β-galactosidase from Durian Seeds *(Durio zibethinus)* and its utilization in ice milk production

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Abstract: Durian fruit *(Durio zibethinus)* which planted especially in South-East Asia, may weight up to 30 kg, The fruits is used for eating when it 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 such as β-galactosidase (3.2.1.23). This enzyme 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. d A progressive increase in activity of the purified enzyme was observed up to 60°C as incubation temperature, rapid it decrease in activity 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 of initial velocity (Vo) across a temperature ranging from 30 to 60°C. The Optimum pH was pH 3.0. The purified enzyme was susceptible to heat treatment as it started loosing activity above 40 °C when heated for 10 min. It lost about 50% its activity around 60°C and completely inactivated at 80°C. Michaelis-constant of (K_m) values of 5.5*mM* and a maximum initial velocity (V_{max}) of 0.9 μmoles/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. On the other hand the use of this enzyme in ice milk production was studied. Different concentration of the enzyme was added to fresh pasteurized milk. Results clear indicate that the addition of the enzyme to ice milk mixes increased the sweetness although the properties of body and texture and appearance of the resultant ice milk slightly decreased.

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Keywords: Durian fruit *(Durio zibethinus),* β-galactosidase, Isolation, Partial characterization, ice milk.

1. Introduction

β-Galactosidase (3.2.1.23) is commonly found in nature (Andresen and Barfoed, 1977) and (Shukla, 1975); 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 world population afflicted with β-galactosidase intolerance. On the other hand, large amount of cheese whey are produced as a 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 the 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 of for 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 is used for eating when it 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. 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).*

2. Materials and Methods

Milk and skim milk powder:

Fresh Buffalo's milk and skim milk powder were obtained from the Research Institute of Animal Production, Agricultural Research Center, Ministry of Agriculture, Dokki, Giza. Egypt. Sucrose and vanilla was obtained from local market. Gelatin was obtained from BDH Chemicals Ltd Pool England.

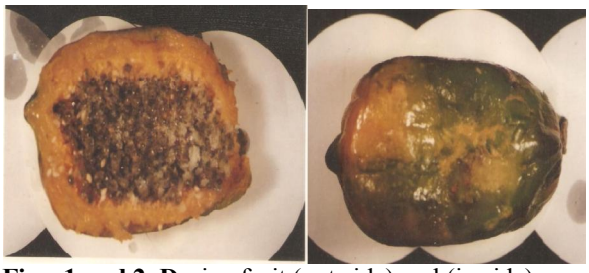
Chemicals:

All reagents were purchased from Sigma Chemical Co.; USA) except for Sephadex G-100 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 *(Durio zibethinus)* was obtained from the Ministry of Agriculture, Cairo, Egypt (Figs 1 and 2).



Figs. 1 and 2. Durian fruit (out side) and (in side)

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 in 0.05 M citrate phosphate buffer, pH 5.0.The mixture was filtrated through a Whatman filtrate paper No. 4 to remove the precipitation and the filtrate solution was as an extract used 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, the 60% pellet was dissolved in 0.05 M citrate phosphate buffer, pH 5.0, dialyzed against the same buffer for 24 h and filtered prior to chamatography on a column (45 x 2.5 cm) of Sephadex G -100 (Pharmacia, Uppsala, Sweeden) with the same buffer for further purification. Fractions of 5 ml were collected at a flow rate of 1ml / min and analyzed for protein and protease activity. Enzyme fractions with high specific activity were pooled, and stored at 4^{o}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 µmol ONP from Orthonitrophenyl -β-D-galactopyranoside (ONPG) per min under assay conditions.

Protein content determination (PC):

Protein content (PC) was determined colourmeterically at 650 nm according to Ohnistti and Barr (1978) using bovine serum albumin as standard.

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

Characterization of purified enzyme

Effect of incubation temperature:

Standard reaction mixtures set at different temperature were incubated at 10^{o}C intervals from 30 to 90^{o}C for 10 min. Energy of activation of enzyme was determined from the slope of an Arrhenius plot of activity measurements.

Effect of reaction time on the activity:

Standard reaction mixtures set at different intervals for a period of 45 minutes, at incubation temperatures 30^{o}C.

Heat stability of purified enzyme:

The purified enzyme was exposed to different temperature at 5^{o}C interval from 30 to 80^{o}C for 10 min holding time at each temperature prior to the enzyme assay which was carried out at 30^{o}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^{o}C.

Michaelis - Menten constant:

ONPG solution was diluted with 0.1*M* phosphate buffers pH 7.0 ranging between 0.11 *mM* and 10 *mM*/ml in the reaction mixture. Values for Km and Vmax were determined from the initial velocity (V_o) of the reaction at pervious concentration. The data were treated graphically by the procedure of Lineweaver 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:

In this experimental a number of metal ions, in the form of chlorides or sulfates compounds at a concentration of (10^{-2} Moles/l) on the enzyme activity.

Ice milk preparation:

The Ice milk was made as described by Ismail *et al*. (1997). Ice milk mixture consisted of 3% fat, 12% skimmed milk powder, 12% sucrose, vanilla and 0.5% gelatin. Before addition of mixes, levels of extracts of β-gal used were 12.3 (TI), 24.6 (TII) and 36.9 (TIII) units of β-gal /ml pasteurized milk, were added and incubated for 2 hr at 40^{o}C. The treated milk was refrigerated at 4^{o}C overnight. A control without β-gal was treated similarly.

