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PURIFICATION AND CHARACTERIZATION OF MCase FROM *MOMORDICA CHARANTIA* SEED EXTRACT

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ABSTRACT

Purification and characterization of MCase from *Momordica Charantia* Seed Extract was carried by fractionation, on DEAE-Cellulose column chromatography and sephadex G-75 column chromatography. When peak II was fractionated on anion exchange, four peaks were resolved (Peak I-IV), but only peak II exhibited strong anticoagulant activity. In addition, peak II showed sharp single band on SDS-PAGE with an apparent molecular weight of 100kDa in both non-reducing and reducing conditions. Purity of that protein was adjudged using RP-HPLC. The purified proteins showed strong proteolytic activity with the specific activity of 14.84 units/mg/min. Protease inhibition studies were performed using specific inhibitors such as PMSF, IAA, EDTA and 1,10-Phenanthroline. Interestingly, protease was susceptible to PMSF but insensitive to remaining, suggesting the purified protein is of serine family. Hence, it named as MCase**.** MCase hydrolysed fibrinogen and fibrin as well. The optimum activity of serine proteases was observed in the temperature range 37-40 ^{0} C and P^{H} between 8-8.5. MCase found to exhibit anticoagulant activity was confirmed by plasma recalcification time and mouse tail bleeding assay.

Keywords: MCase, *Momordica Charantia*, SDS-PAGE, RP-HPLC

INTRODUCTION

Proteases are group of hydrolytic enzymes widely distributed all organisms including plant kingdom, they play a key role in several biological processes due their peptide bond cleaving ability **[1]**. They primarily regulate gene expression, cell growth, development and reproduction **[2]**. Moreover, they also found to exhibit several function namely meiosis, gametogenesis, cell senescence, apoptosis, development of epidermal cell, stomata, synthesis of chloroplast, removal of unwanted proteins, post translational modification, activation of zymogens and peptide hormones **[3]**. The most typical properties of these enzymes is associated with thermal stability, thus plant proteases have been widely exploited as a potential therapeutic agents **[4]**. The key target for the plant derived proteolytic enzymes could be due to the low cost, very easy to extract and they do not trigger an adverse effect to humans and animals while working with them **[5]**. Recent studies revealed that they have been extensively used in the treatment of wounds, cancer, infectious diseases, digestive disorders and diseases associated with liver **[6, 7]**. To illustrate few of them that have been widely used as potential therapeutic agents; papain, bromelain, ficin and cucumisin. Papain was purified from latex of papaya (*Carica papaya*) and bromelain isolated from fruit and stem of the pineapple (Bromeliaceae family) belongs to the family of cysteine protease **[8, 9]**. Ficin, isolated from latex of fig tree (*Ficus s*pp.) is being used as a vermifuge **[10]**. However, Cucumisin, is the only serine protease characterized for the first time from melon fruits (Cucumismelo). In addition, cucumisin like serine proteases found ubiquitously throughout the plant kingdom. Despite those proteases have been considered to be the highly characterized proteases, most of their therapeutic usage is least explored. Serine proteases were once thought to be rare in plants; in recent years, however, several of those enzymes have been isolated from several plant species **[11]**. The research from several group reported the serine proteolytic enzymes from seeds of soybean, barley and rice, from the latex of dandelion, jackfruit, African milk bush and maize **[12-14]**. Furthermore, serine proteolytic enzymes were also identified from the flowers, stems, leaves and roots of mouse ear cress(*Arabidopsis thaliana*) sweet potato, leaves of tobacco and tomato **[15]**. Nevertheless, proteolytic enzymes have been less studied enzymes in bitter gourd. While, trypsin inhibitors, translation inhibitors and a well-

known glycoprotein momordin an antidiabetic agents have been extensively studied **[16-18]**. While, in our previous studies we have demonstrated on the presence of serine and metalloproteolytic enzymes, those exhibited anticoagulant. Thus, the current study focuses on the purification and characterization of high molecular weight serine protease from bitter gourd seed extract and the results are presented.

