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Partial characterization of digestive enzymes in the larvae of the fall armyworm, *Spodoptera frugiperda*

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Abstract

The fall armyworm (FAW), *Spodoptera frugiperda* is one of the most destructive pests of corn in Egypt and over all the world. FAW has a wide range of host due to the variation in its digestive enzymes. In the current study, the digestive enzymes including trypsin, chymotrypsin, papain and alpha-amylase were partially characterized in the different larval stages of the fall armyworm (FAW). Serine proteases were found to have higher activity compared to cysteine and alpha-amylase enzymes. The first and fourth instar larvae found have elevated enzymes activity compared to other larval stages. FAW digestive enzymes show stability at wide range of temperatures and pHs. Kinetic studies revealed that chymotrypsin enzyme exhibited highest *Vmax* value followed by trypsin, alpha-amylase and papain enzymes. On the other hand, papain enzyme showed higher affinity with its substrate followed by chymotrypsin, trypsin and alpha-amylase showed low substrate affinity. The results of the current study provide essential knowledge about the composition, feature and activity of digestive enzymes of FAW that can be used to design effective management program based on distribution of digestive enzymes.

Keywords: *Spodoptera frugiperda*, trypsin, chymotrypsin, papain, α-amylase, Kinetic studies.

INTRODUCTION

Fall armyworm (FAW), *Spodoptera frugiperda* (J.E. Smith, 1797) (Lepidoptera: Noctuidae), often known as "Quarantine Pest" is a new invasive destructive insect pest; causing significant economic loss in several crops by feeding on stems and leaves, particularly those of maize. FAW first appeared in Africa exactly on Nigeria in 2016, and according to the Food and Agriculture Organization of the United Nations (FAO), it invaded the African continent via a ship or a plane. It then quickly spread to more than 28 countries in southern and eastern Africa (Goergen *et al*., 2016; Day *et al*., 2017; Cock *et al*., 2017; FAO, 2018; Dahi *et al*., 2020 and Gamil, 2020), damaging more than 70% of maize production and to a less extent to sorghum and other crops (Baudron *et al*., 2019 and FAO, 2019). In May 2019, FAW was recorded for the first time on maize fields in a village in Kom-Ombo city, Aswan, Upper Egypt (Dahi *et al*., 2020). From there, it moved to Luxor, Qena, Sohag Governorates and later to Assiut Governorate in 2021 (Mohamed *et al*., 2022). By the end of 2021 based on its current invasion rate, the pest covered all governorates in Egypt (Mohamed *et al*., 2023). The high reproduction rate, powerful migration, great dispersal ability, and vigorous flight were associated to the economic value of FAW where the adult can fly up to 100 kilometers in a single night (Prasanna *et al*., 2018). More than 350 economically essential crops, which includes maize, rice, sorghum, sugarcane, wheat, millet, tomato, cotton, peanut, soybean, cabbage, beet, alfalfa, onion, pasture grasses and potato, are all damaged by caterpillars of the FAW, which also have a wide range of hosts (Montezano *et al*., 2018 and Chormule *et al*., 2019). The physiology of insect digestion has been investigated intensively over the last decades and a lot of different strategies have been developed to deal with different kinds of pests creating a new area of research. Despite the intensive research there are still a lot of questions about the fast adaptation of insects to environmental changes, and the physiology and evolution of the mechanisms of adaptation. To develop new methods of pest control the investigation of these mechanisms as well as our knowledge on the physiology of insect digestion including characterization of digestive enzymes has to be expanded.

