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Dubiumin, a chymotrypsin-like serine protease from the seeds of *Solanum dubium* Fresen

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ABSTRACT

A serine protease was purified 6.7-fold and with 35% recovery from the seeds *Solanum dubium* Fresen by a simple purification procedure that combined ammonium sulfate fractionation, cation exchange and gel filtration chromatographies. The enzyme, named dubiumin, has a molecular mass of 66 kDa as estimated by gel filtration and SDS–PAGE. Carbohydrate staining established the existence of a carbohydrate moiety attached to the enzyme. Inhibition of enzyme activity by serine protease inhibitors such as PMSF and chymostatin indicated that the enzyme belongs to the chymotrypsin-like serine protease class. Dubiumin is a basic protein with p*I* value of 9.3, acts optimally at pH 11.0, and is stable over a wide range of pH (3.0–12.0). The enzyme is also thermostable retaining complete activity at 60 °C after 1 h and acts optimally at 70 °C for 30 min. Furthermore, it is highly stable in the presence of various denaturants (2.0% SDS, 7.0 M urea and 3.0 M guanidine hydrochloride) and organic solvents [CH_3CN–H_2O (1:1, v/v) and MeOH–H_2O (1:1, v/v)] when incubated for 1 h. The enzyme showed a high resistance to autodigestion even at low concentrations.

1. Introduction

In eukaryotes and prokaryotes, proteolysis is a key regulatory mechanism involved in many aspects of cell growth and development (Callis, 1995). In fact, proteolytic enzymes regulate protein processing and intracellular protein levels and remove either abnormal or damaged proteins from the cell, working as a cellular housekeeper (Fontanini and Jones, 2002). Moreover, proteolytic enzymes account for approximately 60% of all enzyme sales, because of their broad substrate specificities and activities over a wide range of pH and temperature values. An additional reason is that they have been widely applied in pharmaceutical, medicinal, food, detergent, leather and biotechnological industries (Nallamestty et al., 2003). In the food industry, proteases are used for coagulation of milk in the cheese industry, as well as for improvement of functional and nutritional properties of proteins, hydrolysis of gelatin, soy protein, casein and whey protein (Sumantha et al., 2006).

Serine proteases are one of the largest groups of proteolytic enzymes involved in numerous regulatory processes. In plants, they are widely spread among different taxonomic groups and are found to be involved in a number of physiological processes such as protein degradation and processing, microsporogenesis, symbiosis, hypersensitive response, signal transduction and differentiation, and senescence (Antao and Malcata, 2005). Despite being the largest class of proteases in plant, the functions and regulatory roles of plant serine proteases are poorly understood, probably due to a lack of identification of their physiological substrates (Antao and Malcata, 2005). Once thought to be rare in plants, in recent years, several serine proteases have been isolated and purified from different plant parts of various plant species including seeds, latex and fruits (Antao and Malcata, 2005).

Apparently, most of the isolated and characterized plant proteases have been classified as cysteine proteases, which are widely used in several processes in the food industry (Uchikoba et al., 1998). The major drawback in the use of cysteine proteases is that their activity is readily reduced by air oxidation and metal ions. Therefore, application of these enzymes requires reductants and chelating agents and, thus, are not so economical and handy (Tomar et al., 2008). By contrast, plant serine proteases are both stable and active under harsh conditions of raised temperatures

Abbreviations: SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride; GuHCl, guanidine hydrochloride; EDTA, ethylenediaminetetraacetic acid; TCA, trichloroacetic acid; BSA, bovine serum albumin; SBTI, soybean trypsin inhibitor; AEBSF, 4-(2-aminoethyl) benzene sulfonyl fluoride hydrochloride; *p*-CMB, *p*-chloromercuric benzoate; E-64, L-trans-epoxysuccinyl-leucylamide-(4-guanidino)-butane; PAS, periodic acid Schiff's; CBB, Coomassie brilliant blue; IEF, isoelctric focusing; PVDF, polyvinylidene difluoride.

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and high pH, as well as in the presence of either surfactants or oxidizing agents. Thus, they are more useful and economical for industrial applications (Tomar et al., 2008). Therefore, the search for new potential plant serine proteases still continues in order to make these industrially applicable and cost effective as well as to understand their physiological role in plants.

Solanum dubium Fresen is a woody herb that belongs to the plant family *Solanaceae* and bears thorny fruits characterized by their shiny yellow color (when mature) and their globos-like shape. It is locally known in Sudan as Gubbein and is a major noxious weed that grows in vast areas of the country, flourishes during the rainy season (June–August) and usually bears green fruits at around January. It is reported that some nomadic groups in Sudan use the fruits of Gubbein as a fermenting agent that is added to warm milk (Magid, 1989). It is also reported that a liquid-mixture of water and the ground Gubbein plant has been used for the removal of hair from animal hides (Uphof, 1968). In the course of screening for milk-clotting enzyme from different part of *S. dubium*, high clotting activity was found in the seeds; therefore, this study was carried out to isolate and purify the enzyme named dubiumin according to the nomenclature of proteases.

2. Results and discussion

2.1. Purification of the enzyme

Ammonium sulfate fractionation was performed as a first purification step of the enzyme. The precipitate obtained from the crude extract of *S. dubium* seeds, using 35–55% saturated ammonium sulfate and separated by ion-exchange chromatography on a CM-Toyopearl column, gave two peaks (Fig. 1a). The second peak contained the active form of the enzyme which was purified 2.7-fold with 64.3% yield and a specific activity of 82.1 U/mg protein (1368.6 nkat/mg). The partially purified enzyme obtained using cation-exchange chromatography was further subjected to fractionation by ammonium sulfate (35–85% saturated). To obtain a pure ammonium sulfate fraction with high specific activity, the fractions were collected at 5% saturation intervals over a range of 35–55% saturation. The results obtained showed that ammonium sulfate fractions in the range of 40–55% saturation had high specific activity (data not shown). Gel filtration chromatography was used as a polishing step to purify the enzyme. Only one peak with protease activity was obtained when the dialyzed pooled fraction was loaded onto Superdex 75 column equilibrated with 0.1 M KCl in 50 mM sodium acetate buffer (Fig. 1b). Purification results of the enzyme from *S. dubium* seeds using different purification means resulted in a 6.7-fold purity with a yield of 35% and specific activity of 205.4 U/mg (3424 nkat/mg) (Table 1). A simple purification procedure was developed in this study to obtain a very active and stable enzyme from *S. dubium* seeds.