MATERIALS

ADP type-I, ADP, epinephrine, collagen were purchased from Sigma Chemicals Company, St. Louis, USA. UNIPLASTIN, LIQUICELIN-E and FIBROQUANT were purchased from Tulip Diagnostics Pvt. Ltd., Goa, India. Sephadex G-100 and DEAE cellulose were purchased from Sigma Chemicals Company (St. Louis, USA). All other chemicals and reagents used were analytical grade. Fresh blood sample was collected from healthy human donors. Swiss Wister albino mice weighing 20–25g from the central animal house facility, Department of Studies in Zoology, University of Mysore, and Mysore, India. Animal care and handling complied with the National Regulation for Animal Research.

Momordica Charantia seed and Preparation of Momordica Charantia seed extract

Momordica charantia (bitter gourd) fruits were purchased from the local market of Tumkur and the seeds were collected from the fruit. The outer coat was removed from the seeds, thoroughly chopped, homogenized using double distilled water and centrifuged at 5000g for 20 min at 4^0C .The supernatant was collected and proteins were precipitated using 30% of ammonium sulfate. The precipitated protein sample was again centrifuged at 10000g for 20min; the supernatant was collected and dialyzed overnight. The protein sample obtained was stored at -20^0C until it was used for further studies. Protein concentration was determined **[19]**, using bovine serum albumin (BSA) as standards.

DEAE Cellulose column chromatography

DEAE cellulose column (1.5 X 18cm) was pre-equilibrated with 10mM Tris-HCl buffer pH 7.5 and MCSE 100mg in 1mL equilibrating buffer was loaded on the same. By passing different molarities of eluent NaCl in 10mM Tris-HCl buffer (10mM Tris-HCl buffer, 0.1M NaCl in10mM Tris-HCl pH7.5, 0.2M NaCl in 10mM Tris-HCl, 0.3M NaCl in10mM Tris-HCl pH7.5, 0.4M NaCl in10mM Tris-HCl pH 7.5 and 0.5M NaCl

in10mMTris-HCl pH7.5) sub fractions 2.0ml were collected at the flow rate of 20ml/h of fractionation. Proteolytic activity and anticoagulant activity for every alternate tube were done, later fractions showing above said activities were pooled and concentrated by lyophilizing. Ten peaks were resolved of which Peak II and Peak III showed proteolytic activity but not remaining peaks. In the current study Peak-II was selected for further studies.

Sephadex G-75 column chromatography

Peak II fraction was recovered (20mg in 1mL of 0.1M NaCl) and loaded on to a pre-equilibrated Sephadex G-75 column with 0.1M NaCl (1 x 90cm). The fractions were collected with a flow rate of 20mL/h and 2mL fractions using 0.1M NaCl. At 280nm Protein elution was monitored. Alternate tubes were screened for anticoagulant and proteolytic, Fractions exhibited positive results were pooled and concentrated.

Reversed phase –HPLC (RP-HPLC)

The purified fraction of protein obtained from Sephadex G-75 column chromatography was subjected for RP-HPLC using shimadzu LC20AD prominence HPLC with PDA detector. C18 column (150mm × 4.60mm, particle size 5µm) that was pre-equilibrated with 0.1% Trifluoro Acetic Acid (TFA) in water. By using linear gradient from 0.1%TFA in water (solution A) to 0.1% TFA in acetonitrile (solution B) for 20min. The protein was eluted at a flow rate 0.5 mL/min and it was monitored at 280nm.

Protein estimation

Concentration of Protein was determined **[19]**, using Bovine serum albumin (BSA) as standards.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried[20] Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10%) and periodic acid-Schiff (PAS) staining were carried out **[20, 21]**. The crude MCSE (100µg) prepared under reducing and nonreducing conditions was used for SDS-PAGE. The electrophoresis was carried out using Tris (25mmol/l), glycine (192mmol/l) and SDS (0.1%) for 3h at room temperature. After electrophoresis, the SDS-PAGE gels were stained with 0.1% Coomassie brilliant blue R-250 for detection of the protein bands and destained with 25% ethanol in 8% acetic acid and water (30 : 10 : 60, v/v). Molecular weight standards from 200 to 14.3kDa were used. For PAS staining, after electrophoresis, the gel was fixed in 7.5% acetic acid solution and stored at room temperature for 1h. Then the gel was washed with 1% nitric acid solution and kept in 0.2% aqueous periodic acid solution and stored at

40C for 45min. After that, the gel was placed in Schiff's reagent at 4^0C for 24h and was destained using 10% acetic acid to visualize a pink color band.