MATERIALS AND METHODS

1. Materials

Bovine serum albumin fraction v (sigma), sodium phosphate monobasic (NaH_2PO_4), sodium phosphate dibasic (Na_2HPO_4), maltose, α-amylase from porcrine pancreas, starch, dinitro salicylic acid (DNS), BA*p*NA (Nα-Benzoyl-DL arginine 4-nitroanilide hydrochloride 98%), BT*p*NA (N-benzoyl-l-tyrosine-p-nitroanilide), BANA (Nα-Benzoyl-DL-arginine β-naphthylamide hydrochloride), PMSF (phenylmethylsulfonyl fluoride, a serine protease inhibitor), EDTA (a metaloprotease inactivator), trypsin inhibitor (ex. Soyabean extra pure for biochemistry), glycine, sodium chloride (NaCl), calcium chloride ($CaCl_2$), sodium carbonate (Na_2CO_3), sodium hydroxide (NaOH), copper sulphate ($CuSO_4$), folins phenol reagent, sodium acetate ($C_2H_3NaO_2$), glacial acetic acid (GAA), sodium potassium taratrate and *p* (Dimethylamino) cinnamaldehyde (DMAC, DMACA) were purchased from Sigma Chemicals Co.(ST. Louis, MO.USA).

2. Insect culture and rearing

FAW rearing technique was conducted according to the methodology described by Kruger *et al*. (2012) and Mohamed (2022) with minor modification. The egg masses and larvae of the fall armyworm, *S. frugiperda* were collected from maize fields at Sohag Governorate, Upper Egypt. Field collected egg masses were kept in plastic container until hatching. The newly hatched larvae were reared in plastic containers (40 × 20 × 15 cm) with aerated cover (muslin) and provided with fresh maize (shoot system) leaves and kept in BOD incubator at 27° C ± 1° C, 65 ± 5% RH, and 12L: 12D hours (Light: Dark) photoperiod. Maize (shoot system) leaves were replaced at two-day intervals. When the Larvae reached the 3rd instar, they were reared individually in

small plastic cups (7 cm in height × 2 cm in diameter) covered with muslin until pupation. Pupae were collected and placed on wooden cages (35 × 35 × 35 cm^3) until adult moth emergence. Moths were fed by 10% honey solution soaked on cotton pads, hanged inside the cage and renewed daily till. For egg laying, the inner walls of the cage were covered with zigzag-sheets of A4 white paper and also branches of oleander (*Nerium oleander L.*) or maize stems placed in a plastic bottle filled with water and provided with small stones were used as oviposition site and inspected daily for egg batches. The egg batches were transferred to a plastic container until hatching, the newly hatched larvae were reared as described above using maize leaves.

3. Enzyme extraction

The whole larvae for the first, second and 3rd instar and the gut of the 4th, 5th, and 6th instar (free of food) were homogenized in 500 µl of cold fresh prepared sodium phosphate buffer (0.1 M, pH 7). The homogenate was centrifuged for 20 minutes at 10,000 rpm and 4° C. The supernatant was transferred to a new Eppendorf tube and stored at -20° C for further use and considered as the enzyme stock. All handling of dissecting was done on ice.

4. Enzyme assay

4.1. Determination of serine proteases activity

Trypsin and chymotrypsin activities were determined according to Erlanger *et al.* (1961) with minor modifications using synthetic substrates BA*p*NA (N-benzoyl-dl-arginine-p-nitroanilide) and BT*p*NA (N-benzoyl-l-tyrosine-p-nitroanilide), respectively. Trypsin activity in the larval crude extract was measured by adding 1 ml of 1 mM BA*p*NA in pre-warmed (37° C) 0.01 M Tris-HCl buffer (pH 8.0) containing 0.02 M $CaCl_2$ and incubated for 15 min at 37° C. The reaction was stopped by adding 200 µl of 30% glacial acetic acid and absorbance was read by using UV/VIS single beam spectrophotometer at 410 nm. The chymotrypsin activity was also measured in a similar way except that the substrate used was BT*p*NA. The standard curve of trypsin and chymotrypsin was done using pure trypsin and chymotrypsin enzymes to calculate trypsin and chymotrypsin activity.

