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The β -galactosidase System of a Novel Plant from Durian Seeds (*Durio zibethinus*) I. Isolation and Partial Characterization

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Abstract: β -galactosidase was partially purified 2.16-fold with a total yield of 21.32% of the original activity by sequential use of ammonium sulfate precipitation and gel filtration through Sephadex G-100 from Durian fruit. A progressive increase in activity of the purified enzyme was observed up to 60°C accompanied by a decrease thereafter and the enzyme activity was linear with time at least up to 10 min reaction time, the maximum activity reached it after 20 min and still constant thereafter. An energy of activation of 3.04 Kcal/mole for the enzyme activity was derived from the Arrhenius plot. The optimum pH was 3.0. The purified enzyme started losing activity above 40°C when heated for 10 min. and became completely inactive at 80°C. Michaelis-constant of (K_m) values of 5.5mM and a maximum velocity (V_{max}) of 0.9 μ moles/mg/min. A Molecular weight (MW) determination of ~122 kDa was estimated by gel filtration methods using a Sephadex G-100. Fe^{+++} , Zn^{++} and Cu^{++} strongly inhibited the enzyme. However, Mg^{++} , Ca^{++} and Mn^{++} inhibited negligibly.

Key words: Durian fruit (*Durio zibethinus*), β -galactosidase, isolation, partial characterization.

Introduction

β -galactosidase (3.2.1.23) is commonly found in nature (Andresen and Barfoed, 1977; Shukla, 1975); and in the small intestines of young mammals, in seeds of almonds, peaches, apricots, apples, as well as in a large number of microorganisms. Among these are both bacteria (e.g. *E. coli*, various *Bacillus* and *Lactobacillus* species), yeast (e.g. *Kluyveromyces fragilis*) and mould (e.g. *Mucor* and *Aspergillus* species). Of special interest is its use in the treatment of milk to meet the needs of the large percentage of the worlds, population afflicted with β -galactosidase intolerance. On the other hand, large amount of cheese whey are produced as by-product of cheese industry all over the world. The problem of lactose in whey is mainly related to environmental pollution created, when large quantities of whey are discharged in drain. Hydrolysis of lactose in milk and milk products to glucose and galactose would solve the problem of milk intolerant people and in whey it would avoid environmental pollution and an interesting possibility of by-product utilization. Durian fruit (*Durio zibethinus*) which planted especially in South-East Asia, may weight up to 30 kg. The fruits are used for eating when reached the soft stage. Recently this plant has been planted in the Botanical Garden at Aswan, Egypt. Whereas, from preliminary experiments, it found several important enzymes in Dairy field (Abd-Rabou and Ismail, 1995 and Ismail *et al.*, 1999). The aim of this paper was to present a novel plant β -galactosidase after partial purification and characterization of β -galactosidase produced by Durian seeds (*Durio zibethinus*).

Materials and Methods

Chemicals: All reagents were purchased from Sigma Chemical Co. : USA) except for Sephadex G-100 which was obtained from Aldrich Chemical Co. Ltd., Gillingham, Dorest, England; Folin and Ciocalteu's Phenol reagent was obtained from BDH limited Poole, England.

Durian fruit (*Durio zibethinus*): Durian fruit (Figs. 1 and 2) was obtained from the Ministry of Agriculture, Cairo, Egypt.

Isolation of enzyme: One hundred grams of fresh seeds Durian fruit (*Durio zibethinus*) were ground in a mixer for 15 min at room temperature containing 500 ml of 0.05 M citrate

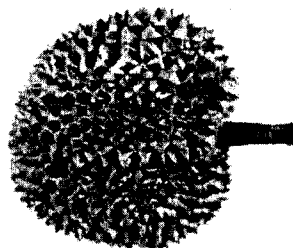


Fig. 1: Durian fruit (*Durio zibethinus*) plant (out side)



Fig. 2: Durian fruit (*Durio zibethinus*) plant (in side)

phosphate buffer, pH 5.0. The mixture was filtered through a Whatman filtrate paper No. 4 to remove the precipitation and the filtrate solution was used as an extract for purification procedure.

