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Research Article

Biochemical Characterization of Protease Isoforms in Cucumber Sap Extract

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Abstract

Cucumber (*Cucumis sativus*. L) fruit is a popular vegetable, which has been widely used in folk medicine as cosmetics, to treat skin-disorders and reduce heat and inflammation. All parts of cucumber fruit are being used for various purposes but the fruit sap although always associated with the fruit pulp, its biochemical and medicinal properties are unexplored. The juicy fluid obtained from the skin peels of cucumber fruit was subjected to acetone precipitation. The precipitate was dissolved in saline to obtain Cucumber Sap Extract (CSE). CSE was assayed for proteolytic activity on several natural protein substrates such as casein, gelatin, hemoglobin, bovine serum albumin, type-I collagen and human fibrinogen. Biochemical properties such as effect of pH, temperature, inhibitors, metal ions, organic solvents and detergents on the proteolytic activity were studied. Medicinal properties such as effect on the formation of plasma clot, dissolution of clot and degradation of collagen type I was determined. Activity staining on casein substrate gel revealed several translucent activity bands that are distributed in the wide molecular mass range indicating several isoforms and further their differential sensitivity and stability towards pH, temperature, protease inhibitors, metal ions, organic solvents and detergents. CSE was devoid of toxic properties such as edema and hemorrhage. In this preliminary study an attempt is made to show the presence of variety of proteases isoforms for commercial exploration in food, drug, cosmetic, leather and detergent industries.

1. INTRODUCTION

Cucumis sativus. L belongs to the family Cucurbitaceae distributed in tropical, subtropical and temperate regions and is commonly known as melon or gourd or cucurbit. It is a popular plant with numerous beneficial effects in human health and diseases and as well as in cosmetic field. The different parts of cucumber are being used to treat various skin disorders such as wrinkles, chink, skin mold, freckles, swelling under the eyes, sunburn, and hyper-pigmentation, hyperdipsia, burning sensation, thermoplegia, fever, insomnia, cephalalgia, bronchitis, jaundice, hemorrhages, and as an anti-oxidant, anti-hyaluronidase, anti-elastase, anti-microbial, anti-inflammatory, anti-proliferative, analgesic, anti-diabetic and anti-cancer agents [1-6]. Water being the major content, the fruit contains a variety of phytochemicals such as flavonoids, lignans, triterpenes, carbohydrates, lipids, proteins, vitamin C, vitamin A, thiamine, riboflavin, niacin, dietary fibers, and minerals like molybdenium, sodium, calcium, phosphorus, iron, manganese, magnesium and silica [7,8]. In addition, chitinase, peroxidase, subtilase, xylem sap protein-30 and proteases are present in different parts of the plant [9, 10]. Several proteases with potential beneficial properties have been isolated from different plants. Papain, bromelain and ficin respectively from *Carica papaya*, *Ananas comosus* and *Ficus carica* are being used extensively in food industry [11, 12]. A cysteine protease from the latex of *Calotropis gigantea* [13] and a serine protease from the leaves and roots of *Moringa oleifera* [14] have been shown to interfere strongly in hemostasis. Similarly few proteases have also been isolated and studied from the plants belonging to the family Cucurbitaceae. An aspartyl protease[10], a metalloprotease [15] from the seeds/cotyledons of *Cucumis sativus* and serine proteases from *C. ficifolia* and *C. melo*, cucumisin from *C. melo*, protease D from *C. melo*, kiwano protease from *C. metuliferus* [16] have been isolated and studied.
Cucumber is extensively used along with the juicy material as a softening and cleansing agent by the traditional healers and as well as the beauticians to remove plasma clot, bad skin and for conditioning acne and other facial wounds. Proteolytic activity is presumed to be one of the significant contributors for the observed beneficial properties. The fresh juicy material, the sap from the cucumber fruit which is generally removed before consumption due to its bitter taste has not been studied for the proteolytic activity. Thus, the aim of the present study was to evaluate the biochemical and medicinal properties of the proteolytic activity of cucumber fruit sap with the intension of providing the possible scientific basis for the use of cucumber as a skin cleanser and conditioner in cosmetic industry.

2. MATERIALS AND METHODS

2.1 MATERIALS

Cucumbers were purchased from Krishna Raja Market, Mysore. Fat free casein, gelatin, hemoglobin, bovine serum albumin, ethylene diamine tetra acetic acid (EDTA), ethylene glycol-N,N,N',N'-tetra acetic acid (EGTA), 1,10,phenonthroline, phenyl methyl sulphonyl fluoride (PMSF), iodoacetic acid (IAA), human plasma fibrinogen and collagen type I were purchased from Sigma Chemicals Company (St. Louis, USA). Molecular weight markers were purchased from Bangalore Genei private limited, India. The *Vipera russelli (VR)* venom was obtained from Hindustan Park, Kolkotta, India. All other chemicals and reagents purchased were of analytical grade. Plasma was prepared from fresh human blood collected from healthy donors. Swiss albino mice weighing 20–25 g (from the central animal house facility, Department of Zoology, University of Mysore, Mysore, India) were used for studies. Animal care and handling compiled with the National Regulation for Animal Research.

