

Purification of Chymotrypsin-Trypsin Inhibitor from Winged Bean Seeds using Single Step Immunoaffinity Column

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Abstract: Problem statement: Winged bean is a rich source of protease inhibitors. The seeds of winged bean contain several serine protease inhibitors of which winged bean chymotrypsin-trypsin inhibitor (WbCTI) is of significant importance due to its dual inhibition of both chymotrypsin and trypsin. The purification of WbCTI has always been difficult and a time taking process that involved large number of steps and various purification columns. **Approach:** This study was focused on the single step method of purification of WbCTI to homogeneity from winged bean seeds using immunoaffinity column chromatography. **Results:** WbCTI was purified to homogeneity using anti-WbCTI antibody column in a single step which gave a single band on SDS-PAGE analysis and with almost one and half times more yield of purified protein than obtained from previously mentioned purification methods. **Conclusion:** This method using immunoaffinity column is high yielding and time saving process of purification.

Key words: *Psophocarpus tetragonolobus*, serine protease inhibitor, single step purification, Immunoaffinity column, Western blot analysis

INTRODUCTION

Protease inhibitors are ubiquitous in nature and are found to be involved in various important biological functions like digestion of proteins, control of blood clotting, apoptosis, signaling receptors interaction in animals and in plant defense against insect attack^[11]. They are present in significant amounts in plants and belong to two major groups, namely Bowman-Birk type and Kunitz type protease inhibitors.

Winged bean (*Psophocarpus tetragonolobus* (L.) DC.) seeds are a rich source of Kunitz type protease inhibitors and are reported to contain at least nine trypsin or chymotrypsin inhibitors^[5,12]. Its high protein and oil content has earned it a name "soybean of tropics^[10]". Kortt first reported the isolation and characterization of protease inhibitors from winged bean seeds obtained by low pH elution of trypsin-sepharose or chymotrypsin-sepharose columns^[7,8]. However, it was found that part of the reactive sites of the isolated inhibitors got cleaved by the cognate proteases due to prolonged exposure during purification using trypsin/chymotrypsin sepharose columns. To avoid such unwanted cleavage of the inhibitors,

Yamamoto *et al.*^[14] purified two inhibitors from winged bean using DEAE cellulose column followed by SP-Sephadex C-25 column. Shibata *et al.*^[12] purified seven inhibitors including their iso-inhibitors from winged bean using a large number of conventional ion-exchange and gel filtration columns. All these inhibitors were Kunitz type having molecular weights of around 20 kDa, including four half cysteine residues. Earlier in our lab, three different protease inhibitors from winged bean have been purified, namely a chymotrypsin inhibitor (WbCI), a trypsin inhibitor (WbTI) and a dual chymotrypsin-trypsin inhibitor (WbCTI) using the conventional trypsin/chymotrypsin sepharose columns and FPLC gel filtration columns^[3]. Despite their presence in large amounts in seeds, isolation of individual inhibitors free from each other using affinity chromatography has always posed difficulties. This was mainly due to their functional similarities, binding of more than one inhibitor for their mutual affinity and identical sizes. Purification of these inhibitors from winged bean using trypsin/chymotrypsin affinity chromatography would result in cleavage of the inhibitors or would require a large number of column chromatography steps that are really arduous and time

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consuming. To overcome all these problems, the need of a simple purification method became essential. The present study describes about a rapid single step purification method of WbCTI using an immunoaffinity column chromatography with the high yield of purified biologically active protein. This immunoaffinity column is conceived with an aim that it would aid in purification of the WbCTI from other inhibitors present in seeds with high yield and without any interference. WbCTI, a dual inhibitor of 18.6 kDa molecular weight, inhibits both trypsin and chymotrypsin in 1:1 molar ratio but does not form a ternary complex with the cognate proteases^[3].

MATERIALS AND METHODS

Winged bean plants were grown in institute field. The seeds were collected from fully matured pods. Chymotrypsin (TLCK treated), trypsin (TPCK treated), bovine serum albumin, TAME and BTEE were from Sigma (St. Louis, USA). Sepharose CL-4B was from Pharmacia (Uppsala, Sweden). Affi-Prep HZ hydrazide support and PVDF membrane were purchased from BioRad Laboratories, CA. All other reagents used were of analytical grade.

