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Isolation, expression, and characterization of the serine protease inhibitor gene (*600Hbpi*) from *Hevea brasiliensis* leaves, RRIM600 cultivar

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Abstract

First-strand cDNA encoding a serine protease inhibitor was synthesized from RNA extracted from *Hevea brasiliensis* leaves, RRIM600 cultivar. A full-length cDNA of RRIM600 *H. brasiliensis* protease inhibitor (*600Hbpi*) (GenBank accession no. KJ471471) was obtained from reverse transcription polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends (RACE). The primers for *600Hbpi* were created from alignments of *H. brasiliensis* RRIM600 latex protease inhibitor (Hb-PI) (GenBank accession no. EU295479) and *H. brasiliensis* protease inhibitor protein 1 (PI1) (GenBank accession no. AY221985). 600HbPI encodes a 70 amino acid protein and is a member of the potato inhibitor I (PI-I) family of serine protease inhibitors. Multiple sequence alignment of homologous PI-I family proteins revealed one motif WPEL of 600HbPI conserved across the PI-I family. The coding region for the active site of 600HbPI was predicted as Met^{46}-Glu^{47}. *600Hbpi* was cloned into the pFLAG-ATS vector. Recombinant 600HbPI was expressed as 11 kDa proteins in *Escherichia coli* strain BL21. Protease inhibition analysis showed that recombinant 600HbPI is more effective at inhibiting subtilisin A than chymotrypsin but did not inhibit trypsin protease. These results indicate that the recombinant 600HbPI encoded a functional protease inhibitor that specifically targets the chymotrypsin and subtilisin classes of serine proteases.

Keywords: cDNA; cloning; gene expression; potato inhibitor I family; rubber tree.
Abbreviations: 600HbPI_RRIM600 *Hevea brasiliensis* protease inhibitor; RT-PCR_ Reverse transcription polymerase chain reaction; RACE_ Rapid amplification of cDNA ends; Hb-PI_*Hevea brasiliensis* RRIM600 latex protease inhibitor; PI1_*Hevea brasiliensis* protease inhibitor protein 1; PI-I_Potato inhibitor I; 251HbPI_RRIT251 *Hevea brasiliensis* protease inhibitor; SDS-PAGE_Sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Introduction

Protease inhibitors appear to function by impairing the proteolytic activity of target proteins. They are produced in organisms that include microorganisms, plants, and animals. Protease inhibitors are classified into at least six types (Bode and Humer, 1992). Serine protease inhibitor is in the class of protease inhibitors found in many plants such as sweet potatoes (Wang et al., 2003), wheat (Poerio et al., 2003), and tomatoes (Lee et al., 1986). Potato inhibitor I (PI-I) family is a widespread family of serine protease inhibitors (Wang et al., 2003) that is generally monomeric and has a molecular mass of 8 kDa (Habib and Fazili, 2007). Inhibitors in the PI-I family lack a disulphide bond (Habib and Fazili, 2007). The PI-I protein is found in many plant species, including potato tubers (Ryan and Balls, 1962), tomato leaves (Lee et al., 1986), tomato fruit (Margossian et al., 1988; Wingate et al., 1989), and squash phloem exudates (Murray and Christeller, 1995). Moreover, the PI-I family of serine protease inhibitor has been found in latex of *Hevea brasiliensis*, RRIM600 cultivar (Sritanyarat et al., 2006) and leaves of *H. brasiliensis*, RRIT251 cultivar (Chinnapun et al., 2016).

H. brasiliensis is an important plant in the economy of Thailand. RRIM600 and RRIT251 cultivars of *H. brasiliensis* are popularly grown plants in Thailand. The RRIM600 cultivar has less capacity to resist pathogens than the RRIT251 cultivar. A previous study found that the RRIT251 cultivar of *H. brasiliensis* leaves produces a serine protease inhibitor designated as RRIT251 *H. brasiliensis* protease inhibitor (251HbPI) (Chinnapun et al., 2016). In this study, we hypothesize that serine protease inhibitor characteristics of the RRIM600 cultivar of *H. brasiliensis* leaves are different from those of 251HbPI because the capacity to resist pathogens of the RRIT251 and RRIM600 cultivars is different.

