DIGESTIVE PROTEOLYTIC PROFILE IN *STROMATIUM FULVUM* VILLERS (COLEOPTERA: CERAMBYCIDAE)

ARASH ZIBAEE*

Department of Plant Protection, Faculty of Agricultural Sciences, University of Guilan, Rasht, Iran 41635-1314

(Received January 12, 2014)

Digestive proteolytic profile was determined in the midgut of *Stromatium fulvum* Villers adults by using the site of activity, specific substrates, inhibitors and pH dependency. Optimal pH of soluble protease was found at pH 9, with a small peak at pH 5, while membrane-bound protease showed the highest activity at pHs 8-10 and a peak at pH 6. Soluble and membrane-bound proteases of *S. fulvum* adults demonstrated the highest activities at temperatures of 25 and 30˚C. Verification of specific proteases by using negative control, inhibitors and activators revealed the presence of all specific proteases in the soluble fraction, except for membrane-bound trypsin and cathepsin L. pH dependency of specific proteases by using their substrates revealed alkaline pHs for serine proteases and exopeptidases, as well as acid pHs for cysteine proteases. Specific inhibitors, including AEBSF, TLCK, TPCK, E-64, DTT and phenanthroline, caused significant effects on general proteolytic activities in the midgut of *S. fulvum*. Results of the current study expand our knowledge on proteolytic activity of a wood-boring beetle which was less studied in insects.

Keywords: digestive protease characterization, *Stromatium fulvum*.

INTRODUCTION

Proteases have several functions in insects physiological processes, *e.g.*, digestion, vitellogenesis, immunity, intercellular signaling, etc. Insect digestive proteases are complex and diverse enzymes that hydrolyze protein molecules to amino acids. Exopeptidases contain amino- (EC 3.4.11.2) and carboxypeptidases (EC 3.4.17.1) that attack external bonds of proteins from N- and C-terminals, respectively (1). Serine proteases contain trypsin (EC 3.4.21.4), chymotrypsin (EC 3.4.21.1) and elastase (EC 3.4.21.36), which are active at alkali pHs and have serine, histidine and aspartic acid residues in their active sites. Cathepsins B (EC 3.4.22.1) and L (EC 3.4.22.15) are the two main groups of cysteine proteases that are active at pH values of 5-6. The sulfhydryl group of a cysteine side chain in the active site acts as a nucleophile to hydrolyze peptide bonds (2).

* Corresponding author (Ph.D, Assistant Professor of Insect Physiology; Email: arash.zibaee@gmx.com; arash.zibaee@guilan.ac.ir; Tel: +98-0131-6690485; Fax: +98-0131-6690284)

Digestive proteolytic activities of lepidopterans have been elucidated, and several endogenous and exogenous inhibitors have been suggested to be used in pest control, but proteases in beetles have been less studied so far and research has been restricted to some species of *Tribolium*, curculionoidae, Chrysomelidae, etc. As a well studied example, Vinokurov *et al*. (3) determined the digestive protease profile in the larvae of *Tenebrio molitor* L (Coleoptera: Tenebrionidae). The authors reported that the pH of midgut contents, ranging from 5.2–5.6 to 7.8–8.2, reflected the optimal pH of the total proteolytic activity in anterior and posterior midgut (3). Cysteine proteases accounted for 2/3 of the total proteolytic activity in the anterior midgut, while serine proteases were responsible for 76% of the total proteolytic activity in the posterior midgut. Besides, there is no report on digestive proteolytic activity of wood-boring beetles like *Stromatium fulvum* Villers (Coleoptera: Cerambycidae). These beetles have a unique digestive habit because of shortage in nutritional sources. Most of them depend on microorganisms from their own gut to provide nutritional requirements, mainly fatty acids, vitamins, etc. (4). As a wood-boring beetle, *S. fulvum* Villers (Coleoptera: Cerambycidae) is a widely distributed Cerambycidae beetle that utilizes dead and dry woods (5). Therefore, the current study aimed to identify and characterize general and specific proteolytic activities in the midgut of *S. fulvum*, in order to achieve a better understanding of its protein digestion.

MATERIALS AND METHODS

INSECT COLLECTION

Adults were collected from infested woods in urban areas of Rasht, north of Iran and identified by Dr. Jalil Hajizadeh in the Department of plant protection, University of Guilan.

SAMPLE PREPARATION

Adults were randomly selected and their midguts were removed by dissection, using a stereomicroscope in ice-cold saline buffer (NaCl, 10 mM). Pronotum and undesirable organs were removed and midguts were gently separated, then cut from the body and rinsed in 1 mL of ice-cold distilled water. To obtain appropriate samples, five midguts were placed in one Eppendorff tube (www.eppendorf.com) containing 1 mL of distilled water. Tissues were ground by a homogenizer and then centrifuged at 13,000 rpm for 20 min at 4°C. Supernatant was carefully removed, transferred to new tubes and stored at –20°C for no more than one week, until the onset of experiments. For solubilization of membrane-bound enzymes (such as glycosidases and exopeptidases) in Triton X-100, membrane preparations (*via* precipitation from primitive centrifuge, above) were exposed to Triton X-100 for 20 h at 40˚C, in a ratio of 10 mg of Triton X-100 per

mg of protein, before being centrifuged at 13,000 rpm for 30 min. No sediment was visible after supernatant centrifugation at 10,000 rpm for 60 min. Enzyme activity remained unchanged at −20 °C for at least one month (6).

