2.0 Bioactive peptides and its classification

Peptides with biological activities generally contain three to twenty amino acid units, which are proteins synthesized in the form of large prepropeptides in the cell. Bioactive peptides in plants supposedly involved in defence mechanisms that confer resistance against phytophagous predators and infection by viruses, bacteria, fungi, nematodes and other organisms (Carlini and Grossi-de-Sa, 2001). The best known plant proteins are lectins, ribosome-inactivating proteins (RIPs), arcelins, alpha-amylase canatoxin and protease inhibitors.

2.0.1 Lectins

Lectins are class of a protein of non-immune origin that possess at least one non-catalytic domain that specifically and reversibly binds to mono or oligosaccharide (Lis and Sharon, 1986; Peumans and Van Damme, 1995; Carlini and Grossi-de-Sa, 2001). The seeds of the *Leguminoseae* are rich sources of lectins, but the same lectin and homologs are also found in other parts of the plant, such as the bark, stem, and leaves. This family includes lectins such as ConA, soybean agglutinin, and lentil lectin. Two other smaller families of plants whose lectins have been characterized are the Gramineae; cereals, such as wheat germ and Solanaceae; potatoes and tomatoes.

The function of lectins in plants is still controversies with their biological roles in the parent organisms (Carlini and Grossi-de-Sa, 2001). The binding site for carbohydrate in some lectins involves a combination of hydrophobic interactions and van der Waals contacts to which small plant growth regulators such as adenine can bind to (Roberts and Goldstein,

1983; Carlini and Grossi-de-Sa, 2001). Although plant lectins have an ability to bind carbohydrate, evidence exists that these proteins may have additional activities. For example, some lectins, such as *Dolichus biflorus*, bind adenine residues with high affinity and specificity in regions of the protein outside the common carbohydrate-binding site.

Some plant lectins have shown entomotoxic effects when fed to insects from Coleoptera, Homoptera, and Lepidoptera orders (Peumans and Van Damme, 1995; Carlini and Grossi-de-Sa, 2001). Transgenic expression of the gene encoding *G. nivalis* agglutinin in rice plants decreases survival and fecundity of insects attacking the transgenic plants (Schuler *et al.,* 1998). Therefore this class of protein should be studied more for their potential.

2.0.2 Ribosome-inactivating proteins (RIPs)

Ribosome-inactivating proteins or RIPs are the proteins that are capable of inactivating ribosomes in many plants (Peumans *et al.,* 2001). RIPs are a group of cytotoxic N-glycosidases that specifically cleave nucleotide N-C glycosidic bonds. RIPs were first identified more than 100 years ago. Their biological functions are determined to play a role in plant defence mechanism.

RIPS have been described into three types, types I is composed of a single polypeptide chain, whereas type II is a heterodimer consisting of a chain, which is attached to a sugar-binding B chain that functionally equivalent to a type I while type III is unknown function that forms single chain containing an extended carboxyl-terminal domain (Park *et al.,* 2004).

The effect of ribosome show differential sensitivity to RIPs in plant cells and isolated ribosomes, while ribosomes of protozoans and fungi seem to be highly sensitive. Ricin, the toxic principle of castor bean was identifried as a protein at the 19th century was shown to be ineffective to a variety of insects of different orders, although it was able to inhibit protein synthesis by insect ribosomes in cell-free preparations (Gatehouse *et al*., 1990; Carlini and Grossi-de-Sa, 2001). Since the isolation and characterization of ricin, many structurally and functionally related proteins have been identified in a wide variety of plants (Peumans *et al.,* 2001).

2.0.3 Alpha-Amylase inhibitors

Alpha-amylases are found in microorganisms, animals and plants, whixh catalyze the initial hydrolyses of alpha-1,4-linked sugar polymers into shorter oligosaccharides, an important step towards transforming sugar polymers into single units that can be assimilated by the organism. The first alpha-amylase inhibitor characterised was that of the monomeric 13 kD known as 0.31 form, from wheat (Carlini and Grossi-de-Sa, 2001).