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2.2 METHODS

2.2.1 Preparation of cucumber sap

The peeled outer green layer of cucumber was crushed in saline and stirred overnight using magnetic stirrer, centrifuged for 10 min at 8000 rpm. Supernatant was collected and pellet discarded. Chilled acetone was added to supernatant in the ratio of 2:1 and kept for overnight in cold condition and centrifuged for 10 min at 8000 rpm. Pellet was collected and supernatant discarded. Pellet was dissolved in saline and dialyzed in saline. The dialyzed sample was centrifuged for 10 min at 8000 rpm and the supernatant was collected and designated as cucumber sap extract(CSE) and stored at -20 ^{0}C.

2.2.2 Proteolytic activity, optimum pH and temperature, and inhibition studies

Proteolytic activity was determined according to the method of Satake [17] using casein, gelatin, hemoglobin and bovine serum albumin as substrates. Briefly, different substrates (0.4 ml, 2% in 0.2 M Tris-HCl buffer pH 8.5) were incubated independently with 50 µg CSE in a total volume of 1 ml for 2 h 30 min at 37 ^{0}C. Addition of 1.5 ml 0.44 M trichloroacetic acid (TCA) and left to stand for 30 min in room temperature to stop the reaction. The mixture was then centrifuged at 2500 rpm for 10 min. Sodium carbonate 2.5 ml (0.4 M) and 0.5 ml Folin ciocalteus reagent (diluted to 1/3 of the original strength in water) were added sequentially to 1 ml of the supernatant and the color developed was read at 660 nm. One unit of enzyme activity was defined as the amount of enzyme required to cause an increase in optical density of 0.01 at 660 nm/min at 37 ^{0}C. The specific activity was expressed as the units/min/mg protein. Optimum pH and temperature were respectively determined using different buffers (0.2 M) of pH 3.6 to 12.5 and temperatures (4^{0}C to 90 ^{0}C) independently.

For inhibition studies, CSE (50 µg) was pre-incubated for 30 min with 5 mM each of EDTA, EGTA, 1,10,phenonthroline, PMSF and IAA independently and then activity was determined. In all the cases appropriate controls were kept.

2.2.3 SDS-PAGE and activity staining

SDS-PAGE (7.5%) was carried out according to the method of Laemmli [18] under both reduced and non-reduced conditions. Molecular weight standards from 205 to 3.5 kDa were used. Electrophoresis was carried out using Tris (25 mM), glycine (192 mM) and SDS (0.1%) for 3 h at room temperature. After electrophoresis, gels were stained with 0.1% Coomassie brilliant blue R-250 and destined with 25% ethanol in 8% acetic acid and water (3:1:6 v/v).

For activity staining, CSE (25 µg) prepared under non-reduced condition was subjected for electrophoresis on gels containing 0.2% casein, gelatin, hemoglobin and bovine serum albumin as substrate independently. After electrophoresis, gels were washed with phosphate buffered saline (PBS) containing 2.5% of Triton X-100 with constant agitation for 1 h to remove SDS. The gels were incubated for 72 h at 37 ^{0}C in Tris-HCl buffer (50 mM) pH 7.6 containing 10 mM $CaCl_2$ and 150 mM NaCl. Gels were then stained with 0.1% Coomassie brilliant blue R-250 and destined with 25% ethanol and 8% acetic acid in water (3:1:6 v/v) to observe the translucent activity bands. Activity staining was also performed independently using various pH buffers and as well as in presence of 5 mM each of proteases inhibitors such as EDTA, EGTA, 1,10-phenanthroline, PMSF and IAA.

2.2.4 Effect of metal ions, solvents and detergents

CSE (50 µg) was pre-incubated independently with 5 mM each of metal ions Mg^{2+}, Cu^{2+}, Li^{+}, Zn^{2+}, Fe^{2+}, K^{+} Co^{2+} and Ca^{2+}, 0.5% solvents each of DMSO, isopropanol, ethanol, hexane and benzene, and 0.5% detergents each of SDS and Triton X-100 for 30 min at 37 ^{0}C. Casein (2%) was added and incubated for 2 h 30 min at 37 ^{0}C and assayed as described by Satake [17].

2.2.5 Activity on collagen type I

Collagen type I (50 µg) was incubated with different amounts of CSE (0.5 to 20 µg) for 48 h and time intervals (30 min to 72 h) in a total volume of 40 µl 10 mM Tris HCl buffer pH 7.4 at 37 ^{0}C. The reaction was terminated by the addition of 20 µl sample buffer containing 1 M urea, 4% SDS and 4% β-mercaptoethanol and analyzed on 7.5% SDS-PAGE under reduced condition.

2.2.6 Plasma re-calcification time

Plasma re-calcification time was determined according to the method of Quick [19] using plasma prepared from citrated human blood collected from healthy individuals. The plasma 0.2 ml was pre-incubated with different concentration of CSE (2.5 to 100µg) in 20 µl 10 mM Tris HCl buffer pH 7.4 for 5 min at 37 ^{0}C, and then clotting time was determined by adding 20 µl 0.25 M $CaCl_2$ against a light source.

