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Isolation, purification and characterization of a protease inhibitor-Hayanin from horse gram [*Macrotyloma uniflorum* (LAM.) VERDC.] seed coat

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Abstract

A novel protease inhibitor, named as Hayanin exhibiting a molecular mass of ≈ 26 kDa in SDS-PAGE was isolated from Horse gram **(***Macrotyloma uniflorum* (Lam.) Verdc**.)** seed coats. The isolation procedure involves extraction, ammonium sulphate precipitation, Sephadex G-50 column chromatography; it exerts protease inhibitory activity up to 88 % at 50 µg protein dose against Serine proteases. The protein was stable up to a temperature of 65 ^{0}C, active over a wide range of pH from 2 to 12. NaCl concentration at 0-3 % did not influence the inhibitory activity of the purified inhibitor, whereas in milk agar assay there was complete inhibition of milk hydrolysis induced by proteases. The horse gram seed coat protease inhibitor appears to be a member of Kunitz type inhibitors. This is the first report on isolation of a protein protease inhibitor from Horse gram seed coat.

1. INTRODUCTION

Protease inhibitors (PIs) are extremely wide spread throughout the plant kingdom. PIs are involved in several physiological processes, such as reserve control, ontogeny regulation of protein turnover, apoptosis, stress tolerance, cell proliferation, defense mechanism against pathogens and pests[1,2,3,4]. Further, PIs have been shown to be developmentally expressed in seeds and reserve organs[5,1] or induced by wounding[6]. The PIs are specific to each of six classes of proteolytic enzymes i.e., Serine-, Cysteine-, Metallo-, Aspartate[7], Threonine- and Glutamic- proteases. PIs have been most extensively studied in the Leguminosae, Gramineae and Solanaceae[8], probably because of the large number of species in these families which form important source of food[9].

Pulses are important source of protein in many developing countries[10], some having disadvantage of poor protein digestibility[11]. This is due to the presence of anti-nutritional factors including PIs considerably reduces the nutritional potential of the legume protein also induces stomach distension, flatulence and some are toxic[12]. PIs have been purified and characterized from a variety of legume seeds[13,14,8,15,16]. Legume seed PIs normally contain trypsin/chymotrypsin inhibitors, which belong to two families, Kunitz and Bowman-Birk[17]. The inhibitor content and type in legumes vary with many factors, e.g. the cultivar, maturity, fermentation, and heat treatment[8]. The presence of trypsin inhibitor affects the protein efficiency ratio thereby inhibit the activity of the protein digesting enzymes in the digestive tract, reducing the body's ability to utilize protein in food. However, PIs isolated from leguminous seeds are important due to possible applications in medicine, agriculture, food technology[16].

Horse gram belongs to the family Leguminosae is a branched, sub-erect and downing herb, native to most parts of India and is found up to altitudes of 1000 m[18]. It is a fast growing annual vine with trifoliate leaves and brown, flat, curved pods filled with seeds; In *Ayurveda*, the seed is

used in the treatment of piles, pain, constipation, wounds, urinary infections, cough, edema and asthma[18]. The prearation from seeds is also beneficial in enlarged liver and spleen. Seeds are difficult to digest because of the tough seed coat and may be due to the possible presence of potent PIs[19] in seed coat. Hence it was of interest to isolate, purify and characterize protease inhibitor present in seed coat.

2. MATERIALS AND METHODS

2.1. Chemicals

Acrylamide, bisacrylamide, N, N, N´, N´-tetramethylethylenediamine (TEMED), Trypsin (EC 3.4.21.4), Chymotrypsin (EC 3.4.21.1), Phenylmethanesulfonyl fluoride (PMSF) were purchased from sigma chemicals (St Louis, MO, USA). Sephadex G-50 was procured from Pharmacia, Sweden. Bovine serum albumin, Casein was from Himedia Private Ltd (India). Ammonium sulphate and all other chemicals unless otherwise mentioned were of analytical grade procured from Merck (Germany). Solvents were distilled before use.

