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Biochemical Characterization of Alpha-amylase from the Digestive Fluid of Larvae of the Rhino Beetle, *Oryctes owariensis*

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ABSTRACT-- ***Alpha-amylase was purified from the digestive fluid of larvae of the Rhino Beetle (Oryctes owariensis) by chromatography on ion-exchanges and hydrophobic interaction columns. The preparation was shown to be homogeneous on polyacrylamide gel. The enzyme was purified 260.20-fold to a specific activity of 128.50 (U/ mg of protein) and an overall yield of 26.22 %. SDS-PAGE revealed a single polypeptide of 53.8 ± 1.2 kDa, thus indicating apparent homogeneity of the final enzyme preparation. Gel filtration chromatography showed that the enzyme was a 50.4 ± 2.2 kDa monomeric protein. The purified enzyme exhibited pH and temperature optima at 7.0 and 55°C, respectively. The alpha-amylase was stable at 37 °C and its pH stability was in the range of 6.6-7.6. This enzyme exhibited a high affinity towards soluble starch with Km values of 0.6 ±0.04. The alpha-amylase activities were stimulated by Ca^{2+}, K^{+}, Mg^{2+} and Na^{+} and were inhibited by EDTA, Fe^{2+} and Cu^{2+}. The analysis of hydrolytic products after soluble starch hydrolysis by alpha-amylase revealed that maltose, maltotriose and maltotetraose were the major products. This indicated that this enzyme can be classified as the alpha-amylase (the endoamylase). From a physiological point of view, alpha-amylase play a fundamental role in energy production for this insect larvae. Consequently, the digestive tract of this larva could be a good source of alpha-amylase for starch saccharification.***

Keywords-- *Oryctes owariensis*, alpha-amylase, Enzyme, monomeric protein, Energy production, Starch saccharification

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1.INTRODUCTION

Most living organisms are able to exploit environmental polysaccharides. Alpha-amylases (α-1,4-glucan-4-glucanohydrolase, EC 3.2.1.1), which hydrolyze starch and other polysaccharides in maltose, maltotriose and maltodextrins, are key enzymes in this process [1]. These enzymes constitute a family of endo-amylases that catalyze the hydrolysis of α-D-(1,4)-glucan linkages in starch components, glycogen and other carbohydrates. In many cases, they form multigene families. The presence of several, sometimes divergent amylase molecules enables organisms to digest a broad range of substratse, in a broad range of environmental conditions [2]. The enzymes play a key role in carbohydrate metabolism of microorganisms, plants and animals [3]. These properties are use in a wide number of industrial processes such as food, fermentation, textile, paper, detergent, and pharmaceutical industries. Moreover, they have almost completely replaced chemical hydrolysis of starch in starch processing industry [4]. These enzymes are among the most

important enzymes and are of great significance for biotechnology, constituting a class of industrial enzymes having approximately 25% of the world enzyme market [5], [6]. They can be obtained from several sources, such as plants, animals and microorganisms. To date, the majority of alpha-amylases used in industry are generally produced by bacteria and fungi because of their ease of cultivation and the desirable physicochemical properties of the secreted enzymes [7], [8]. Among insects, α-amylase has been purified or characterized in a number of species including *Tenebrio molitor* [9], *Rhizoperta dominica* [10], *Protephanus truncates* [11], *Lygus hesperus and L. lineolaris*. [12], *Zabrotes subfasciatus* [13], *Morinus funerus* [14], *Periplaneta americana* [15], *Eurygaster maura* [30] and *Gryllodes sigillatus* [16].

Rhino Beetle (*Oryctes owariensis*) is major pest of raphia palm. Though, they are very destructive, their nutritional potential have endeared them to man [17], [18]. Consequently, its reproduction, nutritionally values and habitat ecology have been well studied [19], [20], [21]. However, the digestive physiology involving digestive enzymes, such as the alpha-amylase, of this larva remains largely unexplored.