4.2. Determination of papain activity

The papain enzyme activity was determined according to Abd El-Latif (2015) with minor modification. Papain activity in the larval crude extract was measured by adding of 500 µl of 1 mM BANA and the volume was made to 700 µl by adding 0.1 M phosphate buffer pH 6.0 containing 2.5 mM EDTA and 3 mM DTT. After incubation at 37 °C for 15 minutes, the reaction was started by the addition of 1ml of 2% HCl/ethanol then 1ml of 0.06% (w/v) *p*-dimethylamino cinnamaldehyde prepared in ethanol was added. The mixture was left for 30 minutes in water bath at 30 °C to enable the color to develop. After that, a UV/VIS single beam spectrophotometer was used to measure the products absorbance at 540 nm. To calculate papain activity, a papain standard curve was prepared using papain enzyme.

4.3. Determination of alpha amylase activity

The α-amylase activity of was measured using the method of Bernfeld (1955) with slight modifications. A mixture of larval crude extract and 400 µl of 1 % starch solution, in 0.1 M sodium phosphate buffer, PH 6.9, containing 20 Mm NaCl and 0.1Mm $CaCl_2$ were incubated in water bath at 30° C for 20 minutes. The color was developed by adding 250 µl of 3,5- DNSA reagent (1 % 3, 5-dinitrosalicylic acid, 0.4 M sodium hydroxide and 1.06 M potassium sodium tartrate), followed by boiling for 10 minutes in the water bath. The reaction mixture was diluted with 3 ml of distilled water and the absorbance was measured at 540 nm and α-amylase activity was determined using maltose standard curve.

5. Protein determination

Protein content of the sample was determined by following the method of Lowry *et al.* (1951) and using bovine serum albumin fraction V (Sigma Aldrich) as a standard.

6. Stability of digestive enzymes at different temperatures and pHs

The effect of temperatures and pHs on enzymes activity was determined using the crude extract of the sixth instar larvae reared on maize. The effect of temperature on enzymes activity was determined by pre-incubating of the sample at 20, 40, 60, 80 and 100° C for 1 hour followed by centrifugation then measurement of activity as described before. The effect of pH using the following buffers at final concentrations of 0.1 M, was measured at various pHs ranging from 2 to 12: glycine-HCl for pH 2, Na-acetate-acetic acid for pH 4, phosphate buffer for pH 6, Tris-HCl for pH 8, glycine-NaOH for pH 10, and glycine-NaOH for pH 12.

7. Kinetic studies

Kinetics enzymes activity of the extra pure sixth instar larvae reared on maize was determined at different substrate concentrations using Lineweaver-Burk plots, in which the inverse of the initial velocity was plotted against the inverse of the substrate concentration, *Km* and *Vmax* were calculated.

RESULTS AND DISCUSSION

Age -related enzyme activity

The digestive enzymes vary in its activity in different ages. Ademolu and Idowu (2011) reported that enzyme activity increase with insect age of the *grasshopper Z. variegatus*. The authors explain that this increase is due to the increase in the gut size which probably stimulate enzymes. However different trends were noticed in activity of digestive enzymes in different insect species (Kotkar *et al*., 2009; Baghery *et al*., 2014; Abd El-latif, 2020; Intayung *et al*., 2021 and Lv *et al*., 2022). In the current study, the specific activity of digestive enzymes was compared in different larval instars of FAW reared on maize (Table 1). The first instar larvae of FAW found to have the highest enzyme specific activity of trypsin (99.60 unit/mg protein), chymotrypsin (520.70 µmole/mg protein) and papain (0.32 unit/mg protein) compared to other larval instars. The enzymes activity was dramatically decreased in the second and third larval instars. The activity of trypsin, chymotrypsin and papain enzymes was highly increased in the fourth larval instar. The highest alpha-amylase activity was recorded in the fourth instar larvae with specific activity of 50.49 unit/mg protein. Moderate alpha-amylase activity was noticed in the first and sixth larval instars with specific activity of 39.39 and 40.44 unit/mg protein, respectively. Serine protease enzymes (trypsin and chymotrypsin) seem to be dominant in all larval instars followed by alpha-amylase while papain enzyme had the lowest activity in all larval instars.

