STUDY OF PROTEASE INHIBITOR IN RUBBER LATEX

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Protease inhibitors (PI) were detected in the latex of rubber trees (*Hevea brasiliensis*). The PI was located mainly in the C-serum by screening assays with pronase while only little activity could be detected in the B-serum. Characterization of the C-serum protease inhibitor indicated that it was a thermostable protein with a very strong heat stable property. The PI in C-serum (CS-PI) was precipitated out from other proteins at a high concentration of acetone (80-95% v/v) and designated as *Hevea* protease inhibitor (HPI). It was shown as a single protein band with a calibrated subunit MW of 5.5 kD upon SDS-PAGE analyses. Heating of this HPI in boiling water for 15 and 30 min resulted in almost no PI activity loss, indicating it is a very thermostable protein with low subunit MW nature. Screening experiments on different protease classes of HPI showed that HPI was the most effective inhibitor for pronase (57.59 % enzyme activity inhibition), followed by chymotrypsin, trypsin (18.05 % and 14.47 % enzyme activity inhibition, respectively). A weaker inhibitor on papain (7.39 % enzyme activity inhibition) was observed. On the contrary, very mild PI activity was found for thermolysin inhibition (4.61% enzyme activity inhibition) and no PI activity against pepsin and protease from *Aspergillus saitoi* was observed in this comparative study. The HPI was further purified by passing through the Sephadex G-75 column. Two protein peaks with PI activity were obtained and designated as HPI-1 and HPI-2 , respectively. Calibration for subunit MW determination showed both HPI-1 and HPI-2 possess the same MW of 5.5 kD upon SDS-PAGE. By gel filtration chromatography, the native MW of HPI-1 and HPI-2 were 20.8 kD and 11.7 kD, respectively. The results thus suggested that native HPI-1 existed as tetrahomomeric form and HPI-2 existed as dihomomeric form. The PI activity for both HPI-1 and HPI-2 after heating in boiling water for 30 min was decreased to 90% and 88% of the control inhibition, respectively. Both HPI-1 and HPI-2 have quite a broad range of pH stability. No effect or loss of the PI activity was observed between pH 3-11. However, their activities were rapidly decreased to 38% inhibition (HPI-1) and 50% inhibition (HPI-2) of the control level at pH 12. The pI values were determined to be 4.24 for HPI-1 and 4.17 for HPI-2, respectively.

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จากการศึกษา พบตัวยับยั้งโปรตีเอสในน้ำยางสดจากยางพารา (*Hevea brasiliensis*) โดยพบมากในส่วนของ C-serum ในขณะที่พบเป็นส่วนน้อยใน B-serum เมื่อศึกษาคุณสมบัติของตัวยับยั้งโปรตีเอสใน C-serum พบว่าทนความร้อนได้ดี ตัวยับยั้งโปรตีเอสใน C-serum ถูกแยกออกจากโปรตีนอื่นโดยวิธีตกตะกอนโปรตีนด้วยอะซีโตนที่เปอร์เซนต์ความเข้มข้นของอะซีโตนในระดับสูง (80-95% โดยปริมาตร) สารสกัดที่ได้เรียกว่า HPI ซึ่งมีน้ำหนักโมเลกุลที่ได้จากการทำ SDS-PAGE เท่ากับ 5.5 kD เมื่อทดสอบ HPI ที่ได้ หลังการแช่ในน้ำเดือดเป็นเวลา 15 และ 30 นาที พบว่าความสามารถในการยับยั้งโปรตีเอสยังคงเดิม ดังนั้น HPI จึงเป็นโปรตีนที่ยับยั้งโปรตีเอสที่มีขนาดโมเลกุลของหน่วยย่อยที่เล็กและคงทนต่อความร้อนได้ดีมาก การทดสอบ HPI กับเอนไซม์โปรตีเอสหลายกลุ่ม พบว่า HPI สามารถยับยั้งเอนไซม์ต่างๆได้ดีจากมากไปหาน้อย ดังนี้ โปรเนส (57.59%), ไคโมทริปซิน, ทริปซิน (18.05% และ 14.47% ตามลำดับ), ส่วนการทดสอบกับปาเปอิน พบว่า HPI สามารถยับยั้งการทำงานได้เล็กน้อย (7.39%) ในขณะที่ HPI มีผลยับยั้งการทำงานของเทอร์โมไลซินน้อยมาก (4.61%) และไม่มีผลในการยับยั้งการทำงานของเปบซินและโปรตีเอสจาก *Aspergillus saitoi* เลย เมื่อนำ HPI ไปทำบริสุทธิ์ต่อโดยการแยกผ่านคอลัมน์ Sephadex G-75 โดยอาศัยข้อแตกต่างของขนาดโมเลกุล พบว่ารูปแบบในสภาพธรรมชาติของ HPI น่าจะแบ่งออกเป็น 2 รูปแบบคือ HPI-1 และ HPI-2 โดยทั้งสองรูปแบบประกอบไปด้วยโปรตีนหน่วยย่อยที่มีน้ำหนักโมเลกุลที่ได้จากการทำ SDS-PAGE เท่ากันคือ 5.5 kD ส่วนน้ำหนักโมเลกุลที่ได้จากวิธีแยกผ่านคอลัมน์โดยอาศัยข้อแตกต่างของขนาดโมเลกุล พบว่า HPI-1 และ HPI-2 มีน้ำหนักโมเลกุลเท่ากับ 20.8 kD และ 11.7 kD ตามลำดับ จากข้อมูลที่ได้แสดงว่า รูปแบบสภาพธรรมชาติของ HPI-1 น่าจะประกอบด้วยโปรตีนหน่วยย่อยที่เหมือนกันอยู่ 4 หน่วยย่อย ส่วน HPI-2 เป็นโปรตีนที่ประกอบด้วยโปรตีนหน่วยย่อยที่เหมือนกัน 2 หน่วยย่อย พบว่าหลังผ่านการต้มในน้ำเดือดเป็นเวลา 30 นาที ความสามารถในการยับยั้งโปรตีเอสของ HPI-1 และ HPI-2 ลดลงเหลือ 90% และ 88% ตามลำดับ ทั้ง HPI-1 และ HPI-2 มีความคงทนต่อสภาวะกรด-ด่างอยู่ในช่วงที่กว้าง คือ pH 3-11 และความสามารถในการยับยั้งโปรตีเอส ของ HPI-1 และ HPI-2 ลดลงเหลือ 38% และ 50% ตามลำดับ ที่ pH 12 ค่า pI ของ HPI-1 และ HPI-2 มีค่าเท่ากับ 4.24 และ 4.17 ตามลำดับ

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LIST OF ABBREVIATIONS

cm	centrimetre
µm	micrometre
nm	naonmetre
g	gram
mg	milligram
µg	microgram
ml	millilitre
µl	microlitre
min	minute
hr	hour
N	normality
M	molarity
mM	millimolar
mol	mole
mA	milliampere
V	voltage
v/v	volume/volume
w/v	weight/volume
%	percentage
kD	kilodalton

LIST OF ABBREVIATIONS (CON.)

°C	degree Celsius
x g	gravitational acceleration
Vo	void volume
Vt	total volume
Ve	elution volume
Rf	relative mobility
OD	optical density
Tris	Tris(hydroxymethyl) aminomethane
SDS	sodium dodecyl sulfate
IEF	isoelectric focusing
PAGE	polyacrylamide gelelectrophoresis
EDTA	ethylenediamine tetraacetic acid
TEMED	N,N,N′,N′-tetramethylene-ethylenediamide
TCA	trichloroacetic acid
DMSO	dimethyl sulfoxide
BAPNA	benzoly-DL-arginine-*p*-nitroanilide
BSA	bovine serum albumin
PI	protease inhibitor
PIs	protease inhibitors

CHAPTER I

INTRODUCTION

1. Rubber Tree

Para rubber tree (*Hevea brasiliensis*) belong to Euphrobiaceae's family is the best rubber producer, be cultivated and has economic importance. Native rubber trees grow in the hot humid intertropical regions with a life span of almost 100 years and over 40 meters in height. However, the economic life of the rubber tree is in the range 25-35 years due to being replanted after yield fall to an uneconomic level (1).

Native rubber is obtained by using a systematic "tapping" procedure, which consist of marking an incision in the bark to the latex vessel rings (laticifers) and the outflow of latex occurs. Upon regular tapping lead to systemic induction for wounding response in rubber tree, this includes the expression of multiple protease inhibitors. Then rubber latex should be a good source to produce protease inhibitors.

2. The Composition of the Natural Rubber

Natural rubber latex consists of approximately: 34% rubber cis-1,4-polyisoprene, 2-3% proteins, 0.1-0.5% sterol glycosides, 1.5-3.5% resins, 0.5-1% ash, 1-2% sugars and 55-65% water (2). For the part of proteins, approximately 20% is adsorbed on rubber particles, 20% associated with the sedimentable fraction and thus essentially

with the lutoid, the remaining 60% is reported as being cytosolic or the serum fraction (3).

The fresh latex can be separated into 4 main fractions after subjected on high-speed centrifugation (Fig.1). The top fraction consists almost entirely of rubber which lie above the orange-colored layer containing Frey-Wyssling particles, the middle zone is colorless which made up of the aqueous phase of the latex (C-serum) and yellowish bottom fraction consists mainly of membrane bound organelles called lutoid particle. Details of these fractions were described in the following part.

2.1. Rubber Particle

In addition to the rubber hydrocarbon (cis-polyisoprene), the rubber fraction contains other substances, mainly proteins and phospholipids, forming a protective surface coating around the rubber particles (1). It has been calculated that a rubber particle of diameter 0.1 μm would contain several hundred molecules of hydrocarbon (4). The size of rubber particles usually ranges from 5 nm to about 3 μm and a surface film, approximately 10 nm thick, surround each. This phospholipoglycoproteinic film carries a negative charge and contributes to the colloidal stability of the rubber particles (1).

2.2. Frey-Wyssling Particles

The Frey-Wyssling particles were found after the first ultra-centrifugation of fresh latex. They were separated by ultra-centrifugation on a density gradient. They from 1 to 3% of latex by volume and are therefore quantitatively less important lutoids (3). These particles are mainly composed of lipid material and are yellow or orange in color due to the presence of carotenoids. Using electron microscopy revealed the structural complexity of these organelles and led to use the terms of the "Frey-Wyssling complex" which consists of one or more inclusions containing the lipid-carotenoid complex accompanied by another type of inclusion (a single one in general) with a complex system of branching single-membrane tubules associated withseveral concentric double-membrane lamellae. The Frey-Wyssling complex thus described is 4 to 6 μm in diameter and enclosed in a typical double membrane (3). However, the Frey-Wyssling complex is still the organelle in the laticiferous cell whose physiological role remains the most mysterious.

2.3. C-serum

The aqueous phase of the laticiferous cytoplasm contains most of soluble compounds normally found in plant cells such as inositols, carbohydrates, amino acids, proteins, inorganic anions and metal ions, together with the enzymes and intermediates of various biochemical process, including rubber biosynthesis (1). C-serum is the majro non rubber part in the latex and contains proteins approximately 60% of total proteins in latex. A great number of proteins have low isoelectric point which are anionic at the normal pH (6.9) of the serum.

2.4. Lutoid Particles

Lutoid particles are the most numerous of the larger organelles in the latex next to the rubber particle, since they form 10 to 20% by volume of fresh latex, whereas rubber particles from 20-45% (3). They are membrane-bound vesicles, which are fairly spherical in shape with 0.5 to 3.0 μm in diameter, bounded by a semi-permeable membrane about 8 nm thick, consists mainly of phosphatidic acid in which unsaturated and saturated fatty acids are present in equal proportions (1, 5).

The lutoids are sensitive to the tonicity of suspension media, have a liquid content called B-serum. The B-serum has a pH of about 5.5 which consists of an acid serum enriched with divalent cations (Mg^{2+}, Ca^{2+}) and positively charged proteins which may neutralize the negatively charged of rubber particles and resulted in destabilization of the colloidal solution which is latex (3). In addition, lutoids contain a wide range of hydrolytic enzymes and some of their enzymatic properties are analogous to those of lysosomes of animal cells (5). Approximately 20% of the dry matter in the lutoids is water-soluble protein, of which about 70% is hevein. This substance is anionic protein, has a very low molecular weight of about 5000 and showed to contain no less than 5% sulfur, all as cystine (3). In the light of its low molecular weight and high disulfide content, Archer considered that it could be a protease inhibitors but several test carried out by Walujono *et al.* do not appear to confirm this hypothesis (3, 6, 7).

Fig.1 Ultracentrifuged freshly latex

Fresh latex from rubber trees of *Heavea brasiliensis* was subjected to ultracentrifugation (Beckman model L8-70M ultracentrifuge) at 59,000 x g for 45 min at 4 °C and 4 main layers depend on different density of compounds containing in the latex were obtained.

3. Plant Defense

Throughout their life cycle, plants have to react to various threats coming from the outside environment. They have developed a broad range of strategies, collectively known as "defense" or "stress" responses, to protect themselves against biotic and abiotic stresses (8). Plants react to wounding and pathogen attack by activating a set of genes, most of which play a role in wound healing and prevention of a subsequent pathogen invasion (9). Since wound sites are known to be important entry points for many pathogens, when a plant is infected with a pathogen to which it is susceptible (disease), the pathogen replicates and frequently spreads throughout the plant, often causing considerable damage and even death of the host (10). Plant disease resistance is often associated with changes in the composition and physical properties of cell walls, the biosynthesis of secondary metabolites that serve to isolate and limit the spread of the invading pathogen and necrotic lesion at the site of invasion, known as "hypersensitive response"(HR) (11) or local acquired resistance (12). Often associated with this local response is the development, over a period of several days to a week, of enhanced resistance of a secondary infection by the same or even unrelated pathogens (10), the capacity to resist pathogens gradually becomes distributed throughout the entire plant and is known as systemic acquired resistance (SAR) (11).

The fundamental processes involved in the HR and SAR are not yet well understood but a large number of changes have been noted that correlate with one or both of these responses. These include (10):

i. Synthesis and incorporation of hydroxyproline-rich glycoproteins (HRGPs), cellulose, callose, and phenolic polymers such as lignin in to the cell wall to fortify this physical barrier.

ii. Production of low molecular weight antimicrobial compounds called phytoalexins (phytoalexins are chemically heterogeneous compounds, which are induced, as a consequence of the pathogen/host relation, in plant metabolism and inhibit the growth of microorganisms with unspecific inhibition (13)).

iii. Enhanced expression of genes encoding enzymes in the phenylpropanoid pathway, such as phenylalanine ammonia lyase (PAL), which often lead to the production of phytoalexins and other phenolic compounds.

iv. Production of antiviral activities, some of which appear to be due to novel proteins.

v. Synthesis of proteinase inhibitors that block the activity of microbial and insect proteinases.

vi. Enhanced peroxidase activity, which is necessary for lignification and may be involved in crosslinking of cell wall protein.

vii. Expression of genes encoding hydrolytic enzymes such as chitinases and β-1,3-glucanases that degrade the cell wall of microbes and may be involved in release of elicitor molecules.

viii. Synthesis of pathogenesis-related (PR) proteins.

Note: Exogenous elicitors, arising from hydrolysis of pathogen components by plant enzymes.

: Endogenous elicitors, arising from hydrolysis of plant components by

pathogen enzymes.

: A variety of elicitors such as glycoproteins, carbohydrates, fatty acids, and peptides. Once a particular plant molecule recognizes and reacts with elicitor, aseries of biochemical reactions and structure changes are set in motion in the plant cells in an effort to fend off the pathogen and its enzymes, toxin, etc.(12, 14).

3.1. Proteinaceous Protease Inhibitors (PIs) in Plant

Protease inhibitor proteins are among the defensive chemicals in plant tissues that are both developmentally regulated and induced in response to injury of plant due to phytophagous insects or mechanical damage (15, 16). Accumulation of protease inhibitors occurs both locally, at the site of injury, and systemically, in other organs of the plant distal to the primary wound site (15-18). Wounding, either mechanically or by induces a rapid increase in protease inhibitor activity throughout the plants. (9, 16, 17, 19-24).

The plant proteinase inhibitors are generally small proteins having molecular weights under 50,000 and more commonly under 20,000 (25-27). A number of the inhibitors from corn, potatoes and several legumes have minimum molecular weights of below 10,000 and are often present as dimers or tetramers (25). An inhibitor of mammalian carboxypeptidases A and B that is unique in inhibiting carboxypeptidases was isolated from potatoes (25, 28, 29) and is the smallest proteinase inhibitor isolated to date and has a molecular weight of 3,500 (26). Many inhibitors are products of multigene families and it is not uncommon to find several isoinhibitor species exhibiting different specificities toward proteases (15). Some of the inhibitors were

found to control the activity of endogenous proteases. However most of them do not have an effect on endogenous proteases but inhibit the activity of exogenous enzymes, e.g. serine proteinases of animal origin or of microorganisms (30).

3.2. Roles of Protease Inhibitors in Plant

The physiological functions of protease inhibitors in plants have been somewhat of a puzzle. They are thought to have certain regulatory or protective roles in plants, including the regulation of endogenous proteinase levels before and during seed germination for storage protein digestion and the control of protein turnover, and the inactivation of digestive proteolytic enzymes of invading insect pests (31). While the inhibitor's role as regulator of plant proteinases and plant protection is becoming more strongly supported by evidence, they may also have other important physiological functions. Due to their high content of cysteine residues, serine proteinase inhibitors may also be seen as storage proteins (storage of sulfur amino acids during dormancy) which are degraded during germination and seedling growth (32-34). It has evidences for the storage role of inhibitors, they are stored in tissues, utilized, and thus must be susceptible to proteolytic degradation, although their mode of disappearance in tissue is unknown (25, 31).

3.3. Protease Inhibitors Families in Plant

In plants at least 10 protease-inhibitor families have been recognized and found that are specific for each of the four mechanistic classes of proteolytic enzymes, e.g. serine, cysteine, aspartic and metallo-proteases (15). Table 1 shows families of plant protein protease inhibitors (PIs) that categorized according to the class of protease that they inhibit (35).

Members of the serine and cysteine proteinase inhibitor families have been more relevant to the area of plant defense than metallo- and aspartyl proteinase inhibitors (15). Some plant serine protease inhibitors are bifunctional, typically possessing trypsin and α-amylase inhibiotr activities (36), other PIs exist as multidomain proteins in which eachdomain possesses functional PI activity (35). Serine PIs are the most extensively characterized class of plant PIs, a detrimental effect of these families of PIs on insect larvae has been reported (15, 37). They are currently subdivided into eight families based on primary sequence data (Table 1). The seeds of many plants belonging to the legume family are rich sources of serine PIs and two types of them are widely distributed in legume seeds: the Kunitz type inhibitors and the Bowman Birk type (32, 38). However, the effectiveness of any given protease inhibitor with the same class of protease from different species can vary enormously (15).

Plant cysteine protease inhibitors are typified by the phytocystatins, which inhibit proteases of the papain superfamily. Most of them have been found in animals, but several have been isolated from plant species as well (15). The complete amino acid sequence has been elucidated for a number phytocystatins, all contain the highly conserved putative binding region "QVVAG" which characteriz es cystatins whether they have a plant or animal origin and are structurally similar (39). In plants, it seems

that cysteine PIs are involved in plant defense against insects, particularly those in the coleopteran and hemipteran orders and phytopathogenic nematodes, since major digestive proteolytic activities of them are apparently the result of papain-like cysteine proteases that are susceptible to inhibition by plant cysteine PIs (16, 35).

Table 1 Families of plant protein protease inhibitors[†]

Family	Protease inhibited
Serine protease inhibitors Soybean trypsin inhibitor (Kunitz) family Bowman-Birk family Barley trypsin inhibitor family Potato inhibitor I family Potato inhibitor II family Squash inhibitor family Ragi I-2/maize trypsin inhibitor family Serpin family	Trypsin and chymotrypsin
Cysteine protease inhibitors (phytocystatins)	Papain, cathesin B, H, L
Metallo-protease inhibitors	Carboxypeptidase A, B
Aspartic protease inhibitors	Cathesin D

[†]See Ref. 15, 35.

4. Protease Inhibitor (PI)

Proteinaceous protease inhibitors (PIs) are present in multiple forms in numerous tissues of animals and plants as well as in microorganisms. Their gross physiological function is the prevention of unwanted proteolysis, but detailed physiological function are not clear. Limited knowledge of physiological function due to most laboratories were studied using readily available proteinase, e.g. bovine trypsin, rather than physiological target enzymes (40). High concentrations of PIs are often found in fluids are tissue that are particularly vulnerable to foreign proteases, such as blood serum, pancreatic acinar cells, and storage tissues of plant (15). In each inhibitor molecule there exists on the surface one peptide bond, the reactive site, which combines with the enzyme in a substrate-like manner and serves as a substrate for the enzyme (40). In general, PIs are specific for the active site of a specific class of protease; the exception is the mammalian plasma PI α-2 macroglobulin, which is non-specific for the enzyme or the class of protease. Then PIs can be useful tools in pest control, in the prevention and treatment of diseases such as cancer and AIDS, in controlling proteinases involved in a number of disorders (such as pancreatitis, shock, and emphysema), in the elimination of unwanted proteinase activity in food processes and in the laboratory. Biochemists have also profitable studied plant PIs as model systems to explore the mechanism of action and inhibition of proteinases (25, 41-49).

5. Proteases

Protease or proteolytic enzymes are enzymes that catalyze the cleavage of peptide bonds in other proteins. They are presumed to have arisen in the earliest phases of biological evolution since even the most primitive organisms must have required them for digestion and for the metabolism of their own proteins (50). Proteases have important roles in various physiological processes through the modification of proteins: mobilization of tissue protein, neuropeptide, hormone and proenzyme processing, Table 2 showed representative physiological reactions by proteolytic enzymes. They are involved in the digestion of food proteins and are important components for protecting from pathogen (35, 51). Proteolytic enzymes are not only a physiological necessity but also a potential hazard, since, if uncontrolled (e.g. cancers and hypertension), they can destroy the protein components of cells and tissues. The action of proteolytic enzymes themselves is regulated in several ways involving gene control, zymogen production, enzyme activation and protease inhibitors (51).

Table 2 Representative physiological reactions trigged by proteolytic enzymes[†]

Type of reaction	Example
Multiple cleavage of precursor "side chains"	Adrenocorticotropin Oxytocin-neurophysin
Transmembrane processes	Bacterial outer membrane and periplasmic protein
Hormone precursors	Bacterial toxins Proinsulin Proglucagon
Macromolecular assembly	Proparathyroid Picornaviruses Collagen Fibrin
Zymogen activation	Phage head protein Pancreatic protease zymogens Blood coagulation Complement (C1r, C1s, C3 convertase)
Fibrinolysis	Plasminogen proactivator Plasminogen
Release of physiologically active peptides	Angiotensinogen Kininogen
Development	Proacrosin Prococoonase Prochitin synthetase Procollagenas

[†]See Ref.50.

The term protease is used broadly for all enzymes that cleave peptide bonds (EC 3.4), can be subdivided into exopeptideases or peptidases (EC 3.4.11-19), endopeptideases or proteinases (EC 3.4.21-24). Exopeptideases act on the amino- or carboxy-terminus of the peptide whereas endopeptideases cleave peptide bond internally in the peptide (51-52). Three major criteria are currently in use for the classification of the proteases:

5.1. Classification by reaction catalyzed, by reference to the reactions they catalyze. Classification according to the Enzyme Commission (EC), based on a systematic name and a four-digit code related to the type of activity catalyzed by the enzyme, and not according to its molecular properties. So, enzyme molecules of different origins may be classified by the same code as long as they catalyze the same reaction, including in the case of isoenzymes.

Proteases belongs to the class 3 or hydrolases (enzymes which split chemical bonds using water), subclass EC3.4.x.x, by the second digit showed those hydrolyzing peptide bonds. The third digit showed subsubclass, for proteases is 11-19, 21-24, and 99. The first group,11-19, are those enzymes hydrolyzing peptide bonds between amino acids at the amino and carboxylic end of the protein, they are named peptidases or exoproteases, by EC 3.4.11-14.x enzymes hydrolyze peptide bonds next to the amino terminus of the protein are called aminopeptidases and EC 3.4.15-17.x do this at the carboxy terminus of the protein are called carboxypeptidases. The second set, 21-24, are enzymes hydrolyzing internal peptide bonds, called proteinases or endoproteases, The last group, 99, is employed for enzymes whose mode of action remains unknown (51).

5.2. Classification according to chemical nature of the catalytic site the presence of essential catalytic residues at their active sites (which is the site of substrate binding). Four distict classes are known: serine-, cysteine or sulfhydryl or thiol-, aspartic or carboxyl or acidic- and metallo-proteases. For proteinases, they are classified in to 4 classes, while carboxypeptidases are divided in to 3 classes according to this criterion as showed in Table 3 (53).

Table 3 Subdivision of carboxypeptidases and proteinases according to catalytic type†

Group of Peptidases	EC sub-subclass
Carboxypeptidases	
Serine-type carboxypeptidases	3.4.16
Metallocarboxypeptidases	3.4.17
Cysteine-type carboxypeptidases	3.4.18
Proteinases	
Serine proteinases	3.4.21
Cysteine proteinases	3.4.22
Aspartic proteinases	3.4.23
Metalloproteinases	3.4.24
Proteinases of unknown catalytic mechnism	3.4.99

†See Ref.53.

5.3. Classification by evolutionary relationship, as revealed by structural relationships among the enzymes from the comparison of the sequences (53). Rawlings and Barrett used the term "family" to describe a group of enzymes, established a numbering system in which each family of peptidases was assigned a code constructed of a letter denoting the catalytic type (S, C, A, M or U for serine, cysteine, aspartic, metallo or unknown), follow by an arbitrarily assigned number. These will summarize the distinctive characteristics of serine peptidases, nearly as many families of cysteine peptidases, about 30 families of metallopeptidases, and also a number of families of aspartic peptidases and those of as yet unknown catalytic type (53). However, proteinases are currently grouped into six families (Table 4) that are broad groups of proteins for which there is evidence of an evolutionary relationship and whose sequences have about half of their amino acid residues in common (50, 51). There are two major families of serine proteinases, the chymotrypsin (mammalian) family and the subtilisin (bacterial) family. Although both of them have similar amino acids at the catalytic sites, their three-dimensional conformation show quite different (50, 52). Similary, the mammalian pancreatic carboxypeptidases and the bacterial enzyme thermolysin, each containing a catalytic indispenable zine atom, have similar active site configurations but bear no other structural or evolutionary relation to each other (50).

Table 4 Families of proteolytic enzymes according to evolutionary relationship†

Family	Example	Characteristic active site residues
Serine I (mammals)	Chymotrypsin* Trypsin	Asp(102);Ser(195);His(57)
Serine II (bacterial)	Subtilisin*	Asp(32);Ser(221);His(64)
Cysteine	Papain* Ficin	Cys(25);His(159);Asp(158)
Aspertic	Penicillopepsin* Pepsin Chymosin	Asp(33); Asp(213)
Metallo I (mammals)	Carboxypeptidase * Collagenase	Zn; Glu(270);Try(248)
Metallo II (bacterial)	Thermolysin* (*B thermoproteolyticus* neutral protease)	Zn;Glu(143);His(231)

* The sequence corresponds to this enzyme.

†See Ref.51.

6. Rubber Tree and Proteases Inhibitor(s)

As mentioned earlier, protease inhibitors are of great interest because they provide useful tools for its applications in many fields such as therapeutic agents in medicine, food technology, agriculture and in study of various physiological processes to improve knowledge of their mechanism. Then, the discovery of new protease inhibitors with particular kinetic properties might prove useful as biochemical tools in practical applications. Para rubber trees (*Hevea brasiliensis*), one of the most important commercial plants of Thailand, are very interesting to be good source of protease inhibitors, since they have been regularly induced for wounding response by a systematic "tapping" procedure in latex collection. Since rubber trees have economic life span in the range 25-35 years, this imply that we can regularly collect protease inhibitors for a long time until replanting of the rubber tree is necessary after yield fall to an uneconomical level. This also increased the value of rubber latex, particularly in non-rubber part of latex that is being wasted at present in industrial application of rubber.

7. Aims of Thesis

This project is aimed to address and answer these key questions;

1. To study the presence of proteases inhibitor (s) in the latex and its localization.
2. To characterize potency of PI activity and its specificity among the protease classes.
3. To characterize properties of purified PI (chemical nature, MW size, optimum conditions).