This endosperm protein is relatively abundant in seeds that suggesting a role as a storage or reserve protein, as regulators of endogenous enzyme or as defensive mechanisms against the attacks of animal predators and insect or microbial pests. These inhibitors were also relevant in several aspects of human health such as to control of diabetes and obesity, diagnosis of pancreatic hyperamylasemia disorders and nutritional and toxicological aspects of foods (Turcotte *et al.,* 1994; Bisschoff *et al.,* 1994; Carlini and Grossi-de-Sa, 2001). The amylase inhibitors present in seeds used as food present some toxicological

significance in the diets of infants who have a lower production of pancreatic alpha-amylase than adults and for patients with impaired peptic or gastric function (Richardson, 1991).

Alpha-amylase inhibitors also show great interest as potentially important tools of natural and engineer resistance of crop plant against pests that are improved through the use of transgenic technology (Gatehouse and Gatehouse, 1998, Carlini and Grossi-de-Sa, 2001). Focused on lectin-like inhibitors present in common bean *Proteus vulgaris* seeds have been shown that toxic effects to several insect pests (Ishimoto and Chrispeels, 1996). The effect was well determined not only in different oraganisms by enzymatic activity but also in feeding assay experiments (Ishimoto *et al.,* 1996).

Transgenic pea and azuki bean seeds expressing the inhibitor, Alpha-amylase-1, of the domesticated common bean *Proteus vulgaris* was completed resistance against bruchids (Ishimoto *et al.,* 1996; Morton *et al.,* 2000). Therefore these proteins can be safely introduced into food plants because the transgenic grains showed minimal effects on mammalian digestion system (Pusztai *et al.,* 1999; Carlini and Grossi-de-Sa, 2001).

2.0.4 Arcelins

Arcelins are lectin-related proteins detected only in wild beans (*Phaseolus vulgaris*) and exists in six electrophoretic variants which supposedly involved in defence mechanisms to confer resistance against predators such as bruchid beetles (Chrispeels and Raikhel, 1991;

Carlini and Grossi-de-Sa, 2001), although the precise mechanism behind the toxicity of arcelin is as yet unknown.

Arcelins can be divided into three subgroups,group one are arcelin 1, arcelin 2 and arcelin 6, group two is arcelin 4 and group three are arcelin 5a and arcelin 5b. Sequence data of arcelin 6 was indicated that this protein is closely related to arcelin 1 and arcelin 2. Biochemical data indicate that arcelin 3 belongs to the same subgroup of arcelin 4.

Arcelin 5 has been reported to consist of a mixture of two major protein fractions, termed arcelin 5a, Arc5a, 32.2 kDa and arcelin 5b, Arc5b with 31.5 kDa (Goossens *et al.,* 1994). Arc5b and Arc5a contain one and two glycans, respectively, while Arc5c is not glycosylated. Two different arcelin-5 cDNA sequences were reported (Goossens *et al.,* 1994), called *arc5-I* and *arc5-II*. They encode two polypeptides of 240 amino acids (26.8 and 27.0 kDa) with a high identity (96.9%, a difference of 8 residues in the N-terminal part of the chain).

The *arc5-I*-encoded protein contains three potential glycosylation sites, while the*arc5-II* encoded protein contains only two. Arc5a and Arc5b are encoded by *arc5-I* and *arc5-II*, respectively, while Arc5c could be encoded by *arc5-II* or by a third copy of the *arc5* gene with a much lower rate of expression. Arcelins are thought to provide resistance against the bean bruchid pest *Zabrotus subfasciatus* (Osborn *et al.,* 1988). Among the arcelin variants, Arc1 and Arc5 appear to be the most promising in conferring insect resistance (Kornegay *et al.,* 1993) discovery of putative novel enzymatic (Goossens *et al.,* 1994).

