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Extraction, purification and characterisation of a milk-clotting protease from 'kesinai' (*Streblus asper* Lour.) leaves

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Abstract

Extracts from 'kesinai' (*Streblus asper*) leaves were investigated as a potential source of enzymes that can serve as an alternative to calf rennet in cheese making. Different types of extraction buffers were investigated namely sodium acetate buffer (pH 4.2-5.0), phosphate buffer (pH 6.0-7.0) and Tris-HCl buffer (pH 7.0-9.0). Finally, the milk-clotting enzyme was extracted using 100 mM Tris-HCl buffer (pH 7.4) with and without 5.0 mg/mL polyvinylpyrrolidone, 0.015 mL/mL Triton X-100 and 2 mM sodium metabisulphite. Purification was carried out using acetone precipitation, and ion-exchange and size-exclusion chromatographic techniques. Results showed that 100 mM Tris-HCl buffer (pH 7.4) was the most efficient extraction buffer among the buffers used in the extraction study. After the final purification step of size-exclusion chromatography, the enzyme was purified 3.3-fold with 42.3% of recovery. The enzyme showed an optimum temperature and pH at 60°C and pH 7.4, respectively. The enzyme was stable up to 70°C for one hour and the partially purified enzyme retained 83% and 96% of its original activity at pH 6.0 and 8.0, respectively. The molecular weight of the partially enzyme was estimated to be 75.8 kDa on SDS-PAGE. The milk-clotting activity of 'kesinai' enzyme was found to be lower than that of commercial *Mucor* rennet.

Introduction

Rennet is a milk-clotting enzyme used in the cheese industry (Jacob *et al*., 2011; Llorente *et al*., 2014). It is obtained from the abomasum of un-weaned calf (Cavalcanti *et al*., 2004). Worldwide reduced supply of calf-rennet and increased production and consumption of cheese have led to a systematic investigation of calf-rennet substitutes (Roseiro *et al*., 2003; Llorente *et al*., 2014). Pepsin from bovine, chicken and porcine, fish chymotrypsins and microbial rennet produced from genetically modified organisms have been proven to be suitable as calf-rennet substitute (Sousa and Malcata, 1997; Rogelj *et al*., 2001; Senthilkumar *et al*., 2006; Mazorra-Manzano *et al*., 2013). Due to religious restrictions, vegetarian practices and incidences of animal diseases, attentions are directed towards natural rennet extracts from plant sources (Senthilkumar *et al*., 2006; Chazarra *et al*., 2007; Egito *et al*., 2007). Several plant extracts have been found to be potential substitutes for calf-rennet, for example papain (EC 3.4.4.10) from the latex of papaya (*Carica papaya* L.), ficin (EC 3.4.4.12) from the latex of fig (*Ficus carica* L.), bromelain (EC 3.4.4.24) from pineapple (*Ananas comosus* (L.) Merr.), and cardosin from *Cynara* sp. (Roseiro *et al*., 2003). The use of plant coagulants contributes to improving the nutritional intake of people whose use of animal rennet is restricted (Chazarra *et al*., 2007; Kumari *et al*., 2012).

Vegetable coagulants have been used for many years in cheese-making (Roseiro *et al*., 2003). However, only a few milk-clotting enzymes of plant origin are used in the cheese industry (Llorente *et al*., 2004). The aqueous extracts of *Cynara* sp. have been widely used for many years in the manufacture of several traditional Portuguese and Spanish ewe's milk cheeses (Roseiro *et al*., 2003; Vairo-Cavalli *et*

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al., 2005; Beka *et al*., 2014). Similarly, Sodom apple (*Calotropis procera* (Aiton) W.T.Aiton) extract has been used for traditional cheese making in West African countries (Mohamed Ahmed *et al*., 2009b).

The leaves of *Streblus asper* Lour., commonly known as 'kesinai' in Malaysia, have also been reported to possess milk-clotting properties (Manap *et al*., 1992). It is a tropical plant widely distributed in Malaysia, India, Sri Lanka, Philippines and Thailand (Rastogi *et al*., 2006). 'Kesinai' is also known as a medicinal plant used for several pharmaceutical functions. For instance, extracts from the bark have been used to reduce fever, dysentery, relief of toothache and antigingivitis (Hashim and Devi, 2003; Taweechaisupapong *et al*., 2005). Manap *et al*. (1992) and Idris *et al*. (1999) reported that 'kesinai' leaf extract contains a thermostable milk-clotting protease, which could be a potential rennet substitute in cheese-making. However, a detailed study on the extraction and purification of the proteases is not yet available. Therefore, the present work was undertaken to extract, purify and characterise the milk-clotting protease from the leaves of 'kesinai'.