2.2.7 Fibrinogenolytic activity

Fibrinogenolytic activity was determined according to the method of Ouyang and Teng [20]. Different concentrations of CSE (1 to 30 µg) were incubated with 50 µg human plasma fibrinogen in a total volume of 40 µl 10 mM Tris-HCl buffer pH 7.5 for 12 h. For time dependent activity CSE (10 µg) was incubated for different lengths of time (30 min to 24 h). Reaction was terminated by adding 20 µl denaturing buffer containing 1 M urea, 4% SDS and 4% β-mercaptoethanol. It was then analyzed by 10% SDS-PAGE.

2.2.8 Fibrinolytic activity

Citrated human plasma (100 µl) was mixed with 20 µl 10 mM Tris HCl buffer pH 7.4 and 20 µl 0.25 M $CaCl_2$ for 1 h at 37 ^{0}C to get soft fibrin clot. The fibrin clot was washed thoroughly 5-6 times with phosphate buffered saline (PBS). Fibrin clot was then incubated with different concentration of CSE (5 to 40 µg) for 12 h and time intervals (1 h 30 min to 24 h) in a final volume of 40 µl 10 mM Tris HCl buffer pH 7.4 at 37 ^{0}C for 12 h. The reaction was stopped by adding 20 µl sample buffer containing 1 M urea, 4% SDS and 4% β-mercaptoethanol. The samples were boiled for 10 min and centrifuged to settle the debris. An aliquot of 20 µl supernatant was analyzed in 10% SDS-PAGE.

2.2.9 Hemorrhagic activity

The hemorrhagic activity was determined according to the method of Kondo [21] using groups of mice (n=4). The samples in a final volume of 50 µl saline were injected intra-dermally on the back of mice. The mice were anaesthetized after 3 h and sacrificed. The dorsal patch of the skin was removed, the inner surface was observed for the hemorrhage and the diameters of the hemorrhagic spots were measured. The minimum hemorrhagic dose (MHD) was defined as the amount of protein required to induce a hemorrhagic area of 10 mm diameter. The group received *Vipera russellii* venom served as positive control.

2.2.10 Protein estimation

Protein content was determined using biuret reagent according to the method of Harrison [22] where bovine serum albumin was used as standard.

2.2.11 Statistical analysis

All the experimental data are presented as the mean ± SEM of three independent experiments.

3. RESULTS

The CSE, upon SDS-PAGE, displayed several protein bands in the wide molecular mass range from 3.5 to 205 kDa under both non-reduced and reduced conditions (**Fig. 1A**). However, many thick and prominent bands are seen in the mass range less than 43 kDa as compared to only few thin bands seen in the range of mass over 43 kDa. CSE showed proteolytic activity on various protein substrates such as casein, gelatin, hemoglobin, and BSA with the specific activities of 2.27 **±** 6, 1.4 **±** 6, 1.13 **±** 3 and 0.27 ± 0 units / mg / min respectively. Casein was hydrolyzed better than rest of the substrates tested. The activity staining/zymogram/substrate gel assay on afore said substrates revealed varied proteolytic activity bands. In each case a significant variations in activity bands where distinct pattern of translucent activity bands with different molecular masses were observed. The activity bands seen were however more prominent in casein zymogram than in BSA, gelatin and hemoglobin zymograms. A highly translucent activity band in the molecular mass region of 97.4 kDa, two overlapping bands in the region of 66 kDa, a band in the region little less than 43 kDa and one more band in the region around 29 kDa were seen in casein zymogram. Three noticeable activity bands in molecular mass around 97.4 kDa and a faint activity band in the region around 43 kDa were seen in gelatin zymogram. Activity band in the region of

mass around 97.4 kDa is less conspicuous while two noticeable bands in the mass region of about 43 kDa were seen in hemoglobin zymogram. In contrast, two activity bands one each in the mass region of 205 kDa and 66 kDa but less prominent as compared to other cases were observed in BSA zymogram (**Fig. 1B**).

In a pH versus activity profile, the activity seen was in the wide pH range from pH 5.5 to 12.5 and with two pH optima respectively at pH 8.5 and 10.5 (**Fig. 2A1**). Interestingly, casein substrate gel assay revealed the differential stability and sensitivity of individual activity bands towards pH. At pH 5.5 (lane 2) activity bands were not prominent; the high molecular mass activity band is slightly visible. However, at pH 7.5 (lane 3) and above, till pH 10.5 (lane 6) all activity bands seen were conspicuous. At pH 11.5 (lane 7), and the other two bands positioned on either side are also not conspicuous, at pH 12.5 (lane 8) all the activity bands vanish except for a light activity band seen in the mass range of about 205 kDa (**Fig. 2A2**). The temperature versus activity profile revealed the optimum temperature of 45^{o} C (**Fig. 2B1**), and this has been further supported by substrate gel assay where at 45^{o} C the translucent activity bands are more prominent (lane 5) than in rest of the cases and at 90^{o} C none of the activity bands were seen in the gel (lane 7) (**Fig. 2B2**).

