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EXTRACTION, PARTIAL PURIFICATION AND SOME FACTORS AFFECTING MILK CLOTTING ACTIVITY OF A MILK COAGULANT PREPARED FROM SUNFLOWER (*Helianthus annus*) SEEDS

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ABSTRACT: Crude extract of sunflower seeds was prepared using different extractants (distilled water, 5% NaCl in distilled water, 5% NaCl in acetate buffer pH 5.0) and certain mixing ratios (1:2, 1:5 and 1:10 seeds powder : extractant, respectively). Using of 5% NaCl in acetate buffer pH 5.0 at mixing ratio of 1:5 gave an extract with reasonable efficient clotting properties. Ammonium sulphate at 40-60% concentration was used in one single step for the partial purification of the extract. Ammonium sulphate, (40-60%) fraction showed milk clotting and specific activities of 440 sec and 2.30 U/mg protein, respectively. Meanwhile a yield percentage and a fold of purification of 28.58 and 2.02 were achieved. Clotting/proteolytic activity, water holding capacity and curd synersis of the partially purified extract approved those of commercial calf rennet indicating the suitability of the extract as a rennet substitute. The optimum temperature, pH, $Cacl_2$ and GDL concentration of the resultant extracts were found to be 65°C, 5.0, 0.04 % and 0.4%, respectively.

Key words: Sunflower seed, milk clotting activity, crude protein extracts, proteolytic activity, rheological analysis

INTRODUCTION

Using calf rennet for milk coagulation is the most procedure used in cheese making. However, the worldwide increase in cheese production coupled with the reduced supply and increasing prices of calf rennet has led to search for alternative milk clotting enzymes as an appropriate rennet substitute (**Anusha *et al.,* 2014, Shah *et al.,* 2014**). A part from this some religious factors (Islam and Judaism) and others related to vegetanism of some consumers have limited their use (**Shah *et al.,* 2014**).

Chymosin (EC 3.4.23.4) the main enzymatic component of calf rennet is characterized by its high specificity for K casein and low general proteolytic activity. This enzyme predominantly cleaves Phe-105-Met 106 bond in K casein deplicating the casein micelle and inducing the milk clotting (**Piers *et al.,* 1999; Vishwanatha *et al.,* 2010**). Microbial milk coagulants and genetically engineered chymosin remain the major categories of milk coagulating enzymes (**Barry and Tamime, 2010**). Although the biotechnological prepration of recombinant chymosin has diminshed the cost of this product, certain sectors of the population still prefer a natural alternative.

Plants are used as source of proteases due their easy availability, efficient purification processes and isolation of natural coagulants. Furthermore, plant coagulant usage increases the acceptability by vegetarian population and has an advantage of improving their nutritional intake. An inherent drawback of vegetable enzymes is that they usually possess high proteolytic activity leading to undesirable flavour and cheese textures (**Sousa and Malcata, 2002**). This could be explained on the basis that enzyme preparation had low ratio of milk clotting/proteolytic activity (**Anusha *et al.,* 2014; Shah *et al.,* 2014**). Therefore the search of a rennet substitute having a high ratio of milk clotting/proteolytic activity is extremely needed

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to be used for the production of cheese with better quality.

Sunflower (*Helianthus annus*) seeds are edible and are used for extraction of cooking oils. Seeds of this plant were used for the isolation of aspartic protease (**Park *et al*., 2000**) with high sequence similaty to Cynarase of *Cynara cardunculus* (**Cordeiro *et al.,* 1994**). In addition (**Egito *et al.,* 2007**) studied the milk clotting activity of ammonium sulphate precipitated extract of sunflower seeds and investigated its mode of action of different casein. Meanwhile, **Darwish *et al.* (2016)** used the partially purified extract of sunflower seeds in the production of Domiati cheese with acceptable quality.

Nasr *et al.* (2016) developed a simple purification procedure for the production of substantial amounts of active milk clotting enzymes from sunflower seeds as a cheap milk clotting preparation for cheese making. The partially purified enzyme demonstrated great milk coagulation specificity and curd formation comparable to that of commercial rennet indicating its potentiality in cheese making industry as rennet substitute. Despite the a aforementioned studies on milk clotting enzyme from sunflower seeds, evaluation of the potentiality of milk clotting enzymes of this plant as rennet substitute in depth is still scare. Therefore the main aim of the present study was extract milk clotting enzyme preparation from sunflower seeds, partial purification of the extract and investigate factors affecting milk clotting properties potentiality of this extract to be used as rennet substitute.