Materials and methods

Material and reagents

Tris (hydroxymethyl) aminomethane, azo-casein, skim milk powder, polyvinylpyrrolidone, Triton X-100, sodium metabisulphite, blue dextran, apoferritin, alcohol dehydrogenase, cytochrome C, vitamin B12, porcine myosin, β-galactosidase, phosphorylase b, bovine serum albumin (BSA), ovalbumin, carbonic anhydrase, acrylamide, and commercial rennet (*Mucor* miehei-lyophilised) were purchased from Sigma-Aldrich, Saint Louis MO, USA. Trichloroacetic acid (TCA), ammonium sulphate, acetone and sodium dodecyl sulphate (SDS) were purchased from Fisher Scientific UK Limited, Loughborough, UK., HiPrep desalting Sephadex G-25 pre-packed column (column dimension 2.6 × 10 cm), HiTrap diethylaminoethyl Sepharose Fast Flow (DEAE Sepharose FF) pre-packed column (column dimension 0.7 × 2.5 cm), Sephacryl S-100 High Resolution pre-packed column (column dimension 2.6 × 60 cm) and Superose 12 10/300 GL [column dimension 10 × 300 mm with molecular range (Mr) from 1×10^3 to 3×10^5] pre-packed column were purchased from GE Healthcare Bio-Sciences, Uppsala, Sweden.

Enzyme extraction

'Kesinai' leaves were collected from the University Science Park, Universiti Putra Malaysia, Malaysia. After cleansing with distilled water, 20 g of fresh leaves were homogenised in a Warring blender (Model 32BL80, Dynamic Corporation of America, New Hartford, Connecticut, USA) with 100 mL of cold 100 mM Tris-HCl (pH 7.4) buffer for 2 min. The homogenate was filtered through a piece of muslin cloth, and the filtrate was centrifuged at 10,000 rpm for 30 min at 4°C using a refrigerated centrifuge (Model 3-18 k, Sartorius AG, Weender Land Strasse, Gottingen, Germany). The supernatant was collected and stored at 4°C before proteolytic activity was assayed and the colour of extract determined. Further, the most efficient pH buffer solution (ranging from pH 4.2 to 9.0) on extraction of the enzyme was investigated. The buffers (all at 100 mM) used were sodium acetate buffer pH 4.2-5.0, phosphate buffer pH 6.0-7.0 and Tris-HCl buffer pH 7.0-9.0. Proteolytic activity and colour of the crude enzyme were also determined on extracts obtained using 100 mM Tris-HCl buffer containing 5.0 mg/mL polyvinylpyrrolidone (PVP), 0.015 mL/mL Triton X-100 and 2 mM sodium metabisulphite (MBS).

Purification of 'kesinai' enzyme

Acetone precipitation

Pre-cooled acetone (-15°C) was slowly added to 500 mL of 'kesinai' crude extract obtained using 100 mM Tris-HCl buffer until the ratio between the supernatant and acetone became 1:0.75. The mixture was gently stirred for 60 min, and the precipitate formed was separated from the supernatant by centrifugation at 12,100 rpm (Sartorius Model 3-18 k, Sartorius AG, Weender Land Strasse, Gottingen, Germany) for 10 min at 4°C. The precipitate was dissolved in 125 mL of 100 mM Tris-HCl buffer (pH 7.4). After standing for about 2 h at 4°C, the enzyme preparation was centrifuged at 12,100 rpm for 15 min. The supernatant was collected and stored at 4°C before assaying for protease activity (Zotos and Taylor, 1996). The procedure was repeated for the ratio of supernatant and acetone of 1:1 and 1:1.25.

Desalting of acetone-precipitated 'kesinai' enzyme extract

Desalting of acetone-precipitated 'kesinai' enzyme extract was carried out using a Desalting HiPrep Sephadex G-25 pre-packed column (column dimension 2.6 x 10 cm) that has been pre-equilibrated with 50 mM Tris-HCl (pH 7.4) buffer. The extract (15 mL) was loaded onto the column fitted to a fast protein liquid chromatographic (FPLC) system (UNICORN 4.12, Amersham Bio-Sciences, Uppsala, Sweden). Protein was eluted with the equilibrating

buffer at a flow rate of 2.5 mL/min. Sixteen fractions of 1.0 mL each were collected. The eluted protein content was detected at 280 nm. Protease activity and protein concentration were determined for each fraction. Active fractions were pooled and stored at 4°C.

Ion-exchange chromatography

Ion-exchange chromatography was carried out according to Senthilkumar *et al*. (2006) with some modifications. HiTrap diethylaminoethyl Sepharose Fast Flow (DEAE Sepharose FF) pre-packed column (column dimension 0.7 × 2.5 cm) was pre-equilibrated with 50 mM Tris-HCl (pH 7.4) buffer, and was used for the chromatography. The active enzyme after desalting (5 mL per replicate injection) was applied onto the column and 1.0 mL fractions were collected at a flow rate of 1.0 mL/min using a linear gradient of 0.0–1.0 M NaCl. Elution was monitored at 280 nm. Protease-active fractions were pooled and stored at 4°C until they were applied in gel filtration chromatography.

