Isolation of the Astacin-like metalloprotease coding gene (*astl*) and assessment of its insecticidal activity against *Spodoptera littoralis* and *Sitophilus oryzae*

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Abstract

Astacin- like metalloprotease (*astl*) is a multi-domain metalloproteinase that has
protease activity against a number of organisms; including fish, frogs, birds and
insects. In this present investigation, the full length of *astl* cDNA was cloned
from spider species, *Hasarius adansonii*. Sequencing of the cloned *astl* cDNA
proved that its full length including 802 bp with 714bp open reading frame
encoding for 238 amino acids. The catalytic domain comprised of 489 nts was
cloned and expressed by the yeast expression system *Pichia pastoris* and its
insecticidal activity was determined against two species of agricultural insects
Spodoptera littoralis (Lepidoptera:Noctuidae) and *Sitophilus oryzae*
(Coleoptera:Curculionidae). Bioassay was performed using three

concentrations (100,500 and 1000 ppm) for four days for *S. littoralis* and 14 days for *S. oryzae*. In addition, the astl was fused to the GNA snowdrop lectin in the same frame and expressed in *P. pastoris*. The synergistic effect of astl and GNA was examined on the *S. littoralis* larvae and *S. oryzae* adults. The mortality percentages of the fused protein (Ha-astl/GNA) "1000 ppm " after 4 days, were 78.6%± 4.16 and 71.66% ±3.51 for first and second spodpotera larval instars, respectively. While, lower mortality of the fused protein of the same concentration was observed on *S. oryzae* adults, 49.3±2.08 %.

Introduction

The spiders and predacious beetles are considered the most venomous terrestrial animals (Windley et al., 2012). Spider venoms are a cocktail of thousand peptide toxins, and most of these are likely to have insecticidal activity against a wide range of insect orders (Escoubas et al., 2006; Windley et al., 2012; and King et al., 2002). Many of the insecticidal peptide toxins have been isolated from spider venoms and their activities have been demonstrated. Three families of insect-specific peptide toxins (atractotoxins), from *Hadronyche versuta*, are specific blockers of arthropods voltage gated calcium channels (Mukherjee et al., 2006). Orally active venom (OAIP-1) was isolated from the Australian tarantula spider, *Selenotypus plumipes*, (Hardy et al., 2013). Some of spider toxins show no adverse effect on economically important insects such as Hv1a that is harmless to the pollinating insect honey bee *Apis mellifera* (Nakasu et al. 2014). Proteins belong to family of astacin-

like metalloprotease were described as toxins that exist in the venom of 46
different spider species of *Loxosceles* (Trevisan-Silva et al., 2010). 47
Astacin (*astl*) is a multi-domain metalloprotease that is distinguished by the 48
presence of zinc binding motif (HEXXHXXGXXH) and methionine-turn 49
(MXY) (Gomis-Rüth et al., 2012). They are either secreted or membrane- 50
anchored as inactive zymogen which is activated by cleaving the inhibiting 51
pro-segment (Yiallourous et al., 2002 and Guevara et al., 2010). The *astl* gene is 52
expressed as hatching enzyme in the oocyte and in the developing embryo 53
(Gomis-Rüth et al., 2012). It is suggested that hatching enzyme is responsible 54
for degrading embryonic envelopes of crustaceans, fish, frogs, and birds 55
(Gomis-Rüth et al., 2012). It is also known as ovastacin in mammals (Quesada 56
et al., 2004) and plays a role in egg-sperm interaction (Sachdev et al., 2012). 57
The *astacin* gene copy number in the genome is different from organism to 58
another and it is suggested that its number determines its roles in the organism 59
(Gomis-Rüth et al., 2012). According to MEROPS database 60
(<http://merops.sanger.ac.uk>), Nematode genome (*Caenorhabditis elegans*) 61
contains the largest copy number, around 40 copies of *astacin* gene, because it 62
plays role in breaking down the host connective tissue (Möhrle et al., 2003). 63
However in mammals and birds, the *astacin* gene is represented by a limited 64
copy number, six, compared to those of lower vertebrate and invertebrate 65
genomes (Stöcker et al., 2013). 66
Based on the fusion protein technology, a carrier protein such as lectin could be 67
used, thus allowing the spider venom toxins to act orally. Fitches et al. (2012) 68