This study describes the isolation, expression, and functional characterization of a serine protease inhibitor of the PI-I family gene from the RRIM600 cultivar of *H. brasiliensis* leaves designated as RRIM600 *H. brasiliensis* protease inhibitor (*600Hbpi*). The major serine proteases targeted by 600HbPI are studied: chymotrypsin, subtilisin A, and trypsin.

Results

600HbPI gene encodes a homolog of the PI-I family

A full–length *600Hbpi* sequence was obtained from RT-PCR and two RACE-PCR reactions that were deposited at the National Center for Biotechnology Information GenBank under accession number KJ471471. Using ExPASy-Tools, the full-length *600Hbpi* cDNA sequence was identified as an open reading frame of 213 bp that corresponds to a translated product of 70 amino acids. After searching the InterPro database, it was determined that the amino acid sequence of 600HbPI was a member of the PI-I family of serine protease

inhibitor (InterPro IPR000864). Analysis using the SignalP 4.1 server revealed that the 600HbPI amino acid sequence has no signal peptide. The 600HbPI amino acid sequence is highly similar to the amino acid sequence of serine protease inhibitor. The 600HbPI amino acid sequence showed 87% sequence identity with 251HbPI when analyzed using the protein-protein BLAST (blastp) program with only nine differences in the amino acids (Fig. 1).

Multiple sequence alignment of homologous PI-I family proteins from 600HbPI (KJ471471), 251HbPI (KJ471470), *Glycine max* (ACA23204), *Vitis vinifera* (AAN85825), *Oryza sativa* (AAK73145), *Zea mays* (CAA55588), and *Triticum aestivum* (P82977) revealed one motif WPEL of 600HbPI conserved across the PI-I family (Fig. 2). Based on the other known members of PI-I family, Met^{46}-Glu^{47} was predicted as the active site of 600HbPI amino acid residues (Fig. 2)

Expression and purification of recombinant 600HbPI

The recombinant 600HbPI was expressed in *Escherichia coli* strain BL21 using the pFLAG-ATS vector. Recombinant protein was purified using a gravity column packed with anti-FLAG M2 affinity gel. The purified recombinant 600HbPI was run on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The molecular weight of recombinant 600HbPI was identified using GenColor prestained protein marker (11-180 kDa) as 11 kDa (Fig. 3). The electrophoresis gel was stained with Coomassie Brilliant Blue.

Recombinant 600HbPI inhibits chymotrypsin and subtilisin A protease

Protease inhibition of recombinant 600HbPI was analyzed with chymotrypsin, subtilisin A, and trypsin proteases using the Colorimetric Quanti-cleave protease assay kit. Protease activities were measured at 450 nm by spectrophotometry. Results from independent triplicate analysis showed that recombinant 600HbPI weakly inhibited chymotrypsin, was highly effective at inhibiting subtilisin A, but did not inhibit trypsin protease (Fig. 4). The results indicate that specific targets of recombinant 600HbPI are chymotrypsin and subtilisin A. Chymotrypsin and subtilisin A activity remained at about 92.77% and 26.91%, respectively, after inhibition with recombinant 600HbPI (Fig. 4).

These results indicate that the recombinant 600HbPI encoded a functional protease inhibitor that specifically targets the chymotrypsin and subtilisin classes of serine proteases. The results also show that recombinant 600HbPI has a greater effect on protease subtilisin than chymotrypsin.

