* DRDFS13 MN726447 under solid-state fermentation. Catalysis Letters, 152:2033–2046. https://doi.org/10.1007/s10562-021-03777-4

Martínez-Ruiz, N.D.R, Enriquez, S.F., Vázquez-Nájera, R.E. and López-Díaz, J.A. (2013). Microbiological quality of Asadero cheese manufactured with a plant based coagulant from *Solanum elaeagnifolium*. *Nutrition & Food Science*, 4, 75–81. https://doi.org/10.4236/fns.2013.47a009

Mazorra-Manzano, M.A., Perea-Gutiérrez, T.C., Lugo-Sanchez, M.E., Ramirez-Suarez, J.C., Torres-Llanez, M.J., González-Córdova, A.F., et al. (2013). Comparison of the milk-clotting properties of three plant extracts. *Food Chemistry*, 141(3), 1902–1907. https://doi.org/10.1016/j.foodchem.2013.05.042

McMahon, D.J., Motawee, M.M. and McManus, W.R. (2009). Influence of brine concentration and temperature on composition, microstructure, and yield of feta cheese. *Journal of Dairy Science*, 92, 4169–4179. https://doi.org/10.3168/jds.2009-2183

Michael Wehr, H. and Frank, J.F. (2004). Standard Methods for the Examination of Dairy Products. 18th Ed., American Journal of Public Health, Washinton, DC., USA. https://doi.org/10.2105/9780875530024

Nájera-Domínguez, C., Gutiérrez-Méndez, N., Carballo-Carballo, D.R., Peralta-Pérez, M.R., Sánchez-Ramírez, B., Nevarez-Moorillón, G.V., Quintero-Ramos, A., García-Triana, A. and Delgado, E .(2022). Milk-gelling properties of proteases extracted from the fruits of *Solanum Elaeagnifolium* Cavanilles. *International Journal of Food Science*, 4625959. https://doi.org/10.1155/2022/4625959

Narwal, R.K., Bharat B., Ajay, P., Anil, P. and Sarla, M (2016). Purification, physico-chemico-kinetic characterization and thermal inactivation thermodynamics of milk-clotting enzyme from *Bacillus subtilis* MTCC 10422. *LWT - Food Science and*

Technology, 65, 652-660. https://doi.org/10.1016/j.lwt.2015.08.065

Nasr, A.I., Ahmed, M.I.A. and Hamid, O.I. (2016). Characterization of partially purified milk-clotting enzyme from sunflower (*Helianthus annuus*) seeds. *Food Science & Nutrition,* 15, 4(5), 733-41. https://doi.org/10.1002/fsn3.338

Nicosia, F.D., Puglisi, I., Pino, A., Caggia, C. and Randazzo, CL. (2022). Plant milk-clotting enzymes for cheese making. *Foods,* 18, 11 (6), 871. https://doi.org/10.3390/foods11060871

Pagthinathan, P. (2020). Influence of partially purify enzyme in experimental cheddar cheese production. *European Journal of Agriculture and Food Sciences*, 2, 1-10. https://doi.org/10.24018/ejfood.2020.2.2.27

Re, R., Pellegrini, N. and Proteggente, A. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology and Medicine*, 26, 1231–1237. https://doi.org/10.1016/s0891-5849(98)00315-3

Rincon, A.A., Pino, V., Fresno, M.R., Jimenez-Abizanda, A.I., Alvarez, S., Ayala, J.H. and Afonso, A.M. (2017). Influence of vegetable coagulant and ripening time on the lipolytic and sensory profile of cheeses made with raw goat milk from Canary breeds. *Food Science and Technology International*, 23, 254–264. https://doi.org/10.1177/1082013216685202

Silva, G.M.de S.; da Costa, J.S.; Freire, J.O.; Santos, L.S. and Bonomo, R.C.F. (2021). Artichoke leaf extracts: Proteolytic activity, coagulant and HPLC analysis, Ciênciae Agrotecnologia, 45, e001721. https://doi.org/10.1590/1413-7054202145001721