2.2. Homogeneity and physical properties of the enzyme

Polyacrylamide gel electrophoresis (SDS–PAGE) showed that the enzyme was highly purified as a single band, which suggested a monomeric nature of the enzyme (Fig. 2a). The molecular mass of the purified enzyme was calculated as 66 kDa, which coincided with the band of bovine serum albumin. The molecular mass of the enzyme is similar to that of the well-known plant serine protease cucumisin (Yamagata et al., 1989), and with other cucumisin-like serine proteases (Asif-Ullah et al., 2006; Uchikoba et al., 1998; Rudenskaya et al., 1998). The molecular mass of the purified enzyme was also similar to those of subtilisin-like endoproteases from the tomato plant (Messdaghi and Dietz, 2000; Tornero et al., 1996). The molecular masses of plant serine proteases so

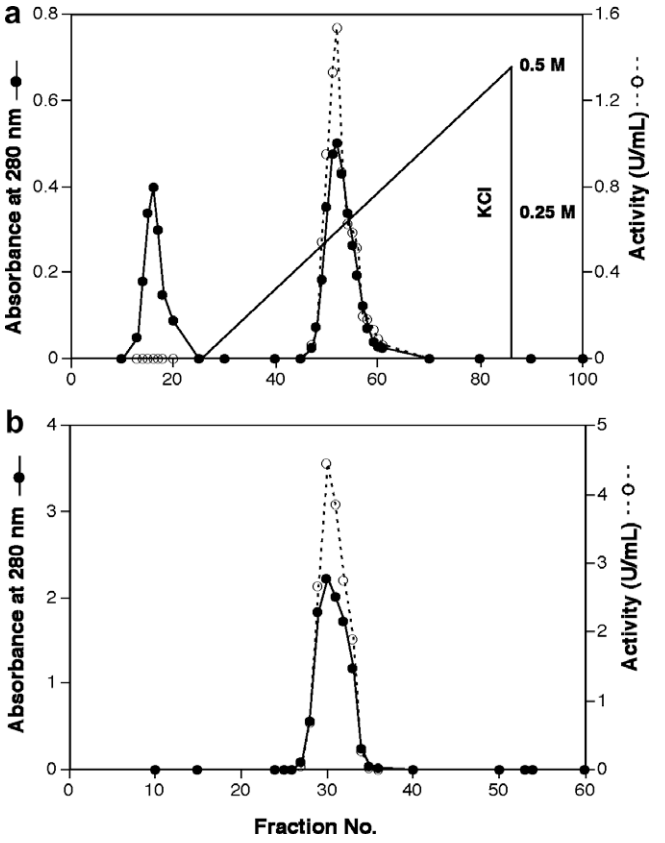

Fig. 1. Elution profile of dubiumin on (a) cation-exchange resin. The CM-Toyopearl column (2.6 cm × 85 cm) was pre-equilibrated with 50 mM sodium acetate buffer, pH 5.0. Then the column was washed with the same buffer and eluted at a flow rate of 3.0 mL/min with a linear gradient of 0.0–0.5 M KCl in the same buffer. (b) Gel filtration chromatography on a Superdex 75 column. The concentrated sample (3.0 mL) after second ammonium sulfate fractionation was loaded onto a Superdex 75 column (1.6 cm × 58 cm), and the column was washed, and eluted with 0.1 M KCl in 50 mM sodium acetate buffer, pH 5.0, at a flow rate of 1.0 mL/min. All fractions were assayed for protein (●) and protease activity (○).

far known vary from 19 to 110 kDa; however, the majority lie between 60 and 80 kDa (Antao and Malcata, 2005). As shown in Fig. 2b, zymogram activity staining on 12.5% SDS and non-reducing polyacrylamide gel using casein as a substrate established that a well-resolved band on SDS–PAGE was obtained corresponding to the protease activity. Although the zymogram gel contained 0.1% SDS and the sample was treated with 1% SDS, the enzyme still displayed activity, indicating that it was resistance to SDS denaturing. As recently reported, SDS resistance is a property often associated with heat-stable proteases of thermo-stable *archaea* and *bacteria* (Joo and Change, 2005). SDS resistance is the most striking property of purified enzyme among all reported plant serine proteases except for cucumisin (Yamagata et al., 1989). As shown in Fig. 2c, the purified enzyme was apparently resolved as a single band on isoelectric focusing with an isoelectric point (p*I*) of 9.3 indicating that it is a basic protein. Noda et al. (1994) found similar results for plant serine protease from *Cucumis melo* L. with a basic isoelectric point (p*I*) of 9.5. In contrast, most of the recently isolated serine proteases from plants have isoelectric points in the range of 4.0–7.0 (Tomar et al., 2008). Furthermore, the native molecular mass of the enzyme was determined using gel filtration chromatography on Superdex 75. The retention time of the purified enzyme on the column was compared to that of the standard proteins. The molecular mass of the native enzyme was estimated to be 66 kDa (Fig. 3), which is similar to that obtained with SDS–PAGE. A single band obtained on SDS–PAGE, IEF electrophoretograms as well as a single peak on gel filtration of the enzyme demonstrated its high purity.

Table 1
Purification of dubiumin from *Solanum dubium* seeds.

Purification means	Protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification (fold)
Crude extract	2.1×10^3	65.4×10^3	30.5	100.0	1.0
$AmSO_4$ (35–55%)[a]	968.5	56.4×10^3	58.2	86.2	1.9
CM-Toyopearl[b]	512.0	42.0×10^3	82.1	64.3	2.7
$AmSO_4$ (40–55%)	444.6	40.7×10^3	91.6	62.3	3.0
Superdex 75[c]	111.5	22.9×10^3	205.4	35.0	6.7

[a] Ammonium sulfate.
[b] Ion-exchange chromatography.
[c] Gel filtration chromatography.

2.3. Carbohydrate detection

Evidence for glycosylation was obtained by SDS–PAGE and blotting to a PVDF membrane followed by subsequent carbohydrate staining. As shown in Fig. 4a, the protein bands stained with Schiff's reagent indicated that a carbohydrate moiety was attached to the purified enzyme. Staining of the membrane with CBB (Fig. 4b) after carbohydrate staining confirmed that the band stained with PAS and CBB is that of a purified enzyme with molecular mass of 66 kDa. The result demonstrated that the purified enzyme is a glycoprotein. The glycocarbohydrate moiety may be part of the functional architecture of the purified enzyme, and might be responsible for its thermal stability. More work is needed to elucidate the linkage nature between the carbohydrate(s) and protein in the purified enzyme and its function. Generally, glycosylation was detected in most of the isolated plant serine protease such as cucumisin, and taraxalisin, cryptolepain, milin and wrightin (Yamagata et al., 1989; Rudenskaya et al., 1998; Pande et al., 2006; Yadav et al., 2006; Tomar et al., 2008).

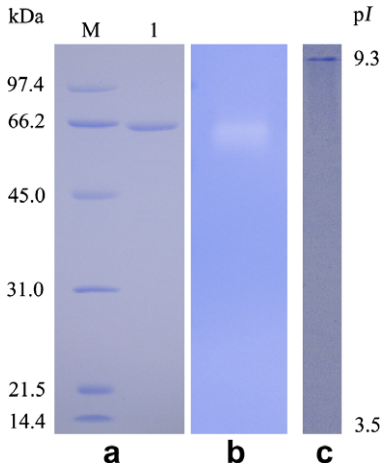

Fig. 2. Analysis of the purified enzyme by (a) SDS–PAGE: lane M, molecular weight standards from high molecular weight descending; Phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme. Lane 1, (30 μg) inactivated purified enzyme. (b) Activity staining (Zymography) clear zone showed the hydrolysis of casein by the enzyme. (c) Isoelectric focusing of purified enzyme.

