

Effect of Extraction Method, Ammonium Sulphate Concentration, Temperature and pH on Milk-Clotting Activity of *Solanum dubium* Fruit Extract

¹M.O.M. Abdalla, ²S.E.O. Kheir and ¹O.A.O. El Owni

¹Department of Dairy Production, Faculty of Animal Production, University of Khartoum, Shambat, P.O. Box 32, Postal code 13314, Khartoum North, Sudan

²Department of Animal Production, Faculty of Agriculture, Abu Naama, University of Sinnar, Sudan

Abstract: *Solanum dubium* fruits were collected and used as a plant source for extracting milk coagulating enzymes. The enzyme was extracted by four methods and the activity of each extract was determined. *Solanum dubium* fruits were kept at 4 and 37°C for five months, while aqueous extracts of the *Solanum dubium* fruits were kept at 4 and 37°C for three months. The crude extract of *Solanum dubium* fruit was precipitated by ammonium sulphate using different concentrations (0-90%). Partial characterization of the milk coagulant was carried out. The results showed that maximum milk-activity ($p < 0.001$) was obtained from *Solanum dubium* fruit extracted with freeze-drying. The milk-clotting activity significantly decreased ($p < 0.001$) from 3.65 U/mL when *Solanum dubium* was extracted with 1% NaCl in distilled water to 1.74 when *Solanum dubium* was extracted with 5% NaCl. The loss in activity of *Solanum dubium* fruits stored in liquid and solid forms increased significantly ($p < 0.05$) at room temperature compared to refrigerator storage. The saturation with ammonium sulphate (60%) gave higher milk-clotting activity (5.03 mg/mL) and protein content. The partially purified *Solanum dubium* fruit extract had the highest activity at 70°C and pH 10. Twenty five milliliters (25 mL/50 L milk) of a partially purified *Solanum dubium* fruit extract was recommended for cheese making.

Key words: Extraction method, milk-clotting activity, *Solanum dubium*, temperature

INTRODUCTION

Rennet is a natural complex of enzymes produced in mammalian stomach to digest mother's milk, and it contains a proteolytic protease enzyme that coagulates milk causing it to separate into solid curd and liquid whey. The active enzyme in rennet is called chymosin but there are other important enzymes such as pepsin. Today, there are two major sources of chymosin; from animals and different kinds of fungi, in addition to chymosin derived from genetically engineered microbes. The most common rennet substitutes include bovine, porcine and to a lesser extent chicken pepsin and microbial proteases from *Mucor miehei*, *Bacillus subtilis* and *Cryphonectria parasitica* (Shieh *et al.*, 2009; Trujillo *et al.*, 2000). Plant proteases have been investigated as milk coagulants, but only a small number of aspartic proteases from plant origin have been isolated and partially characterized (Tavaria *et al.*, 1997). A unique feature shared by most of these plant proteases is an extra segment of about 100 amino acid residues bearing no sequence similarity with proteinases of mammalian or microbial origin (Faro *et al.*, 1995).

Many aspartic and other proteinases are obtained from plants and some of them have been studied as

coagulants i.e., proteinases from *Benincasa cerifera* (Gupta and Eskin, 1977), *Calotropis procera* (Ibama and Griffiths, 1987; Sharagi *et al.*, 2009), *Dieffenbachia maculate* (Padmanabhan *et al.*, 1993), fruit parts of *Solanum dubium* (Yousif *et al.*, 1996), *Centaurea calcitrapa* (Tavaria *et al.*, 1997). The difficulties experienced with these preparations result mainly from the unique composition of the plant extracts which contain a complex cocktail of enzymes whose activity is difficult to control.

Solanum dubium Fresen, an indigenous plant in northern and central Sudan, is a woody herb with solid erect stem and green in colour, about 30 cm in height. The fruits are grouped in clusters with exile alternately bent to bring all clusters to one side of the stem or the branch. Unripened fruits are green and almost enclosed in spiny calyx, while the ripened ones are yellow. The seeds are dark brown in colour and animals do not eat *Solanum dubium* because of its bitter taste and thorny leaves (Salih, 1979; Yousif *et al.*, 1996). Recently, *Solanum dubium* extract has been reported to have a maximum activity at 45-50°C and 60°C with a gradual decrease in activity as temperature increased (Abdalla *et al.*, 2010; Osman, 1996). The maximum activity was observed at pH 4.5-5.5 and the activity

decreased with increasing pH value (Abdalla *et al.*, 2010; Mohamed and Habbani, 1996).