OPTIMAL pH DETERMINATION OF GENERAL PROTEOLYTIC ACTIVITY

General proteolytic activities in the midgut of *S. fulvum* were assayed by using hemoglobin (2 mg/ml, BBL co., Maryland USA, 11872) as a substrate in both soluble and membrane-bound fractions (7). The reaction mixture consisted in 50 µl of universal buffer solution (20 mM containing succinate, glycin and 2-morpholinoethanesulfonic acid), 20 µl of hemoglobin and 5 µl of enzymatic samples. After incubation for 120 min, proteolysis was stopped by adding 100 µl of 30% trichloroacetic acid (TCA). Then, reaction mixtures were centrifuged at 13,000 rpm for 5 min. Absorbance was recorded at 340 nm. The blank solution consisted of all mentioned portions, except for the enzyme solution. The peptides liberated from hemoglobin were estimated using Folin–Ciocalteu reagent at 650 nm.

DETERMINATION OF SPECIFIC PROTEASES

A negative control containing boiled samples of soluble and membrane-bound fractions (for 30 min) was used to prove the presence of specific proteases in the midgut of *S. fulvum* (see below). Moreover, 10 mM concentrations of specific inhibitors – including AEBSF [4-(2-Aminoethyl)benzenesulfonyl fluroride hydrochloride; Thermo Scientific, USA, 78431], TLCK (Nα-*p*-Tosyl-L-lysine methyl ester hydrochloride, Sigma-Aldrich, T5012), TPCK (N-tosyl-L-phenylalanine chloromethyl ketone, Sigma-Aldrich, T7254), E-64 E-(L-trans-epoxysuccinyl-leucylamido(4-guanidino)-butane; Sigma-Aldrich, E3132), phenanthroline (Sigma-Aldrich, 131377) and DTT (dithiothreitol; Sigma-Aldrich, D0632) – were incubated by specific substrates to have better perspectives.

SERINE PROTEINASES

Trypsin-, chymotrypsin- and elastase-like activities (as three subclasses of serine proteases) were assayed using a concentration 1 mM of Na-benzoyl-L-arginine-p-nitroanilide hydrochloride (Sigma-Aldrich, 19362), 1 mM of N-succinyl-alanine-alanine-proline-phenylalanine-p-nitroanilide (Sigma-Adrich, S7388) and 1 mM of N-succinyl-alanine-alanine-alanine-p-nitroanilide (Sigma-Aldrich, S4760) as substrates, respectively. The reaction mixture included 35 µl of Tris-HCl buffer (20 mM, pH 8, as literature recommended pH for serines), 5 µl of each mentioned substrate and 5 µl of enzyme solution. The reaction mixture was incubated at 30°C for 0–10 min before adding 30% TCA to terminate the reaction. The absorbance of the resulting mixture was then measured by spectrophotometry at 405 nm, evaluating the *p*-nitroaniline release. To prove the specific proteolytic

activity, a negative control was provided separately for each substrate containing all the above mentioned components, except for enzyme pre-boiled at 100°C for 30 min (8).

CYSTEINE PROTEINASES

Cathepsin B, L and D activities (as three subclasses of cysteine proteases) were assayed using a concentration of 1 mM Z-Ala-Arg-Arg 4-methoxy-β-naphthylamide acetate (Sigma-Aldrich, C8536), N-Benzoyl-Phe-Val-Arg-p-nitroanilide hydrochloride (Sigma-Aldrich, B2133) and cathepsin D (Sigma-Aldrich Co. Switzerland, SCP0109) as substrate, respectively. The reaction mixture consisted of 35 µl Tris-HCl buffer (pH 5, as literature recommended for cysteines), 5 µl of each mentioned substrate and 5 µl of enzyme solution. The reaction mixture was incubated at 30°C for 0–10 min before adding 30% TCA to terminate the reaction, and then absorbance was read at 405 nm. To prove the specific proteolytic activity, a negative control was provided separately for each substrate containing all the above mentioned components, except for enzyme pre-boiled at 100°C for 30 min (8).

EXO-PEPTIDASES

Activities of the two exo-peptidases in the midgut of *P. versicolora* were obtained by using Hippuryl-L-Arginine (Sigma-Aldrich, H2508) and Hippuryl-L-Phenilalanine (Sigma-Aldrich, H6875) for carboxy- and aminopeptidases, respectively. The reaction mixture consisted of 35 µl of Tris-HCl buffer (pH 7, as literature recommended for exo-peptidases), 5 µl of each mentioned substrate and 5 µl of enzyme solution. The reaction mixture was incubated at 30°C for 0–10 min before adding 30% TCA to terminate the reaction, and then absorbance was read at 340 nm. To prove the specific proteolytic activity, a negative control was provided separately for each substrate containing all the mentioned components, except for enzyme pre-boiled at 100°C for 30 min (8).

OPTIMAL pH DETERMINATION OF SPECIFIC PROTEASES

Tris-HCl buffer (20 mM, pH range 3–12) was used to obtain the optimal pH of each specific protease. The reaction mixtures were mentioned earlier, but the buffer pHs varied from 3 to 12. The blank mixture consisted of all the compounds, excepting the enzyme.

SPECIFIC INHIBITORS

Different concentrations (2, 4, 6, 8 and 10 mM) of specific inhibitors were used to find changes in the digestive protease (hemoglobin as substrate) of *S. fulvum*, including AEBSF TLCK, TPCK, E-64, DTT and phenanthroline.

PROTEIN DETERMINATION

Protein concentrations were assayed according to the method described by Lowry *et al*. (9).

STATISTICAL ANALYSIS

All data were obtained from a complete randomized design and those were further compared by one-way analysis of variance (ANOVA), followed by Tukey's test and t-test when significant differences were found at $P \leq 0.05$.

