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Zymograms as a Tool to Detect PPIs in Host Plants of *Antheraea assamensis*

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Abstract

Antheraea assamensis (vernacular: 'muga') larvae is commercially reared on two tree species of the Lauraceae family, Persea bombycina and Litsea monopetala for its golden yellow, lustrous cocoon silk. Biochemical and molecular studies suggested that the midgut digestive enzymes and their transcripts in larvae feeding on P. bombycina differ from those found in larvae feeding on L. monopetala indicating that host plant 'choice' affects the digestive physiology of this insect. Ingestion of plant proteinase inhibitors is known to influence expression of digestive enzymes. Using reverse zymography technique trypsin and chymotrypsin inhibitors were detected in herbivore-induced leaves of L. monopetala and P. bombycina that could inhibit midgut proteinases of A. assamensis. Such interactions may affect proteolytic digestion in larvae reared on different host plant species. This work may have significance in quality of silk produced by muga silkworm, ultimately benefiting the silkworm rearers/industry.

Keywords: *Host Plant 'Choice', Digestive Enzymes, Reverse Zymogram, Midgut Proteinases, Plant Proteinase Inhibitor (PPIs)*

1. Introduction

Plant proteinase inhibitors (PPI) are well known defense proteins which are induced upon herbivory. Ingestion of plant proteinase inhibitors is known to influence expression of digestive enzymes. Literature studies have cited innumerable cases of midgut proteases and PPI interaction in agricultural pests (1-23). Although midgut proteinases have been widely studied in Lepidopteran pests but little is known about PPI-gut protein interaction in the economically important silk producing lepidoptera *A. assamensis.* A few reports published indicates that PPIs are stable in the alkaline environment of the gut and can potentially interact with gut proteinases [23-26]. Also, the expression levels of PPIs differed in *A. assamensis* larvae when feeding on different host plants. [12,27]. Given below is a schematic representation of interaction of *A. assamensis* midgut proteinases and PPIs (Figure:1).

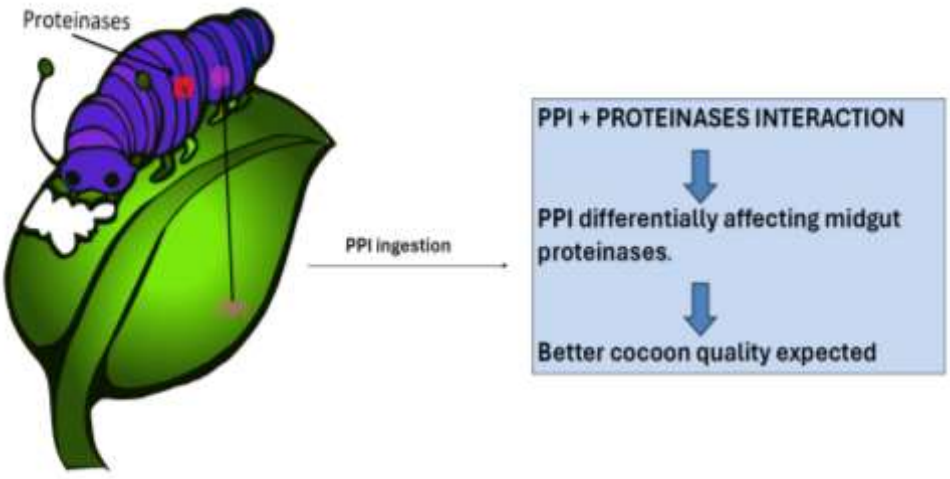

Figure 1: Schematic representation of *A. assamensis* midgut proteinases and PPI interaction*.

