Purification and Some Properties of a Protease from Sorghum Malt Variety KSV8-11

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ABSTRACT

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A protease from sorghum malt variety KSV8-11 was purified by a combination of dialysis against 4 M sucrose, ion-exchange chromatography on Q-Sepharose (Fast flow), gel filtration chromatography on Sephadex G-100 and hydrophobic interaction chromatography on Phenyl Sepharose CL-4B. The enzyme was purified 5-fold to give a 14.1% yield relative to the total activity in the crude extract and a final specific activity of 1348.9 U mg^{-1} protein. SDS-PAGE revealed a single migrating protein band corresponding to a relative molecular mass of 16 KDa. Using casein as substrate, the purified protease had optimal activity at 50°C and maximal temperature stability between 30°C and 40°C but retained over 64% of its original activity after incubation at 60°C for 30 min. The pH optimum was 5.0 with maximum stability at pH 6.0 but 60% of the activity remained after 24 h between pH 5.0 and 8.0. The protease was inhibited by Ag^+, Ca^{2+}, Co^{2+}, Fe^{2+}, Mg^{2+}, iodoacetic acid (IAA) and p-chloromercuribenzoate (p-CMB), stimulated by Cu^{2+}, Sr^{2+}, phenylmethylsulfonyl-fluoride (PMSF) and 2-mercaptoethanol (2-ME) while Mn^{2+} and ethylenediaminetetraacetic acid (EDTA) had no effect. The purified enzyme had a K_m of 18 mg · mL^{-1} and a V_{max} of 11.1 μmol · mL^{-1} · min^{-1} with casein as substrate.

Key words: Characterization, malting, protease, purification, sorghum.

INTRODUCTION

The proteolytic enzymes of the germinated sorghum grain have been studied by a number of workers[9,10,12,16,22,35]. Recent studies[11,24,25] have shown some improvements in sorghum enzyme character and other malt quality properties arising from appropriate cultivar selection and manipulation of steep programmes and treatment during malting.

Garg and Virupaksha[12], purified and characterized an acid protease from an Indian sorghum variety, M-35-1, while Taylor[30], reported the determination of the molecular weight of a prolamin (Kafirin) from a South African variety, Bernard Red. Also, Mazhar et al.[19], employed differential extractabilities to purify α-, β- and γ-kafirins from another Indian sorghum variety, SB-2413. Recently, Agu and Palmer[1], reported the determination of the molecular weights of different extracts of crude proteins from a Nigerian sorghum variety, ICSV400, using SDS–polyacrylamide gel electrophoresis. Despite these efforts, proteolysis and other biochemical processes of sorghum malting remain incompletely understood compared with barley malt[31]. Besides, little has been reported on the purification and characterization of the proteolytic enzymes from an array of improved sorghum varieties for brewing purposes. Empirical data derivable from the study of the physico-chemical, molecular and catalytic properties of the purified sorghum malt proteases are necessary for the development of a novel and workable malting procedure that will ultimately enhance proteolysis and make sorghum malt a better and sustainable alternative to barley malt.

The objective of this study was to purify and characterize, based on the properties enumerated above, a protease from an improved sorghum malt variety.

MATERIALS AND METHODS

Sorghum malting

An improved sorghum variety (KSV8) was obtained from the National Seed Service, Zaria, Nigeria. A 200 g aliquot of the grains was surface-sterilized by immersion for 40 min in sodium hypochlorite solution having 1.0% (v/v) available chlorine, washed repeatedly in tap water and malted as described by Morrall et al.[21].

Enzyme extraction

Sorghum malt grists, 16.67 g, were extracted with 250 mL of 0.1 M citrate-phosphate buffer, pH 7.0, containing: 0.5% (w/v) cysteine hydrochloride and 1.075 g NaCl for 2 h at 30°C using a Gesellschaft rotary shaker (Model D3006 Burgweded) at 120 rev · min^{-1}. Enzyme extraction is usually carried out at low temperatures due to the problem of thermal denaturation[34]. However, as plant proteolytic enzymes are unusually temperature stable[13] the use of a moderately high extraction temperature of 30°C for 1 to 2 h appeared worthwhile in view of the improved extractability of sorghum malt proteolytic enzymes using the above extraction conditions as earlier reported[9]. The extract was centrifuged at 5000 g for 30 min at 4°C and the supernatant fraction was retained for enzyme purification.

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Enzyme assay

Using 1.0% (w/v) casein as the substrate, proteolytic activity was assayed by a modification of the methods of Lowry et al.[18] as described by Upton and Fogarty[32]. The enzyme/substrate mixture was incubated for 30 min at 50°C and pH 4.6. The reaction was stopped by the addition of 6 mL of 5% (w/v) trichloroacetic acid (TCA) and tyrosine equivalents in the TCA-soluble fraction were determined at 660 nm using the Folin-Ciocalteau reagent. One unit of activity is the amount of enzyme releasing 1 mg of tyrosine from the substrate (casein) per minute at 50°C under the conditions of the assay.