This study aims to undertake the purification and biochemical characterization of the alpha-amylase sourced from the digestive tract of *O. owariensis*, in order to gain a better understanding of its digestive physiology and to investigate a new potential good source of alpha-amylases for starch saccharification

2.MATERIALS AND METHODS

2.1 *Chemicals*

Substrates: starch, glucose, Glucose, Maltose, Maltotriose were purchased from Sigma Aldrich. DEAE-Sepharose Fast Flow, CM-Sepharose CL-6B, Sephacryl S-200 HR and Phenyl-Sepharose 6 Fast Flow were obtained from Pharmacia Biotech, bovine serum albumin (BSA) from Fluka Biochemika and standard molecular weight proteins from Bio Rad. The chemicals used for polyacrylamide gel electrophoresis (PAGE) were from Bio-Rad. All the other reagents used were of analytical grade.

2.2 *Biological material*

Rhino Beetle (*Oryctes owariensis*) larvae were obtained from the oil palm Raffia, *Raphia hookeri* G. (Arecales: Arecaceae), plantation near Daloa (Côte d'Ivoire). The larvae were kept without feed for 24 hours, after which they were stored at -20°C until analysis.

2.3 *Enzyme samples*

O. owariensis larvae were rinsed in cold water and blotted with filter paper. Guts were dissected in cold 0.9% NaCl (w/v) solution and digestive content was removed and stirred in the presence of 0.9% NaCl (w/v) solution before centrifugation at 6,000 x g for 30 min. The supernatant was then stirred with 100 mM phosphate buffer pH 7.0 for 30 min. The homogenate was centrifuged at 10,000 x g for 30 min. The collected supernatant constituted the crude extract. After freezing at −180°C in liquid nitrogen, the crude extract was stored at −20°C.

2.4 *Alpha-amylase assay*

This enzyme assay was performed as described below [22]. Briefly, purified alpha-amylase extract (50 µl) was incubated with 0.5 % solution starch (80 µl) on 100 mM phosphate buffer pH 7.0 (170 µl) at 37°C for 30 min. The amount of reducing sugars produced was determined by dinitrosalicylic acid (DNS) method with maltose as the standard. One unit of the enzyme activity (UI) is defined as the amount that liberated 1 µmol of maltose equivalent per min under the above conditions. Specific activity was expressed as units per mg of protein.

2.5 *Protein determination*

Protein was determined according to Lowry method [23] using bovine serum albumin as standard.

2.6 Purification of enzymes

All the purification procedure was carried out in the cold room. The crude extract of the Rhino Beetle (*Oryctes owariensis*) larvae was loaded onto an anion-exchange chromatography using a DEAE-Sepharose Fast Flow column (2.5 cm x 4.5 cm), equilibrated with 20 mM sodium phosphate buffer (pH 7.0). The column was washed at a flow rate of 3 mL/min with two bed volumes of equilibration buffer to remove unbound proteins. Bound proteins were then eluted with a stepwise salt gradient (0.1, 0.3, 0.5, 0.7 and 1 M) of NaCl in 20 mM sodium phosphate buffer (pH 7.0), and fractions of 3 mL were collected. One peak of alpha-amylase activity was obtained.

On the one hand, the unbound alpha-amylase activity was loaded onto a cation-exchange chromatography using a CM-Sepharose CL-6B column (2.6 cm x 4.0 cm), equilibrated with 20 mM sodium phosphate buffer (pH 7.0). The column was washed with the same buffer at a flow rate of 1 mL/min. Alpha-amylase activity was eluted with a stepwise salt gradient (0, 0.2, 0.5 and 1 M) NaCl in 20 mM sodium phosphate buffer (pH 7.0). Fractions of 2 mL were collected and, to the pooled active fractions, solid sodium thiosulphate was slowly added to give a final concentration of 1.7 M and the resulting enzyme solution was subsequently applied on a Phenyl Sepharose 6 Fast Flow column (1.5 cm × 3.2 cm) previously equilibrated with 20 mM sodium phosphate buffer (pH 7.0) containing 1.7 M of sodium thiosulphate salt. The column was washed with a reverse stepwise gradient of sodium thiosulphate concentrations (from 0–1.7 M) dissolved in the same sodium acetate buffer at a flow rate of 1 mL/min and fractions of 1 mL were collected. The pooled active fractions were dialyzed overnight against 20 mM sodium phosphate buffer (pH 7.0) and constituted the purified enzyme solution.