Gel filtration chromatography

The active enzyme preparation (8 mL each replicate injection) obtained from the ion-exchange chromatography was applied on to a Sephacryl S-100 High Resolution pre-packed column (column dimension 2.6 × 60 cm) which had been pre-equilibrated with 50 mM Tris-HCl (pH 7.4) buffer containing 0.15 M NaCl. Protein was eluted from the column at a flow rate of 0.75 mL/min, and 2.0 mL portions were collected until no more protein was eluted. The elution was monitored at 280 nm. Each fraction was analysed for protease activity. Active enzyme fractions were pooled and concentrated at 4°C by ultra-filtration using a 10,000 Da molecular weight cut-off membrane (Amicon, USA) (Senthilkumar *et al*., 2006).

Protease assay

Protease activity in extracts and chromatographic fractions were estimated following the method described previously (Zotos *et al*., 1996). The reaction mixture contained 1.0 mL of 5 mg/mL azocasein (except otherwise stated) prepared in 100 mM Tris-HCl (pH 7.4) buffer, and 0.1 mL of extract/fraction. The mixture was incubated in a water bath at 60°C for 20 min, and 0.3 mL of 0.1 g/mL trichloroacetic acid (TCA) was added to terminate the reaction, followed by centrifugation at 10,000 rpm for 10 min (Microfuge 18 centrifuge, Germany). The absorbance of the TCA-soluble supernatant was determined at 410 nm using a spectrophotometer (BioMate™-3, Thermo Scientific, Alpha Numerix, Woodfield Dr, Webster, NY). One unit of caseinolytic activity is defined as the amount of enzyme causing an increase in absorbance by 0.01. The control was similarly treated as above except that extract/fraction that has been heated at boiling temperature for 2 min replaced the active extract/fraction.

Protein determination

The total protein was determined according to Lowry *et al*. (1951) method using bovine serum albumin (BSA) as the protein reference.

Characterisation of partially purified 'kesinai' enzyme

Optimum temperature and thermal stability

The optimum temperature of the enzyme was determined using the assay method described above with the reaction mixture incubated at temperatures ranging from 20 to 100°C at 10°C interval for 20 min. The pH of reaction was pH 7.4 and the source of protease was partially purified enzyme obtained after gel filtration chromatography. The thermal stability of the partially purified enzyme was determined by incubating the enzyme at different temperatures ranging from 20-100°C for 1 h at pH 7.4. This was followed by measurement of residual proteolytic activity as previously described at the optimum pH and temperature (7.4 and 60°C, respectively). A non-heated partial purified enzyme extract served as control (relative activity as 100%) (Bougatef *et al*., 2007).

Effect of pH on activity and stability of enzyme

The effect of pH on protease activity of partially purified enzyme was investigated between pH 3.0–11.0 at the optimal temperature (60°C). The following buffer systems were used to obtain different pH range: 100 mM sodium acetate for pH 3.0 - 5.0, phosphate buffer for pH 6.0- 7.0, Tris– HCl buffer for pH 7.0-9.0 and carbonate buffer for pH 10.0–11.0 (Bougatef *et al*., 2007). For the determination of pH stability, the enzyme was incubated at the optimum temperature (60°C) for 1 h at different pH's (using different buffers) and then the residual proteolytic activity was determined under standard assay conditions.

Estimation of native molecular mass by gel filtration chromatography

The molecular mass of native enzyme (protease) was estimated by gel filtration chromatography using Superpose 12 10/300 GL (column dimension 10 × 300 mm). Apoferritin (443 kDa), alcohol dehydronase

(150 kDa), carbonic anhydrase (29 kDa), cytochrome C (12.4 kDa) and vitamin B12 (1.355 kDa) were used as reference. Blue dextran (2,000 kDa) was used to determine the void volume. The elution buffer was 20 mM Tris-HCl buffer (pH 7.4) and the flow rate was 0.75 mL/min. The absorbance of elute was monitored at 280 nm. The estimated molecular weight of the protease was obtained from the plot of molecular weights of standard proteins against their retention time (elution volume) (Zotos and Taylor, 1996; Mohamed Ahmed *et al*., 2009b).