RESULTS AND DISCUSSION

OPTIMAL pH OF GENERAL AND SPECIFIC PROTEOLYTIC ACTIVITY

The optimal pH of general proteolytic activity was determined, as shown by the two peaks in both soluble and membrane-bound fractions (Fig. 1). The highest activity of soluble protease was found at pH 9, followed by a small peak at pH 5 (Fig. 1; F: 82.92, Pr>F:0.0001). Similar results were found in case of membrane-bound protease, where two peaks were observed (at pH 5 and 8-10), but in this case, the region of pH values from 8 to 10 was dominant and showed the highest activity in the alkali domain (Fig. 1; F: 108.31, Pr>F:0.0001). Results of the current study revealed a significant proteolytic activity in the midgut of *S. fulvum*. Almost all the identified proteases were detected in the midgut content by various activities. Many insects synthesize more than one proteinase or multiple molecular forms of the enzymes that may reflect various biochemical characteristics (10). Two peaks in both soluble and membrane-bound fractions could be attributed to the presence of various specific proteases, active at acid and alkaline pHs. The various pH ranges of proteolytic activity have been reported for other coleopteran insects. For example, Zibaee and Hajizadeh (11) observed two peaks in acid and alkali pH of digestive proteolytic activities in the midgut of *Plagiodera versicolora* Laicharting (Coleoptera: Chrysomelidae). Oppert *et al*. (12) reported optimal values of 8.5–9 for proteolytic activity in *Lasioderma serricorne* Fabricius (Coleoptera: Anobiidae), while Oppert *et al*. (13) reported an acidic pH for general proteolytic activity in *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae). According to Vinokurov *et al*. (3), there is a maximal proteolytic activity of the anterior midgut in the acidic region (with a peak at pH 5.2) and a flat shoulder in the neutral and alkaline regions, with a slight increase at pH 8.0 of *T. molitor* L. (Coleoptera: Tenebrionidae). In the posterior midgut, maximal activity was reported at pH values of 9.0 and 5.5–6.0 (3).

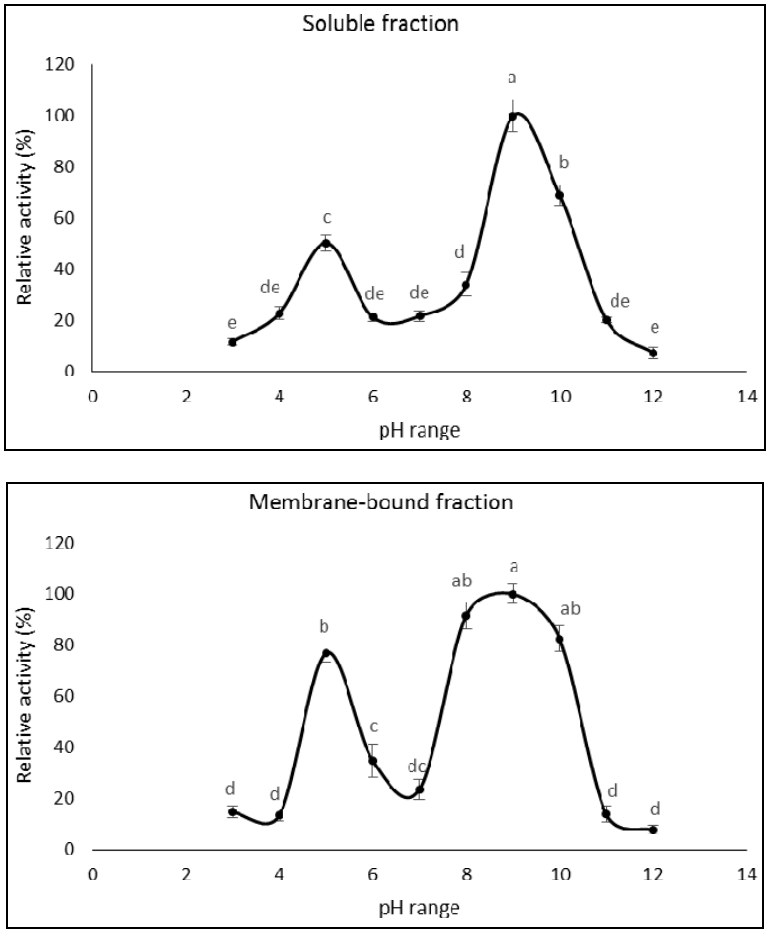


Fig. 1 – Determination of optimal pH for soluble and membrane-bound proteases in the midgut of *S. fulvum*. Statistically significant differences have been marked by different letters (Tukey test, $p \leq 0.05$).

Since specific substrates of proteases contain amino acids, they could react in various ways to the pH of different biochemical media. Activities of serine proteases sharply increased from pH of 5 to the optimal value. The highest activities of membrane-bound trypsin, chymotrypsin and elastase were observed at pH 8 (F: 113.29, Pr>F:0.0001) and 9 (F: 58.57, Pr>F:0.0001, F: 81.53, Pr>F:0.0001), respectively (Fig. 2). Optimal activity of soluble cathepsin B and membrane-bound cathepsin L was found at pH 9 (Fig. 4, F: 85.14, Pr>F:0.0001, F: 50.72, Pr>F:0.0001), while the two exopeptidases showed the highest activity at pH 10 (Fig.4; F: 126.1, Pr>F:0.0001, F: 63.24, Pr>F:0.0001). These results might re-confirm the presence of mentioned proteases in the midgut of *S. fulvum* based on the suggested pH.