2.2. Sample preparation

A single batch of Horse gram **(***Macrotyloma uniflorum* (Lam.) Verdc. Or *Dolichos biflorus***)** seeds (variety Hebbal Hurali-2) were procured from local fields and authenticated by Prof. Lohithaswa H. C., Department of Genetics and Plant breeding, University of Agricultural Sciences, Mandya, Karnataka. The seeds were cleaned and soaked for 8 hours in distilled water. The coat was separated from seeds manually, seed coats was dried in shade and powdered, kept in air tight container at 4 ^{0}C prior to use. 50 g of coat powder was homogenized with 500 mL distilled water and allowed to equilibrate at room temperature in a rotary shaker at 120 rpm for 4 hours. The supernatant obtained after centrifugation at 10000g at 4 ^{0}C for 20 min was used as crude protein.

2.3. Purification

The supernatant protein was subjected to ammonium sulphate precipitation (20%, 40%, 65%, 80% and 100% saturation) and allowed to precipitate overnight at 4 ^{0}C. The precipitated protein was collected by centrifugation at 10000g for 20 min at 4 ^{0}C and the pellet was resuspended in small volume of distilled water and was extensively dialyzed using dialysis tubing of pore size 2000 NMWCO against water for 24 hours and lyophilized to concentrate the protein. The protein content and the inhibitory activity of the ammonium sulphate fractions were analyzed; the ammonium sulphate fraction which showed the highest specific activity was selected for further study.

The ammonium sulphate fraction was loaded on to the column (205 x 1.2 cm) of Sephadex G-50, distilled water was used as the mobile phase, and the samples eluted were collected at the rate of about 1.5 mL for 5 min and were read at 280 nm using UV-visible spectrophotometer. The peak fractions showing protease inhibitory activity were pooled, dialyzed against distilled water and concentrated by centrifugation using 5 kDa MWCO centrifugal filter devices (Sartorius Vivaspin® 20). The active peak was further fractionated on same column and by using same parameters to obtain a homogenous preparation.

2.4. Protein Estimation

The protein content was determined using Bradford's reagent[20] using Bovine Serum Albumin as standard. Different aliquots of the extract were made up to 100 µL with distilled water, to this 900 µL of Bradford's reagent was added and the color developed was read spectrophotometrically at 595nm (UV 1601, SHIMADZU, Japan).

2.5. Sodium dodecyl sulphate- Gel electrophoresis

To determine the purity of the protease inhibitor during purification, 12.5% SDS-PAGE was done[21]. Gels were stained in 0.1% (w/v) coomassie blue - 30% (v/v) methanol - 10% (v/v) acetic acid in water, followed by destaining using 30% (v/v) methanol - 10% (v/v) acetic acid in water.

2.6. Protease inhibitory activity

The protease inhibitory activity was assayed according to the method of [22]; Fifty µL aliquot of trypsin and chymotrypsin was pre incubated separately with different concentrations of Protease inhibitor. To the above add denatured casein as substrate of 0.4 mL (2%) in a final volume of 1 mL using 0.2 M Tris-HCl buffer of pH 8.5 for 2 h at 37 ^{0}C. after incubation the reaction was stopped by adding 1.5 mL of 0.44 M trichloroacetic acid (TCA) and the mixture was allowed to stand for 30 min. The reaction mixture was centrifuged at 1500g for 15 min. An aliquot (1 mL) of the supernatant was mixed with 2.5 mL of 0.4 M sodium carbonate and 0.5 mL of Folin–Ciocalteu reagent (1:2, v/v). The color developed was read at 660 nm. Activity was expressed as units/h. One unit of enzyme activity was defined as the amount of enzyme required to increase the absorbance by 0.01 at 660 nm/h. Protease inhibitor unit is defined as the amount of protease inhibitor that inhibited one unit of respective enzyme activity. Protease inhibitor activity of the enzyme is finally expressed in terms of percent inhibition.

2.7. Thermal and pH stability of Protease inhibitor

The effect of temperature on trypsin inhibitory activity of extracts from horse gram seed coat were tested by incubating at 37, 40, 50, 60, 70, 80, 90, 100 ^{0}C for 30 min. after cooling the samples to room temperature the residual trypsin inhibitory activity was determined as described earlier. The effect of pH on the trypsin inhibitory activity was examined at pH ranging between 2-12 for 30 min at room temperature using following buffers: glycine-HCl (pH 2-3), sodium acetate-acetic acid (4-5), Sodium phosphate buffer (pH 6), Tris-HCl (pH 7-9) and glycine-NaOH (pH 10-12). The residual inhibitory activity was measured as described earlier and the final concentration of used buffers is of 50mM.

