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Salivary amylase from *Chilo partellus* (Swinhoe) — Characterization and mode of inhibition through analysis of double reciprocal, fractional velocity and combination plots

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The stem borer *Chilo partellus* (Swinhoe) is the most devastating pest that causes huge losses to agricultural productivity. Being a herbivorous insect, the starch degrading enzyme, salivary amylase, plays a crucial role in its digestive system. The characterization of salivary amylase and targeting it with potent inhibitors could help in managing the pest by hindering its normal digestive process. Therefore, we have made an attempt to characterize the enzyme and analyze its nature of interaction with organic acids and inorganic salts. The salivary amylase was purified by G-100 column chromatography to 16.02 folds and biochemically characterized. The purified fraction consisted of α-amylase activity with a single isoform of 59.26 kDa. It showed an optimum pH between 6.0 and 7.0. Its optimum temperature was 40 °C and was thermally stable up to 70 °C. Starch was the preferred substrate of salivary amylase. By critical analysis of Lineweaver-Burk, Eadie- Hofstee and Hanes plots, the Km and Vmax values of salivary amylase for starch were confirmed to be 0.49 mg/mL and 1.67 nmoles of reducing sugars formed/min/mL, respectively. Zinc chloride, calcium nitrate, salicylic acid, citric acid and oxalic acid were found to be potent inhibitors of salivary α-amylase activity. From Lineweaver-Burk, fractional velocity and combination plots, it was inferred that citric acid was a pure non-competitive inhibitor of salivary α-amylase while salicylic acid, oxalic acid and zinc chloride caused complete mixed non-competitive- competitive inhibitions. The inhibition of salivary α-amylase with calcium nitrate was of complete mixed non-competitive- uncompetitive type.

Keywords: Crop pests, Deadheart, Inhibition kinetics, Pest management, Salivary α-amylase, Stem borer

Biotic stresses pose a major threat to crop productivity worldwide. They include the constraints caused by pests and pathogens and have been reported to cause heavy yield losses[1]. Among these stresses, crop pests are of great significance. They damage the plant through feeding or egg laying and also act as vectors that transmit viruses to the plants through their stylets[2]. *Chilo partellus* (stem borer) is the most devastating pest in Asia and Africa with a broad range of host plants, such as maize, sorghum, rice, sugarcane, pearl millet and several grasses including sudan grass and napier grass[3,4]. It damages the crop from seedling to the harvest stage and causes 13-25% yield losses[5,6]. The first instars of *C. partellus* move into the leaf whorls where they feed on the leaf tissues by scrapping off chlorophyll and develop on the bases of the leaves, causing lesions[7]. The late third and early fourth instars make bores into the stem, followed by tunneling and feeding within the stem. Finally, the larvae in the leaf whorl or stem, cut through the central leaves which dry up to produce the deadheart symptom, resulting in death of the plant, and thus significant yield loss[7].

As it causes heavy damage to the crops, its management is highly crucial. One of the strategies for its management could involve targeting the enzymes of the digestive tract with potent enzyme inhibitors that could hinder the normal digestive process of the insect[8]. Since, *C. partellus* is a herbivorous insect, the starch degrading enzyme, amylase, plays a crucial role in its digestive system. Though, major part of the food digestion occurs in the gut, it initiates in the mouth with the aid of salivary amylase that hydrolyzes starch to produce dextrin, maltose, maltotriose and glucose as the main products. Salivary amylase is synthesized and secreted by the salivary glands into the saliva of the insect[9]. The characterization of saliva from different lepidopteran insects revealed the presence of amylases in them[10]. This shows that larval saliva plays a major role in the successful feeding on the host plant.

Insect amylases (E.C.3.2.1.0) constitute a multigene family that participates in digestion of

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broad range of substrates in wide range of environments[11]. Among these amylases, α-amylase (E.C. 3.2.1.1) is one of the starch/polysaccharide degrading enzymes that catalyzes the cleavage of random glycosidic bonds and give maltose, maltotriose, glucose as the main products along with dextrin. Therefore, to control the pest, there is a crucial need to study the properties of the enzyme along with its interaction with a wide range of amylase inhibitors. In this context, we have made an attempt to characterize salivary amylase and study its inhibition kinetics against a range of inhibitors that could help in pest management studies.

Materials and Methods

Insect rearing and isolation of salivary amylase

C. partellus larvae and pupae were collected from the agricultural fields and used for mass rearing on the green gram (*Vigna radiata* L.) based artificial diet[12]. Third instar larvae were selected randomly and their salivary glands were isolated[13]. For extraction of salivary α-amylase, the tissue was homogenized in 0.02 M sodium phosphate buffer (pH 7.0) containing 10 mM NaCl followed by centrifugation at 10,000 ×*g* for 30 min at 4°C. The supernatant was collected for determination of α-amylase activity. About 1.0 mL of the reaction mixture contained 0.1 mL enzyme extract, 0.5 mL of 1% starch solution and 0.4 mL of 0.02M sodium phosphate buffer (pH 7.0) containing 1 mM $CaCl_2$[14]. The reaction mixture was incubated at 30°C for 60 min. The α-amylase activity of crude extract was measured by determining the reducing sugars formed from starch[15]. The specific activity was determined by measuring the protein content[16].

