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Single Step Purification of a Winged Bean Trypsin Inhibitor by Using Immunoaffinity Column Chromatography

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Abstract: *We report a very efficient, rapid and easy protocol for purification of a trypsin inhibitor protein from leguminous plant source. Primarily the homogeneous preparation of winged bean trypsin inhibitor (WbTI-2) was obtained by using trypsin sepharose column chromatography, followed by HPLC-gel filtration column chromatography. Purified WbTI-2 was then used for raising anti-WbTI-2 antibody in rabbit. After due time the antibody was collected from rabbit, purified and cross-linked with Affi-Prep HZ hydrazide support and antibody column was prepared. Immunoaffinity column chromatography was performed for the first time with the protein WbTI-2. This technique appeared a much rapid, easy and efficient method compared to the coupled trypsin-sepharose and HPLC column chromatography and yielded absolute homogeneity of protein in single step.*

Keywords: *protease inhibitor, immunoaffinity Chromatography, Trypsin sepharose column, inhibition assay, western blot.*

1. Introduction

Protease inhibitors have been a focus of study for several workers over a long period of time due to their versatile therapeutic potential in a number of diseased conditions[1-5]. Winged bean (*Psophocarpus tetragonolobus)* is a rich source of kunitz type serine protease inhibitors, most of which have a molecular mass of about 20 kDa and contain four half cysteine residues. WbTI-2 is one of such trypsin inhibitor with blocked N-terminal end that shows complete inhibition of trypsin at 1:2 molar ratios. This acidic trypsin inhibitor shows no activity against chymotrypsin. Complete amino acid sequence of WbTI-2 revealed presence of four methionine residues at 30th, 128th, 145thand 146th positions[6]. In our previous work we have done cDNA cloning of WbTI-2 gene using degenerate primer designed from the amino acid sequence and shown retention of partial activity (about 50%) of CNBr digested largest fragment (30th to 128th) of expressed protein[7].This result establishes active but shorter fragment of the protein which indicate towards a probability of WbTI-2 being an efficient potential drug in future. We also reported an in-silico study conducted by us to understand the correlation between structure and function of some of those protease inhibitors including WbTI-2[8]. Since development of an efficient and easy method of purification is the primary necessity in order to work towards understanding any applicatory utility of the protein, this report should be a pathfinder for functional studies pertaining to WbTI-2 in future. Cleavage at the reactive site loop of the trypsin inhibitor by catalytic amount of trypsin at acidic pH was reported earlier in 1966[9].WbTI-2 was reported to get cleaved at Arg^{64}-Sre ^{65} during its elution from trypsin sepharose column at pH 2[6].This result on the one hand reveals the probability of Arg ^{64} to be the P_1 residue of the protein, on the other hand it indicates the necessity of preparing immuno-affinity column for the elution of intact functional protein.

In present study, the WbTI2 protein was primarily purified to homogeneity from seed extract using trypsin sepharose column and then sepharose-12 10/30 FPLC gel filtration column respectively. This purified protein preparation was used for raising antibody from rabbit and antibody column was prepared using highly specific anti-WbTI2 antibody. The resulting antibody column gave a protein recovery yield of about 60% from the seed extract (prepared from 70% acetone cut). The homogeneity of the protein was confirmed by SDS-PAGE. The purified protein was characterized by using trypsin inhibition assay and western blot technique.

2. METHODS

2.1. Purification of Protein

Preparation of crude Extract: 30 g of soaked seeds of winged bean was homogenized after removing the seed coats in homogenization buffer[100mM Tris HCL(pH 7.8), 5 mM EDTA, 0.5mM PMSF(dissolved freshly in 100% dehydrated alcohol, and 2 drops of antifoam emulsion]. The PMSF was added to the buffer drop wise with swirling motion. The homogenate was then centrifuged twice for 10 minutes at 12000 x rpm. After first spin the clear supernatant was collected by discarding the pellet and filtering out the top floating fat and the same was done after second spin.

Acetone Fractionation of protein: 100 ml of chilled acetone was slowly added to the homogenate to make 0-25% acetone cut. The precipitate was separated by centrifuging at 12000 x rpm for 15%. The supernatant was further given 25-70% acetone cut by adding 600 ml of chilled acetone. The protein was allowed to precipitate for 30 minutes at -20°c and then centrifuged at 8000 x rpm for 20 minutes. The pellet was dissolved in minimal volume of buffer [50mM Tris HCL (pH 7.8), and 500 mM NaCl] and then dialyzed against the same buffer.

CNBr-Activation of Sepharose and preparation of trypsin-sepharose column: CNBr activation was carried out as described by Kohn and Wilchek.[10] About 30 g of wet sepharose was activated by CNBr in a concentration of 20 mg/g of gel matrix following the above mentioned protocol, and finally 10 g of trypsin/g of gel matrix was taken for crosslinking. After the crosslinking reaction the slurry was washed with 0.1 M $NahCO_3$, containing 500 mM NaCl and water. The slurry was finally washed with equilibrium buffer i.e. 50 mM Tris HCl (pH 7.8), containing 500 mM NaCl before packing it into the glass column.

