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Purification of protease inhibitors of *Vigna hainiana*, *Vigna aconitifolia* and *Vigna sublobata*

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Abstract
Plant Protease inhibitors (PIs) are widely studied defense proteins of the Plants. In seed and other tissues of the plant, protease inhibitors are found which represents up to 10% of the total protein (Casaretto and Corcuera 1995). PI inhibits tryptic and chymotryptic gut proteinases which affect protein digestion in the insects. This leads to the overproduction of gut proteases to compensate for the inhibited activity leading to deficiency of essential amino acids. This creates physiological stress and affects growth of the insect pests. The different Vigna Accessions as *Vigna hainiana*, *Vigna aconitifolia* and *Vigna sublobata* found to have potent *Helicoverpa armigera* insect gut proteolytic activity. Hence, efforts have been made to isolate and purify the protease inhibitors from these 03 Vigna Species. Ammonium Sulphate salt precipitation, Ion Exchange Chromatography and PAGE techniques have been used for the Purification of Protease Inhibitors. The single protein purified bands of different *Vigna* such as *V. hainiana*, *V. aconitifolia* and *V. sublobata* showed molecular weight of 8.18 KDa, 10.66 KDa and 10.26 KDa respectively when compared with standard molecular weight markers.

Keywords: purification, protease inhibitors, *Vigna*

Introduction
Protease Inhibitors are abundant proteins in plant tissues and are highly active against insects, bacteria & fungi. The genes encoding PI may be transferred in plants which may yields promising results in the field of agriculture. The approach has several advantages over the standard method to control insect pests and is ecologically safe (Dunaevsky et al., 2005). The genus Vigna belongs to Fabaceae, formerly called Leguminosae. It is found that, seeds of the Leguminosae generally contain large quantities of PIs active against insect serine proteases such as trypsin and chymotrypsin (Ryan, 1974; Dhande, 2022) [8, 3]. The different Vigna Accessions as *Vigna hainiana*, *Vigna aconitifolia* and *Vigna sublobata* found to have potent *Helicoverpa armigera* insect gut proteolytic activity (Dhande, 2017) [2]. Hence, efforts have been made to isolate and purify the protease inhibitors from these 03 Vigna Species. Transgenic crops encoding proteinase inhibitors are going to play an eminent role in future in insect pest management systems.

Material and Methods
Protease inhibitor purification was done by salt precipitation and ion exchange chromatography and the analysis of purified protease inhibitors was done electrophoretic ally.

1. Salt Precipitation
Requirements
1. Ammonium sulphate (solid)
2. Ice bath at 4^0 C
3. Magnetic stirrer
4. Crude enzyme solution

Method
1. The 30% $(NH_4)_2SO_4$ i.e., 1.17g was slowly added to a 7 ml Crude enzyme solution while gently stirring. The amount of $(NH_4)_2SO_4$ was added to precipitate the protein of interest will have to be initially determined empirically. The concentration of $(NH_4)_2SO_4$ needed to precipitate a particular protein in usually expressed as a percent of saturated $(NH_4)_2SO_4$. For large volumes, adding solid is more convenient.
2. Once dissolved, the $(NH_4)_2SO_4$/protein suspension was allowed to slowly stir at 4^0C (usually while kept cold) for an hour.
3. The precipitated proteins were collected by centrifugation. Typically, 6000 rpm for 30 minutes was sufficient to pellet precipitated proteins.

4. The protein pellet was dissolved in the 100 mM KCl, 50 mM Tris HCl, pH 8 and again 30 to 60% $(NH_4)_2SO_4$ i.e., 1.24g was then added to the supernatant to bring the concentration to the minimal % saturation that completely precipitates the protein.
5. The precipitated proteins were collected by centrifugation at 6000 rpm for 30 minutes.
6. The pellet was retained in the 100 mM KCl, 50 mM Tris HCl, pH 8 and the supernatant was used for saturation from 60 to 100%. 2.17g of ammonium sulphate was added to the supernatant. The precipitate was collected by centrifuging at 6000 rpm for 30 mins.
7. The activity of three 0-30%, 30-60% and 60-100% precipitate enzyme solution was checked after salt precipitation.