Raising of antibody and preparation of immunoaffinity column: The initial purification of WbCTI from winged bean seeds was done as described previously^[3]. The purity of the protein was checked by silver staining on SDS-PAGE. Polyclonal antibodies against purified WbCTI were raised in rabbit by subcutaneous injections of 0.8 mg protein. The anti-WbCTI enriched sera were checked against other inhibitors of winged bean by immuno-blot analysis. Western blot analysis showed that there was no immunological cross-reactivity between the sera rich in anti-WbCTI antibody and other protease inhibitors present in winged bean seeds. Thus, these sera were then considered for preparation of the anti-WbCTI antibody column. For the immunoaffinity column preparation, decomplexed sera were subjected to a 70% ammonium sulfate cut and the immunoglobulins (IgG) were recovered after centrifugation at $5,000 \times g$ for 20 min. The pellet was dissolved in a minimum volume of phosphate buffer saline, pH 7.4 (1X PBS) and dialyzed extensively and were further purified through protein-A sepharose column pre-equilibrated with 1 M potassium phosphate, pH 9.0. Purified immunoglobulins were cross linked through its Fc portion with Affi-Prep HZ hydrazide support according to the vendor's protocol (BioRad). In brief, the purified immunoglobulins were oxidized with 0.5 M periodate at

room temperature and were coupled with Affi-Prep hydrazide support (15 mg IgG/2 mL slurry of Affi-Prep hydrazide) in coupling buffer (0.1 M sodium acetate, pH 4.5 containing 1 M NaCl) for 24 h at 4°C. Finally the slurry was washed with washing buffer (20 mM sodium phosphate, 0.5 M NaCl, pH 7.0) and packed in a column (1×3 cm).

Purification of WbCTI from seeds using a single step immunoaffinity column: WbCTI was purified from the crude extract of winged bean seeds directly using single step anti-WbCTI antibody column. Crude extract was prepared and processed according to the published method^[3]. The dialysate obtained was loaded on to the anti-WbCTI antibody column equilibrated with 1X PBS, pH 7.4. The column was washed extensively with the same buffer and WbCTI was eluted with 0.1 N HCl. The fractions collected were neutralized with 1 M Tris-HCl, pH 7.5 and concentrated using Centriprep-10 (Amicon Inc., MA).

Homogeneity of purification: The homogeneity of purified WbCTI eluted from the immunoaffinity column was checked by 15% SDS-PAGE^[9]. Western blot analysis of the purified protein was carried out according to Towbin^[13].

Inhibition assays of purified WbCTI with different proteases: The inhibitory activity of purified WbCTI against trypsin and chymotrypsin was carried out by measuring the residual esterolytic activity of trypsin and chymotrypsin towards the substrates Na-p-Tosyl-L-Arginine Methyl Ester (TAME)^[4] and N-Benzoyl-L-Tyrosine Ethyl Ester (BTEE)^[11] respectively.

Other methods: Protein estimation was done according to Bradford, using BSA as standard^[2]. The protein concentration was also monitored by measuring absorbance at 280 nm.

RESULTS

Specificity of anti-WbCTI antibodies towards WbCTI: Polyclonal antibodies were raised in rabbits against WbCTI and were attested to be monospecific by immuno-diffusion (data not shown) and also by immuno-blot analysis. Western blot analysis using this antibody against other inhibitors purified from winged bean showed that there was no immunological cross-reactivity between anti-WbCTI antibody and other protease inhibitors present in winged bean seeds (Fig. 1A).

Table 1: Comparison of purification methods of WbCTI from winged bean seeds. Specific protein content is defined as the amount (mg) of WbCTI in 1mg of total protein

Steps	Total protein (mg)	Total WbCTI (mg)	Specific protein content (mg mg ⁻¹)	Yield (%)
Previous method:				
Crude extract (From 30 g seeds)	940	92	0.097	100.00
Trypsin Sepharose column elution	75	68	0.906	73.90
FPLC gel filtration	62	58	0.935	63.04
Present method:				
Crude extract (From 22 g seeds)	746	68	0.091	100.00
Anti-WbCTI antibody column elution	64	62	0.968	91.17