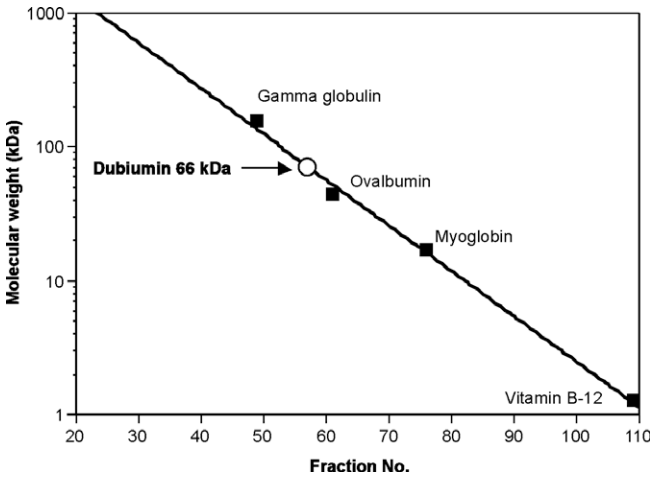

Fig. 3. Estimation of native molecular mass of purified enzyme by gel filtration (Superdex 75 column, 1.6 cm × 12 cm) chromatography. Proteins were eluted with 0.1 M KCl in 50 mM sodium acetate buffer, pH 5.0 at a flow rate of 1.0 mL/min (2.0 mL/tube). Standard proteins used were; gamma globulin (158 kDa), ovalbumin (45 kDa), myoglobin (17 kDa), and vitamin B_{12} (1.35 kDa).

2.4. Effect of pH and temperature on the activity of dubiumin

The purified enzyme was stable under a wide range of pH (4.0–11.0) conditions and retained all its activity within the same pH range when incubated for 1 and 25 h (Fig. 5a). Moreover, in the pH 3.0 and 12.0 more than 80% of activity retained after 1 h incubation at 30 °C. The stability of proteins and enzymes is usually a factor that limits their usefulness in many applications. The isolated enzyme is more stable at a wide range of pH (3.0–12.0), and its stability is more comparable to other cucumisin-like serine proteases from *Cucumis trigonus* Roxburghi, *Cucumis melo* L. var.

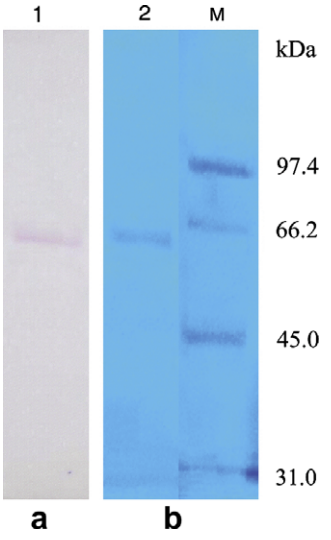

Fig. 4. Carbohydrate detection of the purified protease electroblotted to PVDF membrane (a) PAS staining, and (b) CBB staining. A 30 μg purified enzyme was applied to 10% SDS–PAGE and electroblotted to PVDF membrane. First, the membrane was stained with periodic acid/Schiff's reagents for carbohydrate detection and thereafter stained with CBB for protein detection. Lane M, standard proteins, lane 1 and 2 purified protease stained with PAS and CBB, respectively.

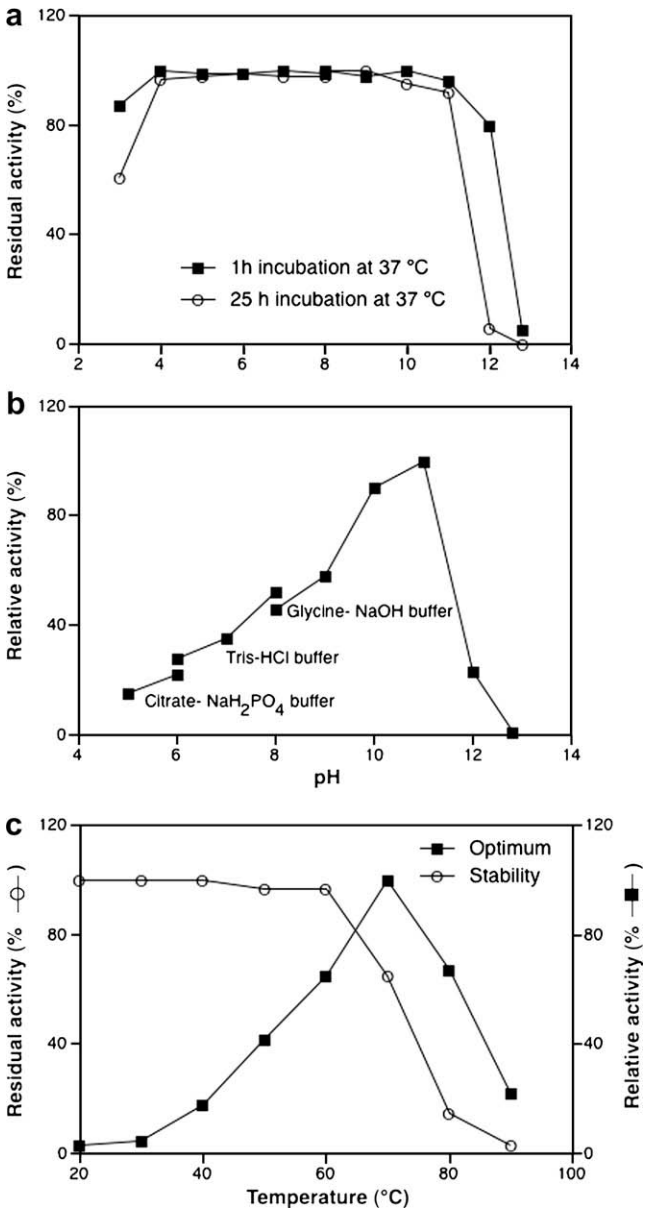

Fig. 5. Effects of pH and temperature on catalytic activity and stability of dubiumin. (a) pH stability, (b) effect of pH on catalytic activity, and (c) effects of temperature on activity and stability of isolated protease. Experimental conditions are as specified in the methods.

Prince, *Euphorbia milii*, and *Trichosantus kirrilowi* A. (Asif-Ullah et al., 2006; Yamagata et al., 1989; Yadav et al., 2006; Uchikoba et al., 1990). As shown in Fig. 5b, the purified protease acted optimally at pH 11.0. Such optimum activity at high pH was not reported for most of the isolated plant serine proteases (Table 2). In this regard, the isolated enzyme is unique, and therefore, might be suitable for uses in industry under alkaline conditions. These characteristics are important, because most enzymes are catalytically unstable at alkaline pH values, thus limiting their usefulness in the food industry especially as cheese-making coagulants (Lamas et al., 2001). An exception to this general rule is represented by the aqueous extract and aspartic proteases from the flower of *Cynara cardunculus*, which have been employed successfully for the manufacture of traditional cheeses from ovine and caprine milk (Sousa and Malcata, 2002). The results in Fig. 5c showed that the enzyme activity increased as the temperature increased from 20 to 70 °C. The activity at 70 °C was 5- and 10-fold higher than that of the activities at 40 °C and 20 °C, respectively. However, the activity decreased steadily as the temperature rose over 80 °C. There was 100 and 70% activity retention after 1 h incubation at 60 and 70 °C, respectively. The thermostability of the enzyme was found to be up to 70 °C. The temperature profile of the purified enzyme was similar to those of subtilisin/cucmisin like plant serine proteases from *Cucumis trigonus* Roxburghi, *Cucumis melo* L. var. Prince, *Benincasa cerifera*, *Trichosantus kirrilowi* A. and *Trichosantus cucumeroides* (Asif-Ullah et al., 2006; Yamagata et al., 1989; Kaneda and Tominaga, 1977; Uchikoba et al., 1990; Kaneda et al., 1986).

