Kasetsart J. (Nat. Sci.) 42 : 240 - 244 (2008)

Purification and Characterization of C. *maculatus* α-amylase

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ABSTRACT

Callosobruchus maculatus causes damaging of storage mungbean seeds. Understanding control mechanism of a-amylase activity is essential for developing method for the insect control. In this study, C. *maculatus* a-amylase was purified and characterized. Purification was carried out using self-coupled β-cyclodextrin sepharose 6B affinity column. It was found that C. *maculatus* α-amylase had one isoform with a molecular weight of 50 kDa. The purified enzyme with specific activity of 182.78 U mg protein^{-1}, showing optimum pH at around 5-6 and optimum temperature at 50-60 °C.

Key words: mungbean seeds, α-amylase, characterization, C. *maculates,* purification

INTRODUCTION

Callosobruchus maculatus causes damaging of storage mungbean seeds, leading to economical losses. These insect larvae utilize α-amylase (α-1, 4-glucan-4-glucanohydrolase, EC 3.2.1.1) to hydrolyze starch, glycogen and related α-D-1, 4-glucan by randomly cleaving an internal α-1, 4-glycosidic linkage to produce maltose and glucose for metabolic energy. Other insects which feed on storage seeds include *Callosobruchus chinensis*, *Tenebrio molitor* and *Zabrotes subfaciatus*. Researches on the starch digestion as a target for controlling of starch-dependent insects have been stimulated in recent years. Many results showed that α-amylase inhibitors from *Phaseolus vulgaris* seeds suppressed growth and development of *C. maculatus* and *C. chinensis* (Ishimoto and Kitamura, 1989 and Shade *et al.*, 1994).

Understanding the control mechanism of α-amylase activity in *C. maculatus* is essential for developing method for insect control, such as the use of an α-amylase inhibitor as a biocontrol agent and overexpression of α-amylase inhibitor in transgenic plant. The different forms of α-amylases in the insect midgut lumen have been observed in *C. maculatus* and *Z. subfasciatus* (Campos *et al.*, 1989 and Silva *et al.*, 1999). The patterns of the α-amylase expression varied in *Z. subfasciatus* feeding on different diets, apparently in response to the presence of antimetabolic proteins such as α-amylase inhibitors, rather than as a response to structural differences in the starch granules.

In this study, the *C. maculatus* α-amylase was purified by an affinity chromatography. Then, the purified enzyme was used to determine optimum conditions for enzyme activity and the molecular weight of the protein.

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METERIALS AND METHODS

Source of materials

Callosobruchus maculatus (cowpea weevil) was obtained from Insect Section of Stored Products Laboratory, Division of Entomology and Zoology, Department of Agriculture.

Purification of C. *maculatus* α-amylase

After 30 min freezing at -20 °C, 2 grams of C. *maculatus* were finely grounded in a deep cold mortar with 8 ml of 20 mM sodium phosphate buffer pH 7 and centrifuged at 10,000 xg for 20 min. 5 ml of supernatant was applied to an affinity chromatography column with a self-couple β-cyclodextrin sepharose 6B (1 x 5 cm). After the initial wash with 20 mM sodium phosphate buffer pH 7, the elution was carried out using 8 mg/ml β-cyclodextrin in 20 mM sodium phosphate buffer pH 7 as an eluting buffer.

α-amylase assay

α-amylase activity was measured using a method of Bernfeld (Bernfeld *et al.*, 1955). 100 μl α-amylase and 250 μl of 2% starch solution in 20 mM sodium phosphate buffer pH 6 containing 20 mM NaCl and 0.2 mM $CaCl_2$ were incubated at 50°C for 10 min. The reaction was terminated by adding 250 μl of 3, 5-dinitrosalicylic acid reagent, followed by boiling in water bath for 5 min. The reaction mixture was cooled and diluted with 2 ml of water and the absorbance was measured at 540 nm. One unit of enzyme activity was defined as the amount of enzyme that produced 1 μmol of reducing sugar in 1 min under the condition defined.

The α-amylase activity was also detected on a 12% native polyacrylamide gel electrophoresis (native PAGE). Proteins were placed on the native PAGE and run at 150 V for 1 h. Then the gel was immersed in 1% starch dissolved in respective optimum pH buffer for 1 h, followed by 2 min washing with distilled water. The gel was stained with 10 mM iodine in 14 mM potassium iodide for 5 min. Excess iodine was washed off with cold distilled water and the gel was soaked in 1% acetic acid after visualization of α-amylase activity bands.