2.8. Salt stability

The purified inhibitor was incubated at room temperature for 30 min in the presence of NaCl ranging from 0% to 3% and was tested for inhibitory activity against trypsin and chymotrypsin, the residual inhibitory activity was measured.

2.9. Milk agar assay

The isolated protein was assayed for inhibitory activity by milk agar disc diffusion method of [23]; Agar was prepared along with 1% (w/v) skimmed milk and poured into petri dishes after autoclaving. The plates were allowed to solidify for 30 min, autoclaved Whatman No. 1 filter paper disc (5mm in diameter) were placed equidistantly from the rim of the plate that were pretreated with Proteases aseptically. Aliquot of protease inhibitor of different concentrations was applied to the disk and sterile distilled water was added as a negative control. PMSF (10mM-Phenylmethanesulfonyl fluoride) a standard protease inhibitor was used; the petriplates were incubated at 37 ±2^{0}C for 48 hours.

Table 1: Specific activity, Yield and Fold of purification of protease inhibitor at different stages of purification

Sample	Protein (mg)	Activity (Units)	Specific activity (U/mg)	Yield (%)	Fold of purification (%)
Crude extract	1210.12	31432	26	100	1.00
$(NH_4)_2SO_4$ fraction (65%)	698.33	32317	46	71.9	1.76
Dialysed	404.72	28193	69	53.8	2.65
Sephadex G-50	29.63	26982	910	38.6	35.00
Sephadex G-50 (Rechromatographed)	4.91	25669	5238	24.5	201.40

10. Statistical analysis

All experiments were carried out in triplicate to check the reproducibility of results. The data presented here are the averages of triplicate determinations and the standard deviations for all the values were <±5%.

3. RESULTS AND DISCUSSION

The crude protein obtained from the seed coat extract of Horse gram was initially subjected to Ammonium sulphate (w/v) fractionation, and it was found that 65% $(NH_4)_2SO_4$ saturation was efficient for precipitating the protease inhibitor having highest specific activity compared to other fractions and shows strong serine protease (Trypsin and Chymotrypsin) inhibitory activity. This active ammonium sulphate fraction on Sephadex G-50 column chromatography resulted in two protein peaks (Peak-I and Peak-II) as monitored at 280nm (Fig. 1a).

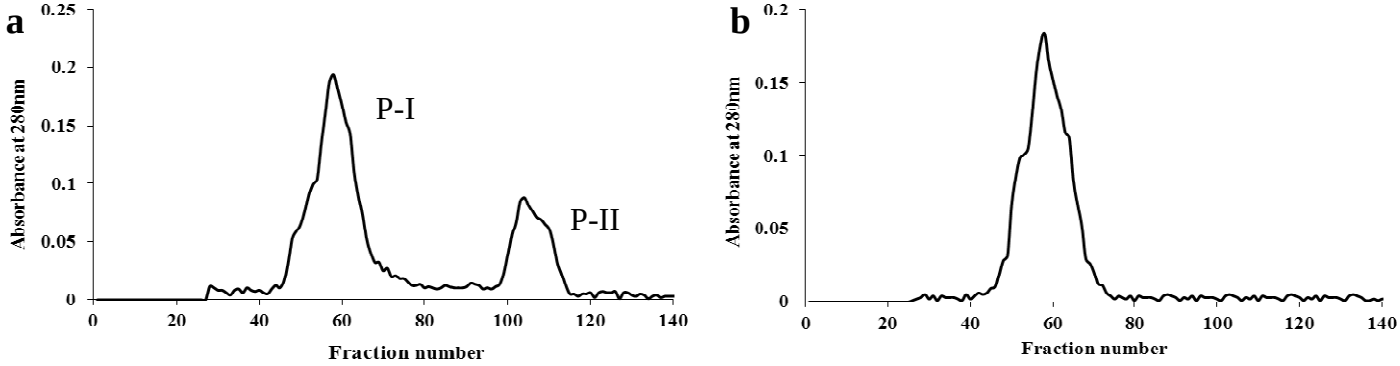

Fig. 1 (a) Gel filtration profile of ammonium sulphate precipitated protein of crude extract of horse gram seed coat on Sephadex G-50 column [8mg of crude protein was loaded to the Sephadex G-50 chromatography, (V_t=205 mL; V_o= 68 mL; Mobile phase= Water; Flow rate= 1.5mL/5min)]. Two peaks (Peak-I and Peak-II) eluted from the column and protease inhibitory activity of each peak was determined. (b) The active peak-I fractions rechromatographed on Sephadex G-50 column, the chromatogram shows single peak.