Protein measurement

Protein content was determined by the dye-binding method of Bradford[4] as detailed by Hammond and Kruger[14], using BSA as the standard and measuring the absorbance at 595 nm.

Enzyme purification

Ion-exchange chromatography on Q-Sepharose. The purification process was initiated by concentrating the enzyme solution through an overnight dialysis at 4°C against 4 M sucrose solution[28]. Thereafter, the concentrated crude enzyme (50 mL), was applied on a Q-Sepharose (Fast flow) column (Pharmacia: 1.8 × 14.0 cm) equilibrated with 100 mL of 0.1 M citrate-phosphate buffer, pH 7.0 and eluted with 270 mL of the same buffer and equal volume of 0.5 M NaCl solution at a flow rate of 90 $mL \cdot h^{-1}$. A total of 36 fractions (10 mL each) were collected and assayed for both enzyme activity and protein content. Fractions which showed high enzyme activity were pooled and re-concentrated by overnight dialysis at 4°C against 6 M sucrose solution.

Gel filtration chromatography on Sephadex G-100. The re-concentrated, partially purified enzyme sample (30 mL) was subjected to gel filtration in a column of Sephadex G-100 (Pharmacia: 2.5 × 60.0 cm) having a bed height of 45 cm and pre-equilibrated with 200 mL of deionised water followed by 200 mL of 0.1 M citrate-phosphate buffer, pH 7.0. Elution was done with the same buffer at a flow rate of 50 $mL \cdot h^{-1}$. A total of 45 fractions of 10 mL per tube were collected. Fractions 30–45, which showed high enzyme activity, were designated KSV8-Peak 11 (or KSV8-11), pooled and re-concentrated by overnight dialysis at 4°C against 6 M sucrose solution. Proteolytic activity and protein content of each dialyzate were estimated and subjected to further purification using the hydrophobic interaction resin.

Hydrophobic interaction chromatography on Phenyl Sepharose CL-4B. For hydrophobic interaction chromatography, the column (1.8 × 14. 0 cm) was packed with Phenyl Sepharose CL-4B gel (Pharmacia), washed with 200 mL of deionised water and made hydrophobic by the application of 2 M $(NH_4)_2SO_4$ solution in 70 mL 0.1 M citrate-phosphate buffer, pH 7.0. Thereafter, the enzyme sample (20 mL), was applied to the column and eluted using varying concentrations of the ammonium sulphate solution but equal volumes of the buffer as follows: 2 M $(NH_4)_2SO_4$ for fractions 1–15, 1.5 M $(NH_4)_2SO_4$ for fractions 16–20, 1 M $(NH_4)_2SO_4$ for fractions 21–24, 0.5 M $(NH_4)_2SO_4$ for fractions 25–27 and, finally, using the elution buffer alone for fractions 28–36, all at a flow rate of 60 $mL \cdot h^{-1}$. Fractions 28–32 which showed high enzyme activity were pooled and re-concentrated by overnight dialysis at 4°C against 6 M sucrose solution and then subjected to characterization.

Homogeneity test

The homogeneity of the purified protease was tested by polyacrylamide slab gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE) as described by Chung[6]. The purified enzyme was first concentrated with cold acetone and recovered by centrifugation at 5000 g for