2.5. Effect of denaturant and organic solvent on the activity of dubiumin

The purified enzyme exhibited remarkable stability under various conditions. Complete retention of proteolytic activity was observed in 7.0 M urea, 3.0 M GuHCl (guanidine hydrochloride), and 3.0 M NaCl for 1 h incubation as shown in Table 3. Moreover, the enzyme retained more than 95% of its activity when incubated for 1 h in MeOH–H_2O (1:1, v/v) and CH_3CN–H_2O (1:1, v/v), and about 70% in 2% SDS. The most striking property of the purified enzyme is its high stability with respect to pH, temperature, denaturants and organic solvents. The stability of the purified enzyme in the presence of SDS was superior to that of recently isolated plant serine proteases, such as milin (Yadav et al., 2006) and wrightin (Tomar et al., 2008). High stability in GuHCl (4 M) and urea (8 M) was reported for many plant cysteine proteases, such as procerain (Dubey and Jagannadham, 2003), papain, ficin, stem bromelin (Glazer and Smith, 1971), ervatamin C (Sundd et al., 1998) and ervatamin B (Kundu et al., 2000).

2.6. Effect of inhibitors and metal ions on activity of dubiumin

As shown in Fig. 6, typical inhibitors against aspartic, and metallo-proteases had no effect on the enzyme activity, indicating that the protease was neither an aspartic nor a metallo-protease. However, the inhibitors for cysteine protease (10 μM E-64, and 1 mM iodoacetate) inhibited the enzyme by 30%, and 20%, respectively. The inhibitor for trypsin-like protease, soybean trypsin inhibitor, also had no inhibitory effect on activity. The strongest inhibition was observed with PMSF, a general inhibitor for serine proteases, and chymostatin, an inhibitor of chymotrypsin-like serine protease. Inhibition studies can provide a first insight into the nature of the enzyme, its cofactor requirement, and the nature of the active center (Sedmak and Grossberg, 1977). The above-mentioned inhibition profile classified the isolated protease as a member of the chymotrypsin-like serine protease class, with a cysteine residue near its active site. The inhibition profile of the purified enzyme is consistent with those reported for bamboo serine protease (Arima et al., 2000a) and cucumisin (Uchikoba et al., 1995). Strong inhibition by PMSF was also reported for some plant serine proteases, such as cucumisin-like protease from latex of *Euphorbia supina* (Arima et al., 2000b), subtilisin-like protease from *Cucumis trigonus* Roxburghi (Asif-Ullah et al., 2006), and milin (Yadav et al., 2006). It is noticeable that a proteinaceous inhibitor such as soybean trypsin inhibitor (SBTI), which is present in a typical protein-rich food such as soybean, did not inhibit activity of the purified enzyme. This property could, therefore, pave the way for the application of the purified enzyme in food industries. Generally, proteinaceous inhibitors known so far inhibit activity of either bacterial or animal serine proteases, but fail to do so in the case of plant serine proteases such as cucumisin, bamboo sprout proteases, milin, wrightin, etc. (Uchikoba et al., 1995; Arima et al., 2000a; Yadav et al., 2006; Tomar et al., 2008). The effect of differ-

Table 2
Physiochemical properties of purified enzyme and other plant serine proteases.

Plant source	Mol mass (kDa)	Optimum		Stability		Glycoprotein	pI
		pH	Temperature (°C)	pH	Temperature (°C)		
S. dubium (Dubiumin)[a]	66	11	70	3–12	60–70	Yes	9.3
W. tinctoria (Wrightin)[b]	57.9	7.5–10	70	5.5–11.5	75–80	Yes	6.0
E. milli (Milin)[c]	51.4	8	60	5–12	65	Yes	7.2
C. melo (Cucumisin)[d]	54	7.1	70	4–11	50	Yes	NR
I. carnea (Carnein)[e]	80.2	6.5	65	3–10	35–75	NR	5.6
C. trigonus Roxburghi[f]	67	11	65	4–10	65	NR	NR
T. officinale (Taraxalisin)[g]	67	8	40	6–9	40	Yes	4.5
S. grantti Hook[h]	76 ± 2	7	60	5–10	65	NR	NR
H. vulgare L (SEP-1)[i]	70	6.5	50	4–7	50	NR	NR
C. buchanani (Cryptolepain)[j]	50.5	8–10	65–75	2.5–11.5	80	Yes	6.0

NR: not reported.
[a] This report.
[b] Tomar et al. (2008).
[c] Yadav et al. (2006).
[d] Kaneda et al. (1975).
[e] Patel et al. (2007).
[f] Asif-Ullah et al. (2006).
[g] Rudenskaya et al. (1998).
[h] Menon et al. (2002).
[i] Fontanini and Jones (2002).
[j] Pande et al. (2006).

Table 3
Stability of purified enzyme under various denaturant and organic solvents.

Condition	Relative activity (%)
Urea (7.0 M)	100
GuHCl (3.0 M)	100
NaCl (3.0 M)	100
CH_3CN–H_2O (1:1, v/v)	98
MeOH–H_2O (1:1, v/v)	96
SDS (2.0%)	70

The purified enzyme (12 μg) was incubated with different organic solvents and denaturants at the indicated concentration for 1 h at 30 °C. Remaining activity was measured using 1% azocasein in 50 mM sodium acetate buffer, pH 5.0, as substrate at 30 °C for 30 min.

ent metal ions was determined by incubating the enzyme with metal ions at 30 °C for 30 min. The remaining activity was then assayed under the standard assay methods using azocasein as a substrate. As shown in Table 4, the monovalent cations (K^+, Rb^+, and Li^+), and the divalent cations (Ca^{2+}, Mg^{2+}) had no inhibitory effect on enzyme activity. However, the activity was inhibited by some divalent cations such as Hg^{2+}, Co^{2+}, Zn^{2+}, and Cu^{2+}. The results indicated that metal ions are not required for the activity of *S. dubium* serine protease. Moreover, a slight inhibition of the purified enzyme by E-64, Hg^{2+}, and iodoacetate, indicated involvement of cysteine residue near the active site of the enzyme. Similar observations were reported for a protease from melon fruit (Kaneda et al., 1975), and subtilisin-like protease from *Bacillus cereus* (Moriyama et al., 1998).

2.7. Autodigestion

Autocatalysis of dubiumin was monitored at pH 8.0 with a protein concentration range from 0.01 to 0.08 mg/mL (Fig. 7). The enzyme retained all of its activity at a concentration of 0.04 mg/ml,

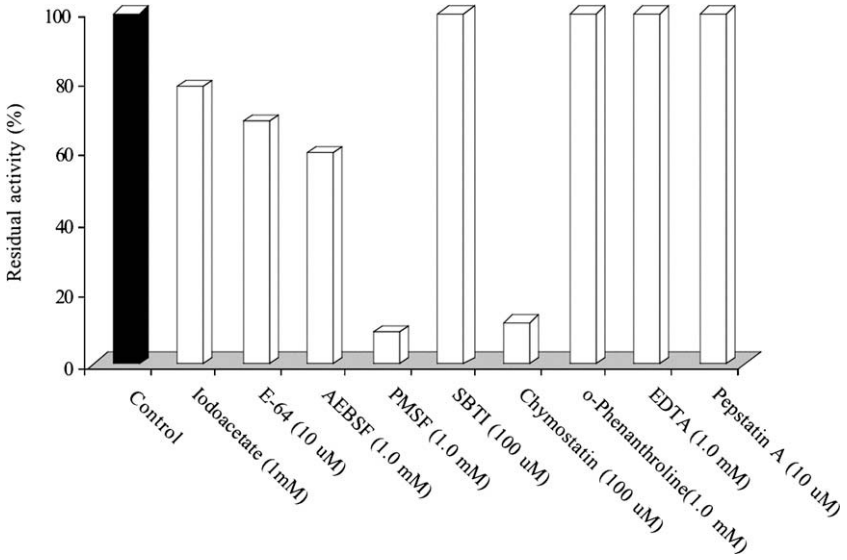

Fig. 6. Effects of various inhibitors on activity of dubiumin. The enzyme (12 μg) was pre-incubated with various inhibitors of the indicated concentration for 30 min, and then the residual activities were measured using 1% azocasein as substrate at 30 °C for 30 min.