The protein content of fractions was also simultaneously estimated by Bradford method, which corresponded with the peaks at A_{280} curves, Protease inhibitory activity of each fraction from the peaks against serine proteases was determined. The fractions falling under Peak-I displayed the maximal inhibitory activity and it was designated as active peak, this was pooled and was rechromatographed on Sephadex G-50 column resulted in single peak (Fig. 1b) indicating the homogeneous preparation of the protein from horse gram seed coat and the resultant protein was named as Hayanin (Haya in Sanskrit means Horse). The purification pattern through different sequential steps resulted in 201.4 fold purification of Hayanin with a recovery of 24.5% was summarized in Table 1.

Electrophoretic separation of Hayanin showed a single band with an approximate molecular mass of 26 kDa in SDS-PAGE (Fig. 2). A higher molecular mass (> 20kDa) may either be due to monomer-dimer equilibrium[24] or due to oxidation of cysteine residues as it has been reported by [25]. The molecular weight of the trypsin inhibitor from bambara groundnuts was 13 kDa[8]. Two trypsin inhibitory activity bands were observed for cowpea (10 and 18 kDa) and pigeon pea (15 and 25 kDa)[8]. It is reported that the molecular weight of a trypsin inhibitor purified from *Calliandra selloi Macbride* seeds was 20 kDa[26]. Kunitz type PIs having different molecular masses than that of the Hayanin have been reported in Soyabean (19kDa), Mustard seeds (20kDa) and *Cajanus cajan* (14kDa)[27]. The Graminaceae, Leguminosae and Solanaceae families differ from each other in mass, cysteine content, and number of reactive sites[9].

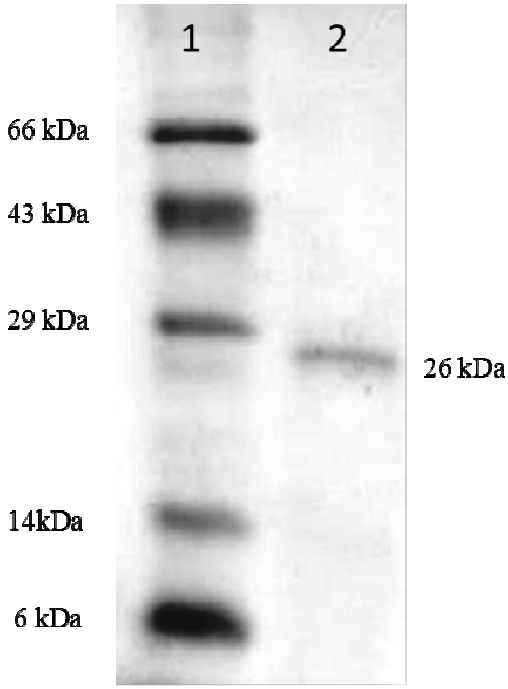

Fig. 2 Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis of the active, chromatography purified, Protease inhibitory fraction from Horse gram seed coat (Lane 2). Lane 1: Molecular weight standards.

Generally, the Bowman- Birk type of inhibitor has a lower molecular weight compared with the Kunitz-type. The Kunitz type inhibitors are proteins of a molecular weight of >20 kDa, with low cysteine content and a single reactive site, whereas the Bowman-Birk type inhibitors have a molecular weight of 8–10 kDa, as well as a high cysteine content and two reactive sites[9]. It is found that two PIs, including trypsin-chymotrypsin inhibitor and trypsin inhibitor (Bowman-Birk type), with molecular weights of 15,000 and 10,500 Da, respectively, were found in Pigeon pea[14]. From the results, it is consistent to predict that the protease inhibitor from horse gram seed coat is likely to be of Kunitz type.