MATERIALS AND METHODS

Materials

Skim milk powder

Imported skim milk powder from USA extra grade, supplied by Egyptian Company for Dairy Products and Food Additives "EGY- DAIRY" 10th of Ramadan City, Sharkia Governorate, Egypt, was used for the preparation of reconstituted skim milk that used for evaluating clotting activity of both crude and partially purified sunflower seed extracts.

Sunflower seeds

Sunflower (*Helianthus annus*) seeds were obtained from the local market at Zagazig, Egypt. Seeds were air dried at room temperature for 2 weeks. The seeds were carefully cleaned and then coarsely ground using an electric grinder and kept in polyethylene bags at refrigerator (4-5°C) until being used for enzyme extraction.

Animal rennet

Standard rennet (Activity ≈ 49.5 IMCU/ ml, Dosage: 1.0 ml 50 ≈IMCU/L milk) was obtained from Chr. Hansen's Laboratories, Copenhagen, Denmark. It was diluted with distilled water to a standard rennet solution before using.

Preparation of Crude Enzyme Extract

The crude enzyme extracts were prepared using different extractants namely; distilled water, 5% NaCl in distilled water and 5% NaCl in acetate buffer pH5.0. Sunflower seeds powder were mixed with each extractant at different mixing ratios including 1:2, 1:5 and 1:10 (*W/V*) seed powder and extractant respectively. Sunflower seeds powder in each case was immersed in the extractant for 24 hr., at 4°C.The extract was then filtered through double layer of cheesecloth and centrifuged at 5000×g for 20 min. The supernatant was collected and considered as a crude enzyme extract (**Ahmed *et al.,* 2009**).

Evaluation of milk clotting activity (MCA)

Milk clotting activity was determined according to the method described by (**Ahmed *et al.,* 2009**). The substrate (10% reconstituted skim milk in 0.01 M $CaCl_2$) was prepared and the pH was adjusted to 6.5. The substrate (5ml) was preincubated at different temperatures for 5 min, the temperature was adjusted to 50°C 55°C, 60°C, 65°C, 70°C or 75°C using a thermostatically controlled water bath. Briefly, 1 ml by volume of the crude extract was added into 5ml of the milk samples and unit of milk clotting activity (MCU) was determined by rotating the test tube at regular interval times and checking for visible clot formation on the wall of the test tubes. The following formula was used for calculating the milk clotting unit

(MCU) (**Nouani *et al.,* 2009**). Results are expressed as MCU/ml.

Unit of milk clotting activity (U/ml)=(2400/t) × (S/E)

Where:

t is the time required for clot formation,

S is the volume of skim milk,

E is the volume of crude enzyme extract.

Partial purification of crude enzyme extract

Crude enzyme extract was partially purified using ammonium sulphate fractionation as described by **Egito *et al.* (2007)**, Ammonium sulphate was added to the crude extract (25 ml) at 40% saturation and the mixture was kept at 4°C for 45 min before centrifugation (10000 ×g for 15 min). The pellets were discarded. Ammonium sulphate was then added to the supernatant to reach 60% saturation. After 45 min of incubation at 4°C, the mixture was again centrifuged (10000 ×g at 4°C for 15 min). The pellets were dissolved in acetate buffer (5 ml). The process was repeated several times to obtain sufficient amount of the partially purified extract, Extract was dialysed against acetate buffer pH 5.0 at 4°C for 24 hr., and used during this study.

Protein content

The protein contents of both crude and partially purified extracts were estimated through the determination of nitrogen content according to **AOAC (2000)**.

Proteolytic activity

The proteolytic activity of coagulant extracts allows for the evaluation of the rate of the degradation rate of casein (Sigma, Biochemical, and reagents) during the primary reaction. It consists of measuring, the increase of non-protein nitrogen (NPN) in 12% trichloro acetic acid (TCA) of the final mixture (**Nouani *et al.,* 2009**) as modified by **Abdel Raouf *et al.* (2017)**. Briefly, 1.0 ml of crude enzyme extract was added to 10 ml of 3% soluble casein (pH 5.0) in a test tube and incubated at 65°C for 1, 2, 3, 4, 5 and 6 hours.

Some Factors Affecting Milk Clotting Properties of Partially Purified Extract

The effect of some factors including temperature, pH, $CaCl_2$ and glucono delta lactone (GDL) concentrations on the milk clotting properties of the extract was evaluated.

Effect of temperature

Temperature range of 55°C-75°C was used to determine the optimum temperature for clotting activity using 1ml of enzyme extract.

Effect of pH

The optimum pH for milk coagulation by 1ml of the enzyme extract was determined at different pH values (5 - 6.5) incubated at 65°C. The pH value at which the milk coagulated in the shortest time was recorded as the pH optimum value required for milk coagulation using the extract.