pointed out that the neurotoxin protein from the Australian funnel web spider 69
Hadronyche versuta, α -hexatoxin-Hv1a was lethal to many insect species 70
when injected, while it is non-toxic through feeding. However, the α - 71
hexatoxin-Hv1a/GNA fusion protein showed oral insecticidal activity against 72
insects from different orders. 73
The agricultural and horticultural pest insects cause 40% loss of the crop yield 74
worldwide (Oerke et al., 1994) that is estimated by 17.7 billions dollars 75
annually (Oliveira et al., 2014). *Spodoptera littoralis* one of the most 76
destructive agricultural lepidopteron insect pests that can damage many 77
economical crops such as cotton, maize, tomato and vegetables (Salama et al., 78
1970). The larvae of *S. littoralis* feed on the leaves, fruits, flowers, buds and 79
bolls of cotton making them useless (Bishari, 1934). *Sitophilus oryzae* is one of 80
the important insect pests of stored grains such as rice, wheat and their products 81
(Baloch, 1992). *S. oryzae* causes loss of weight, reduction in the nutritional 82
value and increasing the infection with other mites and fungi, therefore the 83
commercial value of the stored grains decreased (Madrid et al., 1990). These 84
agriculture pests are controlled by different methods such as chemical 85
insecticides which are extensively used in Egypt (Issa et al., 1984a; Issa et al., 86
1984b; and Abo-El-Ghar et al., 1986). Therefore, resistant strains of insect 87
pests appeared in the field (Sawicki, 1986). An alternative control method that 88
used in control strategy is biological agents such as natural enemies, nuclear 89
polyhedrosis virus (Elnagar and El-Sheikh, 1990 and Jones et al., 1994 and 90
Atia et al., 2016) and *Bacillus thuringiensis* and its derivative (Navon et al., 91

1983 and Moussa et al., 2016). However, *S. littoralis* and *S. oryzae* developed resistant against biological control agents (Salama et al., 1989). New biopesticides are always needed to be involved in control strategy. In this article, the astacin like metalloprotease coding gene (*astl*) has been isolated from spider *Hasarius adansoni*, expressed in yeast and the toxicity of the *astl* protein from and *astl*/GNA fused protein were demonstrated against *S. littoralis* and *S. oryzae*.

Material and methods

Spider collection

The spider samples were collected from different locations including human houses. The spider species used in the current study was identified as *Hasarius adansoni* by amplification of 889 bp of ribosomal RNA (*rRNA*) gene large subunit and 705bp in length of the 5'-end of mitochondrial cytochrome c oxidase subunit 1 (*COI*) gene. The fragments of *rRNA* and *COI* were amplified using specific primer sets Sp28SN/Sp28SC (Starrett and Hedin, 2007), and SP-LCO 1490FJJ/SP-HCO2198RJJ (Astrin et al., 2016), respectively. The spider species was confirmed with complete homology of the two fragments with *H. adansoni* using “BLASTN” NCBI-BLAST alignments tool. The cephalothorax region was dissected in paraffin wax plate and isolated from the rest of the body.

Total RNA extraction

The total RNA was extracted from the cephalothorax region of the spider by 115
Trizole[®] reagent (Invitrogen, USA, Cat.#15596-026). First strand cDNA was 116
synthesized using SuperScript[™]II reverse transcriptase (Invitrogen Cat.# 117
18064014) according to the manufacturer's instructions. 118

Cloning of *astl* cDNA 120

A set of degenerate primers (Table 1) was designed to amplify a part of the 121
nucleotides sequence of the astacin like metalloprotease cDNA in a standard 122
PCR reaction using the first strand cDNA as a template. Then, two sets of 123
specific primers (Table 1) were designed to amplify the 5' and 3' using First 124
Choice[®] RLM-RACE kit (Ambion, Austin, TX, USA). All PCR products 125
were cloned into pGEM-T Easy vector (Promega, Madison, WI, USA) and 126
transformed into TOP10F' chemically competent cells (Invitrogen). 127