Antheraea assamensis Helfer (Vernacular: "Muga") is famed for the production of superior quality muga silk. The commercially reared muga larvae produces golden hued thread for which it is much sought after (Figure 2 a,b). *A. assamensis* feeds mainly on the leaves of *Litsea bombycina* (Vernacular: "Som") and *Litsea monopetala* (Vernacular "Soalu") from Lauraceae (Figure 3 a,b) although it has a large number (around 28) of other secondary and tertiary host plants [28,29]. There are no reports of proteinase inhibitors (PI) from leaves and seeds of Lauraceae family. A first – hand account on presence of herbivore -induced serine proteinase inhibitor in the leaves of the host plants is reported here. Knowledge of host plant PIs and their interaction with gut proteinases of *A. assamensis* would help in manipulating host plant choice and improving food ingestion by the larvae. The significance of this work may be manifested in quality of silk produced by this economically important insect, with potential implication for silkworm rearer's.

(a) (b)

Figure 2 (a) Rearing of *A. assamensis* larvae at the State Sericulture Board Guwahati, Assam; (b) *A. assamensis* cocoon

(a) (b)

Figure 3 (a): Three-year old *Persea bombycina* tree growing at the State Sericulture Board, Guwahati, Assam (b) Two-year-old *Litsea monopetala* sapling growing in University of Delhi, Botanical garden, Delhi.

2. Materials And Methods

Preparation of *A. assamensis* midgut homogenates

A hundred larvae from a single egg mass were reared from 1st instar to maturity on *P. bombycina* var. Naharapatiya and *L. monopetala* (commercially favored varieties) of Lauraceae family. A three-year-old tree of each species growing at the Government Basic Muga Seed Farm, Khanapara, Guwahati,

Assam was used. Larvae were reared on all leaves (basal, apical, small and large) of each tree and collected. Midgut samples were collected during the months of September-December (Katiya Crop). Midguts of fourth instar larvae of *A. assamensis* feeding on *Persea bombycina* and *Litsea monopetala* were dissected and homogenized in 100mM Hydrochloric acid (HCl), 100mM *N*-2-hydroxyethylpiperazine-*N*-2-ethane sulphonic acid (HEPES) and 100mM Tris hydroxymethyl- amino methane (Tris) buffer pH 8.0. The homogenate was centrifuged in an Eppendorf Centrifuge 5415 D (Eppendorf AG, Hamburg, Germany**)** at 13,200 rpm for 10 minutes and the supernatant was stored at -20°C. Total protein was determined by the standard Bradford's method [30] and the absorbance was measured at 595nm with a UV VIS Spectrophotometer 119 (Systronic Inc., India). All samples obtained from larvae reared on *P. bombycina* will be referred to as **AGP1** and all samples obtained from larvae reared on *L. monopetala* will be referred to as **AGP2**.

SDS-PAGE of leaf proteins and gut extracts of *A. assamensis* incubated with PPIs

Fresh leaves (3-4 cm) of *P. bombycina* and *L. monopetala* proximal to those on which larvae were feeding were collected from the third branch of a tree growing at the Government Basic Muga Seed Farm, Khanapara, Guwahati (Assam). These were labelled as herbivore-induced leaf samples. Similar leaf samples were collected from trees on which no larvae had been reared. These were labelled as uninduced leaf samples. Protein from the de-veined leaves of *P. bombycina* and *L. monopetala* were extracted according to Hirano [31]. In brief, fresh leaves were crushed in liquid nitrogen. 1gm of the frozen tissue powder was homogenized in 10 ml of homogenizing buffer (0.0625 M Tris-HCl, pH 6.8, containing 8M urea, 2% SDS, 5% β-mercaptoethanol and 10% sucrose) and heated at 90 ºC for 5 minutes. The homogenate obtained was centrifuged at 12,000 rpm for 25 min, and the supernatant used as experimental sample. Total protein estimation of the samples was done by the method of Bradford [30]. The Laemmli system [32] was used to separate the proteins according to their molecular weight. Equal amounts of the crude protein extracts of AGP1 and AGP2 were mixed with STI (5mg/ml) in the ratio 1:1 and incubated at 37ºC. After incubation, the samples were mixed with sample buffer (0.5M Tris-HCl, pH-6.8, 10% glycerol, 2% SDS, 0.1% bromophenol blue and β-mercaptoethanol) and boiled in water for 5-10 minutes. About 15µg of protein was loaded in each well. Controls (with and without inhibitor) were kept and electrophoresis was carried out at 120V till the dye front reached the bottom of the gel. The gel was then stained in 0.25% Coomassie brilliant blue solution for 30 minutes and destained by repeated washing in the destaining solution (45% methanol, 10% glacial acetic acid, 45% ddH_2O) for 2 hours until fine bands appear. The gels were viewed under white light.