2. Ion Exchange Chromatography

Preparation of exchange medium.

1. It was usually carried out by suspending the dry resins in 5V of distilled water and allows to settle for 1 hour and the settled volume of resins was measured. The resin was suspended in 0.5 M NaCl buffer for 10 mins and the slurry was pour in funnel while in gentle suction and was allowed to flow the buffer. All the resins were added to the funnel.
2. Removal of very small particles of exchangers: The exchangers are repeatedly suspended in a large volume of water and after the large polymers have settled down, the slow sedimenting material was decanted.
3. Finally, the exchanger had to be equilibrated with suitable counters ions. This was accompanied by washing the exchangers with different reagents depending upon the desired counter ion to be introduced.
4. Following conversion of the exchanger to be desired form, excess countries were removed by washing the exchanger with large volumes of diluted buffer of specific PH.

Method

1. Prior to setting up the column, DEAE-cellulose matrix was washed with 0.5N HCl and then 0.5 N NaOH.
2. This was done by gentle centrifugation. Next the matrix is washed with 5 volumes of 0.02 M sodium acetate buffer (PH 5,2)
3. The pooled fraction containing maximum activity obtained from salt precipitation was loaded on to the column. The separation of protein was done by stepwise elution with buffer containing increasing concentration of NaCl.
4. The 3.0 ml of fraction was collected manually in different test tube and absorbance was monitored at 280 nm using Shimadzu 1800 UV-VIS spectrophotometer. The fractioned with protein were analyzed for enzyme activity.

3. Electrophoretic Analysis of Purified PI

Non reductive SDS Electrophoresis for visualization of purified trypsin proteases inhibitors and chymotrypsin protease inhibitors was done and assayed with 1% of trypsin and chymotrypsin separately. Using gel documentation system, the molecular weight of each band was characterized. The alpha imager 2000 was the computer software used for the analysis purpose. The standard molecular bands were added to data and in reference to that molecular weight, relative mobility of each set of samples with the position was calculated. The comparisons were made between the control set of protein bands with the bands obtained from the sample.

Results and Discussion

1. Ammonium Sulphate Salt Precipitation

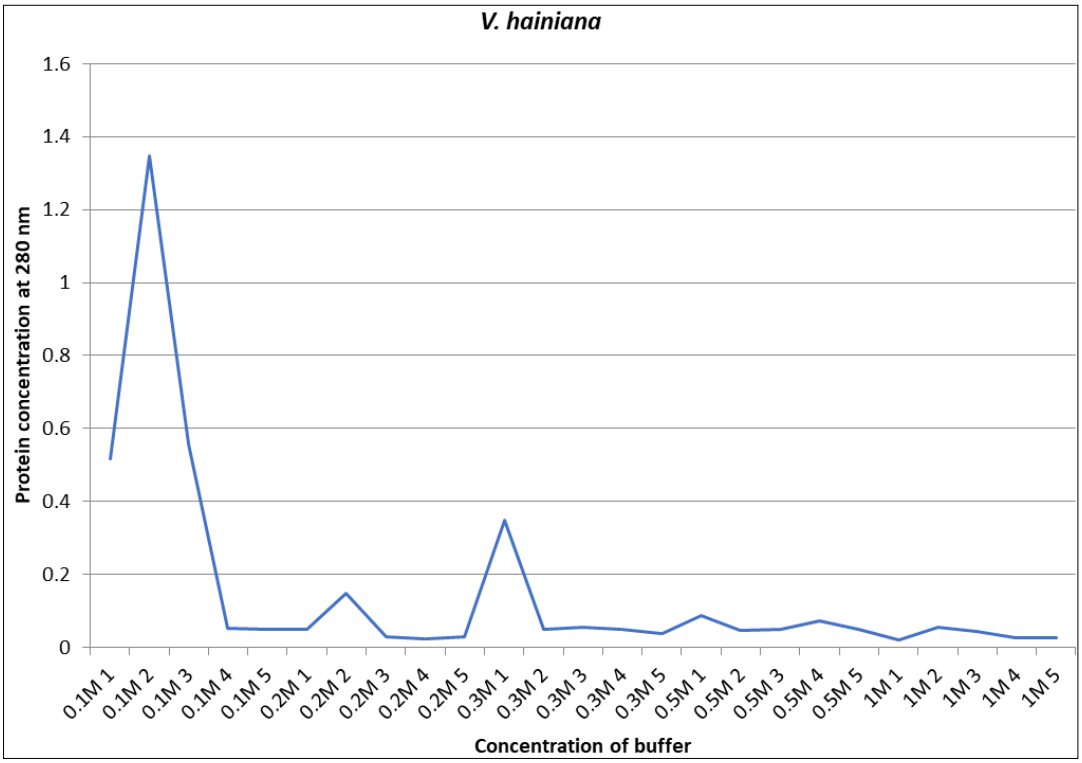
After checking the inhibitory activity of all the three precipitate samples, the precipitate obtained after 30% saturation does not show activity hence the excess protein was removed and the precipitate obtained after 60% saturation show activity and the precipitate obtained after 100% does not show any activity hence the protein was precipitated at 60% saturation. The 30-60 % saturation was found to have an inhibitory activity of all the three Vigna species such as *V. hainiana, V. aconitifolia* and *V. sublobata*. The inhibitory activity of 60% precipitate protein of *V. hainiana* was found to be 73% and that of *V. aconitifolia* and *V. sublobata* was found to be 67% & 70% respectively. These precipitates were subjected to Ion exchange chromatography for further purification process.