Sequences and phylogenetic analysis 129

The sequence of *astl* cDNA was aligned with the other published sequences in 130
GenBank using the “BLASTN” and “BLASTX” tools at the National Center 131
for Biotechnology Information (NCBI) web site 132
(<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The amino acid sequences from cDNA 133
clones were deduced using ExPASy translate tool 134
(<https://web.expasy.org/translate/>). Alignment between the amino acid 135
sequences and other amino acid sequences from other spiders and a scorpion 136
and the phylogenetic tree were performed using the phylogeny.fr software 137

(<http://www.phylogeny.fr/index.cgi>). The molecular weight (MW) and isoelectric point (pI) were calculated computationally on ExPASy Proteomics website (http://web.expasy.org/cgi-bin/compute_pi/pi_tool). The deduced amino acid sequence was scanned for motifs against the PROSITE database (<http://prosite.expasy.org>). Glycosylation site was predicted using NetNGlyc 1.0 software (<http://www.cbs.dtu.dk/services/NetNGlyc/>). O- β -glycosylation sites were predicted using YinOYang program (<http://www.cbs.dtu.dk/services/YinOYang/>).

Snow drop lectin (GNA) assembly

A 357 bp fragment encoding the *Galanthus nivalis* snow drop lectin (LECGNA 2) was designed according to published sequence accession no. M55556.1. GNA coding fragment was synthesized *in silico* by designing ten overlapping smaller fragments. Each of the small fragments was designed by two complementary single strands of 35-39 nucleotides strip and each strip had extra few nucleotides on the 5' end to be used as sticky ends. After annealing, these sticky ends were used for ligation with the following neighbor fragments using T4 DNA ligase enzyme. Finally, the synthesized 357bp fragment was amplified by PCR and then cloned into pGEM-T- easy vector and subjected to sequence. GNA nucleotide sequence was confirmed by alignment to the original GNA sequence.

Cloning of *astI* catalytic region and *astI*/GNA fused fragment into the yeast 160

expression vector pPICZαA 161

The 489bp fragment of *astI* catalytic region was amplified using specific 162
primer set “astIFHE/astRX” (Table 1). Both the *astI* fragment and pPICZαA 163
vector (Invitrogen, USA) were double digested with *XbaI* and *EcoRI* restriction 164
enzymes and then ligated using T4 DNA ligase enzyme (Promega, USA). The 165
ligated DNA was transformed into TOP10F competent cells and colonies were 166
screened for positive pPICZαA-*astI* plasmid. 167

In addition, the *astI* and GNA sequences were fused as a single sequence frame; 168
firstly cloned in pSK⁺ and then subcloned into pPICZαA vector. The *astI* (489 169
bp) was amplified and cloned into *HindIII*/*PstI* double digested pSK⁺ vector 170
forming pSK-*astI*. The amplified 357bp GNA fragment was double digested 171
with *PstI* and *XbaI* and then cloned downstream *astI* in the corresponding 172
restriction sites forming pSK-*astI*/GNA. The 846 bp *astI*/GNA fused sequence 173
was amplified using astIFHE and GNARX primers (Table 1) and then 174
subcloned into pPICZαA vector with *EcoRI* and *XbaI* restriction sites, creating 175
the pPICZαA-*astI*/GNA. 176

Transformation of the pPICZαA-*astI* and pPICZαA-*astI*/GNA into yeast 178

"*Pichia pastoris*", KM71H strain. 179

pPICZαA-*astI* and pPICZαA-*astI*/GNA (3-5μg) were digested using *SacI* 180
followed by purification and transformation into yeast cells, *Pichia pastoris* 181
yeast competent cell strain “KM71H”. The transformed cells were incubated 182

at 28°C for 2-3 days on YPDS medium plates containing 100mg/ml Zeocin. 183
Then, the colonies were numbered and transferred to YPD plates, and 184
incubated for two more days and allowed to grow. A number of colonies was 185
screened by universal primers “Alfa factor/3'AOX1” followed by nested PCR 186
by specific primers (astlFHE/astlRX and astlFHE/GNARX) (Table 1). 187
The grown yeast colonies were numbered and dissolved in 20mM NaOH then 188
boiled for 45 min at 95°C then centrifuged for 10 min and 2µl supernatant was 189
used as a template. The screening was performed by two sequential rounds of 190
PCR reaction. The first round was performed using the universal primers “Alfa 191
factor/3'AOX1” while the second round was conducted using the specific 192
primers. 193