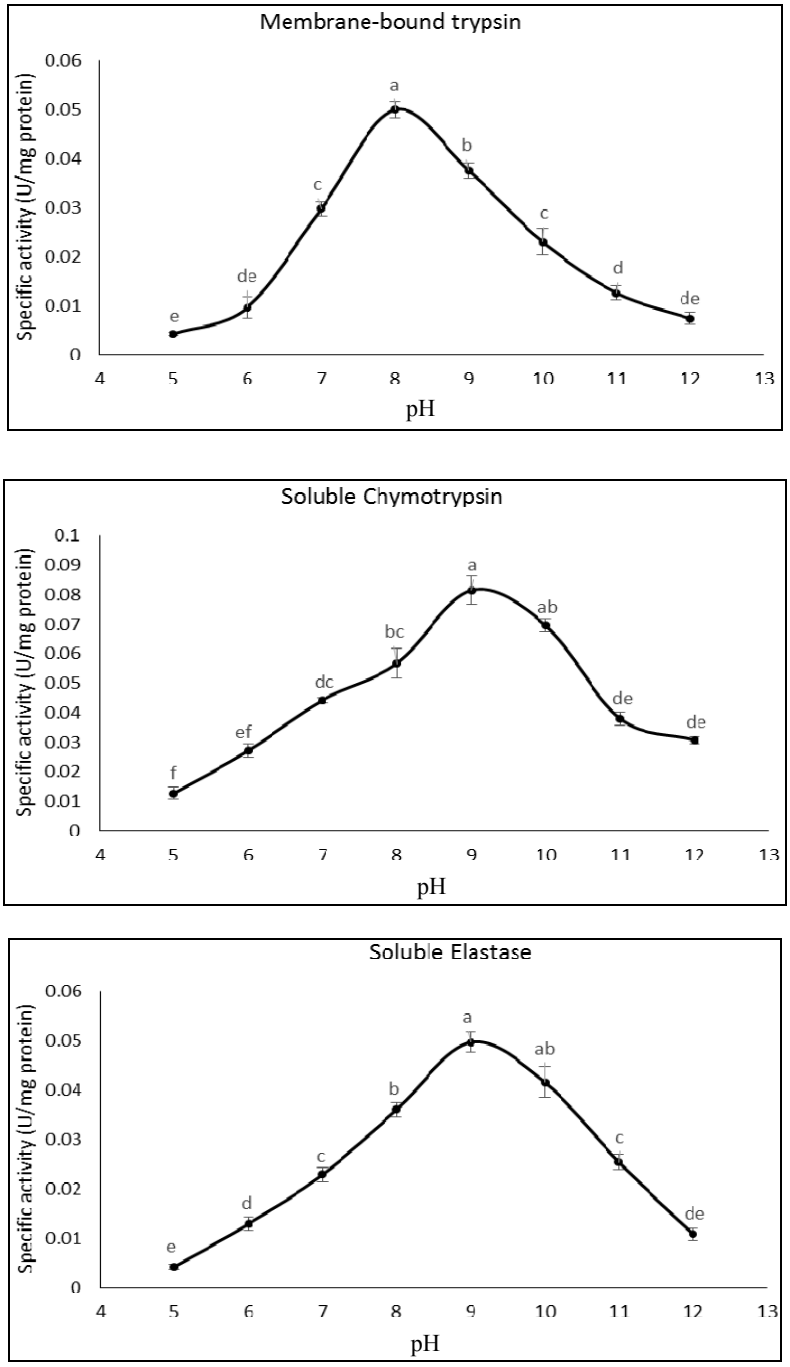


Fig. 2 – The pH dependency of serine proteinase activities in the midgut of *S. fulvum*. Statistically significant differences have been marked by various letters (Tukey test, $p \leq 0.05$).