Hayanin showed inhibitory activity against both trypsin and Chymotrypsin (Fig. 3), a characteristic feature of Bowman Birk Inhibitors type and Kunitz type PIs[28]. The inhibitory activity of Hayanin was more pronounced against trypsin when compared to chymotrypsin, this may be due to the presence of two different reactive sites[29].

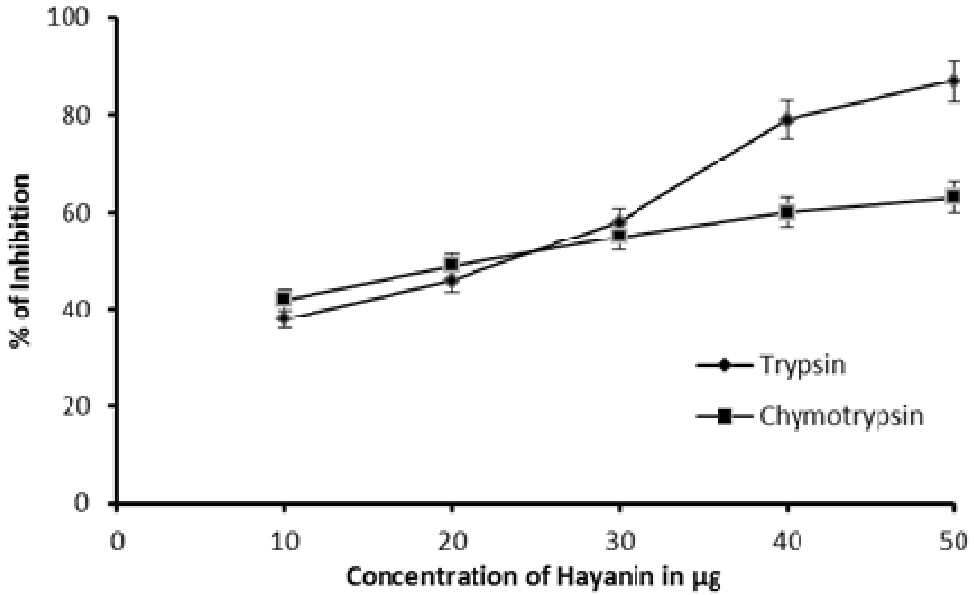

Fig. 3 Dose dependent inhibition of Trypsin and Chymotrypsin in the presence of Hayanin, Bar indicates standard deviation from triplicate determination.

The protease inhibitory activity of Hayanin was stable up to 65 ^{0}C without any loss in its activity, as the temperature increased from 70 ^{0}C to 100 ^{0}C the inhibitory activity decreased gradually (Fig. 4). These temperature induced conformational changes are reversed when it was cooled down to 20 ^{0}C from 100 ^{0}C (data not shown), thereby demonstrating that the inhibitor possesses some degree of flexibility. The inhibitory activity of trypsin and chymotrypsin was tested at different pH's between 2.0 and 12.0 (Fig. 5), the purified protein is stable over a broad range of pH. However there was some decrease in activity at low and high pH's, although it was generally stable at neutral pH ranges. The possible occurrence of cysteine residues forming disulphide bonds may account for this striking stability and inhibitory properties[29] of Hayanin.