Effect of concentration of $CaCl_2$

The optimum concentration of $CaC1_2$ was determined by observing the milk coagulation time as affected by $CaC1_2$, at concentrations between 0.01 to 0.04% in a test tube incubated at 65°C with added 1ml enzyme extract.

Effect of concentration of Glucono-Delta-Lactone (GDL)

The optimal concentration of Glucono-Delta-Lactone (GDL) was determined by observing the milk coagulation time as affected by GDL, at concentrations between 0.2, 0.4, 0.6 and 0.8% in a test tube incubated at 65°C and pH 5.0 with added 1ml enzyme extract.

Rheological analysis

Water holding capacity (WHC) and susceptibility to syneresis (STS) of the coagulam were evaluated according to **Isanga and Zhang (2009)**. One ml of enzyme extract was added to 100 ml of the substrate (10% reconstituted skim milk in 0.03% $Cacl_2$, 0.04% GDL). The substrate was subsequently incubated at 65°C and the formation of curd was observed. The coagulation end point was recorded when discrete particles were discernible. The experiment was carried out in triplicate.

The water holding capacity (WHC) was determined according to the following formula:

$$WHC(\%) = (1 - \frac{W_1}{W_2}) \times 100$$

Where:

W_1= Weight of whey after centrifugation.

W_2= Initial sample weight.

Coagulated milk was centrifuged at 4°C for 30 min at 1000 rpm.

Susceptibility to Syneresis

The susceptibility to syneresis (STS) was measured by placing a 100 ml of the coagulated sample on a filter paper placed on the top of a funnel. After 6 hr of drainage, the volume of the whey was collected and measured. The index of syneresis is calculated as follows:

$$STS(\%) = \frac{V_1}{V_2} \times 100$$

Where:

V_1 = Volume whey after drainage.

V_2 = Initial volume sample.

Statistical Analysis

The data were analyzed by ANOVA according to the appropriate experimental designs and reported as means (± standard deviations), which were separated by Duncan's Multiple Range Test at $p \leq 0.05$ (**Cochran and Cox, 1992**) and least significant difference (LSD) test using SPSS computer program, version 20 (SPSS Inc., Chicago, IL, USA). Triplicate measurements were performed for each analysis.

RESULTS AND DISCUSSION

Effect of Extractants and Mixing Ratio on Milk Clotting Activity

Table 1 shows the results of clotting activities of sunflower seeds crude extracts as affected by extractant type and mixing ratios. Results indicated that crude extracts prepared using 5%Nacl in acetate buffers pH 5.0 had the highest clotting activities as assessed by shorter clotting time, and higher milk clotting activity compared with extracts using distilled water and 5% NaCl in distilled water. In addition, the clotting activities of extracts were found to be in proportional with the level of sunflower seeds powder used in preparing the extract. However crude extract prepared using 5% NaCl in acetate buffer and mixing ratio of 1:5 (B) had an efficient and reasonable clotting activity. In addition this mixing ratio takes into account production costs together with effectiveness. Thus extracts using 5% NaCl in acetate buffer and a mixing ratio of 1:5 were selected for the preparation of crude extract throughout this investigation.

Several investigators reported similar results whereas **Ahmed *et al.* (2009) and Talib *et al.* (2009)** recommended the use of 5% NaCl in sodium acetate buffer as an effective extractant in the preparation of crude extracts from *S. dubium* berries.

Meanwhile **Nasr *et al.* (2016)** used 5% NaCl in acetate buffer pH 5.0 for the preparation of crude extracts from sunflower seeds with potent clotting activities. They added that increasing the salts strength in the extracting solution was accompained with enhancing the extraction officially and the milk clotting activity of the producing extracts.

Partial Purification of Sunflower Seeds Crude Extract

Crude extract of sunflower seeds was subjected to ammonium sulphate fractionation. The fraction of 40-60% ammonium sulphate saturation was separated in a single step. The purification profile of the extract as indicated with clotting time, clotting activity, total activity, specific activity, yield and purification fold is illustrated in Table 2. Partial purification using ammonium sulphate had led to a clear enzyme preparation with an efficient clotting properties. Thus clotting time, clotting activity and specific activity of the partial purified extract were 440 sec, 27.26 U/ml and 2.30 U/mg protein, respectively. A purification fold of 2.02 with a yield of 28.58% were achieved. **Barros *et al.* (2001)** showed that the use of ammonium sulphate as a simple purification procedure resulted in an effective removal of the partial proteases and coloured materials existing in the crude extract. Meanwhile this procedure concentrated the enzyme preparation to a workable volume that could be used in enzyme characterization and cheese making.