Purification of α-amylases

The enzyme extract was partially purified by gel permeation chromatographies using Sephadex G-25 and G-100. Two mL of crude extract was first passed through sephadex G-25 column equilibrated with 0.02M sodium phosphate buffer (pH 7) to remove the various contaminants from the crude extract. The eluent having amylase activity was then loaded on Sephadex G-100 column for further purification and molecular weight determination of the enzyme extract. Sodium phosphate buffer (pH 7) was used as the elution buffer. Thirty five fractions each of 2 mL were collected. The amylase activity and protein content of each fraction were determined[14]. The fraction having amylase activity was used for characterization of salivary α-amylase.

Characterization of salivary amylases

Nature of partially purified enzyme

The nature of partially purified enzyme was determined by incubating the reaction mixture containing partially purified protein with 1% starch at 37°C for 2-10 h[14]. The products formed were separated by ascending paper chromatography using butanol: acetic acid: water (4:1:5) solvent followed by treatment with saturated $AgNO_3$ solution. The paper was then immersed in 0.5M alcoholic NaOH solution to visualize the spots. Thiosulphate (20%) treatment was given to clear the dark background. The separated products were identified from the standards applied[17].

In gel visualization of amylase isoforms

The partially purified fractions of salivary amylase along with crude extract were run on the native PAGE (10%). The gel was then incubated with 1% starch solution for 60 min at 37°C to visualize amylase activity. The amylolytic activity was stopped by transferring the gel into the staining solution (10 mM I_2 in 14 mM KI) for 5 min. Gel was washed with distilled water to remove excess of I_2. The isoforms of amylase were noticed in the form of light bands on the dark blue background of starch zymogram[18].

Determination of molecular weight

The molecular weight of salivary amylase was determined by analysis of SDS-PAGE and Sephadex G-100 gel permeation chromatography. The crude and purified enzyme extracts were loaded on 12% SDS-PAGE along with high range protein molecular weight markers (14-220 kDa from Sisco research laboratories). The electrophoresis was carried out at constant voltage of 100 V. After the completion of electrophoresis, the resolved proteins were fixed by transferring the gel in 12.5% trichloroacetic acid for 1.0 h followed by immersing the gel in staining solution (0.1 g coomassie blue, 100 mL of methanol, 20 mL of acetic acid and 80 mL of distilled water). The gel was then immersed in destaining solution (125 mL methanol, 35 mL acetic acid and 340 mL distilled water) to clear the background[19]. The log molecular weight of protein molecular weight markers was plotted against the relative migration distance (Rf) values to determine the molecular weight of the purified protein.

The molecular weight of partially purified protein was also determined by plotting the log molecular weight versus elution volume of the standards (β-amylase, 250 kDa; alcohol dehydrogenase, 150 kDa; albumin bovine, 66 kDa; carbonic anhydrase, 29 kDa)

(Table 1) on sephadex G-100 gel permeation chromatography.

Determination of substrate specificity

The substrate specificity of salivary amylase was determined by incubating it with 1% solution of different polysaccharides viz., starch, amylose, amylopectin, α-cyclodextrin, β-cyclodextrin, dextrin-I, dextrin-IV and pullulan[14,20].

Determination of pH and temperature optima and thermal stability of salivary amylase

Assay buffers of pH ranging from 3.0-10.5 were used to determine the pH optima of salivary amylase. The buffers used were 50 mM glycine-HCl (pH 3.0-4.0), sodium acetate (pH 4.5-5.5), sodium phosphate (pH 6.0-8.0) and glycine-NaOH (pH 9.0-10.5). The amylase activity was monitored at temperatures ranging from 30-80°C to determine its temperature optima. The thermal stability of salivary amylase was determined by pre-incubating it at the same temperature range for different time intervals (10-60 min). The amylase activity was then determined by the method described earlier.

Effect of different chemicals on salivary amylase activity

The purified salivary amylase was pre-incubated with different chemicals (5 mM) for 10 min before the addition of the substrate[14]. The concentration of the chemical used in the reaction mixture was selected from the literature[14].

Table 1 — Molecular weights and v_e/v_o of standards used in molecular weight determination of *C. partellus* salivary α-amylase

Standards	v_e/v_o	Molecular wt. (kDa)
β-amylase	1.02	205
Alcohol dehydrogenase	1.13	150
Albumin bovine	1.32	66
Carbonic anhydrase	1.62	29
Chilo partellus salivary α-amylase	1.39	59.25

Kinetic analysis

The salivary amylase activity was determined using different concentrations of soluble starch. The Lineweaver-Burk, Eadie-Hofstee and Hanes plots were plotted to accurately determine the Km and Vmax values. The mode of amylase inhibition was analyzed by constructing Line weaver-Burk, fractional velocity and combination plots at different substrate and inhibitor concentrations. The type of enzyme inhibition was determined from the shape of plot in the presence of inhibitor, Ki (Inhibitor constant), αKi, α and logKi/αKi values. 'α' is described as a factor by which Km value changes when inhibitor interacts with the enzyme.