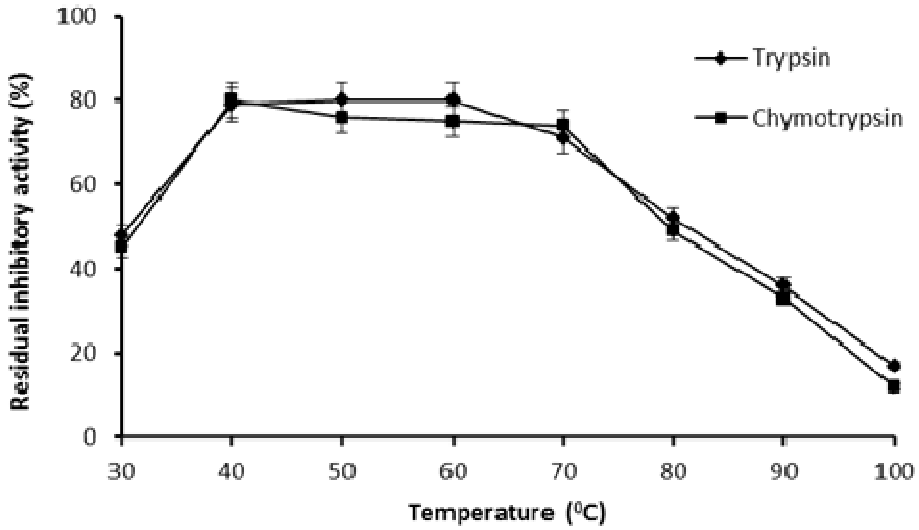

Fig. 4 Temperature stability of Hayanin after incubation for 30 min at the indicated temperatures and the residual inhibitory activity against trypsin and chymotrypsin, Bar indicates standard deviation from triplicate determination.

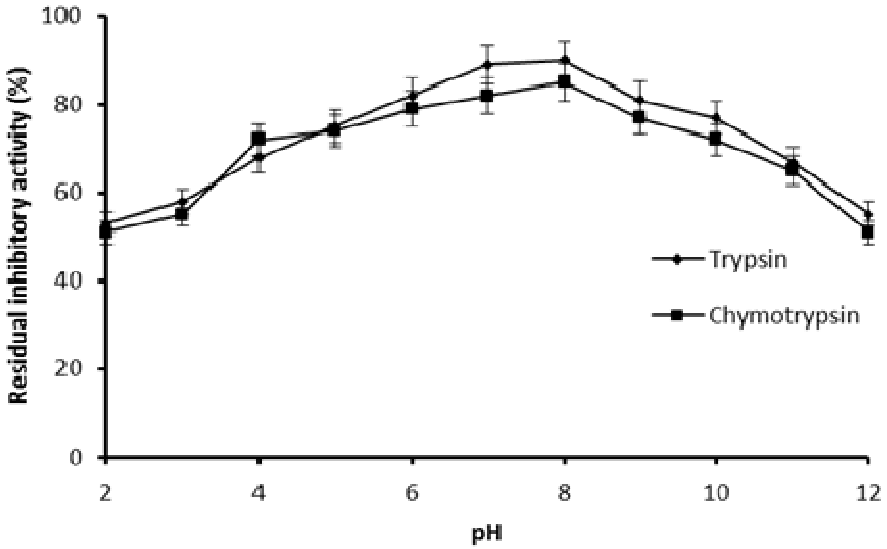

Fig. 5 Stability of Hayanin after incubation at various pH ranging from 2.0 to 12.0 for 30 min and the residual inhibitory activity was determined against Trypsin and Chymotrypsin, Bar indicates standard deviation from triplicate determination.

The effect of NaCl on the inhibitory activity of purified trypsin inhibitor is depicted in Fig.6. No marked changes in the relative inhibitory activity were found when NaCl was added up to 3.0%. NaCl at 0–3% concentration did not affect the inhibitory activity of the trypsin inhibitor purified from adzuki bean seeds[16].

