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Isolation, Purification and Partial Characterization of a Serine-Like Protease from *Solanum dubium* Seeds

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Abstract

The aim of this study was to extract, purify and characterize an enzyme from *Solanum dubium* (*S. dubium*) seeds and to investigate the seed extractability in milk clotting activity. The extracted were using different buffers of sodium phosphate (pH 7.1), Tris HCl (pH 8.5), Sodium Acetate (pH 5.2) in addition to a distilled water treatment. The results shows that buffer extraction (sodium phosphate pH 7.1) and presoaking produced more extractable protein with better clotting ability. The extracted enzymes were filtered and fractionated on Sephadex G-75, anion exchange, and gel filtration chromatography columns. The purified enzyme is characterized by being thermostable (up to 70ºC) with a pH range of 6-7 (for 1% casein substrate). The enzyme is inhibited by serine protease inhibitors. It is, therefore, concluded that the enzyme is of the chymotrypsin-like serine protease class. Gel filtration and SDS-PAGE methods showed that the purified enzyme consists of three bands with molecular masses of 11KD, 35KD, and 40KD. The enzyme has a Km value of 0.6 mM and a Vm value of 66.7unit/min. mg. Crude extracts obtained from *S. dubium* seeds were shown to exhibit milk clotting activity.

Keywords: *Solanum dubium* seeds; Milk clotting activity; Extraction; Enzyme; Chromatography; Chymotrypsin; Serine protease

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Introduction

Milk production in Sudan is economically important due to its contribution to human nutrition. The Ministry of Animal Wealth estimates the annual production of 5.1 million tons of milk as appears in the FAO report [1]. Cheese-making has survived, as an art, for more than 7000 years [2]. The advancement of scientific knowledge has led to a better understanding of the raw material milk and the cheese-making and ripening process. In Sudan "given bayda" is unique among cheese varieties as having a high concentration of table salt added to milk before processing [3]. Since cheese is manufactured from raw or heated milk [4]; it becomes important to subject it to the salting process to avoid microbial contamination especially under tropical conditions [5]. The worldwide increase in cheese production, alongside the reduced supply of calf rennet, had promoted research towards a search for alternative sources of milk coagulants [6,7]. Plant or microbial rennet produced by genetically engineered bacteria and molds provided substitutes for rennin [8]. Early, much research interests have been directed towards discovering a milk-clotting enzyme from natural plants [9,10] that could satisfactorily replace commercial rennet in cheese making [11]. Rennet substitutes of plant origin have been increasingly used to manufacture cheese, especially at the putative artisanal level. Application of plant coagulants allows targetted cheese production and hence contributes to improving the nutritional input of those populations on whom restrictions are imposable by the use of animal rennets [12]. Aqueous fruit extracts from *S. dubium* were used traditionally as coagulants in the making of white and soft cheese in Sudan [13]. Other species of the Solanum genus such as *S. incanum. L, S. esculentum, S. macrocarpon. L and S. melongena. L* have shown the ability to clot milk [14,15]. *Solanum dubium* Fresen is a well-known species belonging to the Solanaceae family. Therefore, this study focused on the establishment of the extracted purification enzyme from *S. dubium* plant seeds which play a role in milk coagulation activity.

Materials and Methods

S. dubium seeds, used in this investigation, were collected from west of Omdurman city, Khartoum state, Sudan. Two types of seeds were collected; one in a full ripe stage (yellow seeds) and the other in an unripe condition (green seeds). The seeds were cleaned, dried under room condition, and kept in paper bags until use.

Sample preparation of extraction

The clotting activity was measured according to the method described by Arima and Iwassaki [16] with slight modification which excluded the addition of calcium. A small amount of the crude extract (50-100 ml) was taken the extracts and mixed with 2 ml of 50% milk (fresh milk diluted in sterile distilled water pH 5.4) at 55°C until clotting was achieved. Milk-clotting activity (MCA) was calculated according to equation (1).

$$MCA(U/ml) = \frac{2400}{clotting\ time(s)\ X\ dilution\ factor} \quad (1)$$

where U is the unit activity which is defined as the quantity of crude protein in ml needed to coagulate 2 ml of 50% milk at 55°C within 40 min.

Partial purification of rennin-like enzyme: Ammonium sulphate precipitation of rennin-like enzyme

The seed extract of *S. dubium* was further subjected to separate steps of precipitation using 0-30%, 30-50%, and 50-80% concentration by the gradual addition of solid ammonium sulfate and was allowed to stand submerged in ice for 30 min. It was then centrifuged at 13,000 rpm for 20 minutes at 4°C. The obtained protein pellets were dissolved in 50 mM sodium phosphate buffer (pH 7.1) and the protein obtained was collected and combined as one fraction. Ammonium sulphate (30% - 55%) precipitation was repeated and the protein produced was pooled again in one fraction and dialyzed for 24 hours at 4°C and then follow by desalting, The membranes containing the 30% - 55% concentration precipitate were immersed in a beaker containing sodium phosphate buffer pH (7.1) for 8 hours with continuous stirring and occasional change of the buffer. The precipitated enzyme samples were subjected to Sephadex G-75, anion exchange, and superdex G-75 column chromatography.