Results

Purification and nature of salivary amylase

Salivary amylase showed 2.35 folds purification on sephadex G-25. After loading the eluant on G-100 sephadex column, salivary amylase eluted at v_e/v_o of 1.39 with 16.02 folds purification in a single fraction that had the maximum activity of 185.45 nmoles of reducing sugars formed /min/mg protein. This fraction was taken for further studies of α-amylase characterization. The results of paper chromatography revealed the presence of α-amylase activity in the purified extract as the products formed were glucose, maltose, maltotriose and other oligosaccharides (Fig. 1A).

Isozyme pattern and molecular weight of salivary α-amylase

Starch zymogram studies revealed the presence of one isoform of salivary amylase as only one band appeared on the starch zymogram (Fig. 1B). A single band was obtained on SDS-PAGE for crude extract and purified fractions from G-25 and G-100 column chromatography showing the presence of single

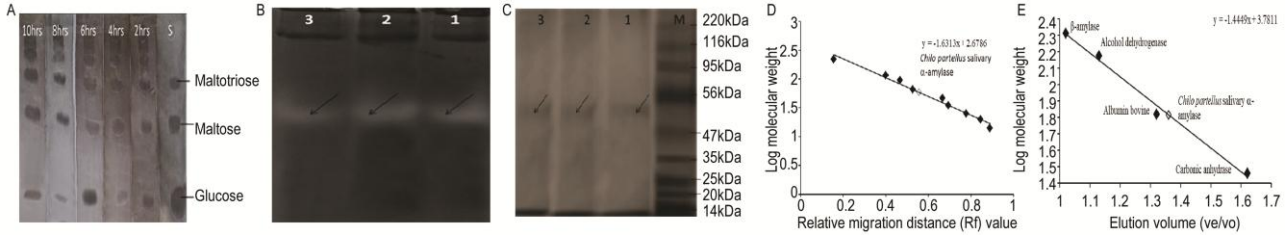

Fig. 1 — (A) Paper chromatogram showing the products formed on hydrolysis of starch by *Chilo partellus* salivary α-amylase after 2, 4, 6, 8 and 10 h incubation, S: standards; (B) Starch zymogram of salivary α-amylase (1: crude extract; 2: partially purified enzyme by sephadex G-25; 3. partially purified enzyme by sephadex G-100; arrows show the single isoform of α-amylase in all the cases; (C) SDS-PAGE of salivary amylase extracts (M: protein molecular weight marker, 1: crude extract; 2: partially purified enzyme by Sephadex G-25; 3. partially purified enzyme by sephadex G-100; arrows show the polypeptide band of α-amylase; (D) Determination of molecular weight of *C. partellus* α-amylase from SDS-PAGE; and (E) Determination of molecular weight of α-amylase from gel permeation chromatography using proteins of known molecular weights (i. β-amylase, 205 kDa; ii. alcohol dehydrogenase, 150 kDa; iii. albumin bovine, 66 kDa and iv. carbonic anhydrase, 29 kDa).

polypeptide of salivary amylase of *C. partellus* (Fig. 1C). The SDS-PAGE and gel permeation chromatography analysis revealed the molecular weight of 59.26 kDa for salivary α-amylase (Fig. 1 D & E).

Optimim pH, temperature optima and thermal stability of salivary amylase

The results showed that salivary amylase had a pH between 6 and 7 where it exhibited its maximum activity (Fig. 2A). Salivary amylase showed more than 90% activity from 30-50°C (Fig. 2B). Optimum temperature was found to be 40°C where salivary α-amylase showed its highest activity. At 80°C, activity decreased by more than 50%. The purified extract of salivary amylase was highly stable when pre incubated at 30 and 40°C for 10-60 min before the addition of the substrate (Fig. 2C). Above 40°C, its activity decreased with the increase in pre incubation time. With increase in incubation period at 50 and 60°C, amylase activity decreased gradually. At 80°C, amylase activity reduced to 56% just after 10 min of pre incubation while after 60 min, only 11.83% activity was left in the extract. The results thus showed that the enzyme tend to retain more than 60% activity even at 70°C when pre incubated for 30 min.

Substrate specificity of salivary amylase

Salivary amylase activity was studied with different substrates to find out the most preferable substrate (Table 2). Among the various substrates analyzed, it was observed that salivary amylase had the maximum activity with starch (185.46 nmoles of reducing sugars formed/min/ mg of protein which was considered as 100% activity) followed by amylopectin and amylose that showed 155.53 and 121.18 nmoles of reducing sugars formed/min/mg of protein, respectively. Salivary amylase showed less than 25% activity with the other substrates used (Table 2).