Table 1: Summary of PIs purification

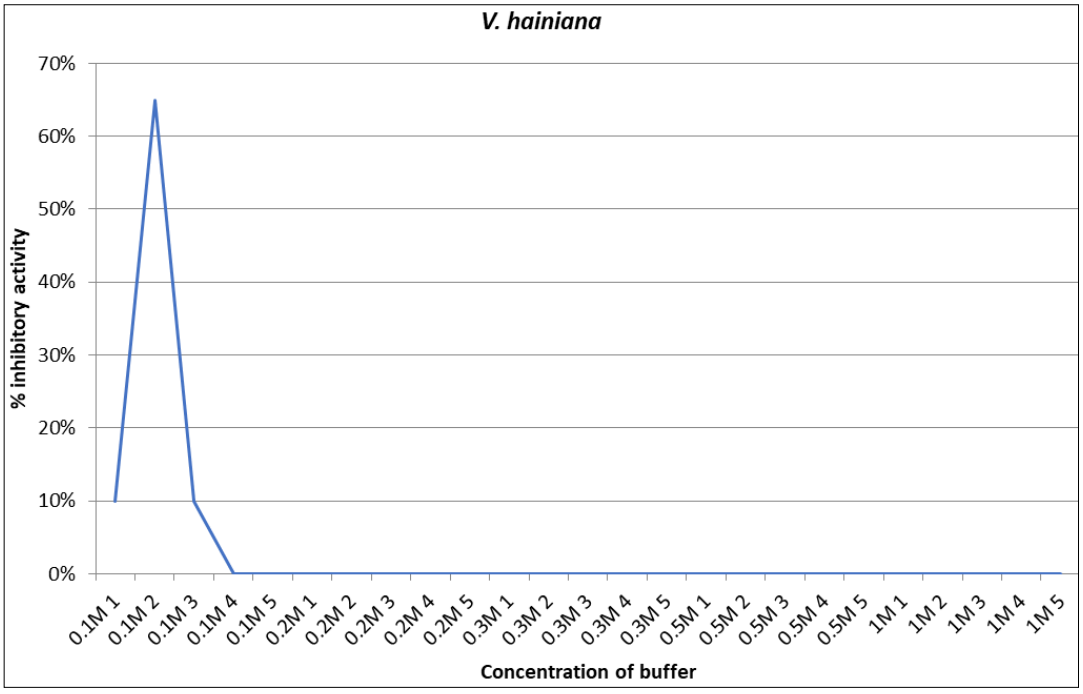
Sample	*V. hainiana*	*V. aconitifolia*	*V. sublobata*
Crude extract (% proteinase inhibitory activity)	71%	65%	68.84%
Ammonium Sulphate 30-60 % saturated fraction (% proteinase inhibitory activity)	73%	67%	70%
Ion-exchange Chromatography (% proteinase inhibitory activity)	Fraction 1: 10% Fraction 2: 65% Fraction 3: 10%	Fraction 2: 55% Fraction 3: 15%	Fraction 2: 70%