2.0.5 Protease inhibitors

Protease inhibitors (PIs) are found in animals, plants and microorganisms (Laskowski and Kato, 1980). Protease inhibitors adopt many different structures, ranging in size from mini-proteins to large macromolecular structures, much larger than the target enzyme (Otlewski *et al*., 1999). PIs are classified into two large groups based on their structural dichotomy which include the low molecular weight peptidomimetic inhibitors and protein protease inhibitors (Fear *et al.,* 2007).

PIs are divided into five groups of serine, cysteine, threonine, aspartyl and metalloprotease inhibitors according to the mechanism employed at the active site of proteases they inhibit (Fear *et al.,* 2007). Two mechanisms can occured prroteolytic inhibition by protease inhibitors by irreversible trapping reactions and reversible tightbinding reactions (Rawlings *et al.,* 2004).

From previous studies, plants seed are widely distributed and a rich source of protease (Richardson, 1991; Mello and Silva-Filho, 2002; Chaudhary *et al.,* 2008). Protease inhibitors (PIs) have evolved to inhibit proteolytic enzymes. They are classified according to their types of enzyme they inhibit (Mosolov, 1998; Otlewski *et al.,* 1999; Carlini and Grossi-de-Sa, 2001). The molecular mass of these inhibitors can vary from 4 to 85 kDa, with majority in the range of 8 to 20 kDa (Hung e*t al.,* 2003).

PIs play different roles in their action as storage proteins (Xavier-Filho, 1992), as regulators of endogenous proteolytic activity (Ryan, 1990), as participants in program cell death, or

as components with extraordinary properties that protective naturally to defense against pathogens and pests attack such as viral, bacterial, fungal and others, or play regulatory roles during plant development, involve as markers in studies of plant diversity and evolution in relation to host co-evolution and other properties (Ryan, 1990; Lu *et al.,* 1998). PIs also are known to be involved in clinical studies, such as blood coagulation, immune regulation, platelet aggregation and anti-carcinogenesis (Kennedy, 1998; Chaudhary *et al.,* 2008).

In the past decade, PIs are used as therapeutic agents for the treatment of human immunodeficiency virus (HIV) and hypertension. Research done by Hilder et al. In 1987 shown the first success experiment of using the stable genes that encoded the stable inhibitors was transferred to plants to improve their resistance to pests or fungi (Hilder, 1987; Ryan, 1990). They transferred trypsin inhibitor gene from *Vigna unguiculata* to tobacco, which conferred resistance to wide range of insect pests including Lepidopterans, Coleopterans and Orthoptera to protect the plants.

Since proteinase inhibitor genes are primary gene products, they are excellent candidates for engineering pest-resistance into plants and extremely overcome the potential risk of proteolysis. From the latest studies show that PIs also influence insect development by causing amino acid deficiency, reducing the ability of essential amino acids for the production of the other proteins and remain active under different gut pH (Pompermayer *et al.,* 2001; Bhattacharya *et al.,* 2007).

2.1 Classification of protease inhibitors

They are classified according to their mechanism of catalysis and the amino acid present in the active center such as cysteine proteinases, with a cysteine, aspartic proteinases, with an aspartate group, metalloproteinases, with a metallic ion such as Zn^{2+}, Ca^{2+} and $Mn^{2+}$and serine proteinases, with serine and histidine (Neurath, 1984; Carlini and Grossi-de-Sa, 2001). Attachment of the proteases to a certain group depends on the structure of catalytic site and the amino acid essential for its activity.

2.1.1 Cysteine protease inhibitors

Cysteine protease inhibitors or cystatin are known as phytocystatins in plants. The cysteine protease class includes papain, calpain and lysosomal cathepsins and have been recognized in maize, soybean, and potato and in many variety of plant (Gruden *et al.,* 1997; Ryan *et al.,* 1998). Phytocystatins are 5 to 87 kDa proteins, usually found in cystatins subfamilies I and II (Arai *et al.,* 1998; Carlini and Grossi-de-Sa, 2001). One group of phytocystatins contains a single domain and second group has multiple domains that are found in sunflower seeds and potato tubers (Walsh and Strickland, 1993; Pernas *et al.,* 1999; Carlini and Grossi-de-Sa, 2001).