Estimation of molecular mass polyacrylamide gel electrophoresis (SDS-page)

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a mini-vertical gel electrophoresis unit (Amersham Biosciences) using 15% polyacrylamide in the presence of 1.0 mg/ mL SDS separating gel and 4% acrylamide stacking gel containing 1.0 mg/mL SDS following the method previously described (Laemmli, 1970) The sample buffer and tank buffers were 50 mM Tris-HCl (pH 6.8) containing 2.0 mg/ mL of SDS and Tris-Glycine (pH 8.3) in the presence of 1.0 mg/mL of SDS, respectively. Electrophoresis was done at room temperature where the current and voltage were set at 15 mA and 250 V, respectively, for stacking gel, and 30 mA and 250 V, respectively, for resolving gel. The protein sample was denatured by heating in a sample buffer at 100°C for 5 min before loading into the gel. Protein stranded markers, porcine myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase b (97 kDa), BSA (66 kDa), albumin (45 kDa) and carbonic anhydrase (29 kDa) were used for calculating the molecular mass of the protein. Following electrophoresis, protein bands on the gel sheets were visualised by silver staining procedure previously described (Heukeshovan and Dernick, 1985). The standard curve was plotted using molecular marker against their relative mobility. The molecular mass of protease from 'kesinai' leaves was determined from the standard curve.

Milk-clotting activity assay

The milk-clotting activity was determined following the method described by Shieh *et al*. (2009) with slight modification. The substrate was prepared by reconstituting 12.5% (w/v) skim milk in 0.01 M CaCl2 before use. The assay was performed in a test tube by adding 0.2 mL of partially purified 'kesinai' enzyme to 2.0 mL of reconstituted milk that had been pre-incubated for 5 min at 60°C. Mixing was done by manually rotating the test tube from time to time. Clotting time was determined by the appearance of visible clots at 60°C. The milk-clotting activity was calculated by using the following formula: SU = 2400 × 5 × D/T × 0.5; SU = Soxhlet units; T = clotting time (s); D = dilution testing material (Shieh *et al*., 2009). *Mucor* rennet was prepared at 0.1 mg of enzyme/mL, where the enzyme was dissolved in deionised water immediately prior to use. The activity for *Mucor* rennet was determined by adding 0.2 mL of *Mucor* rennet to 2.0 mL of reconstituted milk that had been pre-incubated for 5 min at 60°C. The clotting activity was measured similarly as partially purify 'kesinai' enzyme.

Statistical analysis

Samples were randomly collected, and measurements were made in triplicate. The results were expressed as means and standard deviations. Experimental data were subjected to analysis of variance (ANOVA) using the SAS Statistical Computer Package Version 9.1.3 (SAS Institute, Inc., Cary, NC, USA, 2003). Duncan's multiple range tests was used to make the significant variation among means. Significance was defined at $p < 0.05$.

Results and discussion

Extraction of enzyme

The specific activity of crude protease from 'kesinai' leaves extracted at different pH range was from 4.2 to 9.0. The results indicate that the highest protease activity was significantly ($p < 0.05$) obtained when the pH of the extraction buffer was between pH 7.2 and 7.4. Lowering the extraction pH to 6.0 reduced the activity by 26.5%. A similar effect (reduction by 30.1%) was observed when the enzyme was extracted at pH 8.0. Hence, the extraction buffer subsequently used for the extraction of the protease from 'kesinai' leaves was 100 mM Tris-HCl (pH 7.4). This result is in agreement with the previous report of Idris *et al*. (1999), who found the suitable extraction buffer system for this enzyme was a 100 mM Tris-HCl. Although the addition of 2 mM metabisulphite (MBS) to the extraction buffer was found to be most effective in reducing browning of the crude extract, it led to a significant decrease in the protease activity of the extract by 29.9%. The presence of PVP and Triton X 100 in the extraction buffer led to a reduction in protease activity. Moreover, they did not cause any significant changes in colour of the crude extract. PVP is a phenol-adsorbing agent which was added to the extraction buffer to remove the phenolic components. Triton X 100 may influence the solubilisation of membrane bound enzyme.

Partial purification of 'kesinai' enzyme

In a preliminary study, ammonium sulphate precipitation was used at the first step in the partial purification of enzyme from 'kesinai' leaves where the effect of different degrees of ammonium sulphate saturation was examined. Results showed that much of the enzyme activity was lost when using this protein precipitant. Subsequently, cold acetone was used to precipitate proteins from the extract, and it was observed that higher yields of proteolytic 'kesinai' enzyme were consistently obtained as compared to when ausing ammonium sulphate (result not shown). These results appear to be in agreement with Fernández-Lahore *et al*. (1998) who reported that milk-clotting proteases from microorganisms were better recovered using acetone precipitation as compared to ammonium sulphate precipitation. Wang *et al*. (2002) and Wang (1993) also reported a similar observation for protease extraction from lobster.