2. Ion Exchange Chromatography Results for *V. hainiana*

The protease inhibitors precipitated by salt precipitation was further attempted to purifying by passing through DEAE cellulose column. Unbound proteins were collected by passing buffer. The proteins bound to the matrix were eluted with gradient buffer NaCl. The protein concentration of each of 25 fractions was further analyzed by taking absorbency at 280 nm spectrophotometric ally. Fraction number 1, 2 and 3 of 0.1M elution buffer showed maximum concentration of proteins as 0.516, 1.346 & 0.556 mg/ml respectively. The proteinase inhibitory activity of fraction 1, 2 and 3 was found to be 10%, 65% and 10% respectively. Fraction number 11 showed 0.349 mg/ml concentration of protein but does not show proteinase inhibitory activity like other 21 fractions. As fraction 2 of 0.1 M elution buffer showed maximum proteinase inhibitory activity, this fraction was further utilized for SDS-PAGE.



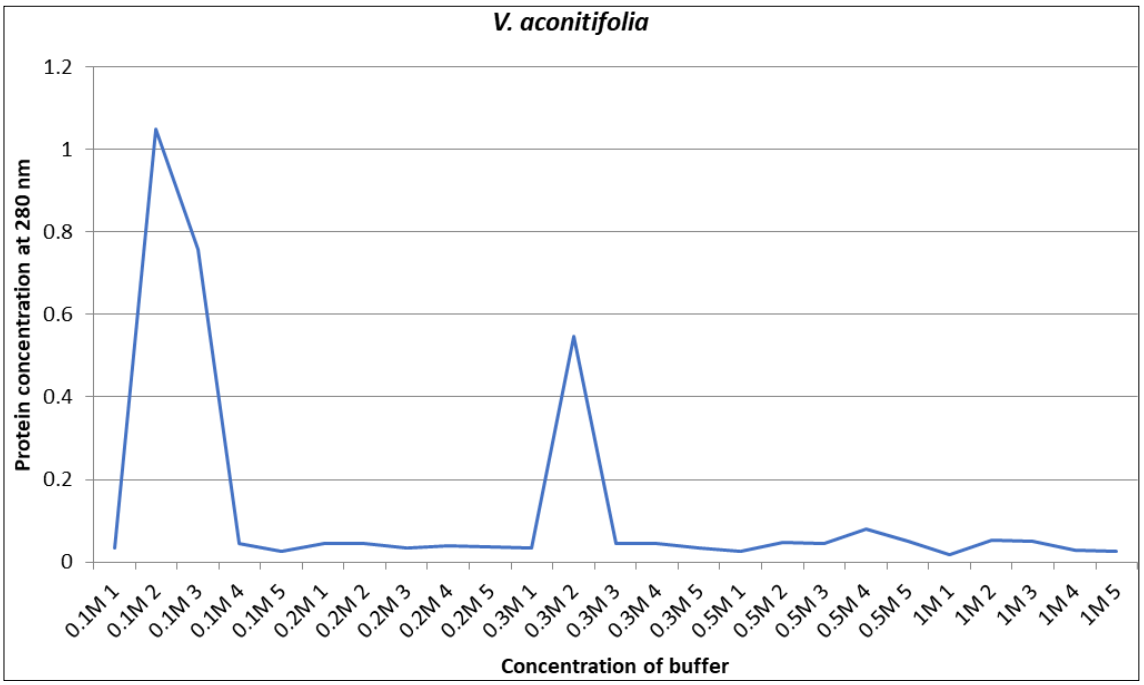
Graph 1: Determination of protein concentration after Ion exchange chromatography of 30-60% saturated fraction of *Vigna hainiana*