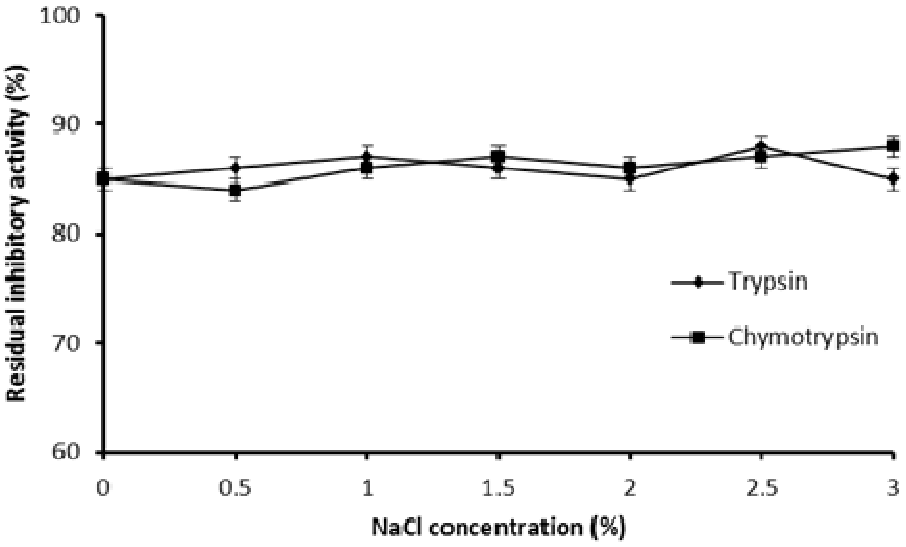

Fig. 6 Effect of salt concentration on the stability of Hayanin from horse gram seed coat, and its inhibitory activity against proteases. Bar indicates standard deviation from triplicate determination

Skimmed milk agar plate assays allow principally for the determination of protease activity. A transparent ring around the paper disc signifies milk hydrolysis zone which depicts the protease activity and the reduced zone or no zone of hydrolysis indicates its inhibitory activity[30].

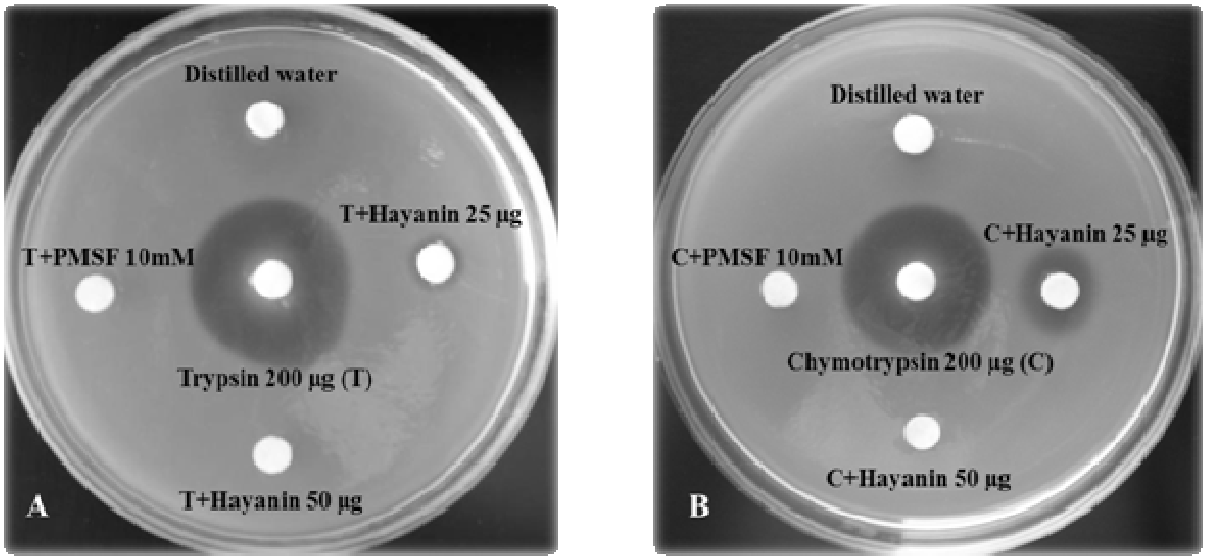

Fig.7 Milk agar diffusion assay, effect of Hayanin on milk hydrolysis of (A) Trypsin and (B) Chymotrypsin. 10mM PMSF was used as standard protease inhibitor and sterile distilled water served as negative control.

4. CONCLUSION

This is the first report of isolating protease inhibitor from seed coat of Horse gram, the current study revealed the presence of a serine protease inhibitor-Hayanin, which belongs to the Kunitz type PIs family. The purified Hayanin showed molecular mass of 26kDa in SDS-PAGE and it showed a potent inhibitory activity against both trypsin and chymotrypsin. Therefore, future studies in this direction have to be performed to completely elucidate the characteristic features of Serine type protease inhibitor-Hayanin from Horse gram seed coat. The presence of protease inhibitor may be the cause for difficulty in human digestion of Horse gram. An inexpensive method of negating the protease inhibitory activity will increase the availability of a good protein source at a lower cost, thereby, making it a much needed protein source to meet the middle class and lower middle class needs.

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