CHAPTER II

MATERIALS & METHODS

1. Chemicals

Chemicals and enzymes were obtained as follows: acrylamide, 2-mercaptoethanol Folin reagent , ammonium sulfate, citric acid, $NaH_2PO_4 \cdot 2H_2O$ were from Merck. Ammonium persulfate was from Carloerba. N,N′-methylene-bisacrylamide,TEMED, and Coomassie brilliant blue R-250 were from Fluka. EDTA, trichloroacetic acid (TCA),and sodium acetate were from Riedel-deHaen. Benzoyl-DL-arginine-*p*-nitroanilide(BAPNA), bovine casein, bovine hemoglobin, trypsin (bovine pancreas, EC 3.4.21.4), chymotrypsin (bovine pancreas, EC 3.4.21.1), papain (papaya latex, EC 3.4.22.2), pepsin (pepsin A from porcin stomach mucosa, EC 3.4.23.1), thermolysin (from *Bacillus thermoproteolyticus* rokko, EC 3.4.24.27), protease type XIII (from *Aspergillus saitoi*, EC 3.4.23.18), sodium dodecyl sulfate (SDS), Tris-HCl, glycine, isoelectric focusing marker kits, aprotinin from bovine lung (6.5 kD marker), bovine serum albumin (BSA), Bromophenol blue, and dimethyl sulfoxide(DMSO) were from Sigma Chemical Co. Acetic acid, acetone, and methanol were from Lab-Scan. Sephadex G-200, Sephadex G-75, standard molecular weight protein (for gel electrophoresis and gel filtration) were from Pharmacia. Azocoll and pronase (from *Streptomyces griseus*) were from Calbiochem-Behring corp. Glycerol, cysteine-HCl, and NaOH were from BDH-Chemicals Ltd. NaCl was from Univar.

Sodium carbonate was from Carlo Erba. $Na_2HPO_4 \cdot 2H_2O$ was from Ferak. All other reagent were of analytical grade.

2. Collection of Latex from Rubber Trees

All experiments were carried out with fresh latex from rubber trees of *Hevea brasiliensis* (clone RRIM 600) age about 20 years grown at Songkhla Rubber Research Center, Hat-Yai, Songkhla. The trees were tapped regularly on a half-spiral, alternate-day tapping system. Fresh latex was collected into ice-chilled plastic containers under the collecting tube of latex drops for 30 minutes per tapping tree. The pooled latex was kept in an ice container, brought to the laboratory and separated latex fractions at the same day.

3. Separation of Latex by Centrifugation

The pooled latex was sieved through cheese cloth to remove particulate materials and centrifuged on a Beckman model JA 2-21 centrifuge at 5,000 x g for 15 minutes at 4°C to separate bottom fraction from latex serum as soon as possible. The latex fractionated into 3 layers: the top zone (white and cream rubber layer), the middle zone is made up of the aqueous phase (milky serum) of the latex called C-serum and the relatively heavy bottom zone contain lutiod particles.

The bottom zone was further subjected to ultracentrifugation (Beckman model L8-70 M ultracentrifuge) at 59,000 x g for 45 minutes at 4°C and 4 main layers depend

on different density of compounds containing in the latex were obtained. The top white creamy layer consisted mainly of rubber particles, a thin layer of yellow Frey-Wyssling particles underneath the top layer, less of colorless serum fraction called C-serum and yellowish bottom fraction (largest part) containing largely lutoids.The middle zone was separated by the same method as the bottom zone but the largest part was obtained is C-serum whereas less of bottom fraction was appeared. Fig.1 showed separation of fresh latex by ultracentrifugation.

4. Preparation of C-serum and B-serum

4.1. C-serum

A spatula was used to make an opening in the rubber cream layer of the ultra-centrifuged latex. The milky top layer of the serum phase was carefully sucked out and discarded while the clear serum fraction was collected through a Pasteur pipette.

4.2. B-serum

A spatula was used to make an opening in the rubber cream layer, small volume of aqueous phase was discarded, and the rubber layer was then scooped out. A spatula was inserted to collected the pellet (bottom fraction) and lightly rinsed with 50 mM Tris-HCl, pH 7.5 containing 0.9% (v/w) NaCl to remove other parts of latex components. The bottom fractions were pooled and then subjected to freezing and thawing until most of them were lysed. After ultracentrifuged on a Beckman model L8-70 M ultracentrifuge at 59,000 x g for 45 minutes at 4°C, the yellow aqueous called B-serum was collected.

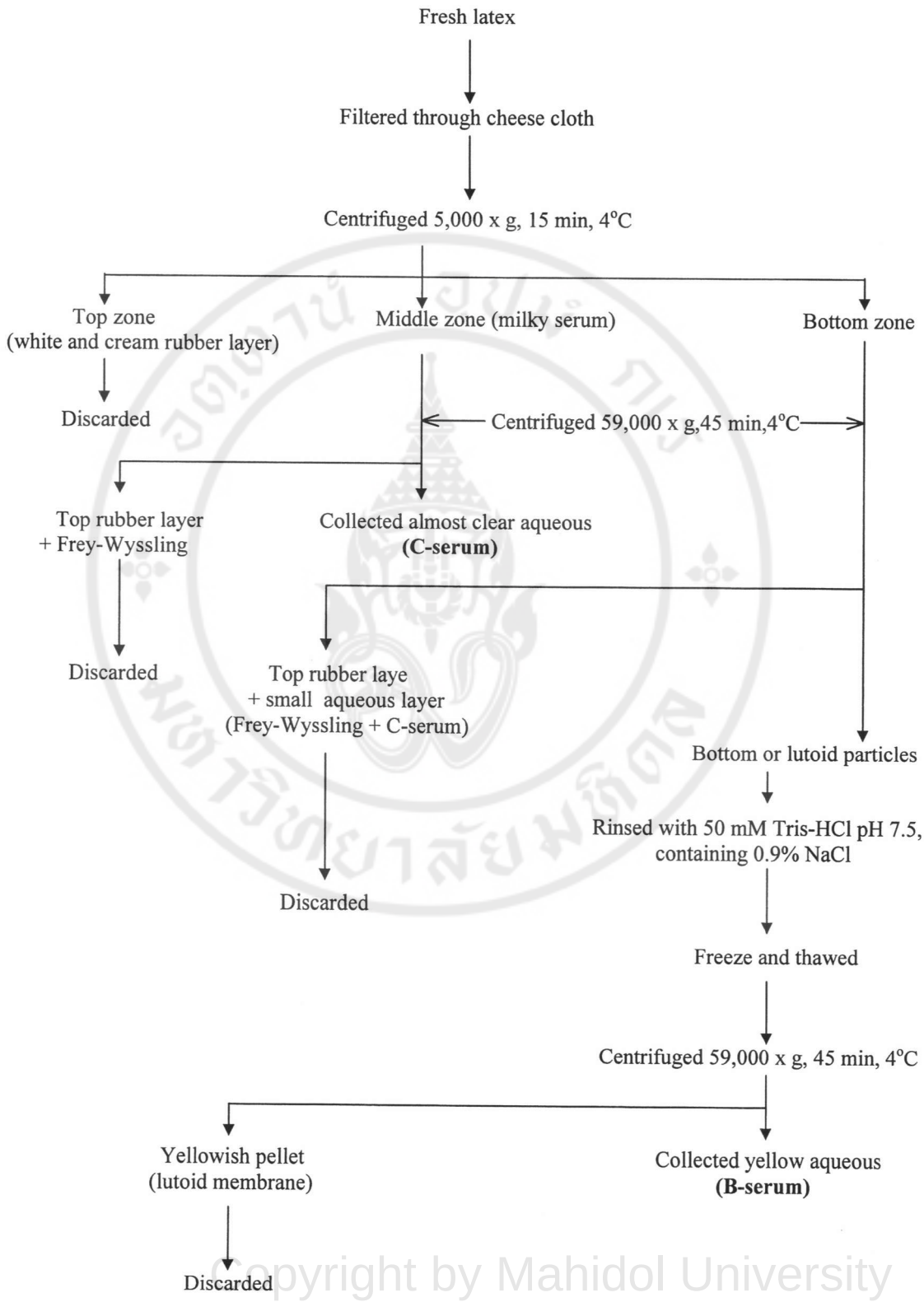

Fig 2. Summarized diagram of preparation of C-serum and B-serum.

5. Preparation of Protein Fractions from Ammonium Sulfate Precipctation of C-serum

Ammonium sulfate powder was added in C-serum up to percentage (w/v) that required, stirred in ice bath for 1 hr. and subjected to centrifuge (Beckman Model Avanti^{TM} 30 centrifuge) at 10,000 x g for 10 minutes at 4°C. The supernatant was subjected to sequential extraction with ammonium sulfate. The pellet was then resuspended in 50 mM Tris-HCl pH 7.5, desalted by passed through PD-10 column (Amersham Pharmacia), concentrated to appropriate volume by Speed Vac concentrator (Savant model Speed Vac SVC 100) and used for further studied.

Note: The amount of ammonium sulfate be added to the solution to give desired final concentration was showed in Table 5.

Table5 The amount of ammonium sulfate ($(NH_4)_2SO_4$) to be added to the solution (54)

Ammonium Sulfate, Grams to be Added to 1 Liter

From S_1% \ To S_2% →	5	10	15	20	25	30	35	40	50	55	60	65	70	75	80	85	90	95	100
0	27	55	84	113	144	176	208	242	314	351	390	430	472	516	561	608	657	708	761
5		27	56	85	115	146	179	212	282	319	357	397	439	481	526	572	621	671	723
10			28	57	86	117	149	182	251	287	325	364	405	447	491	537	584	634	685
15				28	58	88	119	151	219	255	292	331	371	413	456	501	548	596	647
20					29	59	89	121	188	223	260	298	337	378	421	465	511	559	609
25						29	60	91	157	191	227	265	304	344	386	429	475	522	571
30							30	61	126	160	195	232	270	309	351	393	438	485	533
35								30	94	128	163	199	236	275	316	358	402	447	495
40									63	96	130	166	202	241	281	322	365	410	457
45									31	64	97	132	169	206	245	286	329	373	419
										32	65	99	135	172	210	250	292	335	381
55											33	66	101	138	175	215	256	298	343
60												33	67	103	140	179	219	261	305
65													34	69	105	143	183	224	266
70														34	70	107	146	186	228
75															35	72	110	149	190
80																36	73	112	152
85																	37	75	114
90																		37	76
95																			38

6. Preparation of Protein Fractions from Actone Precipitation of C-serum and B-serum

Cold acetone was added into the sample solution (C-serum or B-serum) up to percentage (v/v) that required, stirred in ice bath for 10 minutes and subjected to centrifuge (Beckman model Avanti^{TM} 30 centrifuge) at 10,000 x g for 10 minutes at 4^{o}C. The supernatant was further extracted with cold acetone. The pellet was blowed with N_2 for a minute to remove acetone, resuspended in 50 mM Tris-HCl pH 7.5 and used for further studied.

Note: The amount of acetone to be added to the solution to give desired final concentration was showed in Table 6.

Table 6 The amount of acetone to be added to the solution (54)

Volume of Miscible Solvent, ml to be Added to 1 Liter

From C_1% \ To C_2% →	5	10	15	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95
0	52	111	176	250	333	428	538	666	818	1,000	1,222	1,500	1,857	2,333	3,000	4,000	5,666	9,000	19,000
5		55	117	187	266	357	461	583	727	900	1,111	1,375	1,714	2,166	2,800	3,750	5,333	8,500	18,000
10			58	125	200	285	384	500	636	800	1,000	1,250	1,571	2,000	2,600	3,500	5,000	8,000	17,000
15				62	133	214	307	416	545	700	888	1,125	1,428	1,833	2,400	3,250	4,666	7,500	16,000
20					66	142	230	333	454	600	777	1,000	1,285	1,666	2,200	3,000	4,333	7,000	15,000
25						71	153	250	363	500	666	875	1,142	1,500	2,000	2,750	4,000	6,500	14,000
30							76	166	272	400	555	750	1,000	1,333	1,800	2,500	3,666	6,000	13,000
35								83	181	300	444	625	857	1,166	1,600	2,250	3,333	5,500	12,000
40									90	200	333	500	714	1,000	1,400	2,000	3,000	5,000	11,000
45										100	222	375	571	833	1,200	1,750	2,666	4,500	10,000
50											111	250	428	666	1,000	1,500	2,333	4,000	9,000
55												125	285	500	800	1,250	2,000	3,500	8,000
60													142	333	600	1,000	1,666	3,000	7,000
65														166	400	750	1,333	2,500	6,000
70															200	500	1,000	2,000	5,000
75																250	666	1,500	4,000
80																	333	1,000	3,000
85																		500	2,000
90																			1,000

7. Gel Filtration

7.1. Sephadex G-200 Column Chromatography

The 0-60% ammonium sulfate fraction of C-serum proteins was applied to the Sephadex G-200 column (1.2 x 68 cm.). Column was equilibrated and eluted with 50 mM Tris-HCl pH 7.5 at a flow rate of 9 ml/hr. at 4°C. Fractions of 1.5 ml were collected until the absorbency at 280 nm fell below 0.05. Each 4 fractions were pooled, concentrated by Speed Vac concentrator (Savant model Speed Vac SVC 100) to an approximate volume 1 ml, and assayed protease inhibition (PI) activity.

Molecular weight estimation by gel filtration was determined, standard proteins used as references were catalase(MW 232,0000), aldolase(MW 158,000), albumin (67,000), ovalbumin(MW 43,000) and chymotrypsinogen(25,000). Blue dextran (MW 2×10^6) was used to determine the void volume (Vo) of the column. Total column volume (Vt) was determined by using potassium dichromate ($K_2Cr_2O_7$) (MW 294.19 g/mol). The Kav values were obtained by

$$Kav = \frac{Ve - Vo}{Vt - Vo}$$

The Kav values were plotted against their respective log molecular weights, and the molecular weight of the PI activity fractions were estimated.

7.2. Sephadex G-75 Column Chromatography

Proteins from 80-95% acetone fraction (HPI) were applied to the Sephadex G-75 column (1.6 x 85 cm.). Column was equilibrated and eluted with 50 mM Tris-HCl pH 7.5 at a flow rate 9 ml/hr, at 4°C. Fractions of 1.5 ml were collected until the absorbency at 280 nm fell below 0.01. Peak and valley fractions were concentrated by Speed Vac concentrator (Savant model Speed Vac SVC 100) to an approximate volume of 0.5 ml, changed with the same buffer pass through PD-10 column (Amersham Pharmacia) prior to determining protein content, PI activity and SDS-PAGE.

Molecular weight estimation by Sephadex G-75 was determined, standard proteins used as references were bovine serum albumin (MW 67,000), ovalbumin (MW 43,000), chymotrypsinogen A (MW 25,000) and ribonuclease A (MW 13,700). Blue dextran (MW 2 x 10^6) was used to determined the void volume (Vo) of the column. Total column volume (Vt) was determined by using potassium dichromate ($K_2Cr_2O_7$, MW 294.19 g/mol). Standard curve was plotted and MW estimation were determined in the same method as described in part 7.1.

8. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to the modified method of Laemmli (55). The separation slab gel (10 x 8 x 0.1 cm.) was casted in an exponential linear gradient fashion from 10% to 20% (or 7% to 15%) of acrylamide concentration using a

standard 2 chambered plexigels gradient mixer and containing 3% (w/v) acrylamide of staking gel (10 x 2 x 0.1 cm.). The compositions of both separation and stacking gels were summarized in the following table:

Table 7 Recipes for polyacrylamide separating and stacking gels

Stock solutions (µl)	Stacking gel (3%)	Linear gradient of separating gel			
		7%	15%	10%	20%
30%acrylamide+0.8%bisacrylamide	300	700	1,500	1,000	2,000
1.5 M Tris-HCl pH 8.9	-	750	750	750	750
0.5 M Tris-HCl pH 6.8	750	-	-	-	-
10% SDS	30	30	30	30	30
Distilled water	1,745	1,440	640	1,140	140
0.2 M EDTA pH 7.0	20	-	-	-	-
*1% ammonium persulfate	150	75	75	75	75
*TEMED	5	5	5	5	5
Total volume	3,000	3,000	3,000	3,000	3,000

* Added to chemically the gel within 10 minutes.

Preparation of Sample for SDS-PAGE

The protein samples were mixed with sample buffer (with final concentration of 0.0627 M Tris-HCl buffer pH6.8, 10% (v/v) glycerol, 1% (w/v) sodium dodecyl sulfate (SDS), 1% (v/v) 2-mercaptoethanol and 0.0005% (w/v) bromophenol blue as the dye (56), heated sample for 10 minutes in boiling water, spun down protein solution for a minute and loaded into well.

Running Condition

Electrophoresis was performed at room temperature with the anode in the lower chamber, 14 mA of constant current per slab gel was applied until the tracking dye was approached the bottom of the separating gel.

Note: Electrophoresis buffer pH 8.3 was contained 0.025 M Tris-HCl,
0.192 M glycine and 0.1% (w/v) SDS.

Coomassie Staining

The electrophoresis gel was fixed and stained with staining solution (0.2% (w/v) Coomassie brilliant blue R-25, 50% (v/v) methanol and 10% (v/v) acetic acid) for 2 hr. Destaining of excess dye was performed by repeating changes of destaining solution (20% (v/v) methanol and 10% (v/v) acetic acid) until the background was clear.

8.1. Subunit Molecular Weight Determination by SDS-PAGE

To determine molecular weight of each protein, sample was performed using linear gradient 10% to 20% of acrylamide concentration. The standard markers consisted of phosphorylase b (94 kD), bovine serum albumin (67 kD), ovalbumin (43 kD), carbonic anhydrase (30 kD), soybean trypsin inhibitor (20 kD), and α-lactalbumin (14 kD). Relative migration values (Rf) were against the log MW of standard marker and the molecular weight of sample was estimated.

$$Rf = \frac{\text{distance protein has migrated from origin}}{\text{distance from origin to reference point (the tracking dye)}}$$

9. Polyacrylamide Gel Isoelectric Focusing

Isoelectric focusing was performed in BIO-RAD minigel IEF apparatus (model 111 Mini IEF Cell) according to the manufacturer's instructions (BIO-RAD) (57).

Casting Polyacrylamide Gel

Monomer- ampholyte solution 3.2 ml contained 0.64 ml of monomer concentrated stock solution (24.25% (w/v) acrylamide and 0.75% (w/v) bis (N,N′- Methylene-bis-acrylamide)), 0.64 ml of 25% (v/v) glycerol, 0.16 ml of Bio-lyte ampholytes 3/10 and deionized water 1.76 ml was mixed gently and degassed for 5 minutes. The catalyst solution 31 µl contained 10 µl of 10% (w/v) ammonium persulfate, 16 µl of 0.1% (w/v) riboflavin and 5 µl of TEMED was added to the degassed monomer and swirl

gently. The solution was carefully introduced into the casting tray using a pipette. The gel was photopolymerized for 45 minutes at room temperature. The protein sample and standard markers were directly applied to the gel surface and left to diffuse into the gel for 5 minutes.

Running Condition

Focusing was carried out under constant voltage conditions in a stepped fashion, started at 100 V for 15 minutes, increased voltage to 200 V for 15 minutes and finally 450 V for 60 minutes.

Band Detection

After electrofocusing was completed, the focusing gel was fixed immediately in 10% (w/v) trichloroacetic acid (TCA) for 10 minutes, replaced with 1% (w/v) TCA and soak for 2 hr. to remove ampholytes. The protein band in focusing gel were stained in staining solution contained 0.04% (w/v) Coomassie brilliant blue R-250, 27% (v/v) isopropanol, 0.5% (w/v) $CuSO_4$ and 10% (v/v) acetic acid for 1-2 hr. Destaining was performed by immerged the gel in destaining solution I contained 12% (v/v) isopropanol, 7% (v/v) acetic acid and 0.5% (w/v) $CuSO_4$ with 2-3 changes of this solution until the background was nearly clear. The gel was immersed in destaining solution II contained 25% (v/v) isopropanol and 7% (v/v) acetic acid to remove the last traces of stain and $CuSO_4$. Determination pI value was performed according to the method in Sigma General catalog, 1995.

The IEF markers (Kit for isoelectric focusing rang 3.6-9.3, IEF-M1A, Sigma) were used for determination pI value of protein sample, contained amyloglucosidase

from *Aspergillus niger* (pI 3.6), soybean trypsin inhibitor (pI 4.6), β-lactoglobulin A from bovine milk (pI 5.1), carbonic anhydrase II from bovine erythrocytes (pI 5.9), carbonic anhydrase I from human erythrocytes (pI 6.6), myoglobin from horse heart (pI 6.8 and 7.2), lectin from *Lens culinaris* (pI 8.2, 8.6 and 8.8), trypsinogen from bovine pancreas (pI 9.3) and Methyl Red (marker dye, pI 3.8). The relative pI value of protein sample was estimated from the standard protein marker calibration curve. This curve was constructed by plotting the distances from anode (cm) of standard marker proteins against its pI.

10. Protein Determination

Protein concentration was determined according to the method of Lowry *et al.* (1951), with bovine serum albumin as standard (58).

11. Protease Inhibition Assays

11.1. Screening and Detection for the Presence of Protease Inhibitors in C-serum and B-serum

Protease inhibition activity in both samples source (C-serum and B-serum) were detected by measuring the decrease in activity of pronase on the Azo dye imprenated collagen (Azocoll) when compared to the control. The method of manufacturer's instruction (Calbiochem-behring corp.(59), 60) was used, with modifications as described. C-serum or B-serum 300 µl was preincubated with 100 µl of pronase (0.1

mg/ml) in a total volume of 500 µl containing 50 mM Tris-HCl pH 7.5 for 30 minutes at 37°C. Using buffer instead of C-serum or B-serum performed control. The enzyme reaction was initiated by the addition of 500 µl Azocoll solution (0.5% w/v in 50mM Tris-HCl pH 7.5), further incubated for 15 minutes at 37°C. The reaction was terminated by immerged tubes in an ice bath for 5 minutes, centrifuged for a minute to sediment substrate, the supernatant that contained soluble products produced during Azocoll degradation was estimated by measuring the OD at 520 nm (Beckman model DU® Series 650 i spectrophotometer). An appropriate sample blanks were prepared for each sample. One unit of pronase inhibitory activity (PI) was defined as the amount of inhibitor, which depresses absorbancy at 520 nm by 0.1 OD of the control value under the assay condition.

11.2. Trypsin Inhibition Assay

Trypsin inhibition was detected by measuring the decrease in enzyme activity on the chromogenic substrate benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) when compared to the control. The method of Kakade *et al.* (61) was used, with modifications as described. The BAPNA (30 mg) was dissolved in 1 ml of dimethyl sulfoxide (DMSO) as substrate stock solution and subsequently diluted to 100 fold with 50 mM Tris-HCl pH 8 before used. The suitable amounts of inhibitor-containing sample was preincubated for 30 minutes at 37°C with 75 µl of 0.1 mg/ml (w/v) trypsin in a total volume of 300 µl containing 50 mM Tris-HCl pH 8. Using buffer instead of the sample performed control.The enzyme reaction was initiated by the addition of 500 µl substrate solution, further incubated for 20 minutes at 37°C. The reaction was terminated by added 130 µl of 30% (v/v) acetic acid and the release of *p*-nitroaniline

(*p*NA) was estimated by measuring the OD at 410 nm (Beckman model DU® Series 650 i spectrophotometer). An appropriate sample blanks were prepared for each sample. One unit of trypsin inhibitor activity (PI) was defined as the amount of inhibitor, which decreases absorbancy at 410 nm by 0.1 OD of the control value under the assay condotion.

12. Thermal Stability of *Hevea* Protease Inhibitor

12.1. Thermal Stability of C-serum Protease Inhibitor (CS-PI)

For studying the effect of heat treatment on the protease inhibitory activity of *Hevea* protease inhibitor in crude C-serum (CS-PI), the aliquots of C-serum were incubated at various temperatures at 65, 75, 85, and 95 °C for 15 and 30 minutes in a Reciprocating shaking water bath model RW 1812. The contents were immediately immerged in an ice bath, centrifuged at 5,800 x g for 10 minutes (Beckman model Avati™ 30 centrifuge). The supernatant of samples was assayed for residual inhibitory activity in the same method as described in 11.1. The C-serum (kept at 4 °C before assayed) was used as 100% PI activity control and expressed the results as percentages of the control.

In addition, heat stability of protease inhibitors in crude C-serum was studied as a function of time. The aliquots of C-serum were heated in boiling water for various times ranging from 30, 45, 60 and 90 minutes. The contents were cooled and assayed for residual inhibitory activity in the same method as described in 11.1.

12.2. Thermal Stability of Purified *Hevea* PI

The PI proteins of paek1 and peak2 from Sephadex G-75 column 23 µg in a volume 120 µl of 50 mM Tris-HCl pH 7 were incubated at various temperatures at 50, 60, 70, 80, 90 and 100°C for 30 minutes in a Reciprocating shaking water bath model RW 1812. The contents were cooled, added 355 µl of 50 mM Tris-HCl pH 8 and assayed for residual inhibitory activity in the same method as described in 11.2. The PI proteins of peak1 and 2 (4°C) were used as 100% PI activity control for each of them. The results were expressed as percentages of its control.

13. pH Stability of *Hevea* Protease Inhibitors HPI-1 and HPI-2

Effect of pH on the inhibitory activity of HPI-1 and HPI-2 derived from Sephadex G-75 column were studied by preincubating at various different pH from pH 3 to pH 12 of 100 mM buffers (acetate pH 3-6, citrate-phosphate pH 3-7, phosphate pH 5-8, Tris-HCl pH 7-10 and glycine-NaOH pH 10-12). Each of 10 µl HPIs proteins solution, HPI-1 (3.25 mg/ml) and HPI-2 (4.31 mg/ml) was mixed with 60 µl of each buffer and 50 µl of distilled water (DW). After incubation for 24 hr. at 37°C, each of the mixture was readjusted to pH 8 with 355 µl of 50 mM Tris-HCl pH 8. Then 75 µl of 0.1 mg/ml trypsin was added and assayed for residual inhibitory activity as described in 11.2. The HPI-1 and HPI-2 (kept in 50 mM Tris-HCl pH 7.5, 4°C) were used as 100% PI activity control for each of them. The results were expressed as percentages of its control.

14. Screening Experiment on Different Protease Classes of HPI

Commercially available proteases (trypsin, chymotrypsin, thermolysin, pronase, papain, pepsin and protease type XIII (from *Aspergillus saitoi*)) were used in the assays

14.1. Using Casein as Substrate

Inhibition activity against trypsin, chymotrypsin, thermolysin, pronase and papain were determined by caseinolytic assay (36, 62, 63) with the following modification. The suitable amounts of inhibitor-containing samples were preincubated with each enzymes in a total volume of 50 µl containing buffer each enzyme at 37°C for 20 minutes. Using buffer of each enzymes instead of the sample performed controls. The enzymes reaction were initiated by the addition of 100 µl substrate solution (2%(w/v) of casein in each buffer), further incubated for 20 minutes at 37°C. The reactions were terminated by added 200 µl of 2% (w/v) TCA, centrifuged at 8,900 x g for 10 minutes (Beckman model Avati^{TM} 30 centrifuge). Then 100 µl samples of the supernatant were mixed with 500 µl of 0.4 M sodium carbonate,left for 10 minutes , added 100 µl of Folin reagent (diluted 1:4) and mixed thoroughly. The mixtures were left for 30 minutes and measured the OD at 660 nm (Beckmanmodel DU® Series 650 i spectrophotometer). An appropriate sample blank was prepared for each sample. One PI unit was defined as amount of inhibitor, which decreases absorbancy at 660 nm by 0.1 OD of its original value (enzyme activity or positive control) under the assay conditions.

Note:

- Trypsin and chymotrypsin used 50 mM Tris-HCl pH 8 with 0.02 M $CaCl_2$ as buffer.
- Thermolysin used 50 mM Tris-HCl pH 8 with 0.1 M NaCl and 0.01 M $CaCl_2$ as buffer.
- Papain used 100mM Phosphate pH 6 with 3.8 x 10^{-4}M EDTA, 1.9 x 10^{-3}M Cysteine-HCl as buffer.
- Pronase used 50 mM Tris-HCl pH 7.5 as buffer

14.2. Using Hemoglobin as Substrate

Inhibition activity against pepsin and protease type XIII (from *Aspergillus saitoi*) were determined according to Anson method (62), using hemoglobin as substrate with the following modification. The suitable amounts of inhibitor-containing samples were preincubated for 20 minutes at 37 °C with each enzymes (pepsin or protease type XIII) in a total volume of 175 µl containing 100 mM acetate buffer pH 4.5. Using buffer instead of the samples performed controls.The enzymes reaction were initiated by the addition of 125 µl substrate solution (2% (w/v) hemoglobin), buffer incubated for 10 minutes at 37°C. The reactions were terminated by added 500 µl of 5% (w/v) TCA, centrifuged at 4,000 x g for 20 minutes (Beckman model Avati™ 30 centrifuge). Then 250 µl samples of the supernatant were mixed with 500 µl of 0.5 N NaOH, left for 10 minutes, added 100 µl of Folin reagent (diluted 1:2) and mixed thoroughly. The mixtures were left for 30 minutes and measured the OD at 750 nm (Beckman model DU® Series 650 i spectrophotometer). An appropriate sample blank was prepared for each sample. One PI unit was defined as amount of inhibitor, which decreases

absorbancy at 750 nm by 0.1 OD of its original value (enzyme activity or positive control) under the assay conditions.