Table 4
Effect of metal ions (1.0 mM) on the enzyme activity.

Reagent	Relative activity (%)	Reagent	Relative activity (%)
None	100	$MgCl_2$	97
RbCl	102	$BaCl_2$	93
$CaCl_2$	116	$Fe_2(SO_4)_3$	89
LiCl	106	$AL_2(SO_4)_3$	75
$MgSO_4$	103	$CuSO_4$	71
NaCl	110	$ZnSO_4$	56
KF	101	$CoSO_4$	51
KCl	98	$HgCl_2$	38

while at 0.01 mg/mL it was observed that more than 80% of the activity was retained when incubated for 48 h. Dubiumin showed exceptionally high resistance to autodigestion. This, in turn, indicates its high stability, and thus its possible application in food, textile, and biotechnological industries. Most of the isolated proteases studied undergo autodigestion at low protein concentrations such as procerain (Dubey and Jagannadham, 2003). Recently isolated plant serine proteases were also found to resist autodigestion, for example milin (Yadav et al., 2006), carnein (Patel et al., 2007), cryptolepain (Pande et al., 2006), and wrightin (Tomar et al., 2008).

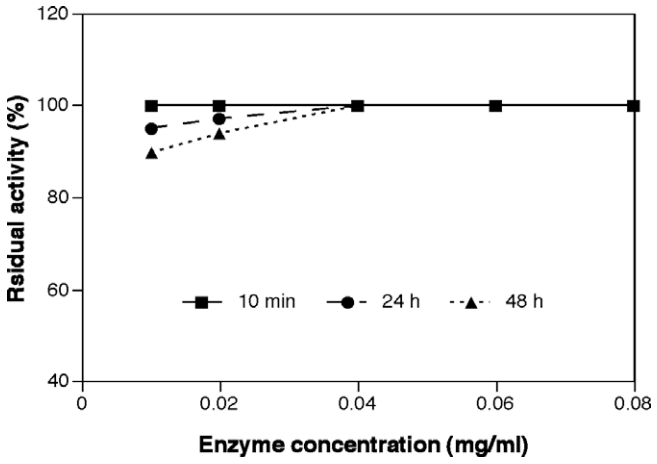

Fig. 7. Autodigestion of dubiumin as a function of protein concentration. The enzyme was incubated for 10 min, 24, and 48 h at different concentrations (0.01–0.08 mg/mL) at 37 °C in 50 mM Tris-HCl buffer, pH 8.0. The residual activity was measured at standard assay conditions as described in the Materials and Methods. The activity of the enzyme after 10 min was taken as 100% activity.

2.8. N-terminal sequence

The sequence of 14 amino acids at the N-terminus of dubiumin was determined, and compared with other plant serine proteases (Table 5). The N-terminal sequence of dubiumin showed high similarity with that of plant serine proteases. The highest similarity was (92%) with Tomato P69B (Tornero et al., 1997) and (85%) with Tomato P69A (Tornero et al., 1996), and significant similarity (64%) with Alnus ag12 (Ribeiro et al., 1995). Thus, it appears that dubiumin may be a new member of the plant serine proteases.

2.9. Milk-coagulation

The enzyme dubiumin greatly coagulated skimmed milk and formed a white and firm curd (Fig. 8). Furthermore, the ratio of milk-clotting activity to proteolytic activity of dubiumin was determined and compared with those of some commercial rennets (Arima et al., 1970). It was found that the ratio of milk-clotting activity to the proteolytic activity of dubiumin was (2490 U/OD 660 nm) comparable to those of (4990, 4650, 2590 U/OD 660 nm) Calf rennet, *Mucor* rennet and *Endothia parasitica* rennet, respectively. The ratio of milk-clotting activity to proteolytic activity is a useful indicator of the protease efficiency to be used as a coagulant for cheese making (Arima et al., 1970). The capacity of this protease to produce milk curds, beside its high ratio of milk clotting to proteolytic activity, could make it useful as a new milk coagulants, although, more studies about quality of both milk

Fig. 8. Coagulation of skimmed milk with dubiumin. Milk curd was formed by the reaction of 0.25 mg dubiumin with 100 ml of skimmed milk at 37 °C for 1 h.

Table 5
Comparison of N-terminal sequence of dubiumin with other known plant serine proteases.

Enzyme	Source	Amino terminal sequence (first 14residues)	Identity (%)
Dubiumin[a]	*S. dubium*	**T T H T P R F L G L Q Q N M**	100
P69B[b]	*L. esculentum*	**T T H T P** S **F L G L Q Q N M**	92
P69A[c]	*L. esculentum*	**T T H T** S S **F L G L Q Q N M**	85
Alnus ag12	*A. glutinosa*	**T T H T P R F L** S **L** N P T G	64
Lily LIM 9	*L. longiflorum*	**T T H T P** D Y **L G** I **Q** T G V	57
Carnein	*I. carnea*	**T T H** S **P** E **F L G L** A E S S	57
Protease B	*E. supina*	**T T** R **T P** N **F L G L** V D D S	57
ARA12	*A. thaliana*	**T T** R **T P** L **F L G L** D E H T	57
Bamboo protease	*P. hindsii*	**T T** R **T P** S **F L** R **L** S A V G	50
Cucumisin	*C. melo*	**T T** R S W D **F L G** F P L T V	35

The amino acids are abbreviated with one-letter symbols, and the identical amino acid residues are presented in bold.
[a] This study.
[b] Tornero et al. (1997).
[c] Tornero et al. (1996), all other sequence were from Patel et al. (2007).

curds and the cheese formed should be carried out in the future to confirm its usefulness in the dairy industry.

3. Conclusion

To our knowledge, this is the first report of purification and characterization of a putative serine protease from seeds of *S. dubium* Fresen. Compared to other purification procedures carried out previously, we succeeded in developing a simple purification procedure in this study. The procedure is economic and, combined with the availability of the plant seeds, could possibly be used for large-scale production of the enzyme, allowing a broad study of its various aspects and hence probable applications. Moreover, the high stability of dubiumin against autodigestion and under various conditions, in accordance with the availability of raw materials, in addition to its high milk-clotting ability, could therefore pave the way for its uses in the cheese industry as well as other food and biotechnological industries.

4. Experimental

4.1. Materials

Fruits of *S. dubium* Fresen were collected during the growing season 2004/2005 from the Shambat area of Khartoum North, Sudan. These were sun-dried and hand-crushed to obtain the seeds. *Solanum dubium* Fresen seeds were checked and Plant Protection Directorate, Ministry of Agriculture and Forestry, Sudan issued the Phytosanitary Certificate No. 003236. The seeds were preserved in the laboratory in dissector. Standard proteins for gel filtration and SDS–PAGE were from Bio-Rad laboratories (Hercules, CA, USA). CM-Toyopearl was from Tosoh, Tokyo, Japan, and Superdex 75 was from Amersham (Amersham Bioscience, Uppsala, Sweden). Unless otherwise stated, all chemicals used in this study were of reagent grade.