Silvestre, M.P.C., Carreira, R.L., Silva, M.R., Corgosinho, F.C., Monteiro, M.R.P. and Morais, H.A. (2012). Effect of pH and temperature on the activity of enzymatic extracts from pineapple peel. *Food and Bioprocess Technology*, 5, 1824–1831. https://doi.org/10.1007/s11947-011-0616-5

Siow, H. L., Lim, T. S. and Gan, C. Y. (2017). Development of a workflow for screening and identification of α-amylase inhibitory peptides from food source using an integrated bioinformatics-phage display approach: case study – cumin seed. Food Chemistry, 214, 67–76. https://doi.org/10.1016/j.foodchem.2016.07.069

Soltani, M., Sahingil, D., Gokce, Y. and Hayaloglu, A.A. (2019). Effect of blends of camel chymosin and microbial rennet (*Rhizomucor miehei*) on chemical composition, proteolysis and residual coagulant activity in Iranian ultrafiltered white cheese. *Journal of Food Science and Technology*, 56(2), 589–598. https://doi.org/10.1007/s13197-018-3513-3

Sorin, N.I.T.U., Mihaela, G. and Florentina, M. (2021). Milk-clotting enzymes obtained from plants in cheesemaking-A review. *Scientific Bulletin, Series F. Biotechnologies,* XXV(1), 66-75. https://doi.org/10.1007/s13197-018-3513-3

Srinivasan, K. (2018). Cumin (*Cuminum cyminum*) and black cumin (*Nigella sativa*) seeds: traditional uses, chemical constituents, and nutraceutical effects. *Food Quality and Safety*, 2, 1, 1–16. https://doi.org/10.1093/fqsafe/fyx031

Sun, Q., Wang, X. P., Yan, Q.J., Chen, W. and Jiang, Z.Q. (2014) Purification and characterization of a chymosin from *Rhizopus microsporus* var. *rhizopodiformis*. *Applied Biochemistry and Biotechnology*, 174, 174-185. https://doi.org/10.1007/s12010-014-1044-6

Vairo-Cavalli, S., Claver, S., Priolo, N and Natalucci, C (2005) Extraction and partial characterization of a coagulant preparation from Silybum marianum flowers. Its action on bovine caseinate. *Journal of Dairy Research* , 72 (3) , pp. 271 – 275. https://doi.org/10.1017/s0022029905000749

Wadhwani, R. and McMahon, D.J. (2012). Color of low-fat cheese influences flavor perception and consumer liking. *Journal of Dairy Science*, 95, 2336-2346. https://doi.org/10.3168/jds.2011-5142

Wang, Y., Qiaolin, C., Zaheer, A., Xiaoxue, J. and Xiaojia, B. (2009). Purification and partial characterization of milk-clotting enzyme extracted from glutinous rice wine mash liquor. *Korean Journal of Chemical Engineering*, 26(5), 1313-1318. https://doi.org/10.1007/s11814-009-0225-4

Wu, S., Sun, J., Tong, Z., Lan, X., Zhao, Z. and Liao, D. (2012). Optimization of hydrolysis conditions for the production of angiotensin-I converting enzyme-inhibitory peptides and isolation of a novel peptide from lizard fish (*Saurida elongata*) muscle protein hydrolysate. *Marine Drugs*, 10(5), 1066-1080. https://doi.org/10.3390/md10051066

Yasar, D. and Kose, S. (2024). The effect of packaging methods and storage temperatures on the antioxidant and antimicrobial activities of Malatya cheese. *GIDA The Journal of Food,* 49 (5), 960-967. https://doi.org/10.15237/gida.gd24026

Zhang,Y., Hu,J .,Wang,J Liu,C., Liu,X., Sun,J., Song,X and Wu,Y (2023) Purification and characteristics of a novel milk-clotting metalloprotease from *Bacillus velezensis* DB219. *Journal of Dairy Science*, 106, 6688–6700. https://doi.org/10.3168/jds.2023-23450