In the present work, the enzyme from 'kesinai' was partially purified using the following protocol: cold acetone precipitation followed by ion exchange chromatography using DEAE cellulose and gel filtration chromatography using Sephacryl S-100. The purification fold and recovery of enzyme at each step of the purification procedure are summarised in Table 1. To precipitate the enzyme, different ratios of crude enzyme extract to acetone 1:0.5, 1:0.75, 1:1 and 1:1.25) were investigated. The ratio 1:1.25 was found to be the most effective for precipitation as it yielded the highest specific activity (225.8 U/mg) and recovery (81.8%) of protease activity, and was subsequently used for further purification. Michail *et al*. (2006) reported the efficiencies of cold acetone as an initial purification step for proteolytic enzyme from protease extracts. He also reported that the recovery of protease activity using the ratio of acetone to crude extract of 1.25:1 was 99%.

When the acetone-precipitated protease solution was subjected to HiPrep desalting, 74.9% recovery and specific activity of 339.1 U/mg proteins were obtained from pooled protease active fractions. The pool protease active fraction was then loaded onto DEAE ion-exchange cellulose column pre-equilibrated with starting buffer and gave single protease active peak (Figure 1). The recovery and specific activity of protease was 73.4% and 648.6 U/mg proteins, respectively. The active fractions of this step were subsequently chromatographed using a Sephacryl S-100 gel filtration column giving a 3.3-fold purification with a recovery of 42.3% and specific activity of 577.7 U/mg protein. The recovery of 'kesinai' milk-clotting enzyme obtained in the present work was higher than that reported by Senthilkumar *et al*. (2006)

Effect of temperature on enzyme activity and stability

The effect of temperature on the activity of the purified enzyme was determined at different temperatures ranging from 20°C to 100°C (Figure 2a). The activity was found to increase as the temperature increased from 20°C to 60°C. The relative activities of 20°C and 90°C were approximately 2.5% and 40.2%, respectively. The optimum temperature for the enzyme activity was found to be at 60°C. This result is in agreement with that reported by Senthilkumar *et al*. (2006) for 'kesinai' enzyme. In addition, the optimum temperature of 'kesinai' enzyme was also found to be similar to other milk-clotting enzyme, such as *Solanum dubiu* (55°C) (Mohamed Ahmed *et al*., 2009a), ginger protease (60°C) (Hashim *et al*., 2011), *Penicillium oxalicum* (65°C) (Hashem, 2000), *Cynara scolymus* (70°C) (Heimgartner *et al*., 1990) and *Centaurea calcitrapa* (52°C) (Raposo and Domingos, 2008). The 'kesinai' enzyme was highly stable for one hour at temperature below 70°C. However, the enzyme activity was completely lost when the temperature increased to 100°C (Figure 2b). After one hour of incubation at 70°C and 80°C, the activity of enzyme was retained 96.6% and 80.5% of its initial activity, respectively. The enzyme lost 86.0% of its initial activity after one hour incubation at 90°C.

Table 1. Purification of 'kesinai' protease.

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification fold	Yield (%)
Crude extract	56160.0 ± 31.2	320.1 ± 1.9	175.6 ± 1.2	1.0 ± 0.0	100.0 ± 0.0
Acetone-precipitation	45942.5 ± 18.7	228.9 ± 1.4	200.7 ± 1.2	1.1 ± 0.1	81.8 ± 0.6
Desalting	42035.7 ± 26.9	123.9 ± 0.9	339.1 ± 2.3	1.9 ± 0.1	74.9 ± 0.7
Ion-exchange	41234.9 ± 24.2	63.6 ± 0.3	648.6 ± 2.9	3.7 ± 0.1	73.4 ± 0.7
Gel-filtration	23780.9 ± 26.1	41.2 ± 0.2	577.7 ± 3.2	3.3 ± 0.1	42.3 ± 0.5

One unit of enzyme activity is measured as the amount of enzyme causing an increase in absorbance by 0.01 at 410 nm at pH 7.4 and 60°C at different steps of purification. Data are means ± SD of the triplicate measurement (n = 3).

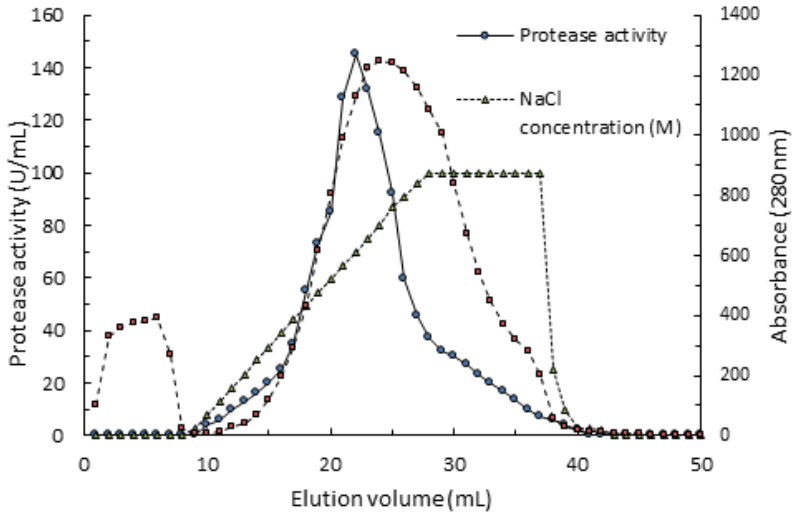

Figure 1. Anion-exchange chromatography of 'kesinai' protease using HiTrap diethylaminoethyl Sepharose Fast Flow (DEAE Sepharose FF) pre-packed column (column dimension 0.7 × 2.5 cm). The enzyme preparation after desalting was applied to column and fractions (1 mL) were collected from the column. Continuous line represents the absorbance at 280 nm; dashed line the NaCl concentration and data points of protease activity.