Table 1. Effect of extractant type and mixing ratio on the milk clotting properties of sunflower seeds of crude extracts

Extractant	Mixing ratio	Properties	
		Clotting time (second)	Milk clotting activity (U/ml)
Distilled water	A	4203.30±5.77[a]	2.82±0.02[f]
	B	3816.70±15.27[b]	3.14±0.01[e]
	C	3633.30±30.55[c]	3.23±0.05[e]
5% NaCl in distilled water	A	735.00±5.00[d]	16.30±0.10[d]
	B	676.70±5.77[e]	17.70±0.17[c]
	C	646.70±11.54[f]	18.56±0.33[b]
5% NaCl in acetate buffer pH 5.0	A	721.70±2.88[d]	16.57±0.05[d]
	B	636.70±5.77[f]	18.85±0.16[b]
	C	485.00±5.00[g]	24.70±0.30[a]

A, B and C: 1:10 , 1:5 and 1:2 seeds powder/extractant (*W/V*) respectively.

Means having the same letters in the same column are not significantly different.

Table 2. Partial purification profile of sunflower seeds extract

Extracts	Volume	Protein content (mg/ml)	Clotting time (second)	MCA* (U/ml)	Specific activity (U/mg protein)	Total activity MCA x volume	Yield (%) (a)	Purification fold (b)
Crude extract	25.00	16.50±0.50	636.67±5.78	18.84±0.17	1.14±0.01	471.17±4.18	0.00±0.00	1.00±0.00
Partially purified extract (40-60% ammonium sulphate fraction)	5.00	11.80±0.30	440.00±20.00	27.26±1.21	2.30±0.10	97.30±72.73	28.58±1.17	2.02±0.65

a: Yield (%) = Total activity of partially purified extract/total activity of cured extract.

b: Purification fold = Specific activity of partially purified extract/specific activity of crude extract.

* MCA: Milk clotting activity.

Egito *et al.* (2007) showed that Albizia and sunflower seed crude extracts and their corresponding ammonium sulphate precipitated protein extract (40-60% ammonium sulphate saturation) exhibited milk clotting activity suggesting that the two plants possessed one or more enzyme with rennet like activity. **Ahmed *et al.* (2009)** showed that the 35-55% ammonium sulphate saturation fraction of *Salanum dubium* gave the highest milk clotting activity. By applying 35-55% ammonium sulphate fractionation as a first purification step, over 86% of the total protease in the crude extract was salted out. **Cavalcanti *et al.* (2004)** showed that 40-60% ammonium sulphate fraction of *Nocardiopsis* sp crude extract showed the most milk clotting activity.

Ammonium sulphate precipitation was performed by **Nasr *et al.* (2016)** as the sole step for the partial purification of sunflower seeds extract. They reported that 30-50% ammonium sulphate fraction showed the highest milk clotting activity compared with other fractions. This level of ammonium sulphate concentration was recommented for partial purification of sunflower seeds crude extract, whereas it resulted in 4.3 fold purity with a yield of 10.87%.

Proteolytic Activity of Partially Purified Extract

The levels of non protein N (NPN) liberated during incubation of soluble casein with the crude and partially purified extract were taken as an index for the proteolytic activity, and compared with that of commercial calf rennet (Table 3). From these results, it could be seen that the level of NPN in the partially purified extract was somewhat higher than that of commercial rennet but it was lower than that of crude extract. This could be explained by the presence of partial proteases in the crude extract which might contribute to the excessive proteolysis (**Barros *et al.,* 2001; Nasr *et al.,* 2016**). Several investigator have showen that many plant coagulants had higher proteolytic activity compared with calf rennet (**Abd El-Gelil and Zawahary, 2004; Chazarra *et al.,* 2007; Ahmed *et al.,* 2009; Elmazar *et al.,* 2012; Abdel-Raouf *et al.,* 2017**).

Factors Affecting Milk-Clotting Activity (MCA) of Partially Purified Extract

Effect of temperature

Table 4 shows the effect of temperature on clotting activity of sunflower seeds protease. Results indicated that raising temperature from 55 to 70°C was associated with an increase in clotting activity as indicated by shorter clotting time as well as increased milk clotting and specific activities. The clotting activity began to decrease when temperature increased to 75°C. The thermophilic nature of plant proteases was reported by many investigators (**Sidrach *et al.,* 2005**) and (**Chazarra *et al.,* 2007**) on Cynarase (70°C), **Raposo and Domingos (2008)** on the protease of *Centaurea calcitrapa* (52°C), **Lo piero *et al.* (2002)** on the lettucine from *lactuca sativa* (50°C) and **Abdel-Raouf *et al.* (2017)** on artichoke flower (*Cynara scolymus*) (65°C).