Table 1: Specific activity of digestive enzymes in different larval instars of *S. frugiperda* reared on maize.

Larval instar	Digestive enzymes			
	Trypsin specific activity (unit/mg protein)	Chymotrypsin specific activity (µmole/mg protein)	Papain specific activity (unit/mg protein)	α-amylase specific activity (unit/mg protein)
L1	99.60	520.70	0.32	39.39
L2	29.03	249.21	0.24	10.83
L3	24.15	67.98	0.05	27.51
L4	88.85	198.01	0.29	50.49
L5	79.55	28.35	0.23	13.71
L6	81.45	94.64	0.15	40.44

Stability of digestive enzymes at different temperatures and pHs

The influence of temperature and pH on the activity of digestive enzymes was studied in the 6th instar larvae of FAW. All the digestive enzymes showed stability at different temperatures (table 2 and figure 1). Trypsin and chymotrypsin enzyme exhibited highest activity at 40° C degree while the activity seriously decreased at 80° C and above Papain enzyme showed high sensitivity against temperature as it lost 50% of its activity when temperature increased from 20° C to 40° C.

Table 2: Stability of digestive enzymes of the 6th instar larvae of *S. frugiperda* at different temperatures.

Temperatures	Trypsin activity (unit/ml)	Chymotrypsin activity (µmole/ml)	Papain activity (unit/ml)	α-amylase activity (unit/ml)
20° C	583.5	2378.5	0.042	111.5
40° C	602	2448.5	0.020	71.5
60° C	468	1942.5	0.019	66.5
80° C	169.5	1331	0.013	46
100° C	131	1026.5	0.004	28.5