Discussion

The full-length *600Hbpi* cDNA sequence is 213 bp and contains 70 amino acids (Fig. 1). Compared with the full-length 251*Hbpi* cDNA sequence from a previous study (Chinnapun et al., 2016), the 600HbPI amino acid sequence shows 87% sequence identity with 251HbPI (Fig. 1). This result indicates that the RRIM600 and RRIT251 cultivars of *H. brasiliensis* produce different amino acid sequences of serine protease inhibitor. Moreover, the conserved sequence across the PI-I family of 600HbPI is different from that of 251HbPI. Multiple sequence alignments of homologous PI-I family proteins from various plant species revealed one motif WPELVG of 251HbPI conserved across the PI-I family (Chinnapun et al., 2016) and one motif WPEL of 600HbPI conserved across the PI-I family (Fig. 1). However, the 600HbPI amino acid sequence lacks a signal peptide and has an active site at Met^{46}-Glu^{47} (Fig. 2) like the 251HbPI amino acid sequence (Chinnapun et al., 2016). The active site of 600HbPI was predicted based on the other known members of PI-I family as Met-Glu (Svendsen et al., 1980), Met-Asp (Richardson, 1977), Leu-Asp (Richardson, 1977), Ala-Asp (Svendsen et al., 1984), and Lys-Asp (Wingate et al., 1989).
Protease-inhibitory activity of recombinant 600HbPI (Fig. 4) is similar to recombinant 251HbPI in that it inhibits chymotrypsin and subtilisin A but does not inhibit trypsin protease. However, the inhibitory capacity of recombinant 600HbPI and recombinant 251HbPI to inhibit chymotrypsin and subtilisin A is different. Recombinant 600HbPI weakly inhibits chymotrypsin but is highly effective at inhibiting subtilisin A. Recombinant 251HbPI has greater chymotrypsin-inhibitory activity than subtilisin A-inhibitory activity (Chinnapun et al., 2016). These results may be related to a difference between the PI-I family conserved sequences of recombinant 600HbPI and those of recombinant 251HbPI. Conserved sequences of protease inhibitors are important in binding and formation (Habib and Fazili, 2007). For example, a conserved tripeptide sequence, Phe-Ala-Val, near the C-terminus and a conserved dipeptide, Phe-Tyr, near the N- terminus are important in binding to the target proteases for family-2 cystatin inhibitors (Habib and Fazili, 2007; Machleidt et al., 1983; Turk et al., 1997). Further, the conserved sequence Gln-Val-Val-Ala-Gly of all cystatins is likely involved in complex formation with enzymes (Alan, 1987).

Materials and methods

Plant material

Rubber plants (RRIM600 cultivar) were grown in pots with a photoperiod of 12 h of light and 12 h of dark at 25ºC. Eight-week-old leaves of rubber plants were collected for RNA extraction.

Isolation of 600Hbpi

Total RNA from rubber plant leaves was extracted using the RNeasy plant mini kit (Qiagen). First-strand cDNA was synthesized from 3 µg of total RNA using the SuperScript III reverse transcriptase RT-PCR system (Invitrogen). The first-strand cDNA was diluted to 1:10 and then used as a template for PCR. The CLUSTAL-X program was used for alignment to design DNA primers of *600Hbpi*. The conserved regions from alignments of *H. brasiliensis* RRIM600 latex protease inhibitor (Hb-PI) (GenBank accession no. EU295479) and *H. brasiliensis* protease inhibitor protein 1 (PI1) (GenBank accession no. AY221985) were used to create primers for *600Hbpi*. Both Hb-PI and PI1 sequences were obtained from the NCBI database (www.ncbi.nlm.nih.gov). RT-PCR was performed using oligonucleotides (5'-TCGGGACAAACGGGGACATTGCAGCG-3') and (5'-CCCGATTWTCATCCACGAAAACC-3') for the forward primer and degenerate reverse primer, respectively. RACE-PCR was performed after obtaining a partial *600Hbpi* sequence from RT-PCR using the Smart RACE cDNA amplification kit (Clontech). A full length of the *600Hbpi* sequence was obtained from RACE-PCR using forward primer (5'-CGGGACAAACGGGGACATTGCAGCG-3') and reverse primer (5'-CCCGATTATCATCCACGAAAACC-3'). PCR products from RACE-PCR were transformed to *E. coli*

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600HbPI    MASQCPVKDAWPELIGTNGDIAAGIIETENANVKAIVVKE
251HbPI    MTSQASVENSWPELVGTNGDIAAGIIQTENANVKAIVVKE
           *  **  *    **** ************ **************

600HbPI    GSPMTMEYNLCRVLVFVDDNRVVTQAPAIG
251HbPI    GSPMTMEYNLCRVLVFVDDNRVVTQVPAIG
           ***************************** ****
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Fig 1. Amino acid sequence line-up of 600HbPI and RRIT251 *H. brasiliensis* protease inhibitor (251HbPI) (GenBank accession no. KJ471470). The asterisks indicate amino acid residues conserved between the two sequences.