CHAPTER III

RESULTS

Plants react to wounding and pathogen attacks by a combination of two defense mechanisms: [1] structural characteristics that act as physical barriers and prevent the pathogens from gaining entrance and spreading though the plant and [2] biochemical reactions that take place in the cells and tissues of the plant and produce substances which either are toxic to the pathogen or create conditions that inhibit growth of the pathogen in the plant (12). During the massive shift in cellular metabolisms and genes expression, plant synthesizes many novel proteins following attack by pathogens, stress, or wounding, collectively called pathogenesis-related proteins (PR proteins). The several groups of proteins have been classified according to their function, serological relationship, amino acid sequence, molecular weight and certain other property (12). Proteinaceous inhibitors of proteases or proteinases can serve as one of the PR-proteins in defense mechanisms of plants.

Tappings of rubber trees for rubber latex collection cause wounding to their trunks, This will lead to systemic induction for defense wounding response and inhibition of pathogen attack. The presence of subtilisin as protease inhibitor in *Hevea* rubber latex was first reported in 1982 but the defense role was not noted (64). In the present study, we made an attempt to look for novel proteases inhibitor(s) in rubber

latex as being induced by wounding response. The results in this study were quite conformed to our working hypothesis and we have found a novel proteinaceous proteases inhibitor with small molecular weight in C-serum of the rubber latex. The induced protease inhibitor (PI) was then purified, characterized for its various different properties as described in the following studies and the resulted data in each part.

Part 1. Screening and Detection for the Presence of Protease Inhibitors (PIs) in *Hevea* Latex

Fresh natural rubber latex derived from tapping of rubber trees (*Hevea brasiliensis*) contains about 1-1.8 % proteins depending on the sample sources of which 27.2 % is in the rubber fraction, 47.5 % in the aqueous serum phase (C-serum) and 25.3 % in the bottom fraction (lutoid particles) which contain B-serum (65). Proteins content of the latex was found to have clonal variations. The search for the presence of protease inhibitor (PI) was focused on the non-rubber or the soluble component of latex. Since both B-serum and C-serum are major components of the non-rubber constituent which contain most of the soluble proteins mixtures in rubber latex. They are being screened for the presence and localization of the PI. Natural endogenous protease inhibitors from various different specimens appear to be of mostly proteins in nature. We started the investigation of the protease inhibitors from these two fractions(B and C-serum) for both of the protein as well as the non-protein nature for its presence in latex.

1.1. Screening for the Presence of Protease Inhibitors (PIs)

Non rubber fractions, C-serum and B-serum (form lutoid particles), prepared by ultracentrifuge of fresh latex were used as the starting sources for detection of the protease inhibitory activity. Since pronase is the mixture of several proteases in the same preparation, it is therefore very suitable for the screening experiments. Azocoll is dye-impregnate protein hide powder that is suitable as a non-specific substrate for various proteases activity assay. They were then used for screening experiments for the assays detecting PIs in *Hevea* latex.

The results showed both C-serum and B-serum have PIs activity that can be detected. The PIs activity was expressed as pronase activity inhibition unit (1 unit is defined as the amount of inhibitor which decreases absorbancy at 520 nm by 0.1 OD of the control value under the assay condition). The differences of PIs activity between C-serum and B-serum were observed which could be caused by variability of latex and the source of samples. However, the presence in C-serum of much more PIs activity than B-serum was consistently observed and noted as shown in Table 8, Fig.3 and Fig.4. These results thus suggested that PI was induced as wounding response from tapping cuts and being accumulated in the latex. These results are quite different from the previous report by Archer (64) where no PIs activity could be detected in the lutoid (or B-serum). Possibly, this is due to using different assay methods (such as enzyme, substrate and other assay conditions) resulted in different sensitivity and spectrum of PI detection. Since, our results clearly indicated that C-serum had much more PIs activity than B-serum coupled with previous report that little or no PI can be detected in B-serum, the scope of further study in this thesis will be mainly focused on C-serum. The data presented in this study are of the C-serum protease inhibitor

(CS-PI) properties and its characterization, unless otherwise indicated if B-serum is referred to for comparison.

Table 8 Inhibition of pronase activity by C-serum and B-serum from different samples of latex as to different rubber tree collection

Sample	Type of sample	Inhibitor activity* (units)	%Inhibition	% Remaining enzyme activity
1	Control	0	0	100
	C-serum	3.51	89.73	10.27
	B-serum	2.78	70.99	29.01
2	Control	0	0	100
	C-serum	5.91	92.02	7.98
	B-serum	3.86	60.01	33.99
3	Control	0	0	100
	C-serum	6.33	99.56	0.44
	B-serum	5.18	81.45	18.55

*1 unit is defined as the amount of inhibitor, which decreases absorbancy at 520 nm by 0.1 OD of its original value in the assay condition.

The results were plotted as showed in Fig.3 and 4.

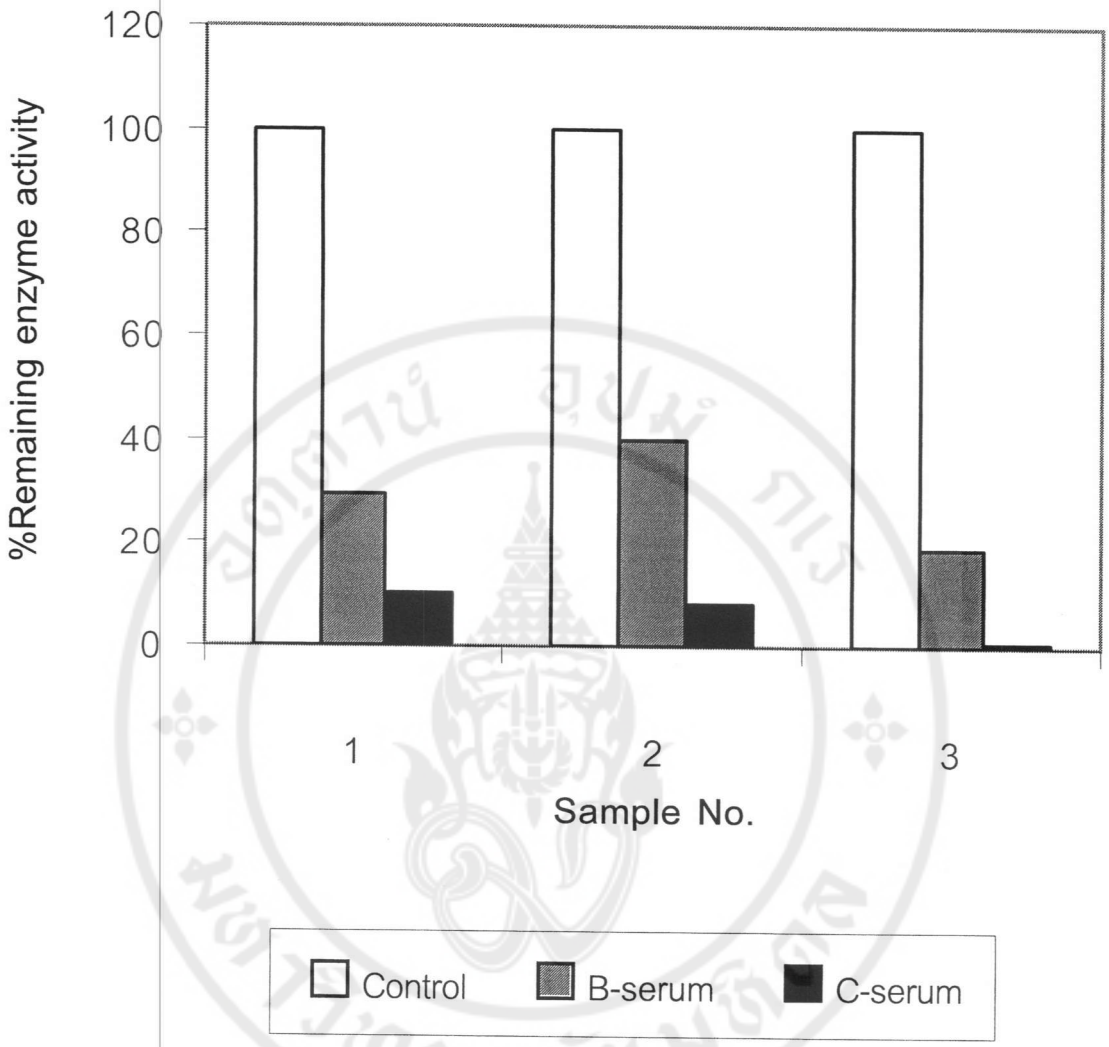

Fig 3. Comparison of pronase activity inhibition by C-serum and B-serum

C-serum and B-serum were each preincubated with pronase for 30 minutes at 37°C first and the assay was started by addition of the Azocoll substrate, further incubated for 15 min before % remaining enzyme activity was determined. The results were expressed as the percentage of remaining enzyme activity when compared to the control which used 50 mM Tris-HCl pH 7.5 buffer instead of B-serum or C-serum and expected to be 100% enzyme activity.

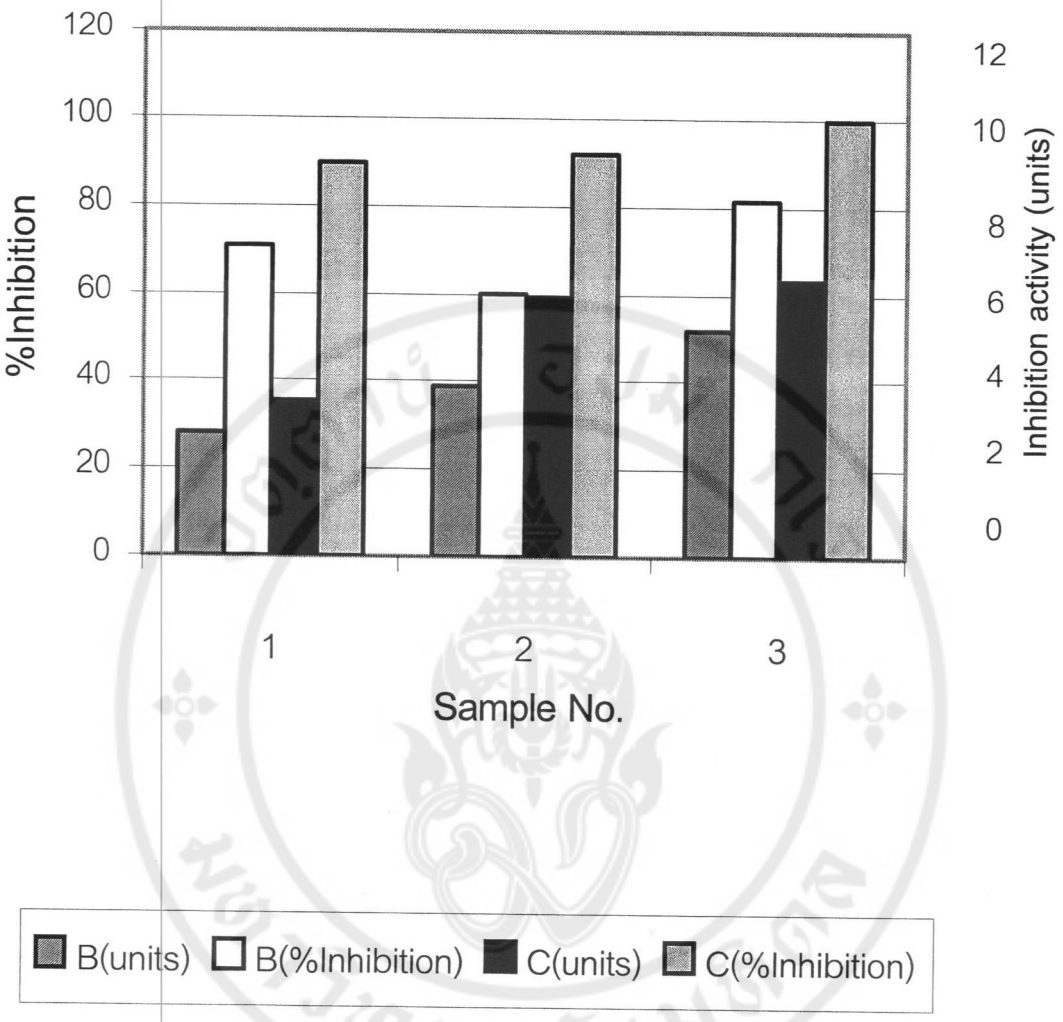

Fig.4 Correlation between PI units and % inhibition of pronase activity by B-serum and C-serum

The results were shown correlation between pronase activity inhibition unit (1 unit is defined as the amount of inhibitor which decreases absorbancy at 520 nm by 0.1 OD of the control value under the assay condition) and % pronase activity inhibition (when compared to the control which used 50 mM Tris-HCl pH 7.5 buffer instead of B-serum or C-serum and expected to be 100% enzyme activity.or 0% inhibition) of B-serum and C-serum.

1.2. Properties of the C-serum Protease Inhibitors (CS-PI)

1.2.1. Heating Effect on the C-serum Protease Inhibitors

The effect of temperature on protease inhibitory activity of CS-PI was studied by heating pretreatment of C-serum at various temperatures ranging from room temperature (RT) up to 95 °C. The duration of heating was either 15 min or 30 min then being cooled to RT. Pronase was tested for the remaining activity of CS-PI after the heat treatments. The results are shown in Fig.5 and summarized for both temperature variations at different times in Table 9. The results for heating times either at 15 min or 30 min were almost the same. It was found that the CS-PI was stable up to 65 °C without any loss of the activity. At temperature above 65 °C the CS-PI started to lose certain amount of its activity. A loss of 40% PIs activity occurred at 75 °C heating. Further heating up to 95 °C showed no further loss with about 60% remaining PIs activity at 95 °C similar to that observed at 75 °C. The results suggested that there might exist 2 forms of PIs in the C-serum. One is stable up to 65 °C and constituted about 40% of the total PIs activities. The other PI is a very heat stable component even at 95 °C, which comprises 60% of the total PIs activities in the C-serum. These results were compared for both 15 min and 30 min heating at various temperatures in Fig.5 and are summarized with calculated remaining PIs activity for comparison as shown in Table 9.

1.2.2. Effect of Boiling on C-serum Protease Inhibitors Stability

To test further the heat stable property of the CS-PI, it was subjected to heating in the boiling water for varying time up to 90 min. and checked for the remaining PIs activity. The results are shown in Fig.6 and summarized in Table 10. It

was found that the heat stable fraction of the CS-PI could withstand the boiling effect even up to 90 min with still 50% PIs activity remaining. This result as shown in Fig.6 and Table 10 indicated that the CS-PI was the thermostable molecule with a very strong heat stable property. The molecular and type of the CS-PI will be further characterized and identified for the detailed properties. The results from these studies suggested at least two possibilities or even more. First possibility is that there might exist 2 groups of PIs in the C-serum, a moderate and a very high heat stable PIs. The second possibility is that only one group is present but the heating effect could only destroy 50% of the PIs activity. However, this is less likely for any good explanation of the observed results. Therefore, we will concentrate more on the first possibility and carry out further experiments on the heat stable fraction of CS-PI. This is quite obvious for the advantage in working with this heat stable group, both in purification protocol as well as the case of handing and processing and superior quality of the heat stable PI as derived from natural source for possible application in various different uses in biotechnology and other practical implications.

Table 9 Pronase activity inhibition of C-serum remained after heating at varying temperatures

Temperature (^{o}C)	%Remaining PI activity	
	Time (min)	
	15	30
Control	100	100
65	100	98
75	62	63
85	64	68
95	63	65

Note: The aliquots of C-serum were incubated at various temperatures for 15 and 30 minutes and immediately immerged in an ice bath before test PI activity. The control was C-serum that kept at 4^{o}C before test and its activity was defined as 100% remaining PI activity. The results are plotted as shown in Fig.5.

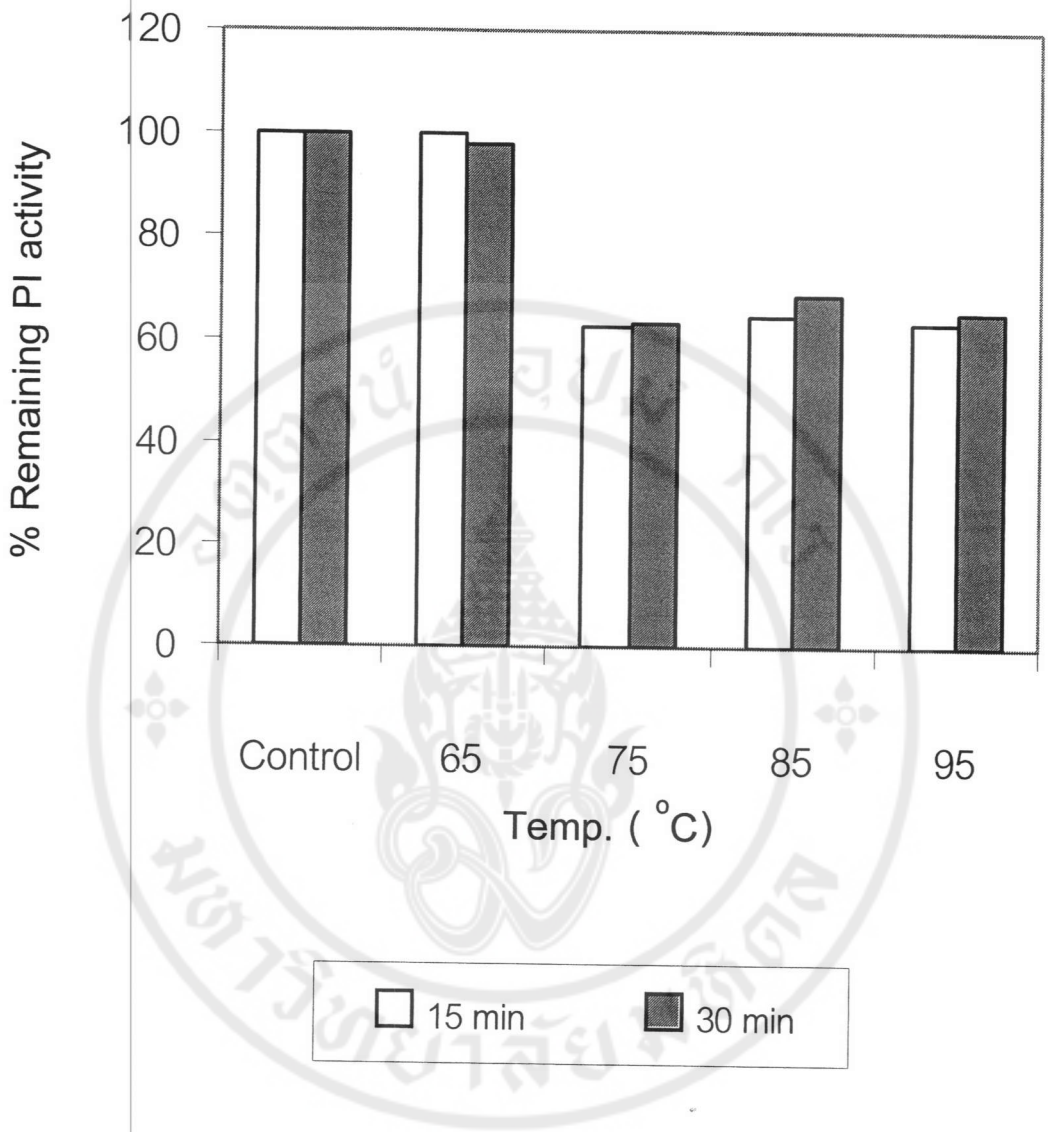

Fig.5 Pronase activity inhibition of C-serum remained after heating at varying temperatures

The C-serum was incubated at various temperatures ranging from room temperature (RT) up to 95 °C. The duration of heating was either for 15 min or 30 min then being cooled to RT before determined % remaining PI activity. The results were expressed as the percentage of remaining PI activity when compared to the control which C-serum (kept at 4 °C before assayed) was used as 100% PI activity.

Table 10 Pronase activity inhibition of C-serum remained after heating sample in boiling water at varying time

Time (min)	%Remaining PI activity
0	100
30	54.18±5.62
60	49.57±4.63
90	49.73±5.16

Note: The aliquots of C-serum were immerged in boiling water at varying time as indicated in the method and immediately immerged in an ice bath before test PI activity. The results are expressed as the percentage of remaining PI activity when compared to the control. The average values from three experiments are plotted as shown in Fig.6.

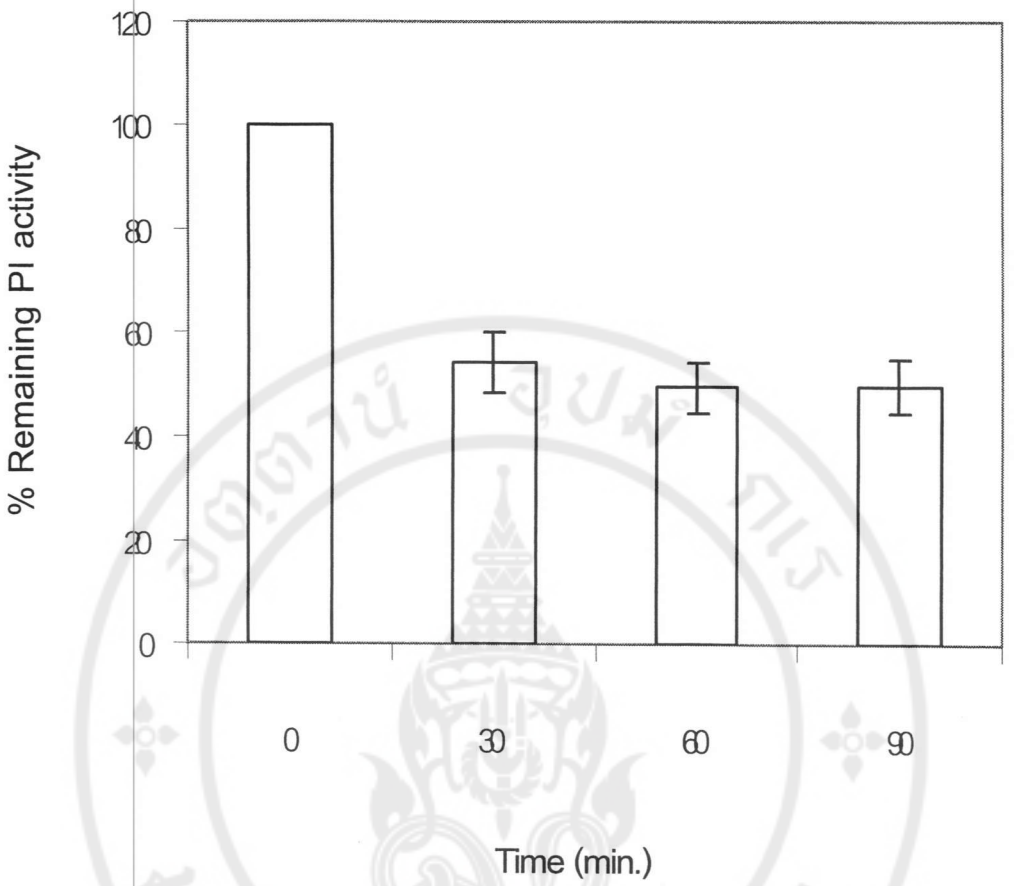

Fig.6 Pronase activity inhibition of C-serum remained after heating sample in boiling water at varying time

C-serum was heated in boiling water for varying time up to 90 min then being cooled to RT before determined % remaining PI activity. The results are expressed as the percentage of remaining PI activity when compared to the control which C-serum (kept at 4 ^{o}C before assayed) was used as 100% PI activity.

Note: The standard deviations in the figure were calculated from the data with three experiments.

Part 2. Characterization of Properties of PIs in the C-serum

2.1. Macromolecular Nature of PIs

The molecular nature of CS-PI was first characterized for its sizes to designate it as macromolecule or small organic compound. The size exclusion of Sephadex gel filtration was used for this simple differentiation.

In general, Amersham Pharmacia column PD-10 was used for desalting and buffers exchange of protein samples which will be eluted out as the first few fractions with almost 100% recovery of the proteins. To identify whether PIs in C-serum are macromolecule or a low molecular weight compound, the C-serum was loaded on to Amersham Pharmacia column PD-10 and fractions were collected and detected for the PIs activity. The results are shown in Fig.7 and summarized for further details in Table 11. The result showed that the elution with protein bulk had strong PIs activity in the early fractions of eluted protein using BSA as protein marker. (Table 11 and Fig.7). Thus, the PIs in C-serum were clearly shown to be macromolecule, not a small organic molecule as no PIs activity could be detected in the later fractions. The results of this experiment indicated that the CS-PI is protein in nature which would be further tested and employed for the purification protocol.

Table 11 Pronase activity inhibition of C-serum after eluted from PD-10 column

Fraction No.	Inhibition activity (units*)	%Inhibition	%Remaining enzyme activity
Control	0	0	100
1	0	0	100
3	0	0	100
5	1.98	54.98	45.02
7	2.17	60.07	39.93
9	2.27	62.89	37.11
11	1.90	52.65	47.35
13	1.23	34.15	65.85
15	1.35	37.39	62.61
17	0.99	27.30	72.70
19	0.57	15.65	84.35
21	0.41	11.42	88.58
23	0.46	12.78	87.22
25	0.48	13.30	86.70
27	0.41	11.48	88.52
29	0.28	7.77	92.23
31	0	0	100
33	0	0	100
35	0	0	100
C-serum	0.27	73.62	26.38
BSA[#]	0.08	2.10	97.90

*1 unit is defined as the amount of inhibitor, which decreases absorbancy at 520 nm by 1 OD of its original value in the assay condition.

[#]Stock solution = 20 mg/ml

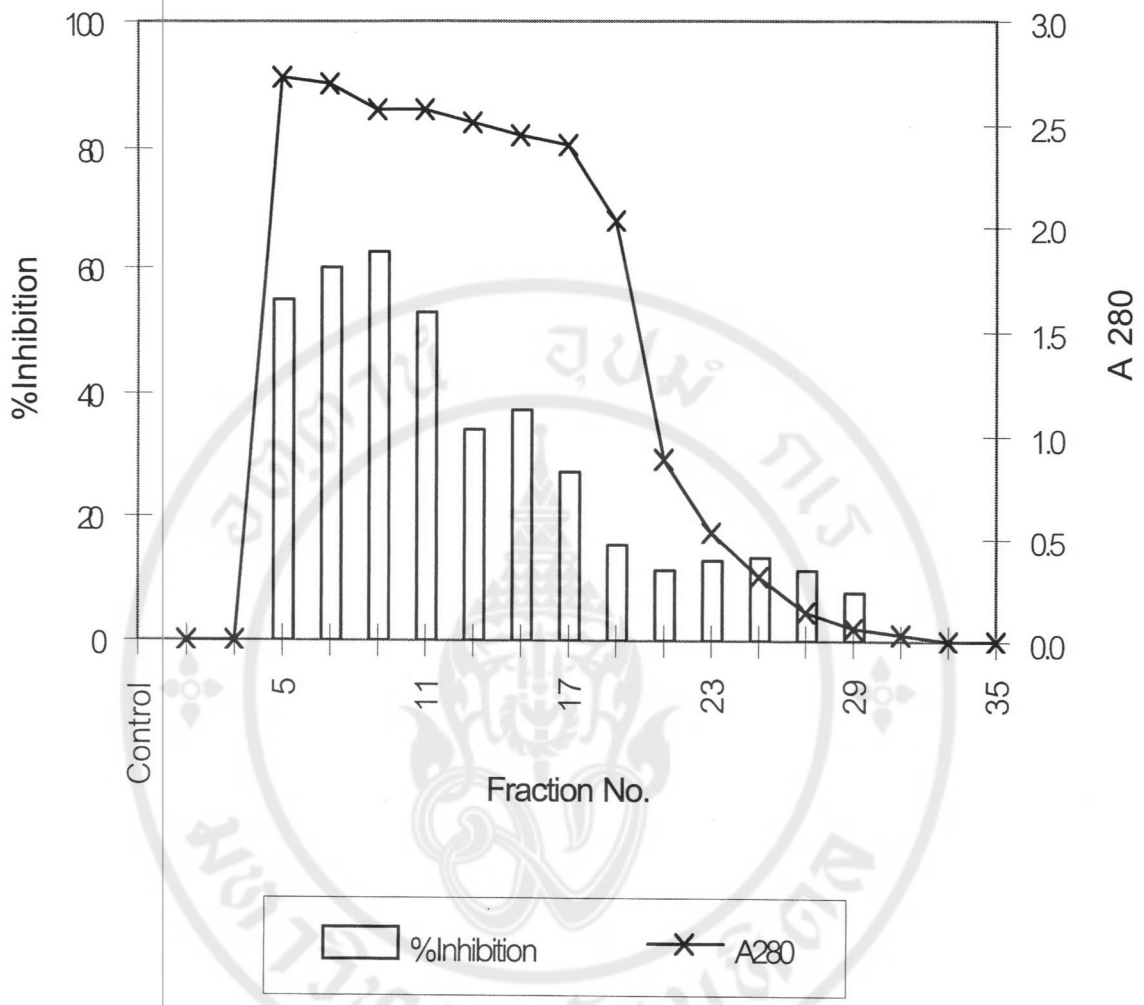

Fig.7 Elution profile of C-serum on a PD-10 column and pronase activity inhibition.