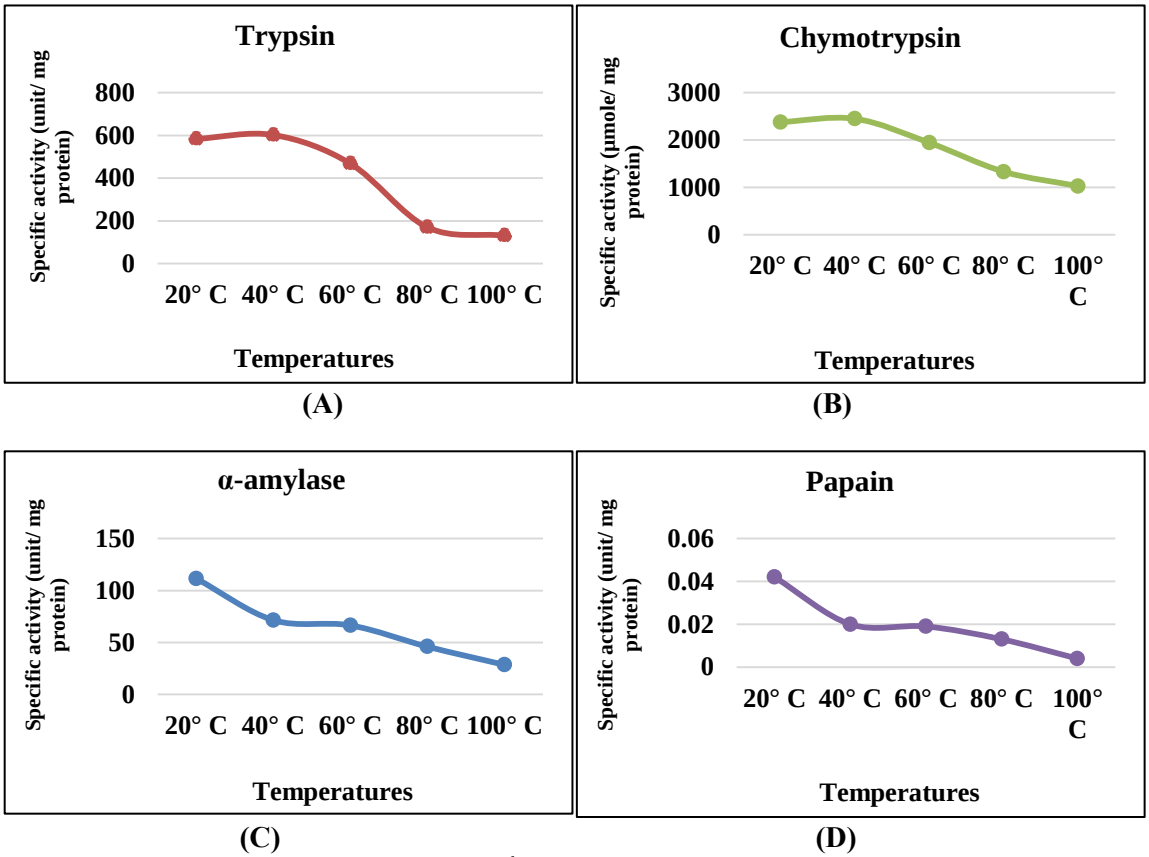


Figure 1: Stability of digestive enzymes of 6th instar larvae of *S. frugiperda* at different temperatures, (A)Trypsin, (B) Chymotrypsin, (C) Papain, and (D) α-amylase.

The highest α-amylase activity (111.5 unit/ ml) was achieved at 20° C while the activity gradually decreased at the higher temperature. At 100° C, α-amylase enzyme lost about 74.80% of its activity. Most of insect have a midgut pH in the range of (6-10). Gut pH is likely to have a major influence on the efficiency of nutrient extraction in insect (Sanatan *et al*., 2013). Digestive enzymes of FAW seems to work at a wide pH range (table 3 and figure 2). pH 8 was found to be optimum for the activity of chymotrypsin and α-amylase enzymes while pH12 was found to be optimum for the activity of trypsin and papain enzymes

which indicate that the digestive enzyme of FAW gut to work efficiently in the alkaline condition.

Table 3: Stability of digestive enzymes of the 6th instar larvae of *S. frugiperda* at different pHs.

pH	Enzyme activity			
	Trypsin activity (unit/ml)	Chymotrypsin activity (µmole/ml)	Papain activity (unit/ml)	α-amylase activity (unit/ml)
2	561	523.33	0.061	370.33
4	868	569	0.125	342.67
6	787.67	1192.67	0.091	493.33
8	807.33	1211.33	0.10	509.67
10	1080	1071.67	0.222	430.67
12	1472.67	1087	0.226	426.33