The susceptibility of the protease activity to various protease inhibitors revealed varied degrees of inhibition. The percent inhibition varied as EDTA (29 ± 2.5%), EGTA (27 ± 5%), 1, 10, phenanthroline (35 ± 5%), PMSF (40 ± 7%) and IAA (12 ± 5%). Among the inhibitors, IAA showed least inhibition on casein substrate (**Fig.3A**). **Fig. 3B** shows the effect of various monovalent and bivalent cations. Mg^{2+}, Cu^{2+}, and Li^{+} enhanced the activity of CSE, while K^{+}, Zn^{2+} and Fe^{2+} decreased the activity significantly whereas Na^{+}, Ca^{2+} and Co^{2+} did not affect. However, Hg^{2+} abolished the proteolytic activity of CSE.

Further, the stability of CSE proteolytic activity towards organic solvents varied significantly. At 0.5% in the reaction mixture, solvents such as DMSO and Isopropanol enhanced the activity by 24 ± 7% and 12 ± 5% while hexane and benzene decreased the activity by 6 ± 2.5 % and 18 ± 2.5% respectively as compared to the control value whereas ethanol had no effect on the activity (**Fig. 4A1 and 4A2**). Similarly, at 0.5%, SDS and Triton X100 decreased activity by about 30 ± 8% and 20 ± 4 % respectively was observed (**Fig. 4B1 and 4B2**).

Upon storage at below 0^{o}C for a period of 9 months, CSE showed a marginal reduction in proteolytic activity. The 15, 30, 90 days and 9 months old samples showed the respective specific activities of 1.94 ± 7 units, 1.72 ± 3 units, 1.50 ± 3 units, and 1.43 ± 3 units as compared to fresh sample which revealed 2.27 ± 6 units/min/mg. The activity bands especially in the region of molecular masses of about 43 kDa and 27 kDa are seen up to 15 days old sample and disappeared in 30 days and above old samples. While, the activity bands corresponding to other high molecular masses (above 60 kDa range) remained stable throughout the storage period as revealed by casein activity staining (**Fig. 5**).

Interestingly, CSE hydrolyzed type I collagen, **figures 6A** and **6B** shows the dose and time dependent response respectively. All the chains such as α1α2βγ are susceptible for degradation. During prolonged incubation all chains are degraded as the bands vanish with the appearance of new bands in the low molecular mass range.

When tested on citrated human plasma, CSE decreased the clotting time dose dependently; the clotting time was declined from 266 ± 3.5 sec to 141 ± 1.7 sec (**Fig. 7**). CSE hydrolyzed human fibrinogen dose dependently (**Fig. 8A**) and time dependently (**Fig. 8B**). All, such as Aα, Bβ and γ chains are hydrolyzed. The intensity of respective bands gradually decreases with increasing doses of CSE and with the appearance of low molecular mass degradation peptide end products. The Bβ chain is preferentially hydrolyzed over Aα chain while the γ chain is hydrolyzed with a less preference (**Fig. 8A**). Further, CSE also hydrolyzed the hard fibrin clot dose dependently (**Fig. 9A**) and time dependently (**Fig. 9B**). All chains of fibrin clot are degraded; however α polymer and α chain are hydrolyzed in preference nearly to a similar extent over β chain, and γγ dimers are hydrolyzed much slowly. However, CSE was non hemorrhagic up to a tested dose of 200 µg in a mouse model while *Vipera russelli* venom was highly hemorrhagic and served as a positive control (**Fig. 10**), further, CSE was devoid of other toxic properties such as edema, dermonecrosis and myonecrosis (data not shown).

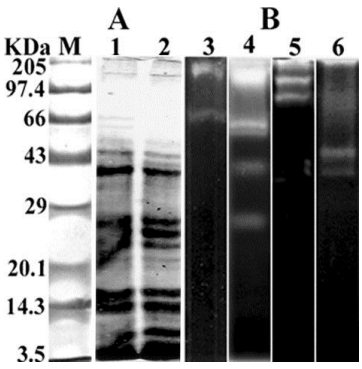

Fig.1: SDS-PAGE pattern and substrate gel assay of CSE proteolytic activity: **A** represents the protein banding pattern of CSE (100 µg) on SDS PAGE, lane 1(non-reducing) and lane 2 (reducing condition).**B** represents substrate gel assays of CSE (25 µg) on different substrates, lane 3 (BSA zymogram), lane 4 (casein zymogram), lane 5 (gelatin zymogram), lane 6 (haemoglobin zymogram) and M represents the molecular weight markers (kDa): rabbit muscle myosin (205), phosphorylase B (97.4), bovine serum albumin (66), carbonic anhydrase (29), soybean trypsin inhibitor (20.1), lysozyme (14.3) and insulin (3.5).