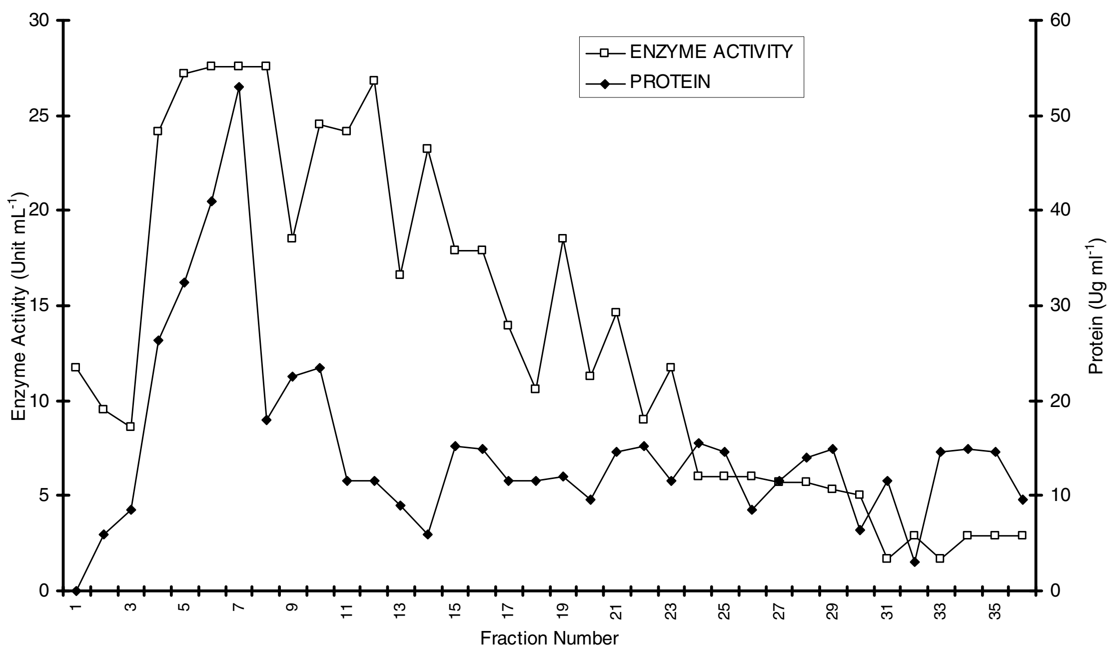

Fig. 1. Elution profile of sorghum malt variety KSV8 protease on Q-Sepharose (Fast Flow) ion-exchange chromatograph.

30 min. Thereafter, the enzyme was dissociated by boiling for 5 min in 6.25 mM Tris-HCl buffer, pH 6.8, containing: 40% (w/v) SDS, 20% (v/v) glycerol and 10% (v/v) 2-mercaptoethanol. Electrophoresis was run at a constant current of 18 mA for 2 h when the tracking dye, bromophenol blue, emerged. Separated protein bands were visualized by staining with 0.1% (w/v) Coomassie Brilliant Blue R250 followed by destaining.

Estimation of relative molecular weight

The molecular weight of the purified protein was estimated by SDS-PAGE as described by Weber et al.[33], using bovine serum albumin (66 KDa), egg albumin (45 KDa), carbonic anhydrase (31 KDa), haemoglobin (16 KDa), and lysozyme (14.6 KDa) as markers.

Temperature activity and stability determination

The temperature activity profile of the purified protease was determined over the range: 30°C–100°C by adding 0.2 mL of the enzyme to 0.2 mL of 1.0% (w/v) casein in 0.3 M citrate-phosphate buffer, pH 4.6, and incubating for 30 min at the test temperatures. Then the reaction mixture was cooled on ice before enzyme activity was assayed as described earlier.

To determine the thermostability, the enzyme was pre-incubated at various temperatures between 30°C and 100°C for 30 min and then chilled on ice, after which residual activity was determined under normal conditions as described earlier.

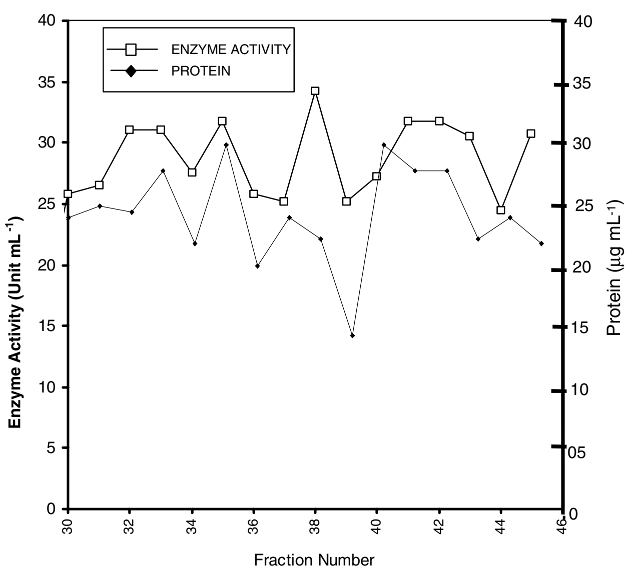

Fig. 2. Elution profile of sorghum malt variety KSV8 on Sephadex G-100 gel filtration chromatography.

pH activity and stability determination

The effect of pH on the activity of the purified protease was studied over a pH range of 3.0–7.0 using citrate-phosphate buffer and at pH 8.0–9.0 using Tris-HCl buffer. The reaction mixture was comprised of 0.2 mL of the purified enzyme preparation and 0.2 mL of 1.0% (w/v) casein in appropriate buffer. The mixture was incubated for 30 min at 50°C and the activity of the enzyme was determined using the standard method as earlier described.

To determine the pH stability profile of the protease, 0.2 mL of the enzyme solution was added to 0.2 mL of the appropriate buffer at pH 3.0–9.0 and pre-incubated for 3 h at 30°C. Thereafter, residual enzyme activity was assayed as described earlier.