Purification of the enzyme using chromatography column

The protein samples obtained after Sephadex G-75 were subjected to anion exchange chromatography using HI Trap 2 cm × 5 cm column (Bio-Rad Laboratories Inc.), previously equilibrated with a mixture of 50 mM sodium phosphate and 1 mM EDTA (pH 7.1). The protein fractions were loaded and subsequently eluted using a sodium chloride gradient (1M) in the same buffer with a flow rate of 1.3 ml/min. 1 ml fractions were collected and monitored at a wavelength of 280nm. The peaks were collected and small aliquots (100 µl) were evaluated for milk-clotting activity. Active peak fractions were pooled and lyophilized and were used in the next step of purification in superdex G-75. Protein samples (300 µl) obtained, after anion exchange chromatography, was subjected to gel exclusion chromatography. The column (1.8 cm+25 cm) was packed with superdx G-75, equilibrated with a mixture of 20 mM sodium phosphate buffer and 0.15 M sodium chloride (pH 7.1). Elution was performed at a flow rate of 0.2 ml/min and 1ml fractions were collected. The absorption effect of the eluent was monitored at a wave-length of 280 nm.

Polyacrylamide Gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE) of the enzymes was performed by the method of Lammli [17], using 15% polyacrylamide gel, sodium dodecyl sulfate (SDS), and β-mercaptoethanol (SDS-PAGA). The protein bands in the gels were stained with Coomassie Brilliant Blue. The relative molecular mass of the protein was estimated by a standard protein marker.

Protease activity

Protease activity was assessed using the spectrophotometric method reported by Sarath et al. [18]. The enzyme solution (100 ml) was added to 100 ml of 1% casein in 50 mM sodium phosphate buffer (pH 7.1), followed by gentle mixing and incubation at 37°C for 30 min. The reaction was stopped by adding 1.5ml of TCA, and the mixture was incubated at room temperature for 30 min and was then centrifuged at 13,000 rpm for 5 min at 4°C. A control was prepared in the same way, but with the addition of TCA to stop the enzyme activity. The absorption of the enzyme was examined at a wavelength of 280 nm. One unit of the enzyme activity was defined as the amount of the enzyme which increases the absorption by 0.001 nm at a wavelength of 280 nm under assay conditions.

Protease activity by the agar plate

The agar plate method [19] may be used for the determination of the protease activity. The Agar 15g was dissolved in 150 ml of a mixture which consisted of 20 mM Tris-HCl buffer (pH 8.5), 2 mM calcium chloride and 0.5% casein solution. The mixture was autoclaved, cooled down to a reasonable temperature, distributed into 25ml Petri dishes, and was allowed to cool down and solidify. Wells of 0.8cm diameter were made in the solidified agar. Each well in a separate agar plate was treated by the addition of 100ml of either ammonium sulfate precipitate or Superdex G-75 purified enzyme; a standard was made which contained a micro quantity of chymosin. The plates were incubated at 37°C for 20 hours.

Protease activity detection zymography

Enzyme activity was detected by the zymography technique described by Vladimir et al. [20]. In this technique a purified enzyme (20 ml) was added to 1 ml of 0.125 M Tris HCl buffer (pH 8.0) containing 5% (w/v) SDS, 1% (w/v) sucrose, and 0.05% (w/v) bromophenol blue. Electrophoresis (SDS-PAGE) was conducted in a resolving gel and a stacking gel having all specifications described earlier in section 2.8.2 and 2.8.3; in addition to 1% casein. The gel was washed twice with 2% (v/v) Triton X-100 for 30 min. The gel was then transferred into 0.05 M Tris–HCl buffer, (pH

7.5) containing 15 mM $CaCl_2$. The enzyme activity was revealed as translucent bands in the gel. The band was further visualized by subjecting the gel to staining and destaining procedure.

Effect of temperature on the enzyme activity

The optimum temperature of protease activity was determined by conducting the assay at different temperatures in 50 mM sodium phosphate buffer (pH 7.1). The reaction mixture was incubated for 30 minutes at various ranges of temperatures from 20°C to 90°C, taking readings every 5°C. The temperature of the incubation mixture was maintained at 60°C and was left for 1, 2, 3, and 4 hours before the activity was re-measured as a test for temperature effect on enzyme stability.