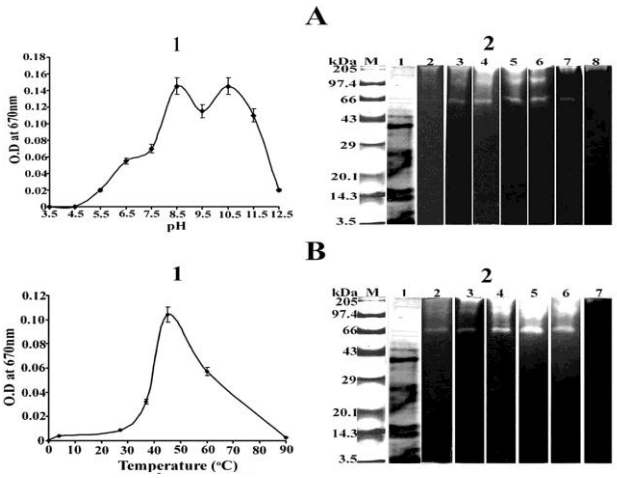

Fig. 2: Effect of pH and temperature on the proteolytic activity of CSE: **A1** *represents the proteolytic activity on casein at different pH values. CSE (25* µg*) was incubated with 0.4 ml of casein substrate for 2 h 30 min at 37* ^{0}*C with different pH conditions.* ***A2*** *represent the proteolytic activity of CSE in casein substrate gel assay, lane 1 (protein banding pattern in SDS-PAGE under non-reducing condition), lanes 2 to 8 the gels were incubated in buffers of pH (5.5), (7.5), (8.5), (9.5), (10.5), (11.5), and (12.5) respectively.* ***B1*** *represents the activity profile from 0*^{o} *C to 90*^{o} *C. CSE (50* µg*) was incubated with 0.4 ml of casein substrate for 2 h 30 min at different temperature conditions.* ***B2*** *represents the proteolytic activity of CSE in casein substrate gel assay, lane 1 (protein banding pattern in SDS-PAGE under non-reducing condition), lanes 2 to 7 the gels were incubated at 4* ^{0}*C, 27* ^{0}*C, 37* ^{0}*C, 45* ^{0}*C, 60* ^{0}*C, and 90* ^{0}*C respectively. M represents the molecular weight markers (kDa): rabbit muscle myosin (205), phosphorylase B (97.4), bovine serum albumin (66), carbonic anhydrase (29), soybean trypsin inhibitor (20.1), lysozyme (14.3) and insulin (3.5).*

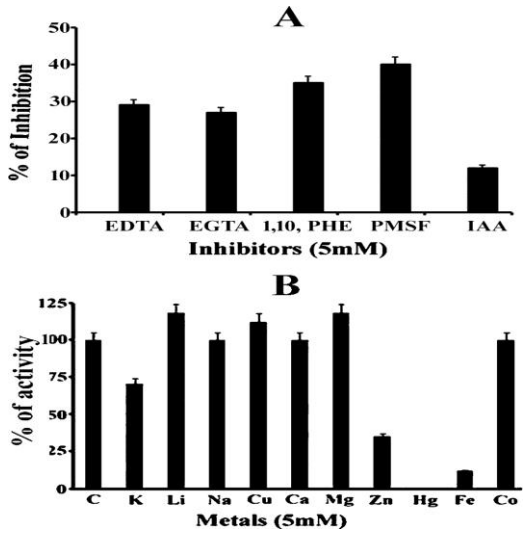

Fig. 3. Effect of protease inhibitors and metal ions on proteolytic activity of CSE:***A*** *represents the effect of known protease inhibitors on proteolytic activity of CSE. Fifty microgram of CSE was preincubated independently with or with out protease known inhibitos (5 mM) for 30 min at 37°C.* ***B*** *represents the effect of metal ions by pre- incubating the CSE (50 µg) with 5 mM metal ions independently for 2 h 30 min at 37 ^{0}C. The percentage of activity varied as K (70.6 ± 4.7%), Li (118 ± 4.7%), Na (100 ± 4.7%), Ca (100 ± 0%), Cu (112 ± 5%), Mg (118 ± 4.7%), Zn (35.3 ± 7%), Hg (0 ± 0%), Fe (11.8 ± 4.7%) and Co (100 ± 4.7%).*

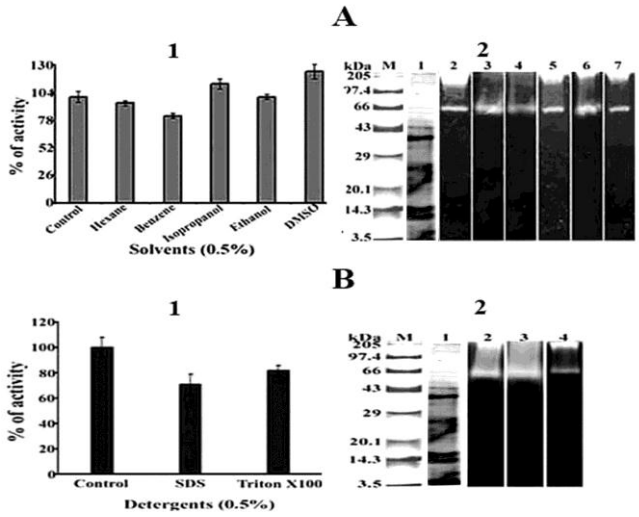

Fig. 4: Effect of solvents and detergents on proteolytic activity of CSE:***A1*** *represents the effect of solvents on proteolytic activity, CSE (50 µg) was pre-incubated with 0.5% of solvents independently for 30 min at 37 ^{0}C and then incubated with the substrate for 2 h 30 min.* ***A2*** *represent the effect of solvent on casein substrate gel assay, lane 1 represents (protein banding pattern in SDS-PAGE under non-reducing condition), lane 2 represent control CSE, from lanes 3 to7 effects of hexane, benzene, Isopropanol, ethanol and DMSO respectively on casein substrate gel assay.* ***B1*** *represents the effect of detergents on proteolytic activity, CSE (50 µg) was pre-incubated with 0.5% of detergents independently for 30 min at 37 ^{0}C and then incubated with the substrate for 2 h 30 min.* ***B2*** *represent the effect of detergents on casein substrate gel assay, lane 1 represents (protein banding pattern in SDS-PAGE under non-reducing condition), lane 2 control CSE, lane 3 (SDS) and lane 4 (Triton-X-100) on casein substrate gel assay. M represents the following molecular weight markers (kDa): rabbit muscle myosin (205), phosphorylase B (97.4), bovine serum albumin (66), carbonic anhydrase (29), soybean trypsin inhibitor (20.1), lysozyme (14.3) and insulin (3.5).*