Expression of astl catalytic domain and the fused recombinant astl/GNA 194 proteins 195

A single colony of KM71H strain harboring either pPICZαA-astl or pPICZαA- 196
astl/GNA was inoculated in 25 ml of minimal glycerol medium ±histidine 197
(MGYH) “1.34% yeast nitrogen base YNB, 1% glycerol, 4x10⁻⁵% biotin and 198
±0.004% histidine” in a 250 ml flask. The culture was grown at 28-30°C in a 199
shaking incubator (200 rpm) until the intensity of the culture reached 2-6 on an 200
OD₆₀₀. The cells at log phase growth were harvested by centrifugation at 3000 201
xg for 5 min at RT. The supernatant was decanted and the cell pellet was 202
resuspended in 200 ml minimal methanol ±histidine (MMH) 1.34% 203
YNB, 4x10⁻⁵% biotin and 0.5% methanol” to reach to an OD₆₀₀=1 to induce 204
the expression. The flask nozzle was capped with many layers to reduce the 205

evaporation of the methanol. To maintain the induction through 4 days 206
expression, a volume of 100% methanol was added every 24h to the culture to 207
reach a final concentration of 0.5% methanol. 208

The expression levels were analyzed from day one to day four to determine the 209
optimal harvest time post induction. The expression analysis was performed on 210
secreted proteins in the supernatant. SDS-PAGE and ELISA were used to 211
analyze the expressed proteins. 212

Insect cultures 214

The *S. littoralis* colony was reared in the insectory of the Agriculture Genetic 215
Engineering Research Institute (AGERI), Agricultural Research Center (ARC) 216
under standard conditions. The larvae were reared on castor leaves at a 217
temperature of 25±2°C and light period of 16 h light/8 h dark. 218

S. oryzae was kindly supplied by the Plant Protection Research Institute (PPRI), 219
ARC. The adults of the same age were reared on wheat seeds in glass jars 220
under standard conditions and then used to assess the insecticidal activity. 221

Bioassay 222

The insecticidal activities of astI and astI/GNA fused proteins were assessed by 223
feeding the *S. littoralis* larvae and the *S. oryzae* adults on three different 224
concentrations (100, 500 and 1000 ppm) of each individual protein. The 225
castor leaves were immersed in solution containing the assayed amount of 226
protein with agitation for half an hour. The leaves were then picked up 227
from the protein solution and allowed to air dry. Twenty five of either 228

first or second stadium of *S. littoralis* larvae were added treated castor 229
leaves which were replaced daily with fresh treated leaves for four days. 230
Each treatment was repeated three times. The larval mortality was 231
recorded daily during experiment period. The sensitivity of *S.oryzae* 232
adults to astI and astI/GNA were assayed for 14 days period. Ten gm of 233
wheat were immersed in protein solution for 30 minutes. The treated 234
wheat was then filtered and allowed to dry. Twenty five adults were 235
added to each 10 gm of treated wheat in glass jar with three replica for 236
each concentration. The jars were incubated at 27°C for 16h/8h light/dark 237
periods. The adult mortalities were recorded by day 14. For all bioassays, 238
control larvae were treated as the same as experimental larvae except 239
water was used instead of protein solutions. 240

Statistical analysis of the mortality data was performed with Student's t- 241
test in the Excel program. 242