Production and purification of enzyme: The filtrate (21 mg protein) was fractionated in two steps with ammonium sulfate. Precipitates, formed at 50 and 60% saturation, were collected by centrifugation at 4000 x g for 20 min in a refrigerated centrifuge. The 50% pellet was discarded while

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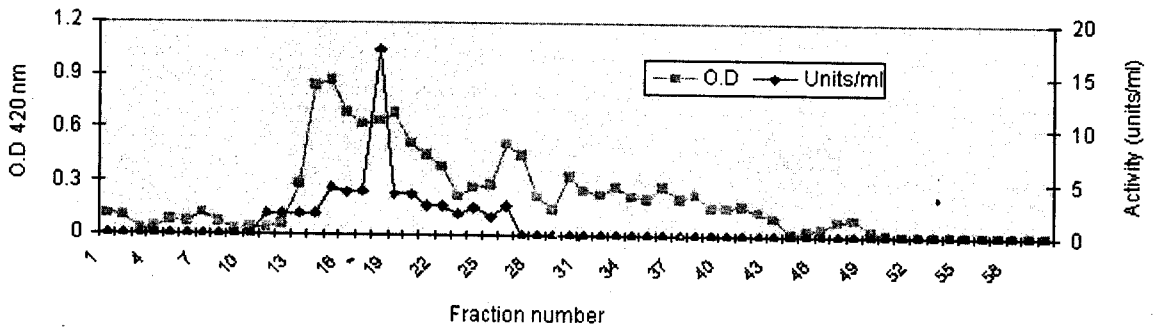


Fig. 3: Purification of β -galactosidase precipitation by ammonium sulfate on Sephadex G-100 from Durian seeds (*Durio zibethinus*)

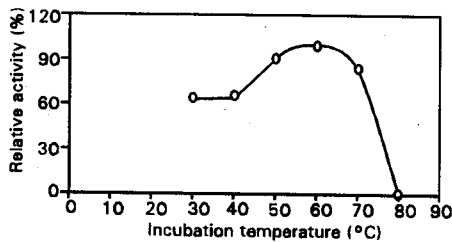


Fig. 4: Effect of incubation temperature on enzyme activity

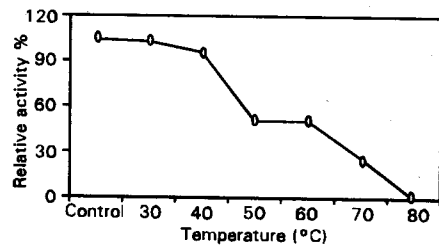


Fig. 7: Effect of heat on enzyme activity

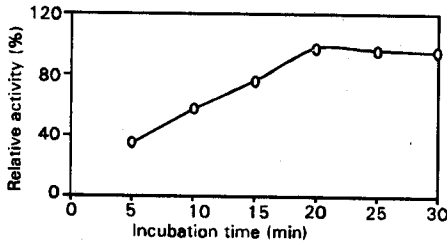


Fig. 5: Effect of incubation time on enzyme activity

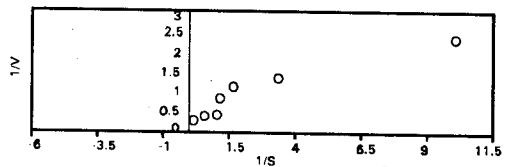


Fig. 8: Line weaver-Burk plot for ONPG hydrolysis

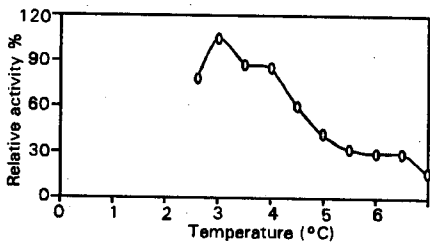


Fig. 6: Effect of pH on enzyme activity

the 60% was dissolved in 0.05 M citrate phosphate buffer, pH 5.0, dialyzed against the same buffer for 24 h and filtered prior to chromatography on a column (45x2.5 cm²) of Sephadex G-100 (Pharmacia, Uppsala, Weeden) with the same buffer for further purification. Fractions of 5 ml were collected at a flow rate of 1 ml / min and analyzed for protein and protease activity. Enzyme fractions with high specific activity were pooled, and stored at 4°C.