The objective of this investigation is to extract the enzyme with different methods and characterize it for milk-clotting activity in order to use it in cheese making as an alternative to rennet.

MATERIALS AND METHODS

This study was conducted at the Department of Dairy Production, Faculty of Animal Production, University of Khartoum and Department of Animal Production, Faculty of Agriculture, University of Sinnar during the period April-December, 2008.

Enzyme extraction: Four extraction methods were tried to select the one with a reasonably high activity.

Extraction with distilled water: Yellow fruits of *Solanum dubium* plant were powdered using laboratory mortar, then five grams were soaked in a conical flask for 24 h at 5°C using distilled water (30 mL) with frequent shaking for the first 3 h and solutions were then filtered through filter paper. The aqueous filtrate was used for testing milk clotting activity.

Drying in a current of warm air: The filtrate of *Solanum dubium* extract was spread on a shallow glass basin and exposed to a current of air at 45°C till completely dry (Osman, 2001).

Soaking in sodium chloride and evaporation in a current of warm air: Hundred grams of coarsely ground plant powder were soaked in 1, 2, 3, 4 and 5% sodium chloride for 24 h at 5°C. The solution was filtered and the filtrate was finally spread on a shallow glass basin and exposed to a current of warm air at 45°C (Osman, 2001).

Freeze-drying: The powdered yellow fruits (100 g) were soaked in a conical flask for 24 h using distilled water with occasional shaking for the first 3 h and the solution was kept at -25°C and freeze-dried.

Stability of *Solanum* extract: Powdered extract of *Solanum dubium* fruits (dried in a current of warm air) was kept at 4°C and 37°C for 5 months, while aqueous extract of the *Solanum dubium* fruits was kept at 4°C and 37°C for 3 months, and samples from each were tested every month for activity.

Determination of crude *Solanum* extract activity: The activity of *Solanum dubium* fruit extract was determined according to the method described by Mohamed and Habbani (1996).

Concentration of *Solanum dubium* extract by ammonium sulphate: The crude extract of *Solanum dubium* fruit was precipitated by ammonium sulphate using different concentrations (0-90%). Precipitation was carried out at 5°C, and the precipitate was recovered by centrifugation. The supernatant was discarded and the sediment from each concentration was re-suspended in buffer solution (pH 10) and dialyzed against distilled water for 24 h, changing the distilled water six times. The enzyme was then dialyzed against buffer (pH 10) for 12 h. The volumes of the dialyzed enzyme concentration and protein contents were measured (Lowry *et al.*, 1951).

Characterization of partially purified enzyme:

Effect of incubation temperature on enzyme activity:

The effect of incubation temperature on the enzyme activity was determined at 40, 50, 60, 70, 80, 90 and 100°C. Casein solution (2%) in buffer at pH 10 was used as enzyme substrate. The enzyme concentrate was diluted to 100 fold, and 1 ml of the diluted enzyme solution was added to 1 mL of casein solution and incubated at the required temperature for 10 minutes. Proteolysis was stopped by the addition of 2 mL of 5% TCA (Anson, 1938).

Effect of pH on enzyme activity: Casein solution (2% w/v) was suspended in a buffer of different pH values (4, 5, 6, 7, 8, 9, 10, 11, 12). Both enzymes and substrate were allowed to equilibrate with the incubation temperature of 37°C before adding the enzyme to the substrate. Enzyme activity was measured according to Anson (1938).

RESULTS AND DISCUSSION

Extraction of the milk-clotting coagulant: The results show that, maximum milk-clotting activity ($p < 0.001$) was obtained from *Solanum dubium* fruit extracted with freeze-drying (3.60 ± 0.43 U/mL), followed by *Solanum dubium* fruit extracted with distilled water and evaporated at 45°C (3.39 ± 0.74 U/mL) and *Solanum dubium* extracted by distilled water (1.30 ± 0.17 U/mL). The lowest activity (0.05 U/mL) was obtained by extraction with 5% NaCl, and this extract was subjected to a current of warm air (45°C). The results show that milk-clotting activity significantly decreased ($p < 0.001$) from 3.65 U/mL when *Solanum dubium* was extracted with 1% NaCl in distilled water to 1.74 when *Solanum dubium* was extracted with 5% NaCl. Our results disagree with Yousif *et al.* (1996) who found that 5% NaCl solution extracted more of the compound associated with clotting in *Solanum dubium*. Abdalla *et al.* (2010) reported that the higher activity was obtained by *Solanum dubium* fruit extracted with distilled water, followed by *Solanum dubium* fruit extracted with freeze-drying, then *Solanum dubium* fruit extracted with