Results 244

Amplification of the full length of astI cDNA sequence 245

The first strand cDNA, prepared from total RNA of the *H. adansoni* 246
cephalothorax, was used as a template for amplification of Astacin-like 247
metalloprotease coding gene (*astI*). One degenerate primer set was designed for 248
astacin-like metalloprotease coding gene (*astI*) from the conserved regions of 249
the same gene in the spider *Latrodectus hesperus* and the scorpion *Tityus* 250

serrulatus. A 119 bp fragment was firstly amplified using the degenerate
primers. This was followed by amplifying the 5' and 3' ends using two PCR
rounds of RACE based on the sequence of the amplified fragments, it was
deduced that the full length of *H. adansonias* (*Ha-astl*) cDNA is 802
nucleotides (accession no.MN453831) with an open reading frame of 714 nts
encoding 238 amino acids. The predicted molecular weight of encoded protein
is 27.33 kDa with an isoelectric point (pI) of 8.29. Analysis of *Ha-astl* protein
reveals presence of two disulfide bridges between cysteine residues at positions
87-238 and 108-128. In additions, three zincbinding sites at histidine residues
were found at positions; 136,140 and 146. Also, a N-glycosylated amino acid
was predicted at aa 149 and 0- β -glycosylation sites were predicted (T56, S70
and T94) (Fig. 1).

The deduced amino acid sequence was aligned using the BLASTp tool in the
NCBI website. The covered score was 88-99% and the identity ranged between
74-84% in spiders as *Trichonephila clavipes*, *Latrodectus hesperus*, *Stegodyp*
husmimosarum, *Parasteatodate pidariorum* and scorpion as *Tityus serrulatus*
with accession no. PRD25795.1,ADV40108.1,KFM63176.1,XP_015909675.1
and CDJ26716.1, respectively. Alignment results of the deduced amino acids
sequence with other published astacin like metalloprotease toxin are shown in
Fig. (2). The phylogenetic tree performed by the phylogeny.fr software is
shown in Fig.(3). These results demonstrated that the *Ha-astl* aa sequence is
closely related to that of the spider *T. clavipes* and the scorpion *T. serrulatus*.

Cloning the catalytic region of Ha-astl and the fused (Ha-astl/GNA)	274
fragments into the yeast expression vector (pPICZαA)	275
A 357 bp lectin fragment was prepared as described in materials and methods	276
and fused to the 489bp of astl. The 489bp of the Ha-astl and the fused (Ha-astl-	277
GNA) fragments were cloned in pPICZ α A through the digestion by <i>EcoRI</i> and	278
<i>XbaI</i> then ligation. The colonies were screened using the universal and specific	279
primers (Table 1) and the positive colonies were confirmed by sequencing.	280
	281
Transformation of pPICZαA-astl and pPICZαA-astl/GNA into the yeast	282
"<i>Pichia pastoris</i>" "KM71H strain"	283
The positive recombinant vectors were digested by <i>SacI</i> and transformed in	284
KM71H competent cells. Two sequential rounds of PCR reaction were	285
performed to distinguish between the transformed and non-transformed yeast	286
colonies by the desired fragment. The first round was carried out using the	287
universal primers (alfa factor and 3'AOX1) (Fig. 4A and 5A). The second PCR	288
was nested to the first. For pPICZ α A-Ha-astl, it was conducted using the	289
specific primer astl F1 and the universal primer 3'AOX1 that would amplify a	290
200 bp fragment (Fig. 4B). While, for nested PCR for the fused fragment	291
(pPICZ α A-Ha.astl/GNA) was performed using the astl F1and GNA R5 primers	292
to cover about 144 and 175 nucleotides from the astl and GNA fragments,	293
respectively, thus revealing a 319 bp fragment in the positive lanes (Fig. 5B).	294
The positive colonies were confirmed by different combinations between the	295
universal and specific primers (Fig. 5C).	296

297

Expression of pPICZαA-astl and pPICZαA-astl/GNA into yeast "*Pichia* 298
***pastoris*" "KM71H strain"** 299

Both pPICZαA-astl and pPICZαA-astl/GNA, expressing target proteins bound 300
to His tag, were transformed into the yeast *P. pastoris* "KM71H" competent 301
cells. The cell cultures were induced by methanol to express Ha-astl and Ha- 302
astl/GNA proteins. The expressed proteins were demonstrated on SDS-PAGE 303
gel (Fig 6). Expressed Ha-astl showed faint band on the SDS-PAGE at 304
expected molecular weight. On the other hand, the fused Ha-astl/GNA band 305
was hardly visible on the gel. However, the chimeric protein was detected by 306
sandwich ELISA that targets His Tag with His-specific antibody with different 307
reading values at OD 450 (Fig. 7). 308

