

# Cloning and Characterization of Protease Inhibitor from Latex of Rubber Tree (*Hevea brasiliensis*)

## การโคลนและศึกษาสมบัติของยีน Protease Inhibitor จากน้ำยางพารา (*Hevea brasiliensis*)

Aining Musor (ไอนิง มูซอ)\* Dr. Wilaiwan Chotigeat (ดร.วิไลวรรณ โชติกีรติ)\*\*  
Dr. Amornrat Phongdara (ดร.อมรรัตน์ พงศ์дарา)\*\*

### ABSTRACT

cDNA library of *Hevea brasiliensis* was performed and found full-length of protease inhibitor (*Hb-PI*) cDNA. An open reading frame encoding 210 bp of *Hb-PI* gene was cloned to express in *E. coli* via pQE-40 expression vector. The trypsin inhibition by the recombinant protein was tested and the result showed that 0.25 mg protein from recombinant *Hb-PI* had the ability to inhibit 0.2 mg/ml trypsin for 9.5% and the percentage of inhibition was increased to 36, when *Hb-PI* protein concentration was increased up to 0.75 mg protein.

### บทคัดย่อ

การศึกษา cDNA library ของน้ำยางพารา ได้พบยีนเส้นเดี่ยมของตัวยับยั้งเอนไซม์โปรตีอีส (*Hb-PI*) ซึ่งมีขนาด 210 เบส เมื่อนำมาแสดงออกในแบคทีเรีย *E. coli* โดยโคลนเข้ากับเวคเตอร์ pQE-40 และกระตุ้นการสังเคราะห์โปรตีนลูกผสม *Hb-PI* ผลจากการศึกษาการยับยั้งเอนไซม์ทริปชิน พบว่าโปรตีนลูกผสม ดังกล่าวปริมาณ 0.25 มก. สามารถยับยั้งเอนไซม์ทริปชินปริมาณ 0.2 มก/มล. ได้ 9.5% ค่าเบอร์เซ็นต์การยับยั้งมีค่าเพิ่มขึ้นเป็น 36 เมื่อปริมาณโปรตีนลูกผสม *Hb-PI* เพิ่มขึ้นเป็น 0.75 มก.

**Key Words:** *Hevea brasiliensis*, Latex of rubber tree, Protease inhibitor  
**คำสำคัญ :** *Hevea brasiliensis* น้ำยางพารา ตัวยับยั้งเอนไซม์โปรตีอีส

\* Master Student of Department of Biochemistry, Prince of Songkla University

\*\* Assoc. Prof., Center for Genomics and Bioinformatics Research, Prince of Songkla University.

## Introduction

Latex is the cytoplasm of laticiferous cell from latex plants. Most component of latex para rubber is organic substrate including protein (Yeang et al., 2002). Martin (1991) reported, various enzymes have been detected in the latex and they have a function to degrade the cell wall of widespread microorganism, that agreeable with Han et al. (2000) discovered the defense-related genes expressing in the latex. Protease inhibitors are the one group of defense-related genes which have been found in cDNA library of latex para rubber (Duangchu, 2007).

Protease inhibitors are generally small proteins, accumulation upto 10 % of the total protein content in seed and storage tissue of plant (De Leo et al., 2002). They can contribute in other plant tissue following a variety of different stimuli such as wounding, insect feeding or pathogenic microorganism attacks, and therefore are considered to play a key role in some of the most important defense mechanisms (Hermosa et al., 2006)

Protease inhibitors can be divided into four classes, categorized according to the protease that they inhibit and the classes of protease are serine, cysteine, aspartate and metallocarboxy protease (Ryan, 1990). Many of protease inhibitors have already isolated and characterized from a variety sources, but serine and cysteine protease inhibitor are the two most studies classes, because of their ability to inhibit the growth of widespread insects, nematodes and plant pathogens (Haq et al., 2004).

In this study, we have identify and cloning a full-length of protease inhibitor and

characterize the ability to inhibit trypsin of recombinant protein.

## Methods

### Nucleotide sequence alignment of Hb-PI gene

The nucleotide of *Hb-PI* sequence was used in blast search with sequence in the National Center for Biotechnology Information (NCBI) database.