The decrease in clotting activity as a result of increasing temperature over certain limit could by explained by the changes in salt equilibricum and the complex formed between K-casein and β-lactoglobulin or α- lactoglobulin (**Balcones *et al.,* 1996**).

Effect of pH

Results presented in Table 5 show the effect of pH on clotting activity of the partially purified extract of the partially purified extract of sunflower seeds. The results clearly indicated that a considerable increase in the clotting activity was accompanied with reducing the pH value from 6.5 to 5.0. In view of these results, the optimum pH for the clotting activity was found to be 5.0, which resulted in a higher clotting activity as indicated by shorter clotting time and higher clotting and specific activities.

Castillo *et al.* (2000) showed that pH affect both the enzymatic and aggregation phase of milk coagulation, although the effect of pH on the former phase is less than it is on the latter one. **Sidrach *et al.* (2005)** reported that the optimum pH for artichoke protease was 5.0 while (**Chen *et al.,* 2003**) found that the optimum pH for artichoke protease was 6.0. **Hashem (2000)** observed that an increase in the pH of reaction mixture was associated with a gradual decrease in milk clotting activity, but at pH 7.0 the milk clotting activity of penicillium oxalicum still possessed 38% of its original activity.

Lamas *et al.* (2001) showed that most plant protease are catalytically unstable at alkaline pH value. **Sidrach *et al.* (2005)** found that an increase in pH of milk was accompanied with a loss of milk clotting activity and at pH 7.0, 87% of the enzyme activity was lost. The general trend of the results are in agreement with (**Nouani *et al.,* 2009; Abdel-Raouf *et al.,* 2017**).

Effect of Calcium Chloride Concentration

Table 6 shows the effect of increasing calcium chloride ($Cacl_2$) concentration on the clotting activity of the partially purified extract of sunflower seeds. The results clearly indicated that the addition of $Cacl_2$ enhanced the clotting activity of the partially purified extract. This decreasing the clotting time and increasing both milk clotting and specific activities. In the light of these results, a concentration of 0.04% $Cacl_2$ was considered sufficient to increase the clotting activity to an efficient level. The obtained results could be explained on the basis that calcium concentration influences the coagulation of milk and textural properties of cheese (**Lucey and Fox, 1993**). The primary (enzymatic) stage of coagulation is independent on calcium. However, the addition of calcium reduces the pH of milk *via* the exchange of Ca^{++} for H^{+} which indirectly

Table 3. Proteolytic activity of different crude extract of sunflower seeds

Extract	Incubation time (hour)	Non protein nitrogen (%)	Non protein nitrogen as (%) of total N
Crude extract	1	0.033±0.01^{fgh}	6.80±1.17^{hi}
	2	0.040±0.00^{efg}	8.16±0.00^{gh}
	3	0.043±0.01^{efg}	8.84±1.17^{fg}
	4	0.057±0.01^{cd}	11.53±1.15^{de}
	5	0.077±0.01^{ab}	15.60±1.21^{b}
	6	0.087±0.01^{a}	17.63±1.15^{a}
Partially purified	1	0.023±0.01^{hi}	4.76±1.17^{jk}
	2	0.027±0.01^{hi}	5.44±1.17^{ijk}
	3	0.033±0.01^{fgh}	6.80±1.17^{hi}
	4	0.060±0.00^{cd}	12.20±0.00^{cd}
	5	0.067±0.01^{bc}	13.53±1.15^{c}
	6	0.077±0.01^{b}	15.60±1.21^{b}
Calf rennet	1	0.015±0.00^{j}	3.06±1.00^{l}
	2	0.025±0.01^{ij}	5.1±1.00^{k}
	3	0.035±0.01^{efg}	7.14±1.00^{ij}
	4	0.045±0.01^{efg}	9.14±1.00^{gh}
	5	0.055±0.01^{de}	11.6±1.00^{ef}
	6	0.065±0.01^{cd}	13.3±1.00^{cd}

Means having the same letters in the same column are not significantly different.

Table 4. Effect of temperature on the milk clotting activity of sunflower seeds partially purified extract

Temperature (°C)	Property		
	Clotting time (second)	Milk clotting activity (U/ml)	Specific activity (U/mg protein)
55	545.00±5.00^{a}	22.00±0.20^{e}	1.83±0.02^{e}
60	413.33±5.77^{b}	28.97±0.40^{d}	2.40±0.00^{d}
65	163.33±5.77^{c}	73.50±2.59^{c}	6.17±0.23^{c}
70	116.67±5.77^{e}	103.00±5.19^{a}	8.67±0.46^{a}
75	138.33±2.88^{d}	86.73±1.78^{b}	7.30±0.17^{b}

Means having the same letters in the same column are not significantly different.