Reverse zymography for detection of protease inhibitors

Endogenous protease inhibitors are potential key regulators of proteases in living organisms. Reverse zymography techniques are effective tools for isolating and characterizing natural protease inhibitors [33-35]. Reverse zymography gels were run as described below. A separating gel was poured, containing gelatine (10mg/ml), 30% (w/v) acrylamide, 0.8% (w/v) bis-acrylamide, 1.5M Tris pH 8.8, 10% (v/v) SDS (10% APS and TEMED. The stacking gel consisted of a mixture of stock solution of 30% (w/v) acrylamide, 0.8% (w/v) bisacrylamide, and 0.5M Tris-HCl pH 6.8, was cast with combs on top of the separating gel. The crude protein extracts of the seeds and leaves of *P. bombycina* and *L. monopetala* were prepared according to Hirano and Kanellis et al [31, 36]. Un-induced and herbivore induced leaf samples were used. The protocol of Hirano [31] has been described in the earlier paragraph. In the protocol of Kanellis et al [36] 4 ml of the extraction buffer containing 50mM Tris (pH 7.4), 0.02M NaCl, 20mM $NaHCO_3$, 20mM $MgSO_4$, 10mM EDTA and 5mM β-Mercaptoethanol was added to 1 gm of grinded seed and leaf tissue of the host plants. The mixture was allowed to stand on ice for 15 minutes with occasional stirring and the centrifuged at 13, 000 rpm for 20 minutes. The supernatant was used immediately without addition of exogenous inhibitors for reverse zymography. Equal amount of protein (20µg) of all the samples were prepared by adding the appropriate non denaturing loading buffer (0.5M Tris pH 6.8, 10% glycerol, 2% SDS and 0.1% bromophenol blue). The gel was run at 120V for about 90 minutes at 4ºC till the dye front reached the bottom of the gel. The gel was washed in 2.5% Triton X-100 (2-3 times) for 45 minutes with gentle shaking. The gel was incubated overnight at 37ºC in *A. assamensis* gut extracts containing active proteases reared on *P. bombycina* (AGP1) and *L. monopetala* (AGP2) and with commercially available pure enzyme preparations (bovine trypsin and bovine chymotrypsin). The gel was stained with 0.5% Coomassie Blue R-250 solution and destained with methanol and acetic acid until fine dark clear bands appeared.

3. Results and Discussion

Resistance of heterologous proteinase inhibitors to proteolytic digestion by midgut extracts from AGP1 and AGP2

There are no reports of proteinase inhibitors (PI) from leaves of Lauraceae family. In order to develop a technique for identifying PI in leaf tissues of three-year-old trees of *Persea bombycina* and *Litsea monopetala*, several preliminary experiments were conducted. The aim of one such experiment was to determine if PIs persist in the gut of *A. assamensis.* If PIs are degraded by insect midgut enzymes, then the effect of ingesting PIs is unlikely to cause differential expression of the insect digestive enzymes. Hence, heterologous PIs were added to midgut extracts containing active serine proteinases, for varying lengths of time. Their persistence in the protease-rich, alkaline environment was monitored by SDS-PAGE. Figure 4 shows that STI was not degraded when incubated with midgut samples from *A. assamensis* reared on *P. bombycina* (AGP1) and *L. monopetala* (AGP2) for 20 minutes. Similar results showing resistance of SBBI and LBTI to midgut proteinases of *A. assamensis* were observed (not shown). These results indicated that serine proteinase inhibitors were not susceptible to proteolysis by midgut enzymes present in *A. assamensis.*