Purification of protein using Trypsin sephsrose column: Trypsin was purified from winged bean crude extract by using trypsin sepharose column as described in an earlier report from our laboratory[11]. 2ml of crude extract (about 40 mg of protein) was loaded onto the column in each run. About 3 Ml of eluent was collected in each tube where 1 Ml of 1M Tris HCL buffer (pH8.0) was added in advance to each eluent to neutralize the acidic pH immediately after collection in the tubes.

Purification of protein using FPLC-Gel filtration column: The protein obtained from Trypsin sepharose column was further purified with Sepharose -12 HR 10/ 30 FPLC Gel filtration column. 200 µl of protein (1 mg/ml concentration) was loaded onto the column and 50 mM Tris HCL (pH 7.8) with 100 mM NaCl was used as equilibrium buffer. The column was run at a flow rate of 0.5 ml/min.

Immunization of rabbit using purified protein, preparation of antibody for immunoaffinity column: This part of work was done as described in our previous publication[7].

Preparation of antibody column: Purified immunoglobulin was crosslinked through its Fc-portion with Affi-Prep HZ hydrazide support (BioRad) keeping the antigen binding site free according to the vendor's protocol.

Purification of WbTI-2 by Immuno-affinity column chromatography: WbTI-2 was then purified in a single step directly from the crude extract by loading about 30 mg of protein in every run. The column was equilibrated in phosphate buffered saline (PBS), pH 7.0 and eluted using 0.1 M HCL buffer. The eluents were collected in tubes where 1M Tris HCL were taken in advance to neutralize the acidic pH of eluting buffer.

2.2. Characterization of Protein

Trypsin inhibition assay: Trypsin activity of WbTI-2 was assayed using N-α-p-tosyl-L-Arginine Methyl ester as substrate. The assay was done by determining the increase in absorbance at 247 nm by adding varying amount of bovine trypsin[12]. The Trypsin inhibitory assay of WbTI-2 was done by using varying amount of WbTI-2 with pre-incubated fixed amount of bovine trypsin (2µg) for 10 min and the residual protease activity was measured[7].

SDS-page: The purity of protein preparation was analyzed by 15% SDS-PAGE[13]. The gel was stained separately by both Coomassie Brilliant Blue R-250 (4x gel loading dye) as well as Silver staining method.[14]

Western blot analysis: SDS-PAGE fractionated protein was transferred from polyacrylamide gel to PVDF membrane in transfer buffer [25mM Tris HCL pH8.3, 192 mM glycine and 20% methanol]

under 300 mA current flows for one and half hour[15]. The PVDF membrane was then soaked in 100% methanol for a few seconds and kept in transfer buffer. The membrane was blocked in 1% solution of fat free milk in TBS [100 mm Tris HCl, pH 7.5 containing 150 mM NaCl] overnight. The immuno-decoration was then carried out according to Blake et al[16].

Determination of the protein concentration: The concentration of the purified protein was estimated by Bradford assay[17].

3. Result and Discussion

In present study WbTI2 is purified in two different methods. Primarily crude extract was prepared from the seeds by 70% acetone fractionation wherefrom WbTI2 was purified through trypsin-sepharose column following a previously reported protocol from our laboratory[11]. Since the crude extract of winged bean contained a number of double headed inhibitors which has different degree of affinity towards trypsin along with chymotrypsin, this method utilizes a differential pH for elution of those inhibitors from immobilized trypsin sepharose column. The WbTI-2 is found to be eluted in moderately low pH (50mM citrate phosphate buffer, pH 3). The trypsin present in trypsin sepharose column is already reported earlier to cleave trypsin inhibitor at its sessile bond during low pH elution[9]. Our group also experienced the same effect in such purification method. The very small amount of fragmented trypsin inhibitor produced after such elution was eliminated by passing the eluent through Sepharose -12 HR 10 / 30 FPLC Gel filtration columns. The purification method standardized in such method is essentially a two-step process, that involves loss of total protein content at every step, thus the yield as well as the specific protein content is lower compared to the other method (refer to table:1). The concentration of the protein obtained by this method is also relatively low with respect to the other one as seen in the figure 1a and 1b. However, the trypsin inhibitory activity of the protein obtained from both the methods is found to be very much similar when equal amount of protein was assayed with different enzyme: inhibitor molar ratios. Complete inhibition by the protein acquired by both methods was obtained at a ratio of 1: 2 (Fig: 2). The second procedure is immunoaffinity chromatography in which WbTI-2 could be purified directly from 70% acetone fractionate in a single step. WbTI-2 obtained in such method is free from any contaminant protein and the concentration obtained from such column was moderately good (Fig.1a and 1b). As indicated earlier, the protein yield obtained here is also much better (60%, refer to table 1). For testing reactivity of the purified WbTI-2 with raised antibody western blot analysis was carried out which revealed similar kind of band pattern for both trypsin sepharose and immunoaffinity column purified WbTI-2 (Fig: 3). The only difficulty which we encountered during repeated use of immunoaffinity column (after 10-12 cycles) was leaching of a trace amount of antibody with the eluent. Since the molecular weight of the anti-WbTi-2 antibody is much higher than WbTI2, the contaminant protein could be easily eliminated by Spin-win filter concentrator.