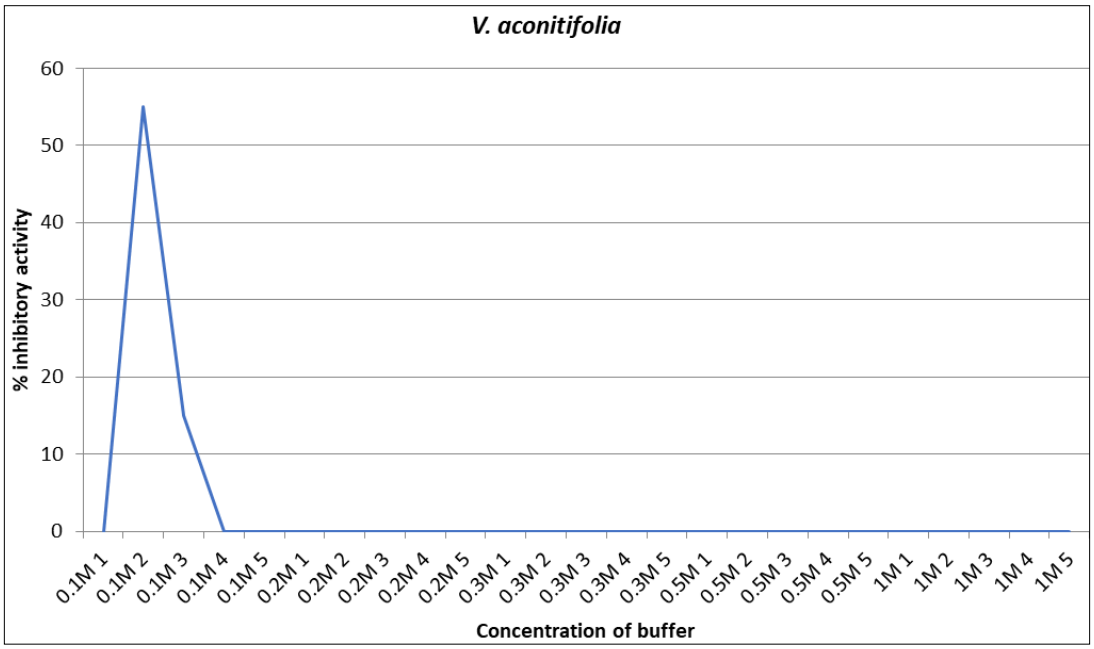
Graph 2: Determination of % inhibitory activity of Ion-exchange chromatography fractions of *Vigna hainiana*

3. Ion exchange chromatography results for *V. aconitifolia*

Fraction number 2 and 3 of 0.1M elution buffer showed maximum concentration of proteins as 1.048 mg/ml & 0.756 mg/ml respectively. The proteinase inhibitory activity of fraction 2 and 3 was found to be 55% and 15% respectively. Fraction 12 of 0.3 M elution buffer showed high protein concentration as 0.548 mg/ml but like other 22 fractions it does not showed proteinase inhibitory activity. As fraction 2 of 0.1 M elution buffer showed maximum proteinase inhibitory activity, this fraction was further utilized for SDS-PAGE.