Proteolytic activity

Colorimetric estimation

Proteolytic activity was carried **[22]**. Fat-free casein (0.4ml, 2% in 0.2mol/l Tris–HCl buffer, pH7.6) was incubated with 50µg of crude M. charantia seed extract (MCSE) in a total volume of 1ml for 2h and 30min at 37^0C. Undigested casein was precipitated by adding 1.5ml of 0.44mol/l trichloroacetic acid (TCA) and left to stand for 30min. The reaction mixture was then centrifuged at 2000g for 10min. Sodium carbonate (2.5ml, 0.4mol/l) and Folin–Ciocalteu 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 660nm. One unit of the enzyme activity was defined as the amount of the enzyme required to cause an increase in optical density (OD) of 0.01 at 660nm/min at 37^0C. The specific activity was expressed as units/min/µg of protein. For inhibition studies, a similar reaction was performed independently after preincubating the crude MCSE (50µg) for 30min with 5mmol/l each of EDTA, 1,10-phenanthroline, EGTA, PMSF and IAA. In all the cases, appropriate controls were kept. One unit of enzyme activity was defined as the amount of the enzyme required to cause an increase in OD of 0.01 at 660 nm/min at 37^0C.

Optimum pH and Optimum temperature

The activity of protease was determined by using different buffers in pH range of 3-12 by the method described earlier. Buffers used were sodium acetate, 0.1M pH (3-4), sodium citrate, 0.1M pH (5-6), phosphate buffer, 0.1M pH (7-8) and Glycine-NaOH, 0.1MpH (9-12). Optimum temperature was determined for protease by measuring its activity in temperature range 20^0-60^0C using the activity assay procedure.

Plasma recalcification time

Plasma recalcification time was determined **[23]**. MCase (0-5µg) was pre-incubated with 0.2ml of citrated human plasma in the presence of 10Mm TrisHCl (20µl) buffer pH 7.4 for 1min at 37^0C. Then 0.25M $CaCl_2$ (20µl) was added to the pre-incubated mixture and clotting time was recorded.

Activated partial thromboplastin time and prothrombin time

100µl of normal citrated human plasma and MCase (0–10µg) were preincubated for 1min. For APTT, 100µl reagent (LIQUICELIN-E phospholipids preparation derived from Rabbit brain with ellagic acid), was used to measure clotting time. For PT,

the clotting was initiated by adding 200µl of the PT reagent (UNIPLASTIN–rabbit brain thromboplastin).

Bleeding time

The bleeding time was assayed **[23]**. MCase (0-5µg) in 30µl of PBS was injected intravenously through the tail vein of a group of five mice. Bleeding time was recorded from the time bleeding started till it completely stopped and it was followed for 10min.

Fibrinogenolytic activity

Fibrinogenolytic activity was determined **[24]**. MCase (0-10µg) was incubated with the human fibrinogen (50µg) for 4h and the reaction was terminated by adding 20µl denaturing buffer. It was then analyzed by 7.5% SDS-PAGE.

Fibrinolytic activity

Colorimetric estimation

Fibrinolytic activity was determined **[25]**. MCase (0-5µg) in 100µl of saline and incubated for 2h 30min at 37^0C. One unit of activity is defined as the amount of enzyme required to increase in absorbance of 0.01 at 660m/h at 37^0C.

Protein banding pattern on SDS-PAGE

Fibrinolytic activity was done **[26]**. Washed fibrin clot was incubated with the MCase (0-5µg) for 8h. The reaction was stopped by adding 20µl of sample buffer. The samples were kept in a boiling water bath for 5min and centrifuged to settle the particulate of the plasma clot. An aliquot of 20µl supernatant was analyzed in 7.5 % SDS-PAGE for fibrin degradation.

Statistical Analysis

The data are presented as mean± SD. Statistical analyses were performed by Student's t test. A significant difference between the groups was considered if P value was less than 0.01.