C-serum was loaded on to PD-10 column pre-equilibrated with 50 mM Tris-HCl pH 7.5. Fractions of 0.5ml were collected until the effluent absorbency at 280 nm approached zero and detected for PI activity toward pronase for each odd number fractions. The results were expressed as the percentage of pronase activity inhibition when compared to the control which used 50 mM Tris-HCl pH 7.5 buffer instead of sample fractions and expected to be 100% enzyme activity or 0% inhibition.

2.2. Ammonium Sulfate Precipitation of the C-serum PIs

The protein nature of CS-PI was employed for separation from the bulk of C-serum proteins by using ammonium sulfate precipitation methods. 20% interval of ammonium sulfate concentration was initially employed for separation of PI and the results are shown in Fig.8, Fig.9 and detailed summary in Table 12. C-serum was sequentially fractionated in 4 wide ranges of ammonium sulfate precipitations (0-20%, 20-40%, 40-60% and 60-80% fractions) and tested for PIs activity. The maximum PIs activity was found in the range 40-60% ammonium sulfate (Fig.8, Fig.9 and Table 12) but was also found in considerable amount in the other two ranges (20-40%, 60-80%). These results indicated that the PI could be easily precipitated by ammonium sulfate from 20% to 80%, suggesting that the separation by ammonium sulfate precipitation is rather indiscriminating. Nonetheless, this result confirmed their macromolecule nature as determined by using PD-10 and suggested that the PIs are proteinaceous protease inhibitors as precipitated by ammonium sulfate. To further characterize the PI, the ammonium sulfate concentration was narrowed down to 10% interval for better separation as shown in the next experiments.

To narrow down the range for better separation of the protein fractions that had high PIs activity, C-serum was sequentially fractionated by ammonium sulfate precipitation using 10% range of concentration and tested for the PIs activity. Distribution of PIs activity in several ranges were detected as shown in Fig.10, Fig.11 and Table 13. The results were still not much improved compared to the 20% interval one. The PI activities were detected all over from 20% up to 95% ammonium sulfate concentration of the 10% interval similar to those observed for the 20% interval. This may have 2 possible aspects for explanation. Firstly, C-serum has more than one type

of protease inhibitors, which can be precipitated by ammonium sulfate over a wide range both at low and high ammonium sulfate concentrations. Secondly, C-serum may has only one type of PI but can be easily salted out by ammonium sulfate over wide ranges. However, it should be kept in mind that pronase being used in these screening experiments is the mixture of several proteases. These different proteases may have different levels of specificity and sensitivity to PIs in C-serum in various ammonium sulfate precipitated PI protein fractions. This pronase nature may cause appearance PIs activity in the many ranges of ammonium sulfate precipitations as depicted in Fig.10, Fig.11 and summarized in Table 13.

Table 12 Pronase activity inhibition of proteins fractions from C-serum, 20% serail ammonium sulfate fractionation

Sample†	% PIs activity*	%Inhibition	%Remaining enzyme activity
Control	0	0	100
0-20%#	4.55	9.02	90.98
20-40%	30.32	60.09	39.91
40-60%	37.92	75.15	24.85
60-80%	27.21	53.89	46.11

*Add up PIs activity of 0-80% ammonium sulfate fractions as 100% PIs activity, and compared with PIs activity of each fractions in the term of percentage.

#0-20% ammonium sulfate had less proteins precipitate out and contaminated with some rubber particles.

†Protein fractions from sequential ammonium sulfate fractionation, represented by % (w/v) of ammonium sulfate to be added.

The results are plotted as shown in Fig.8 and Fig.9.

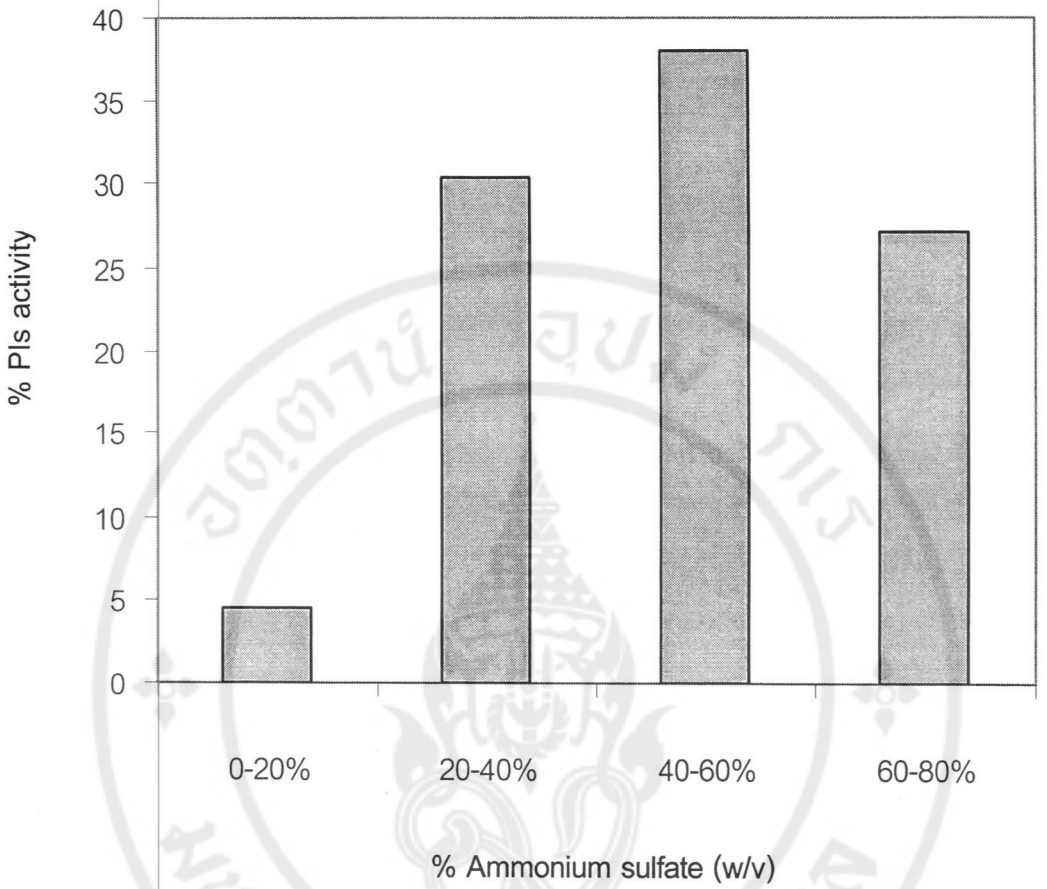

Fig. 8 Pronase activity inhibition of proteins fractions from C-serum, 20% serail ammonium sulfate fractionation

C-serum was sequentially fractionated in 4 wide ranges of ammonium sulfate precipitation and determined for inhibitory activity toward pronase. The results were expressed as % PIs activity when add up PIs activity of 0-80% ammonium sulfate fractions as 100% PIs activity, and compared with PIs activity of each fractions in the term of percentage.

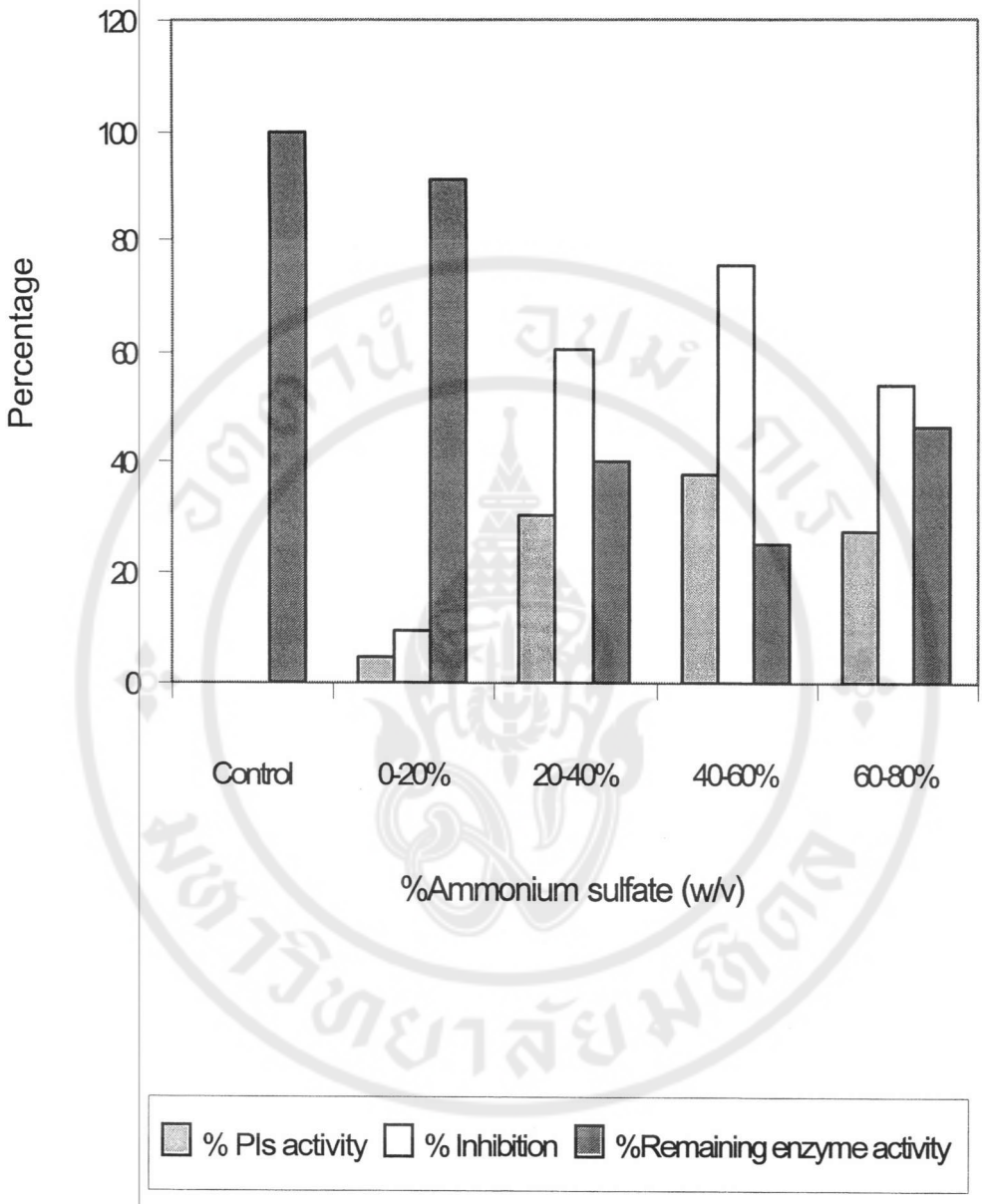

Fig.9 Correlation between % PIs activity, % inhibition of pronase activity and % remaining pronase activity of proteins fractions from C-serum, 20% serail ammonium sulfate fractionation.

Table 13 Pronase activity inhibition of proteins fractions from C-serum , 10% serial ammonium sulfate fractionation

Sample†	% PIs activity *	%Inhibition	%Remaining enzyme activity
Control	0	0	100
20-30%	12.44	69.31	30.69
30-40%	11.71	65.25	34.75
40-50%	13.29	74.07	25.93
50-60%	16.03	89.33	10.67
60-70%	15.98	89.03	10.97
70-80%	15.31	85.32	14.68
80-95%	15.23	84.89	15.11

Note: 0-10% ammonium sulfate had no proteins precipitate out, 10-20% ammonium sulfate had less proteins precipitate out and contaminated with some rubber particles. Therefore, the PI activity was not determined.

†Protein fractions from sequential ammonium sulfate fractionation, represent by % (w/v) of ammonium sulfate to be added.

*Add up PIs activity 20-95% ammonium sulfate fractions as 100% PIs activity, and compared with PIs activity of each fractions in the term of percentage.

The results are plotted as shown in Fig.10 and Fig.11.

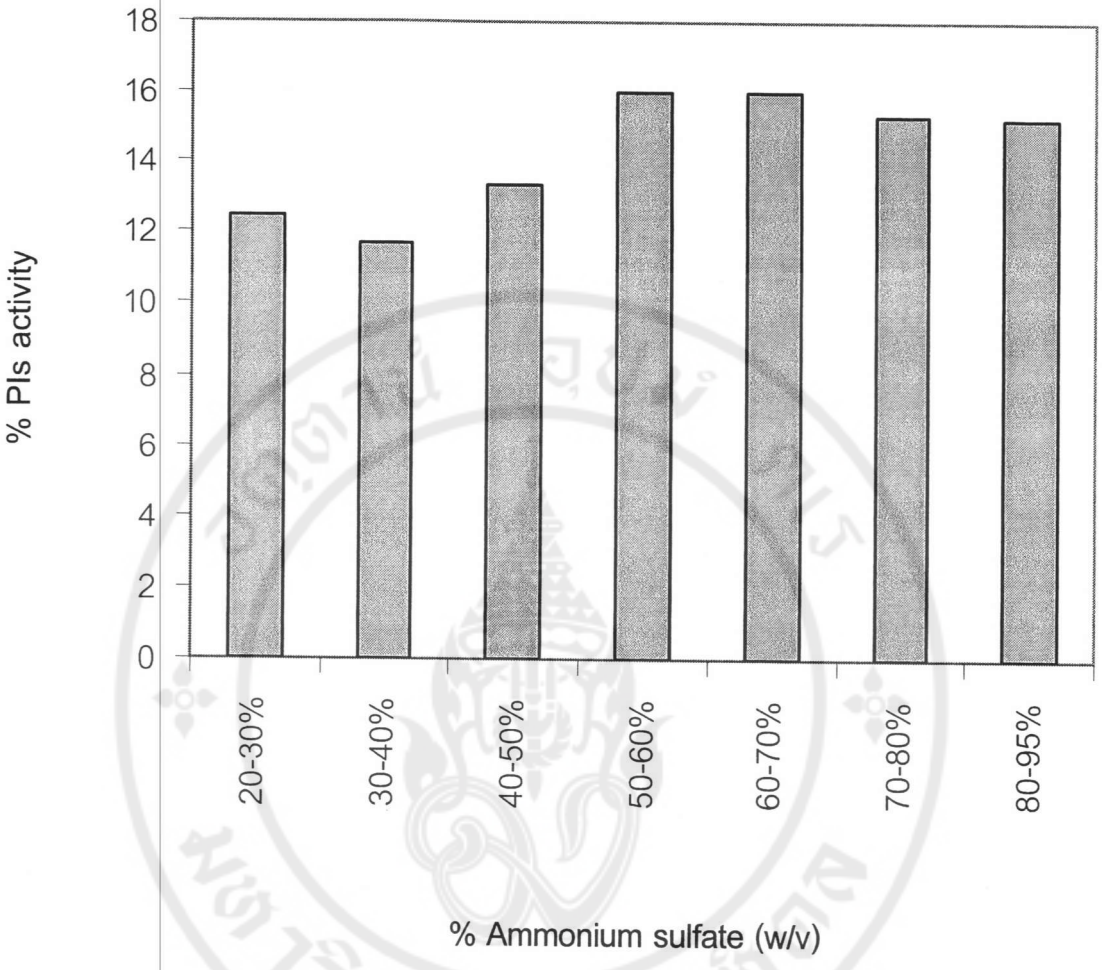

Fig10 Pronase activity inhibition of proteins fractions from C-serum, 10% serial ammonium sulfate fractionation

C-serum was sequentially fractionated by ammonium sulfate precipitation and determined for inhibitory activity toward pronase. The results were expressed as % PIs activity when add up PIs activity of 20-80% ammonium sulfate fractions as 100% PIs activity, and compared with PIs activity of each fractions in the term of percentage.

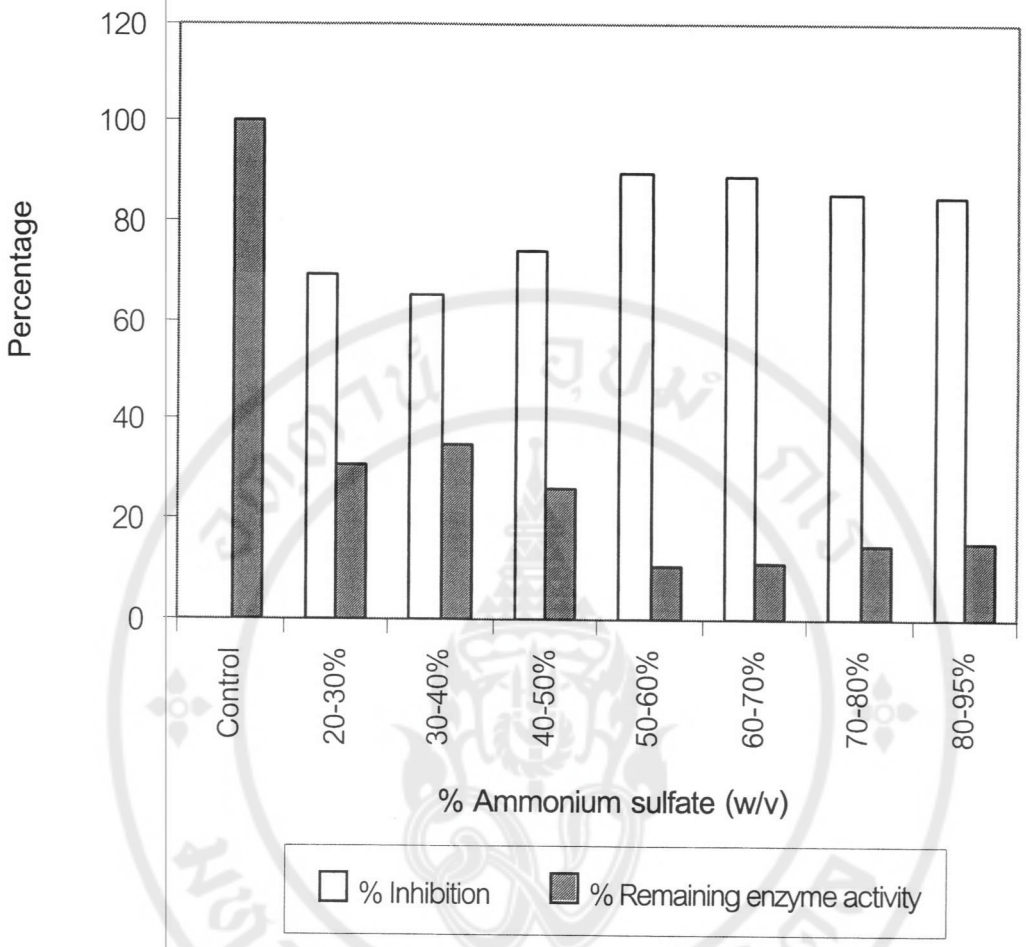

Fig.11 Correlation between % inhibition of pronase activity and % remaining pronase activity of proteins fractions from C-serum, 10% serail ammonium sulfate fractionation

2.3. Protease Inhibitor Activity on Trypsin Inhibition of Ammonium Sulfate Precipitates from C-serum Compared to Pronase

Since pronase is the mixture of several proteases and therefore suitable for experiment to screen proteases inhibitor(s) in the preliminary test but lacking PI specificity. Using the mixture of proteases such as pronase gave distribution of PIs activity in many ammonium sulfate fractions as shown in previous experiments. So, other specific protease such as trypsin was chosen to narrow down the ranges of PI activity and for further step of purification. Besides, this is also more specific for PI characterization.

Protease with substrate specific property such as trypsin was chosen to test PIs activity of various ammonium sulfate precipitant fractions and compared to pronase. The result showed that proteins fractionated by ammonium sulfate precipitated from C-serum could inhibit trypsin activity as shown in Fig.12, Fig. 13 and Table 14. These results showed different sensitivity to ammonium sulfate fractions of trypsin activity inhibition when compared to pronase as previously shown. With pronase, PIs activity were widespread and distributed in fractions ranging from 20-95% ammonium sulfate concentration. On the other hand, trypsin inhibitory activity of the PI fractions had different pattern of PIs activity distribution with the high PIs activity in the ranges of 60-95% ammonium sulfate fractions for the trypsin experiment but still widespread.

From these results, ammonium sulfate fractionation gave wide ranges of PIs activity for both pronase and trypsin and no prominent fractions were particulary of significant or exhibited high specific activity to be of further interest. Therefore, the ammonium sulfate precipitation and separation of PI was not found satisfactory for the initial step in the characterization being employed in the purification protocol.

Alternative method of protein precipitation and separation was sought and adopted for further experiment. Organic solvent precipitation of proteins was chosen to replace ammonium sulfate precipitation method and compared for the possible improvement in separation step. The other protein fractionation method using organic solvent (actone) precipitation was chosen and PI activity was tested on trypsin alone instead of pronase from this point on as the screening experiments were completed.

Table 14 Trypsin activity inhibition of proteins fractions from C-serum, 10% serial ammonium sulfate fractionation

Sample†	% PIs activity*	%Inhibition	%Remaining enzyme activity
Control	0	0	100
20-30%	8.21	49.23	50.77
30-40%	10.56	63.34	36.66
40-50%	15.10	90.56	9.44
50-60%	16.30	97.80	2.20
60-70%	16.56	99.33	0.67
70-80%	16.65	99.88	0.12
80-95%	16.63	99.78	0.22

Note: 0-10% ammonium sulfate had no proteins precipitate out, 10-20% ammonium sulfate had less proteins precipitate out and contaminated with some rubber particles. Therefore, no measurement was made.

†Protein fractions from sequential ammonium sulfate fractionation, represented by % (w/v) of ammonium sulfate added.

*Add up PIs activity 20-95% ammonium sulfate fractions as 100% PIs activity, and compared with PIs activity of each fractions in term of percentage.

The results are plotted as shown in Fig.12 and Fig.13.

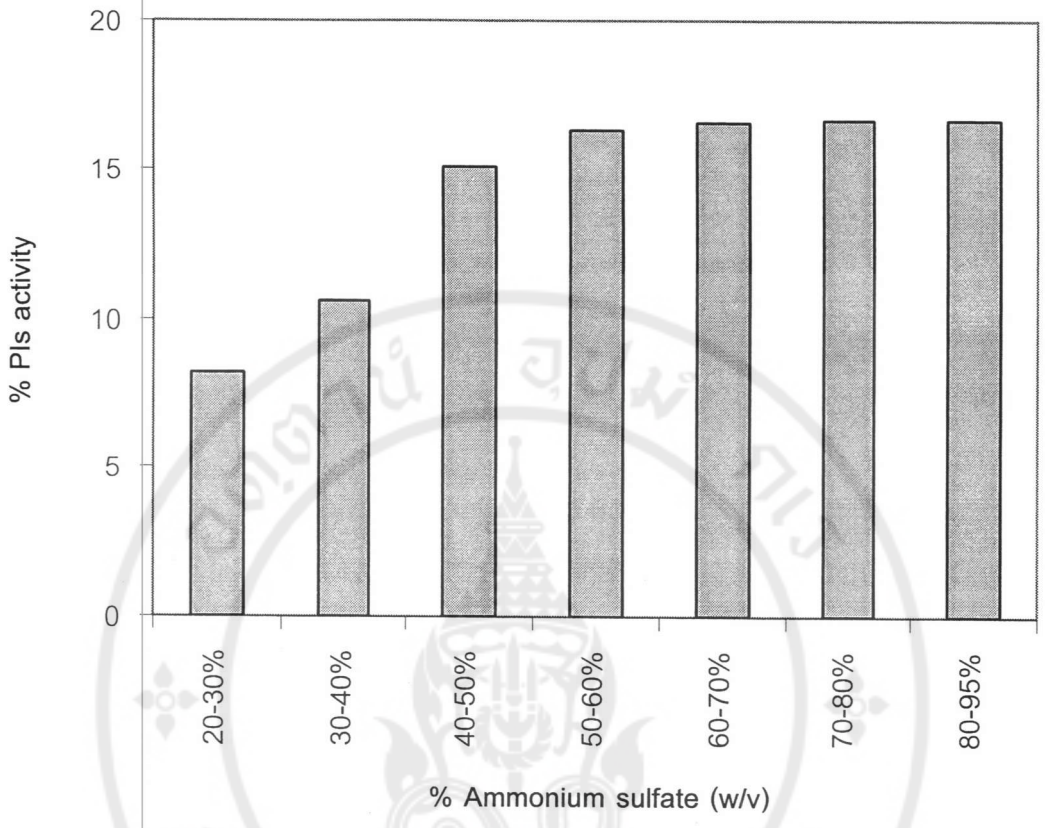

Fig. 12 Trypsin activity inhibition of proteins fractions from C-serum , 10% serial ammonium sulfate fractionation

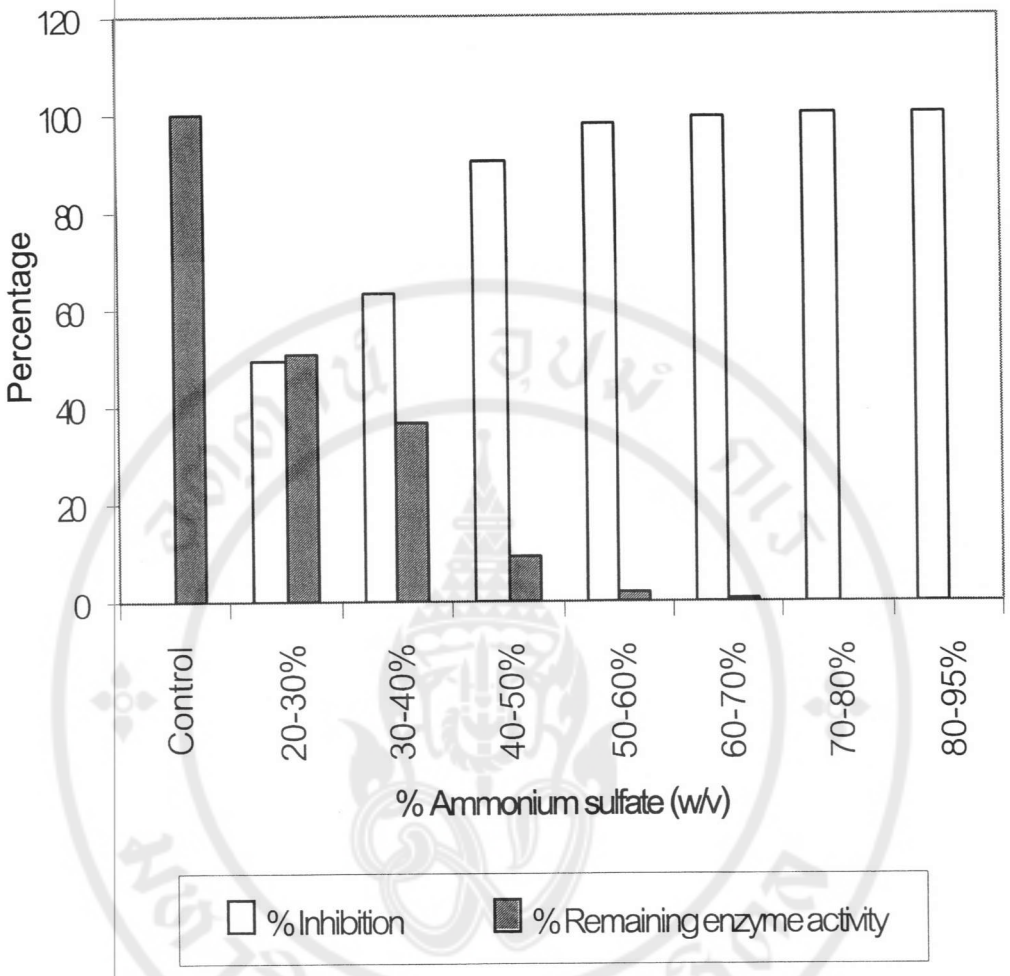

Fig.13 Correlation between % inhibition of trypsin activity and % remaining trypsin activity of proteins fractions from C-serum, 10% serail ammonium sulfate fractionation

2.4. Acetone Precipitation of the PIs Fraction

As mentioned in the previous section, acetone was employed for the sequential precipitation of proteins in the C-serum in this experiment. C-serum was sequentially fractionated into 4 acetone ranges of 20% interval (0-20%, 20-40%, 40-60% and 60-80% acetone) and tested for the PIs activity with trypsin. The results showed maximum PIs activity in the range of 60-80% acetone fraction as shown in Fig.14 and Table 15. The acetone precipitation experiment confirmed and agreed with the result on ammonium sulfate precipitation that PIs in C-serum were of proteinaceous protease inhibitors. However, the results also pointed out that PIs in C-serum need rather high acetone to precipitate out from other proteins as indicated by the increasing PI activity with high percentage of acetone. These observations suggested that the PI might probably be small proteins with rather hydrophobicity in its molecular nature. The requirement for high percentage of acetone to precipitate out and separate the PI from other C-serum proteins was further tested in the subsequent experiment.

Table 15 Trypsin activity inhibition of proteins fractions from C-serum, 20% serial acetone fractionation.