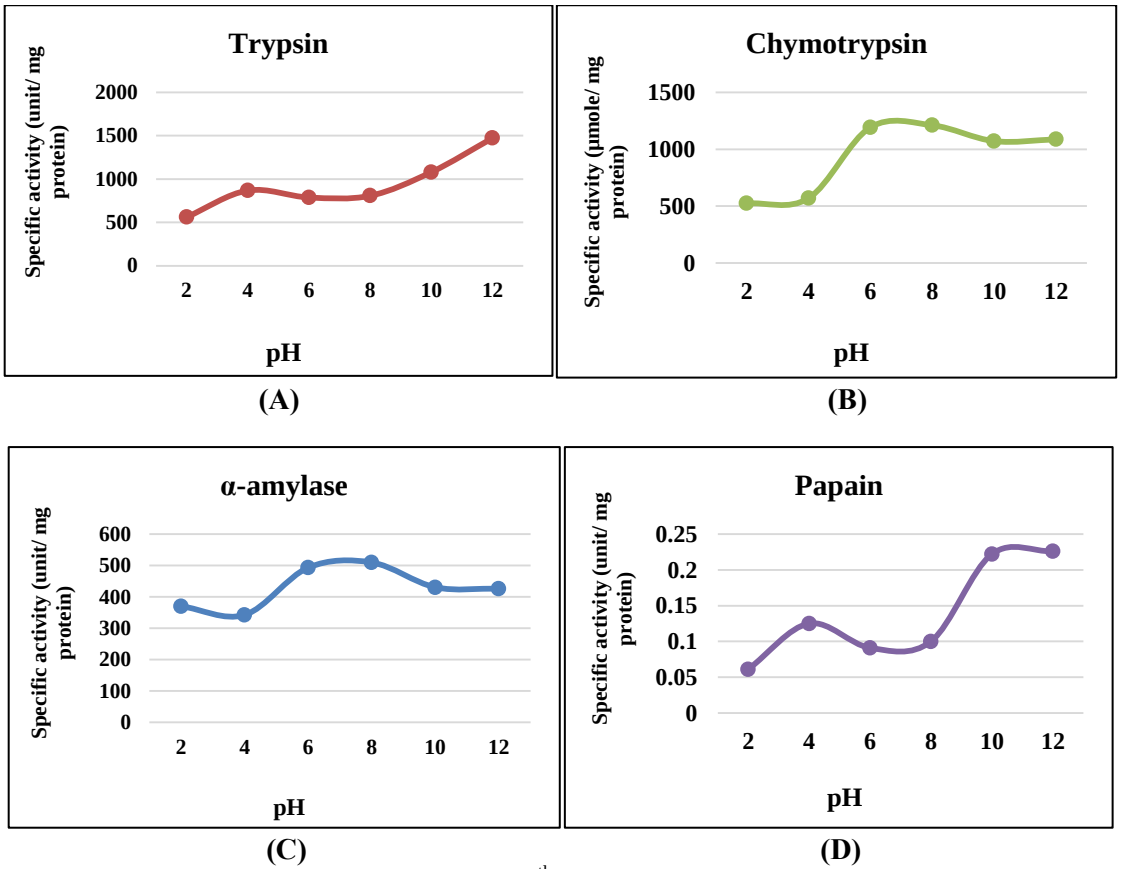


Figure 2: Stability of digestive enzymes of 6th instar larvae of *S. frugiperda* at different pHs, (A)Trypsin, (B) Chymotrypsin, (C) Papain, and (D) α-amylase.

Ferriera *et al*. (1994 b) reported that the optimum pH for trypsin activity is 7.9 while pH 9.6 is optimum for α-amylase of FAW. While pH 8.5-10.5 was reported as the optimum pH for trypsin activity in FAW (Alfonso *et al*., 1997).