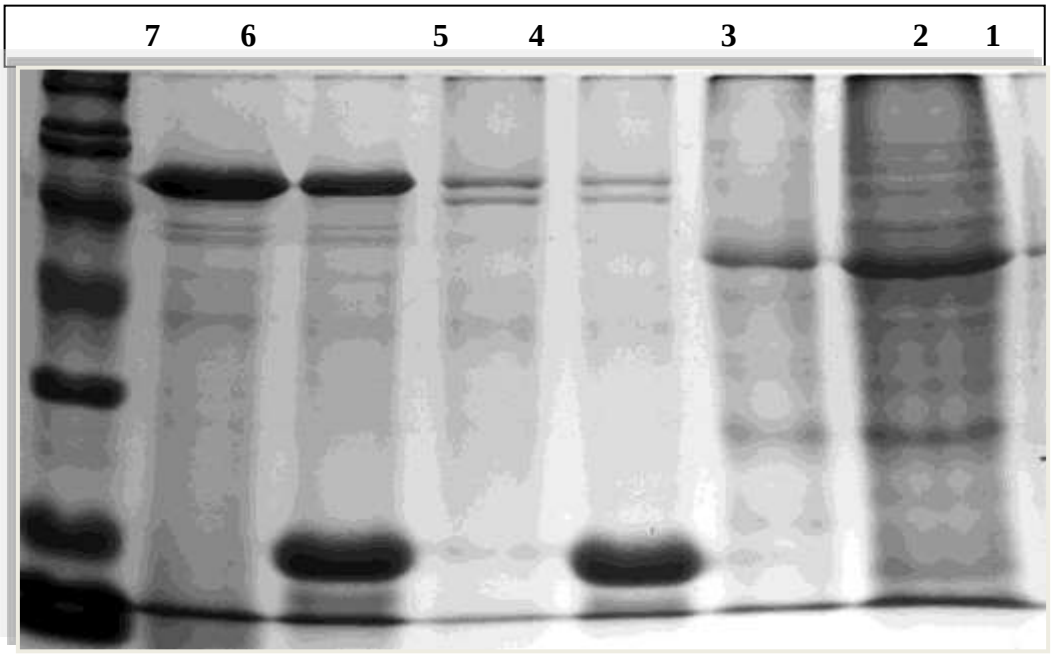

Figure 4: SDS-PAGE showing resistance of Plant proteinase inhibitors (PPIs) to proteolysis by larval midgut proteases. Lane 1: High molecular weight protein marker (BioRad Inc., USA), Lane 2: AGP1, Lane 3: AGP1+STI (5µg/µl), Lane 4: AGP2, Lane 5: AGP2+STI (5µg/µl), Lane 6: *P. bombycina* leaf extract, Lane 7: *L. monopetala* leaf extract.

Detection of serine proteinase inhibitors in seeds and leaves of *P. bombycina* and *L. monopetala* using reverse zymography with mammalian trypsin and chymotrypsin

Published protocols for preparing leaf extracts from *P. bombycina* and *L. monopetala* do not yield discrete bands and do not show up well on SDS PAGE [37]. Hence, two protocols were developed to obtain relatively intact protein extracts of leaf tissues from the two host plants. The first one was modified from Hirano [31]; while the second one was modified from Kanellis et al [36]. The main distinction between the protocols involved the use of 8M Urea in the extraction buffer [31] as compared to the use of sodium acetate buffer at pH 5 with β mercapto-ethanol Kanellis [36]. The main modification employed for both buffers involved exclusion of commercially available inhibitor cocktails usually added to plant extraction buffers. This was logical as the aim of the experiment was to detect endogenous plant proteinase inhibitors. The Kanellis [36] protocol yielded discernable leaf protein extracts from the two host species (Figure 4: Lanes 5 and 6). Figure 5 shows reverse zymography of seed extracts and leaf extracts of *P. bombycina* and *L. monopetala* with porcine trypsin and bovine chymotrypsin. It was interesting to note that the seed extracts prepared by the modified Hirano [31] protocol yielded results for both plant species. Three bands were visible with the seed extracts (Figure 5 Lanes 'S'). No results were obtained with the leaf extracts using this protocol. In contrast, an intense single band was observed with leaf extracts from *P. bombycina* and *L. monopetala* with the Kanellis et al. [36] protocol. As expected, STI used as a positive control was also detected (Figure 5 Lanes 'C'). The occurrence of PIs in seeds and leaves of *P. bombycina* and *L. monopetala* was thus demonstrated.