Effect of metal ions on enzyme activity

The effects of the cations: Ag^{+}, Ba^{2+}, Ca^{2+}, Co^{2+}, Fe^{2+}, Hg^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Pb^{2+}, Sr^{2+} and Cu^{2+}, on the activity of the purified protease were evaluated. The enzyme and each of the cations (5 mM), were pre-incubated in 0.3 M citrate-phosphate buffer, pH 4.6 at 30°C for 3 h and then assayed for residual activity as described earlier.

Effect of some inhibitors on enzyme activity

The effects of the inhibitors: phenylmethyl-sulfonyl fluoride (PMSF), iodoacetic acid (IAA), p-chloromercuribenzoate (p-CMB), ethylenediaminetetraacetic acid (EDTA) and 2-mercaptoethanol (2-ME) on the activity of the purified enzyme were examined.

The enzyme sample (0.2 mL) was pre-incubated with 0.2 mL 1 mM of each inhibitor at 30°C for 10 min. Thereafter, 1.0 mL of 1.0% (w/v) casein in 0.3 M citrate-phosphate buffer, pH 4.6, was added to the reaction mixture and incubated for 30 min at 50°C to determine residual activity and the result compared with an inhibitor-free enzyme control.

Effect of substrate concentrations on enzyme activity

Purified enzyme activity was assayed in a reaction mixture containing various concentrations (0–1.0 $mg \cdot mL^{-1}$) of the substrate (1.0% (w/v) casein) in 0.3 M citrate-phosphate buffer, pH 4.6. The kinetic constants (K_m, V_{max}) were estimated by double reciprocal plots of the data according to Lineweaver-Burk[19].

Table I. Purification summary of a protease from sorghum malt variety KSV8-11.

Purification steps	Vol (mL)	Total activity (U)	Total protein (mg)	Specific activity ($U \cdot mg\ protein^{-1}$)	Yield (%)	Purification (fold)
1. Crude enzyme sample	250	3642.5	13.5	269.8	100	1.0
2. Concentration in 4 M sucrose solution	80	1801.6	4.48	402.1	49.5	1.5
3. Ion-exchange chromatography on Q-Sepharose	50	1192.0	2.3	518.3	32.7	1.9
4. Gel filtration chromatography on Sephadex G-100	30	794.7	0.99	802.7	21.8	3.0
5. Hydrophobic interaction chromatography on Phenyl Sepharose CL-4B	20	512.6	0.38	1348.9	14.07	5.0