Sample†	% PIs activity*	%Inhibition	%Remaining enzyme activity
Control	0	0	100
0-20%	17.85	24.92	75.08
20-40%	23.52	32.84	67.16
40-60%	27.26	38.06	61.94
60-80%	31.37	43.21	56.79

†Protein fractions from sequential acetone fractionation, represent by % (v/v) of acetone added.

*Add up PIs activity 0-80% acetone fractions as 100% PIs activity, and compared with PIs activity of each fractions in the term of percentage.

The results are plotted as shown in Fig.14.

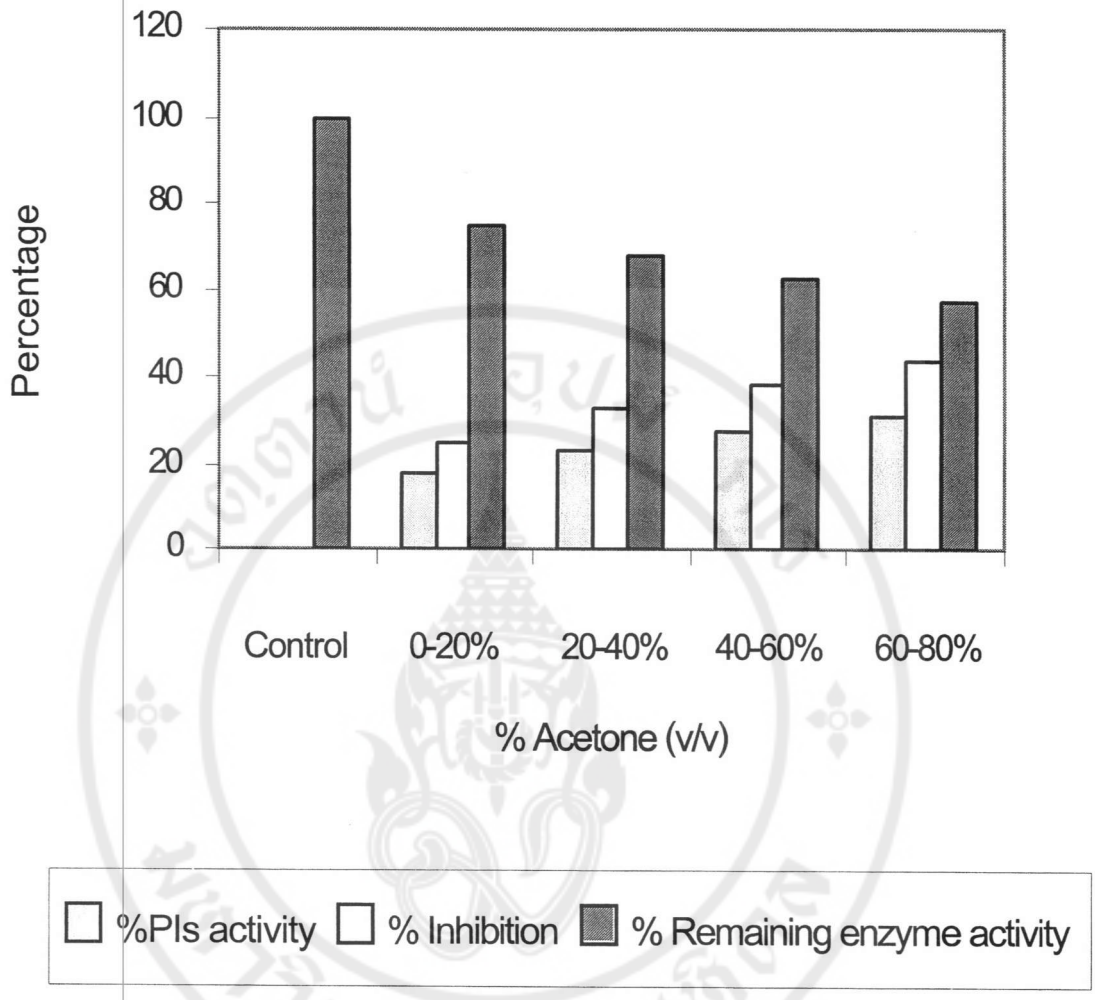

Fig.14 Trypsin activity inhibition of proteins fractions from C-serum, serial acetone fractionation (0-20%, 20-40%, 40-60% and 60-80% acetone)

C-serum was sequentially fractionated into 4 acetone ranges of 20% interval and determined for trypsin activity inhibition. The control was used 50 mM Tris-HCl pH 8 buffer instead of sample fractions in the assay condition and expected to be 100% enzyme activity or 0% inhibition.

%PIs activity = add up PIs activity of 0-80% acetone fractions as 100% PIs activity, and compared with PIs activity of each fractions in the term of percentage.

Part 3. Characterization and Properties of PIs

The main properties of PIs characterization will be on the molecular weight determination and analysis by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for the separation of the isolated PIs protein from C-serum.

3.1. Sephadex G-200 Gel Filtration of the PIs

Separation and partial purification of PIs was carried out with gel filtration column chromatography. The PIs proteins obtained from 60% ammonium sulfate precipitation was employed in this chromatographic experiment. Known molecular weight proteins marker were loaded on to a Sephadex G-200 column (1.2 × 68 cm) for calibration and molecular weight (MW) determination. The Kav values of the standard molecular weight marker and PIs were plotted against their respective log MW as shown in Fig.15. Since several PIs were reported in other plant species and this is the first observation and report on the *Hevea* latex PI, so the latex PI will be designated as *Hevea* protease inhibitor (HPI) from now on to give it specificity and differentiate it from other plant PIs.

The results of Sephadex G-200 chromatography indicated that the HPI has a rather low MW compared to other standard MW marker proteins. The MW value found in this experiment for the protein peak with PI activity was less than 25 kD. The results suggested that the HPI is a low MW protein which was eluted as the later steps of the chromatographic elution profile.

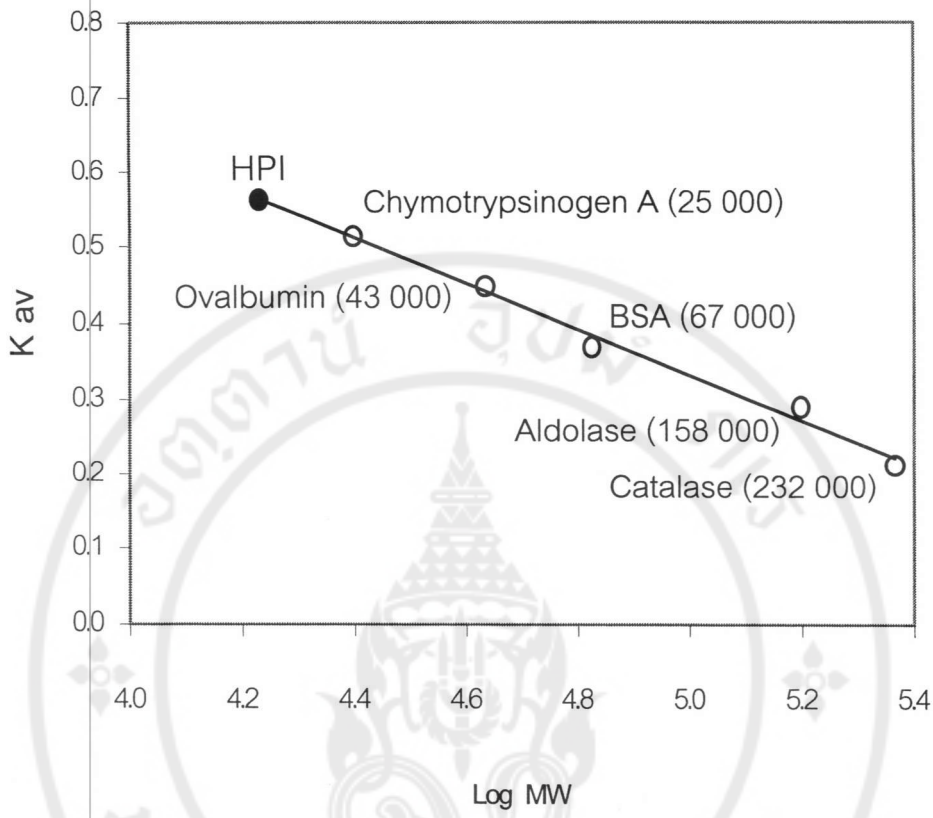

Fig.15 Molecular weight calibration of *Hevea* protease inhibitors (HPI) by gel filtration Sephadex G-200

The calibration curve was plotted between Kav values and their respective log MW. Comparison of the elution peak of HPI was made with the elution profiles of the standard proteins marker for calibration and calculation of HPI native MW. The MW value found in this experiment for the protein peak with PI activity was less than 25 kD.

3.2. Approximate MW Determination of the Protein Peak with HPI Activity

This experiment was carried out to obtain the chromatographic profile of C-serum proteins peak with PI activity by designated as HPI. Sephadex G-200 was employed in this experiment. The 0-60% ammonium sulfate fraction of C-serum proteins was loaded and determined for the eluted PIs activity fractions of the protein peaks with pronase being used for PI activity screening. The chromatographic profile is shown in Fig.16 for the elution. The PIs activity peak was detected with pronase at the arrowed HPI fractions (fraction No.52-57). This protein peak of HPI was calibrated base on the standard curve of known molecular weight proteins as being profiled in Fig.15 shown in the preceding section. The PIs protein appeared to be in the range lower than 25 kD as depicted in the Fig.15 interception designated for HPI. Since the PIs sample was not pure protein, so this result was just a guide for approximating the MW of HPI by this gel filtration separation and calibration profile in the combined results of Fig.16 and Fig.15, respectively. The next step was to characterize the protein peak with SDS-PAGE to gain some idea on the protein number and protein composition in order to develop further purification protocol.

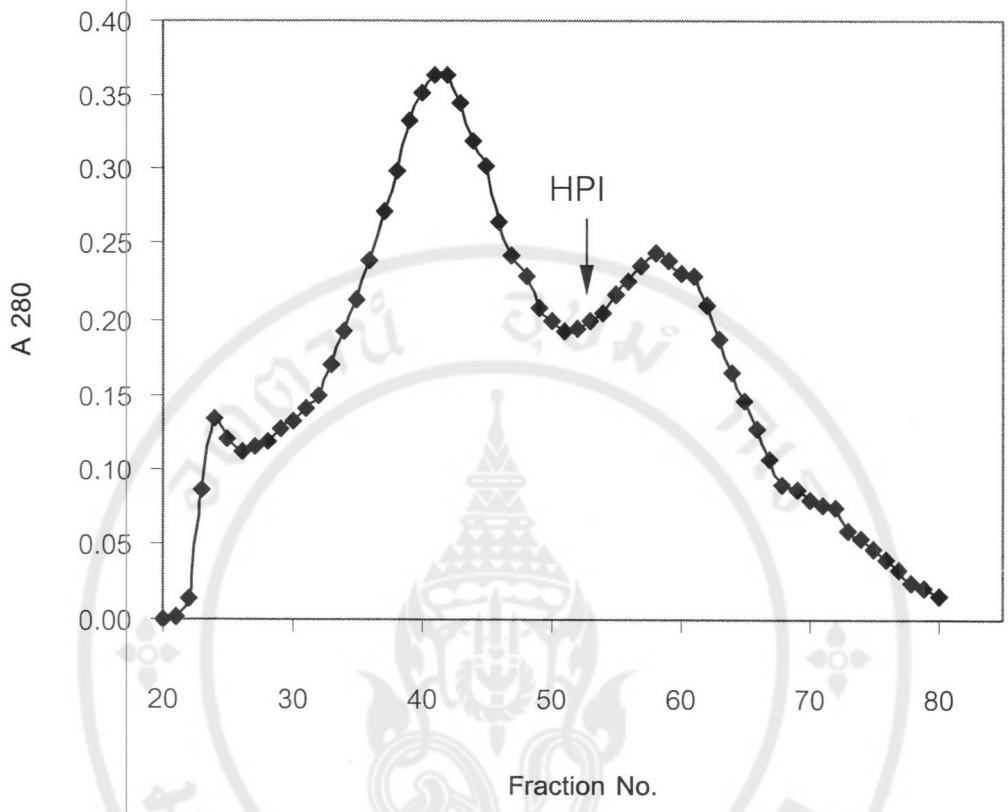

Fig.16 Chromatographic profile for 0-60% ammonium sulfate proteins fractions of C-serum on a Sephadex G-200 column

The 0-60% ammonium sulfate fraction of C-serum proteins was applied to the Sephadex G-200 column (1.2 x 68 cm.). Column was equilibrated and eluted with 50 mM Tris-HCl pH 7.5 at a flow rate of 9 ml/hr. at 4°C. Fractions of 1.5 ml were collected until the absorbency at 280 nm fell below 0.05. Each 4 fractions were pooled, concentrated by Speed Vac concentrator (Savant model Speed Vac SVC 100) to an approximate volume 1 ml, and assayed protease inhibition (PI) activity.

3.3. Gel Electrophoresis (SDS-PAGE) of C-serum Proteins by Ammonium Sulfate and Acetone Fractionation

Analysis of proteins derived from ammonium sulfate and acetone precipitate fraction were carried out by SDS-PAGE. The number of protein bands and approximate MW of the two precipitation methods will then be compared. C-serum was 10% sequential fractionation by either ammonium sulfate or acetone precipitation at 10% interval concentration. Protein bands of each fraction from both precipitation methods were determined by SDS-PAGE. The results are shown in Fig.17 and Fig.18. Protein fractions from ammonium sulfate precipitation showed several protein bands in all precipitant fractions obtained as shown in Fig.17. Interestingly, two fractions from acetone precipitated fractionation of 60-70% and 70-80% acetone (Fig.18) with maximum PIs activity showed quite distinct 2 major bands of the protein. These two major protein bands were between 30-43 kD and smaller than 14 kD as indicated in Fig.18. It was also of interest to note that 80% acetone precipitated proteins showed almost a single protein band of 14 kD or less but with still of very high or maximum PI activity. The results of this experiment as shown in Fig.18 was well agreed with the results reported in part 2.4 and depicted in Fig.14 and Table 15. It was then quite obvious from SDS-PAGE analyses of proteins that the acetone precipitation method was far more suitable and better than ammonium sulfate precipitation to be employed in purification protocol.

Acetone was thus incorporated and employed in the purification of HPI for further characterization and other related studies. Employing acetone in fractionation and separation of HPI from the bulk of C-serum proteins was far more superior and more advantageous than the conventional ammonium sulfate precipitation. Acetone

precipitation was used throughout the subsequent experiment unless otherwise indicated. High percentage acetone precipitation of the HPI also suggested or pointed out one important aspect of the HPI nature, that the HPI might be present as a small protein in C-serum and that it might have hydrophobic character. This was deduced from the results on heat stable property of HPI as well as the environment of the latex in which the HPI has to encounter. These aspects will be later further examined. The next part is an attempt in the purification of HPI to obtain the homogeneous protein.

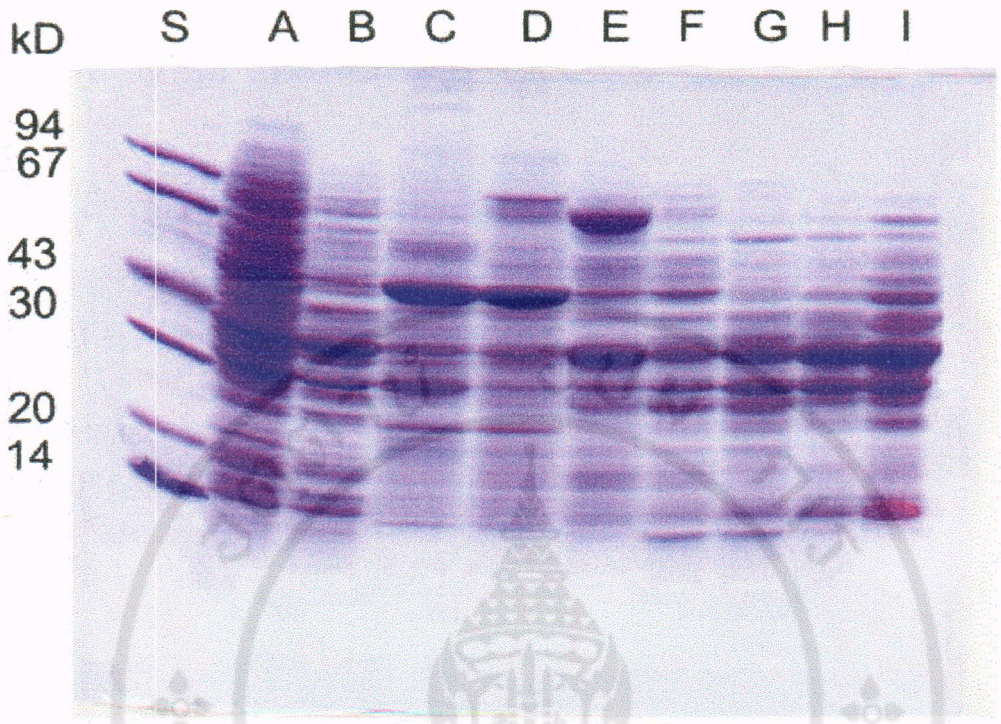

Fig.17 SDS-PAGE (7-15%gel) of proteins fractionation of C-serum by $(NH_4)_2SO_4$ fractionated of C-serum

Electrophoresis was performed in 7-15% gel at a constant current of 14 mA/plate until the tracking dye was approached the bottom of the separating gel. After electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250

Lane S = Standard proteins marker

Lane A = Crude C-serum

Lane B = C-serum, after heated at 65°C for 15 minutes

Lane C-I = 10% sequential fractionation of C-serum; started from 20-30%, 30-40%, 40-50%, 50-60%, 60-70%, 70-80% and 80-95% $(NH_4)_2SO_4$ fractions respectively

Fig.18 SDS-PAGE (7-15%gel) of proteins fractionation of C-serum by acetone fractionated of C-serum

Electrophoresis was performed in 7-15% gel at a constant current of 14 mA/plate until the tracking dye was approached the bottom of the separating gel. After electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250

Lane S = Standard proteins marker

Lane A = Crude C-serum

Lane B = C-serum, after heated at 65°C for 15 minutes

Lane C-I = 10% sequential fractionation of C-serum; started from 10-20%, 20-30%, 30-40%, 40-50%, 50-60%, 60-70% and 70-80% acetone fractions respectively

Part 4. Purification of the HPI

4.1. Acetone Fractionation

From the previous results as shown in part 2.4 and part 3.3, maximum PIs activity was observed in the range of 60-80% acetone fractions and had interesting 2 major protein bands. Particularly, in the 70-80% acetone precipitant protein fraction only one major protein band of small molecular weight was observed among a few very faint bands. This was taken further to refine the small MW protein for PI activity. Increasing the percentage of acetone fractionation was done for trying to cut off other faint protein bands that might be of higher molecular weight. Ranges of acetone fractionation were improved by step-wise protein cut using 0-50%, 50-70%, 70-80% and then addition of more acetone to make it 80-95% acetone. PIs activity of 80-95% acetone precipitation was then tested with trypsin and SDS-PAGE were determined for protein band. The results showed maximum PIs activity in the 80-95% acetone fraction as shown in Fig.19, and Fig.20 and summarized in Table16. Only one band with MW smaller than 6.5 kD was detected in 80-95% acetone fraction whereas the 70-80% acetone f raction showed more protein bands with less PIs activity than 80-95% acetone fraction as indicated in Fig.19 and Fig.21. This mathod of high percentage of acetone (95%) was improved for better recovery of HPI activity which showed only one major protein band that has low MW PI with high PI activity. The results thus obtained in Fig.21 indicated that the HPI was a small protein of less than 6.5 kD and was well precipitated at high acetone concentration for better and cleaner separation from other remaining proteins after 80% acetone precipitation of the

C-serum proteins. It is quite surprising and unexpected to find such an efficient and simple procedure in obtaining almost purified PI with this step-wise acetone precipitation with almost no loss of the HPI activity even at very high acetone concentration. This finding was then adopted in the further subsequent experiment and analyses of the HPI.

Table 16 Trypsin activity inhibition of proteins fractions from C-serum, serial acetone fractionation (0-50%, 50-70%, 70-80% and 80-90% acetone)

Sample†	% PIs activity*	%Inhibition	%Remaining enzyme activity
Control	0	0	100
0-50%	6.44	10.28	89.72
50-70%	11.92	19.44	80.56
70-80%	16.88	29.44	70.56
80-95%	64.13	91.06	8.94

†Protein fractions from sequential acetone fractionation, represented by % (v/v) of acetone added.

*Add up PIs activity 0-95% acetone fractions as 100% PIs activity, and compared with PIs activity of each fractions in the term of percentage.

The results are plotted as shown in Fig.19 and Fig.20.

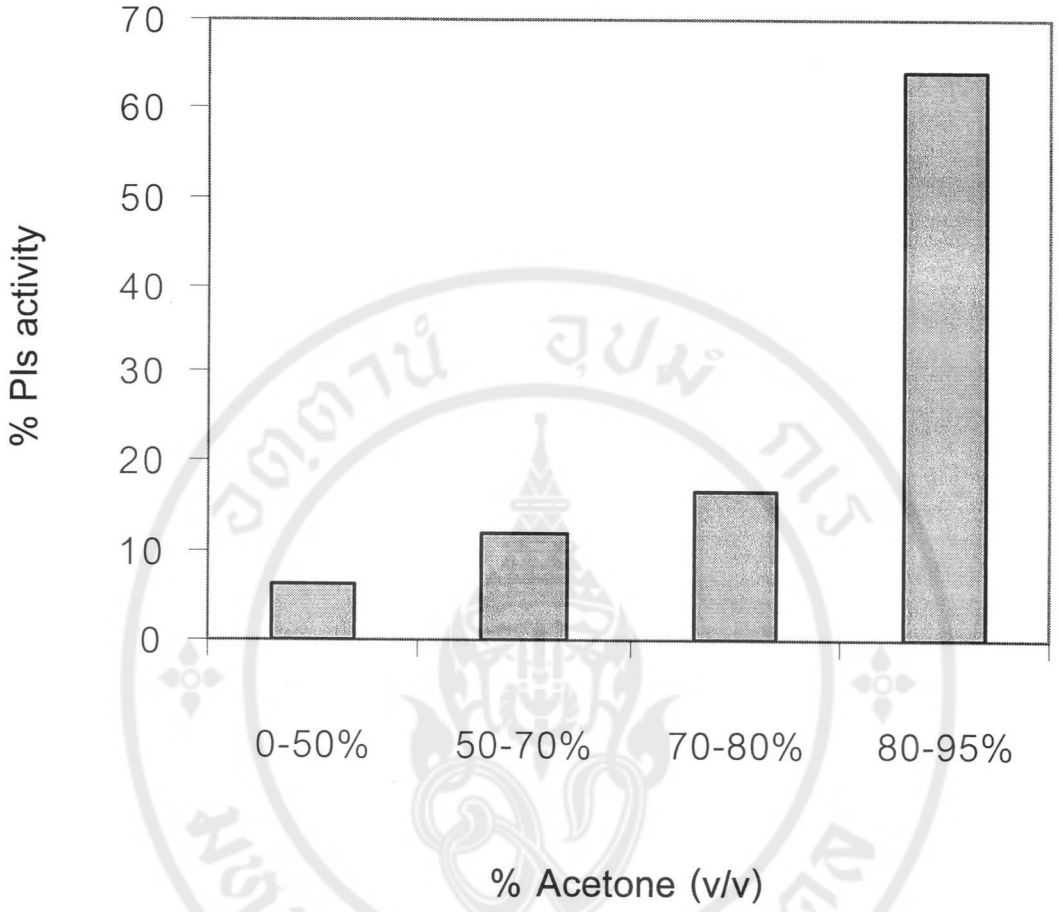

Fig.19 Trypsin activity inhibition of proteins fractions from C-serum, serial acetone fractionation (0-50%, 50-70%, 70-80% and 80-90% acetone)

C-serum was sequentially fractionated by acetone precipitation and determined for trypsin activity inhibition. The results were expressed as % PIs activity when add up PIs activity of 0-95% acetone fractions as 100% PIs activity, and compared with PIs activity of each fractions in term of percentage.

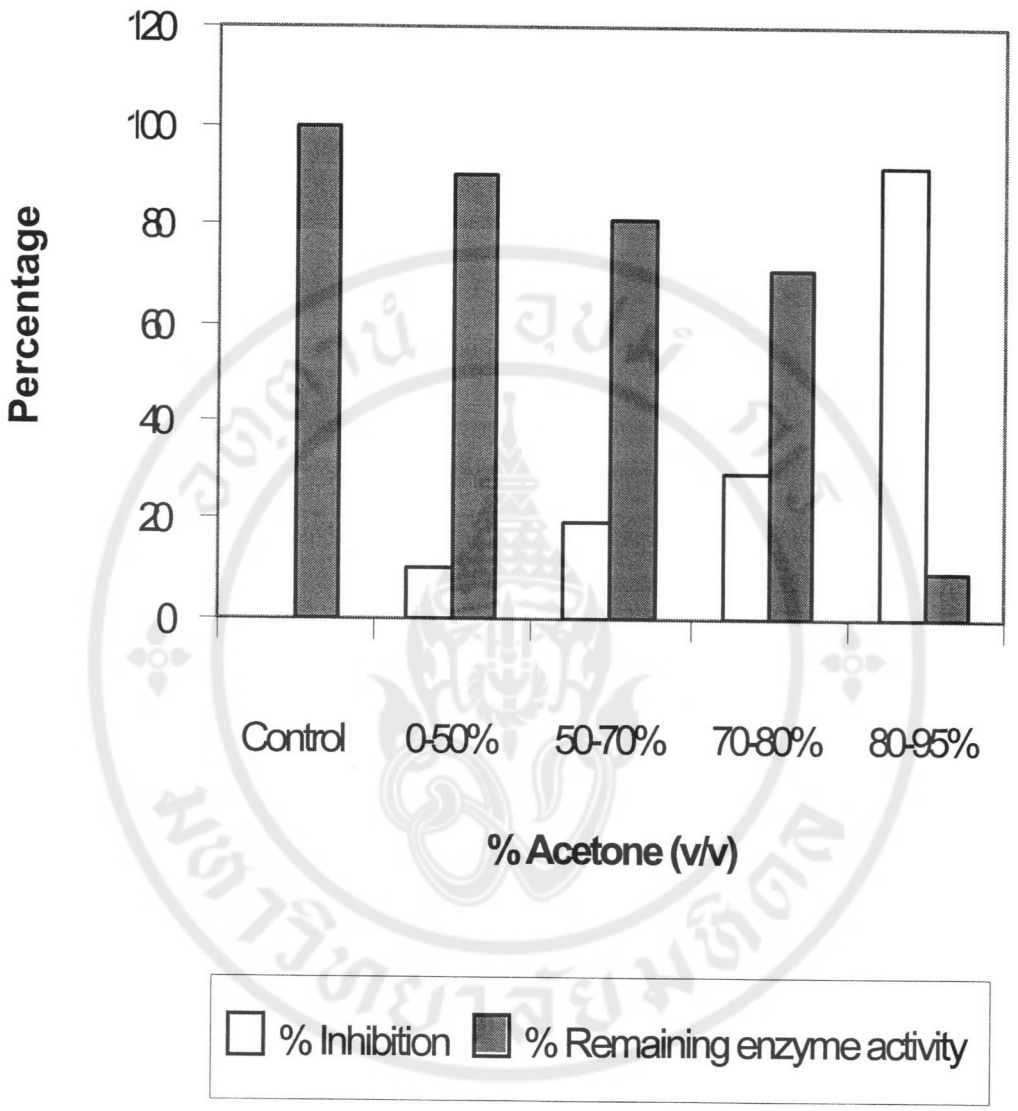

Fig.20 Correlation between % trypsin activity inhibition and % remaining trypsin activity of proteins fraction from C-serum, serial acetone fractionation (0-50%, 50-70%, 70-80% and 80-90% acetone)

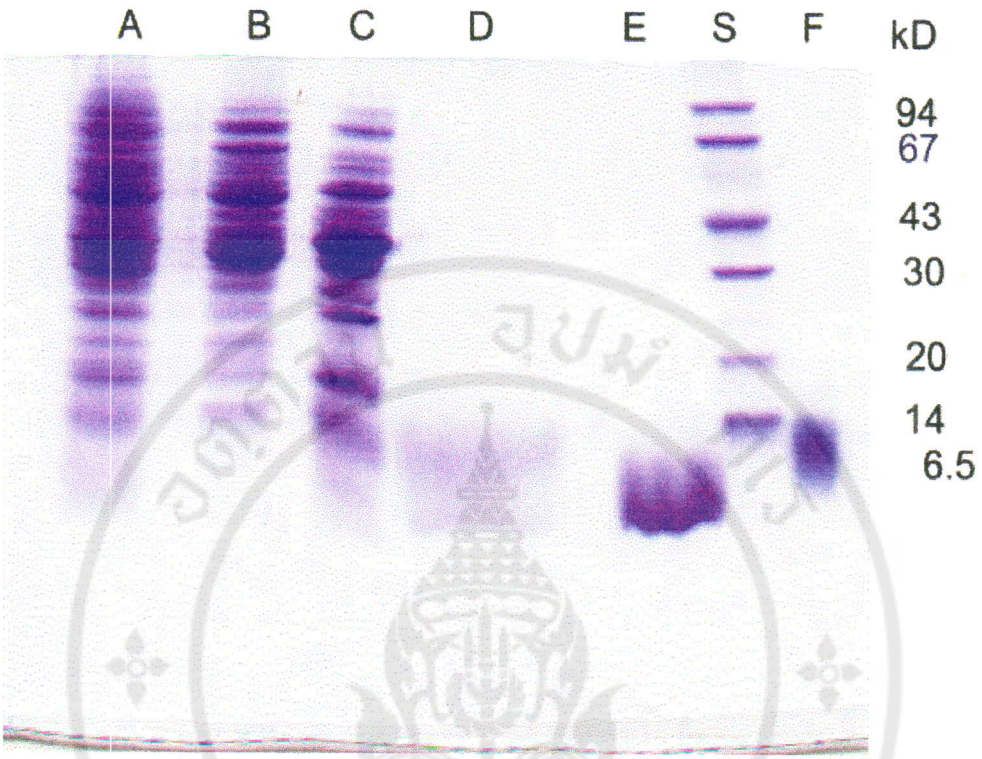

Fig. 21 SDS-PAGE (10-20%gel) of proteins fractionation of C-serum by acetone

Electrophoresis was performed in 10-20% gel at a constant current of 14 mA/plate until the tracking dye was approached the bottom of the separating gel. After electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250

Lane S = Standard proteins marker
Lane A = Crude C-serum
Lane B = 0-50% acetone fraction
Lane C = 50-70% acetone fraction
Lane D = 70-80% acetone fraction
Lane E = 80-95% acetone fraction
Lane F = Protein marker 6.5 kD

4.2. Approximation of HPI Molecular Weight by SDS-PAGE

Proteins from acetone fractionation (from part 4.1) were subjected to SDS-PAGE (10-20% gel) for analyses and characterization of the protein bands. The 80-95% acetone fraction (HPI) showed one single protein band with a calibrated subunit molecular weight of 5.5 kD as shown in Fig.22. The deduced MW of 5.5 kD for HPI was the result of protein analysis in Fig.21 and was subjected to calibration for MW determination in Fig.22 as shown. The result thus obtained, indicated that the HPI was either a monomer of 5.5 kD or multimeric protein of the same monomeric subunit of 5.5 kD. This nature of the native state of the HPI remains to be tested further for its existence in the latex or its active form in the PI activity assay. However, it should be pointed out that several PI of small MW protein has been found and characterized from plant source. It is then quite likely that the native HPI might exist as a monomer of single polypeptide of 5.5 kD.