4.2. Extraction and purification of the enzyme

4.2.1. Extraction

S. dubium seeds (50 g) were coarsely milled in a mortar and extracted with 500 mL of 5% NaCl in NaOAc buffer, pH 5.0, for 1 h with stirring at 4 °C. The extract was filtered through cheesecloth and centrifuged at 10000*g* for 20 min. The supernatant was dialyzed overnight at 4 °C with 0.1 M NaOAc buffer, pH 5.0. Protease activity as well as the protein concentration for extract was determined as described below.

4.2.2. Ammonium sulfate fractionation

The supernatant (510 mL) from the above step was brought to 35% saturation with gradual addition of solid $(NH_2)_4SO_4$ and allowed to stand on ice for 30 min. The resulting precipitate was collected by centrifugation at 10000*g* (HIMAC, type SCR 18B and CR 20B2, Hitachi Koki Co., Ltd, Tokyo, Japan) for 20 min at 4 °C and dissolved in 50 mM sodium acetate buffer, pH 5.0. The above step was repeated twice to obtain 55 and 80% $(NH_2)_4SO_4$ saturation. The supernatant of each step was dialyzed with 50 mM NaOAc buffer, pH 5.0, for 24 h with frequent changes of buffer. After dialysis, the solution was centrifuged to remove any solid particles before the protein concentration and activity were measured.

4.2.3. Ion-exchange chromatography

The active fraction (65 mL) obtained above was further purified by passing through CM-Toyopearl column (2.6 × 85 cm) equilibrated with 50 mM NaOAc buffer, pH 5.0. The column was washed three times with the same buffer until the absorbance at A_{280} nm reached zero. Then, elution was carried out using a linear gradient of 0.0–0.5 M KCl in 50 mM NaOAc buffer, pH 5.0. Fractions of 18 mL each were collected at a rate of 3.0 mL/min, and analyzed for enzyme activity and protein concentration. The partially purified enzyme (320 mL) from the above step was further purified using $(NH_2)_4SO_4$ (second fractionation) with saturation ranged from 35 to 80% (5% interval). Then the most active fractions in the ranges of 40–45%, 45–50%, and 50–55% were pooled and combined as one fraction (40∼55%) and dialyzed overnight at 4 °C with 50 mM NaOAc buffer, pH 5.0. Thereafter, the pooled fraction was gel filtered using Superdex 75.

4.2.4. Gel filtration chromatography

The pooled fractions of the above step were concentrated using an Amicon ultra membrane with 10000 Dalton cut off (Millipore, Carrigtwohil, Co. Cork, Ireland). The concentrated solution (3.0 mL) was applied to a Superdex 75 column (1.6 × 58 cm) equilibrated with 50 mM NaOAc buffer, pH 5.0, containing 0.1 M KCl. Elution was carried out at a flow rate of 1.0 mL/min (3.0 mL/tube). Active fractions were pooled and stored at – 20 °C for further analysis.

4.3. Activity assay

4.3.1. Protease

Protease activity was determined by the colorimetric assay using azocasein as a substrate as described by Sarath et al. (1989). About 0.15 mL enzyme solution was added to 0.25 mL of 1% azocasein in 50 mM Tris-HCl buffer, pH 8.0, mixed gently and incubated at 30 °C for 30 min. The reaction was stopped by adding 1.2 mL of 5% $Cl_3C_2O_2H$, and the mixture was incubated at room temperature for 30 min, then centrifuged at 10,300*g* for 10 min at 4 °C. One mL of the supernatant was mixed with 1.0 mL of 1.0 M NaOH and kept for 10 min at room temperature for color development. A blank was prepared in the same way, but with the addition of $Cl_3C_2O_2H$ to the enzyme before the substrate. The absorbance was read at 440 nm. One unit of the enzyme activity was defined as the amount of the enzyme that increased the absorbance at 440 nm by 1.0/h under the assay conditions (1 U = 16.67 nkat).

4.3.2. Milk-clotting

Milk-clotting activity was determined according to the methods described by Arima et al. (1970) with slight modification. The substrate (10% skimmed milk in 0.01 M $CaCl_2$) was prepared and the pH was adjusted to 6.5. The substrate (2.0 mL) was pre-incubated for 5 min at 37 °C, and 0.2 mL of enzyme extract was added, and the curd formation was observed while manually rotating the test tube occasionally. The end point was recorded when discrete particles were discernible. One milk-clotting unit was defined as the amount of enzyme that clots 10 mL of the substrate in 40 min.

4.4. Determination of the protein content

The protein content of the fractions obtained after the chromatographic purification was estimated by measuring the absorbance at 280 nm. Quantitative determination of the protein content was estimated according to the Lowry method (Lowry et al., 1951). The protein content was calculated from the bovine serum albumin (BSA) standard curve.

4.5. Electrophoresis and activity staining

Homogeneity and intactness as well as molecular mass determination of the enzyme, during and after purification, were determined using SDS–PAGE (Laemmli, 1970). The purified enzyme was

inactivated to avoid autolysis. The gels were stained with 0.1% Coomassie brilliant blue R-250. The molecular weight of the purified enzyme was extrapolated from the plot of log molecular weight vs electrophoretic mobility of markers.

For activity staining (Zymography), the electrophoresis of the purified enzyme was carried as described above except that the sample was treated with buffer containing 2% SDS without boiling to keep the enzyme active. After electrophoresis, the gel was washed with 2.5% Triton X-100 for 30 min with shaking to remove SDS, and then washed three times with distilled H_2O. The gel was then immersed in 50 mL of 2% casein in Tris-HCl buffer, pH 7.5, for 30 min at 4 °C, in order to allow the substrate to diffuse into the gel at reduced enzyme activity (Garcia-Carreno et al., 1993). Then, the gel was incubated at 25 °C for 60 min to digest the substrate. Thereafter, the gel was washed with distilled H_2O, and immediately fixed and stained.

4.6. Detection of carbohydrate

After electrophoresis, the gel was equilibrated with 25 mM Tris-HCl buffer containing MeOH–H_2O (5: 95, v/v) for 15 min. The protein was electroblotted onto a PVDF membrane at a constant current of 126 mA using semidry blotting apparatus (ATTO model AE-6677P/S/N). The PVDF membrane was stained with the glycoproteins-specific Schiff's reagent according to the Kapitany and Zebrowski (1973) method as described by Egito et al. (2001).

4.7. Isoelectric focusing (IEF)

The p*I* value of the purified enzyme was estimated using the Phast sytem (Pharmacia LKB Biotechnology, Uppsala, Sweden) following the manufacture instruction manual. The purified enzyme (4 μg) was centrifuged, filtered (0.45 μm PTFE membrane, Millex-LH, Japan), and then 2 μL per lane was loaded using a sample applicator. The sample and IEF standard (Amersham Bioscience, Sweden) were applied at both the anode and the cathode to ensure that the protein reached its isoelectric point. Pharmacia broad p*I* markers (3.5–9.3) used were trypsinogen (p*I* 9.30), lectins from *Lens culinaris* (p*I* 8.65, 8.45, and 8.15), myoglobin basic band (p*I* 7.35), myoglobin, acidic band (p*I* 6.85), human carbonic anhydrase B (p*I* 6.55), bovine carbonic anhydrase B (p*I* 5.85), α-lactoglobulin A (p*I* 5.20), trypsin inhibitor (p*I* 4.55), methyl red dye (p*I* 3.75), and amylglucosidase (p*I* 3.50). After electrophoresis, the gel was stained and destained for protein as described earlier.