Molecular weight determination

The proteins in the samples were separated on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in discontinuous buffer system as described by Leammli (Leammli, 1970). After the end of electrophoresis, the separated protein bands were visualized by Coomassie brilliant blue R- 250 staining.

Effect of pH and temperature on α-amylase activity

The effect of pH on the α-amylase activity was measured at different pH values. The pH was adjusted using the following buffers: glycine-hydrochloric (pH 2.0), citrate-phosphate (pH 3.0-5.0), phosphate (pH 6.0-8.0) and carbonate-bicarbonate (pH 9.0-10.0). 100 μl α-amylase and 250 μl of 2% starch solution in the buffer containing 20 mM NaCl and 0.2 mM $CaCl_2$ were incubated at 50°C for 10 min and the activity of the enzyme was measured.

The effect of the temperature on the α-amylase activity was determined by incubating 100 μl α-amylase and 250 μl of 2% starch solution in 20 mM sodium phosphate buffer pH 6 containing 20 mM NaCl and 0.2 mM $CaCl_2$ at different temperatures ranging from 20°C to 80°C for 10 min. The reaction was terminated by adding 250 μl of 3, 5-dinitrosalicylic acid reagent, followed by heating in boiling water bath for 5 min. The reaction mixture was cooled and diluted with 2 ml of water and the absorbance at 540 nm was monitored.

RESULTS AND DISCUSSION

Purification of *C. maculatus* α-amylase

The cruded protein extract from the *C. maculatus* larvae was purified by using β-cyclodextrin sepharose 6B affinity chromatography column. The chromatogram indicated that the α-amylase bound to the affinity column and could be eluted with β-cyclodextrin (Figure 1). The purification procedures were summarized in Table 1. The specific activity of the purified α-amylase was182.78 U mg protein^{-1} at 52 fold purification.

Zymogram pattern in the native gel revealed that *C. maculatus* α-amylase had only one isoform (Figure 2A). However, the number of α-amylases identified in different insect species varied from 1 to 8 isoforms e.g., *Helicoverpa armigera*, *Spodoptera litura*, *Callosobruchus chinensis* and *Carcyra cephalonica* exhibited more than five isoforms whereas *Sitophilus oryzae* and *Tribolium castaneum* possessed only one isoform (Sivakumar *et al*., 2006).

The molecular weight of the *C. maculatus* α-amylase was estimated to be 50 kDa on a basis of relative mobility on SDS-PAGE in comparison with standard marker proteins (Figure 2B).

Five milliliters of the crude enzyme was applied to the column. After washing with 20 mM sodium phosphate buffer pH 7, the elution was carried out with 8 mg/ml β-cyclodextrin in the washing buffer at a flow rate of 1ml/min.

Effect of pH and temperature on a-amylase activity

The effect of pH, from 3-10 on the α-amylase activity was measured. The optimum pH for α-amylase activity was found to be with in pH range of 5-6 in the phosphate buffer (Figure 3A). These findings were similar to those of several earlier reports showing pH optima for an α-amylase of 5-6 from *Vigna angularis* (Mar *et al*., 2003), *Z. subfasciatus* (Pelegrini *et*

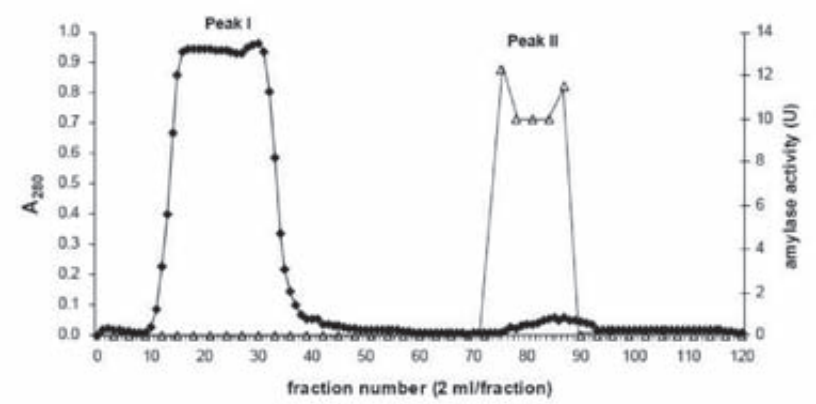

Figure 1 Chromatogram of the curded extract of *C. maculatus* α-amylase in a β-cyclodextrin sepharose 6B affinity chromatography column. The absorbance at 280 nm (♦) and amylase activity (Δ) were monitored.