The primary and tertiary structures of cysteine protease inhibitors have been determined (Kauzuma *et al.,* 2000; Carlini and Grossi-de-Sa, 2001) Homology of cysteine protease inhibitors are similar with serine protease inhibitors, such as Kunitz-type trypsin inhibitor family that belong to potato tuber phytocystatins (Ishikawa *et al.,* 1994). The phytocystatins are displaying high inhibitory activity toward insect gut proteinases making them attractive

to control insect pests (Bode and Huber, 1992; Gatehouse and Gatehouse, 1998; Carlini and Grossi-de-Sa, 2001).

Cysteine protease inhibitors were reduced fecundity, increased motarlity, decreased weight and severe deformations when fed to lepidoptera and coleopteran species (Elden, 2000). Cysteine proteases have been best characterized in the Bruchidae in the Coleoptera, it also occurs in the Curculionoideae, Meloidae and Silphidae (Xavier-Filho, 1992).

Therefore, in this study research are focusing on the function of serine proteases inhibitors by using potential of local plants that are important to control insecticides and thus make them beneficial in agronomical and health relevance.This research study will be involving a thorough screening, isolation, identification and characterization of bi-functional inhibitor of α-amylase and protease inhibitors.

2.1.2 Aspartic protease inhibitors

Aspartic proteases are a family of protease enzymes which inhibits the catalytic activity of an aspartyl protease, a class of proteases that contains active site aspartate residue (Asp). Aspartic proteases include pepsin and rennin. Members of the aspartic protease family have been characterised in humans, plants, fungal and retroviruses. Eukaryotic aspartic proteases include pepsins, cathepsins, and renins.

Aspartic proteases were also involved in defence mechanism. Protease inhibitors active against serine, cysteine and metallocarboxy-proteases are ubiquitous, while inhibitors

active towards aspartic proteases have not been detected in seeds (Valueva and Mosolov, 1999). Research by Dash and friends in 2001 were recognized that the kinetic studies have revealed the bifunctional characteristics of a novel bifunctional inhibitor (ATBI) from an extremophilic *Bacillus* sp., as it was found to inhibit xylanase and aspartic protease. This report had shown a novel class of antifungal peptide, exhibiting bifunctional inhibitory activity (Dash *et al.,* 2001).

2.1.3 Metalloproteinases inhibitors

Tissue inhibitors of metalloproteinases (TIMPs) are the major cellular inhibitors of the matrix metalloproteinase (MMP). Matrix metalloproteinases are a class of enzymes involved in degradation of extracellular matrix that can break down proteins, such as collagen and gelatin. Metalloproteinases include thermolysin and carboxypeptidase A. As they inhibit cell migration they have antiangiogenic effects. They may be both endogenous and exogenous. Exogenous matrix metalloproteinases inhibitors include batimastat and marimastat. The most notorious endogenous metalloproteinases are tissue inhibitor of metalloproteinases (TIMP). There are also cartilage-derived angiogenesis inhibitors.

2.1.4 Serine protease inhibitors

Serine protease inhibitors and their binding to cognate proteinases have been extremely well characterized over the years (Bode and Huber, 1992). Serine protease inhibitors are one of the most diverse families of macromolecules that achieve similar biological functions with entirely different scaffolds. Serine protease inhibitors have been classified about 20 subfamilies, based on amino acid sequence and mechanism of interaction

including a-helical, b-sheet and a/b proteins, as well as small disulfide rich proteins. Based on their mechanisms of action, three types of serine protease inhibitors are now recognized as canonical inhibitors, non-canonical inhibitors and serpins (Laskowski and Kato, 1980).

They are the group of proteolytic enzymes which are characterized by a catalytically active serine residue in their active site. Several serine protease inhibitors are effective protect host plants by against various insect enzymes and therefore have been studied as an alternative approach to pest control (Reckel *et al.,* 1997; Leo *et al.,* 2001). Serine protease inhibitors such as trypsin, chymotrypsin and elastase are the most intensively studied (Otlewski *et al.,* 1999). All three enzymes are synthesized by the pancreatic acinar cells, secreted in the small intestine, and are responsible for catalyzing the hydrolysis of peptide bonds. These enzymes are shown similar in structure through their X-ray structures.