### Cloning of Hb-PI gene

The *Hb-PI* gene was amplified by Polymerase Chain Reaction (PCR) using primers as follows; Forward primer:

5' GGTACCATGGCAAGTCAGTGTCC 3'

Reverse primers:

5' CAAGTCGACTTAGCCAATGACC 3'

Forward primer and reverse primer contained *Kpn*I site *Sa*II site (underlined), respectively. The PCR reaction was carried out in 25  $\mu$ l. reaction mixture consisting of 1  $\mu$ l of 100 ng DNA template, 2.5  $\mu$ l of 10 mM forward and reverse primers, 1  $\mu$ l of 10 mM dNTPs, 2.5  $\mu$ l of 10x Taq polymerase buffer (50mM KCl, 10mM Tris-HCl, pH 9.0 and 0.1% Triton X-100), 2.5  $\mu$ l of 25 mM MgCl<sub>2</sub>, 1  $\mu$ l of Taq polymerase and deionized water. Amplification involved an initial stage at 94°C for 2 min, followed by 30 cycles of 94°C for 2 min, 60°C for 1 min and 72°C for 1 min, then a final extension stage at 72°C for 10 min. The PCR products were purified from 1.2% agarose gel using wizard® SV gel and PCR clean-up system (Promega, Madison, USA). The gene was ligated with pGEM-T Easy vector and transformed to *E. coli* Top10F'. The plasmids from transformants were isolated and digested with *Kpn*I and *Sa*II. The *Hb-PI* gene containing *Kpn*I

and *Sall* sites was ligated into pQE-40 expression vector and transformed to *E. coli* M15 to express the recombinant protein.

#### Expression of recombinant Hb-PI protein in *E. coli*

The *E. coli* M15 transformant harboring the pQE-Hb-PI vector was inoculated in LB broth containing 100  $\mu$ g/ $\mu$ l ampicillin and 25  $\mu$ g/ $\mu$ l kanamycin at 37 °C overnight with shaking. The culture was transferred to fresh medium and incubated until the OD<sub>600</sub> was 0.5 – 0.7, then induced with 1mM IPTG for 3 hours. The cells were harvested by centrifugation at 4,000xg for 20 min and resuspended in Tris-HCl pH 8.5. The cell suspension was then sonicated on ice using a sonicator at 200 – 300 W for 7x 10 sec bursts with a 10 sec cooling period between each burst. The lysate cells were dissolved in Tris-HCl Buffer. The recombinant Hb-PI (rHb-PI) protein was analyzed using 12% SDS-PAGE according to the method of Laemmli (1970). Protein concentrations were determined by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

#### Determination of rHb-PI inhibitory activity

Quantitative assay of Protease Inhibitor activity was determinated with azocasein as substrate by a modified procedure of azocasein digestion method. The recombinant Hb-PI was pre-incubated for 10 min at 37°C with 0.2 mg/ml trypsin in a total volume of 0.2 ml containing 50mM Tris-HCl pH 8.5. Then added 0.1 ml of 1.5% azocasein in 50 mM Tris-HCl pH 8.5 to the solution. The mixture was incubated at 37°C for 30 min and added 0.7 ml of 5% TCA to stop the reaction and placed on ice for 15 min. The solution

was then centrifuged at 4,500xg for 5 min. The supernatant was separated for 0.5 ml then added 0.5 N NaOH and mixed well. Absorbance was measured at 440 nm. Percentage of inhibition was calculated as follows:

$$\text{Percentage of inhibition} =$$

$$\frac{A_{440} \text{ trypsin} - (A_{440} \text{ sample} - A_{440} \text{ sample blank})}{A_{440} \text{ trypsin}} \times 100$$

## Results

#### Nucleotide sequence alignment of Hb-PI gene

The *Hb-PI* gene from cDNA library of *H. brasiliensis* (Duangchu, 2007) was alignment with sequence in the National Center for Biotechnology Information (NCBI) database demonstrated that the *Hb-PI* cDNA are similar to the *H. brasiliensis* protease inhibitor protein1 (PI1) mRNA (accession no. AY221985), *H. brasiliensis* isolate SSH34 mRNA sequence (accession no. DQ3306763) and *H. brasiliensis* isolate SSH7 mRNA sequence (accession no. DQ3306736). The identities of *Hb-PI* with PI1, SSH34 and SSH7 are 90%, 89% and 83%, respectively. (Figure 1).