Table 1: Effect of storage temperature on activity (U/ml) of *Solanum dubium* extract (liquid and solid forms)

Storage (days)	Mean±S.D.			
	Liquid form		Solid form	
	4 (°C)	37 (°C)	4 (°C)	37 (°C)
1	1.85 ^a ±0.59	1.24 ^a ±0.25	4.51 ^a ±1.13	3.32 ^a ±0.65
30	1.33 ^b ±0.12	0.58 ^b ±0.11	2.84 ^b ±0.32	2.76 ^b ±0.42
60	1.08 ^c ±0.26	0.33 ^c ±0.07	2.06 ^c ±0.16	1.97 ^c ±0.15
90	0.20 ^d ±0.05	0.07 ^d ±0.04	1.69 ^d ±0.14	1.62 ^d ±0.24
120	-	-	1.60 ^d ±0.12	1.53 ^d ±0.17
150	-	-	1.21 ^e ±0.28	1.18 ^e ±0.19
L.S	***		***	

Means in each column bearing similar superscripts are not significantly different (p>0.05); LS: Level of significance for interaction; ***: (p<0.001) SD: Standard deviation

Table 2: Effect of ammonium sulphate saturation (%) on milk clotting activity yield and fold of purification in *Solanum dubium* fruits

Saturation of ammonium sulphate (%)	Volume (mL)	Enzyme activity (U/mL)	Protein content (mg/mL) (units/mL)	Specific activity ^a (units)	Total activity ^b	Yield ^c (%)	Fold of purification ^d
Crude enzyme	25	3100	85.5	36.26	77500	100	-
10	5.8	0.08	1.85	45.04	483.31	0.62	1.24
20	7.2	1.50	2.75	545.45	10800	13.94	15.04
30	8.8	3.283	6.25	525.33	28893	73.28	14.49
40	8.2	3.850	9.75	394.87	31570	40.74	10.89
50	8.6	4.33	10.00	433.33	37266	48.09	11.95
60	9.4	5.03	14.25	353.21	47313	61.50	9.74
70	9.8	4.33	13.50	320.99	42466	54.79	8.85
80	8.8	4.08	7.25	563.21	35933	46.37	15.53
90	10.3	3.83	9.50	403.51	39483	50.95	11.13

^a: Specific activity = activity /protein content; ^b: Total activity = activity × volume of fraction; ^c: Yield = total activity of the fraction/total activity of crud enzyme × 100; ^d: Fold of purification = Specific activity of fraction/specific activity of the crude fraction

distilled water and evaporated at 50°C, while the lower activity was obtained by *Solanum dubium* fruit extracted with 5% NaCl in distilled water.

Thermal stability of *Solanum dubium* extract: The effect of storage on activity of *Solanum dubium* extract in liquid and solid forms is presented in Table 1. Activity of *Solanum dubium* extract (liquid) stored at 37°C decreased from 1.24±0.25 at day 1 to 0.07±0.04U/mL at day 90, but when stored at 4°C the activity decreased from 1.85±0.59 at day 1 to 0.20±0.05 U/mL at day 90. In case of the solid form, the activity decreased from 3.32±0.65 at day 1 to 1.18±0.19 U/mL at day 150 when the extract was stored at 37°C and from 4.51±1.13 U/mL at day 1 to 1.21±0.28 U/mL at day 150 when stored at 4°C (p<0.001). It is clear that the loss in activity on storage of solid *Solanum dubium* extract was much less as compared to liquid form. Further, the loss in activity of *Solanum dubium* extract in the liquid as well as solid forms was significantly more at 37°C. In case of storage at 4°C, the loss in activity was appreciably less. Hence, the storage of solid *Solanum dubium* extract at 4°C was favorable for retaining the activity. Similar results were found by Singh *et al.* (1973) who studied vegetable rennet from *Withania coagulans* and found that the loss in activity on storage of solid rennet was much less at both refrigerator and room temperatures. Tejada *et al.* (2008) pointed that refrigerator storage of extract form of *Cynara cardunculus* L. can not be considered a suitable method for prolonged preservation of aqueous cardoon extract.