RESULTS

***The Momordica Charantia* Seed serine protease purification and biochemical characterization**

MCase from *Momordica Charantia* Seed was purified and characterized. MCase upon fractionation on DEAE-Cellulose column chromatography, X peaks were resolved as shown in **Figure 1**. Out of ten peaks only Peak-II and Peak- III showed anticoagulant in **Figure 2.A and 2.B** and proteolytic activity. Therefore, Peak II was selected for further studies. When peak II was fractionated on anion exchange sephadex G-100 column chromatography, four peaks were resolved (Peak I-IV), but only peak II exhibited proteolytic activity observed in **Figure 3.A**. In addition, **Figure 3.B** showed peak II showed sharp single band on SDS-PAGE with molecular weight of 100kDa in

both non-reducing and reducing conditions. Purity of that protein was adjudged using RP-HPLC confirmed in **Figure 4**, chromatograms showed single sharp peak with retention time of 6.74min. The total protein loaded and proteolytic activity recovered in each step and the fold purity is summarized in **Table 2**. Optimum conditions for Catalytic activity of protease pH and temperature were studied. **Figure 5** showed maximum catalytic activity was found to be pH7.9 and at the broad range of 7.5-8.5pH proteases exhibited activity. Further, the enzyme was optimally active at 40^0C and was got inactivated at temperatures above 50^0C as in **Figure 6**. The purified proteins showed strong proteolytic activity with the specific activity of 14.84 units/mg/min. In order to study the nature of protease present inhibition studies were performed using specific protease inhibitors such as PMSF, IAA, EDTA and 1,10-Phenanthroline observed in **Table 1**. Interestingly, protease was susceptible to PMSF but insensitive to IAA, EDTA and 1, 10-Phenanthroline, suggesting the purified protein is of serine family. Hence, it named as MCase.

MCase with strong anticoagulant and fibrinogenolytic

MCase showed strong anticoagulant property, it interfering in plasma coagulation cascade. the clotting time of citrated human plasma was enhanced from the control 146s and the maximum of 1025s at the concentrations of 6µg and remains unaltered even at the increased dose up to 10µg was confirmed by **Figure 7A**. MCase anticoagulant activity was further confirmed by in-vivo mouse tail bleeding assay, i.v injection prolonged the bleeding time Upto >800 s ($p<0.01$) in dose dependent manner at the concentration of 20µgagainst the PBS treated control of 160 ± 8s was shown in **Figure 8**. In **Figure 7B** MCase in-vitro anticoagulant activity was totally abolished only by PMSF and remaining 1, 10, phenanthrolene, IAA, EDTA, EGTA did not affect. Additional, MCase prolonged the APTT clotting time but it did not alter the clotting time of PT observed in **Table 2**.

Fibrin(ogen)olytic activity

MCase exhibited strong fibrino (geno) lytic activity; it hydrolysed fibrinogen and fibrin as well. At dose dependent manner it hydrolyzed preferentially only Aα chain of fibrinogen, but Bβ chain but γ chain was remained resistant for the proteolysis in **Figure 9A**, even at low to high concentration 2-12µg. In addition to MCase when incubated for 24h at 10µgconcentration hydrolysis of only γ chain was observed but Bβ chain but γ chain were not in figure 9B.In

both the cases the appearance of new molecular weight peptides generated by the proteolysis are distributed in the wide range of molecular weight from 29kDa to 14kDa in SDS-PAGE under reduced condition. The fibrinogenolytic activity was completely neutralized by PMSF in figure 9C.MCase in calorimetric method of fibrinolytic activity hydrolysed dose dependent fibrin clot and the specific activity was found to be 7.46 units/mg/min in dose dependent manner(Figure 10D). Although, the fibrinolytic activity was further confirmed by analyzing on SDS-PAGE in **Figure 10A** out of all the chains of fibrin clot α polymer, γ -γ dimer, α chain and β chain only α polymer, α chain and partially γ -γ dimer was degradation was confirmed in figure 10B and the fibrinolytic activity was completely abolished by PMSF in **Figure 10C**. In plasma protein degradation assay, MCase hydrolyzed only fibrinogen and other plasma proteins are not affected in **Figure 11**. The fibrinogen protein band intensity was progressively decreased with increased concentration MCase (0-20µg) as compared to positive control fibrinogen alone.