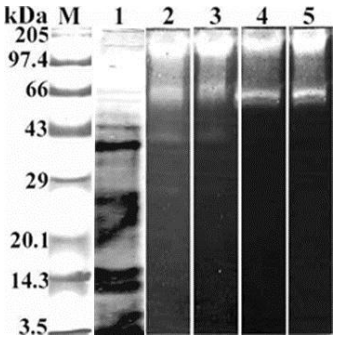

Fig. 5: Stroage stability of CSE proteolytic activity:*Lane 1 (protein banding pattern of CSE (100 µg), 7.5 % SDS-PAGE, non-reducing), lanes 2 to 5, CSE (50 µg) proteolytic activity banding pattern in casein substrate gel assay; lane 2 (fresh sample), lane 3 (15 days old sample), lane 4 (30 days old sample), lane 5 (3 months old sample) respectively and M represents the molecular weight markers (kDa): rabbit muscle myosin (205), phosphorylase B (97.4), bovine serum albumin (66), carbonic anhydrase (29), soybean trypsin inhibitor (20.1), lysozyme (14.3) and insulin (3.5).*

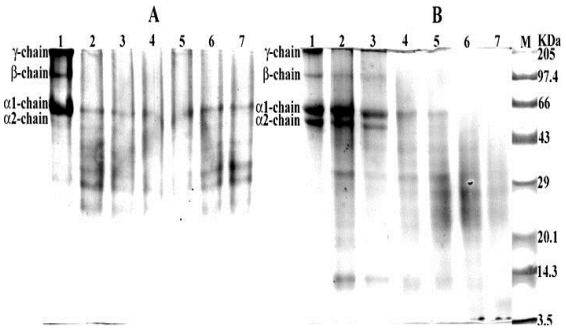

Fig. 6: Hydrolysis of collagen type-I by CSE:***A*** *represents the dose dependent degradation of collagen type-I (50 µg) with different concentration of CSE lane 1 (collagen alone), 2 (0.5 µg), 3 (1 µg), 4 (2.5 µg), 5 (5 µg), 6 (10 µg) and 7 (20 µg) respectively.* ***B*** *represents the time dependent hydrolysis of human fibrin with CSE (10 µg), lane 1 (collagen alone), 2 (3 h), 3 (6 h), 4 (12 h), 5 (24 h) 6 (48 h) and 7 (72 h) respectively. M represents the following molecular weight markers (kDa): rabbit muscle myosin (205), phosphorylase B (97.4), bovine serum albumin (66), carbonic anhydrase (29), soybean trypsin inhibitor (20.1), lysozyme (14.3) and insulin (3.5).*

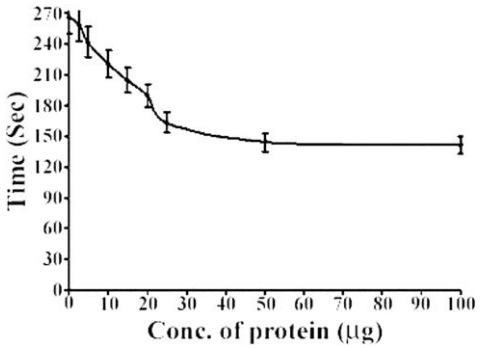

Fig. 7: Effect of CSE on recalcification time of human citrated plasma:*CSE protease (2.5 to 100 µg) was pre-incubated with 0.2 ml of citrated human plasma in the presence of 20 µl of 10 mM Tris-HCl buffer pH 7.4 for 1 min at 37 ^{0}C. Twenty microlitres of 0.25 M $CaCl_2$ was added to the pre-incubated mixture and clotting time was recorded.*