Effect of pH on enzymes activity

The hydrogen ion concentration (pH) for protease activity was determined by conducting the enzyme assay at various pH values. The reaction mixture of the enzyme was incubated for 30 minutes at 37°C in a water bath. Different buffers, such as 50 mM sodium acetate (pH 4.5 to 5.5), 50 mM sodium phosphate (pH 6-7) and 50 mM Tris-HCl (pH 7.5-9) was used separately [21].

Effects of different concentrations of protease inhibitors and metal ions on enzyme activity

The effects of various protease inhibitors such as iodoacetate, phenylmethylsulfonyl fluoride, chymostatin, cocktail (AEBSF, E6, pepstatin A, 1, 10-phenanthroline, bestatin, and ethylenediaminetetraacetic acid (EDTA) on the hydrolyzing ability of purified protease were tested. About 10 µl of the purified enzyme was pre-incubated at 37°C for 30 minutes, separately, with each one of the inhibitors (at different concentrations), followed by the addition of 1% casein and 50 mM sodium phosphate buffer (pH 7.1). The residual protease activity was then measured as was explained before (2.9.3). The following salts were used to determine the influence of metal ions: ($ZnCO_4$), ($MgCl_2$), ($MgSO_4$) ($CuSO_4$), (LiCl), (KCl), (KF), (NaCl), (ZnCl), ($HgCl_2$), ($FeSO_4$), ($COSO_4$), ($AlSO_4$) and ($CaCl_2$). The residual protease activity was measured at a wavelength of 280 nm. The activity without the inhibitors and metal ions was considered as 100%.

Measurement of molecular masses of the purified enzyme

The molecular mass of the purified enzyme was determined by the SDS PAGE electrophoresis method. The purified enzyme (20 ml) and standard proteins with different molecular weights, (10 KD, 15 KD, 20 KD, 25 KD, 50 KD, 75 KD, 100 KD, 150 KD, and 250 KD) were loaded onto SDS-PAGE. The molecular mass of the purified enzyme was estimated from the plot of standard value versus the relative front.

Kinetic activity studies

Michaelis–Menten equation was calculated by plotting the absorbency readings obtained for the effect of different enzyme concentrations on casein. The 1/V and 1/S values of the enzymatic samples were obtained using the Line- Weaver-Burk or double reciprocal equation [22].

Results and Discussion

The result showed that extraction media used in this study, whether distilled water or different buffers, clearly indicated that the latter gave more extractable proteins and consequently enzymes than the former; with the highest values reached when sodium phosphate buffer was used. The results also indicated, that more solid support for the use of the phosphate buffer may be judged by the total amounts of extractable amino-acid as indicated by the amino-acid profiles and their comparison with those obtained by extraction with other media; greater amounts were obtained in case of the phosphate buffer. Accordingly, the phosphate buffer was chosen for protein extraction (both crude and purified extracts) from the seed of *S. dubium*.

The next step, after establishing the phosphate buffer as a medium for the extraction of the best obtainable protein, was to precipitate, purify and characterize the isolated protein. It was found that the best protein precipitation was achieved by the use of 30-50% ammonium sulfate concentration for seed; which makes it within the range of 40-60% concentration for maximum ammonium sulfate precipitation reported by Lakshmi et al. [23] using *Bacillus subtilis* and at the same time closer to those reported by El-Hofi et al. [24] who applied 40-50% ammonium sulfate concentration for the precipitation of Carica papaya protein.

Protein dialysis is another method for increasing the precipitation fold as was reported by Sarah et al. [25,26] and which was applied to attain the results presented in this work leading to a decrease in recovery of proteins, increase in a specific activity, and purification folds characteristic of a pure and active enzyme as shown in **Table 1**. Purification of the enzyme by the use of anion exchange chromatography, which ended up in the separation of the enzyme into two peaks **(Figure 1B),** indicated that the enzyme was still not a homogeneous one. Homogeneity (pure enzyme) is reached after the enzyme was run through superdex G-75 column where only one peak was detected **(Figure 1C).** On the other hand, heterogeneity with the appearance of more than one peak was noticed for the enzymes separated by the sephadex **(Figure 1A)** and ion exchange columns; which, ultimately suggests their impurity. Support for the separation by the pure enzyme with a single peak may be drawn from the work carried out on SDS-PAGA **(Plate 1).** The single homogenous enzyme produced by superdex-G-75 proved to be highly active and leads to complete hydrolysis of casein.

The primary stages of milk coagulation mainly involve the hydrolysis of casein **(Plate 2 and Plate 3).** It is, therefore, important to estimate, quantitatively, how the rate and the coagulant activity are affected by some determining factors. Pure, rather than crude enzyme extracts were subjected to clotting ability test in order to exclude the presence of any possible inhibitors. The time needed for milk clotting pure enzyme seed counterpart reported in this study might be ex-plained by the possibility that some of the extracted enzymes, may still be in the pro-enzyme state as in the case of the soaked seeds which, is a well-established phenomenon. This assumption is consistent with what happens

Table 1 Total proteins, total activity, specific activity, recovery, and purification fold of the enzyme at different concentrations and purification steps.