Kinetic studies of salivary amylase

Kinetics of salivary α-amylase was studied with the different concentrations of starch (Fig. 3). The saturation curve showed an increase in reaction velocity with increase in substrate concentration (Fig. 3A). The Km values were 0.33 and 0.38 mg/mL and Vmax values were 1.42 and 1.52 nmoles of reducing sugars formed/min/mL as obtained from Lineweaver-Burk and Eadie-Hofstee plots, respectively (Fig. 3 B &C). However, Hanes plot showed the Km and Vmax of 0.49 mg/mL and 1.67 nmoles of reducing sugars formed/min/mL respectively (Fig. 3D).

Effect of various chemicals on salivary amylase activity

Effect of various chemicals on salivary amylase activity from *C. partellus* was observed (Table 3). It decreased to 32.81-66.67 nmoles of reducing sugars formed/min/mg of protein (as compared to control with 185.45 nmoles of reducing sugars formed/min/mg of protein) when calcium nitrate, salicylic acid, citric acid, zinc chloride and oxalic acid were used at 5 mM concentrations in the reaction mixture. These chemicals thus caused a reduction of 65-80% in amylase activity.

Table 2 — *C. partellus* salivary α-amylase activity against different substrates

Substrates	Enzyme activity (%)
Starch	$100.00^{e} \pm 8.54$
Amylopectin	$83.86^{d} \pm 6.14$
Amylose	$65.34^{c} \pm 4.50$
Dextrin-I	$13.61^{a} \pm 1.34$
Dextrin-IV	$17.56^{a} \pm 1.22$
α-cyclodextrin	$23.76^{b} \pm 1.89$
β-cyclodextrin	$12.91^{a} \pm 1.25$
Pullulan	$15.07^{a} \pm 1.01$

[Values are means ± SD of three replicates. Different letters show significant difference as analysed by Tukey's test $P \leq 0.05$]

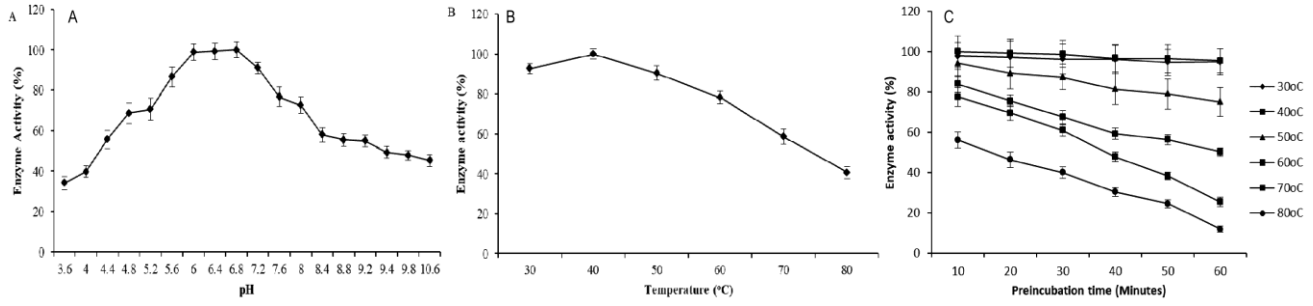

Fig. 2 — (A) Determination of pH optima of *C. partellus* salivary α-amylase using pH in the range of 3-10.5; (B) Determination of temperature optima of *C. partellus* salivary α-amylase. (Reaction conditions were 0.02 M sodium phosphate buffer (pH 7) containing 1.0 mM calcium chloride and a range of temperatures; and (C) Determination of thermal stability of *C. partellus* salivary α-amylase. Reaction conditions were 0.02 M sodium phosphate buffer pH 7.0 containing 1mM calcium chloride, temperature of 30 °C and pre-incubating temperatures ranging from 30-80°C for 10-60 min interval.

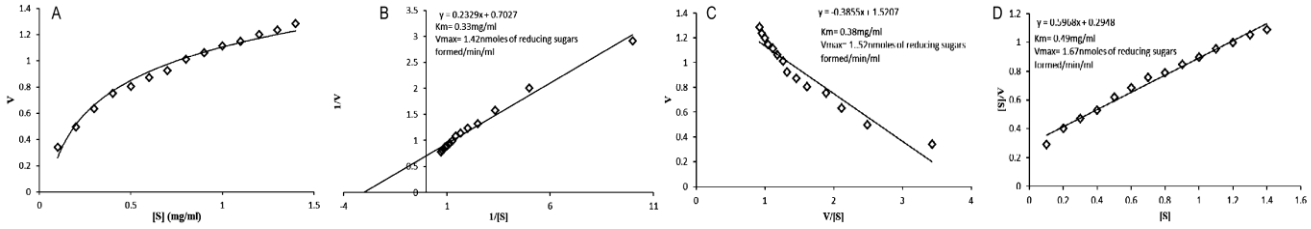

Fig. 3 — (A) Reaction velocity (V) *vs.* [S] (Substrate concentration) plot; (B) Lineweaver- Burk plot; (C) Eadie- Hofstee plot; and (D) Hanes plot for Km (Michaelis constant) and Vmax (Maximum reaction velocity).