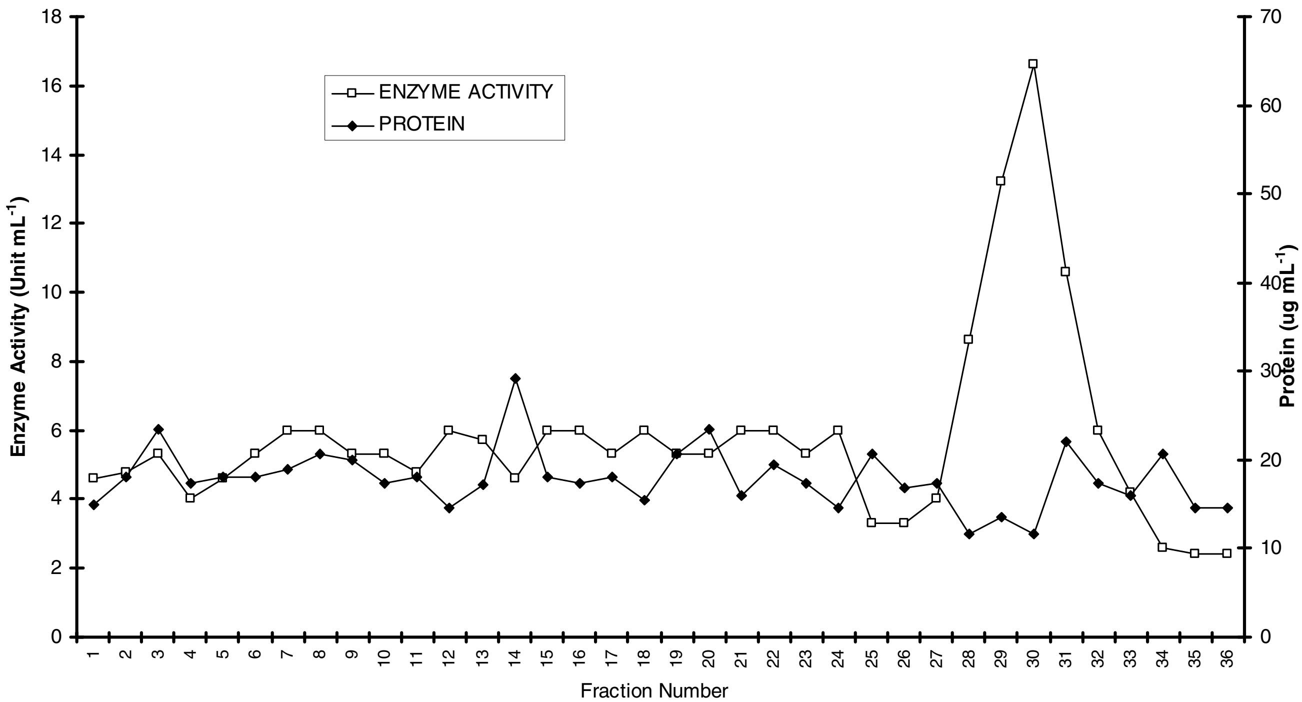

Fig. 3. Elution profile of sorghum malt variety KSV8-11 protease on Phenyl Sepharose CL-4B hydrophobic interaction chromatography. Fractions 28-32 were obtained by eluting with 0.1 M citrate phosphate buffer, pH 7.0 only.

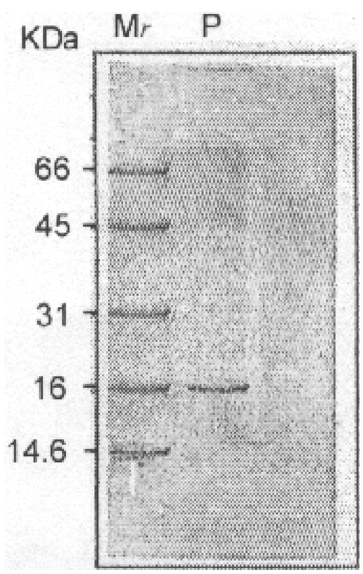

Fig. 4. SDS-PAGE of a purified protease from sorghum malt. Lane M*r* = relative positions of standard molecular weight proteins; Lane P = a purified protease from sorghum malt variety KSV8-11.

RESULTS AND DISCUSSION

Enzyme purification

The elution pattern from Q-Sepharose ion-exchange column chromatography showed a major peak of protease activity between fractions 4–12 and 14 (Fig. 1). Recovery of those fractions led to a 32.7% retention of the overall activity and a 1.9-fold purification (Table I). Similarly, fractions 30–45 (Fig. 2) showed high enzyme activities on Sephadex G-100 gel filtration chromatography. Recovery of those fractions led to a 21.8% retention of the overall activity and a 3.0-fold purification (Table I). A major peak was observed for the protease from fractions 28 to 32 (Fig. 3) on Phenyl Sepharose CL-4B hydrophobic interaction chromatography. The enzyme was purified 5-fold to give a 14.1% yield relative to the total activity in the crude extract (Table I) and a final specific activity of 1348.9 U mg^{-1} protein.

Homogeneity of the purified enzyme

The homogeneity of the purified enzyme was confirmed by the appearance of a single protein band on SDS-PAGE (Fig. 4).

Estimation of molecular weight

The relative molecular weight of the purified protease was estimated to be 16 KDa (Fig. 5). This value is very close to 14.1 KDa obtained for a major component of an acidic endopeptidase from barley malt by Burger[5], using gel filtration on Sephadex G-75. It is also comparable with the value (15 KDa), obtained for one of the hordein fractions from barley and malt by Baxter[3], using gel electrophoresis and gel filtration on Biogel P150 and P200, respectively.

Temperature activity and stability profile

The purified enzyme, demonstrated optimal activity at 50°C (Fig. 6) and maximal stability between 30°C and 40°C but retained over 64% of its original activity after 30 min at 60°C. The optimum temperature of activity for this protease is similar to that of a proteinase[9,12] and a carboxypeptidase[10], from sorghum malt having temperature optima in the range of 45°C–50°C.