Purification Step		Total Protein (mg/ml)	Total Activity (units)	Specific Activity (units/mg)	Recovery (%)	Purification (fold)
Crude Extract		003.2	68.571	21.428	100	1.00
NH4SO4 Precipitation	0-30%	200.0	8.888	44.4	13	2.07
	30-50%	962.0	57.831	60.1	84	02.80
	50-80%	271.0	10.00	36.9	15	01.7
Second NH4SO4	30-55%	512.0	61.40	119.9	89	05.5
Dialysis		477.4	52.00	108.0	76	05.0
Sephadex G-75 Run		390.0	45.00	115.0	65	05.3
Anion Exchange Peaks	Unbound A	300.0	43.00	143.0	62.0	06.7
	Unbound B	277.0	40.00	144.0	58.0	06.7
	Bound C	215.0	38.00	176.0	56.0	08.00
Superdex G-75 Peaks	Unbound A	130.0	34.280	261.53	49	12.1
	Unbound B	132.0	26.666	262.20	38	09.4
	Bound C	115.0	25.945	225.60	37	10.5

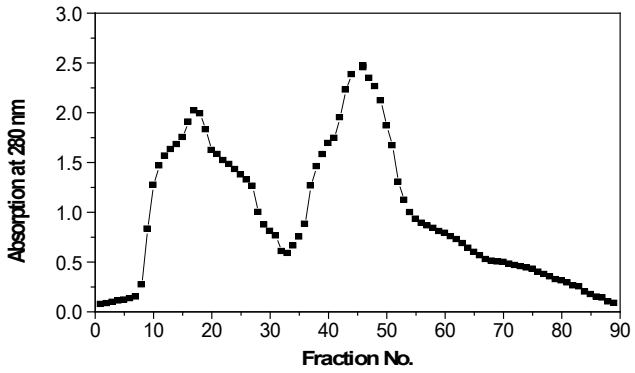


Figure 1A Appearance of unbound fractions A and B after running the purified seed extract in an anion exchange column.

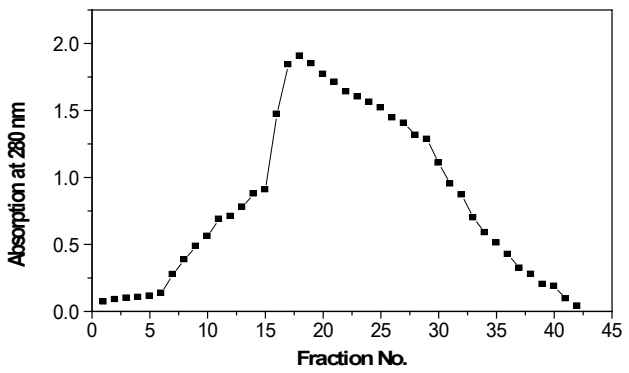


Figure 1B Appearance of unbound fraction B (35 KDa after running the purified seed extract in Superdex G-75) column.

to the enzyme amylase in dormant starchy seeds where only β-amylase shows immediate activity, while α-amylase becomes active as the seed germinates [27].

Temperature plays a central role in the protease or clotting enzyme activity and a wide range of temperatures was suggested as an optimum for high enzyme activity **(Figure 2).** The optimum temperature reported was about 60°C for the enzyme extracted from B. Hispidia var Ryukya [28] and about 55°C for Crustacean Muruid enzyme by Ambrosio et al. The results obtained in this work are similar to those obtained by Mohamed-Ahmed et al. [29] with an optimum temperature upto 70°C which is not far from some other protease of Aspergillus niger with an optimum

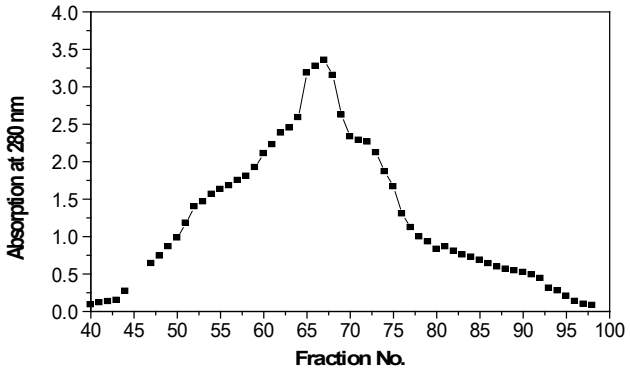


Figure 1C Appearance of unbound fraction A (40 KDa) after running the purified seed extract in Superdex G-75 Column.