Each of these digestive serine proteases targets different regions of polypeptide chain, based upon the side chains of the amino acid residues surroundinsg the site of cleavage. Following a positively-charged amino acid residue, trypsin is responsible for cleaving peptide bonds. Instead of having the hydrophobic pocket of the chymotrypsin, there exists an aspartic acid residue at the base of the pocket. This can then interact with positively-charged residues such as arginine and lysine on the substrate peptide to be cleaved.

Chymotrypsin is responsible for cleaving peptide bonds following a bulky hydrophobic amino acid residue. Preferred residues include phenylalanine, tryptophan, and tyrosine, which fit into a snug hydrophobic pocket. Elastase is responsible for cleaving peptide

bonds following a small neutral amino acid residue, such as Alanine, glycine, and valine. These amino acid residues form much of the connective tissues in meat. The pocket that is in "trypsin" and "chymotrypsin" is now partially filled with valine and threonine, rendering it a mere depression, which can accommodate these smaller amino acid residues.

Serine proteases have been isolated from various seeds that have been isolated and characterized from *Leguminosae, Cucur bitaceae*, *Solanaceae Graminae* and *Rutaceae* families (Garcia-Olmedo *et.al.,* 1987; Oliva *et al.,* 2000; Mello *et al.,* 2002; Oliveira *et al.,* 2002; Shee and Sharma, 2007) and their physiological roles are extensively studied including the regulation of endogenous proteases during seed dormancy, the reserve protein mobilization, the protection against the proteolytic enzymes of parasites and insects and also as storage or reserve proteins.

Most serine PIs is low-molecular mass molecules from 3 to 25 kDa that inhibit trypsin and/or chymotrypsin. Kunitz-type inhibitors are proteins of ~20 kDa, with low cysteine content and a single reactive site, whereas the Bowman–Birk type inhibitors have ~8 to 10 kDa as well as high cysteine content and two reactive sites (Richardson, 1991; Bhattacharyya and Babu, 2007). Kunitz and Bowman–Birk inhibitors are vary in their mode of stability but lack a-helix structurally (Bhattacharyya and Babu, 2007). The linkages of disulfide in the Bowman–Birk inhibitors minimize their conformational entropy and enhance their stability whereas Kunitz inhibitors are stabilized chiefly by hydrophobic interactions of short stretches of hydrogen bonded sheets (Sweet *et al.,* 1974; Ramasarma *et al.,* 1995; Bhattacharyya and Babu, 2007).

The distributions of these two families of inhibitors are in the seeds of *Leguminosae* that contain high amounts of protein that suppress proteolytic activities *in vivo* and *in vitro* by forming stable stoichiometric complexes (Richardson, 1991; Bhattacharyya and Babu, 2007). Kunitz type inhibitors are more common in the seeds of highly primitive Mimosoideae and primitive Caesalpinioideae, in comparison to the recently evolved Papilionoideae which frequently shows the presence of Bowman-Birk inhibitors (Macedo *et al.,* 2002).

2.1.4.1 Bowman-Birk Inhibitor (BBI)

The Bowman-Birk Inhibitor (BBI) is a polypeptide that has ability to inhibit both trypsin and chymotrypsin at independent binding sites. It is characterized by content of high cystine and the absence of glycine. BBI is a soy-derived protease inhibitor with anticarcinogenic and anti-inflammatory properties, has been currently shown to be well tolerated in clinical trials as a human cancer-preventive agent for pre cancerous conditions, such as oral leukoplakia and the inflammatory disease, ulcerative colitis (Gran *et.al,* 2006).