Even though molecular weights cannot be extrapolated from such zymograms, it seemed that the mobility of trypsin and/ or chymotrypsin PIs detected in the seeds and leaves of each species were different. On the other hand, mobility of seed PIs of both host plant species were similar in *P. bombycina* and *L. monopetala*. The mobility of leaf PIs were also similar for *P. bombycina* and *L. monopetala*.

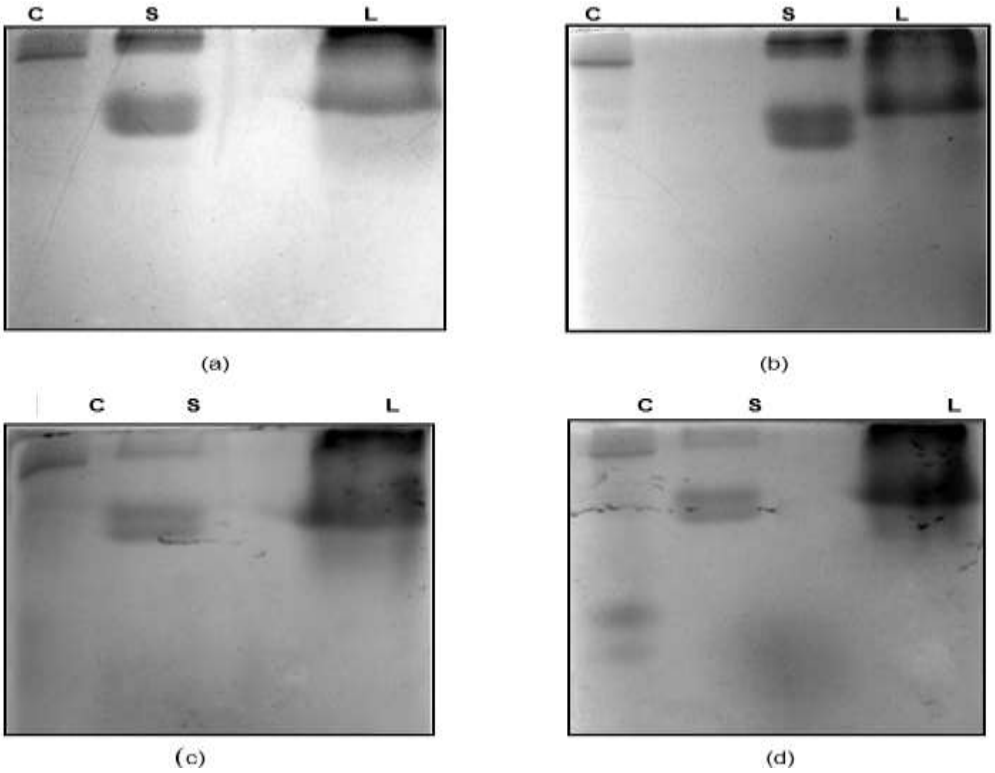

Figure 5: Detection of plant proteinase inhibitors using reverse zymography (a, b) *P. bombycina* herbivore-induced leaf (L) and seed extracts (S) incubated with Bovine Trypsin (BT) and Bovine Chymotrypsin (BC) respectively, (c, d) refer to same experiments with *L. monopetala* extracts. Lane C shows Soybean trypsin Inhibitor (SKTI) as a positive control.