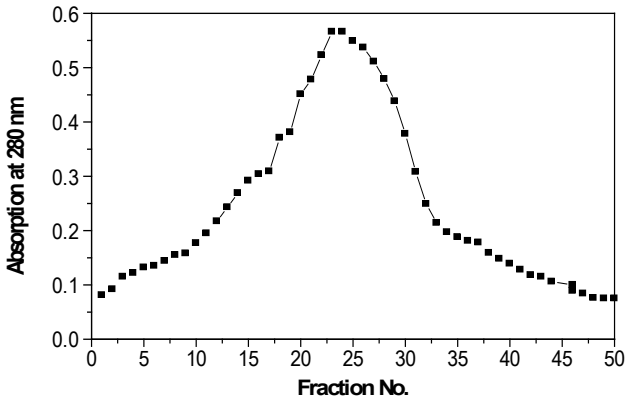


Figure 1D Appearance of bound fraction A (11 KDa) after running the purified seed extract in Superdex G-75 column.

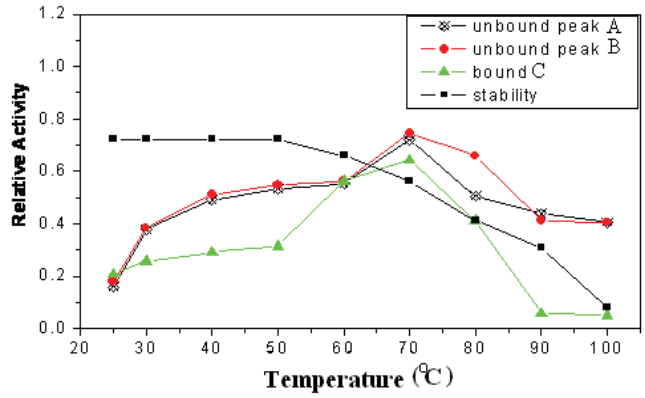


Figure 2 Effect of temperature on the activity and stability of the unbound fraction (peak A and B) and the bound Fraction (C) of the purified enzyme.

enzyme because most enzymes are unstable at alkaline pH. The use of certain inhibitors **(Table 2)** may provide an insight into the nature of the enzyme, its cofactors and the nature of the active center [30-32]. The description of the purified enzyme from *S. dubium* seeds and callus tissues as a serine protease is supported by its inhibition by PMFS [33] and that it reacted to the inhibitor in a way similar to the reaction of chymstain, which is known as a serine protease. On the other hand, iodoacetate and the cocktail mixture (AEBSF, E-6, pepstatin A, 1, 10-phenan throline and bestatin) did not inhibit the enzyme, thus providing a further proof for its serine nature, since the use of these inhibitors affect non-serine proteases [34].

The effects of different metal ions **(Table 3)** on the enzyme activity from seeds gave results that may be divided into three groups. The first group is for the $CaCl_2$, effect which increased the activity and is in agreement with the work of Whitaker on Finin, Sheded on Solanum terbium and Osman on *S. dubium* [35-37]. The effect is attributed to the Ca^{2+} ions which are known to enhance protease activity and stability at various concentrations [38]. The second group ($CuSO_4$, $HgCl_2$, $ZnSO_4$, $Zn\ Cl_2$, and $COSO_4$) exhibited a decrease in enzyme activity; a result which is supported by the work of Habbani [39]. Also, inhibition or reduction of activity was reported by Lynn and Radford [40], for Hg^{2+}, Zn^{2+}, Mg^{2+}, Mn+, and Ca^{2+} ions. The third group is not very much affected by the addition of the metal (Na1+ and K1+) ions and gave results that are more or less similar to the control in case of the seed extract, and consequently in agreement with the results of Mohamed-Ahmed et al. [29]. On the contrary, the callus tissue enzyme reacted differently to the presence of Na^{1+} ion with increased activity of the enzyme.

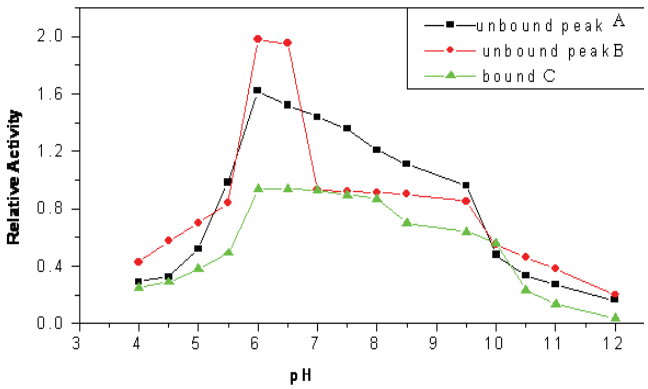
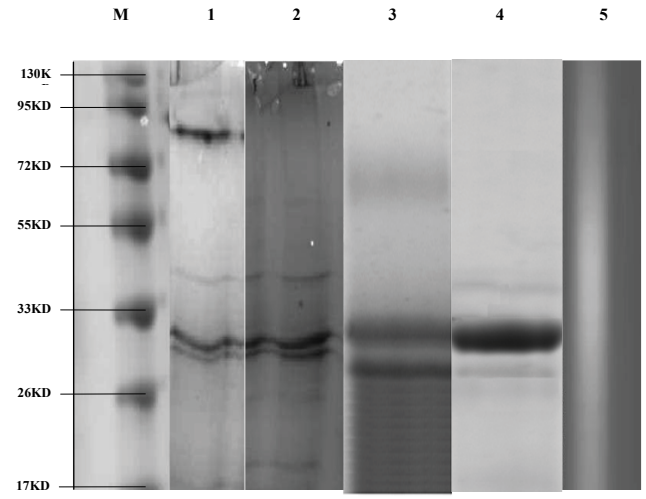