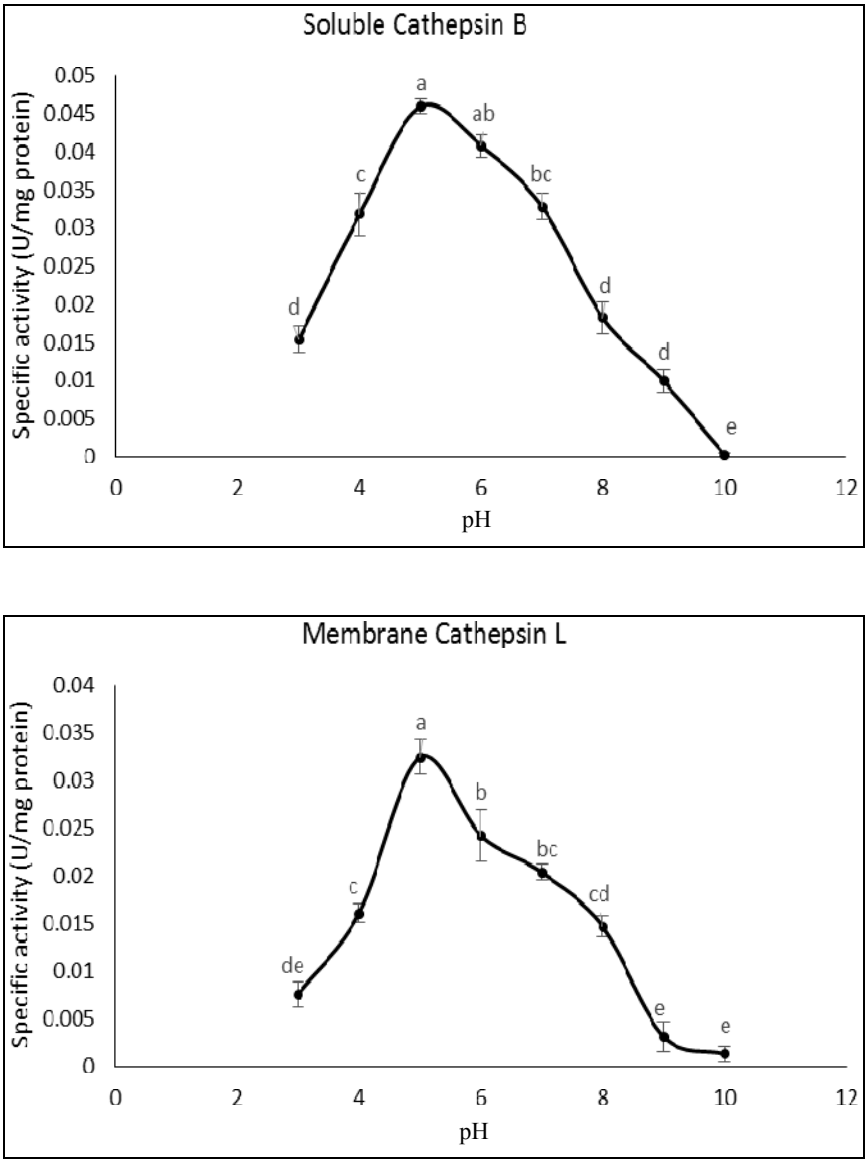


Fig. 3 – The pH dependency of cysteine proteinase activities in the midgut of *S. fulvum*. Statistically significant differences have been marked by various letters (Tukey test, $p \leq 0.05$).

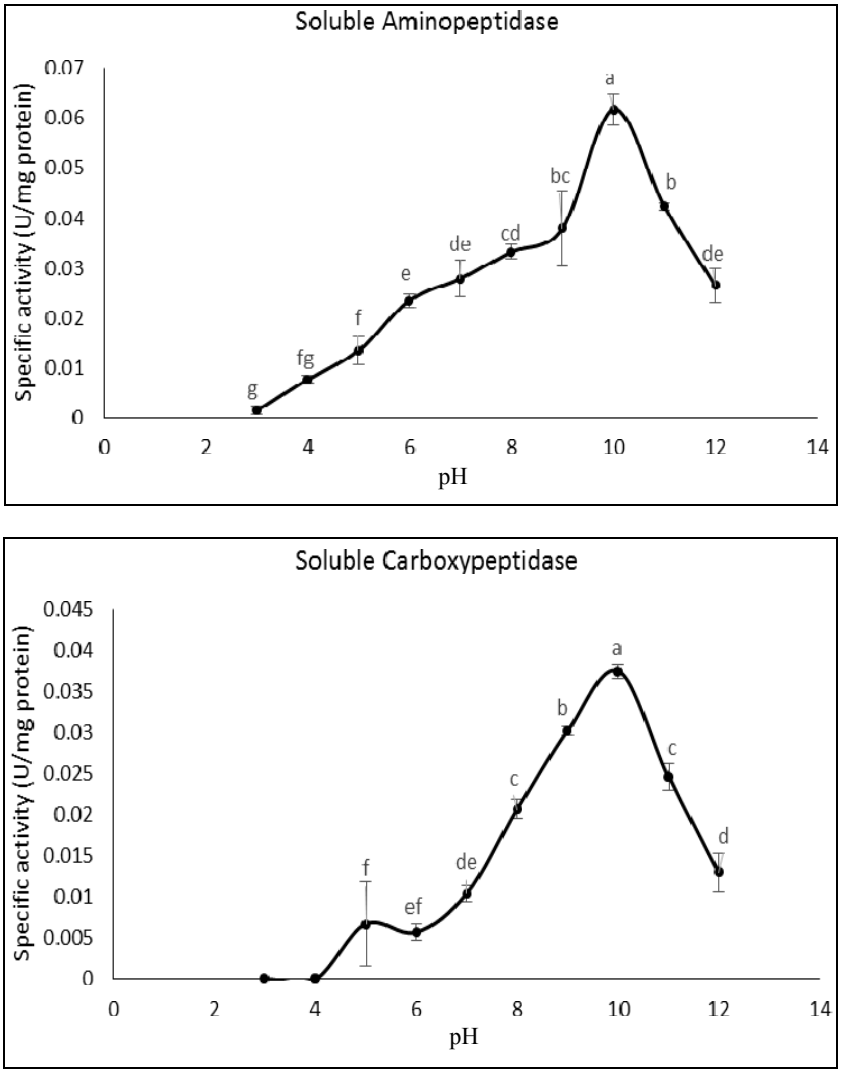
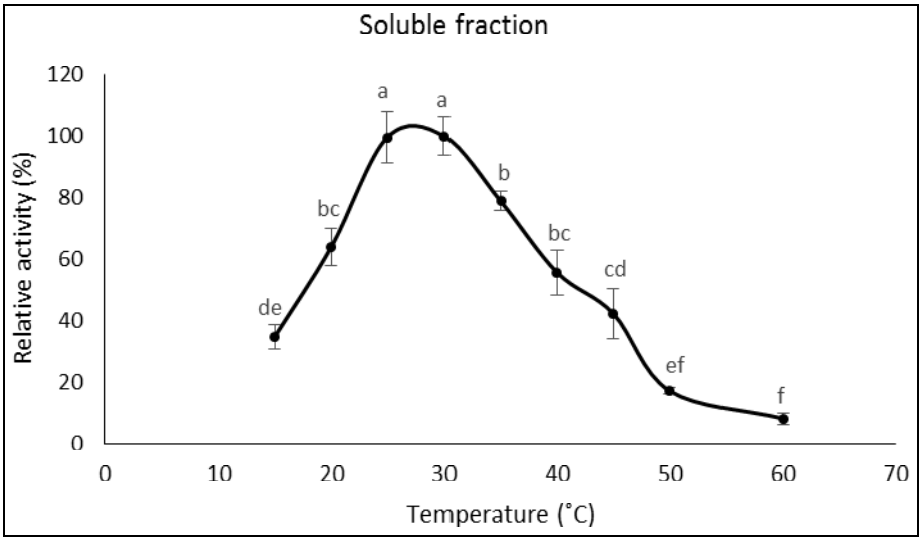


Fig. 4 – The pH dependency of amino- and carboxypeptidase activities in the midgut of *S. fulvum*. Statistically significant differences have been marked by various letters (Tukey test, $p \leq 0.05$).