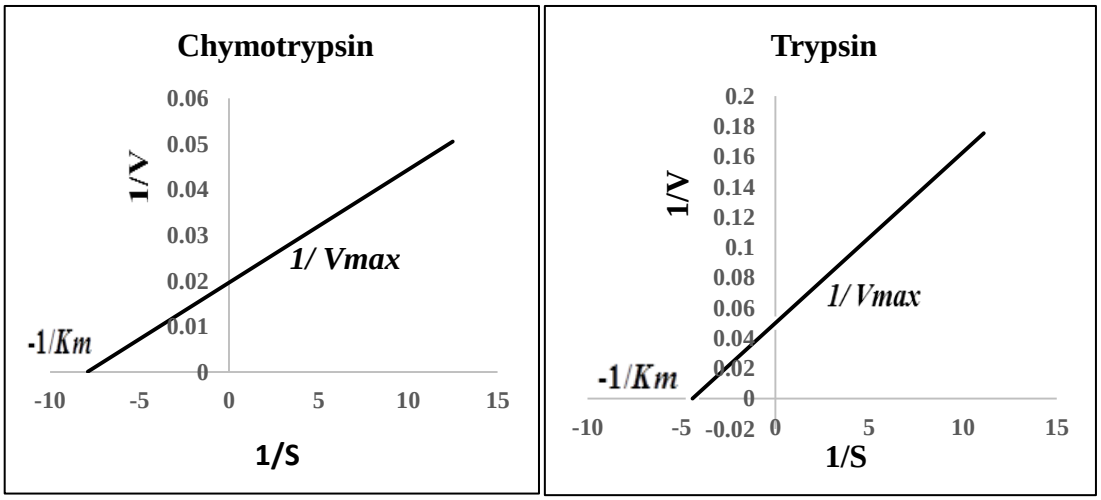
Kinetic analysis

Kinetic analysis of digestive enzymes in the 6th instar larvae of FAW gave line reciprocal Michaelis-Menton (Lineweaver-Burk) plots, enable estimation of *Km* and *Vmax* values, (table 4 and figure 3, 4). Chymotrypsin enzyme exhibited the *Vmax* Value (50.76 µmole/ mg protein) followed by trypsin and α-amylase (20.08 unit/ mg protein) (14.25 unit/ mg protein) while papain exhibited the lowest velocity of (0.13unit/ mg protein). On the other hand, papain enzyme showed higher affinity with its substrate with lowest *km* value (0.01)

followed by chymotrypsin (0.13), trypsin (0.23) while α-amylase showed low substrate affinity (*km* value 0.39). 0.38% was identified as the *km* value for α-amylase in *S. frugiperda* (Ferreira *et al*., 1994 b). Lwalaba *et al*. (2010) reported that trypsin enzyme had *Vmax* value of 3.0 µmole/ min and *km* value of 0.93 Mm while α-amylase has *Vmax* value of 59 µg maltose/ min and *km* value of 0.29% on *S. frugiperda*.

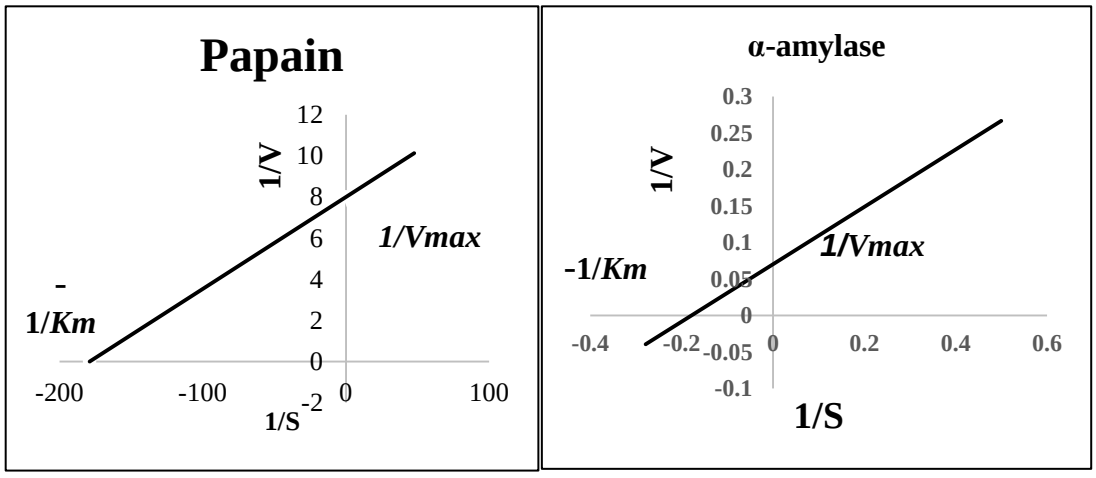
Table 4: Kinetic analysis of the digestive enzymes of the 6th instar larvae of *S. frugiperda*.

Enzyme	*Vmax*	*Km*
Trypsin	20.08	0.23
Chymotrypsin	50.76	0.13
Papain	0.13	0.01
α-amylase	14.25	0.39



(A) **(B)**

Figure 3: Kinetic analysis of the digestive enzymes in the 6th instar larvae of S. frugiperda, (A) Trypsin, and (B) Chymotrypsin.



(A) **(B)**

Figure 4: Kinetic analysis of the digestive enzymes in the 6th instar larvae of S. frugiperda, (A) Papain, and (B) α-amylase.

CONCLUSION

The results of the current study provide essential knowledge about the composition, feature and activity of digestive enzymes of FAW. such results can reflect the consummation ability of FAW to use plant sources and also can be used to design effective management program based on distribution of digestive enzymes.

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