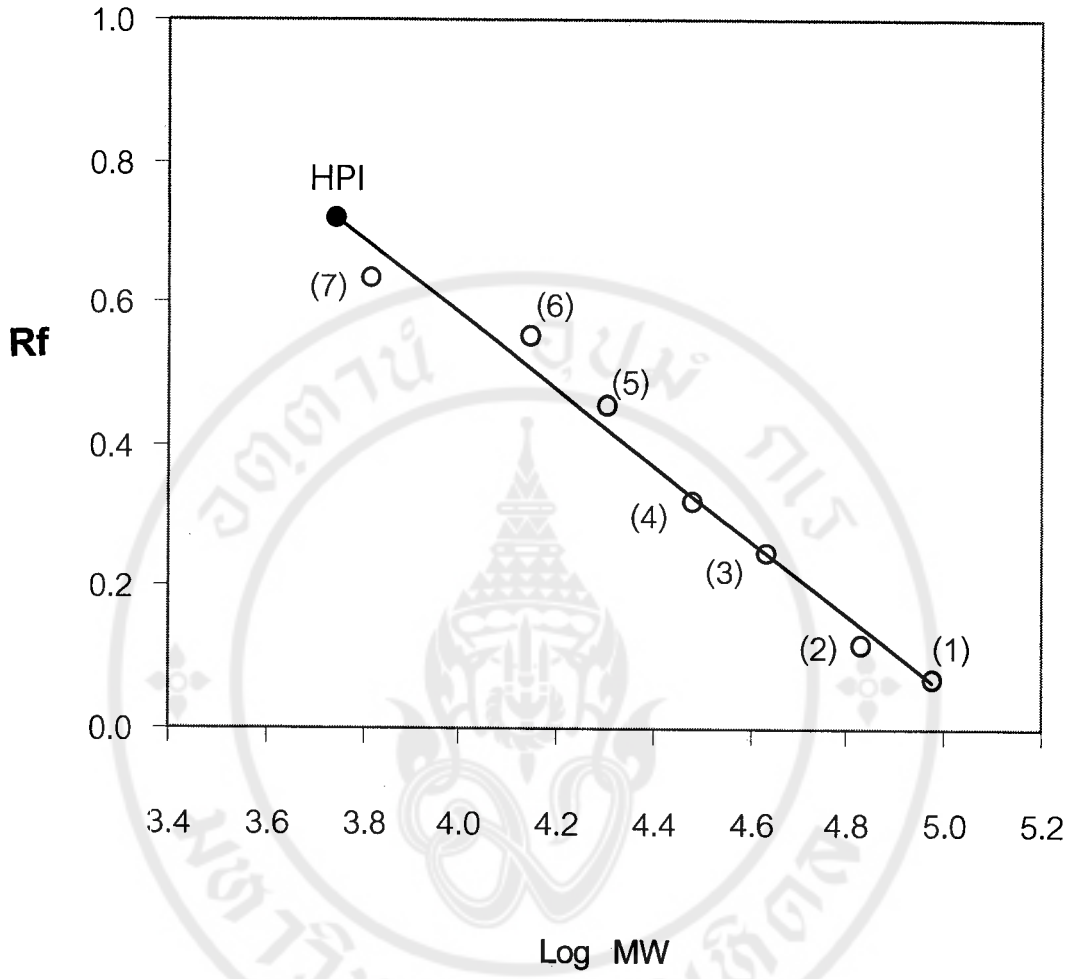

Fig. 22 Molecular weight calibration of 80-95% acetone fractionated of C-serum (HPI) by SDS-PAGE (10-20% gel)

The standard proteins used as reference were: (1) phosphorylase 94 kD, (2) BSA 67 kD, (3) ovalbumin 43 kD, (4) carbonic anhydrase 30 kD, (5) soybean trypsin inhibitor 20 kD, (6) α-lactalbumin 14 kD and (7) apotinin 6.5 kD. The HPI was gave subunit MW of 5.5 kD.

4.3. Comparison of C-serum HPI with B-serum Hevein Protein

It has been reported that hevein was one of the most prominent protein of the lutoid B-serum proteins. The molecular weight of hevein was first estimated to be 10,000 by Archer (6) and later reduced to 5,000 to be more accurate (66). Hevein was very much resembled proteinase inhibitors in general structural characteristics and amino acids composition. However, several attempts were made to prove the PI nature of hevein but they did not observe any inhibition on any proteasees being tested extensively (6, 7). So the idea of hevein as PI was abandoned due to the failure to detect for PI activity either by technical difficulty or error, or the different location of hevein being derived from.

Determination of the small B-serum protein, hevein, was made in this study using acetone precipitation and separation of protein fractions similar to experiment with C-serum. The B-serum proteins was sequentially fractionated into 4 acetone ranges (0-50%, 50-70%, 70-80% and 80-95% acetone) and the protein fractions were analyzed by SDS-PAGE as reported for the C-serum HPI. The results shown in Fig.23 indicated that a single small protein band of lower than 6.5 kD was observed in the 80-95% acetone fraction which was very similar to that observed for C-serum HPI. However, the low MW protein band of B-serum obtained by 80-95% acetone precipitation was very faint band as compared to that observed for the C-serum. The similarity of the two suggested that C-serum HPI might be related to the B-serum hevein. This might result from modification of hevein and rendering it to be localized in different compartment of the latex which differ in the expression of PI activity. However, the speculation needs to be further verified.

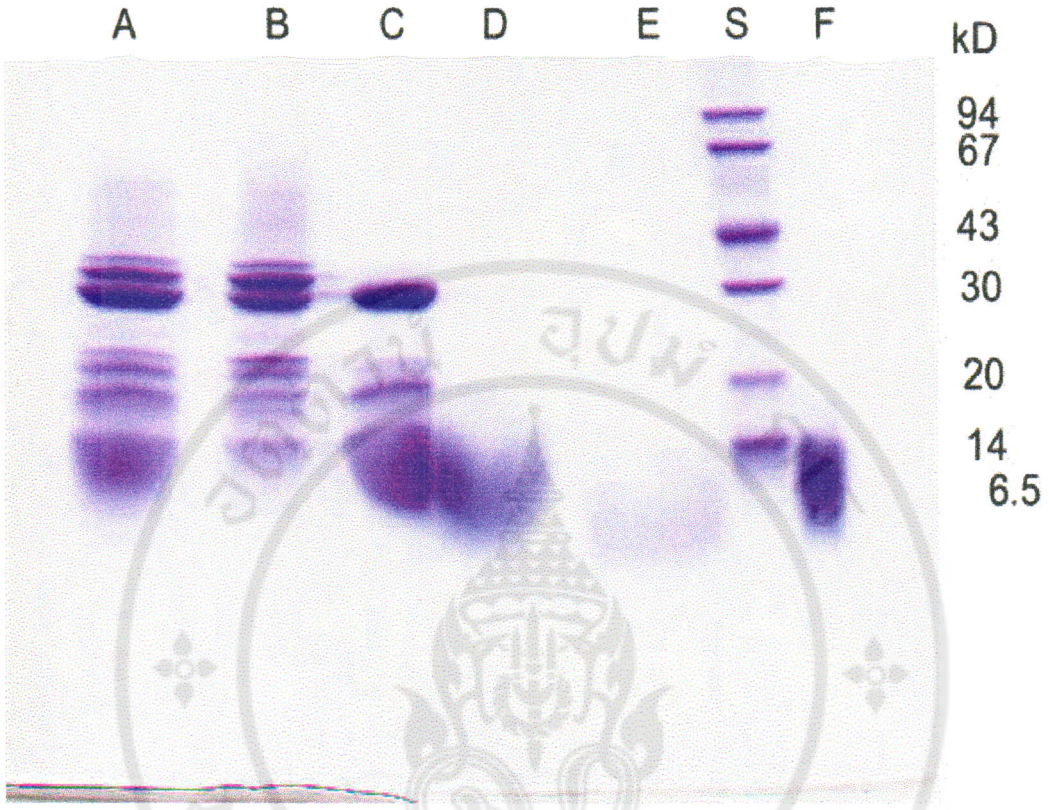

Fig. 23 SDS-PAGE (10-20% gel) of proteins fractionation of B-serum by acetone

Electrophoresis was performed in 10-20% gel at a constant current of 14 mA/plate until the tracking dye was approached the bottom of the separating gel. After electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250

Lane S = Standard proteins marker
Lane A = Crude B-serum
Lane B = 0-50% acetone fraction
Lane C = 50-70% acetone fraction
Lane D = 70-80% acetone fraction
Lane E = 80-95% acetone fraction
Lane F = Protein marker 6.5 kD

4.4. Heat Stable Property of 80-95% Acetone Precipitant HPI

The protein from 80-95% acetone precipitated fraction (HPIs) was subjected to heat treatment at various temperatures ranging from 50 °C to 100 °C for 10 min. The heat pretreated protein fraction was then tested for the remaining PI activity. The results are shown in Fig.24 and are summarized in Table 17. It was found that HPI was a very heat stable protein, only approximately 10% PI activity was lost even at 100 °C heating for 10 min (Fig.24 and Table 17). The results shown in Fig.24 indicated that this HPI was one of the most stable protein in C-serum similar to calmodulin we have previously reported (68). The results was further checked for protein analyses of the heat pretreated 80-95% acetone precipitated fraction (HPI) by SDS-PAGE. The remaining 90% PI activity after boiling for 10 min suggested that the HPI shown still remained in the heated supernatant as only 10% loss of the HPI activity was observed. Since, after heat treatment the band of protein was still present and observed by SDS-PAGE as shown in Fig.25 of SDS-PAGE protein analysis. The results clearly indicated that the protein still remained in solution and that the PI activity as determined and shown in Fig.24 was due to the presence of this 5.5 kD HPI protein in the heated sample.

Table 17 Trypsin activity inhibition remained of 80-95% acetone precipitance (HPI) from C-serum after heating at various temperature for 10 min.

Temperature (°C)	% Remaining PI activity
Control	100
50	98.69
60	98.43
70	95.26
80	96.55
90	94.65
Boil*	93.16

* Heating sample in boiling water

Note: The control was HPI that was kept at 4°C before test and its activity was defined as 100% remaining PI activity.

The results are plotted as shown in Fig.24.

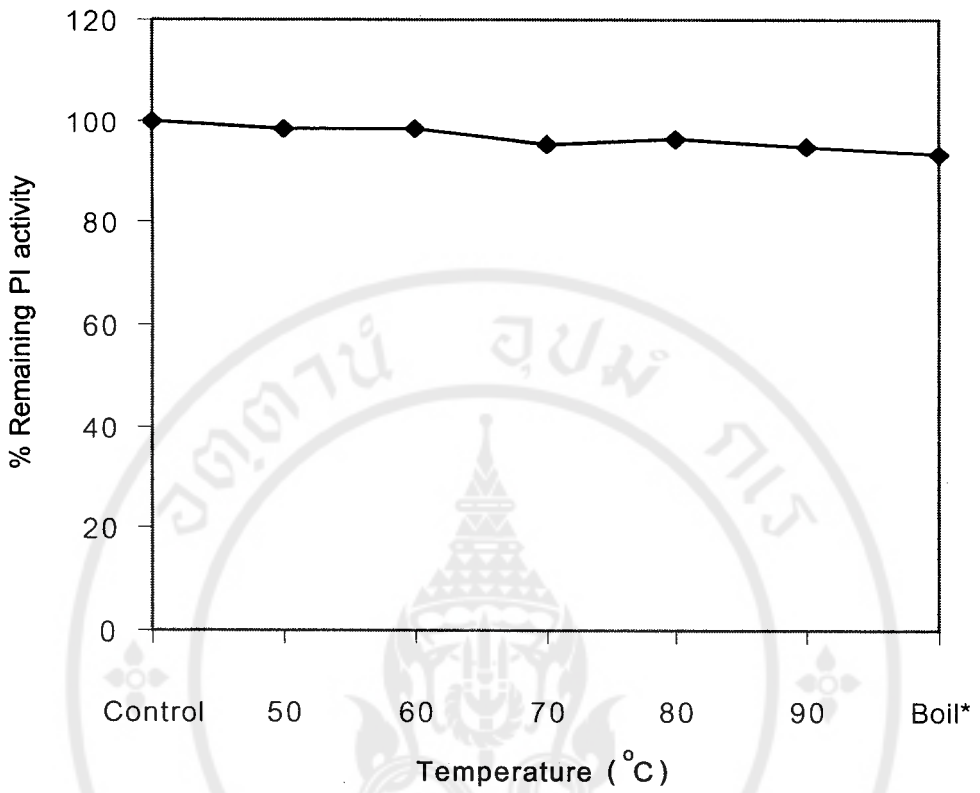

Fig. 24 Trypsin activity inhibition remained of 80-95% acetone precipitant (HPI) from C-serum after heating at varying temperature for 10 min.

The protein from 80-95% acetone precipitant fraction (HPI) was subjected to heat treatment at various temperatures ranging from 50 °C to 100 °C for 10 min then cooled to RT before determining % remaining PI activity toward trypsin target enzyme. The results were expressed as the percentage of remaining PI activity when compared to the control which HPI (kept at 4 °C before assayed) was used as 100% PI activity.

* Heating sample in boiling water.

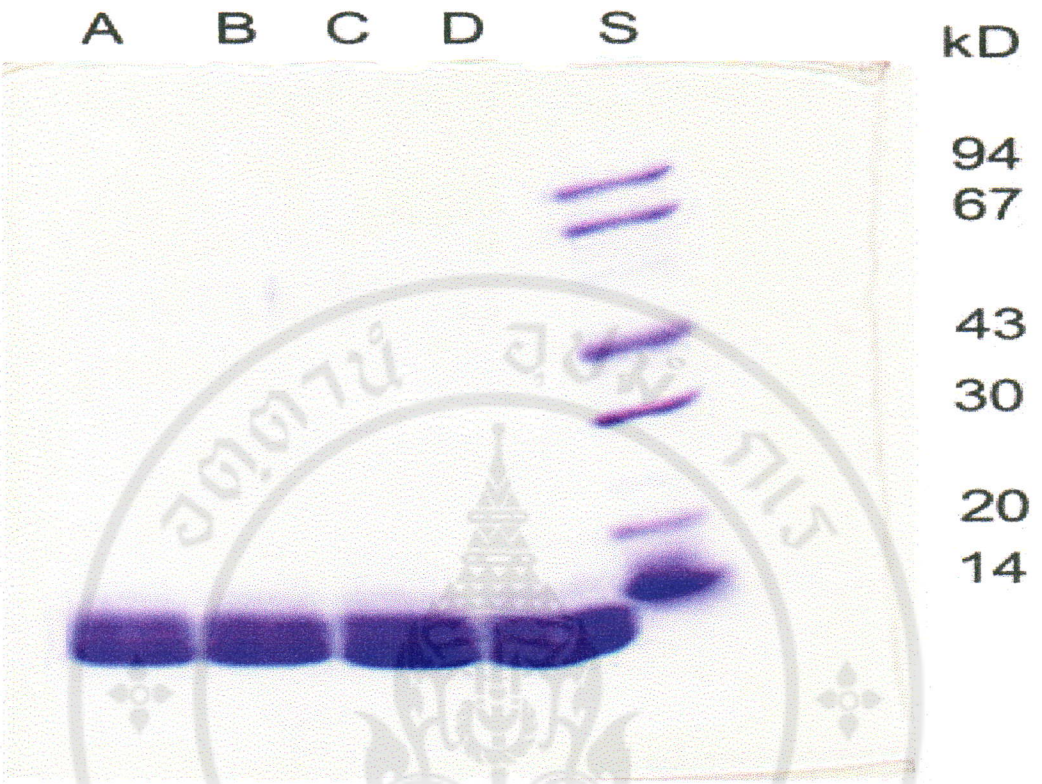

Fig.25 SDS-PAGE (7-15% gel) of HPI after heating at varying temperature for 10 minutes

Lane A = HPI

Lane B = HPI, after heated at 70°C

Lane C = HPI, after heated at 80°C

Lane D = HPI, after heated at90°C

Lane S = Standard proteins marker

4.5. Further Test on the Heat Stability of HPI

As the results in Fig.25 has shown that the small MW of 5.5 kD HPI still remained in SDS-PAGE after heating in boiling water for 10 min and still retained the full PI activity. This thermostable property was further tested to confirm the heat stable nature of HPI by increasing the heating time to 15 and 30 min comparing to the experiment with 10 min heating time. Increasing heat treatment time to 15 and 30 min showed almost no PI activity loss as shown in Fig.26 and summarized in Table 18 for comparison and reference to the 10 min experiment. Although the heat treatment time in boiling water was increased up to 30 min, PI activity was decreased by only approximate 10% (Fig.26 and Table 18) similar to the results shown in Fig.24 and Table 17 for 10 min heating. This strongly indicated that HPI is truly a very thermostable protein, probably the most stable small protein in C-serum of the latex similar to that of calmodulin previously reported by our group (68). This strong thermostable property is quite unique for many possible biotechnological application, including the possibility for industrial scale production as the PI can find many uses in both food technology as well as medical science among several others. With its superior property to tolerate heat, it is certainly of great potential.

Table 18 Trypsin activity inhibition remained of 80-95% acetone precipitance (HPI) from C-serum after heating at varying temperature for 15 and 30 min.

Temperature (°C)	% Remaining PI activity	
	Time (min)	
	15	30
Control	100	100
50	99.07	99.53
60	98.18	97.42
70	98.42	97.82
80	96.64	96.45
90	95.19	96.49
Boil*	93.40	91.62

* Heating sample in boiling water

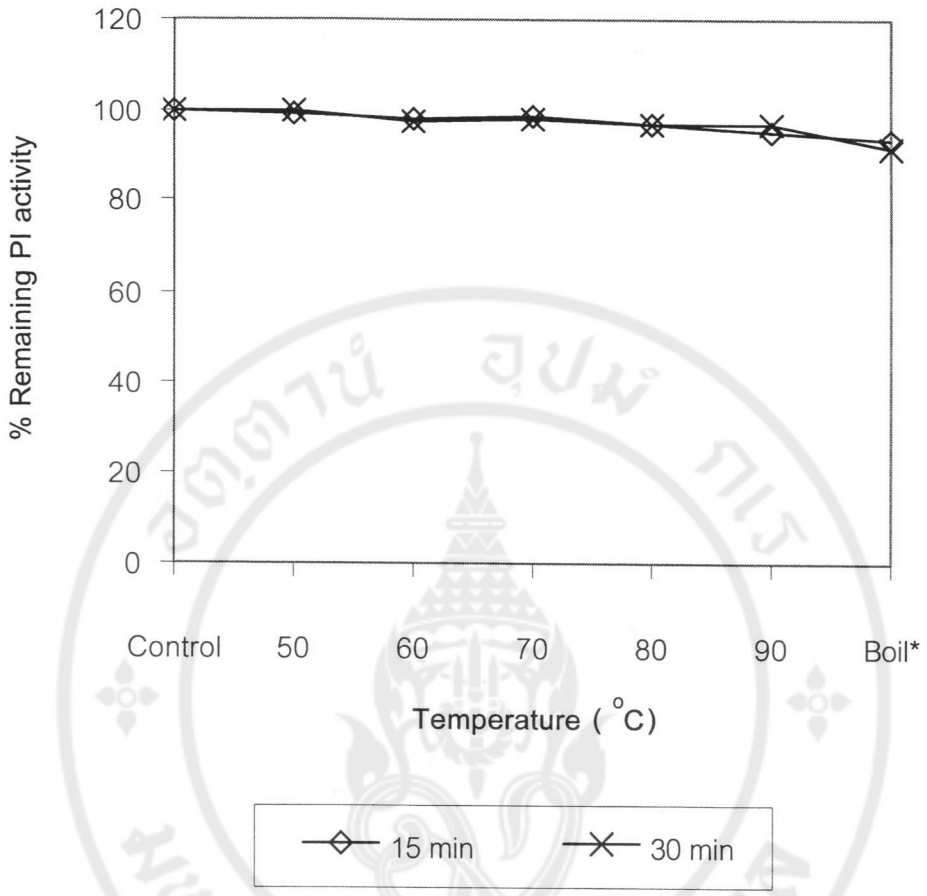

Fig. 26 Trypsin activity inhibition remained of 80-95% acetone precipitant (HPI) from C-serum after heating at varying temperature for 15 and 30 min.

The HPI was subjected to heat treatment by the same method as in Fig.24 but increased heat treatment time to 15 and 30 min. The contents were cooled to RT before determining % remaining PI activity. The results were expressed as the percentage of remaining PI activity when compared to the control which HPI (kept at 4 °C before assayed) was used as 100% PI activity.

* Heating sample in boiling water

Part 5. Chromatographic Purification of HPI

In order to purify HPI for structural characterization and amino acids composition as well as other essential properties of the PI, we need to check specificity and the spectrum of PI activities on different classes of proteases. This is necessary to ascertain that the specific inhibition of protease enzymes activity is due to the direct and stoichiometric interaction between the enzyme and specific recognition by its inhibitor. This is not only useful for classicfication of the PI according to its specific protease but also give information on its spectrum of activities and the degree of its specificity.The term protease is used broadly for all enzymes that cleave peptide bonds (41). Proteinases, which are commonly called endopeptidases, are the major group of industrial proteases that have been extensively studied and well characterized properties are available. They are classified according to the presence of essential catalytic residues of the amino acids at their active sites. Four distinct classes of major important proteinases are known and classified systematically. They are serine-, cysteine- or thiol, aspartic- (carboxyl) or acidic, and metallo-proteinases (51, 52). The well known common example of serine proteinase is trypsin and for cysteine proteinase is papain. As for aspartic proteinase, the common example is pepsin while that of metallo-proteinase is carboxypeptidase and theromlysin which require the presence of divalent metal ions or cations for its activity.

To determine specificity of HPI on different classes of proteases, seven different enzymes were tested in this study which represent each group of the proteases. The different enzymes used were trypsin, chymotrypsin, papain, thermolysin, pepsin, protease from *Aspergillus saitoi* and pronase (mixture of several proteases) . Inclusion

of pronase in this study is to serve as the control and reference for comparison on the degree of specificity. If HPI showed high degree of inhibition on pronase, it might serve as a clue for the HPI activity spectrum that can be further characterized for more details and verified for the HPI specificity.

5.1. Screening Experiment on Different Protease Classes of HPI

The HPI being tested in this experiment was the 80-95% acetone precipitated fraction (HPI) which contained almost a single low MW protein of about 5.5 kD. Seven proteases (trypsin, chymotrypsin, thermolysin, papain, pepsin, protease (*Aspergillus saitoi*) and pronase) were tested with the HPI. They were preincubated with the HPI first and the assay was started by addition of the casein substrate. It was then determined for PI activity of the HPI on each different proteases. The results of this rather complicated experiment are shown in Fig.27 for comparison and summarized for each proteases group in Table 19 for each enzyme chosen for this study. The results showed that HPI were the most effective inhibitor for pronase (57.59 % enzyme activity inhibition), follow by chymotrypsin, trypsin (18.05 % and 14.47 % enzyme activity inhibition, respectively). A weaker inhibition of papain (7.39 % enzyme activity inhibition) was observed. On the contrary, very mild PI activity was found for thermolysin inhibition (4.61% enzyme activity inhibition) and no PI activity against pepsin and protease from *Aspergillus saitoi* was observed in this comparative study (Fig.27 and Table 19). It should be noted that the experiments being carried out in this study were quite complex and some questions still remained on the results obtained. One of the question needed to be corrected is the optimization of the assays for each different enzymes. When they were subjected to the same assay

condition, the optimum for each enzyme will be deviated from the ideal or optimum condition for each specific proteases. Another aspect is the suitablility of the substrate for each enzyme if being arrayed on the same substrate. This would affect the specific interactions of the three components in the assay mixture or assay systems. These three components comprise protease enzyme, substrate, and the HPI which need to be adjusted for optimum assay conditions as well as the optimum stoichiometric relationships. However, this initial experiment is just an exploratory and screening comparative assay only so more factors are needed to be incorporated in each assay of the enzymes. This experiment can only be improved to obtain more meaningful results upon readjustment to take into account variables due to other factors. Besides, more information on the HPI properties are also needed for the better design of the assays and experimental conditions.

The initial interpretation of the results shown in Fig.27 and Table 19 are of twofold that can be made tentatively at the present. Firstly, the high inhibition on pronase suggested that the HPI might have wide spectrum of PI activity. Secondly, the relatively higher inhibition on trypsin and chymotrypsin suggested that the HPI has higher affinity for the serine proteinases but not necessarily mean that it has exclusive specificity for the serine proteinase only. The high protease inhibition on pronase could be taken to mean that the HPI can cross react with other protease groups but with lower affinity toward the active sites of other protease group as compared to the serine proteinase. The more conclusive and more accurate interpretation is therefore awaiting further refinement after assay conditions to be further developed later.

Table 19 Inhibition activity of HPI against various proteases

Type of enzyme	Enzyme	% Enzyme activity inhibition*
Serine protease	Trypsin	14.47
	Chymotrypsin	18.15
Metalloprotease	Thermolysin	4.61
Mix-proteases	Pronase	57.59
Cysteine protease	Papain	7.39
Apatic protease (acid protease)	Pepsin	0
	Protease (*Aspergillus saitoi*)	0

Inhibition activity against trypsin, chymotrypsin, thermolysin, pronase and papain were assayed with 2% casein as the substrate. Pepsin and protease (*Aspergillus saitoi*) were assayed with 2% hemoglobin as the substrate. Enzyme activity used as each proteases control were the amount of enzyme that increases absorbancy at 660 nm (or 750 nm, when used 2% hemoglobin as substrate) by 0.05 OD/min.

*Percentage of enzyme activity inhibition when compared to its control (each enzymes).

The results are plotted as shown in Fig.27.