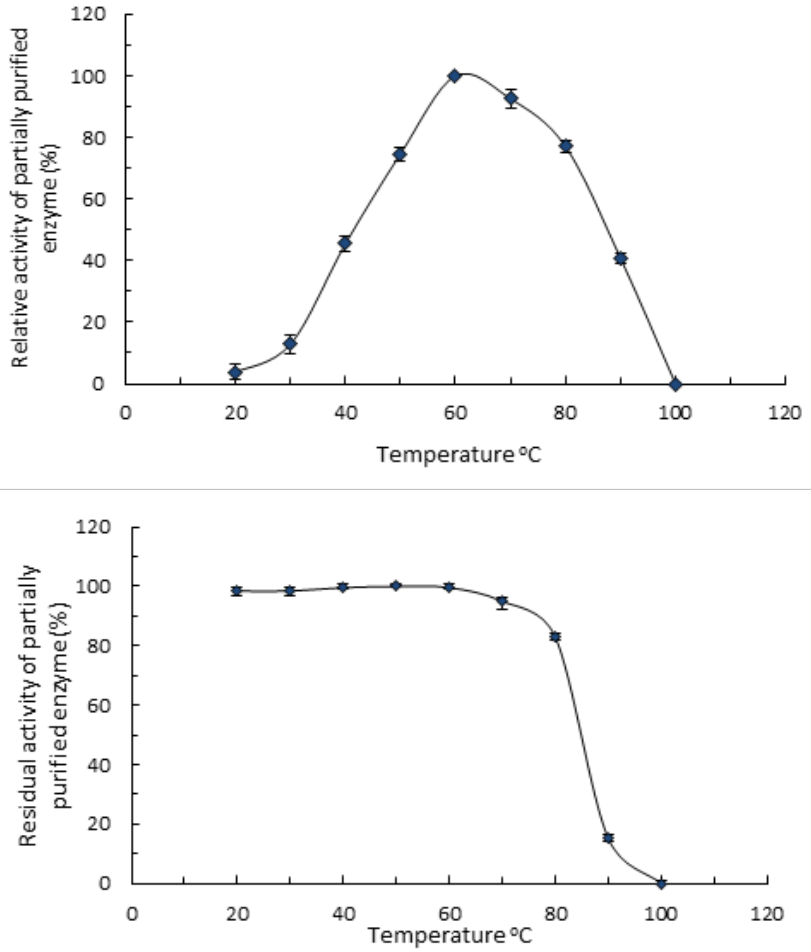

Figure 2. Effect of temperature on activity (a) and stability (b) of partially purified 'kesinai' protease. The temperature profile was determined by assaying protease activity at temperatures between 20 and 100°C at 10°C interval for 20 min. The activity of the enzyme at 60°C was taken as 100%. The temperature stability was determined by incubating the enzyme at different temperatures ranging from 20-100°C for 1 h at pH 7.4 and residual

Effect of pH of on enzyme activity and stability

The effect of pH on purified enzyme activity was determined over a pH range of 3.0 -10.0. The enzyme was active between pH 6.0 and 8.0, with the optimum at pH 7.4 (Figure 3a). The relative activities at pH 6.0 and pH 8.0 were about 62.4% and 66.2%, respectively. The proteolytic activity decreased by 33.7% of its initial activity when the pH of reaction was above 9.0. The optimum activity at alkaline pH has been previously reported for milk-clotting enzyme extracted from *Solanum dubiu* (Mohamed Ahmed *et al*., 2009a), ginger proteases (Huang *et al*., 2011) and *Nocardiopsis* sp. (Cavalcanti *et al*., 2004). However, the pH were slightly higher than those of milk-clotting enzyme produced from various animal sources such as calf chymosin (pH 6.6) (Moschopoulou *et al*., 2006), goat chymosin (5.5) (Kumar *et al*., 2006), recombinant bovine chymosin (pH 6.5) (Walsh and Li, 2000), and buffalo chymosin (pH 5.5) (Mohanty *et al*., 2003).