Table 3 — Effect of chemicals on *C. partellus* salivary α-amylase activity

Chemicals	Enzyme activity (%)
Control	$100.00^{j} \pm 8.12$
$FeCl_2$	$75.65^{defg} \pm 7.12$
$MgCl_2$	$81.47^{fgh} \pm 6.54$
$Ca(NO_3)_2$	$25.61^{ab} \pm 2.13$
$CuSO_4$	$66.59^{cdef} \pm 3.59$
$Fe_2(SO_4)_3$	$60.76^{cd} \pm 4.06$
$FeSO_4$	$84.76^{ghi} \pm 5.48$
Citric Acid	$19.06^{a} \pm 2.12$
Oxalic Acid	$35.41^{b} \pm 4.01$
Boric Acid	$96.21^{hij} \pm 5.15$
$ZnCl_2$	$17.69^{a} \pm 2.29$
Piperazine	$63.46^{cde} \pm 3.15$
Cinnamic Acid	$79.90^{fg} \pm 4.45$
Salicylic Acid	$32.06^{ab} \pm 2.78$
Rutin	$96.85^{ij} \pm 5.15$
Catechol	$98.11^{ij} \pm 8.24$
Succinic Acid	$78.27^{efg} \pm 4.15$
Ferulic Acid	$76.98^{efg} \pm 5.05$

[Values are means ± SD of three replicates. Different letters show significant difference as analysed by Tukey's test $P \leq 0.05$]

Inhibition kinetics of salivary amylase

Kinetic mechanisms were studied for five inhibitors viz. citric acid, salicylic acid, oxalic acid, zinc chloride and calcium nitrate against salivary amylase activity. These inhibitors were selected on the basis of percentage inhibition studies of salivary amylase (Table 3).

Kinetic studies of salivary amylase with citric acid, salicylic acid, oxalic acid, zinc chloride and calcium nitrate

Lineweaver-Burk plot indicated that citric acid acted mainly as non-competitive inhibitior (Fig. 4Ai). The replots of slope versus inhibitor concentration and intercept versus inhibitor concentration were constructed to get Ki and αKi values (Fig. 4 Aii & Aiii). The Ki and αKi values were 1.65 and 1.70 mM, respectively. It was observed that α = 0.97 and logKi/αKi was 0.01. Fractional velocity plot further confirmed the complete/linear pure non-competitive behaviour of inhibition (Fig. 4Aiv). The combination plot provided α and Ki values of 1.0 and 1.7 mM, respectively and further confirmed the type of inhibition (Fig. 4Av).

The Lineweaver-Burk plot displayed mixed non-competitive-competitive inhibition by salicylic acid (Fig. 4Bi). The Ki and αKi values obtained from secondary plots were 7.7 and 45.2 mM (Fig. 4 Bii & Biii). It was noticed that α = 5.87 and log Ki/αKi= -0.77. Fractional velocity plot and replot indicated complete mixed non-competitive- competitive inhibition (Fig. 4 Biv & Bv). It was observed that the combination plot for salicylic acid showed mixed type inhibition with α and Ki values of 6.0 and 7.7 mM, respectively (Fig. 4Bvi).

The mixed non-competitive-competitive inhibition kinetics by oxalic acid was analysed from the double reciprocal plot (Fig. 4Ci). The replots were linear and gave Ki and αKi as 13.9 and 17.1 mM, respectively (Fig. 4 Cii & Ciii). The partial or complete type of mixed inhibition was confirmed by plotting v/v_0-v versus reciprocal of inhibitor concentration in fractional velocity plot (Fig. 4 Civ & Cv). It was found as complete mixed type of inhibition. It was clear from the slope of the fractional velocity replot that inhibition by oxalic acid was of complete mixed non-competitive-competitive type. The combination plot for oxalic acid further confirmed the mixed non-competitive-competitive behaviour of inhibition and gave α and Ki values of 1.2 and 14.2 mM (Fig. 4Cvi).

The mode of salivary α-amylase inhibition by zinc chloride was observed by plotting the Lineweaver-Burk plot and fractional velocity plots. The Lineweaver-Burk plots displayed mixed non-competitive-competitive inhibition by zinc chloride with Ki and αKi values as 3.4 and 5.1 mM respectively (Fig. 4 Di-Diii). The value of α was 1.5 and log Ki/αKi was –0.18. The replot from primary fractional velocity plot was constructed to get the actual nature of enzyme inhibition and was observed