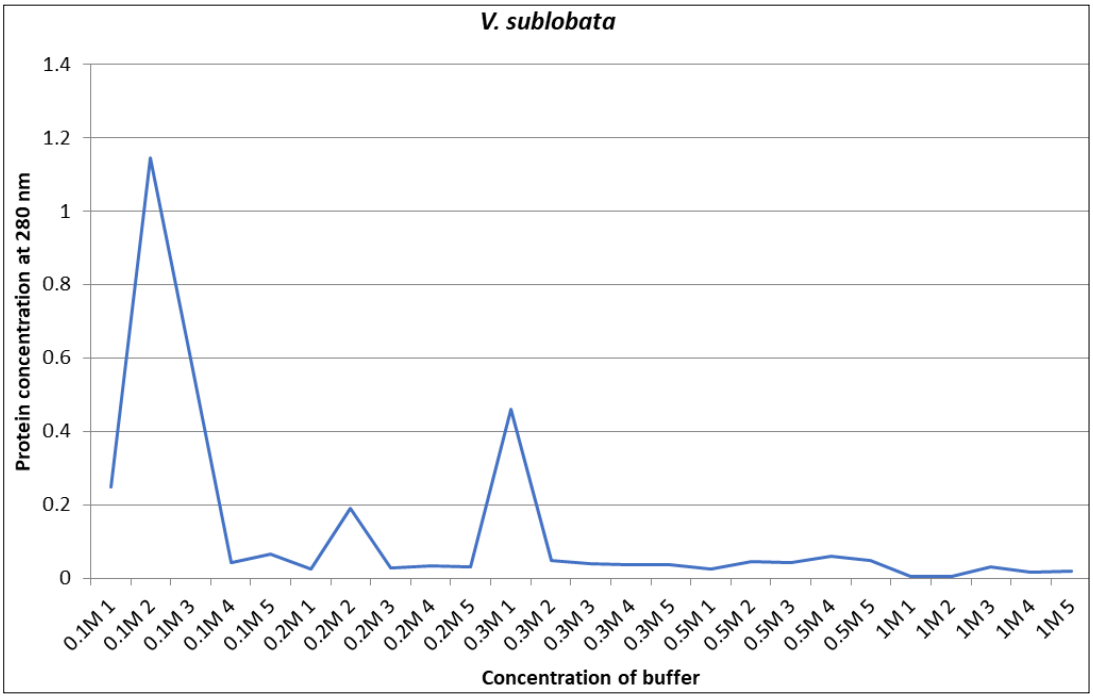
Graph 3: Determination of protein concentration after Ion exchange chromatography of 30-60% saturated fraction of *Vigna aconitifolia*



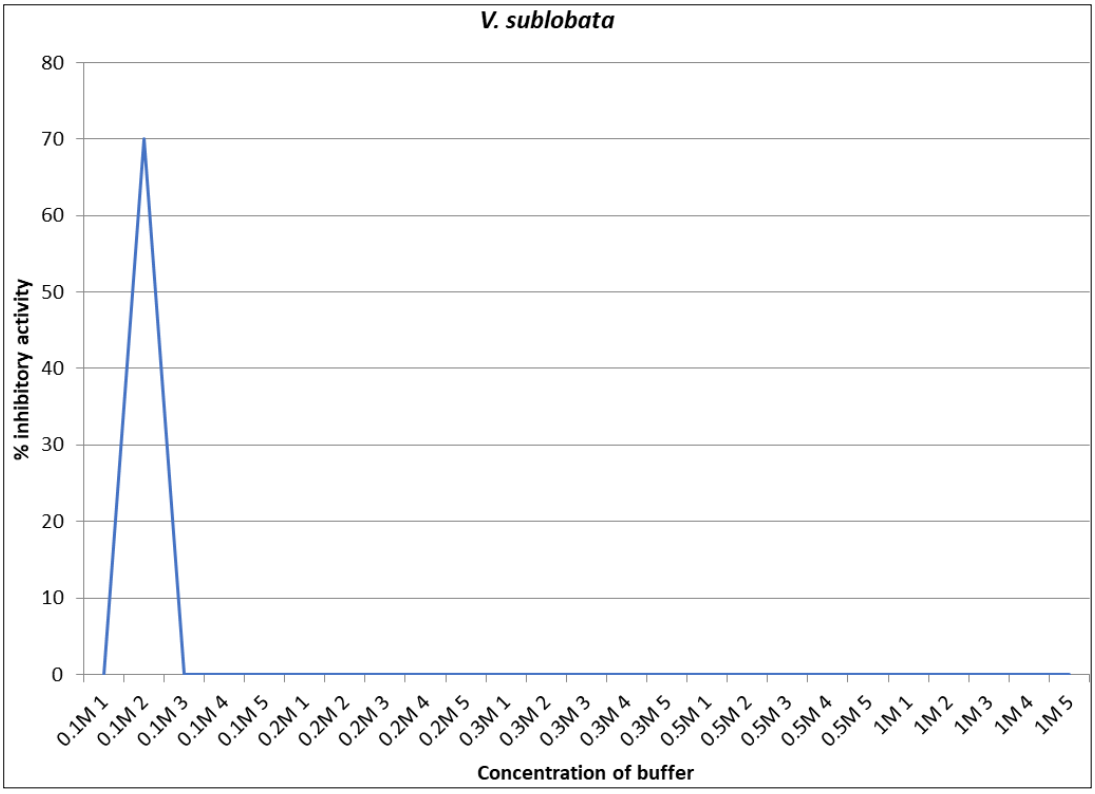
Graph 4: Determination of % inhibitory activity of Ion-exchange chromatography fractions of *Vigna aconitifolia*