309

The insecticidal activity of Astacin-like metalloprotease (Ha-astl) and the 310
fused protein (Ha-astl/GNA) on *S. littoralis* 311

The toxic efficacies of Ha-astl and Ha-astl/GNA fused proteins were evaluated 312
on the first and second instar of *S. littoralis* larvae *per os* using three different 313
concentrations i.e. 100, 500, 1000 ppm of the protein. The oral activity was 314
performed on treated castor leaves which were replaced daily for four days. 315
While the control larvae were fed on washed cleaned untreated castor leaves 316
for the same period. The mortality was recorded daily through out the 317
experiment period. The larval mortality ratio showed daily increment during 318
the four day experiment. As expected, the highest insecticidal effect was 319

recorded for the larvae feed on the fused protein at 1000 ppm concentration. 320
The mortality percentages were 78.6%± 4.16 and 71.66% ±3.51for first and 321
second larval instars, respectively. This was followed by the larvae fed on for 322
astl protein after four days as 69.3%±2.51 and 65%±2.64 mortality recorded for 323
the first and second larval instars, respectively. The statistical analysis of the 324
mortality ratio showed significant (*) and highly significant (**) effects of the 325
protein against the larvae at (p>0.05) and (p>0.01) respectively. Larval 326
mortality of *S. littoralis* and their significance are shown in Fig. (8) and (9). 327

The results of the feeding experiment also showed that the larvae 328
survived in the different treatments expressed a significant retardation in their 329
growth (Fig. 10). Some of them developed to malformed pupae or failed to 330
pupate or retarded to pupate compared to the control (Fig. 11). After a recovery 331
period where larvae were transferred onto untreated castor leaves and allowed 332
to grow for 10 to 15 days, the larvae did not restore normal weight. The 333
difference of consumed castor leaves by control and treated larvae was clearly 334
notable as shown in Fig. (12). The average larval weight of the control and 335
treated larvae was 0.05 and 0.013gm after 10 days and 0.08 and 0.0085 gm 336
after 15 days, respectively. 337

338

The insecticidal activity of Astacin-like metalloprotease (Ha-astl) and the 339 fused protein (Ha-astl/GNA) on *S. oryzae* 340

The toxicity of the astacin-like metalloprotease and the fused protein was 341
evaluated on the adult of *S. oryzae* by *per os*. Experimental adults were fed on 342

treated wheat that was previously immersed in the protein solution for 30 min, 343
then excess solution was filtered and the wheat were allowed to air dry. Three 344
concentrations were used i.e.; 100, 500, 1000 ppm of each protein with three 345
replicates each and 25 adults per replica. The adult mortality was recorded two 346
weeks post-treatment. The mortality ratio for the Ha-astl protein was 347
46.6%±0.577, 48%±1.7 and 64%±3 for the three concentrations 100, 500, 1000 348
ppm, respectively. While, lower mortality ratios were observed on the adults 349
treated with the three fused protein (Ha-astl/GNA) concentrations, i.e., it was 350
41.3%±2.08, 42.6%±2.88, 49.3%±2.08, respectively. The statistical analysis 351
showed that the mortality ratio was highly significant (**) at (p>0.01) for all 352
the concentrations of the Ha-astl and the fused proteins (Ha-astl/GNA) 353
(Fig.13). 354

DISCUSSION 356

Bioinsecticides are being used as potential alternatives to chemical insecticides. 357
The sources of biopesticides are natural organisms, or their metabolic products 358
including insecticidal toxins derived from insect predators and parasitoids. 359
Peptide toxins derived from predatory organisms such as spiders have a great 360
deal of interest for their potent insecticidal activity. In this study, we examined 361
the susceptibility of agricultural insect pests, *S. littoralis* and *S. oryzae*, (belong 362
to two insect orders lepidoptera and coleoptera against expressed 363
metallopeptidase peptide, astacin, derived from the venom gland of the 364
Adanson's house jumper spider, *H. adansonii*, as well as a recombinant fusion 365

protein combining snowdrop lectin (GNA) linked C terminally to astacin. The 366

Ha-astl sequence was identified in the total RNA content of *H. adansoni* spider 367

venom. The presence of metalloproteases as components of spider venom was 368

previously detected in the venom of different *Loxosceles* spider species, *L.* 369

intermedia, *L. gaucho*, *L. deserta*, *L. laeta* and *L. rufescens* (Feitosa *et al.*, 1998; 370