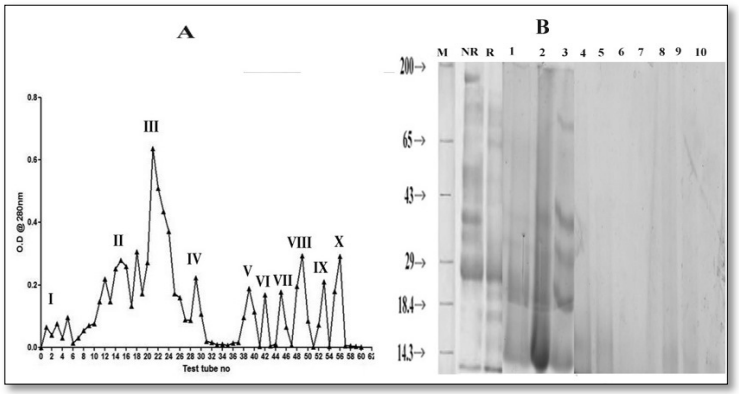

Figure 1 A: DEAE-cellulose column chromatography: MCSE (100mg in equilibrating buffer pH 7.5) was loaded on the column. B. SDS-PAGE of eluted fraction

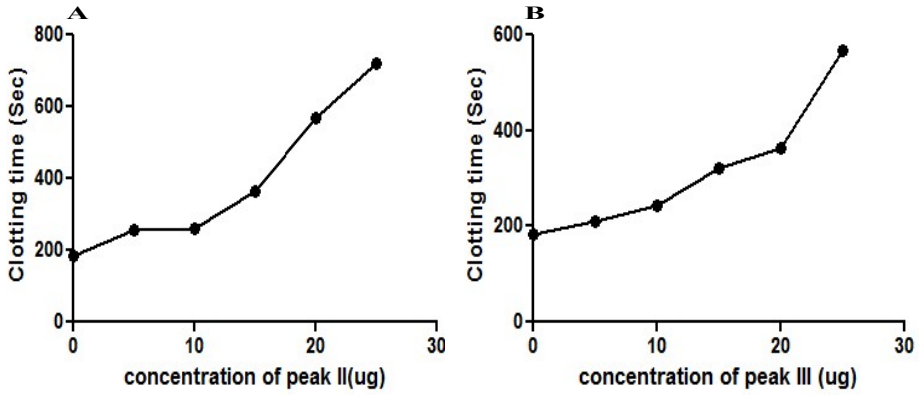

Figure 2: Plasma recalcification time: (A) and (B) peak II and peak III (0-6µg) clotting time was recorded

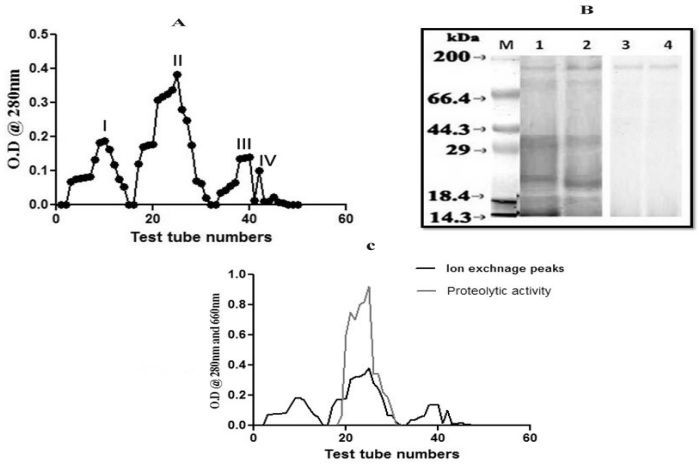

Figure 3: Sephadex G-75 column chromatography: Peak II from previous elution was loaded on to the column reflecting four peaks. B and C Protein content was monitored by absorbance at 280nm and alternate tubes were assayed for anticoagulant activity respectively.