Table 1 Purification of *C. maculatus* α-amylase.

Procedure	Protein (mg)	Activity (U)	Specific activity (U mg protein^{-1})	Yield (%)	Purification (fold)
Crude extract	85.49	301.45	3.53	100	1
β-cyclodextrin sepharose 6B	0.03	12	182.78	2	52

al., 2006) and *C. chinensis* (Podoler and Applebaum, 1971).

The α-amylase activity was determined at different temperatures ranging from 20°C to 80°C. The optimum temperatures were at 50-60°C. The enzyme activity gradually declined at temperatures beyond 60°C (Figure 3B). This condition was different from these found in other insect α-amylases, such as α-amylases from *Zabrotes subfasciatus*, *T. castaneum* and *T. molitor*, which showed higher activities at 37 °C (*Sivakumar et al*., 2006).

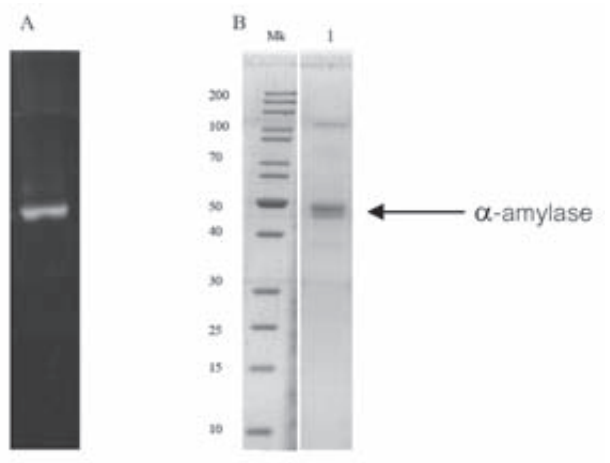

Figure 2 Electrophoretic analysis of *C. maculatus* α-amylase.
A. a zymogram of amylase in the native PAGE using 1% soluble starch as substrate and stained with KI-I_2 staining.
B. SDS-PAGE on 12% polyacrylamide gel followed by Coomassie brilliant blue R- 250 staining. The gels, Mk and 1 indicated the bands for marker proteins and an eluant from β-cyclodextrin sepharose column, respectively.

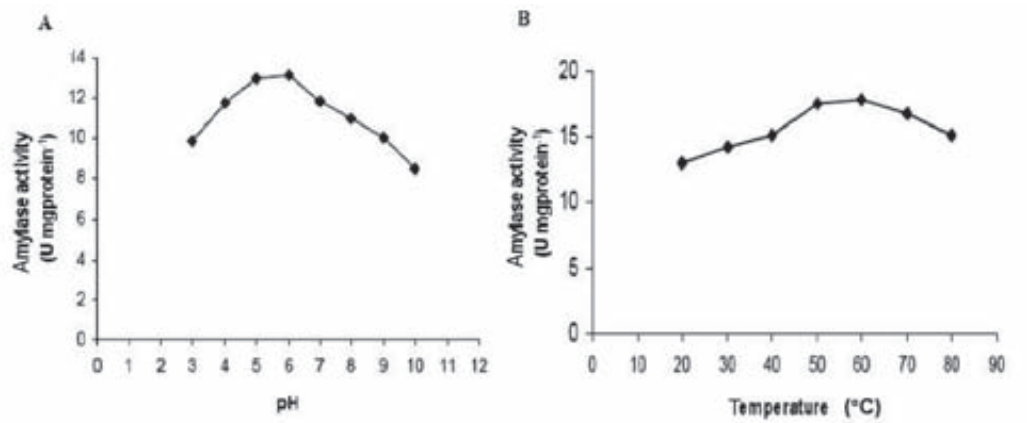

Figure 3 Effect of the pH (A) and temperature (B) on α-amylase activity.

CONCLUSION

The C. *maculatus* α-amylase could be purified by β-cyclodextrin sepharose affinity chromatography. C. *maculatus* had only one isoform of α-amylase with molecular weight of 50 kDa. The specific activity of the enzyme was 182.78 U mg protein^{-1}. The optimal conditions of pH and temperature for the a-amylase activity were 5-6 and 50-60 °C, respectively.

AKNOWNLEDGEMENT

This work was supported by the research grant from the Graduate School Kasetsart University.

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