Effect of ammonium sulphate concentration on activity of *Solanum dubium* extract: The crude extract of *Solanum dubium* fruits was precipitated with ammonium sulphate concentrations ranging from 0 to 90%. The results in Table 2 revealed that the saturation of 60% gave a higher milk-clotting activity (5.03 U/mL) as well as protein content (14.25 mg/mL) with 61.05% yield and 9.74 fold of purification. However, Abdalla *et al.* (2010) found that the saturation range of 40-50% gave the highest milk clotting activity as well as protein content, yield (27.5%) and fold of purification (1.88).

Characterization of partially purified enzymes:
Effect of incubation temperature on milk-clotting activity: It is obvious from Fig. 1 that *Solanum dubium* extract exhibited optimum temperature for milk clotting activity at 70°C. The results are similar to Sidrach *et al.* (2005) who studied the milk clotting activity of *Cynara scolymus* and Ahmed *et al.* (2009a) who found that the enzyme from *Solanum dubium* Fresen seeds is thermostable retaining complete activity at 60°C after 1 h and acts optimally at 70°C for 30 min. However, the results of this investigation are in disagreement with Kumar *et al.* (2005), Aworh and Nakai (2006), Pascaline and Daniel (2006), Raposo and Domingos (2008) and Nouani *et al.* (2009).

Effect of pH on the activity of *Solanum dubium* extract: The activity of *Solanum dubium* extract on a pH

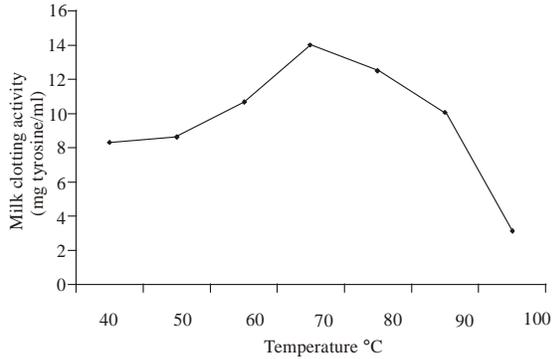


Fig. 1: Effect of incubation temperature on milk clotting activity of partially purified *Solanum dubium* extract

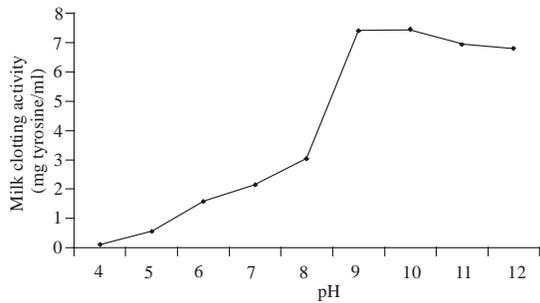


Fig. 2: Effect of pH on milk-clotting activity of partially purified *Solanum dubium* extract

range from 4 to 12 is shown in Fig. 2. Results show that the activity of *Solanum dubium* extract increased with increasing pH value from 4 up to 10 which was the maximum activity, and then the activity declined. The findings disagree with Osman (2001) who reported the maximum activity of *Solanum* extract to be at pH 5.5 and the enzyme activity decreased with increasing pH value, and Kumer *et al.* (2005), Aworh and Nakai (2006), Ishak *et al.* (2006), El-Bendary *et al.* (2007), Raposo and Domingos (2008) and Nouani *et al.* (2009). Ahmed *et al.* (2009b) reported that chymotrypsin-like serine protease from the seeds of *Solanum dubium* Fresen acted optimally at pH 11, and was stable over a wide range of pH 3.0-12.0. The milk clotting activity of a partially purified extract enzyme from glutinous rice wine mash liquor increased with decreasing milk pH from 8.0 to 5.5, and it was active at the wide range of pH 1-7 (Wang *et al.*, 2009).

CONCLUSION

From this study it could be concluded that the storage temperature significantly affected the activity of *Solanum dubium* extract (liquid and solid forms), and that 60% ammonium sulphate saturation gave the best milk-clotting

activity, protein content and yield. Milk-clotting activity increased with increasing incubation temperature and reached its maximum activity at 70°C, while the activity reached its maximum at pH 9.

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