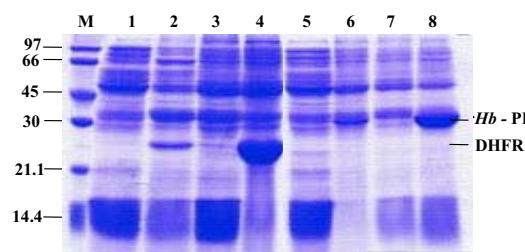
#### Cloning and expression of the Hb-PI in *E. coli*

The recombinant plasmid, pQE-Hb-PI was constructed and verified by PCR and restriction endonuclease digestion analysis. The rHbPI protein was expressed at 37 °C and induced with 1mM IPTG for 3 hours. The rHb-PI was analyzed by 12% SDS-PAGE and compared with low molecular weight marker. The rHb-PI protein (~7 kDa) was fused with dihydrofolate reductase (DHFR) (~26 kDa) from pQE-40 expression vector, so the size of the rHb-PI protein

is approximately 33 kDa. The rHb-PI was shown in part of cytosol (soluble) and inclusion body (insoluble) (Figure.2).

#### Determination of Hb-PI inhibitory activity

The trypsin inhibitory activity of rHb-PI was determined by azocasein digestion method. The rHb-PI crude protein in range of 0.25 – 1.00 mg protein was tested. The inhibition was detected only in soluble part. The percentage was presented at 9.5–36% as shown in Figure 3.

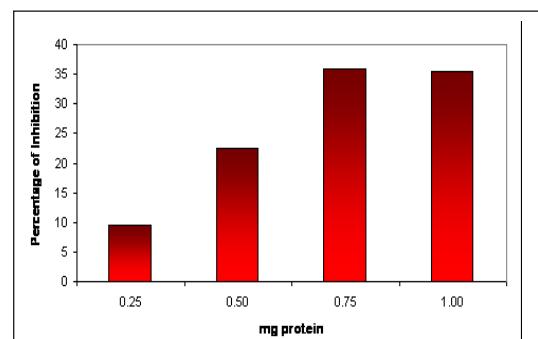


**Figure 2** Protein expression from rHb-PI and pQE: lane M: Low molecular weight marker (GE Healthcare); lane 1: non induced pQE soluble protein; lane 2: induced pQE soluble protein; lane 3: non induced pQE insoluble protein; lane 4: induced pQE insoluble protein; lane 5: non induced rHb-PI soluble protein; lane 6: induced rHb-PI soluble protein; lane 7: non induced rHb-PI insoluble protein and lane 8: induced rHb-PI insoluble protein.

#### Discussion

Protease inhibitors have been isolated and characterized from a large number of organisms, including plants, animal and microorganism (Habib and Fazili, 2007). The most abundant source is the plants and most protease inhibitors are of comparatively low molecular weight varies

from 4–20 kDa (Rao et al., 2007). They can regulate the hydrolysis of proteins inside the cell and also participate in the mechanism of plant defense against herbivore insects and pathogens (Tremacoldi and Pascholati, 2002).



**Figure 3** The percentage of inhibition of rHb-PI.

In this work, we approach to identify the protease inhibitor from cDNA library of latex para rubber. The nucleotide sequence of Hb-PI has been highly identity with sequences of protease inhibitor in NCBI database, PI1, SSH34 and SSH7. The Hb-PI was constructed and characterized the ability to inhibit the trypsin. Azocasein was used as substrate because it was found to be a versatile and sensitive substrate suitable for routine assays (Ramakrishna and Ramakrishna, 2005). The inhibition was increased up according to the amount of crude protein and invariable, when rHb-PI was 0.75–1.00 mg protein.

During the last decade, protease inhibitors have been investigated for the therapeutic agent such as antimicrobial or antiviral (Patick and Potts, 1998). Therefore, Hb-PI will be further

investigate for antiviral property, for example anti white spot syndrome virus (WSSV) or yellow

head virus which could be useful in aquaculture industry in the future.

**Figure 1** Nucleotide sequence alignment of *Hb-PI* with PI1 (accession no.AY221985), SSH34 (accession no. DQ3306763) and SSH7 (accession no. DQ306736). The shading represents degree of homology.

## Conclusion

The nucleotide sequence of *Hb-PI* has been highly identity with PI1, SSH34 and SSH7 and recombinant protein of *Hb-PI* has been able to inhibit trypsin.

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