Reverse zymography reveals proteinase inhibitors in un-induced and herbivore-induced leaf tissues of *P. bombycina* and *L. monopetala.*

In order to detect PIs in *P. bombycina* and *L. monopetala* that may influence the profile of midgut serine proteinases when ingested by larval *A. assamensis*, un-induced (U) and herbivore-induced (HI) leaves of three-year-old trees were collected. Un-induced leaves were collected from trees on which no herbivore had fed, while induced leaves collected were proximal to those on which fourth instar larvae had fed. Equal amounts of leaf extracts prepared in extraction buffer according to Kanellis et al. [36] were evaluated by reverse zymograms using AGP1 and AGP2 midgut extracts. AGP1 and AGP2 midgut extracts contained trypsin and chymotrypsin [12]. Figure 6 a, b showed the presence of trypsin and/or chymotrypsin inhibitors in both induced and un-induced leaf extracts from each species. Three minor bands were seen in un-induced leaf extracts of *P. bombycina* while a prominent band of low mobility was seen in induced leaf extracts when incubated with AGP1. In the case of *L. monopetala*, a prominent band of low mobility was visible in both un-induced and induced leaf tissues when incubated with AGP1 and AGP2. It is of course feasible that the un-induced plant of *L. monopetala* had been wounded inadvertently and was expressing a wound-inducible PI. In any case, trypsin and/or chymotrypsin inhibitors were detected suggesting their possible role in influencing midgut serine proteinases if and when ingested by *A. assamensis* larvae. Similar results were obtained with AGP2. The suggested sizes of induced trypsin and/or chymotrypsin inhibitor (prominent band of low mobility) were similar for both host plant species. Further studies are required to characterize the PIs in these plant species induced in response to *A. assamensis* herbivory.

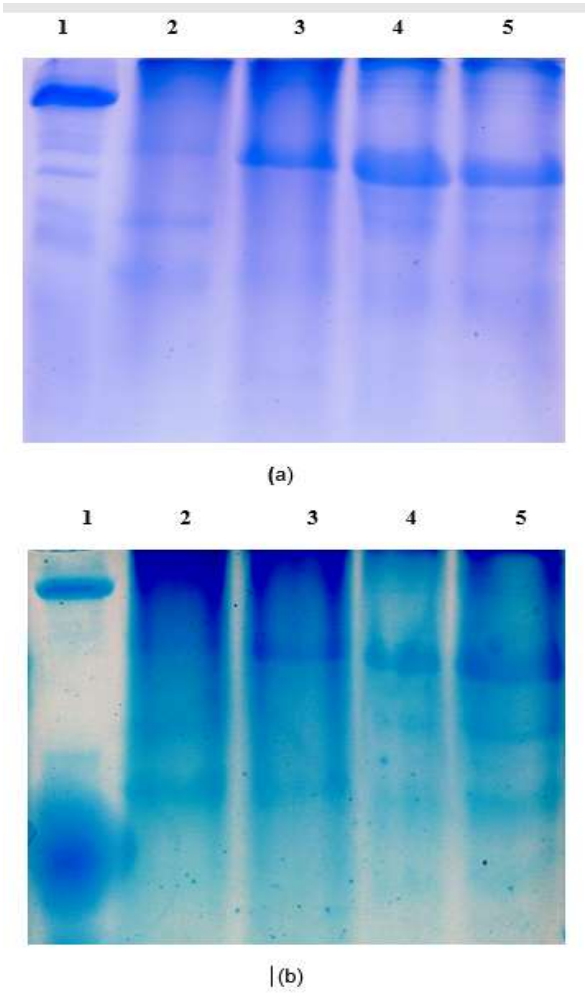

Figure 6: Detection of plant proteinase inhibitors using reverse zymography (A) *P. bombycina* and *L. monopetala* herbivore uninduced and induced leaf extracts incubated with AGP1 and (B) refers to the same samples incubated with AGP2. Lane 1 shows Soybean trypsin Inhibitor (SKTI) as a positive control, Lanes 2 and 3 are uninduced and induced leaf extracts of *P. bombycina*, Lanes 4 and 5 are uninduced and induced leaf extracts of *L. monopetala*.