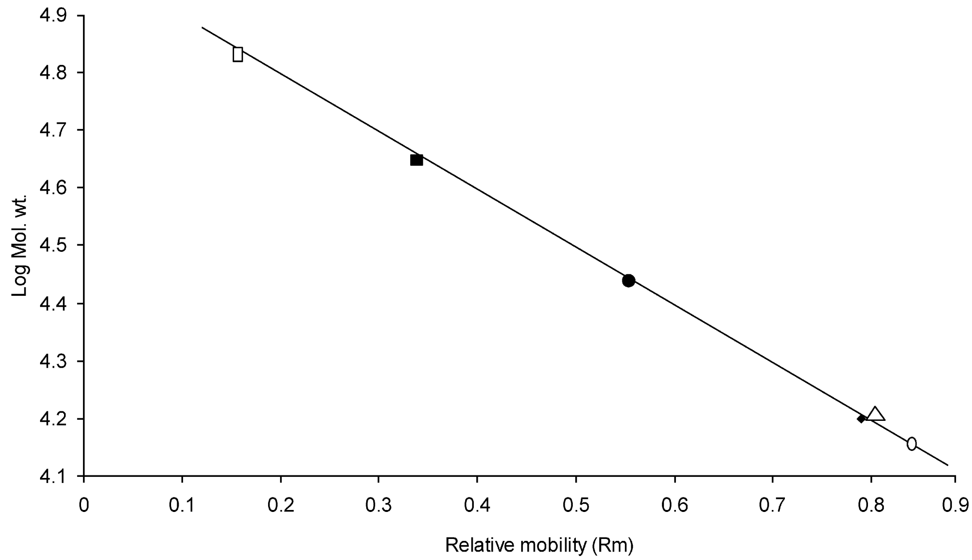

Fig. 5. Estimation of molecular weight of a protease from sorghum malt variety KSV8-11 using SDS-PAGE: △ = a protease from sorghum malt variety KSV8-11. Marker proteins used were: □ = bovine serum albumin (66000); ■ = egg albumin (45000); ● = carbonic anhydrase (31000); ◆ = haemoglobin (16000); ○ = lysozyme (14600).

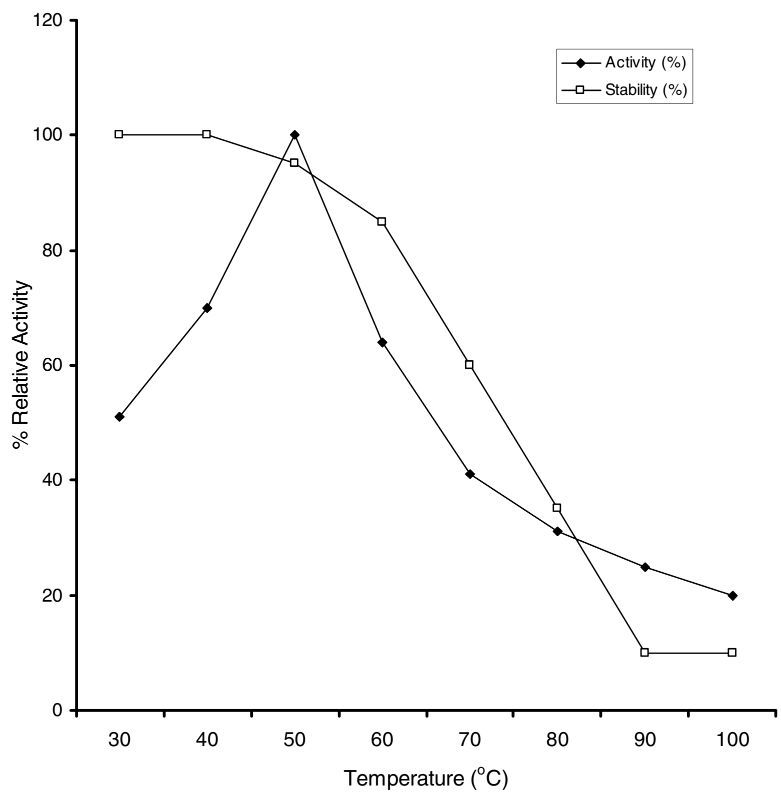

Fig. 6. Effect of temperature on the activity and stability of a protease from sorghum malt variety KSV8-11.

pH activity and stability profile

The protease demonstrated optimal activity at pH 5.0 and was maximally stable at pH 6.0 (Fig. 7), but retained over 60% of its original activity after 24 h at pH 4.0 to 8.0.

It is noteworthy that the pH activity and stability values for this enzyme are higher (i.e. less acidic) than those of a sorghum malt acid protease reported by Garg and Virupaksha[12], that was most stable between pH 3.0 and 6.0 but rapidly inactivated at pH values beyond neutrality. However, the optimal activity recorded for this enzyme at pH 5.0, and pH 6.0 for maximal stability, closely resembled those of a carboxypeptidase from germinated barley with a maximum stability range of pH 3.0 to pH 6.0 using ca-