4. Ion Exchange Chromatography Results for *V. sublobata*

Fraction number 2 of 0.1M elution buffer showed highest concentration of protein as 1.145 mg/ml and 68% proteinase inhibitory activity. The higher concentration of proteins was also found in fraction 1 (0.1 M) about 0.248 mg/ml, fraction 3 (0.1 M) about 0.596 mg/ml, fraction 7 (0.2 M) about 0.189 mg/ml and fraction 11 (0.3 M) about 0.459 mg/ml but they were not possessing proteinase inhibitory activities like remaining fractions. Fraction 2 (0.1 M) showed maximum proteinase inhibitory activity, thus utilized for SDS-PAGE.



Graph 5: Determination of protein concentration after Ion exchange chromatography of 30-60% saturated fraction of *Vigna sublobata*



Graph 6: Determination of % inhibitory activity of Ion-exchange chromatography fractions of *Vigna sublobata*

5. Electrophoretic visualization of purified Protease Inhibitors

The purified protease inhibitors obtained after ion exchange chromatography from the different accessions of *Vigna* such as *V. hainiana*, *V. aconitifolia* and *V. sublobata* with 65%, 55% and 70% maximum proteinase activity respectively were analyzed through polyacrylamide gel electrophoresis. The fractions with maximum protease inhibitory activity were run on PAGE and visualized as single protein bands confirming their purity and homogeneity. The single protein purified bands of different *Vigna* such as *V. hainiana*, *V. aconitifolia* and *V. sublobata* showed molecular weight 8.18 KDa, 10.66 KDa and 10.26 KDa respectively when compared with standard molecular weight markers. Giri et al., 1998 [5] purified trypsin inhibitor from *Cicer arietinum* using 12% preparative gel electrophoresis whereas the different workers as Joshi et al., 1998; Tamhane et al [6]., 2005 and

Kansal et al., 2008 [7] used ammonium sulphate precipitation as one of the methods of proteinase inhibitors purification.