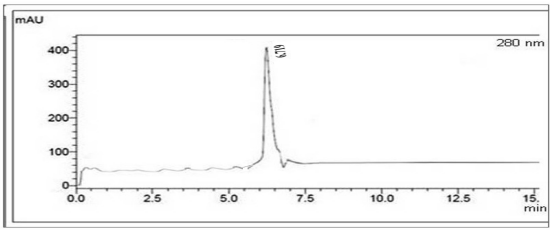

Figure 4: RP-HPLC of fractionized protein: Protein was subjected to RP-HPLC using C_{18} Column (5 mm, 0.21X25 cm) column that had been pre-equilibrated

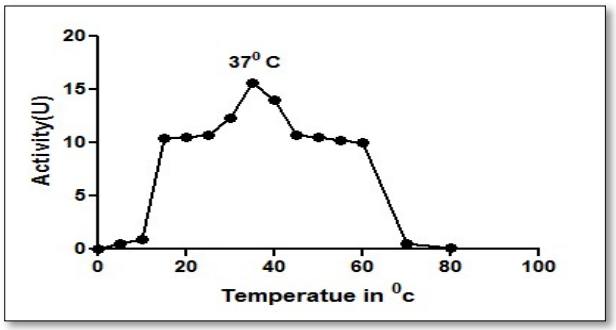

Figure 5: Protease activity - Effect of temperature

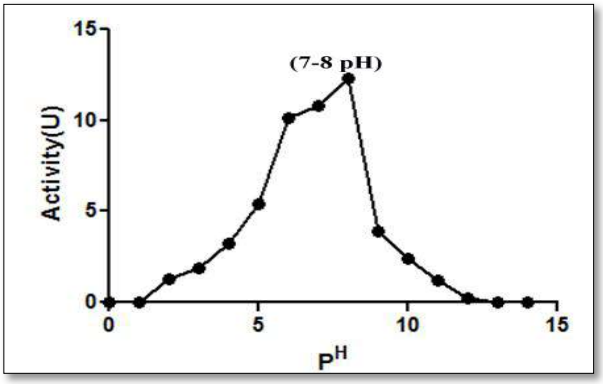

Figure 6: Effect of pH: Proteolytic activity was performed using casein as substrate at 37ºC

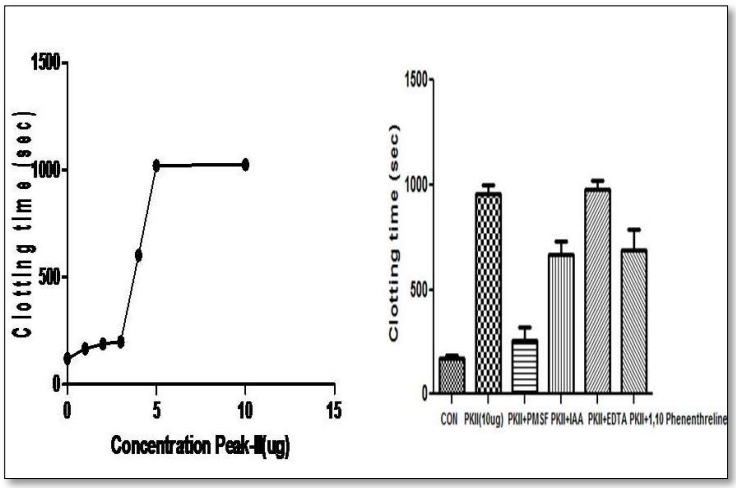

Figure 7: Plasma re-calcification time: (A) MCase (0–10µg) was preincubated with 0.2ml of citrated human plasma was used record clotting time. (B) Effect of protease inhibitors on the anticoagulant activity of MCase