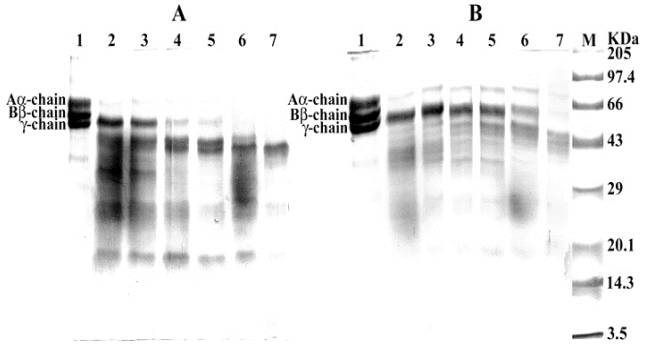

Fig. 8: Hydrolysis of fibrinogen by CSE:***A*** *represents the dose dependent degradation of human fibrinogen (50 µg) , with different concentration of CSE lane 1 (fibrinogen alone), 2 (1 µg), 3 (2.5 µg), 4 (5 µg), 5 (10 µg), 6 (20 µg) and 7 (30 µg) respectively.* ***B*** *represents the time dependent hydrolysis of human fibrinogen (50 µg) with CSE (10 µg), lane 1 (fibrinogen alone), 2 (30 min), 3 (1 h, 30 min), 4 (3 h), 5 (6 h), 6 (12 h) and 7 (24 h) respectively. M represents the following molecular weight markers (kDa): rabbit muscle myosin (205), phosphorylase B (97.4), bovine serum albumin (66), carbonic anhydrase (29), soybean trypsin inhibitor (20.1), lysozyme (14.3) and insulin (3.5).*

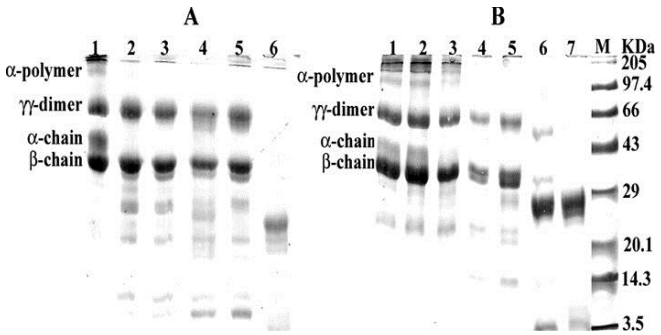

Fig. 9: Hydrolysis of fibrin by CSE:***A*** *represents the dose dependent degradation of human fibrin with different concentration of CSE lane 1 (fibrin alone), 2 (5 µg), 3 (10 µg), 4 (20 µg), 5 (30 µg) and 6 (40 µg) respectively. B represents the time dependent hydrolysis of human fibrin with CSE (10 µg), lane 1 (fibrin alone), 2 (1 h 30 min), 3 (3 h), 4 (6 h), 5 (12 h) and 6 (24 h) respectively. M represents the following molecular weight markers (kDa): rabbit muscle myosin (205), phosphorylase B (97.4), bovine serum albumin (66), carbonic anhydrase (29), soybean trypsin inhibitor (20.1), lysozyme (14.3) and insulin (3.5).*

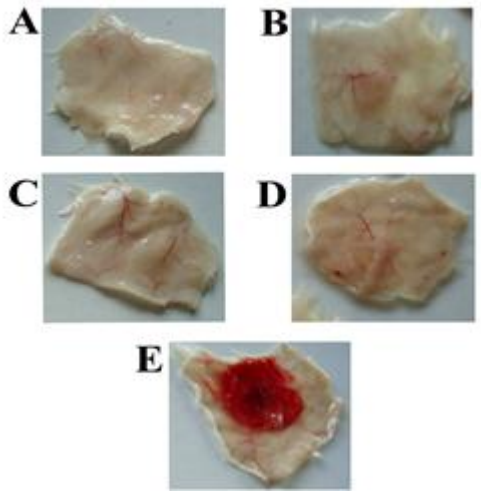

Fig.10. Hemorrhagic activity of CSE: *CSE was injected intradermally into groups of mice (n = 4) in a total volume of 50 µl saline. After 3 h, mice were anesthetized and sacrificed. The dorsal patch of the skin was removed and the skin surface was examined for the hemorrhagic spot and the diameter of hemorrhagic spot was measured in mm. Saline (A), CSE 50 µg (B), CSE 100 µg (C), and CSE 200 µg (D) and D. russellii venom (2 MHD, 5 µg) (E) which served as a positive control.*