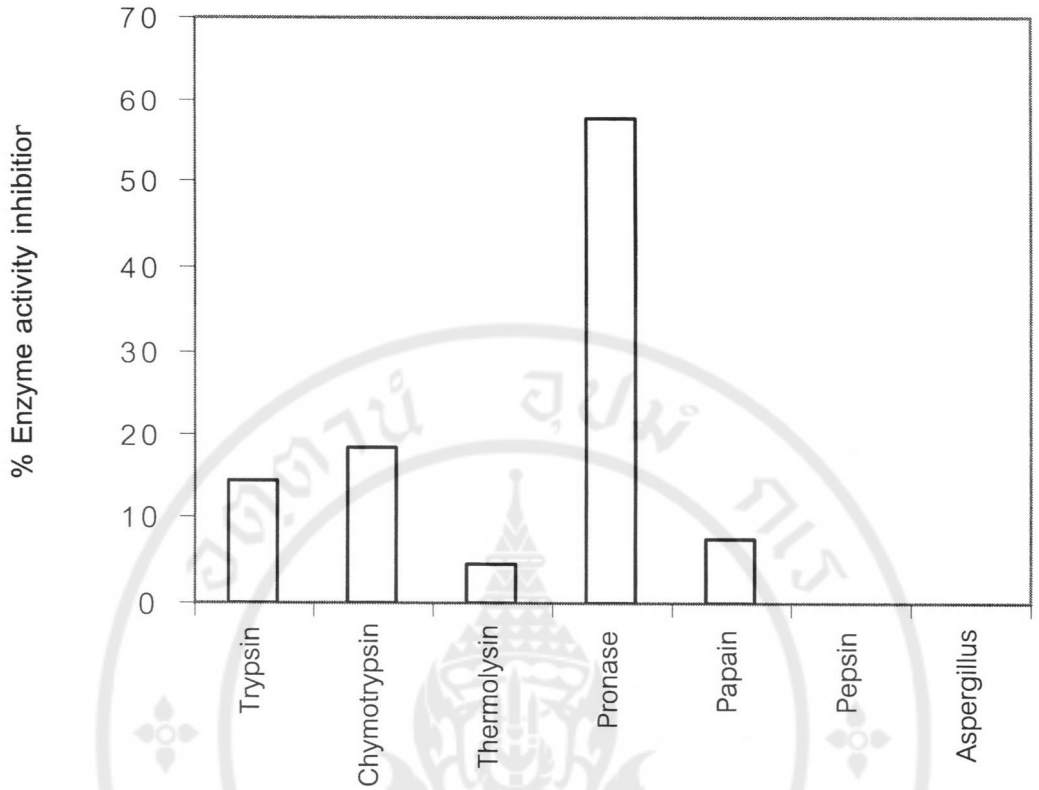

Fig. 27 Comparison of inhibitory activity of HPI against various proteases

The inhibitor (HPI) was preincubated with each proteases for 20 min at 37 °C first and the assay was started by addition of the substrate*, further incubated for 20 min before determined % enzyme activity inhibitions described in the method. The result were expressed as the percentage of enzyme activity inhibition when compared to its control (each enzymes).

*Using 2% casein as substrate, excepted pepsin and protease (*Aspergillus saitoi*) used 2% hemoglobin as substrate.

5.2. Sephadex G-75 Chromatographic Profile of HPI

This experiment was carried out to delineate the molecular nature of small MW protein precipitated by 80-95% acetone as there might be more than one proteins comigrate in the SDS-PAGE. Proteins from 80-95% acetone fraction (HPI) were subjected to Sephadex G-75 column chromatographic separation. The elution profile of HPI gave 2 protein peaks as shown in Fig.28. The first protein peak was designated HPI-1 and the second peak designated HPI-2 as indicated in Fig.28 profile. Both of them were tested for PI activity with trypsin, using BAPNA as enzyme substrate as described in the methods for experiment in this section. The results showed high PI activity in both eluted protein peaks. HPI-1 has higher PI activity than HPI-2 as assayed and compared in Table 20. Protein concentration of HPI-1 was lower than HPI-2 but has higher PI activity, indicating HPI-1 was more active than HPI-2. The results of this study thus indicated that there are two forms of HPIs of similar or the same MW as evidenced by the rather broad band of low MW protein often and regularly noted in SDS-PAGE profiles.

Table 20 Specific trypsin inhibitory activities of HPI-1 and HPI-2

Inhibitor	Specific activity (units/mg)*
HPI-1	106.47
HPI-2	88.23

*1 unit was defined as the amount of inhibitor, which decreases absorbancy at 410 nm by 0.1 OD of its original value in the assay condition .

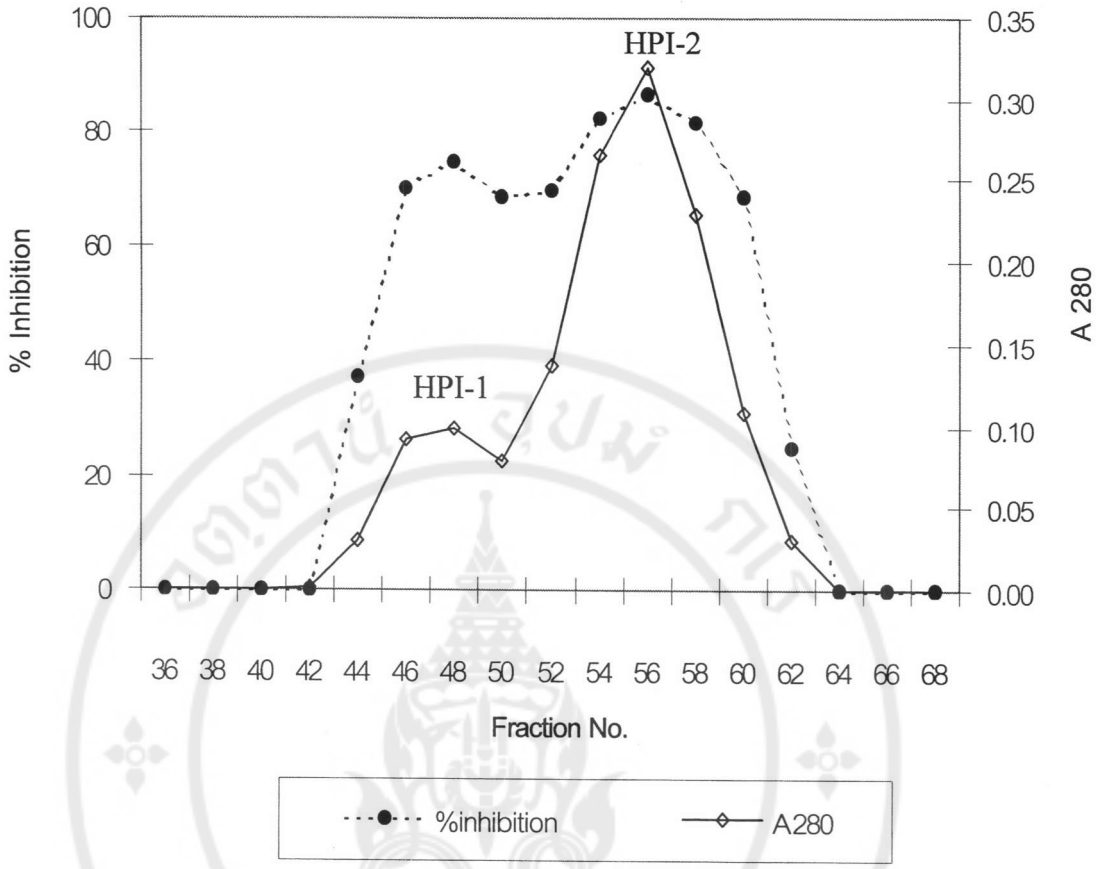

Fig. 28 Chromatographic profile of 80-95% acetone fraction (HPI) on a Sephadex G-75 column

Proteins from 80-95% acetone fraction (HPI) were subjected to Sephadex G-75 (1.6 x 85 cm) prequilibrated with 50 mM Tris-HCl pH 7.5 at a flow rate 9 ml/hr, at 4 ^{o}C. Fractions of 1.5 ml were collected until the effluent absorbance at 280 nm approached zero. The first protein peak was designated HPI-1 and the second peak designated HPI-2. Both of them were tested for PI activity with trypsin as described in the methods for experiment in this section.

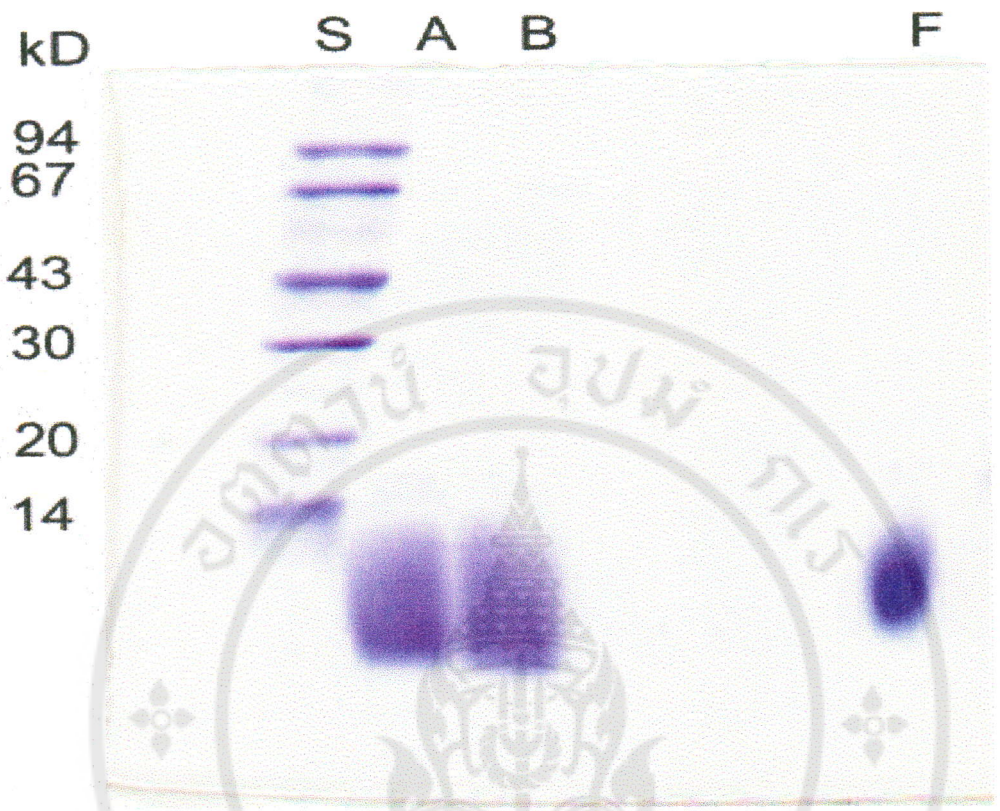

Fig. 29 SDS-PAGE (10-20%gel) of the purified *Hevea* protease inhibitor HPI-1 and HPI-2

Lane S = Standard proteins marker Lane A = HPI-1

Lane F = Protein marker 6.5 kD Lane B = HPI-2

5.3. Molecular Weight Determination of Separated HPI-1 and HPI-2 by SDS-PAGE

The separated HPI-1 and HPI-2 was each subjected to SDS-PAGE (10-20% gel) analysis for MW determination. Both of them showed one single broad protein band probably due to diffusion problem of the low MW protein in the SDS-PAGE as shown in Fig.29. Calibration for MW determination showed both HPI-1 and HPI-2 with similar MW of 5.5 kD (Fig.30). The results showed that HPI resulted from 80-95% acetone precipitation composed of two protein species with similar or the same MW but with different PI activity toward trypsin. The presence of two similar low MW proteins of HPI-1 and HPI-2 might be due to a small different amino acids composition. It might also be tempting to speculate that the two might serve dual functions other than having only PI activity. This is of course remained to be further investigated and characterizated for their properties. It would also be possible that they are serving as the back up for one another or compliment one another for combined effect for higher PI activity but with slightly different specificity toward the protease targets. All these possiblities and properties can be further tested upon suitably designed experiment.

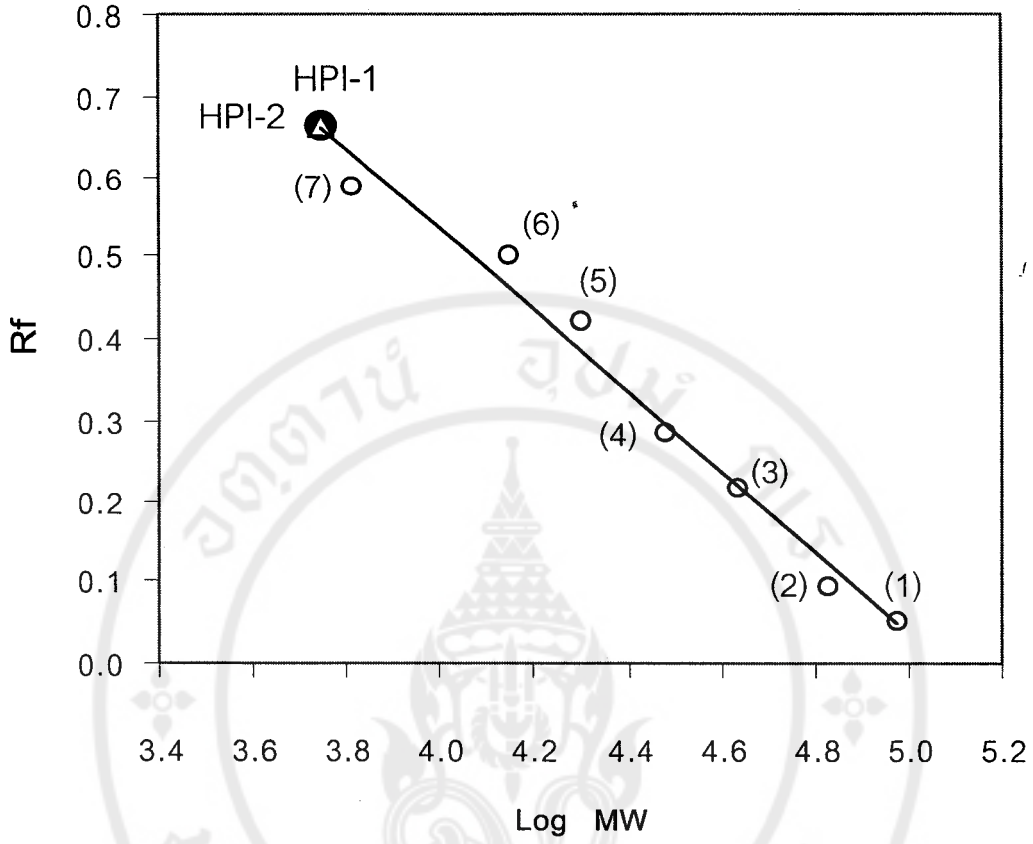

Fig. 30 Molecular weight calibration of HPI-1 and HPI-2 by SDS-PAGE (10-20% gel)

The calibration curve is plotted between relative mobility of the standard proteins and the logarithm of their corresponding molecular weights.

The standard proteins used as reference were: (1) phosphorylase 94 kD, (2) BSA 67 kD, (3) ovalbumin 43 kD, (4) carbonic anhydrase 30 kD, (5) soybean trypsin inhibitor 20 kD, (6) α-lactalbumin 14 kD and (7) apotinin 6.5 kD. Calibration for subunit MW determination show both HPI-1 and HPI-2 with similar Mw of 5.5 kD.

5.4. Molecular Weight Determination of Native HPI-1 and HPI-2 by Gel Filtration

The MW of active form of HPI-1 and HPI-2 were determined for their native states by gel filtration chromatography. This experiment will compliment the results of SDS-PAGE analyses. Standard known molecular weight proteins marker from low molecular weight (LMW) gel filtration calibration kit (Pharmacia) were loaded on to a Sephadex G-75 column (1.6 x 85 cm) for MW determination of the native forms of HPI-1 and HPI-2. The Kav values from the elution profiles were plotted against their respective log MW as shown in Fig.31. Comparison of the elution peaks of HPI-1 and HPI-2 were made with the elution profiles of the standard proteins marker for calibration and calculation of HPIs native MW. The results of calculation for both of them were calibrated for MW based on the standard curve constructed from the LMW markers. The results showed that native MW of HPI-1 and HPI-2 were 20.8 kD and 11.7 kD, respectively as indicated in Fig.31.

The results deduced from data in part 5.3 for subunit MW and part 5.4 for native MW showed that HPI-1 and HPI-2 exhibited two different native forms as analyzed and indicated in Fig.31 of the MW calibration curve. HPI-1 was found to have MW of 20.8 kD representing 4 identical monomeric subunits of 5.5 kD. The results thus indicated that HPI-1 exist as tetrameric form. On the other hand, HPI-2 was found to have native MW of 11.7 kD representing 2 identical monomeric subunits of 5.5 kD. HPI-2 native form thus existed as the dimeric form of the HPIs. It is not certain whether the tetrameric HPI-1 form is composed of the same identical subunits or a combination of the two different subunits. The same can be said of the native dimeric form of HPI-2. The possibility of the multimeric from association of the subunit is

thus an open question to be further verified. This would be of course an interesting molecular study of both the native HPI-1 and HPI-2 for further investigation to be carried out and a better understanding of the subunits affinity and their interactions to form the active form of HPIs.

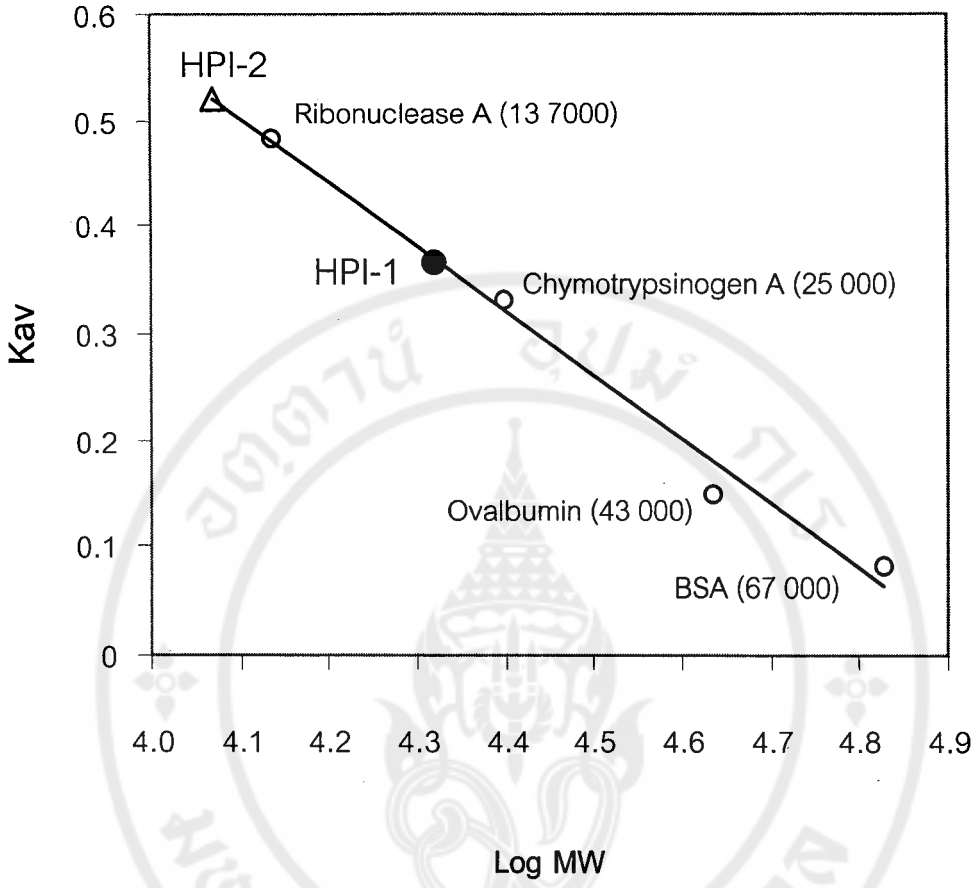

Fig.31 Molecular weight calibration of HPI-1 and HPI-2 by gelfiltration Sephadex G-75

The calibration curve was plotted between Kav values and their respective log MW. Comparison of the elution peaks of HPI-1 and HPI-2 were made with the elution profiles of the standard proteins marker for calibration and calculation of HPIs native MW. HPI-1 and HPI-2 showed native MW of 20.8 kD and 11.7 kD, respectively.

5.5. Characterization of *Hevea* Protease Inhibitors HPI-1 and HPI-2

The purified HPI-1 and HPI-2 were characterized for various important properties. These properties included heat stablility, pH stablility, and their isoelectric point (pI). The results of their various properties are reported in the follwing experiment.

5.5.1. Heat Stability

The heat stable property has been carried out on the 80-95% acetone precipitant fraction in the preceding parts. The experiment in this part will be on the purified HPI-1 and HPI-2 derived from gel filtration chromatography as shown in Fig.28 results.

Samples from peak1 (HPI-1) and peak2 (HPI-2) were subjected to heat treatment at various temperatures ranging from 50 °C to 100 °C for 30 min. The heated HPI-1 and HPI-2 were then tested for the PI activity assay for the remaining inhibitory activity toward trypsin target enzyme. The results showed that the PI activity for both HPIs were not lost up to 90 °C heating for 30 min. PI activity decreased to 90% and 88% of the control inhibition for HPI-1 and HPI-2 respectively, after heating in boiling water for 30 min. as shown in Fig.32. It was thus clear that both HPI-1 and HPI-2 are highly heat stable protease inhibitors similar to the experiments done with the 80-95% acetone precipitant fraction. The results with purified HPIs indicated that they are truly heat stable proteins, which are quite unique and different from other well known protein PI such as soybean trypsin inhibitor which is the heat labile PI. The small contaminant in the acetone precipitant fraction did not interfere or protect HPI from

the heating effect as shown in the preceding part. The HPI heat stability is truely the inherent property of HPI protein by its own virtue.

5.5.2. pH Stability

Effect of pH on HPIs stability is another important property for better understanding of the PI activity. The PI has to encounter different proteases at various extreme pH (pepsin for example) for its efficiency in exerting the PI activity under different pHs. The effect of pH on HPI-1 and HPI-2 were tested by pre-incubating the purified samples at various different pH from 3 to 12 for 24 hr at 37 °C. The pH pretreated samples were then readjusted to pH 8 before PI activity assay for the remaining activity under standard assay condition. The results showed that PI activity of HPI-1 and HPI-2 were stable up to pH 11 without any loss of the activity in the ranges of pH 3-11. The results are shown in Fig.33 for HPI-1 and Fig.34 for HPI-2. However, they were rapidly decreased to 38% and 50% inhibition of the control level at pH 12 for HPI-1 and HPI-2, respectively, as shown and compared in Fig.33 and Fig.34. These results indicated that both HPI-1 and HPI-2 have quite broad range pH stability between pH 3-11 without any effect or loss of the PI activity. The pH stability experiments were carried out with different optimum buffer salts to ascertain the constant buffer capacity at different pH during the pre-incubation period. These different buffers were employed in this experiment as indicated below and are also shown in both Fig.33 and Fig.34. The results clearly showed that both HPIs were quite stable to both acidic and alkaline extreme conditions. This pH stability might be attributed to its low MW subunit composition or its amino acids composition. These

aspects are of interest to be further studied for better understanding of the PI activity under extreme conditions.

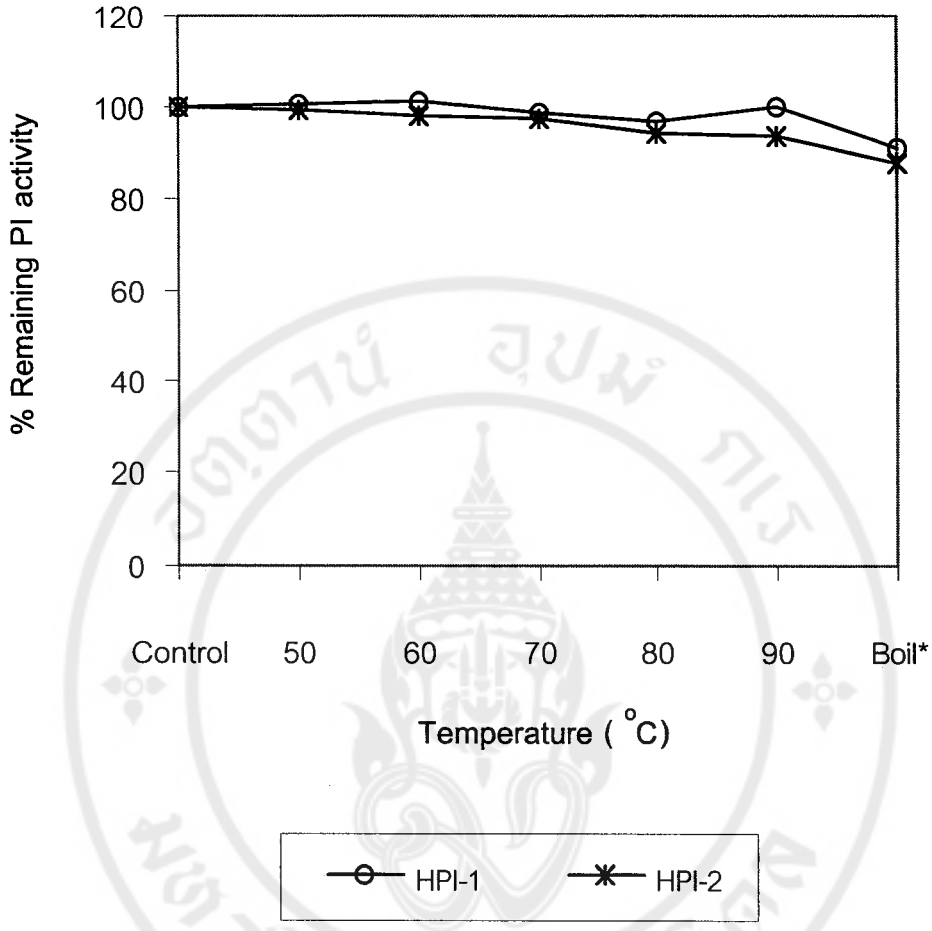

Fig 32 Thermal stability in Tris-HCl pH 7.0 of HPI-1 and HPI-2

Samples from peak1 (HPI-1) and peak2 (HPI-2) were subjected to heat treatment at various temperatures ranging from 50 °C to 100 °C for 30 min. The heated HPI-1 and HPI-2 were then tested for the PI activity assay for the remaining inhibitory activity toward trypsin target enzyme. The results were expressed as the percentage of remaining PI activity when compared to the control which each HPIs (kept at 4 °C before assayed) were used as 100% PI activity.

*Heating sample in boiling water.