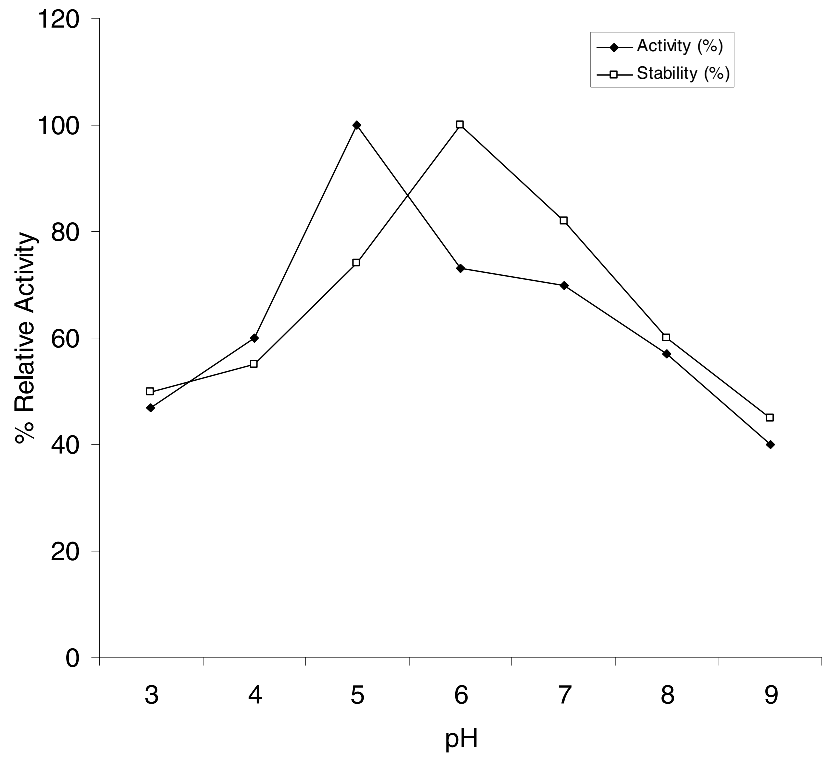

Fig. 7. Effect of pH on the activity and stability of a protease from sorghum malt variety KSV8-11.

Table II. Effect of metal ions on the activity of a protease from sorghum malt variety KSV8-11.

Metal salt (5 mM)	Specific activity ($U \cdot mg\ protein^{-1}$)	Remaining activity (%)[1]	P
1. None	68.14	100	
2. $AgNO_3$	53.57	77	<0.01
3. $BaCl_2$	36.10	53	<0.001
4. $CaCl_2$	53.57	77	<0.01
5. $CoCl_2$	55.29	93	<0.05
6. $CuSO_4$	91.30	141	<0.001*
7. $FeSO_4$	55.29	93	<0.05
8. $HgCl_2$	26.10	27	<0.001
9. $MgCl_2$	56.15	83	<0.01
10. $MnSO_4$	68.14	100	NE
11. $Pb(C_2H_3O_3)_2$	34.55	55	<0.001
12. $SrCl_2$	91.30	141	<0.001*
13. $ZnSO_4$	70.84	111	<0.01*

* = Stimulation; NE = no effect
[1] After a holding time (pre-incubation) of 3 h and temperature of 30°C

Table III. Effect of some inhibitors on the activity of a protease from sorghum malt variety KSV8-11.

Inhibitor (1 mM)	Specific activity ($U \cdot mg\ protein^{-1}$)	Remaining activity (%)[1]	P
None	90.00	100	
PMSF	95.21	106	<0.01*
IAA	73.91	81	<0.01
p-CMB	78.26	84	<0.01
EDTA	90.00	100	NE
2-ME	120.87	136	<0.001*

* = Stimulation; NE = no effect
[1] After a holding time (pre-incubation) of 10 min and temperature of 30°C

sein as a substrate[20]. The differences in the pH values could be due to differences in enzyme assay conditions[31], such as substrate concentrations and assay temperature.

Metal ions and enzyme activity

The protease was slightly ($P < 0.05$) inhibited by Co^{2+} and Fe^{2+} (Table II), appreciably ($P < 0.01$) inhibited by Ag^{+}, Ca^{2+} and Mg^{2+} and highly significantly ($P< 0.001$) inhibited by Ba^{2+}, Hg^{2+} and Pb^{2+}. On the other hand, appreciable ($P < 0.01$) stimulation of the protease was achieved by Zn^{2+}, and a highly significant ($P < 0.001$) stimulation by Cu^{2+} and Sr^{2+}.

The stimulatory effect of Cu^{2+}, on the activity of this protease is striking being at variance with an earlier report[12], where heavy metal ions, including Cu^{2+}, inhibited an acid protease from germinated sorghum to varying degrees.

Effect of some inhibitors

The purified sorghum malt protease was appreciably ($P < 0.01$) inhibited by both IAA and p-CMB (Table III). PMSF and 2-ME were significantly stimulatory to the enzyme at $P < 0.01$ and $P < 0.001$, respectively, while EDTA had no significant effect.

Inhibitors are particularly important in the study of proteolytic enzymes as they give the clearest evidence to the type of catalytic site, which forms the basis for the classification of the enzymes[2,23]. Thus, the protease from sorghum malt, variety KSV8-11 was not inhibited by PMSF, a serine protease inhibitor, indicating a non-requirement for serine for its catalysis[34]. The enzyme was however, inhibited by the thiol (SH)-blocking reagents, IAA and p-