4. DISCUSSION

The cucumber sap extract is a complex mixture of proteins as they distributed in the wide molecular mass range in SDS-PAGE. Many thick protein bands in the mass range less than 43 kD suggests their high relative abundance. Despite lack of thick bands but, appearance of strong translucent activity bands in casein activity staining suggests the presence of highly active proteases in the high molecular mass region. The varied extent and pattern of proteolytic activity bands on various substrates in activity staining not only suggested the complex and a diverse mixture of proteases present. These proteases appear highly active on respective substrates as they revealed prominent and conspicuous translucent activity bands as compared to their existence in trace amounts. Proteases with the molecular masses of 400 kDa [23] and 80 kDa [24] have been isolated and characterized from the leaves of Cucumber. A 42 kDa aspartyl protease [10] and a 200 kDa metalloprotease [15] from the seeds/cotyledons of cucumber have been isolated. Further, proteases in the molecular mass ranges from 26 kDa to 80 kDa have been isolated from different plants belonging to Cucurbitaceae family [16].The larger molecular mass proteases appears to be highly stable under 0^{o} C for a longer period of storage as no much change in the intensity of proteolytic activity bands of nine months old sample in substrate gel assay however, the intensity of activity bands of the medium molecular mass (about 43 and 29 kD) proteases decreased gradually and disappeared in three months old sample and thus, the medium molecular mass proteases appears less stable. PMSF inhibited the proteolytic activity maximum followed by EDTA and 1, 10-phenanthroline and then iodoacetate suggesting the presence of serine, metallo and cysteine proteases. PMSF targets serine proteases, EDTA and 1, 10-phenanthroline inhibits metalloproteases while iodoacetate inactivate cysteine proteases, although these protease inhibitors are highly non-specific, the inhibition pattern clearly suggests that the cucumber sap contain mixture of different class of proteases. PMSF with maximum inhibition and IAA with least inhibition and thus the relative abundance is likely to be varied as serine proteases > metalloproteases > cysteine proteases. Increased activity in presence of Li^{+}, Cu^{++} and Mg^{++} while decreased activity with K^{+}, Zn^{++} and Fe^{++} but, no change in activity in presence of Na^{+}, Ca^{++} and Co^{++} is interesting, different isoforms of proteases may be differentially sensitive to different monovalent and divalent cations. Further studies, whether ions alter the affinity of enzymes or affect the substrates availability appears even more interesting. Mg^{++} and Ca^{++} found to inhibit the activity of serine protease isolated from the cucumber leaves [24]. The protease activity of *Zingiber officinale* Roscoe was inhibited by Hg^{++} and Cu^{++} [25]. The aminopeptidase activity of *Phaseolus vulgaris* seeds was increased 40% by 0.15 M NaCl, inhibited 94% by 2.0 mM Zn^{++} [26]. The protease activity of *Bacillus licheniformis* was inhibited by PMSF, Cu^{++}, Mn^{++} and Ni^{++} [27]. Based on the pH activity profile, it appears that the CSE contain two groups of proteases the first group with the optimal activity at around pH 8.5 and the second with pH 10.5, further supporting the complex nature of CSE protease activity. Similarly, *Eria coronaria* latex protease activity showed two pH optima between pH 6 - 6.5 and pH 7 – 7.5 [28]. The protease from *Bacillus licheniformis* is alkaline active with the optimum activity seen at pH 10 [27]. Interestingly, DMSO and isopropanol enhanced the activity suggesting the co-solvent effect while ethanol had no effect, but hexane and benzene caused marginal inhibition. Similarly the activity varied in presence of SDS and triton X-100. The varied intensity of activity bands in activity staining suggested the varied stability and sensitivity to different solvents and detergents and offer scope for their use in leather, detergent and pharmaceutical industries. The cystein protease from the latex of *Ervatamia coronaria* was stable in 40% acetonitrile, 70% ethanol, and 50% methanol[28]. The *Araujia hortorum* Fourn fruit latex protease *Araujiain* showed the highest residual caseinolytic activity in presence of 50% (v/v) hexane, 50% (v/v) propanone [29]. The crude enzyme preparation obtained from the latex of fruits of *Araujia hortorum* Fourn showed increased activity in presence of different organic solvents[29]. The alkaline protease isolated from Bacillus

pseudofirmus SVB1, showed enhanced activity in presence of non ionic detergents Triton X-100 and Tween -20 and the enzyme retained about 71% activity in 1% SDS [30].

It is interesting to note the fact that most of the proteases from plant sources interfere strongly in the complex blood coagulation process affecting platelet functions, plasma coagulation and or clot dissolution including tissue repair during wound healing process [31]. Understanding the procoagulant property and hydrolysis of human fibrinogen leading to clot formation whether through contact activation pathway, tissue factor pathway or both pathways or through the common pathway is exciting. Isolation of individual proteases and studying their effects on activated partial thromboplastin time (APTT), prothrombin time (PT) and thrombin clotting time (TCT) and understanding their action on platelets would help better understanding of complex properties of CSE. Preferential hydrolysis of Bβ-chain of fibrinogen, and α-chain and α-polymers and less preferential hydrolysis of β-chain of fibrin appears complex. The latex extracts from *Calotropis gigantea*, *Synadenium grantii*, and *Wrightia tinctoria* hydrolyzed fibrinogen and fibrin clot dose-dependently to a varied extent [32,13]. CSE hydrolyzed type-I collagen, an abundant complex protein present in the extracellular matrix of skin, and the precise homeostatic balance in the concentration of this protein is essential for tissue repair during wound healing process. A cysteine protease isolated from the rhizomes of *Zingiber officinale* hydrolyzed bovine and rat type-I collagen appears to find place in meat tenderization process as an alternative to papain [33]. Lack of toxic properties such as edema and hemorrhage and together with collagenolytic and fibrino(geno)lytic activities, CSE may be of interest as an effective and safe cleansing agent in cosmetic industry or as a dressing material in pharmaceutical industries to clean up the debris such as torn out tissues and as well as the fibrin clot of the wound during wound repair and healing process.

5. CONCLUSION

In conclusion, the different parameters studied such as substrate specificity on various protein substrates, like bovine serum albumin, gelatin, hemoglobin, fibrinogen and fibrin clot and effect of pH, temperature, metal ions, organic solvents and detergents including storage stability are successfully disclosing the presence of different isoforms of proteases of different specificities, sensitivities and stabilities. Some of these may find importance in cosmetic, pharmaceutical, food and leather industries. Isolation and detailed structure-function studies of individual proteases although challenging but, highly interesting.

6. ACKNOWLEDGMENTS

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7. CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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