Figure 3 Effect of pH on the activity and stability of the unbound fraction (peak A and B) and the bound fraction (C) of the purified enzyme.

Table 2 Effect of different inhibitors on the purified enzyme activity.

Inhibitors	Concentration(mM)	Relative Activity of Enzymes
Control	None	100
Iodoacetate (1mM)	1.0	83
	2.0	90
PMSF	1.0	35
	2.0	27
Sigma Cocktail	1.0	102
	2.0	112
Chymostain	0.001	51
EDTA	1.0	96
	2.0	100



Lane (M): Protein Reference Marker.
Lane (1): Ammonium Sulphate Precipited Protein (Enzyme).
Lane (2): Sephadex-75 Protein Fraction (Enzyme).
Lane (3): Anion exchange protein fraction (enzyme).
Lane (4): Superdex-75 protein fraction (enzyme).
Lane (5): Zymorgraphy of the purified enzyme activity (after superdex G-75 separation) on casein.

Plate 1 SDS-PAGE protein analysis.

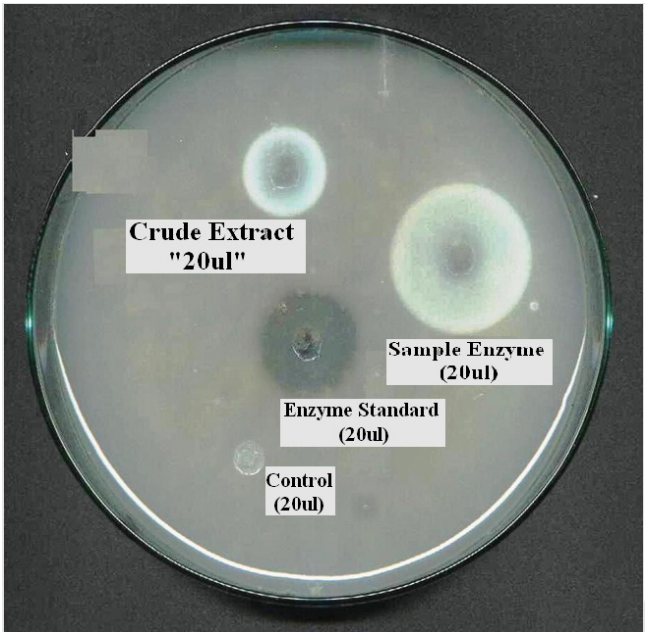


Plate 3a Activity of crude and pure *S. dubium* enzyme by the agar plate methods.

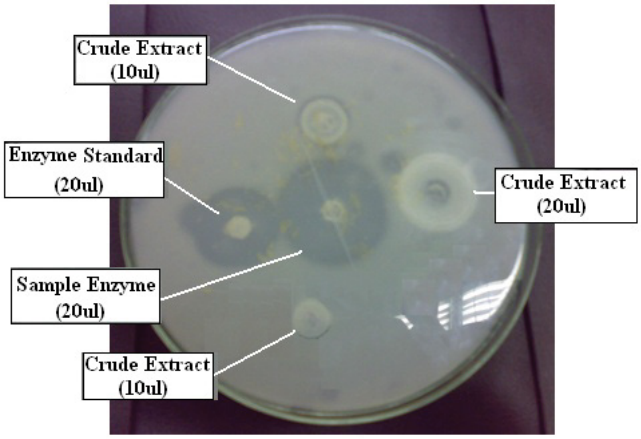
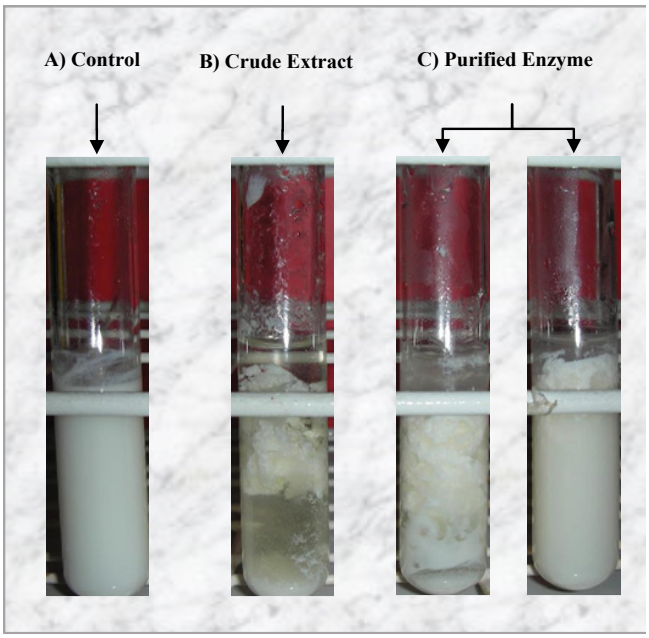