1 2 3

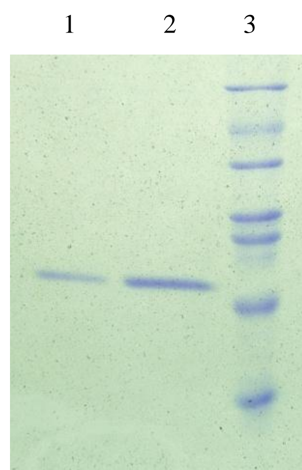

Fig1a. *0.1% coomassie Brilliant Blue (R-250) stained 15% SDS-PAGE profile of WbTI-2. Lane 1: trypsin sepharose column and then FPLC gel filtration column eluted protein (about 3µg). Lane 2: immunoaffinity column eluted protein (about 3µg). Lane 3: Molecular weight marker showing 14, 20, 24, 29, 36, 45, 66 kDa proteins respectively from the bottom (about 6µg of total protein).*

1 2 3

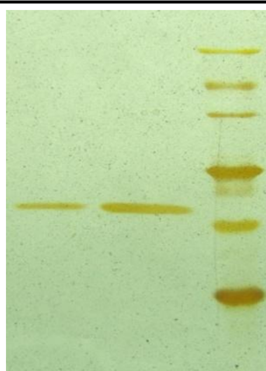

Fig1b. *Silver stained 15% SDS-PAGE profile of WbTI-2. Lane 1: trypsin sepharose column and then FPLC gel filtration column eluted protein (about 1.5µg). Lane 2: immunoaffinity column eluted protein (about 1.5µg). Lane 3: Molecular weight marker showing 14, 20, 24, 29, 36, 45, 66 kDa proteins respectively from the bottom (about 4µg of total protein).*

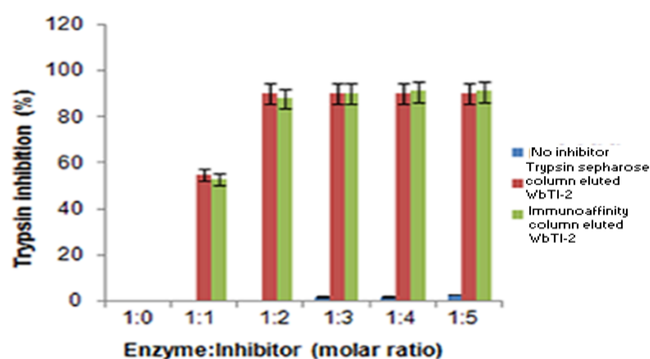

Fig2. *Trypsin inhibition assay with different enzyme: inhibitor molar ratios*

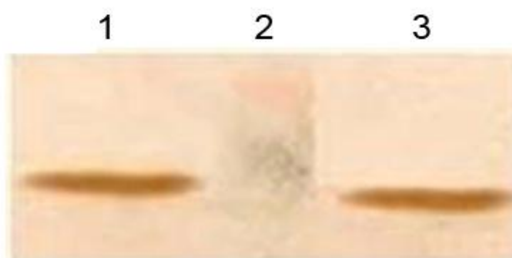

Fig3. *western blot of WbTI-2 using anti WbTI-2 antibody. Lane 1: Trypsin sepharose column and then FPLC Gel filtration column eluted WbTI-2. Lane 2: Molecular weight marker. Lane 3: Immunoaffinity column eluted WbTI-2.*

Table1. *Summary of purification of WbTI-2 from winged bean seeds*

Steps	Total protein (mg)	WbTI-2 present (mg)	Specific protein content (mg/mg)	Yield (%)
70% acetone fractionation	690	50	0.07	100
Acetone fractionate purified by trypsin sepharose column	32	20	0.62	52
Acetone fractionate purified by immuno affinity column	32	30	0.93	60

4. Conclusion

Protease inhibitors from various plant sources have now been proven to be somewhat effective in a number of diseased conditions like disorders of blood coagulation[1], pancreatitis[2], pulmonary emphysema[3], acute respiratory distress syndrome[4] rheumatoid diseases.[5] The results of most of these effects are however in-vitro and requires extensive study to culminate them into potential drugs. Establishing a rapid and efficient purification system is thereby the most fundamental requirement for future progress. This study shows an easy and efficient and new system of purification of one of such protease inhibitors.

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Nilanjana Bhattacharjee graduated with honors in Zoology from University of Calcutta in the year 2000 and completed M Sc in Zoology with specialization in Biochemistry and molecular Biology from Banaras Hindu University in 2002. She qualified National Eligibility Test conducted by CSIR along with Junior research Fellowship (CSIR-NET JRF) in the same year and joined Indian Institute of Chemical Biology, Kolkata as junior research fellow in 2003. She was awarded senior research fellowship at the same institute in 2005, and finally awarded Ph.D in January 2010 and joined as teaching faculty at New Alipore College Kolkata in the same year.