2.7 *Homogeneity and molecular weight determination*

To check purity and determine molecular weight, the purified enzyme was analyzed using SDS-PAGE electrophoresis on a 10% separating gel and a 4% stacking gel (Hoefer mini-gel system; Hoefer Pharmacia Biotech), according to the procedure of Laemmli [24] at 10°C and constant current 20 mM. Proteins were stained with silver nitrate according to Blum *et al*. [25]. The sample was denatured by a 5 min treatment at 100°C. Electrophoretic buffers contained sodium dodecyl sulfate (SDS) and beta-mercaptoethanol. The standard molecular weights (Bio-Rad) comprising myosin (200 kDa), β-galactosidase (116.25 kDa), phosphorylase b (97.4 kDa), BSA (66.2 kDa) and ovalbumin (45.0 kDa) were used.

2.8 *Native molecular weight determination*

The purified enzyme was applied to gel filtration on a Sephacryl S-200 HR column (0.8 cm × 35 cm) equilibrated with 20 mM sodium phosphate buffer (pH 7.0) to estimate the native molecular weights. Elution was done at a flow rate of 0.2 mL/min and fractions of 0.5 mL were collected. Standard molecular weights (SIGMA) used for calibration were β-amylase from sweet potato (206 kDa), BSA (66 kDa), ovalbumin from egg white (45 kDa) and cellulase from *Aspergillus niger* (26 kDa).

2.9 *pH and temperature optima*

The effect of pH on the activity of the enzyme was determined by performing the hydrolysis of 0.5 % starch in a series of buffers (100 mM) at various pH values (5.6–10.0). Buffers used were sodium phosphate (pH 5.6–10.0), Tris-HCl (pH 7.0-9.0) and glycine-NaOH (pH 8.0–10.0). pH values of each buffer were determined at 25 °C. The effect of temperature on alpha-amylase activity was performed in 100 mM sodium phosphate buffer (pH 7.0) over a temperature range of 30–80 °C by using 0.5 % starch as substrate under the enzyme assay conditions.

2.11 *pH and Temperature Stabilities*

The pH stability of alpha-amylase was studied at a pH range of 5.6–10.0 with 100 mM buffers. Buffers used were the same as in pH and temperature optima study. After 2 h incubation at 37 °C, aliquots were taken and immediately assayed for residual alpha-amylase activity. Thermal inactivation of alpha-amylase was performed at 37 and 55 °C by pre-warming the enzyme solution from 15 to 180 min in 100 mM sodium phosphate buffer pH 7.0. Aliquots were removed at different times and immediately cooled. Thermal denaturation of alpha-amylase was determined by preheating aliquots for 15 min between 30 and 80 °C. Residual activity, measured for enzyme at 37 °C under the standard test conditions, was expressed as percentage activity of zerotime control of untreated enzyme.

2.12 *Kinetic parameters determination*

Kinetic parameters (Km and Vmax) of alpha-amylase were determined from a Lineweaver-Burk plot using different concentrations (0.125 to 1.0 mg/ml) of soluble starch. Alpha-amylase activity was determined under the standard test conditions.

2.13 *Effect of Some chemical agents*

The effects of various compounds (ions and EDTA) as possible activators or inhibitors of the alpha-amylase activity were studied at 37 °C for 30 min. The substrate (0.5 % starch) was added to the medium and incubated at 37°C for 30 min. The residual activities, measured under the standard test conditions, were expressed as a percentage of the control without the chemical agent.