In !963, Bowman and Birk were the earliest scientists that identify and characterise a member of this family from soybean (*Glycine max*) (Bowman 1946; Birk *et al.,* 1963). The most well studied member of this family is the soybean inhibitor (Habib and Fazili, 2007). BBI can be found in many plant seeds. From the recent researches the inhibitors have been found in cereals and legumes (Tanaka *et al.,* 1997; Laing and McManus, 2002). The inhibitors of this family are generally found in seeds, but are also wound-inducible in

leaves (Eckelkamp, 1993) and in the grass family Poaceae (Odani *et al.,* 1986; Habib and Fazili, 2007). A small cyclic inhibitor has been identified in sunflower (*Helianthus annuus*) called sunflower trypsin inhibitor 1 (SFTI-1) (Habib and Fazili, 2007).

BBIs have been classified according to their structural features and inhibitor characteristics. The first reactive site in these inhibitors is usually specific for trypsin, chymotrypsin and elastase. The inhibitors have molecular weights ranging from 7000 to 8000, and these inhibitors are stabilized by the presence of disulfide bridges (Chen *et al.,* 1992; Lin *et al.,* 1993). The 14 half-cystine residues are conserved in all BBIs and help to maintain their active conformation, All BBI molecules have two regions of tandem homology and each has a reactive site. Thus, BBIs can inhibit two proteinases simultaneously and independently and are considered as "double-headed" inhibitors (Chen *et al.,* 1992).

The inhibitors from dicotyledonous plants consist of a single polypeptide chain have a molecular mass of approximately 8 kDa and are double-headed, with two homologous domains each bearing a separate reactive site for the cognate proteases (Birk, 1985). These inhibitors interact independently, but simultaneously, with two proteases, which may be same or different (Raj *et al.,* 2002; Birk, 1985). Two types of the inhibitors can be found from monocotyledonous plants. One group consists of a single polypeptide chain with a molecular mass of about 8 kDa. They have a single reactive site. Another group has approximately 16 kDa with two reactive sites (Odani *et al.,* 1986; Tashiro *et al.,* 1987).

The main sturucture of BBI are single polypeptides and comprise a binary arrangement of two sub-domains with a conserved array of seven disulfide bonds. The BBI family of protease inhibitors contains a unique of two disulfide-linked nine-residue reactive site loops that adopts a characteristic canonical conformation (Bode and Huber, 1992) and the positions of the P1 residues are indicated (Odani and Ikenaka, 1976). The loop is called protease-binding loop and binds the protease in a substrate-like manner (Lee and Lin, 1995; Habib and Fazili, 2007).

BBIs are cysteine-rich proteins with inhibitory activity against proteases that are widely distributed in monocot and dicot species (Lin *et al.,* 2006). Recent studies shown that proteinase inhibitors of certain types are anticarcinogenic.The soybean derived BBI with a well-characterized ability to inhibit trypsin and chymotrypsin is particularly effective in suppressing carcinogenesis in a variety of *in vivo* and *in vitro* systems (Kennedy, 1998).

The anticarcinogenic compounds have the ability to reduce the forming of oxygen radicals, to suppress the growth of chemical-induced colon and anal gland tumors, lung tumor in mice, and breast tumor in rat to suppress the chemicalor radiation-induced cell transformation, to reduce spontaneous chromosome abnormality (Kennedy, 1998) and to prevent tumor invasion and metastasis.

2.1.4.2 Kunitz-type inhibitors

Kunitz-type inhibitors are a type of protein which functions as a protease inhibitor (Rawlings, 2004) and mostly active against serine proteases, but may also inhibit other

proteases (Ritonja *et al.,* 1990; Laing and McManus, 2002). Kunitz-type inhibitors are usually in plants and widespread in soybean, legumes seeds, cereals and in solanaceous species (Laskowski and Kato, 1980; Ishikawa *et al.,* 1994). Kunitz-type PIs have been found in potato tubers (*S. tuberosum*) (Plunkett *et al.,* 1982; Park *et al.,* 2005). The inhibitors with antifungal activity have been located in the roots of punce ginseng (*Pseudostellaria heterophylla*) (Wang and Ng, 2006).