The stability of the protease at different pH's was examined by measuring the remaining activity after incubation for one hour at various pH's ranging from pH 3.0 to 11.0. The residual activities were determined at 60°C and pH 7.4. The results showed that the purified enzyme activity was retained in the pH range between 6.0 and 10.0, maintaining over 70% of its original activity and more than 60% of its original activity was retained at pH 11.0 (Figure 3b). The stability of this enzyme was similar to some serine proteases such as those from *Cucumis trigonus* Roxburghi, *Cucumis melo* L. var. Prince, *Euphorbia milii* Des Moul. and *Trichosantus kirrilowi* Maxim. (Asif-Ullah *et al*., 2006; Yadav *et al*., 2006). The stability of the enzyme against pH is an important characteristic because most of the plant enzyme activities are unstable at alkaline pH range. This aspect is important for the cheese industry (Mohamed Ahmed *et al*., 2009a).

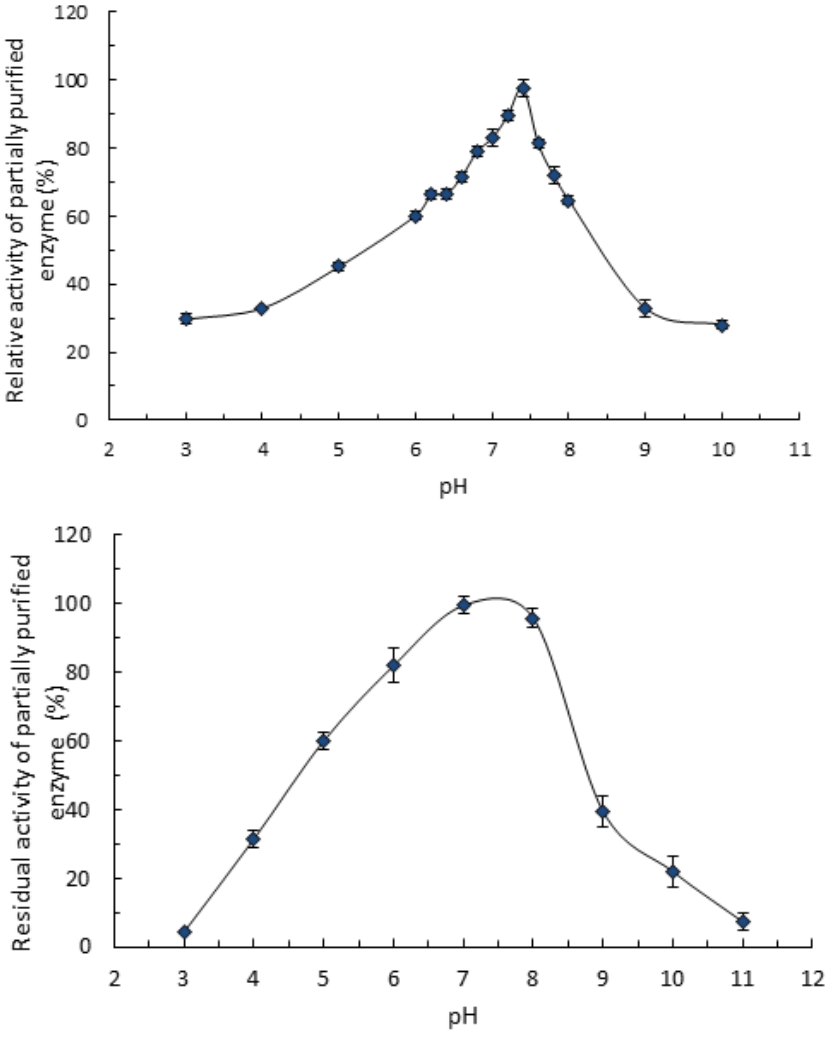

Figure 3. Effect of pH on activity (a) and stability (b) of partially purified 'kesinai' protease. The protease was assayed in the pH range of 3.0–11.0 using buffers of different pH values at 60°C. The maximum activity obtained at pH 7.4 was considered as 100% activity. The pH stability of the enzyme was determined by incubating the enzyme at the optimum temperature (60°C) for 1 h at different pH's, and the residual activity was measured at pH 7.4 and 60°C. The activity of the enzyme before incubation was taken as 100%.