OPTIMAL TEMPERATURE (˚C) OF GENERAL PROTEOLYTIC ACTIVITY

Activities of general protease in both soluble and membrane-bound fractions increased sharply from 15˚C to 30˚C, followed by a rapid decrease (Fig. 5). Differences were found in optimal temperature of 25–30˚C, for soluble protease (F: 51.48, Pr>F:0.0001), and 30˚C for membrane-bound protease (Fig. 5; F: 81.36, Pr>F:0.0001).



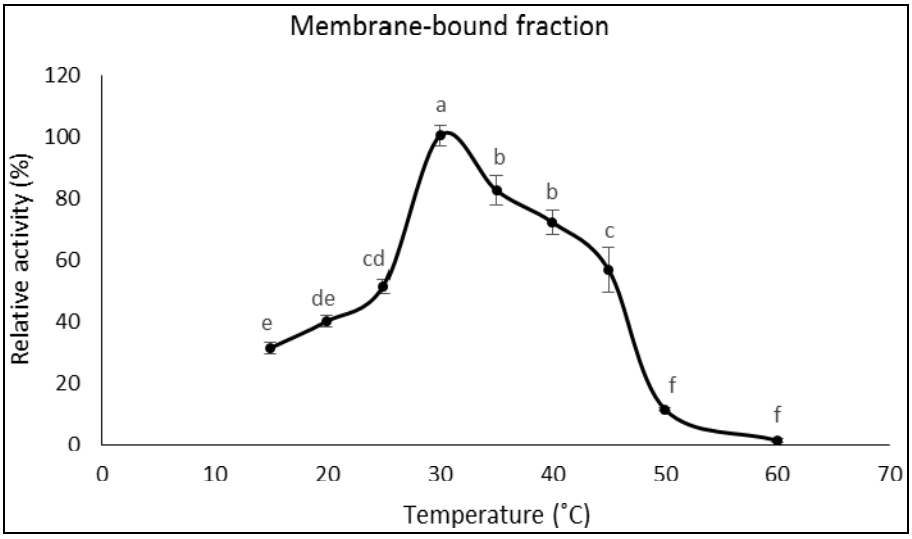


Fig. 5 – Determination of optimal temperature (˚C) for soluble and membrane-bound proteases in the midgut of *S. fulvum*. Statistically significant differences have been marked by various letters (Tukey test, $p \leq 0.05$).

DETERMINATION OF SPECIFIC PROTEASE PROFILE

Using negative control, specific substrates and inhibitors revealed a digestive proteolytic profile in the midgut of *S. fulvum* (Tables 1-3). Although no trypsin-

protease activity was detected in soluble fraction, in membrane-bound fraction the activity was noticed with significant inhibition using TLCK as its specific inhibitor (Table 1; $p \leq 0.0001$). In case of chymotrypsin and elastase, enzymatic activities and inhibitions were obtained in the soluble fraction rather than the membrane-bound one (Table 1, $p \leq 0.017$ for chymotrypsin and $p \leq 0.001$ for elastase). Different results were obtained in case of the assayed cysteine proteases. Cathepsin B was significantly inhibited (E-64) and activated (DTT) in the soluble fraction, while cathepsin L showed the same results in the membrane-bound fraction (Table 2; $p \leq 0.002$, $p \leq 0.011$). Amino- and carboxypeptidases showed the highest activity in the soluble fraction, so that statistical inhibition was also detected in the same fraction (Table 3, $p \leq 0.013$, $p \leq 0.047$). Determination of specific protease activities in soluble and membrane-bound fractions was carried out using specific substrates, inhibitors and negative control. Most of the specific proteases were more active in the soluble fraction, except for trypsin and cathepsin L.

Table 1

Determination of serine proteinase presence in the midgut of *S. fulvum*.
Trypsin activity was found in the membrane-bound fraction, but chymotrypsin and elastase activities were found in the soluble fraction

Serine proteinase	**Soluble-Negative control**	**Soluble+Inhibitor**	**Membrane-negative control**	**Membrane+Inhibitor**
Trypsin	0	0	0.168±0.003	0.102±0.002
Chymotrypsin	0.194±0.022*	0.162±0.009	0.002±0.0009	0
Elastase	0.146±0.007*	0.092±0.002	0.004±0.007	0.004±0.0003

[1-] TLCk, TPCK and AEBSF were used as inhibitors of trypsin, chymotrypsin and elastase, respectively.
[2-] Zero means no activity.
[3-] Samples were boiled and used as negative control.
[4-] Statistical differences have been shown by asterisks (T-test, $p \leq 0.05$).