Measurement of enzyme activity: To determine β -galactosidase activity, the method of Hemme and Jette (1980) was used. Liberated o-nitrophenol (ONP) was measured spectrophotometrically at 420 nm. One enzyme unit (EU) is defined as the quantity of enzyme that catalyzes the liberation of 1 μ mol ONP from Ortho nitrophenyl- β -D-galactopyranoside (ONPG) per min under assay conditions.

Protein content determination (PC): Protein content (PC) was determined colourimetrically at 650 nm according to Ohnisti and Barr (1978), using bovine serum albumin as standard.

Buffers: Citrate phosphate and phosphate buffers were prepared according to Gomori (1955). Moreover, final pH was checked using pH-meter 646 with glass electrodes, Ingold, Knick, Germany.

Characterization of purified enzyme

Effect of incubation temperature: Standard reaction mixtures set at different temperatures were incubated at 10°C intervals from 30 to 90°C for 10 min. Energy of activation of enzyme was determined from the slope of an Arrhenius plot of activity measurements.

Table 1: Purification of β -galactosidase from Durian seeds (*Durio zibethinus*)

Ammonium sulfate Conc. %	Volume of fraction (ml)	Enzyme activity (units/ml)	Total activity	PC (mg/ml)	Sp. activity (units/ml)	Yield (%)	Purification factor
Initial extract	100	11.28	1128	0.207	54.49	100	1
50-60 pp	15	9.577	143.66	0.102	93.89	12.74	1.72
Purified enzyme	30	8.23	246.9	0.07	117.57	21.32	2.16

Table 2: Effect of metal ions on purified enzyme activity

Reagents (1mM)	Enzyme activity (Units/ml)	Inhibition (%)
None	11.07	0
Ca ⁺⁺	9.32	15.81
Mg ⁺⁺	4.55	58.9
Zn ⁺⁺	2.43	78.05
Fe ⁺⁺⁺	2.30	79.22
Cu ⁺⁺	2.36	76.68
Mn ⁺⁺	9.78	11.66

Effect of reaction time on the activity: Standard reaction mixtures were set at different intervals for a period of 45 minutes, at incubation temperature 30°C.

Heat stability of purified enzyme: The purified enzyme was exposed to different temperatures at 5°C interval from 30 to 80°C for 10 min holding time at each temperature, prior to the enzyme assay which was carried out at 30°C

Optimum pH of purified enzyme: In this respect 0.05 M Citrate phosphate buffers with different pH values ranging between 2.6 to 7.0 were used. The reaction mixture was incubated for 10 min at 30°C.

Michaelis-Menten constant: ONPG solution was diluted with 0.1M phosphate buffers pH 7.0 ranging between 0.11 and 10 mM/ml in the reaction mixture. Values for K_m and maximum velocity (V_{max}) were determined from the initial velocity (V_o) of the reaction at previous concentration. The data were treated graphically by the procedure of Line weaver and Burk (1934).

Molecular weight determination: The molecular weight (MW) of the purified enzyme was estimated by gel filtration methods using a Sephadex G-100 under the same conditions; these were Bovine serum albumin (67,000 daltons), crystalline insulin (232,000 daltons) and Catalase (60,000 daltons) were used as standard proteins.

Effect of metal ions: A number of metal ions, in the form of chlorides or sulfates compounds were applied at a concentration rate of (10^{-2} Moles/l) on the enzyme activity.

Results and Discussion

β -galactosidase purification: The ammonium sulfate precipitated enzyme (50-60%) saturation on dialysis yielded 12.74% recovery and 1.72-fold purification of the enzyme. When the ammonium sulfate precipitated enzyme chromatographed on a column of Sephadex G-100, the enzyme was eluted as a highest single peak at fraction number 19 concomitant with the major protein peak (Fig. 3). The other minor protein peak was devoid of β -galactosidase activity. Results from a typical purification procedure show that the enzyme was purified 2.16-fold with a total yield of 21.32% of the original activity (Table 1).