Table 5. Effect of pH values on milk clotting activity of sunflower seeds partially purified extract

pH value	Property		
	Clotting time (second)	Milk clotting activity (U/ml)	Specific activity (U/mg protein)
6.5	345.00±5.00[a]	34.70±0.50[c]	2.87±0.06[c]
6.0	246.67±5.77[b]	48.67±1.15[b]	4.11±0.08[b]
5.5	232.67±6.42[c]	51.59±1.39[b]	4.33±0.11[b]
5.0	73.60±2.42[d]	163.13±5.42[a]	13.77±0.46[a]

Means having the same letters in the same column are not significantly different.

Table 6. Effect of $Cacl_2$ concentration on milk clotting activity of sunflower seeds partially purified extract

$Cacl_2$ concentration (%)	Properties		
	Clotting time (second)	Milk clotting activity (U/ml)	Specific activity (U/mg protein)
0.00	413.33±5.77[a]	28.97±0.40[e]	2.42±0.04[d]
0.01	368.33±7.63[b]	32.57±0.66[d]	2.74±0.07[c]
0.02	349.33±1.15[c]	34.27±0.11[c]	2.83±0.06[c]
0.03	285.00±5.00[d]	42.07±0.75[b]	3.53±0.06[b]
0.04	231.67±7.63[e]	51.82±1.67[a]	4.37±0.15[a]

Means having the same letters in the same column are not significantly different.

increase the rate of enzymatic reaction (**Jen and Ashworth, 1970**). The secondary non enzymatic stage of coagulation is dependent on Ca^{++}, although the process also work with colloridal calcium phosphate (**Pyne and McGann, 1962**). The zeta potential of para casein micelles is reduced further by the addition of Ca^{++} to negatively charged phosphoserine residue or carboxylic acid groups of α-and β-caseins leading to greatly increased aggregation of renneted casein micelles **Lucey and Fox (1993)**. Added calcium bind to casein either directly to negatively charged residue of casein or as CCP. However adding high amount of calcium markedly reduce the firmness of renneted milk gel possibly because excessive calcium binding may interfere with proper gel formation. Several investigators reported that the addition of $Cacl_2$ to milk increases the overall enzymatic coagulation rate, although at lower calcium concentration (**Bencini, 2002; Najera *et al.,* 2003; Lagaude *et al.,* 2004**).

Effect of Glucono-Delta-lactone (GDL) Concentration

Results presented in Table 7 show that addition of GDL (Glucono-Delta-lactone) significantly enhanced the clotting activity of the partially purified extract. This was more remarkable with increasing the level of GDL addition up to 0.4% and then the clotting activity began to decrease. So, it could be recommended the addition of GDL at 0.4% level to enhance the clotting activity. These results could be explained on the basis that the extracts of sunflower seeds showed considerable clotting activity at higher acidity and lower pH as previously shown under the effect of pH. GDL slowly hydrolyse and lactic acid is liberated with consequent reduction in pH. The general trend of the obtained results agree with (**Esposito *et al.,* 2016; Abdel-Raouf, 2017**) on their studies on plant rennet.

Table 7. Effect of GDL concentration on milk clotting activity of sunflower seeds partially purified extract

GDL concentration (%)	Property		
	Clotting time (second)	Milk clotting activity (U/ml)	Specific activity (U/mg protein)
0.0	410.00±10.00[a]	29.23±0.75[e]	1.99±0.44[e]
0.2	330.00±0.00[b]	36.30±0.00[d]	3.07±0.00[d]
0.4	263.33±5.77[c]	45.53±0.98[c]	3.83±0.11[c]
0.6	156.67±5.77[d]	76.67±2.88[b]	6.43±0.23[b]
0.8	118.33±2.88[e]	101.33±2.30[a]	8.53±0.23[a]

Means having the same letters in the same column are not significantly different.

Potentiality of Using the Partially Purified Extract as Rennet Substitute

Ratio of milk clotting to proteolytic activity and the capacity of the extract to produce curd with satisfactory properties was taken as indices to evaluate the usefulness of this extract as a rennet substitute.

The ratio of milk clotting to proteolytic activity

The ratio of milk clotting to proteolytic activity is a valuable indicator of the protease efficiency to be used as a rennet substitute in cheese making (**Arima *et al.*, 1970; Ahmed *et al.*, 2009; Nasr *et al.*, 2016**). Table 8 compare the milk clotting to proteolytic activity of both crude and partial purified extracts of sunflower seeds and compare them with that of commercial calf rennet. Clotting/proteolytic activity ratio of crude extract, partially purified extract and commercial rennets were 216.66, 354.02 and 498.92, respectively. These results clearly indicated that the partially purified extract had a reasonable clotting/proteolytic activity compared with calf rennet indicating the potentiality of the partially purified extract to be used as a rennet substitute.