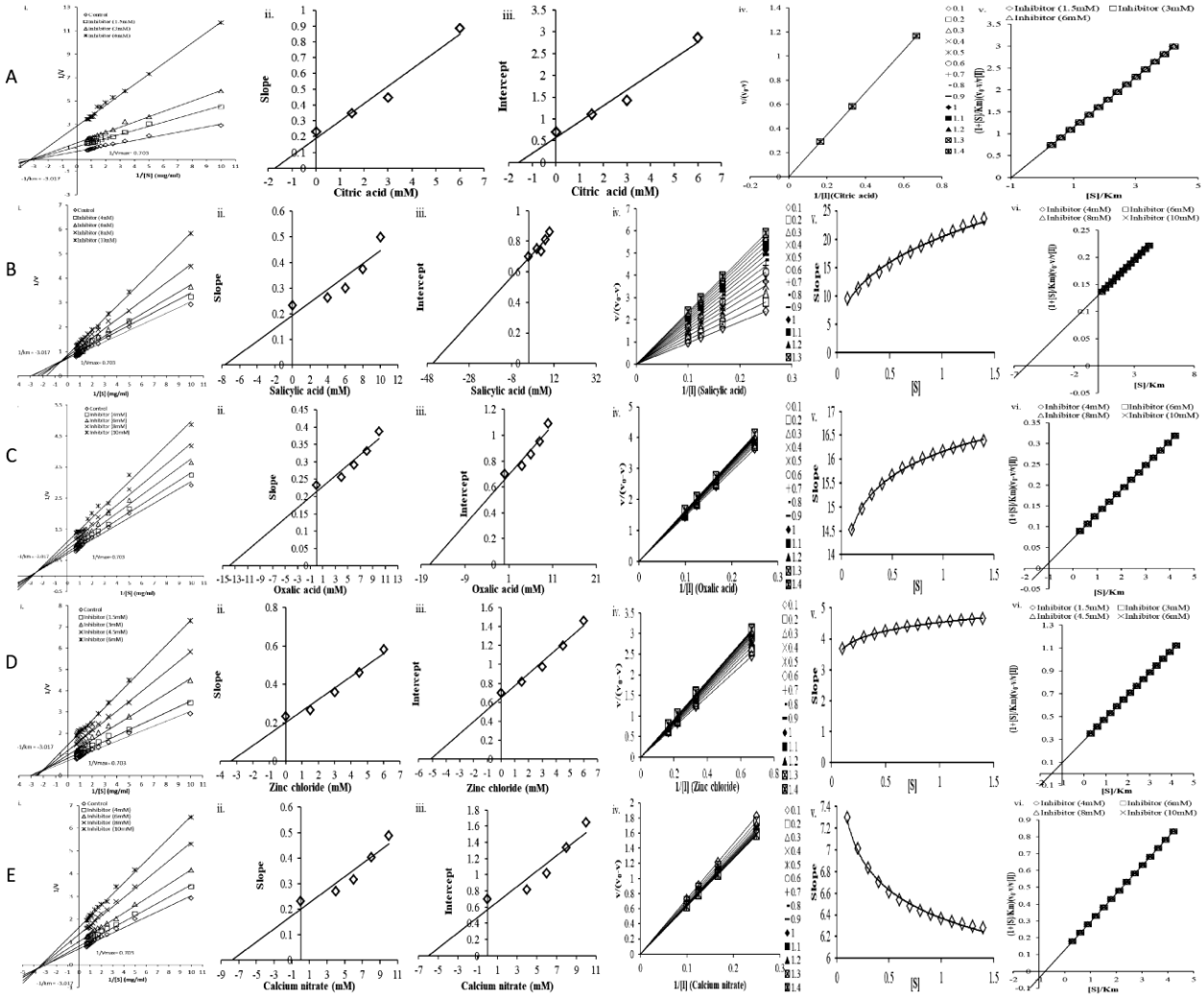

Fig. 4:— (Ai) Lineweaver-Burk plot in the presence of citric acid, (Aii) Replot of slope of primary plot *vs*. inhibitor concentration, (Aiii) Replot of intercept of primary plot *vs*. inhibitor concentration, (Aiv) Fractional velocity plot, (Av) Combination plot of salivary α-amylase inhibition of *C. partellus* by citric acid; (Bi) Lineweaver-Burk plot in the presence of salicylic acid, (Bii) Replot of slope of primary plot *vs*. inhibitor concentration, (Biii) Replot of intercept of primary plot *vs*. inhibitor concentration, (Biv) Fractional velocity plot, (Bv) Fractional velocity replot and (Bvi) Combination plot of salivary α-amylase inhibition of *C. partellus* by salicylic acid; (Ci) Lineweaver-Burk plot in the presence of oxalic acid, (Cii) Replot of slope of primary plot *vs*. inhibitor concentration, (Ciii) Replot of intercept of primary plot *vs*. inhibitor concentration, (Civ) Fractional velocity plot, (Cv) Fractional velocity replot and (Cvi) Combination plot of salivary α-amylase inhibition of *C. partellus* by oxalic acid; (Di) Lineweaver-Burk plot in the presence of zinc chloride. (Dii) Replot of slope of primary plot *vs*. inhibitor concentration, (Diii) Replot of intercept of primary plot *vs*. inhibitor concentration, (Div) Fractional velocity plot, (Dv) Fractional velocity replot and (Dvi) Combination plot of salivary α-amylase inhibition of *C. partellus* by zinc chloride and (Ei) Lineweaver-Burk plot in the presence of calcium nitrate, (Eii) Replot of slope of primary plot vs inhibitor concentration, (Eiii) Replot of intercept of primary plot vs inhibitor concentration, (Eiv) Fractional velocity plot, (Ev) Fractional velocity replot and (Evi) Combination plot of salivary α-amylase inhibition of *C. partellus* by calcium nitrate.

as complete mixed non-competitive-competitive inhibition (Fig. 4 Div & Dv). The combination plots also showed mixed non-competitive-competitive inhibition with α =1.5 and Ki = 3.4 mM which was exactly similar to that obtained from Lineweaver-Burk plots (Fig. 4Dvi).