Young and Pincus, 2001; Da Silveira *et al.*, 2002; Zanetti, 2002; Barbaro *et.* 371

al., 2005 and Da Silveira *et al.*, 2007) Moreover, nine possible isoforms of 372

astacin-like metalloproteases were identified from Peruvian, *L. laeta* venom 373

and validated by *in silico* and *in vitro* experiments (Medina-Santos *et al.*, 374

2019). Presence of metalloproteases in the spider venom provides evidence for 375

its significant biological activity and its conserved feature in the venom of 376

spider species (Feitosa *et al.*, 1998; Young and Pincus, 2001; Da Silveira *et al.*, 377

2002; Zanetti, 2002 and Barbaro *et al.*, 2005). 378

The full length of *Ha-astl* cDNA sequence is a total of 802 nucleotides 379

encoding 238 amino acids peptide. On the basis of the predicted amino acid 380

sequence, *Ha-astl* protein shows the same general features of metalloproteases 381

family members (Dumermuth *et al.*, 1991; Bond and Beynon, 1995 and 382

Mohrlen *et al.*, 2004). *Ha-astl* primary structure includes a prosegment region 383

(M1:R45), a catalytic domain (N46:C238) and a conserved methionine- turn 384

MXR (Met192 and Tyr194). The catalytic domain composes the consensus 385

signature sequence responsible for binding of the catalytic zinc ion, 386

HEXXHXXGXXHE (His136 and Glu147). Around 90% of spider-venom 387

toxins possess several disulfide bridges ranges from one to seven, but nearly 388

60% of all toxins have three bridges (Windley et al., 2012). Two disulfide bridges are potentially present in Ha-astl between Cys108-Cys128 and Cys87-Cys238. The calculated molecular mass and pI of the deduced amino acid of *Hasarius adansoni* (27.3 kDa/8.29) are closely related to other astacin-like metalloproteases in spiders, *Trichonephila clavipes* (27.3 kDa /8.97) , *Latrodectus hesperus* (27.1 kDa /8.81) and a scorpion *Tityus serrulatus* (27.3 kDa /8.9).

The biological significance of native astacin-like venom toxin is not clearly known. However, it is speculated that the presence of this toxin in the venom represents an adaptation of spiders to paralyze and kill insect prey as well as to serve as a defense against predators (DaSilveira et al., 2007). Astacin may play role, in synergism with other venom toxins, in the deleterious effects of the prey after envenomation (Futrell, 1992) as well as activation of peptide derived toxins after proteolysis. However, the toxic efficacy of solely astacin-like metalloprotease on insects is questionable, Does these protease present the venom cocktail could be deployed in insect control? To answer this question, the insecticidal activity of Ha-astl was assayed *per os* on cotton leaf worm and rice weevil. The N-terminal pro-segments of astacin-like metalloproteases are variable in length and inhibit the catalytic zinc domain. Removal of the pro-segment uncovers a deep and extended active-site cleft, which in general shows preference for aspartate residues in the specificity pocket (Gomis-Rüth et al., 2012). Hence, a 571 nts encoding the catalytic region spanning Gln52 and Met28 was used for expression as active astl. To maintain functional