Egito *et al.* (2007), showed that the mode of action of sunflower seeds protease is similar to that of chymosin. They showed that sunflower seed extract similar to chymosin exhibited proteolytic activity towards K-casein, β-casein and αs-casein.

Sunflower seeds protease from mass spectrometry analysis were found to hydrolyse K-casein at the Phe 105-Met 106 bond as chymosin. Meanwhile, several inrestigators have shown that sunflower seeds possess proteases with high clotting/proteolytic ratio which clotted milk readily without developing any undesirable bitterness or excessive proteolytic activity (**Park *et al.*, 2000; Darwish, 2016; Nasr *et al.*, 2016**).

Water Holding Capacity and Curd Synersis

Water holding capacity and curd synersis were taken as a measurement of rheological properties of curd. Figs. 1 and 2 show the water holding capacity and curd syneresis of crude and partially purified sunflower seeds extracts as compared with those of commercial calf rennet. From these figures it could be seen that using ammonium sulphate in the partial purification of crude extract enhanced the water holding capacity and decreased the rate of syneresis.

The levels of water holding capacity and curd syneresis of partially purified extract approached their counterparts in commercial calf rennet. The lower holding capacity and higher curd syneresis observed in sunflower seeds extract might be related to the higher levels of proteolytic activities in these extract (Table 8), **Darwwish (2016)** recorded lower values of hardness, gumminess, chewiness and adhesiveness in Domiati cheese curd produced using sunflower and albizia seeds protein extract.

Table 8. Ratio of milk clotting to proteolytic activity of milk clotting extracts

Extract	Clotting activity (U/ml)	Proteolytic activity (NPN%)	Clotting/proteolytic ratio
Crude extract	18.85	0.087	216.66
Partially purified extract	27.26	0.077	354.02
Calf rennet	32.43	0.065	498.92

Means having the same letters in the same column are not significantly different.