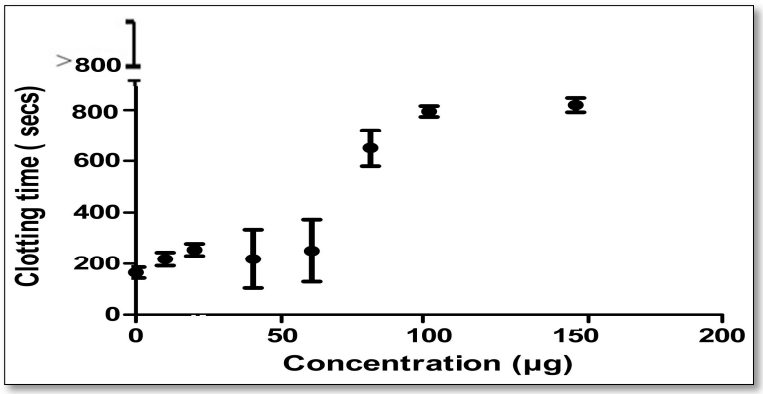

Figure 8: Effect of MCase on the bleeding time: Tail bleeding time was measured 10min after intravenous administration of PBS or various doses of MCase

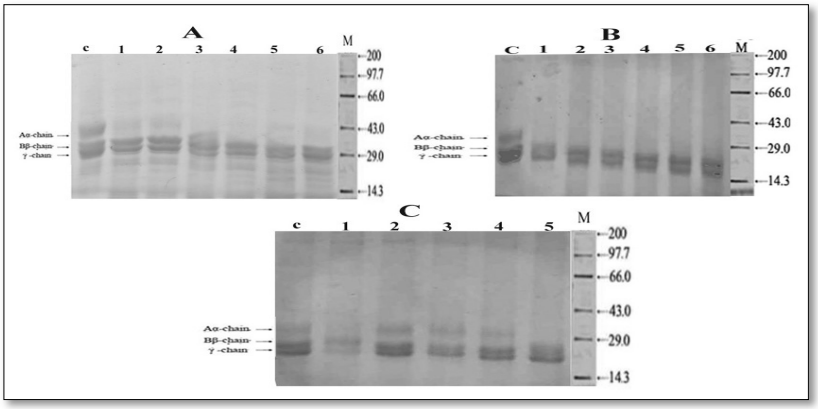

Figure 9: Fibrino(geno) lytic activity: Fibrinogen (50µg) incubated for 4h at 37 ^{0}C and then separated on 7.5% SDS-PAGE under reduced condition. (A) Dose dependent effect, (B) Time dependent effect, (C) Inhibition of fibrinogenolytic activity

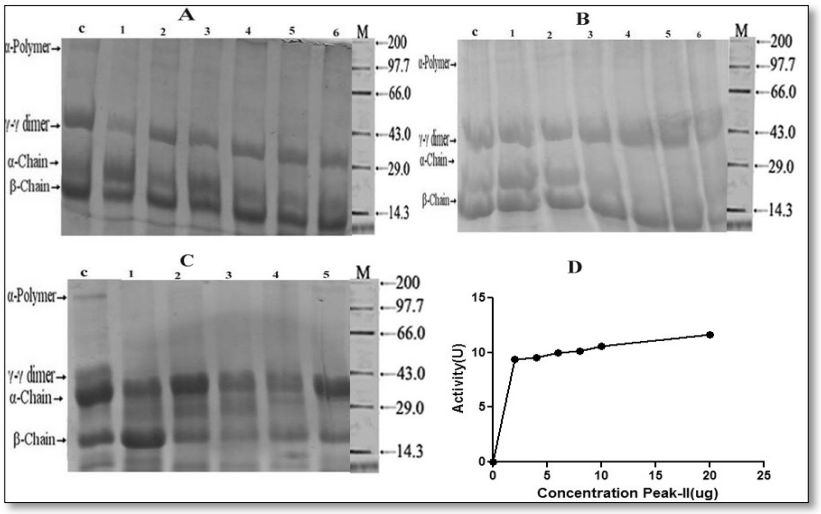

Figure 10: Fibrinolytic activity: (A) Dose dependent effect: Washed plasma clot was incubated with 0–20µg of MCase incubated for 6h at 370C and then separated on 7.5% SDS-PAGE under reduced condition. (B)Time dependent effect (C) Inhibition of Fibrinolytic activity (D)Colorimetric method

Table 1: Effect of inhibitors on the proteolytic activity of MCase

Inhibitor (5Mm Each)	%Activity/residual activity
None	100±0.02
EDTA	90.12±0.09
IAA	85.72±0.03
PMSF	10.53±0.01