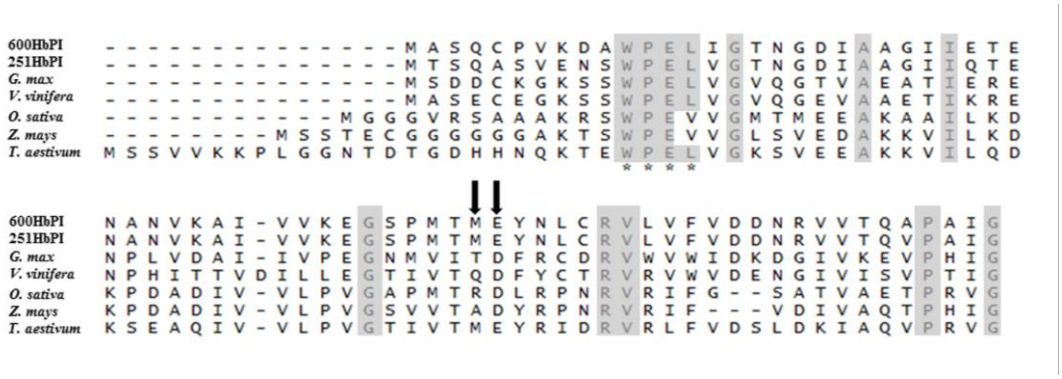

Fig 2. Amino acid alignment of 600HbPI with homologous PI-I family proteins from various plant species. Identical amino acids are shaded in gray. The asterisks represent amino acid residues of 600HbPI conserved across the PI-I family. The arrows indicate amino acid residues at the active site of 600HbPI.

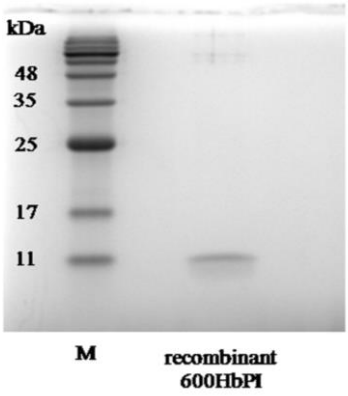

Fig 3. SDS-PAGE of purified recombinant 600HbPI protein. Lane M represents a protein standard marker. Lane recombinant 600HbPI represents purified recombinant 600HbPI protein from the anti-FLAG M2 affinity column.

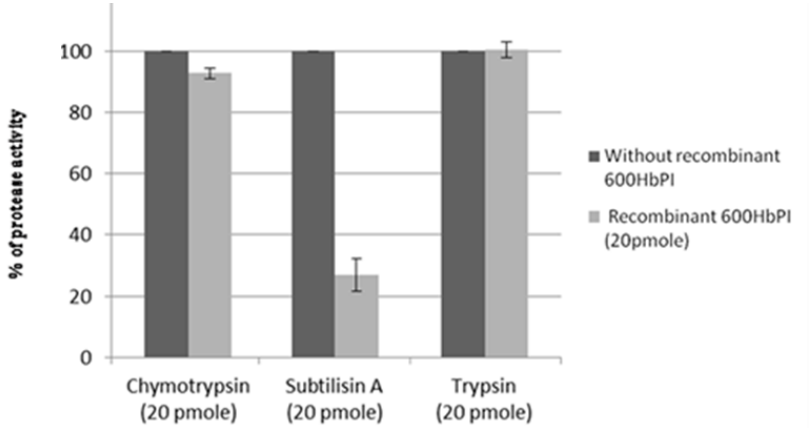

Fig 4. Chymotrypsin, subtilisin A, and trypsin inhibition analysis of recombinant 600HbPI using the Colorimetric Quanti-cleave protease assay kit. Standard error of deviation was calculated from independent triplicate analysis.