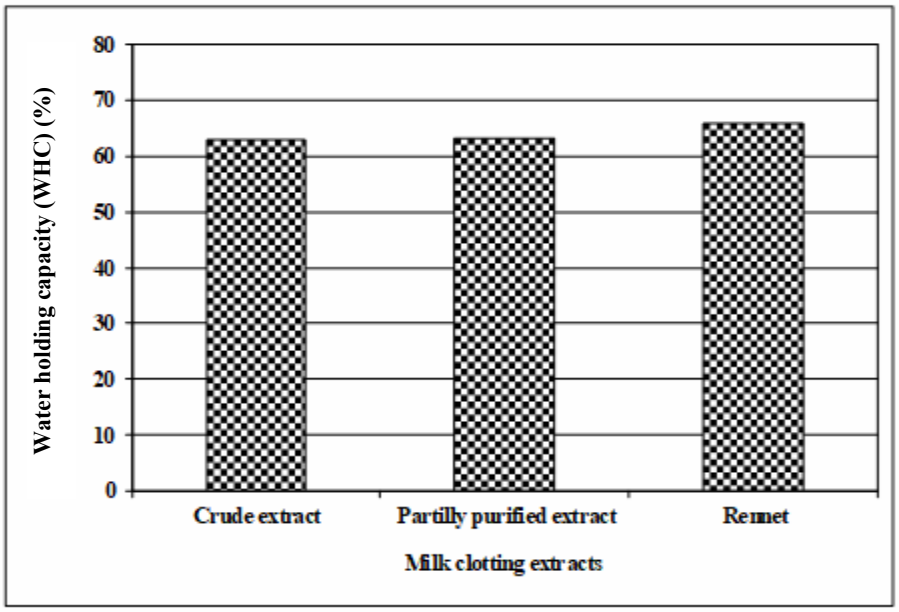

Fig. 1. Water holding capacity

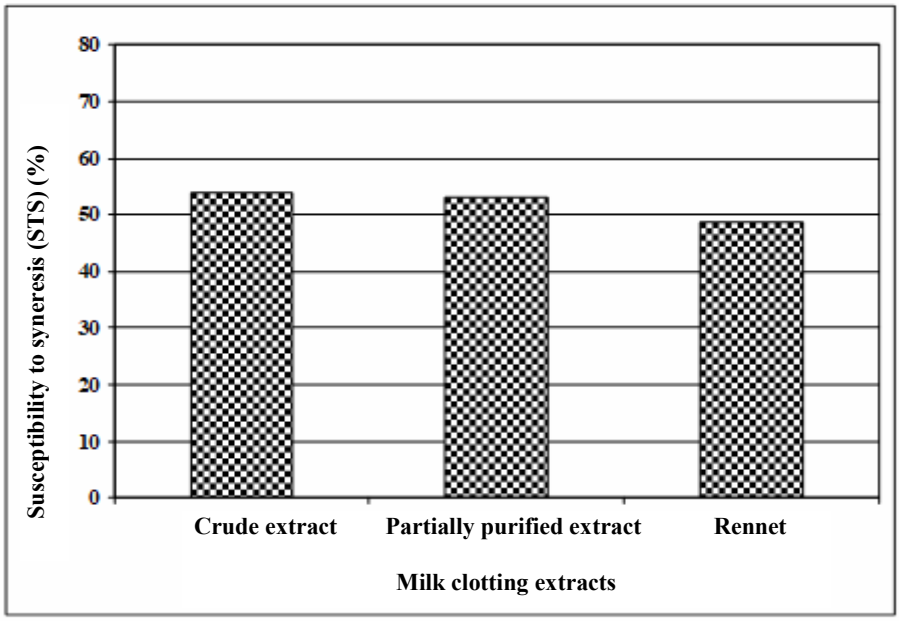

Fig. 2. Curd Syneresis

This could be attributed to the high ability of these protein extracts for the hydrolysis of casein particles compared with chymosin (**Egito *et al.*, 2007**). Thus the matrix of cheese curd is formed by interconnected casein particles. The solubilization of colloidal calcium phosphate and the hydrolysis of these molecules will decreased curd textural characteristics (**Creamer and Olson, 1982; Amira *et al.*, 2017**). The capacity of the extract to produce curd with satisfactory water holding capacity and syneresis, besides its reasonable clotting to proteolytic activity compared with rennet could indicate its usefulness and potentiality as a rennet substitute in cheese making.

Conclusions

The simple procedure for partial purification of sunflower seeds extract using ammonium sulphate combined with the availability of sunflower seeds could be used for large scale production of a milk coagulant of plant source. Moreover , the high specificity of the partially purified extract in term of its high milk clotting /proteolytic ratio and satisfactory curd properties could pave the way for its use in cheese industry as an alternative to calf rennet **.** Additional studies on the complete purification and characterization of this milk coagulant with the intense evaluation of the quality of cheese curd produced by its action will shed more light into its commercial suitability.

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الاستخلاص، التنقية الجزئية وبعض العوامل المؤثرة على القدرة التجبنية لمستحضر انزيمى مجبن للبن من بذور دوار الشمس

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تم تحضير المستخلص الخام من بذور دوار الشمس وذلك باستخدام أوساط استخلاص مختلفة (ماء مقطر، محلول ٥% كلوريد صوديوم، محلول منظم من خلات الصوديوم يحتوى على ٥ % كلوريد صوديوم وذو درجة حموضه =٥) مع نسب خلط مختلفة مع كل مستخلص مع مطحون البذور(١:٢، ١:٥، ١:١٠مسحوق دوار الشمس: المستخلص، وزن/حجم على التوالي)، وقد أظهر مستخلص خلات الصوديوم المحتوى على ٥% كلوريد صوديوم وذو درجة حموضة =٥ مع نسبة خلط ١:٥ قدرة تجبنية مناسبة، لذلك استخدمت تلك الظروف لتحضير المستخلص الخام المستخدم خلال هذه الدراسة، وتم تنقية المستخلص الخام جزئياً باستخدام كبريتات الأمونيوم في خطوة واحدة عند تركيز ٤٠-٦٠% وقد أظهر الجزء الذى تم فصله بكبريتات الأمونيوم (٤٠-٦٠% تشبع) قدرة تجبنية وقدرة تخصصية جيدة (٤٤٠ ثانية ، ٢،٣٠) مع الحصول على عائد بنسبة ٢٨,٥٨ ودرجة تنقية قدرها ٢,٠٢ وكانت درجة الحرارة، ورقم الـ pH، تركيز الكالسيوم، تركيز الجلوكونودلتا لاكتون التى أعطت أفضل قدرة تجبنية للمستخلص كالتالى (٦٥°م، ٥,٠ ، ٠,٠٤ و٠,٤%) على التوالي، وقد تبين من تقييم نسبة القدرة التجبنية/القدرة على تحلل البروتين للمستخلص المنقى جزئياً مع بعض الخواص الريولوجية للخثرة الناتجة من استخدامه (القدرة على الاحتفاظ بالرطوبة/انفصال الشرش) وذلك بالمقارنة مع المنفحة الحيوانية إمكانية استخدامه كبديل للمنفحة فى صناعة الجبن.

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