Table 2

Determination of cysteine proteinase presence in the midgut of *S. fulvum*.
Cathepsin B was present in the soluble fraction, but cathepsin L was active in the membrane-bound fraction

Cysteine proteinase	**Soluble-Negative control**	**Soluble+ Inhibitor**	**Soluble+ Activator**	**Membrane-negative control**	**Membrane+Inhibitor**	**Membrane+ Activator**
Cathepsin B	0.136±0.023	0.123±0.002	0.175±0.008*	0.084±0.002	0.023±0.001	0.098±0.004
Cathepsin L	0.120±0.006	0.120±0.004	0.056±0.001	0.092±0.014	0.021±0.0009	0.093±0.004*

[1]. E-64 and DTT were used as inhibitor and activator, respectively.
[2]. Statistical differences have been shown by asterisks (T-test, $p \leq 0.05$).

Table 3

Determination of exopeptidase presence in the midgut of *S. fulvum.*
Aminopeptidase and carboxypeptidase were active in the soluble fraction

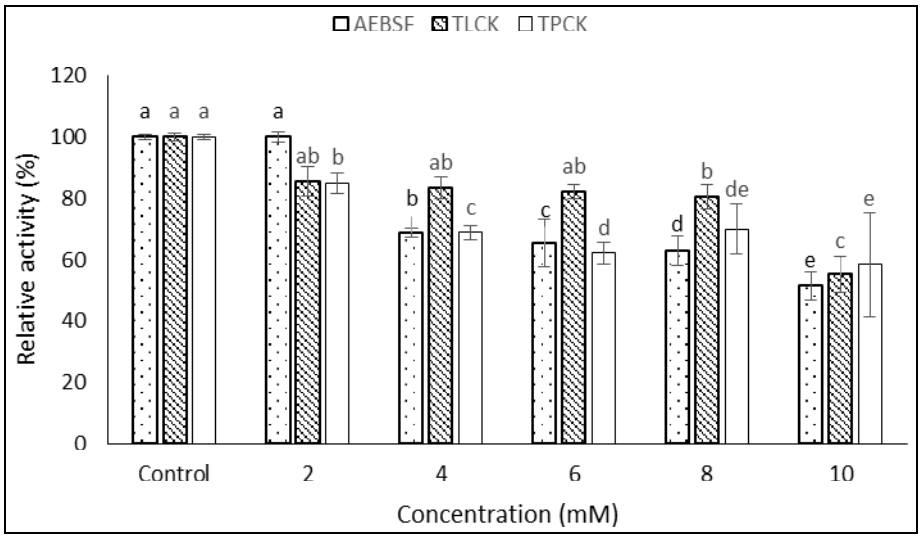
Exopeptidase	Soluble-Negative control	Soluble+Inhibitor	Membrane-negative control	Membrane+Inhibitor
Aminopeptidase	0.139±0.006*	0.064±0.008	0.054±0.004	0.108±0.011
Carboxypeptidase	0.041±0.003*	0.010±0.003	0.051±0.0007	0.0450±0.002

[1]. Phenanthroline was used as inhibitor.
[2]. Statistical differences have been shown by asterisks (T-test, p≤0.05).

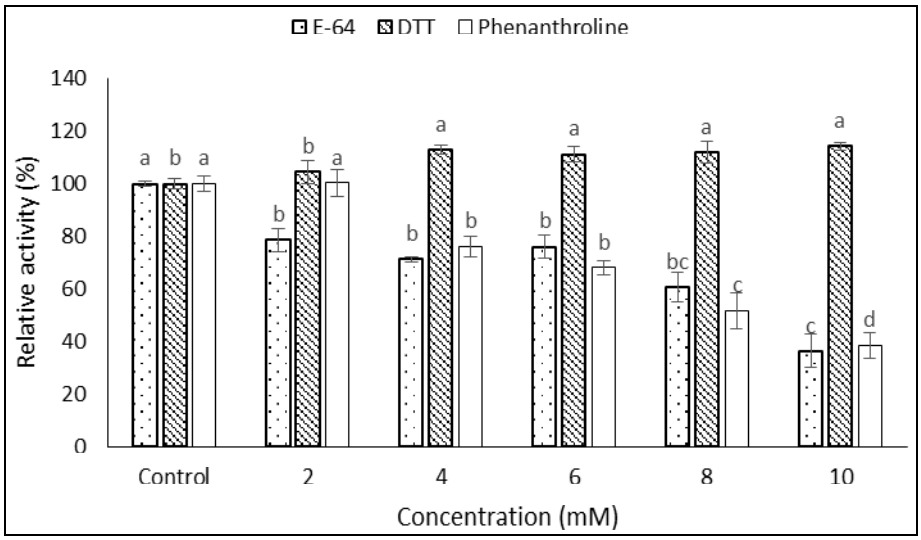
In a similar study, Zibaee and Hajizadeh (2013) reported membrane-bound chymotrypsin in *P. versicolora.* Other serine proteases showed equal activity in both fractions. Although cysteine proteases had the highest activities in the soluble fraction, amino- and carboxypeptidases were shown to be the most active in the membrane-bound fraction (Zibaee and Hajizadeh, 2013). The presence and activity of specific proteases could be explained by feeding of insects on different regimes and their interaction with possible symbiotic microorganisms. Oppert *et al*. (2002) reported eight major proteinase activities in the luminal extracts of *L. serricorne*, in which some might be attributed to trypsin and chymotrypsin. Oppert *et al*. (2003) described the inhibition of digestive protease in *T. castaneum* by E-64 and leupeptin, indicating the presence of cysteine and aminopeptidase activities. Crook *et al*. (2009) showed serine proteases as the major digestive proteases in the midgut of red oak borer *Enaphalodes rufulus* (Haldeman) (Coleoptera: Cerambycidae).