Plate 3b Activity of crude and pure *S. dubium* enzyme by the agar plate methods.



A: Control (No Enzyme),
B: Using crude enzyme extract of *S.dubium* seeds.
C: Using purified enzyme of *S.dubium* seeds.

Plate 2 Coagulation of Fresh Milk

temperature reaching up to 60°C [30]. The stability of the enzyme started to decrease when the temperature approached 70°C after four hours of incubation which is in agreement with work reported by Mohamed-Ahmed et al. [29]. These results indicated that the obtained proteases act at a broad temperature range (50°C -70°C), which offers potential application in many fields.

As for the effect of pH on the enzyme activity **(Figure 3),** the results described as "optimum" (6-8) for extracted unbound apure enzyme are close to those obtained by Ambroso et al., who reported an optimum pH between 6.5 and 8.5. It also coincides with the pH optimum observed for the bound enzyme extracted from callus tissue (7.5-9.5). However, the results presented in this study are lower than those obtained by Yoon et al. [31] who reported a pH value of 7.5-9 concentration. The substantial difference in pH range (6-10) for extracted purified bound may be attributed to the difference of the enzyme precursor, plant part, and the extraction and purification methods. The results conclude that the isolated enzyme from *S. dubium* is a unique

The enzyme affinity to the substrate is measured by the Km value, bearing in mind the fact that smaller values indicate greater affinity [41]. It is clear, from the results obtained in this work, that the Km values are almost 1/3 of those obtained by Hidayatalla [42] for Caesalpinia protease enzyme and hence of greater affinity for the substrate than the latter. The Km values are also lower than those obtained for some other enzymes like field bean seeds enzyme (10.5) and spinach (3.13) reported by Paul et al. [43] and Golbeck et al. [44] respectively **(Table 4).**

The Km represents the maximum rate attained when the enzyme sites are saturated by the substrate of the enzyme Therefore, the enzymes are similar from the enzyme Kinetic point of view. Estimation of crude extract molecular masses revealed that an enzyme from *S. dubium* seeds has five unite of molecular masses (11.3 KDa, 15.7 KDa, 38 KDa, 40 KDa, and 62.8 KDa), while those of the purified enzyme has three only (11.3 KDa, 35 KDa, and 40 KDa) **(Figures 4A and 4B).** These results are similar to those

Table 3 Metal salts compound at 100 mM concentration on the enzyme activity.

Metal Salts	Residual Activity %	Metal Salts	Residual Activity %
Control	100	KF	104
KCl_2	102	$MgSO_4$	098
$CuSO_4$	050	$HgCl_2$	050
Li Cl	109	$FeSo_4$	080
Na Cl	103	$CoSO_4$	060
$CaCl_2$	121	$MgSO_4$	101
ZnCl2	053	$ZnSO_4$	052
MgCl2	099	$AlSO_4$	097

Table 4 Substrate, concentration on the specificity of the pure *S.dubium* enzyme.

Substrate	Concentration (%)	Monitored at Wavelength 280nm	Relative Activity
Casein	1.0	280	100
BSA	1.0	280	30
Gelatin	1.	280	50
Azocasein	1.0	280	62

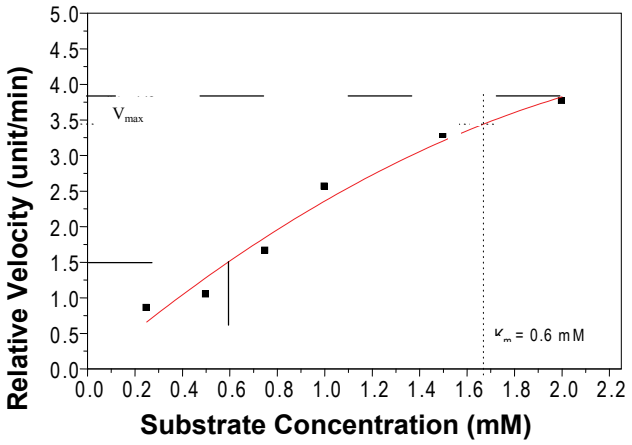


Figure 4A A Plot of the Reaction Velocity (v), as a Function of the Substrate Concentration (S).