The Lineweaver-Burk plot for salivary amylase inhibition by calcium nitrate was noticed to be mixed non-competitive- uncompetitive type (Fig. 4Ei). The secondary plots were linear in shape and Ki and αKi values were 7.8 and 6 mM, respectively (Fig. 4 Eii & Eiii). It was observed from fractional velocity plots that the inhibition was complete mixed non-competitive uncompetitive inhibition (Fig. 4 Eiv & Ev). The combination plot showed α and Ki as 0.8 and 7.5 mM, respectively (Fig. 4Evi).

Discussion

The enzyme was partially purified protein obtained from sephadex G-25 and sephadex G-100 column chromatography. The purification was 2.35 folds with sephadex G-25 which increased to 16.02 folds when passed through sephadex G-100 column. The results of paper chromatography revealed that the purified protein was α-amylase as the products formed were glucose, maltose, maltotriose and other oligosaccharides (Fig. 1A). The salivary α-amylase had only one isoform (Fig. 1B). In contrast to our studies, there were reports of two isoforms of salivary α-amylase in *Naranga. aenescens* on starch zymogram[21]. The SDS-PAGE and gel permeation chromatography analysis revealed the molecular weight of 59.26 kDa for salivary α-amylase (Fig. 1 C-E).

The pH and temperature optima are two important factors for pokilothermic organisms. Salivary amylase showed a pH between 6 and 7 (Fig. 2A). Optimal pH of 6.0 to 9.0 for salivary amylase from *Lygus Hesperus*, *Lygus* lineolaris and *Chilo suppressalis* have been reported in literature[22,23]. Maximum amylase activity at 40°C (Fig. 2B) might be due to the formation of stable enzyme-substrate complex that protect the enzyme from denaturation at this temperature. At 80°C, amylase activity decreased by more than 50%. Our results are similar to those reported earlier that showed an optimum temperature of 40°C for α-amylase activity from rice stem borer[23]. The results further showed that the enzyme tend to retain more than 60% activity even at 70°C when pre incubated for 30 min (Fig. 2C) which further proved that the purified fraction consisted of α- amylase activity.

Starch is the preferred substrate of salivary α-amylase as it showed maximum activity with it (Table 2). Its reduced affinity towards pullulan might be due to the presence of extensive α1-6 glycosidic bonds in pullulan as suggested earlier[24].

The Lineweaver-Burk, Eadie-Hofstee and Hanes plots were constructed for accurate determination of Km and Vmax values. The double reciprocal plot has certain limitations as it is gives undue weightage to the measurements at low substrate concentration where chances of error are more. In Eadie-Hofstee plot, rate is plotted differently on the two axis and if there would be any error in rate then that error gets increased during plotting of this graph. Hanes plot overcomes the limitations of Lineweaver-Burk and Eadie-Hofstee plots. Therefore, the Km and Vmax of 0.49 mg/mL and 1.67 nmoles of reducing sugars formed/min/mL, respectively obtained through Hanes plot (Fig. 3D) were observed to be the most authentic values for the salivary α-amylase. Zibaee (2012) reported Km and Vmax of 1.04% and 7.35 units/mg protein in salivary α-amylase of *A. spinidens*. Kinetic studies on salivary α-amylase of *N. aenescens* indicated its Km and Vmax values as 0.14 mg/mL and 0.08061 µmole/min, respectively[21].

The inorganic ions affect the α-amylase activity significantly (Table 3). The loss of activity by zinc (Table 3) might be due to inactivation of the enzyme as heavy metals have been reported to interact with active site residues and peptide linkages[24]. The reduction in α-amylase activity by calcium nitrate might be due to the blockage of chloride binding sites of the enzyme through the strong electrostatic interactions of NO_3^- ions with α-amylase as was suggested earlier[25]. Citric acid, oxalic acid and salicylic acid have been earlier reported to inhibit α-amylase activity of *Helicoverpa armigera*[14].