2.14 *Analysis of the degradation products*

Hydrolysis of soluble starch by alpha-amylase of *O. owariensis* larvae was tested with 50 µl of purified enzyme mixed with 170 ml of 100 mM phosphate buffer pH 7.0 and 80 µl of 0.5 % starch at 37 °C for 2 and 6 h. Concurrently, aliquots (5 µl) were removed and spotted onto a Thin-Layer Chromatography (TLC) plate. Hydrolyzed products were separated using butanol-acetic acid-water 9: 3.75: 2.25 (v/v/v) and developed using naphto-resorcinol in ethanol and H2SO4 20 % (v/v). The sugar spots were visualised keeping the plate at 110 °C for 5 min.

3.RESULTS AND DISCUSSION

3.1 *Purification procedures*

Purification procedure of Rhino Beetle *(Oryctes owariensis)* larvae is summarized in table 1. The purification protocol involved three steps of chromatography. One peak of alpha-amylase activity was resolved at 0.5 M NaCl concentration in 20 mM sodium phosphate buffer (pH 7.0) on DEAE-Sepharose Fast-flow column (data not shown). The pooled fractions, after this step, were loaded onto cation exchange chromatography on CM-Sepharose CL-6B. One peak showing alpha-amylase was resolved with salt-free in 20 mM sodium phophate buffer (pH 7.0) (data not shown). The Rhino Beetle larvae alpha-amylase activity was finally purified by Phenyl-Sepharose 6 Fast-flow hydrophobic interaction chromatography (data not shown). This result revealed that *O. owariensis* larvae alpha-amylase had only one isoform. However, the number of alpha-amylases identified in different insect species varied from 1 to 8 isoforms e.g., *H. armigera*, *S. litura*, *C. chinensis* and *C. cephalonica* exhibited more than five isoforms whereas *S. oryzae* and *T. castaneum* possessed only one isoform [26]. Active fractions were collected as purified alpha amylase and used for further characterization of the enzyme. The specific activity was 127.5 UI/mg of protein (Table 1). The purification yield (26.22 %) was higher compared to digestive alpha-amylases from *P. truncates* (8 %) [11] and *M. funereus* larvae (15.4 %) [14].

The enzyme showed a single protein band on SDS-PAGE gel electrophoresis staining with silver nitrate (Figure1). This result confirmed that this enzyme was purified to homogeneity.

3.2 *Molecular weight determination*

SDS-PAGE profile of purified enzyme is depicted in figure 1. After SDS-PAGE analysis under reducing conditions, Rhino Beetle *(Oryctes owariensis)* larvae alpha-amylase showed a single protein band.

Its relative molecular weight was estimated to be 53.8 ± 1.2 kDa (Table 2). On the other hand, gel filtration chromatography on Sephacryl S-200 HR column showed an approximately molecular weight of 50.4 ± 2.2 kDa for native enzyme (Table 2). These results strongly suggest that the purified alpha-amylase exist as a monomer as describe by Wisessing *et al*. [27]. The small differences between the molecular weight determined by gel filtration and by SDS-PAGE could be explained by the specific interactions between the glycosyl residues of the enzymes and the gel filtration resin. In comparison to other molecular weights of purified insect alpha-amylases, molecular weight of alpha-amylase of this study was higher than those from *C.maculates* (36 and 33 kDa) [28], *Z. subfasciatus* (28 kDa) [29] and *M. funereus* (33kDa) [14].

3.3 *Effect of pH and temperature*

The optimum values of pH for studying alpha-amylase activity are presented in table 2. The enzyme activity was maximal at pH 7.0.. This optimum pH value was similar on the whole to those reported for other insect alpha-amylases from *E. Maura* [30] and *G. sigillatus* [16]. Insect alpha-amylases are generally more active in neutral to slightly acid pH conditions [14], [30], [31]. Thus, our finding regarding the optimum pH value of this enzyme was in agreement with this result. At 37 °C, the purified enzyme showed best stability over pH values ranging from 6.6 to 7.6 (Table 2), conserving at least more than 90% of total activity. Therefore, a pH of 7.0 is a good compromise between the activity and stability of this enzyme to perform hydrolysis of starch over a long time.