TOP10 cells. Before sequencing, the plasmid was extracted from cloned cells using the QIAprep spin miniprep kit (Qiagen).

Sequence analysis

The InterPro database (http://www.ebi.ac.uk/interpro/) was used to analyze the family of 600HbPI. The signal peptide sequence was predicted by the SignalP 4.1 sever (http://www.cbs.dtu.dk/services/SignalP). The CLUSTAL-X program was used for alignment and predicted the 600HbPI amino acid sequence conserved across the PI-I family. The amino acid sequences of PI-I family members from *H. brasiliensis*, RRIT251 cultivar (KJ471470), *G. max* (ACA23204), *V. vinifera* (AAN85825), *O. sativa* (AAK73145), *Z. mays* (CAA55588), and *T. aestivum* (P82977) (Poerio et al., 2003) were aligned with 600HbPI. The *600Hbpi* sequence was deposited in GenBank under accession number KJ471471. All amino acid sequences described in this paper were obtained from the NCBI database (www.ncbi.nlm.nih.gov).

Expression and purification

A PCR-amplified DNA fragment of *600Hbpi* was cloned into the *Eco*RI and *Kpn*I sites of the pFLAG-ATS vector. The *Eco*RI restriction site was added to the 5'-end of the forward primer, and the *Kpn*I restriction site was added to the 3'-end of the reverse primer after the stop codon. The oligonucleotide forward primer (5'-GCGGAATTCCATGGCAAGTCAGTGTCCAGTTAAG-3') and reverse primer (5'-GCGGGTACCTTAGCCAATGGCAGGAGCTTGAGTGAC-3') were used to amplify the fragments. The introduced *Eco*RI and *Kpn*I restriction site was underlined. The pFLAG-600HbPI plasmid was transformed into *E. coli* strain BL21 using an electroporator at 2500 volts. Recombinant 600HbPI protein was purified using anti-FLAG M2 affinity gel (Sigma). Protein concentration was measured at 280 nm by spectrophotometry and calculated using an extinction coefficient of 7115 M^{-1} cm^{-1}.

SDS-PAGE and Western blot analysis

SDS-PAGE was performed on 15% (w/v) polyacrylamide gel. The molecular weight of recombinant 600HbPI was determined using the GenColor prestained protein marker (11-180 kDa; GeneMark). For Western blot analysis, protein was transferred to nitrocellulose membrane and detected with antigen-antibody complexes using ANTI-FLAG M2-peroxidase (Sigma) and Super Signal West Pico chemiluminescent substrate (Pierce).

Chymotrypsin, subtilisin A, and trypsin inhibition analysis

The Colorimetric Quanti-cleave protease assay kit (Pierce) was used for protease inhibition analysis of chymotrypsin, subtilisin A, and trypsin proteases against recombinant 600HbPI. The quantitative analysis was performed according to the manufacturer's instructions. Briefly, 20 pmol of recombinant 600HbPI was incubated with 20 pmol of chymotrypsin, subtilisin A, or trypsin in a volume of 50 µl for 30 min before analysis. To these reactions, 100 µl of 2 mg/ml succinylated casein was added and incubated at room temperature for 20 min. After the addition of 50 µl of chromogenic reagent 2,4,6-trinitrobenzenesulfonic acid and incubation for 20 min at room temperature, protease activities were measured at 450 nm by spectrophotometry.

Conclusion

In conclusion, 600HbPI from *H. brasiliensis* leaves, RRIT600 cultivar is in the PI-I family of serine protease inhibitor and specifically targets the chymotrypsin and subtilisin classes of serine proteases. Some characteristics of 600HbPI are different from 251HbPI: Namely, there is one motif WPEL of 600HbPI conserved across the PI-I family, and 600HbPI is highly effective for the inhibition of subtilisin A but has only weakly inhibits chymotrypsin.

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