Effect of incubation temperature: A progressive increase in activity of the purified enzyme was observed up to 60°C. A rapid decrease in activity was observed thereafter (Fig. 4). An energy of activation of 3.04 Kcal/mol for the enzyme activity was derived from Arrhenius plot of initial velocity (V_o) across a temperature ranging from 30 to 60°C. Sorensen and Crisan (1974), have also reported a similar optimum temperature of 61°C for a β -galactosidase from *Mucor pusillus*. The activation energy was estimated to be 39.1 KJ/mol (Amarita *et al.*, 1995).

Effect of reaction time on activity: Fig. 5 shows that the enzyme activity was linear with time at least up to 10 min reaction time. It reached the maximum relative activity (100%) after 20 min reaction time and still constant thereafter. The obtained results are in agreement with Abd El-Aty (1989).

Optimum pH: Fig. 6 shows that the purified enzyme exhibited optimum activity at a highly acidic value corresponding to pH 3.0. The activity decreased thereafter with the increase in pH of the buffer reaching about 12.62% of the maximum activity at pH 7.0. The optimum pH is similar to that of Jack bean (*Canavalia ensiformis*) (White and White, 1997), Tomato fruit (Carey *et al.*, 1995) and Kiwi fruit (*Actidia deliciosa*) (Ross *et al.*, 1993 and Moharam, 1991), who found optimum pH 3 for purified enzyme activity from *Mucor humicolus* at the range of 3.0 – 3.5; whereas it differs from the optimum acidic pH in case of *Leuconostoc citrovorum* (Singh *et al.*, 1979), and *Bacillus macerans* (Ismail *et al.*, 1997).

Heat stability: It was observed to be susceptible to heat treatment as it started losing activity above 40 °C when heated for 10 min (Fig. 7). It lost about 50% of its activity around 60°C and completely inactivated at 80°C. These observations are similar to the results reported by Ismail *et al.* (1997) on heat stability of the enzyme preparation from *Mucor pusillus*.

Michaelis-Menten constant: The K_m of purified enzyme was approximately 5.5 mM and the V_{max} of the reactions was 0.9 μ moles/mg when ONPG was used as a substrate (Fig. 8). Similarly K_m values for β -galactosidase from *Mucor humicolus* (Moharam, 1991), and psychrotrophic enterobacterium *Buttiauxella agrestis* (strain NC4) were reported to be 11 μ moles and 85 μ moles/mg protein for K_m and V_{max} , respectively (Amarita *et al.*, 1995).

Molecular weight: The Molecular weight of β -galactosidase was calculated by gel filtration on Sephadex G-100 to be 122 kDa. Ismail *et al.* (1997) and Ross *et al.* (1993) have also reported a similar β -galactosidase molecular weight of approximately 60 kDa by gel permeation and consists of several basic isoforms. Several polypeptides were enriched during purification, with 33-, 46-, and 67-kDa bands being predominant after SDS-PAGE.

Effect of metal ions: The purified enzyme was assayed with

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different metal ions. Fe^{+++} , Zn^{++} and Cu^{++} strongly inhibited the enzyme. However, Mg^{++} , Ca^{++} and Mn^{++} inhibited negligibly (Table 2). The enzyme in the present study was not stimulated by any of cations tested. The enzyme thus behaves similar to the enzyme from *Leuconostoc citrovorum* (Singh *et al.*, 1997).

The results of this study indicate that Durian seeds (*Dour zibethinus*) produce β -galactosidase which can be purified to homogeneity by sequential use of ammonium sulfate precipitation and gel filtration chromatography on Sephadex G-100, and these enzymes could be applied in manufacture of different types of dairy products (sweet milk products e.g. milk, ice cream; cultured milk products e.g. yogurt, cottage cheeses; Ripened cheese e.g., Cheddar, Camembert).

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