The optimum temperature of the purified alpha-amylase of *O. owariensis* larvae was at 55°C (Table2). This optimum temperature value was similar on the whole to those reported for other insect alpha-amylases from *P. americana* [15] and *G. sigillatus* [16].

The thermal inactivation study at pH 7.0 indicated that, alpha-amylase remained fully stable for 180 min at 37 °C (Figure 2). However, at 55 °C (its optimum temperature) the enzyme was less stable and lost about 80 % of its hydrolytic activity after 165 min of pre-incubation. The half-life of the enzyme at 55 °C was obtained at 100 min. The results also indicate that starch saccharification using alpha-amylase from *O. owariensis* larvae could be done at neutral pH and at temperatures lower than 55 °C.

The thermal denaturation was investigated by incubation of the enzyme at various temperatures for 15 min. The result showed that this enzyme was fairly stable at temperatures up to 55 °C. Above this temperature, its activity declined as the temperature increased, but the enzyme was not completely inactivated even at 80 °C (Figure 3).

3.4 *Kinetic parameters values of alpha-amylase*

The kinetic constants (Vmax and km) for α-amylase from *O. owariensis* were determined by incubating fixed amount of enzyme with varied concentrations of soluble starch as a substrate (0.125 to 1.0 %). The enzyme followed the Michealis Menten kinetics of catalysis. In present research work, the Vmax and Km of α-amylase were derived from the Lineweaver Burke plot and found to be 153.7 ± 2.1 UI/mg and 0.6 ±0.04 mg/ml, respectively (table 2). Low values of Km indicate high affinity of the enzyme for the substrate [32]. This Km value was below the average values reported for alpha-amylases purified from other insects such as *T. molitor* (1.8 mg/ml) [9] and *P. americana* (5.0 mg /ml) [15].

3.5 *Effect of metal ions, chelating and reducing agents*

The effect of various metal ions and other chemical agents on purified alpha-amylase from *O. owariensis* larvae are reported in Table 3. Metal ions like K^{+} and Mg^{2+} at 5 mM concentration stimulated alpha-amylase activity by 54 and 65 %, respectively (Table 3). Ca^{2+} however, was stimulatory at both concentrations (1 and 5 mM) whereas other metal ions such as Cu^{2+}, Ba^{2+}, Fe^{2+}were inhibitory. Generally, many alpha-amylases are known to be a calcium metalloenzyme [33] and the role of Ca^{2+} in maintaining stability of structure of alpha-amylases has been demonstrated [34], [35]. This could explain the result obtained for Ca^{2+} which caused a significant increase in alpha-amylase from *O. owariensis* larvae activity at 1 and 5 mM. EDTA also inhibited amylase activity, suggesting that metal ions were required for the amylase activity. This result confirms the presence of Ca^{2+} in alpha-amylase from *O. owariensis* larvae structure.

3.5 *TLC analysis of starch hydrolysis*

The TLC analysis of product formation during starch hydrolysis by alpha-amylase is reported in Figure 4. After 2 and 6 h, maltose, maltotriose and maltotetraose predominated as the products of hydrolysis. On the basis of these major hydrolytic products, alpha-amylase from *O. nasicornis* larvae was classified as the endoamylases as are most of the insect amylases described in the literature. Moreover, considering these major hydrolytic products, we can suggest the application of *O. owariensis* larvae alpha-amylase in the production of syrup of oligosaccharides mixture. Our finding was similar to those reported for *P. americana* alpha-amylase [15] and microorganisms such as the bacteria *B. claussi* LT21 [36] and the yeast *P. burtonii* [37].

4.CONCLUSION

The present study indicated that the *O. owariensis* larvae contain one isoform of alpha-amylase that play a fundamental role in energy production for this larva. From the results, it is clear that the enzyme has the ability to work in a wider temperature and pH range and has high thermostability suggesting that it can be used for starch hydrolysis. The reported pH value indicates it to be similar to those required for efficient starch liquefaction. Therefore, this enzyme can be used in industrial sector.

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