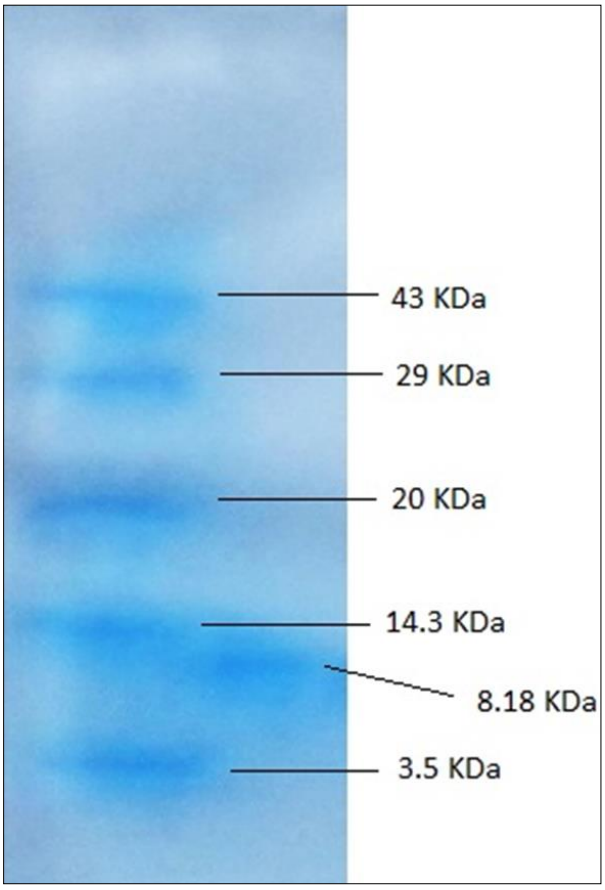


Fig 1: Purified inhibitor from *V. hainiana*

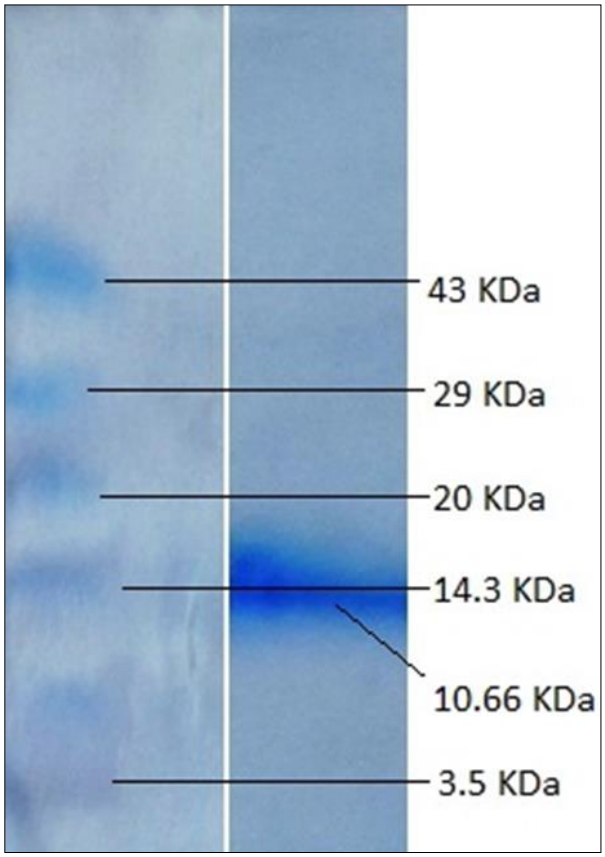


Fig 2: Purified inhibitor from *V. aconitifolia*

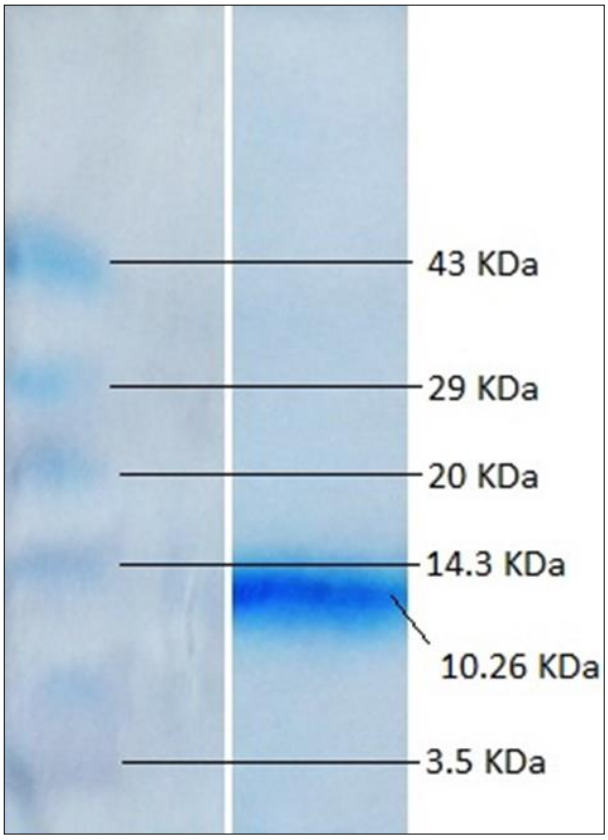


Fig 3: Purified inhibitor from *V. sublobata*

Conclusion

The results obtained in the present study opens new perspectives for utilization of protease inhibitors from the Vigna species in agriculture sector. Vigna genotypes studied found to be the potent tryptic and chymotryptic inhibitors of *H. armigera*. There is also a lot of scope for further research which involves protein engineering as well as development of transgenic crops encoding genes for protease inhibitors.

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