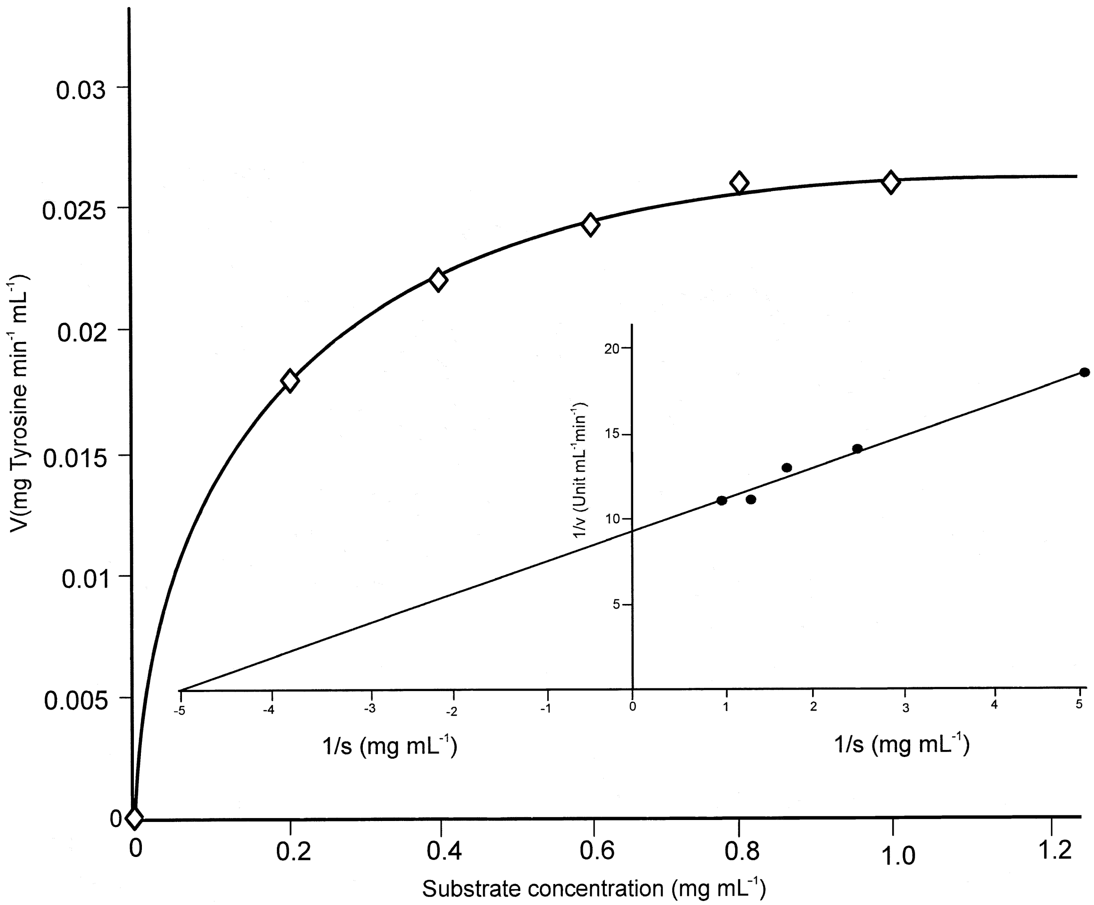

Fig. 8. Effect of casein concentration on the activity of a protease from sorghum malt variety KSV8-11 (◇) and Lineweaver-Burk Plot for casein (●).

CMB, indicating that free thiol groups are essential for its activity[7,12,29,34]. The p-CMB was completely inhibitory to an endopeptidase enzyme from germinated barley even at 0.7 mM concentration[5]. Similarly, Enari and Mikola[8], reported that p-CMB was a very potent inhibitor of an endopeptidase preparation from green barley malt, recording a maximal inhibition of 91% with 0.17 mM in a reaction mixture and 1 mM for 30 min pre-incubation. The enzyme was not affected by EDTA, a metal chelator, suggesting that it is not a metalloprotease[8,34]. Based on the above inhibitory properties, the purified protease from sorghum malt variety KSV8-11 was grouped as a sulphydryl or cysteine protease[2,12,15,27,34].

Effect of substrate concentrations on enzyme activity

The effects of varying concentrations of casein on the activities of the purified enzyme, gave the following kinetic constants: $K_m = 18$ mg · mL^{-1}, $V_{max} = 11.1$ µmol · mL^{-1} min^{-1} (Fig. 8).

The kinetic values for the protease are very low showing a high affinity to casein[26]. The Km value for the sorghum malt protease is however, higher than 14.0 mg · mL^{-1} but lower than the 55.0 mg · mL^{-1} results obtained for an acid proteinase from germinated sorghum[20], using N,N-dimethyl albumin and bovine serum albumin (BSA) as substrates, respectively.

CONCLUSIONS

Our study revealed that sorghum malt variety KSV8 contains two distinct proteases separated by gel filtration chromatography on Sephadex G-100, purified and characterized as KSV8-1 with a higher MW (reports not included here), while KSV8-11 with a lower MW is reported in this paper.

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