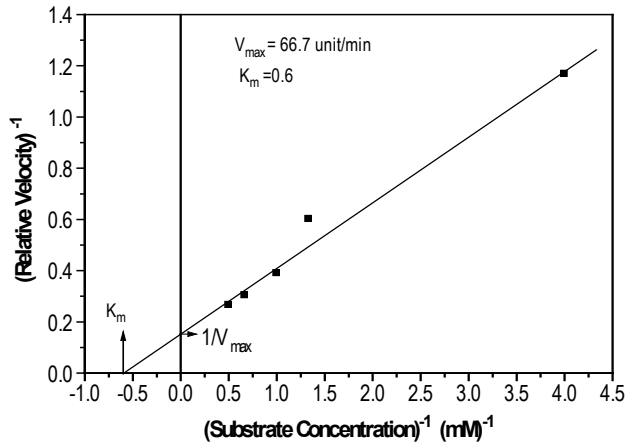


Figure 4B Lineweaver- Burk Double Reciprocal Plot for *S. dubium* purified enzyme at different concentrations.

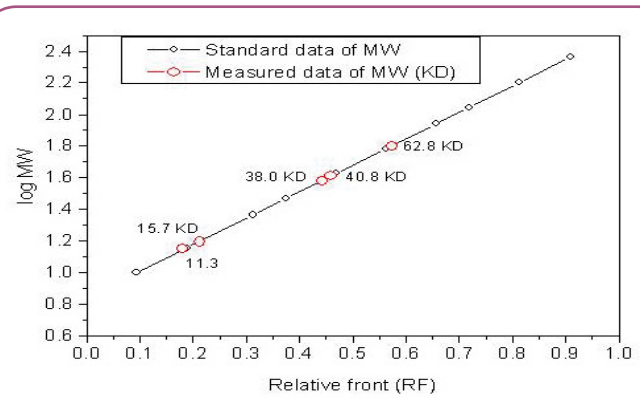


Figure 5A Measurement of molecular masses of the crude extracted enzyme using the Standard Kaleidosope.

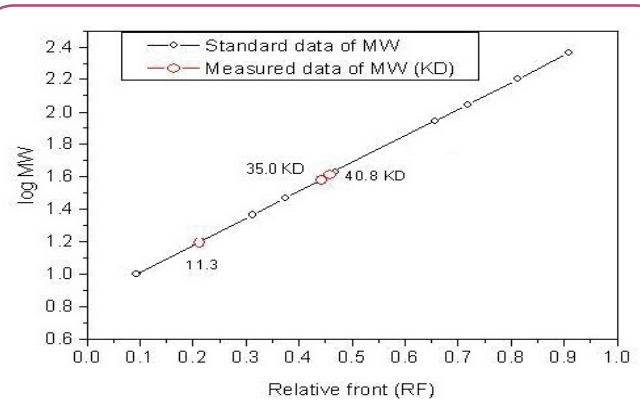


Figure 5B Measurement of molecular masses of the purified enzyme using the Standard Kaleidosope.

obtained by Lynn and Radford [40] who extracted a serine protease from the latex of Hevea brasiliensis. They are also close to those obtained for different parts of *S. dubium* subjected to SDS-electrophoresis with molecular masses within the range of 27-45 KDa [37]. However, other molecular masses reports are available for a well-known plant serine protease, from the Cucumisin family, in the range of 60-70 KDa **(Figures 5A and 5B)** [45-47].

Results obtained from Blast search are highly indicative of the identification of the purified protein as a serine protease. This results is comparable to results recently reported by Mohamed-Ahmed et al. [29]. The authors reported a 14 residue sequence of a dubiumin; a chymotrypsin-like serine protease from seeds of *S. dubium* freshens. The first ten residues of the reported sequence demonstrate a 100% identity to results obtained in this investigation and demonstrate 100% identity to the first ten residues of Lycopersicon esculentum and slightly lower magnitudes of identity to other plant serine proteases, for example, 90% for Alnus species [47] and 80% for Lily and Canein species [48].

Conclusion

Purified enzymes play a highly significant role in the milk-clotting ability and they are agents for use in potential food and bio-industrial raw material. The presented in this study may be concluded as follows: The best buffer medium for extraction is sodium phosphate buffer at a pH 7.1. It was found that $CaCl_2$ enhances the activity of the enzyme; which explains why $CaCl_2$ is used in the cheese industry. The enzyme is thermostable up to 70°C and works well within a pH range of 6-7 concentrations. The purified enzyme was recorded by molecular mass analysis indicates that the enzyme is similar to chymotrypsin-like serine protease.

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