4.8. Measurement of native molecular mass of the purified enzyme

The molecular mass of the purified enzyme was determined by gel filtration chromatography on a Superdex 75 column. The purified enzyme (1.0 mL), and the standard proteins were loaded onto a Superdex 75 column (1.6 × 12 cm), and equilibrated with 50 mM NaOAc buffer, pH 5.0, containing 0.1 M KCl. Elution was carried out at a flow rate of 1.0 mL/min (2.0 mL/tube). Standard proteins used were γ-globulin (158 kDa), ovalbumin (45 kDa), myoglobin (17 kDa), and vitamin B_{12} (1.35 kDa). A plot of the molecular masses substances against their retention time (elution volume) was done.

4.9. Effect of temperature on enzyme activity and stability

Optimum temperature for the enzyme activity was studied using azocasein as substrate. The substrate was pre-incubated at specified temperatures in a range of 20–90 °C for 10 min. Then the enzyme was added, and the reaction was proceeded at the same temperature for 30 min and a control tube was incubated without the enzyme at each temperature. Stability was tested by incubating the enzyme in 50 mM Tris-HCl buffer, pH 8.0, at different temperatures (20–90 °C) for 30 min. The remaining activity was measured at 30 °C for 30 min using azocasein as a substrate and expressed as a percentage of the control.

4.10. Effect of pH on the enzyme activity and stability

The optimum pH of the enzyme was determined by measuring azocasein hydrolyzing activity at 30 °C for 30 min in a pH range of 5.0 to 12.8. The buffers used were; 50 mM citrate-sodium phosphate (pH 5.0–6.0), 50 mM Tris-HCl (pH 6.0–8.0), and 50 mM glycine (pH 8.0–12.8 adjusted with NaOH). Suitable controls at the respective pH values were also set up, comprising the buffered substrate without enzyme. The pH stability of the enzyme was determined by measuring the remaining activity after incubation at 30 °C for 1 h, and 25 h or at 60 °C for 30 min in different buffers (50 mM sodium dihydrogen phosphate/NaOH buffer between pH 3.0 and 8.0, and 50 mM glycine/NaOH between pH 8.0 and 12.8).

4.11. Stability

The enzyme stability in the presence of chemical denaturants such as urea, SDS, and guanidine hydrochloride (GuHCl) or organic solvents such as MeOH, and CH_3CN was also measured. The enzyme was incubated with either 3.0 M GuHCl, 3.0 M NaCl, 7.0 M urea, 2% SDS, MeOH–H_2O (1:1, v/v), or CH_3CN–H_2O (1:1, v/v) at 30 °C for 1 h, and then the residual activity was determined as describe above.

4.12. Effect of various inhibitors and metal ions on the enzyme activity

The effect of various protease inhibitors on the enzyme activity was measured. The protease inhibitors used were serine protease inhibitors [1.0 mM phenylmethylsulphonyl fluoride (PMSF), 0.1 mM soybean trypsin inhibitor, 0.1 mM chymostatin, and 1.0 mM 4-(2-aminoethyl) benzene sulfonyl fluoride hydrochloride (AEBSF)], cysteine protease inhibitors [0.1 mM *p*-chloromercuric benzoate (*p*-CMB), 0.1 mM sodium tetrathianate, 10 μM L-trans-epoxysuccinyl-leucylamide-(4-guanidino)-butane (E-64), and 1.0 mM iodoacetate], metal protease inhibitors [1.0 mM ethylene diamine tetra acetic acid (EDTA), and 1.0 mM *o*-phenanthroline], and aspartic protease inhibitor (10 μM pepstatin). The enzyme (12 μg) was pre-incubated with the inhibitors for 30 min at 30 °C prior to the addition of the substrate. The residual activity was then determined under the standard assay conditions, and expressed as the percentage of the activity measured in the absence of inhibitors.

The effects of metal ions (e.g., Ca^{2+}, Mg^{2+}, Al^{3+}, Ba^{2+}, Fe^{3+}, Na^{+}, Li^{+}, Rb^{+}, K^{+}, Zn^{2+}, Hg^{2+}, Co^{2+}, and Cu^{2+} [1.0 mM] in either chloride or sulfate salts) on the enzyme activity were investigated. The enzyme was incubated with the metal ions for 30 min at 30 °C, then the residual activity was determined under the standard conditions, and expressed as the percentage of the activity measured in the absence of metal ions.

4.13. Autodigestion

Autodigestion of dubiumin was studied as a function of the enzyme concentration (0.01–0.08 mg/mL). The enzyme at each concentration was incubated at 37 °C in 50 mM Tris-HCl buffer, pH 8.0 and then at intervals of 10 min, 24 h, and 48 h an aliquot of the enzyme was withdrawn and assayed for protease activity using azocasein as a substrate. The activity of the enzyme at the first 10 min was used as 100% to estimate the residual activity.

4.14. N-terminal sequence

The purified dubiumin was electrophoresed on 12.5% SDS–PAGE and transferred as a blot onto a PVDF membrane. The N-terminal amino acids sequence was determined by automated Edman's degradation with an Applied Biosystems model 491 PROCISE Sequencing System (Foster City, CA, USA). The N-terminal sequence of dubiumin was compared with other plant serine proteases using DDBJ–BLAST.

4.15. Milk-coagulation

In order to evaluate the milk-clotting activity of the enzyme, milk curd assays were prepared. Coagulations were carried out in 100 ml of skimmed milk (20%) to which 0.25 mg of purified dubiumin was added and incubated for 60 min at 37 °C.

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References

Antao, C.M., Malcata, F.X., 2005. Plant serine proteases: biochemical, physiological and molecular features. Plant Physiol. Biochem. 43, 637–650.

Arima, K., Ya, J., Iwasaki, S., 1970. Milk-clotting enzyme from *Mucor pusillus* var. Lindt. In: Pearlman, E.G., Lorand, L. (Eds.), Methods in Enzymology. Academic Press, New York, pp. 446–459.

Arima, K., Uchikoba, T., Yonezawa, H., Shimada, M., Kaneda, M., 2000a. Isolation and characterization of a serine protease from the sprouts of *Pleioblatus hindsii* Nakai. Phytochemistry 54, 559–565.

Arima, K., Uchikoba, T., Yonezawa, H., Shimada, M., Kaneda, M., 2000b. Cucumisin-like serine protease from the latex of *Euphorbia supine*. Phytochemistry 53, 639–644.

Asif-Ullah, M., Kim, K.-S., Yu, Y.G., 2006. Purification and characterization of a serine protease from *Cucumis trigonus* Roxburghi. Phytochemistry 67, 870–875.

Callis, J., 1995. Regulation of protein degradation. Plant Cell 7, 845–857.

Dubey, K.V., Jagannadham, V.M., 2003. Procerain, a stable cysteine protease from the latex of *Calotropis procera*. Phytochemistry 62, 1057–1071.

Egito, A.S., Girardet, J.-M., Laguna, L.E., Miclo, L., Gaillard, J.-L., 2001. Highly sensitive periodic acid/Schiff detection of bovine milk glycoproteins electrotransferred after nondenaturing electrophoresis, urea electrophoresis, and isoelectric focusing. Lait 81, 775–785.

Fontanini, D., Jones, B.L., 2002. SEP-1-a subtilisin-like serine endopeptidase from germinated seeds of *Hordeum vulgarae* L. cv. Morex. Planta 215, 885–893.