Kunitz-type inhibitors usually have molecular weight approximately 18 to 20 kDa proteins, usually made of two disulfide bridges or contain from 170 to 200 amino acid residues in one polypeptide chain in their single reactive site with low cysteine content. This family have been shown to inhibit trypsin, chymotrypsin and subtilisin (Laing and McManus, 2002; Park et al., 2005) and they also inhibit other proteases. Structurally, Kunitz inhibitors lack a-helix, but vary in their mode of stability. Kunitz inhibitors are stabilized chiefly by hydrophobic interactions of short stretches of hydrogen bonded sheets (soybean Kunitz trypsin inhibitor) whereas the disulfide linkages in the Bowman–Birk inhibitors minimize their conformational entropy and enhance their stability (Sweet *et al.,* 1974; Ramasarma et al., 1995).

Joubert and others scientist found the source of Kunitz-type typsin inhibitor from *Erythrina* seeds to check abilities of inhibition. From the results, the proteins inhibited trypsin strongly and they were poor inhibitors of chymotrypsin (Joubert *et al.,* 1987). Kunitz trypsin inhibitors also effectively inhibit the activity of proteolytic of lepidopterans, such as black cutworm, tobacco, budworm and others.

2.2 Application of serine protease inhibitors

According to research by Bhattacharyya *et al.,* 2007, studied on trypsin and chymotrypsin inhibitor from *Caesalpinia bonduc* (CbTI) seeds. They also studied on the effects of CbTI on insect gut proteases reflect that CbTI is a powerful antifendant of insect herbivores. The deleterious effects of CbTI on larval gut proteinases of *S. litura* were similar to previously observed results with proteinase inhibitors from other leguminous plant (Gomes *et al.,* 2005).

Another research by Bhattacharyya *et al.,* 2007, researched on the roles of serine proteases involved in the digestion mechanism of the cutworm Spodoptera litura (Lepidoptera: Noctuidae) were examined (in vitro and in vivo) following feeding of plant protease inhibitors. A trypsin inhibitor from Archidendron ellipticum (AeTI) was purified by ammonium sulfate fractionation, size-exclusion chromatography (HPLC) and ion-exchange chromatography and its bioinsecticidal properties against S. litura were compared with Soybean Kunitz trypsin inhibitor (SBTI). AeTI inhibited the trypsin-like activities of the midgut proteases of fifth instar larvae of S. litura by over 70%. Dixon plot analysis revealed competitive inhibition of larval midgut trypsin and chymotrypsin by AeTI, with an inhibition constant (Ki) of 3.5×10−9 M and 1.5×10−9 M, respectively.

However, inhibitor kinetics using double reciprocal plots for both trypsin and chymotrypsin inhibitions demonstrated a mixed inhibition pattern. Feeding experiments conducted on different (neonate to ultimate) instars suggested a dose-dependent decrease for both the larval body weight as well as % survival of larva fed on diet containing 50, 100 and 150

μM AeTI. Influence of AeTI on the larval gut physiology indicated a 7-fold decrease of trypsin-like protease activity and a 5-fold increase of chymotrypsin-like protease activity, after being fed with a diet supplemented with 150 μM AeTI. This study suggests that although the early (1st to 3rd) larval instars of S. litura are susceptible to the trypsin inhibitory action of AeTI, the later instars may facilitate the development of new serine proteases, insensitive to the inhibitor (Bhattacharyya *et al.,* 2007).

Research on purification and characterization of a highly stable and potent trypsin inhibitor was purified to homogeneity from the seeds of Putranjiva roxburhii belonging to tree of tropical India by acid precipitation, cation-exchange and anion-exchange chromatography (Chaudhary *et al.,* 2008). SDS page analyses showed that protein consist of a single polypeptide chain with molecular mass of approximately 34 kDa when under reducing conditions. From the report, the structural stability of inhibitor complete at the high temperatures and the complete loss of inhibitory activity were observed above 90^{o}C. N-terminal amino acid sequence of 10 residues did not show any similarities to known serine protease inhibitors, however, two peptides obtained by internal partial sequencing showed significant resemblance to Kunitz-type inhibitors.