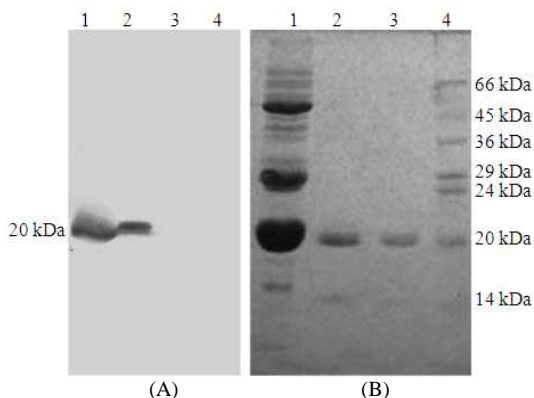


Fig. 1: (A): Western blot analysis. Purified protease inhibitors were resolved by 15% SDS-PAGE followed by transfer to PVDF membrane. Immunodecoration was carried out using rabbit anti-WbCTI immune sera, followed by incubation with protein A-peroxidase and final detection using DAB (3,3'-diaminobenzidine tetrahydrochloride). (Lane 1): Acetone fraction of winged bean seeds; (Lane 2): Purified WbCTI; (Lane 3): Purified WbTI; (Lane 4): Purified WbCI. (B): SDS-PAGE analysis of purified WbCTI. Electrophoresis was carried out in 15% polyacrylamide gel under reducing condition and stained with Coomassie blue. (Lane 1): Acetone fraction of winged bean seeds; (Lane 2): WbCTI purified from trypsin-sepharose column; (Lane 3): WbCTI purified from anti-WbCTI immunoaffinity column; (Lane 4): Standard molecular weight marker.

Purification of WbCTI using anti WbCTI antibody column: WbCTI was purified from acetone fraction using the anti-WbCTI antibody column in a single step process. The homogeneity of the purified protein was then checked by 15% SDS-PAGE which indicated the presence of a single band of desired molecular weight (Fig. 1B) of around 20 kDa. The comparison of the yield of WbCTI from seeds obtained by different purification processes has been summarized in Table 1.

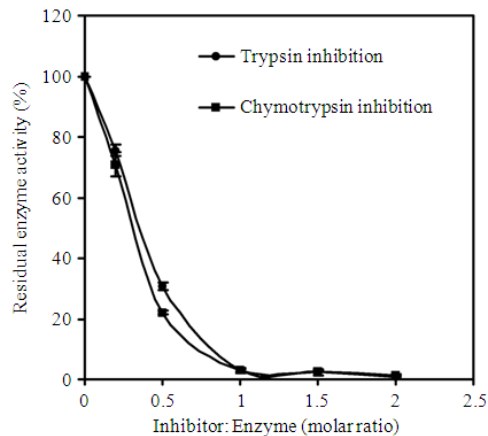


Fig. 2: Inhibitory activity of WbCTI purified from anti-WbCTI antibody column. Increasing amount of the inhibitor was mixed with a fixed amount of trypsin or chymotrypsin and the residual protease activities were measured using TAME and BTEE as substrates for trypsin and chymotrypsin, respectively. The assay of the inhibitory activity was carried out using 2 µg enzymes. The experiments have been done in triplicate and error bars represent standard deviation.

Inhibition assays of purified WbCTI: Inhibitions of trypsin and chymotrypsin activity by WbCTI purified from the immunoaffinity column were carried out using TAME and BTEE respectively as substrates. Figure 2 shows that WbCTI inhibits both trypsin and chymotrypsin in an identical manner and thus retains its activity after elution. It has been determined from graphical extrapolation that WbCTI purified from the immunoaffinity column inhibits both trypsin and chymotrypsin in 1:1 molar ratio.

DISCUSSION

Winged bean seeds are rich source of serine protease inhibitors with varying affinity towards trypsin and chymotrypsin. These inhibitors were first purified

and characterized by Kortt^[7]. The earlier approach for the isolation was based on the intermolecular interaction between the inhibitors and their specific proteases. The WbCTI was earlier purified from our lab through multiple steps using trypsin-sepharose, chymotrypsin-sepharose and FPLC gel filtration columns by differential pH elution. However, the technique suffered from the same serious drawback as that of Kortt that ended up with cleaved inhibitors by the cognate proteases. Again, purification method by Shibata *et al.*^[12] suffered being laborious and time consuming. However, this presently described purification method involves only a single column, is less time consuming and results in high yield of purified protein. The one step purification method used here was satisfactory since the purified protein exhibited a single band in SDS-PAGE analysis. The molecular weight has been found to be around 20 kDa, as reported earlier^[3]. The easy and rapid process of purification of WbCTI described in this study could result in purification of large amount of this inhibitor that would help further to study the molecular mechanisms involved in protein-protein interactions and also in elucidation of its structure. This rapid high yielding purification step results in large amount of purified WbCTI that could be used in studies of host-pathogen interactions as there are reports about the efficiency of protease inhibitors from winged bean as biopesticide against insect attacks^[6]. This method could also be used in purification of other protease inhibitors present in winged bean seeds or from any other different sources with high degree of purity and without any intrusions.

CONCLUSION

This study demonstrated a simple, efficient and rapid purification method of WbCTI from seeds without interference from any other inhibitor proteins with the aid of immunoaffinity chromatography. This strategy could be effectively used in future for purification of any other protease inhibitors from different sources in large amounts and in highly purified form.

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