Garcia-Carreno, F.L., Dimes, L.E., Haard, N.F., 1993. Substrate-gel electrophoresis for composition and molecular weight of proteinases or proteinaceous proteinase inhibitors. Anal. Biochem. 214, 65–69.

Glazer, A.N., Smith, E.L., 1971. Papain and other plant sulfhydryl proteolytic enzymes. In: Boyer, P.D. (Ed.), The Enzymes. Academic Press, New York, pp. 501–546.

Joo, H.S., Change, C.-S., 2005. Oxidant and SDS-stable alkaline protease from halotolerant Bacillus clausii 1–52: enhanced production and simple purification. J. Appl. Microbiol. 98, 491–497.

Kaneda, M., Tominaga, N., 1977. Isolation and characterization of a proteinase from white gourd. Phytochemistry 16, 345–346.

Kaneda, M., Eida, A., Tominaga, N., 1975. Isolation and characterization of a proteinase from the Sarcocarp of melon fruit. J. Biochem. 78, 1287–1296.

Kaneda, M., Sobue, A., Eida, S., Tominaga, N., 1986. Isolation and characterization of a proteinase from the sarcocarp of snake-gourd friut. J. Biochem. 99, 569–577.

Kapitany, R.A., Zebrowski, E.J., 1973. A high resolution PAS stain for polyacrylamide gel electrophoresis. Anal. Biochem. 56, 361–369.

Kundu, S., Sundd, M., Jagannadham, M.V., 2000. Purification and characterization of stable cysteine protease Ervatamin B, with two disulfide bridges, from the latex of *Ervatamia coronaria*. J. Agric. Food Chem. 48, 171–179.

Laemmli, U.K., 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature 277, 680–685.

Lamas, E.M., Barros, R.M., Balcao, V.M., Malcata, F.X., 2001. Hydrolysis of whey proteins by proteases extracted from *Cynara cardunculus* and immobilized onto highly activated supports. Enzy. Microb. Technol. 28, 642–652.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with Folin Phenol reagent. J. Biol. Chem. 193, 265–275.

Magid, A., 1989. Plant domestication in the Middle Nile Basin. An archaeoethnobotanical case study. British Archaeological Reports 523, Cambridge Monographs in African Archaeology 35, Oxford, p. 100.

Menon, M., Vithayathil, P.J., Raju, S.M., Ramadoss, C.S., 2002. Isolation and characterization of proteolytic enzymes from the latex of *Synadenium grantii* Hook, 'f'. Plant Sci. 163, 131–139.

Messdaghi, D., Dietz, K., 2000. Characterization of an extracellular chymostation-sensitive serine protease preferentially expressed in young plant tissues. Biochim. Biophys. Acta 1480, 107–116.

Moriyama, R., Sugimoto, K., Zhang, H., Inoue, T., Makino, S., 1998. A cysteine-dependent serine protease associated with the dormant spores of *Bacillus cereus*: purification of the protein and cloning of the corresponding gene. Biosci. Biotechnol. Biochem. 62 (2), 268–274.

Nallamestty, S., Kundu, S., Jagannadham, M.V., 2003. Purification and biochemical characterization of a highly active cysteine protease ervatamin A from the latex of *Ervatamia coronaria*. J. Protein Chem. 22, 1–13.

Noda, K., Koyanagi, M., Kamyia, C., 1994. Purification and characterization of an endoprotease from melon fruits. J. Food Sci. 59, 585–587.

Pande, M., Dubey, V.K., Yadav, S.C., Jagannadham, M.V., 2006. A novel serine protease cryptolepain from *Cryptolepis buchanani*: purification and biochemical characterization. J. Agric. Food Chem. 54, 10141–10150.

Patel, A.K., Singh, V.K., Jagannadham, M.A., 2007. Carnein, a serine protease from noxious plant weed *Ipomoea carnea* (Morning Glory). J. Agric. Food Chem. 55, 5809–5818.

Ribeiro, A., Akkermans, A., Kammen, A., Bisseling, T., Pawlowski, K., 1995. A nodule-specific gene encoding a subtilisin-like protease is expressed in early stages of actinorhizal nodule development. Plant Cell 7, 785–794.

Rudenskaya, G.N., Bogacheva, A.M., Preusser, A., Kuznetsova, A.V., Dunaevsky, Ya.E., Golovkin, B.N., Stepanov, V.M., 1998. Taraxalisin- a serine protease from dandelion *Taraxacum officinale*. Webb s.l. FEBS lett. 473, 237–240.

Sarath, G., Motte, R.S., Wanger, F.M., 1989. Protease assay methods. In: Beynon, R.J., Bond, J.S. (Eds.), Proteolytic Enzyme – A Practical Approach. IRL Press, Oxford, pp. 25–55.

Sedmak, J.J., Grossberg, S.E., 1977. A rapid, sensitive and versatile assay for protein using Coomassie Brilliant Blue G 250. Anal. Biochem. 79, 544–552.

Sousa, M.J., Malcata, F.X., 2002. Advances in the role of a plant coagulant (*Cynara cardunculus*) in vitro and during ripening of cheeses from several milk species. Le Lait 82, 151–170.

Sumantha, A., Larroche, C., Pandey, A., 2006. Microbiology and industrial biotechnology of food grade proteases: a perspective. Food Technol. and Biotech. 44 (2), 211–220.

Sundd, M., Kundu, S., Pal, G.P., Medicherla, J.V., 1998. Purification and characterization of highly stable cysteine protease from the latex of *Ervatamia coronaria*. Biosci. Biotechnol. Biochem. 62, 1947–1955.

Tomar, R., Kumar, R., Jagannadham, M.V., 2008. A staple serine protease, Wrightin, from the latex of the plant *Wrightia tinctoria* (Roxb) R. Br.: purification and biochemical properties. J. Agric. Food Chem. 56, 1479–1487.

Tornero, P., Conejero, V., Vera, P., 1996. Primary structure and expression of a pathogen-induced protease (PR-P69) in tomato plant: similarity of functional domains to subtilisin-like endoprotease. Proc. Natl. Acad. Sci. 93, 6332–6337.

Tornero, P., Conejero, V., Vera, P., 1997. Identification of a new pathogen-induced member of the subtilisin-like processing protease family from plants. J. Biol. Chem. 272, 14412–14419.

Uchikoba, T., Horita, H., Kaneda, M., 1990. Proteases of the sarcocarp of yellow snake-gourd. Phytochemistry 29, 1879–1881.

Uchikoba, T., Yonezawa, H., Kaneda, M., 1995. Cleavage specificity of cucumisin, a plant serine protease. Phytochemistry 117, 1126–1130.

Uchikoba, T., Yonezawa, H., Kaneda, M., 1998. Cucumisin-like protease from sarcocarp of *Benincasa hispida* var. Ryukyu. Phytochemistry 49, 2215–2219.

Uphof, J.C., 1968. Dictionary of Economic Plants, second ed. Von J. Cramer, Lehre, Germany, 591p.

Yadav, S.C., Pande, M., Jagannadhan, M.M.V., 2006. Highly stable glycosylated serine protease from medicinal plant *Euphorbia milii*. Phytochemistry 67, 1414–1426.

Yamagata, H., Ueno, S., Iwasaki, T., 1989. Isolation and characterization of a possible native cucumisin from developing melon fruits and its limited autolysis to cucumisin. Agric. Biol. Chem. 53 (4), 1009–1017.