EFFECTS OF SPECIFIC INHIBITORS AND ACTIVATORS ON GENERAL PROTEOLYTIC ACTIVITY

Different concentrations of specific inhibitors significantly affected general proteolytic activity of *S. fulvum*. Concentrations of 4–10 AEBSF (F: 160.85, Pr>F:0.0001), TLCK (F: 6.15, Pr>F:0.0047) and TPCK (F: 155.44, Pr>F:0.0001) significantly decreased general proteolytic activity, so that the highest inhibition was observed in case of AEBSF (Fig. 6). E-64 (F: 26.33, Pr>F:0.0002) and phenanthroline (F: 44.75, Pr>F:0.0002) statistically decreased general proteolytic activity, while DTT (F: 10.38, Pr>F:0.003), as activator of cysteine proteases, significantly increased the enzymatic activity to 114% (Fig. 6). Specific inhibitors were used against general proteolytic activity to show their influence on the enzymatic activity in the midgut lumen. Serine inhibitors, including AEBSF, TLCK and TPCK, decreased proteolytic activities up to 55%. E-64 and phenanthroline decreased enzymatic activity by 36% and 38%, but DTT increased it up to 114%. These results have been indicated in studies of Zibaee and Hajizadeh (2013), and Oppert *et al*. (2002 and 2003) on *P. versicolora* and *T. molitor*.



a



b

Fig. 6 – Effects of specific inhibitors (E-64, DTT, Phenanthroline) at different concentrations (2, 4, 6, 8, and 10 mM) on relative proteolytic activity in the midgut of *S. fulvum*: a) Inhibitors' effects on serine proteinase; b) Inhibitors' effects on cysteine and metalloproteinase. Statistically significant differences have been marked by various letters (Tukey test, $p \leq 0.05$).

CONCLUSIONS

The majority of studies on insect digestive proteases have focused on lepidopteran and insects, which utilize plant foliar, but there is few information on wood-boring insects like *S. fulvum*. There are even earlier reports on digestive proteases of Scolytidae, Cerambycidae, Lyctidae, Bostrychidae and Anobiidae (Crook *et al.*, 2009). Results indicated the presence of both serine and cysteine proteinases, as well as aminopeptidases in the midgut contents. It has been suggested that severe heartwood degradation could theoretically be prevented by disrupting proteolytic activities (Crooks *et al*., 2009). In this case, the first steps should be the determination of proteolytic profiles in such insects. In the future, it is suggested to find *in vivo* effects of specific inhibitors on the metabolic activity and digestion of wood-boring beetles to achieve possible control tactics.

REFERENCES

1. Terra WR, Ferriera C, *Biochemistry of digestion*, in: **Insect Molecular Biology and Biochemistry**, Gilbert LI, ed, New York (NY), Elsevier, 2012, pp 365–418.
2. Kanost MR, Clem RJ, *Insect Proteases*, in: **Insect Molecular Biology and Biochemistry**, Gilbert L I, ed, Elsevier, San Diego, California, USA, 2012, pp 346–364.
3. Vinokurov KS, Elpidina EN, Oppert B, Prabhakar S, Zhuzhikov DP, Dunaevsky YE, Belozersky MA, *Diversity of digestive proteinases in Tenebrio molitor (Coleoptera: Tenebrionidae) larvae*, ***Comp. Biochem. Physiol***., ***Part B.***, **145**, 126–137 (2006).
4. Chapman RF, *The insects: structure and function*, in: **Alimentary canal, digestion and absorption**, 5th ed, Simpson SJ, Douglas AE, eds, New York (NY), Cambridge University Press, 2012, p. 929.
5. Soleimani P, Knopf HE, *Stromatium fulvum Vill. (=unicolor Ol.) (Col., Cerambycidae) in Iran*, ***Zeitschrift Angew Entomol.***, **82**, 372–376 (1976).
6. Ferreira C, Terra WR, *Physical and kinetic properties of a plasma-membrane-bound P-Dglucosidase (cellobiase) from midgut cells of an insect (Rhynchosciara americana larva)*, ***Biochem. J.***, **213**, 43–51 (1983).
7. Cohen AC, *Organization of digestion and preliminary characterization of salivary trypsin-like enzymes in a predaceous heteropteran*, *Zalus renardi*, ***Journal of Insect Physiology***, **39**, 823–829 (1993).
8. Oppert B, Kramer KJ, Mc Gaughey WH, *Rapid microplate assay of proteinase mixtures*, ***Biotechnol.***, **23**, 70–72 (1997).
9. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ, *Protein measurement with the Folin phenol reagent*, ***J. Biol. Chem.***, **193**, 265–275 (1951).
10. Nation JL, *Insect physiology and biochemistry*, 2nd ed, CRC press, New York, 2008, pp. 29–69.
11. Zibaee A, Hajizadeh J, *Proteolytic activity in Plagiodera versicolora Laicharting (Coleoptera: Chrysomelidae): Characterization of digestive proteases and effect of host plants*, ***J. Asia-Pacific. Entomol.***, **16**, 329–334 (2013).
12. Oppert B, Hartzer K, Zuercher M, *Digestive proteinases in Lasioderma serricorne (Coleoptera: Anobiidae)*, ***Bull. Entomol. Res.***, **92**, 331–336 (2002).
13. Oppert B, Morgan TD, Hartzer K, Lenarcic B, Galesa K, Brzin J, *et al*, *Effects of proteinase inhibitors on digestive proteinases and growth of the red flour beetle, Tribolium castaneum (Herbst) (Coleoptera: Tenebrionidae)*, ***Com. Biochem. Physiol Part C.***, **134**, 481–490 (2003).
14. Crook DJ, Prabhakar S, Oppert B, *Protein digestion in larvae of the red oak borer Enaphalodes rufulus*, ***Physiol. Entomol.***, **34**, 152–157 (2009).