In order to determine whether PPIs are stable in the midgut of *A. assamensis* as reported in [12, 27] and can also interact differentially with gut proteinases, midgut extracts of AGP1 and AGP2 were incubated with varying amounts of the inhibitors for different periods of time. Figure 4 shows that STI was resistant to proteolysis by enzymes in AGP1 and AGP2. This *in vitro* resistance of STI to proteolysis suggested that PPIs, if ingested, might persist in the gut and influence gene expression of midgut trypsin and chymotrypsins in fourth instar *A. assamensis* feeding on *P. bombycina* or *L. monopetala*.

The presence of herbivore-induced PPIs in leaf tissues of *P. bombycina* and *L. monopetala* are unknown. A sole report of a cysteine proteinase inhibitor is available from the fruit tissues of Avocado, *Persea americana* [36]. In order to determine the presence of PPIs, techniques were developed to isolate intact proteins from tanniferous, mucilaginaous leaf extracts of *P. bombycina* and *L. monopetala* and to detect inhibitors of serine proteinases using reverse zymography. In the first set of experiments with reverse zymography, plant proteinase inhibitors resistant to hydrolysis by commercially available pure porcine trypsin and bovine chymotrypsin were detected in seed and leaf extracts of *P. bombycina* and *L. monopetala* (Figure 5 a,b,c,d). Henceforth, they will be referred to as serine proteinase inhibitors (SPIs). Two bands were observed in seed extracts of each species, probably representing distinct gene products or isoforms. Presence of SPI isoforms have been reported from a variety of plants [38-41]. It was interesting to note that *P. bombycina* and *L. monopetala* seed SPIs had similar mobility on these reverse zymograms. In contrast to the two bands seen with seeds, leaf extracts of both *P. bombycina* and *L. monopetala* contained a prominent single SPI that bound to porcine trypsin and bovine chymotrypsin. The mobility of the leaf SPI was different from the seed SPI. The occurrence of different SPIs in different tissues of the same species has been reported earlier [42,43]. On the flip side, same tissues of different plant species have been known to contain SPIs of similar molecular weight [8,40].

4. Conclusion

P. bombycina and *L. monopetala* are sympatric species with similar range [44]. These species of the Lauraceae family belong to different taxonomic tribes but can support rearing of *A. assamensis* larvae. Insects feeding on each species showed differential expression of midgut proteinases, suggesting that ingested leaf tissues were qualitatively different [12]. This difference may be in the constituent PPIs that interact with midgut proteinases when ingested. In order to investigate the occurrence of PPIs that may interact with larval midgut proteinases, leaf extracts were collected from un-induced and herbivore-induced leaves of each species. Reverse zymograms with these leaf extracts incubated with midgut extracts from AGP1 and AGP2 containing trypsin and chymotrypsin were compared (Figure 6 a, b). In un-induced leaf samples of *P. bombycina*, three bands of PPIs or their isoforms were indicated. In contrast, the herbivore-induced samples contained a prominent band indicating up-regulation of a single PPI or *de novo* synthesis of a wound-inducible PPI. In *L. monopetala* no differences were observed between the un-induced and herbivore induced samples but this may be due to the fact that the plants from which the samples were collected were unknowingly induced! It is particularly interesting that the mobility of herbivore-induced leaf PPI in *P. bombycina* resembled a PPI from *L. monopetala.* In turn, the mobility of herbivore-induced PPI detected in both species by reverse zymography with AGP1 and AGP2 midgut enzymes resembled the mobility of SPI observed in reverse zymograms with purified trypsin and chymotrypsin, suggesting that the PPIs seen in Figure 5 a,b were SPIs. These results suggested that (i) SPIs were present in leaves of *P. bombycina* and *L. monopetala* that could bind to midgut serine proteinases of *A. assamensis* (ii) mobility of leaf SPIs in *P. bombycina* and *L. monopetala* were similar and that (iii) at least one SPI in each species was wound –inducible and responded to herbivory. Further research into the isolation, sequencing and characterization of inhibitors from *P. bombycina* and *L. monopetala* is required.

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