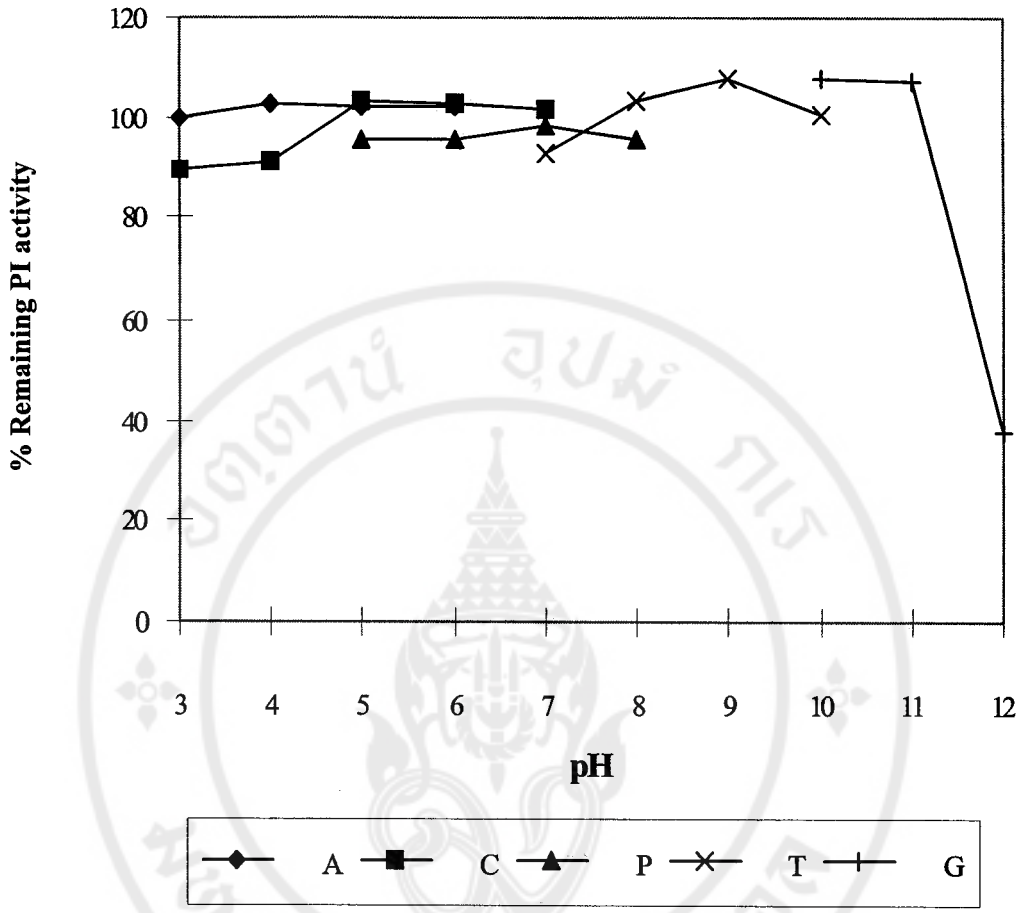

Fig. 33 Effect of pH on the stability of HPI-1

The effect of pH on HPI-1 and HPI-2 were tested by pre-incubating the purified samples at various different pH from 3 to 12 for 24 hr at 37 °C. The pH pretreated samples were then readjusted to pH 8 before PI activity assay for the remaining activity under standard assay condition. The results were expressed as the percentage of remaining PI activity when compared to the control which HPI-1 and HPI-2 (kept in 50 mM Tris-HCl pH 7.5, 4 °C) were used as 100% PI activity control for each of them. (A= Acetate buffer, C = Citrate phosphate buffer, P = Phosphate buffer, T = Tris-HCl buffer, G = Glycine-NaOH buffer)

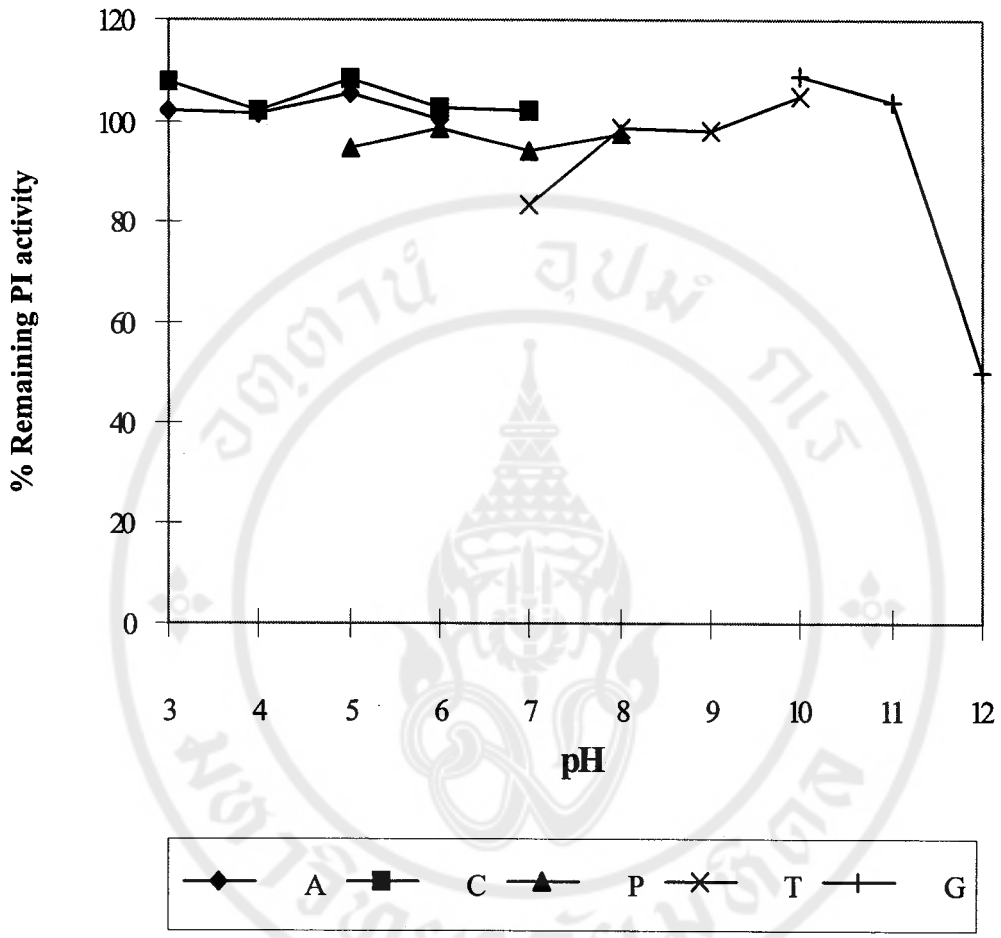

Fig. 34 Effect of pH on the stability of HPI-2

5.5.3. pI Determination of HPIs

Isoelectric focusing was carried out in this experiment to determine the pI values of HPIs. The pH gradients were established in the ranges of 3.6-9.3 using Bio-Rad mini IEF cell as described in the methods. Both *Hevea* protease inhibitors, purified HPI-1 and HPI-2, exhibited acidic isoelectric points as determined by isoelectric focusing (IEF) as shown in Fig.35 with the HPI protein bands located between pH 3.6-4.6 ranges. The results were calibrated from the standard curve constructed from the marker proteins of known pI to obtain pI of HPI-1 and HPI-2 as shown in Fig.36. The pI was determined to be 4.24 for HPI-1 and 4.17 for HPI-2, respectively.

These results indicated that both HPI-1 and HPI-2 are very similar in several aspects, including the pI values which are only slightly small difference of only 0.07 pH unit. This tiny difference of the pI values might reflect a very small difference in amino acids composition of polar nature. It is reasonable to assume that the total amino acids composition must be very conserved and the homology between HPI-1 and HPI-2 was almost 100%. The amino acids composition and sequence analysis will render the answer to this logical speculation.

It should be pointed out that this is the first study and the first report on the induced protease inhibitor in *Hevea* latex as a defense wounding response to tapping of rubber trees for latex collection. A lot of questions still remain to be addressed for a better understanding of the wounding response in the defense mechanism and protective process. Repeated and regular tappings will certainly lead to systemic induction and highly accumulated of the proteins and compounds related to the defense process. Accumulation of HPIs is certainly one of those induced proteins (PR

proteins) for defense. This is certainly warrant for further investigation and detailed study of several open questions to be addressed for a better understanding.

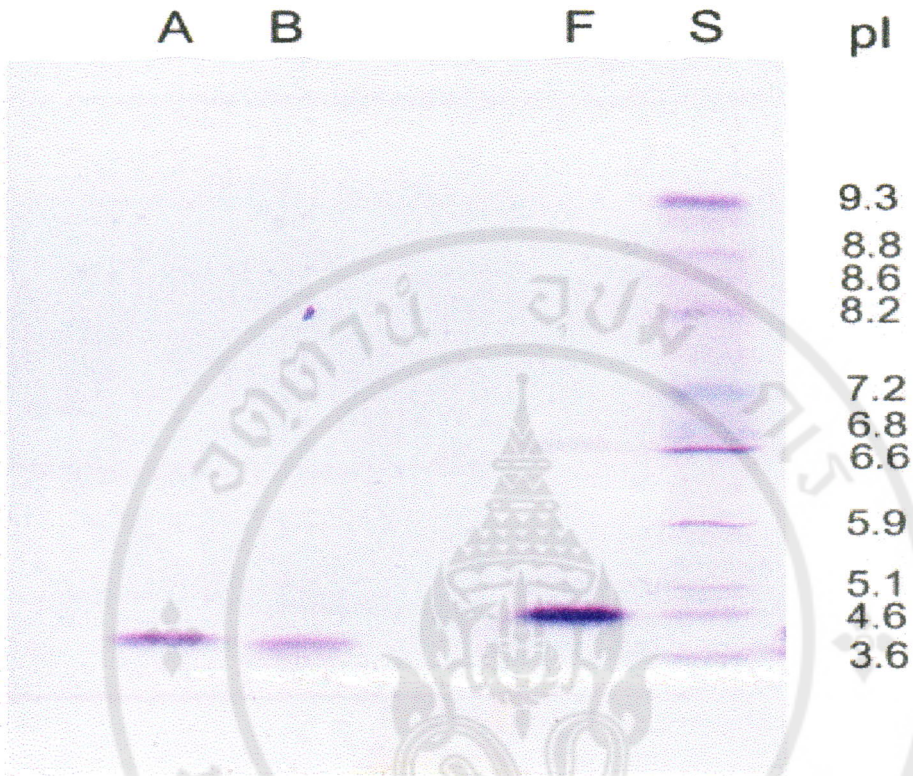

Fig. 35 Isoelectric focusing of HPI-1 and HPI-2

The proteins sample were separated in the model 111 mini IEF cell (Bio-Rad). Focusing was carried out in stepped fashion as described in method section.

Lane S = Standard pI markers (pI 3.9-9.3)

Lane A = HPI-1

Lane B = HPI-2

Lane F = Trypsin inhibitor from soybean (pI 4.6)

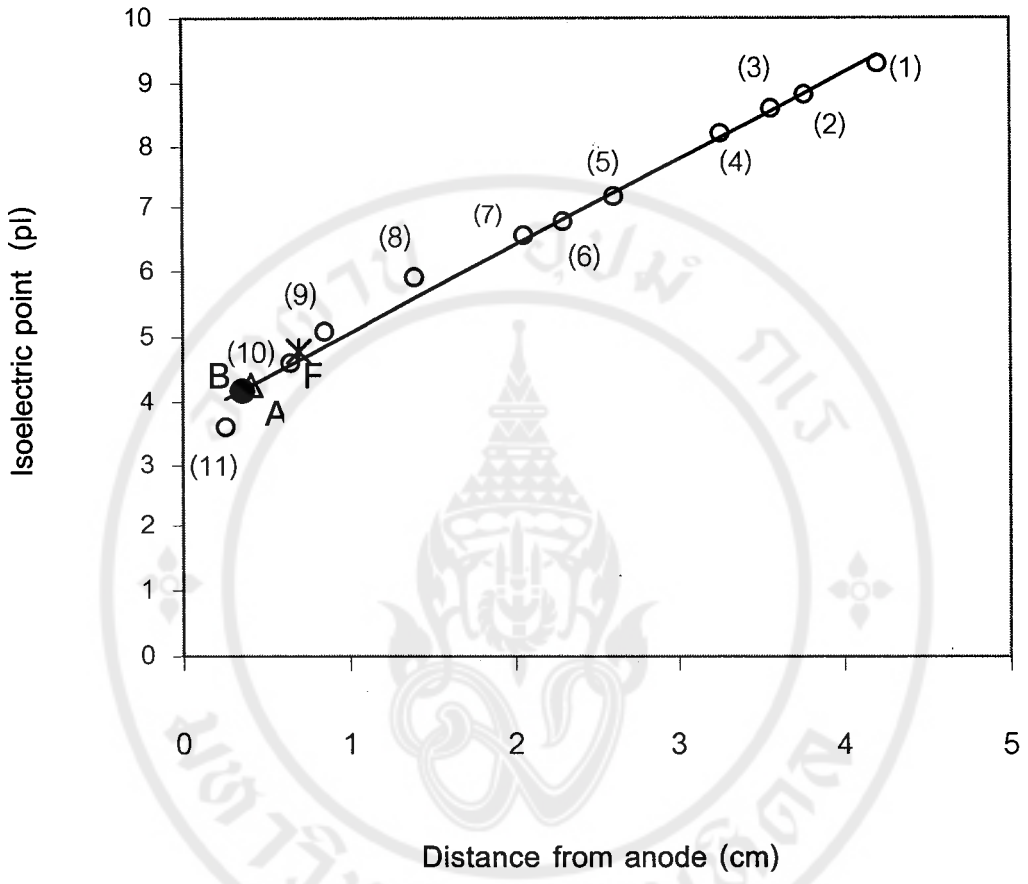

Fig. 36 Calibration curve of standard pI marker for determination of isoelectric pH of the protease inhibitor HPI-1 and HPI-2

The distances from anode (cm)of standard pI markers were plotted against the isoelectric point (pI) values. The standard pI markers were: (1) trypsinogen pI 9.3, (2) –(4) Lectin from *Lens culinaris* pI 8.8, 8.6, 8.2, (5)-(6) myoglobin from horse heart pI 7.2, 6.8, (7) carbonic anhydrase I pI 6.6, carbonic anhydrase II pI 5.9, β-lactoglobulin A pI 5.1, trypsin inhibitor pI 4.6, amyloglucosidase pI 3.6.

A = HPI-1, B = HPI-2, F = trypsin inhibitor from soybean

CHAPTER IV

DISCUSSION

Part 1. Screening and Detection for the Presence of Protease Inhibitors (PIs) in *Hevea* Latex

1.1. Screening for the Presence of Protease Inhibitors (PIs)

Screening for protease inhibitors (PIs)in two main non-rubber parts of rubber latex (B-serum and C-serum) revealed the presence of PIs activity. The observed differences on PIs activity levels between C-serum and B-serum could be attributed to the variability of latex and source of samples. However, the greater PIs activity in C-serum compared to B-serum was consistently observed and noted as shown in Table 8, Fig.3 and Fig.4. These results thus suggested that PI was induced as wounding response from tapping cuts and being accumulated in the latex. However, these results are in contrast to previous negative report on the presence of PIs activity in the bottom fraction (contained most of B-serum) by Archer (64). Similarly, Walujono *et al* .(6, 7) also failed to observe protease inhibitor properties on hevein, which was the main protein in B-serum and has similar molecular weight and high sulfur content to a number of plant protease inhibitors. Hence, our positive PIs detection might be due to the advantageous use of pronase in screening for PIs in this study. Since pronase is the mixture of several protease, so our assay condition was not limited by specificity of enzyme to unknown type of protease inhibitors whereas previous studies used specific

enzyme to detect protease inhibitors. Our preliminary study employing pronase helped detect protease inhibitors (PIs) in B-serum whereas PIs were not found in previous reports. Since our results clearly indicated that C-serum had much more PIs activity than B-serum coupled with previous report that little or no PI can be detected in B-serum, the scope of further study in this thesis is therefore mainly focused on the PI in C-serum.

1.2. Properties of the C-serum Protease Inhibitors (CS-PI)

The results on effect of temperature on protease inhibitory activity of CS-PI suggested that there might exist 2 forms of PIs in the C-serum. One is stable up to 65 ^{o}C and constituted about 40% of the total PIs activities. The other PI is a very heat stable component even at 95 ^{o}C, which comprises 60% of the total PIs activities in the C-serum. Effect of boiling on C-serum protease inhibitor stability indicated that the CS-PI was the thermostable molecule with a very strong heat stable property. The results from these studies suggested at least two possibilities or even more. The first possibility is that there might exist 2 groups of PIs in the C-serum, a moderate and a very heat stable PIs. The second unlikely possibility is that only one group is present but the heating effect could only destroy 50% of the PIs activity. It is quite obvious that there are several advantages in working with this heat stable PIs, both in purification protocol as well as the case of handing and processing and superior quality of the heat stable PI as derived from natural source for many possible application in biotechnology and other practical implications.

Part 2. Characterization of Properties of PIs in the C-serum

The result on gel filtration by using Amersham Pharmacia column PD-10 indicated that PIs in C-serum are clearly macromolecule, not a small organic compound. Since, PIs activity co-eluted with protein bulk, then possibly these macromolecules are protein. When ammonium sulfate precipitation was used to fractionate proteins from C-serum, the result confirmed their macromolecular nature as determined by using PD-10 and suggested that the PIs are proteinaceous protease inhibitors as precipitated by ammonium sulfate. The PI activities were detected for all ammonium sulfate precipitated fractions (20% up to 95% ammonium sulfate concentration) at 10% concentration interval as well as for the 20% interval. This may have 2 possible aspects for explanation. Firstly, C-serum has more than one type of protease inhibitors, which can be precipitated by ammonium sulfate over a wide range both at low and high ammonium sulfate concentrations. Secondly, C-serum may have only one type of PI but can be easily salted out by ammonium sulfate over wide ranges. However, it should be kept in mind that pronase which was used in these screening experiments is the mixture of several proteases. These different proteases may have different levels of specificity and sensitivity to PIs in C-serum in various ammonium sulfate precipitated PI protein fractions. This pronase nature may cause appearance PIs activity in the many ranges of ammonium sulfate precipitations. When Protease with substrate specific property such as trypsin was chosen to test PIs activity of various ammonium sulfate precipitant fractions and compared to pronase, the results showed different sensitivity to ammonium sulfate fractions of trypsin activity inhibition when compared to pronase. However, distribution of PIs activity in several

ranges was detected although specific protease such as trypsin was used instead of pronase and changed substrate from Azocoll with BAPNA (Azocoll was an insoluble substrate and has the following disadvantages: First, it cling to the sides of the tubes upon shaking causing variation of distributing equal amounts of substrate to assay tubes. Second, the non-homogeneity of the solution, thermal motion is not sufficient to insure maximal or equal contact of enzyme with the substrate. Finally, some colored products were released as a function of time.). Therefore, the ammonium sulfate precipitation and separation of PI was not found satisfactory for initial step in the characterization of PIs. Another protein fractionation method using organic solvent precipitation (acetone) was chosen and PI activity was tested on trypsin alone instead of pronase from this point on as the screening experiments were completed.

The acetone precipitation experiment confirmed and agreed with the results from ammonium sulfate precipitation that PIs in C-serum were of proteinaceous protease inhibitors. Acetone precipitated results suggested that the PI protein precipitation gave better clear cut PIs activity fractions than those obtained with ammonium sulfate precipitation. However, the results also pointed out that PIs in C-serum need rather high acetone to precipitate out from other proteins as indicated by the increasing PI activity with high percentage of acetone. These observations suggested that the PI might probably be small proteins with rather hydrophobicity in its molecular nature.

Part 3. Characterization and Properties of PIs

Approximate MW determination of *Hevea* protease inhibitors (HPI) in crude protein from 60% ammonium sulfate fraction by Sephadex G-200 gel filtration, the PIs activity peak appeared to be in the range lower than 25 kD when compared to known molecular weight proteins. Since the PIs sample was not pure protein, so this result was just a guide for approximating the MW of HPI by this gel filtration separation and calibration profile in the combined results of Fig.16 and Fig.15, respectively. They are in the MW range of protease inhibitors from plant sources which are mainly in the range from 3,000 to 25,000 (67).

The pattern of protein bands from 10% sequential fractionation by ammonium sulfate and acetone were determined by SDS-PAGE. Protein fraction from ammonium sulfate precipitation showed several protein bands in all precipitant fractions whereas two fractions from acetone precipitant fractionation of 60-70% and 70-80% acetone with maximum PIs activity showed quite distinct 2 major bands (between 30-43 kD and smaller than 14 kD) of the proteins. . It was then quite obvious from SDS-PAGE analyses of proteins that the acetone precipitation method was far more suitable and better than ammonium sulfate precipitation to be employed in purification protocol.

Acetone was thus incorporated and employed in the purification of HPI for further characterization and other related studies. Employing acetone in fractionation and separation of HPI from the bulk of C-serum proteins was far more superior and more advantageous than the conventional ammonium sulfate precipitation. High percentage acetone precipitation of the HPI also suggested or pointed out one important aspect of the HPI nature, that the HPI might be present as a small protein in C-serum and that it

might have hydrophobic character. This was deduced from the results on heat stable property of HPI as well as the environment of the latex in which the HPI has to encounter with.

Part 4. Purification of the HPI

4.1. Acetone Fractionation

Ranges of acetone fractionation were improved by used 0-50%, 50-70%, 70-80% and 80-95% acetone to precipitate proteins from C-serum. This mathod of high percentage of acetone (95%) was to improve the recovery of HPI which showed only one major protein band of low MW but with high PI activity. The results thus obtained in Fig.21 indicated that the HPI was a small protein of less than 6.5 kD and was well precipitated at high acetone concentration for better and cleaner separation from other remaining proteins after 80% acetone precipitation of the C-serum proteins. It is quite surprising and unexpected to find such an efficient and simple procedure in obtaining almost purified PI with this step-wise acetone precipitation with almost no loss of the HPI activity even at very high acetone. This finding was then adopted in subsequent experiment and analyses of the HPI. Since, this method gave rather pure PIs protein with moderate quantity in quite a few steps and there are some advantages that acetone can be recycled in the system, then it is suitable on the industrial scale.

4.2. Approximation of HPI Molecular Weight by SDS-PAGE

The result suggested that the protein from 80-95% acetone fraction (HPIs) composed of one size of subunit with its molecular weight of 5.5 kD. The result thus obtained, indicated that the HPI was either a monomer of 5.5 kD or multimeric protein of the same monomeric subunit of 5.5 kD. This nature of the native state of the HPI remains to be tested further for its existence in the latex or its active form in the PI activity assay. However, it should be pointed out that several PI of small MW proteins have been found and characterized from plant source. It is then quite likely that the native HPI might exist as a monomer of single polypeptide of 5.5 kD. However, this was quite small and very interesting to further elucidate its structure, properties and application. However, more detail about the number of subunit species and its purity need better method such as HPLC and sequence analysis.

4.3. Comparison of C-serum HPI with B-serum Hevein Protein

It was very interesting to observe that B-serum gave one pale protein band with its size lower than 14 kD in 80-95% acetone fraction which similar to HPI band of C-serum. The similarity of the two suggested that C-serum HPI might be related to the B-serum hevein. However, this protein fraction did not give any PIs activity with trypsin. It has been reported that hevein from B-serum which has molecular weight 5 kD (66) and much resemblance to protease inhibitors in general structural characteristics but did not show any inhibition (6, 7). It has the possibility that the proteins from 80-95% acetone fraction in B-serum and C-serum are the same type but in different form, *eg.* it has inactive form of PIs in B-serum and active form in C-serum. Then, detection of PIs activity in B-serum with pronase was found PIs activity,

may cause from some proteases in pronase being digested and modified inactive PIs to active form during incubation time. This might be resulted from modification of hevein and rendering it to be localized in different compartment of the latex which differ in the expression of PI activity. However, the speculation needs to be further verified.

4.4. Heat Stable Property of 80-95% Acetone Precipitant HPI

The protein from 80-95% acetone fraction or HPI was a very heat stable protein. Heating HPI in boiling water up to 30 min decreased only approximate 10% PI activity. The result was different from previous test in C-serum that PIs activity was decreased to about 45% after heating in boiling water. This was due to other protein in C-serum trapped and co-aggregated PIs protein after heat treatment or C-serum has more than one type of PIs that the non heat stable PIs was denatured after heat treatment. Since HPI was rather pure, then it has less interference from other component. The results indicated that HPI is truly a very thermostable protein, probably the most stable small protein in C-serum of the latex similar to that of calmodulin previously reported by our group (68). This strong thermostable property is quite unique for many possible biotechnology application, including the possibility for industrial scale production as the PI can find many uses in both food technology as well as medical science among several others. With its superior property to tolerate heat, it is certainly of great potential.

Part 5. Chromatographic Purification of HPI

Separation of HPIs by size exclusion chromatography on a Sephadex G-75 column gave 2 peaks with PIs activity, the first peak called HPI-1 and the second peak called HPI-2. The results showed that HPI resulted from 80-95% acetone precipitation composed of two protein species with similar or the same MW but with different PI activity toward trypsin, HPI-1 was more active than HPI-2. The presence of two similar low MW proteins of HPI-1 and HPI-2 might be due to a small difference in amino acids composition. It might also be tempting to speculate that the two might serve dual functions other than having only PI activity. This is of course remained to be further investigated and more characterization for their properties. It would also be possible that they are serving as the back up for one another or compliment one another for combined effect for higher PI activity but with slightly different specificity toward the protease targets. All these possiblities and properties can be further tested upon suitable designs for the desired experiment.

HPI-1 and HPI-2 gave one protein band with similar subunit MW of 5.5 kD on SDS-PAGE. In the case of gel filtration, native MW of HPI-1 and HPI-2 were 20.8 kD and 11.7 kD, respectively. Comparisons of these results suggested that HPI-1 was found to have MW of 20.8 kD representing 4 identical monomeric subunits of 5.5 kD. The results thus indicated that HPI-1 exist as tetrameric form. On the other hand, HPI-2 was found to have native MW of 11.7 kD representing 2 identical monomeric subunits of 5.5 kD. HPI-2 native form thus existed as the dimeric form of the HPIs. It is not certain whether the tetrameric HPI-1 form is composed of the same identical

subunits or a combination of the two different subunits. The same can be said of the native dimeric form of HPI-2. The possibility of the multimeric from association of the subunit is thus an open question to be further verified. This would be of course an interesting molecular study of both the native HPI-1 and HPI-2 for further investigation to be carried out and a better understanding of the subunits affinity and their interactions to form the active form of HPIs.

5.1. Screening Experiment on Different Protease Classes of HPI

It should be noted that the experiments being carried out in this study was quite complex and there are still some questions remained to be answered on the results being obtained. One of the question needed to be corrected is the optimization of the assays for each different enzymes. When they were subjected to the same assay condition, the optimum for each enzyme will be deviated from the ideal or optimum condition for each specific proteases. Another aspect is the suitablility of the substrate for each enzyme if being arrayed on the same substrate. This would affect the specific interactions of the three components in the assay mixture or assay systems. These three components comprise protease enzyme, substrate, and the HPI which need to be adjusted for optimum assay conditions as well as the optimum stoichiometric relationships. However, this initial experiment is just an exploratory and screening comparative assay only so more factors are needed to be incorporated in each assay of the enzymes. This experiment can only be improved for more meaningful results upon readjustment appropriately and accordingly. Besides, more information on the HPI properties are still needed for the better design of the assays and experimental conditions.

The initial interpretation of the results shown in Fig.27 and Table 19 are of twofold that can be made tentatively at the present. Firstly, the high inhibition on pronase suggested that the HPI might have wide spectrum of PI activity. Secondly, the relatively higher inhibition on trypsin and chymotrypsin suggested that the HPI has higher affinity for the serine proteinases but not necessary to mean that it has exclusive specificity for the serine proteinase only. The high protease inhibition on pronase could be taken to mean that the HPI can cross react with other protease groups but with lower affinity toward the active sites of other protease group as compared to the serine proteinase. The more conclusive and more accurate interpretation is therefore awaiting further refinement after assay conditions to be further developed later.

5.2. Characterization of *Hevea* Protease Inhibitors HPI-1 and HPI-2

It was thus clear that both HPI-1 and HPI-2 are highly heat stable protease inhibitors similar to the experiments done with the 80-95% acetone precipitant fraction. The results with purified HPIs indicated that they are truly heat stable proteins, which are quite unique and different from among the well known protein PI such as soybean trypsin inhibitor which is the heat labile PI. The small contaminant in the acetone precipitant fraction did not interfere or protect HPI from the heating effect as shown in the preceding part. The HPI heat stability is true the inherent property of HPI protein by its own virtue.

Effect of pH on HPIs stability is another important property for better understanding of the PI activity. The PI has to encounter different proteases at various extreme pH (pepsin for example) for it efficiency in exerting the PI activity under different pHs. The results clearly showed that both HPIs were quite stable to both

acidic and alkaline extreme conditions. This pH stability might be attributed to its low MW subunit composition or its amino acids composition. These aspects are of interest to be further studied for better understanding of the PI activity under extreme conditions.

The pI was determined to be 4.24 for HPI-1 and 4.17 for HPI-2, respectively. These results indicated that both HPI-1 and HPI-2 are very similar in several aspects, including the pI values which are only slightly small difference of only 0.07 pH unit. This tiny different of the pI values might reflect a very small difference in amino acids composition of polar nature. It is reasonable to assume that the total amino acids composition must be very conserved and the homology between HPI-1 and HPI-2 was almost 100% homology. The amino acids composition and sequence analysis will render the answer to this logical speculation.

It should be pointed out that this is the first study and the first report on the induced protease inhibitor in *Hevea* latex as a defense wounding response to tapping of rubber trees for latex collection. A lot of questions still remain to be addressed for a better understanding of the wounding response in the defense mechanism and protective process. Repeated and regular tappings will certainly lead to systemic induction and high accumulation of the proteins and compounds related to the defense process. Accumulation of HPIs is certainly one of those induced porteins (PR proteins) for defense. This is certainly warrant for further investigation and detailed study of several open questions to be addressed for a better understanding.

CHAPTER V

SUMMARY

The results of this investigation could be summarized as follow :

1. Protease inhibitors (PIs) were found in major components of the non-rubber constituents (B-serum and C-serum). The PI activity in C-serum was much higher than B-serum.
2. High acetone concentration was required to precipitate PI in C-serum out from other proteins.
3. HPI was shown to possess homomeric subunit of a single protein band with MW of 5.5 kD by SDS-PAGE analyzes.
4. HPI was the effective inhibitor for pronase (57.59 % enzyme activity inhibition), followed by chymotrypsin, trypsin (18.05 % and 14.47 % enzyme activity inhibition, respectively). A weaker inhibitor on papain (7.39 % enzyme activity inhibition) was observed. On the contrary, very mild PI activity was found for thermolysin inhibition (4.61% enzyme activity inhibition) and no PI activity against pepsin and protease from *Aspergillus saitoi.*
5. Molecular weight comparisons of C-serum HPI and B-serum hevein protein suggested that C-serum HPI might be related to the B-serum hevein.
6. HPI was composed of 2 and 4 protein subunit monomers with the same MW of 5.5 kD and designated as HPI-1 and HPI-2, respectively.

7. By gel filtration chromatography, HPI-1 and HPI-2 had native MW of 20.8 kD and 11.7 kD, respectively.
8. Both HPI-1 and HPI-2 were thermostable proteins with were stable up to 90 ^{o}C heating for 30 min without loss of activity. PI activity decreased to 90% and 88% of the control inhibition for HPI-1 and HPI-2 respectively, after heating in boiling water for 30 min.
9. Both HPI-1 and HPI-2 have quite broad range pH stability between pH 3-11 without any effect or loss of the PI activity but rapidly decreased to 38% inhibition (HPI-1) and 50% inhibition (HPI-2) of the control level at pH 12.
10. The pI value of HPI-1 and HPI-2 as determined by isoelectric focusing (IEF) were 4.24 and 4.17, respectively.

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