Inhibition kinetics of salivary α-amylase was studied with citric acid, salicylic acid, oxalic acid, zinc chloride and calcium nitrate as these were observed to cause maximum inhibition of salivary amylase activity and thus can be exploited for pest management strategies. The Lineweaver-Burk plots with simultaneous variation in substrate and inhibitor concentration were prepared to find the type of enzyme inhibition. Replots of primary Lineweaver-Burk plot gave Ki, αKi, α and logKi/αKi values which further confirmed the type of enzyme inhibition. However, due to partial information of the type of inhibition and the possibility of errors in reaction velocity determination at low substrate concentrations and the errors in the inhibition constants and α values calculated from the replots of primary reciprocal plots of double reciprocal plots, fractional velocity plots were constructed to distinguish between partial or complete type of inhibition[26]. The combination plot was constructed by plotting (1+[S]/Km) (v_o-v/v [I]) versus [S]/Km to accurately calculate α and Ki values as described by Chan (1995). Combination plots are more advantageous than the other plots as they directly compare all the data points, provide freedom in experimental design and show accurate Ki and α values without the generation of secondary plots[27]. A double reciprocal plot of the data showed that citric acid displayed mainly non-competitive inhibition

kinetics as the plot lines intersect at the surface of 1/[S] axis. It was noticed that there was little difference in Ki and αKi values i.e. 1.65 and 1.70 mM respectively (Fig. 4Ai, 4Aii, 4Aiii). Since, α = 0.97, (approximately 1), the inhibition mode can be considered as non-competitive. The logKi/αKi was 0.01 which was near zero value, it proved to be non competitive inhibition. There was single line for all the inhibitor concentrations which passed through the origin in fractional velocity plot (Fig. 4Aiv). From the inspection of fractional velocity plot, it became clear that the inhibition of salivary α-amylase by citric acid was pure and complete non-competitive type inhibition. The combination plot (Fig. 4Av) further confirmed the type of inhibition. The α and Ki values obtained from combination plot were 1.0 and 1.7 mM, respectively which are near to that obtained from Lineweaver-Burk plot.

The plots crossed the left of the 1/V axis and above the 1/[S] axis in Lineweaver-Burk plot for salivary α-amylase inhibition by varying concentrations of salicylic acid, oxalic acid and zinc chloride (Fig. 4 B-D). The Ki values were smaller than αKi values obtained from secondary plots. α was observed to be more than 1.0 for these chemicals i.e. 5.87 (salicylic acid), 1.23 (oxalic acid) and 1.50 (zinc chloride), respectively. The log Ki/αKi values were negative. All these factors pointed towards mixed non-competitive-competitive inhibition[28,29]. Although mixed type of inhibition was clear from reciprocal plot, but the nature of the inhibition as complete or partial was still unclear. Therefore, for further clarification, fractional velocity plots were obtained by plotting fractional velocity versus reciprocal of inhibitor concentration. The slope of the plots increased with increase in substrate concentration and also converged to the origin (Fig. 4 Biv, 4Civ & 4Div) that showed the nature of inhibition to be complete. The pure or mixed forms of enzyme inhibitions were further distinguished by plotting the slope of primary fractional velocity plot versus substrate concentrations (Fig. 4Bv, 4Cv & 4Dv). The slopes of the replots were hyperbolic, indicating complete mixed non-competitive-competitive type of inhibition. Due to possibility of error in ki and α values obtained from replots of primary reciprocal plot, combination plots were generated. The combination plots for salicylic acid, oxalic acid and zinc chloride showed mixed type inhibition as the plot line crossed [S]/Km axis away from -1. The α and Ki values from combination plot were considered to be the most accurate[27].

The salivary α-amylase inhibition by calcium nitrate was noticed to be of mixed non-competitive uncompetitive type from the Lineweaver-Burk plot as the plots intersected below the 1/[S] axis (Fig. 4Ei). The log Ki/αKi was positive (0.11) for calcium nitrate. The Ki>αKi, α<1 and value of log Ki/αKi also pointed towards mixed non-competitive-uncompetitive inhibition. The replots were found to be linear in shape indicating linear or complete mixed type inhibition (Fig. 4Eii & Eiii). Further confirmation of linear type inhibition was done by generating the fractional velocity plot for calcium nitrate (Fig. 4Eiv). The plot lines converged to the origin of the plot indicating complete/linear inhibition. It was clear from the replot of primary fractional velocity plot that salivary α-amylase inhibition by calcium nitrate was complete mixed non-competitive- uncompetitive inhibition (Fig. 4Ev). The combination plot further confirmed of mode of inhibition and gave α and Ki values as 0.8 and 7.5 mM, respectively (Fig. 4Evi).

Conclusion

C. partellus salivary α-amylase consisted of a single isoform of 59.26 kDa. The enzyme is active between pH of 6 and 7 and is thermally stable up to 70°C. Salivary α-amylase had the highest activity with starch. Citric acid, salicylic acid, oxalic acid, zinc chloride and calcium nitrate strongly inhibited salivary α-amylase activity. The mode of inhibition varied for different inhibitors. Citric acid was a pure non-competitive inhibitor of salivary α-amylase while salicylic acid, oxalic acid and zinc chloride caused complete mixed non-competitive-competitive inhibitions. The inhibition of salivary α-amylase with calcium nitrate was of complete mixed non-competitive-uncompetitive type. These inhibitors can be used to control the pest as α-amylase from salivary glands play an important role in its establishment on the plant. Hence, characterization of the enzyme and its inhibition with potent α-amylase inhibitors would support better pest management strategies.

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Conflict of interest

Authors report no conflict of interest.

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