Table 2: Summary of purification of MCase from MCSE

Procedure	Total Protein (mg)	Protein Recovery (%)	Specific Activity (Unit/mg/min)	Total Activity	Activity Yield (%)	Fold Purity
Sephadex G-100 column	100 20	100 20	0.780±0.02 3.62±0.05	78.0 54.3	100 65.1	1.0 4.4
DEAE-Sephadex column	20 3	20 3	3.62±0.05 14.84±0.08	54.3 46.4	65.1 57.1	4.4 19.0

DISCUSSION

In the present study, purification and characterization of serine protease (MCase) from *Momordica Charantia* was under taken. Gel filtration and ion exchange column chromatographic techniques were employed for the isolation process. The purity of the isolated MCase was confirmed using SDS-PAGE and RP-HPLC. MCase was found to be a monomeric protein with the molecular mass of 100kDa.Mcase hydrolyzed casein suggested its proteolytic efficiency, it was optimally active at the pH7.5and at the temperature 37^0C. The proteolytic activity of

MCase was completely abolished by PMSF suggesting MCase belongs to serine family. In recent years, the interest on plant proteases has been tremendously increasing. In particular serine proteases from plant origin, proved to be more useful in understanding several mechanisms those underline at the subcellular level **[24, 25]**. Serine proteases were extensively studied in plant latex and *Cucurbitaceae* family **[26-28, 12]**. Interestingly MCase exhibited strong anticoagulation property by extending plasma recalcification time by delaying clot formation process both *in-vitro* and *in-vivo*. Interestingly MCase could able to delay only the clot formation process of APTT but PT revealed that the observed anticoagulation of MCase could be due to its interference in intrinsic pathway of blood coagulation but not extrinsic pathway. MCase anticoagulant effect was completely inhibited by PMSE in both in-vitro and in-vivo, suggesting the triggered anticoagulant effect is due to the serine proteolytic activity of the MCase.

Unregulated way of activation of coagulation factors contribute immensely in the pathophysiology of thromboembolic complications **[39]**. Hence, targeting specific pathway that responsible for the unusual clot formation in the artery and veins helps in the better management of thrombotic disorders **[29]**. MCase showed strong anticoagulant effect by interfering in intrinsic pathway of coagulation cascade, could be a promising agent in treating thrombotic disorder. To further reinforce the observed anticoagulation of MCase, fibrinogen degradation ability could be taken into consideration. MCase completely hydrolyzed both Aα and Bβ chains of fibrinogen without degrading γ chain and generates truncated fibrinogen. Protease with fibrinogen hydrolyzing ability similar to thrombin is thrombin like enzymes. They more specifically hydrolyze Aα and Bβ chains of fibrinogen from N-terminal end and generates fibrinopeptide A and B **[35]**. While, proteases those degrade Aα and Bβ chains of fibrinogen from C-terminal end results in the generation of truncated structure which is short of polymerization potential, leads to the anticoagulation **[31, 32]**. Fibrinogenolytic enzymes were highly investigated in plants leaf extract, edible fruit pulp latex, seeds of flax, bitter gourd and jackfruit **[30-34]**.

In addition, MCase was also showed fibrinolytic activity is added advantage being strong anticoagulant agent in the management of thrombosis. As fibrinolytic enzymes have significant therapeutic potency in curing thrombotic disorders, numerous

fibrinolytic proteases were reported from different sources plant latex, earthworms, caterpillar, venoms of snake, spider, and honey bees **[35]**. Patel *et al.,* 2012 reported that serine protease with fibrinolytic activity from the latex of *Euphorbia hirta.* To further acknowledge MCase did not degrade other plasma proteins but selectively degraded plasma fibrinogen, suggesting its substrate specificity.

CONCLUSION

In conclusion, MCase serine protease was purified and characterized form crude aqueous extract of *Momordica Charantia* seed. MCase hinders plasma coagulation time, showed fibrinogenolytic. Hence therapeutic applications of MCase for preventing and/or treating thrombotic cardiovascular disorders found to be promising.

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Conflicts of interest

The authors declared no potential conflict of interest with respect to the authorship and publication.

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