In another case, research done by Kim and friends in 2005 from Republic of Korea were described the purification and characterization of the antimicrobial peptide potamin-1 (PT-1) from potato. A 5.6 kDa trypsin-chymotrypsin protease inhibitor obtained and isolated from tubers of the potato by extraction of the water-soluble fraction, dialysis, ultrafiltration, and C18 reversed-phase high performance liquid chromatography. PT-1 strongly inhibited

pathogenic microbial strains, including *Candida albicans*, *Rhizoctonial solani*, *Clavibacter michiganense subsp. Michiganinse* (Kim *et.al.,* 2005).

The sequence of PT-1 had 62% homology with serine protease inhibitor belonging to the Kunitz family and the peptide inhibited chymotyrpsin, trypsin and papain. These protease inhibitors were composed of polypeptide chains joined by disulfide bridges and reduced PT-1 almost completely lost its activity against fungi. The results suggested that PT-1 is an excellent candidate as a lead compound for the development of novel oral or other anti-infective agents (Kim *et.al.,* 2005).

Serine protease inhibitors have been proposed as a strategy against insect pests. Macedo et al. 2004 have been studied that transgenic grains which expresses gene encoding protease inhibitors may prevent seed damage against Lepidoptera and Coleopteran species without side effects which include reduced fecundity, increased mortality, decreased weight gain and severe deformation though out their developmental phases (Macedo *et al.,* 2002). The potential of these inhibitors that expressed in transgenic plants showed a higher resistance to various insects.

Broadway (1995) were researched on six species of Lepidoptera have also been evaluated for their susceptibility to serine proteinase inhibitors from cabbage. The serine proteinase activity in the midguts of larval *PZutella xylostella* was moderately inhibited (40-50%), and *Trichoplusia ni, Lymantria dispar,* and *Helicoverpa zea* were substantially inhibited (55-85%) by cabbage proteinase inhibitors, while Trypsin and chymotrypsin activity from

larval *Pieris rapae* and *Pieris napi* were not significantly inhibited (0-18%), *in vitro,* by cabbage proteinase inhibitors.

These results shown that the growth and development of the latter three species should be reduced following ingestion of these inhibitors but chronic ingestion of cabbage proteinase inhibitors only reduced the growth and development of *T. ni.* A shift in the relative proportion of digestive enzymes was responded to ingestion of proteinase inhibitors because lack of biological activity of the proteinase inhibitors against the other two species. Following ingestion of cabbage proteinase inhibitors, the trypsin(s) in *T. ni* was moderately susceptible (37% inhibited), while the predominant trypsin-like enzyme(s) in the midgut of larval *L. dispar* and *H. zea* were resistant to inhibition by cabbage trypsin inhibitors (13-18% inhibited). These results were confirmed for *H. zea* and *T. ni* feeding on proteinase inhibitors in tomato foliage (Broadway *et al.,* 1995).

Atkitson and friends (1993) were reported that protease inhibitors may function to protect the reproductive tissue against potential pathogens. Their research had shown the isolation of cDNA clone encoding a protein with sequence similarity to a protease inhibitors type II of potato and tomato. These protease inhibitors were expressed in ornamental tobacco, *Nicotiana alata* stigmas that derived from a precursor protein which is isolated peptide inhibitors with five homologous inhibitors to six regions of the amino acid sequence deduced from the cDNA clone (Atkinson *et al.,* 1993).

Brown and Ryan (1984) shown that serine protease inhibitors in leaves of plants are response to wounding. In leaves of tomato, potato tubers and legume seeds contain inhibitors that 10% or more of the stored proteins (Green and Ryan, 1972; Richardson, 1977; Atkinson *et al.,* 1993). PIs can accumulate to 2% of the soluble protein within 48 hours of insect attack or other types of wounding (Brown and Ryan, 1984; Graham *et al.,* 1986).

The main objective of this research was:

1) To screen plant extracts with inhibitory activities
2) To purify to homogeneity proteins with inhibitory activities
3) To characterise the active proteins