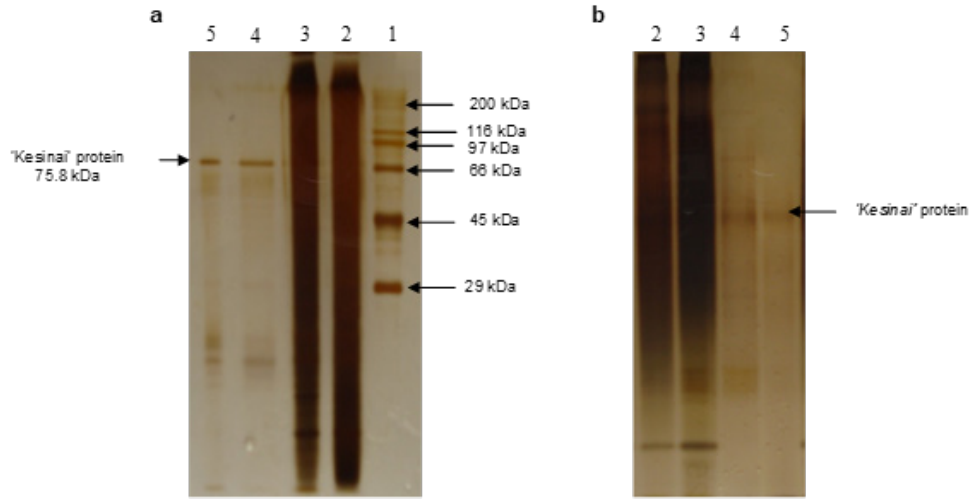

Figure 4. Analysis of 'kesinai' protease using 15% SDS-PAGE (a) and analysis of 'kesinai' protease using 15% NATIVE-PAGE (b). Lane 1 – Marker protein (porcine myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase b (97 kDa), BSA (66 kDa), albumin (45 kDa) and carbonic anhydrase (29 kDa), Lane 2 – crude enzyme, Lane 3 – partially purified enzyme (acetone precipitation), Lane 4 – partially purified enzyme (ion exchange) and Lane 5 – purified enzyme. Electrophoresis was done at room temperature where the current and voltage were set at 15 mA and 250 V, respectively. Protein bands on the gel sheets were visualised by silver staining procedure.

Native molecular mass

The native molecular mass of partially purified 'kesinai' protease was estimated by the gel filtration chromatography using superpose 12 10/300 GL. The elution volume of the purified 'kesinai' enzyme on column was compared to that of the standard proteins, and the molecular mass of the purified 'kesinai' protease was estimated to be 79.4 kDa. This molecular mass was higher than the previously reported 55 kDa by Senthilkumar *et al*. (2006).

SDS-PAGE analysis

The purified 'kesinai' protease showed a single major band and very minor protein band with very low molecular mass, moving along with the dye front in the SDS PAGE profile (Figure 4a). Similarly, a single band protein was also observed on the NATIVE PAGE (Figure 4b). The molecular weight of the purified enzyme was estimated by the SDS PAGE, and the molecular weight of purify 'kesinai' protease enzyme showed a major band with molecular weight corresponding to 75.8 kDa (Figure 4a). This value corresponds to that obtained with gel chromatography. Yamagata *et al*. (1989) reported that the molecular mass of the 'kesinai' enzyme is similar to that of cucumisin, a plant serine protease. Other authors (Antão and Malcata, (2005) reported that the molecular masses of the plant proteases ranged between 19 to 110 kDa. However, a majority of plant serine protease has been shown to be between 60 and 80 kDa in size. The molecular masses of milk-clotting enzymes from *Solanum dubiu*m (66 kDa) and *Centaurea calcitrapa* (66 kDa) have also been reported (Raposo and Domingos, 2008; Mohamed Ahmed *et al*., 2009b). The molecular mass of 'kesinai' protease was found to be much higher than the milk-clotting enzyme obtained from animal sources such as rennin (30.7 kDa), cattle chymosin (35.6 kDa), and buffalo chymosin (23 kDa) (Kumar *et al*., 2006).

Milk clotting activity

In the present work, a comparison of the milk-clotting activity between a partially purified protease extract of 'kesinai' with commercial *Mucor* rennet was performed. It was found that the clotting time obtained for partially purified 'kesinai' enzyme at concentration of 0.1 mg of protein/mL was 96 min while *Mucor* rennet at 0.1 mg of protein/mL was 3.7 min. Suitable rennet substitute should have higher ratio of milk-clotting activity to proteolytic activity (Raposo and Domingos, 2008). The results suggest that the extracts of partially purified enzyme could be used in the production of dairy products of weak pest or together with another commercial enzyme.

Conclusion

The present work showed that the maximum protease activity was obtained by using Tris-HCl buffer at pH 7.4 as an extraction solution for the 'kesinai' leaves. Cold acetone precipitated extract was found to be most suitable techniques for the

initial purification of the 'kesinai' extract. After final purification step, the enzyme was purified 3.3-fold with 42% recovery. The enzyme showed optimum temperature at 60°C and pH 7.4, respectively. It remained stable in a wide range of pH's (from 6.0 to 10.0) and showed high stability up to 70°C for one hour. The 'kesinai' protease yielded a molecular mass of 79 kDa and 75 kDa as estimated by gel filtration and SDS PAGE, respectively. The 'kesinai' protease could serve as an alternative source of milk-clotting enzyme. However, further studies are required for successful application in the cheese-making industry.

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