configuration of the expressed recombinant protein, an expression system that 412
is capable of correctly forming cys-cys cross-links was required. Thus, *Pichia* 413
pastoris was used as expression host for expressing both Ha-astl and the Ha- 414
astl/GNA fusion, with both recombinant proteins engineered for secretion into 415
culture supernatant. The expressed recombinant proteins were exploited for 416
sensitivity assays. A moderate toxic effect of Ha-astl was demonstrated in both 417
insect species. 1000ppm of astacin peptide causes $69.3\% \pm 2.51$ and $65\% \pm 2.64$ 418
for first and second stadium of *Spodoptera* larvae, respectively. On the other 419
hand, $64\% \pm 3$ of *S. oryzae* adults were killed by the same concentration. 420
Similar level of lethality was demonstrated by spider neurotoxins on pest 421
species. The μ -agatoxin-Aa1 toxins from the venom of the Western grass 422
spider, *Agelenopsis aperta*, are insect-selective neurotoxins that cause a 423
convulsive paralysis in insects. They show variant activities to different insect 424
orders as, being very potent in dipterans, moderately potent in orthopterans, but 425
only weakly active in lepidopterans (Adams et al. 1989). The ω -Hexatoxin- 426
Hv1a toxins from the venom of Australian funnel-web spiders have low ED50 427
values in Orthoptera, Hemiptera, Dictyoptera, Diptera, Coleoptera, Acarina and 428
Lepidoptera (Atkinson et al., 1996; Fletcher et al., 1997 and Bloomquist, 429
2003). However, proper manipulation of these toxins revealed the prospect use 430
of spider toxins in insect pest control. Transgenic expression of ω -HXTX-Hv1a 431
in tobacco plants results in protection from *Helicoverpa armigera* and *S.* 432
littoralis larvae (Khan et al., 2006). Topical application of recombinant 433

thioredoxin- ω -HXTX-Hv1a has also been shown to be lethal to these caterpillar species (Khan et al., 2006). Fusion protein technology was exploited to use plant GNA snowdrop lectin as a ‘carrier’ protein allowing proteins such as spider venom toxins to act as orally delivered biopesticides. Comparison of the toxicity of the fully purified recombinant Ha-astI with the Ha-astI/GNA fused protein revealed no significant difference due to GNA fusion on term of killing effect on both insect species. The recombinant Ha-astI caused 65% $\pm$ 2.64 mortality of spodoptera larvae, these mortalities were slightly increased to 71.7% by the fused protein, whereas, susceptibility of rice weevil adults to Ha-astI inclined from 64% $\pm$ 3 to 49.3 $\pm$ 2.08% by Ha-astI/GNA. These data suggest that the toxicity of Ha-astI in the fusion protein somehow was not expected, nonetheless, and the fusion protein is still an effective toxin. However, It is unclear why the two insects reacted differently to GNA fused protein. Cotton leafworm larvae were slightly higher sensitive to fused protein while that of rice weevils was lower. These two pests belong to two different orders which raise questions; do these two orders react differently to GNA fused protein? or is it species-dependent? Previous studies of using recombinant fusion proteins combining GNA snowdrop lectin linked either to the insect neuropeptide (Manse-AS), to an insect spider venom neurotoxin (SF11) or to scorpion neurotoxin (ButaIT) have demonstrated that GNA can be utilised as a transporter to deliver linked peptides to the larval haemolymph (Fitches et al., 2002; 2004 and 2010). Also,

the Hv1a/GNA fusion protein showed oral insecticidal activity against insects 457
from different orders (Fitches et al., 2012). The results presented in this paper 458
are inconsistent with those presented in previous studies. Clearly, the site effect 459
of these toxins is insect nervous system and GNA allowed these toxins to 460
traverse the insect gut epithelium and access its sites of action, producing an 461
orally active insecticidal protein. Action of astacin metalloproteases is more 462
likely on insect tissues rather than nervous system, thus, it does not require 463
reaching hemolymph to be activated as other neurotoxins. Therefore, GNA has 464
no significant effect on astl toxicity as shown in other spider and scorpion 465
toxins. DaSilveira et al. (2007) suggested that the biological mechanism of 466
proteases is to facilitate the diffusion of other important venom toxins through 467
the bodies of victims by rendering tissue structures more permeable, and then 468
acting in parallel with other biologically active toxins. 469
To conclude, evidence that the GNA fusion with metalloprotease is generally 470
provide no advantage as orally insecticidal to a range of insects. The use of 471
metalloprotease, more likely, with insect-selective neurotoxins, to ease tissues 472
penetration, for pest management offers an attractive alternative to broad- 473
spectrum chemical control, particularly in the light of the decreasing numbers 474
of insecticides that are available for use. 475

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