Chemical analysis of Ice milk:

In ice milk samples, lactose content was measured according to (Barrent and Abd El-Tawab (1958). Specific gravity was determined by means of a

pycenometer bottle as described by Winton (1958). Weight per gallon was calculated in pounds according to Bruke (1947) by multiplying the specific gravity of ice milk by the factor 8.34. Overrun was determined by the method mentioned by Sommer (1951).

Organoleptic properties:

Ice milk samples from all treatments were scored for flavors, body & texture and appearance by regular score panels chosen from the staff members of N.R.C., according to the score card given by Nelson and Trout (1951).

3. 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 single peak 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).

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

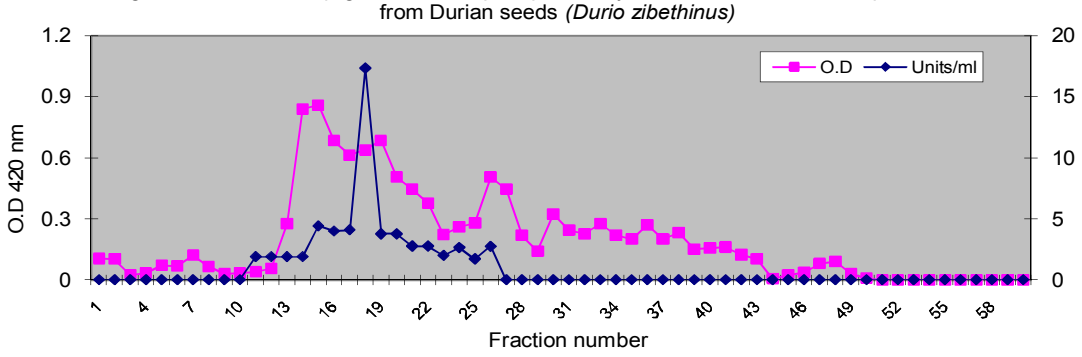


Fig. 4: Effect of incubation temperature on enzyme activity

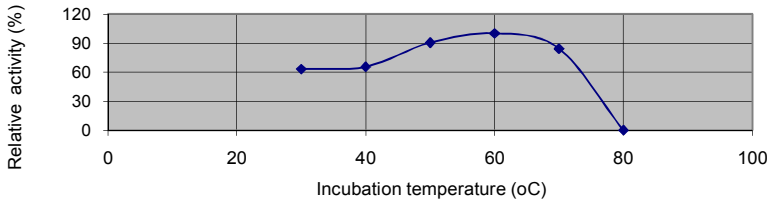
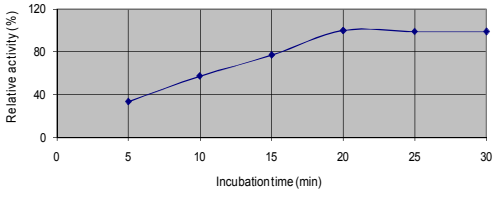


Fig. 5: Effect of incubation time on enzyme activity



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 the 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^{-1} (Amarita *et al*., 1995).

Table 1: Purification of β- galactosedase 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**

Effect of reaction time on the activity:

Fig. (5) shows that the enzyme activity was linear with time at least up to 10 min reaction time. It reached the maximum activity (34.44 Units/ml) 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 of 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, John Stephen 1997), Tomato fruit (Carey *et al.,*1995) and Kiwi fruit (*Actiidia deliciosa*) (Ross *et al.,* 1993) and Moharam M.(1991), who found that the optimum pH for purified enzyme activity from *Mucor Humicolus* was 3.0 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:

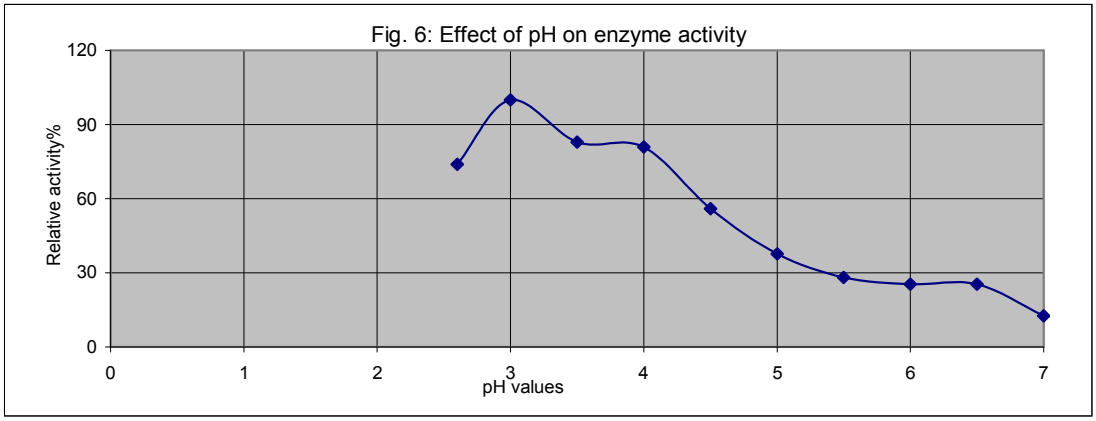
It was observed to be susceptible to heat treatment as it started loosing activity above 40 °C when heated for 10 min (Fig.7). It lost about 50% its activity around 60°C and completely inactivated at 80°C. These observations are similar to the results reported by Ismail *et al.,* (1997) on heat stability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

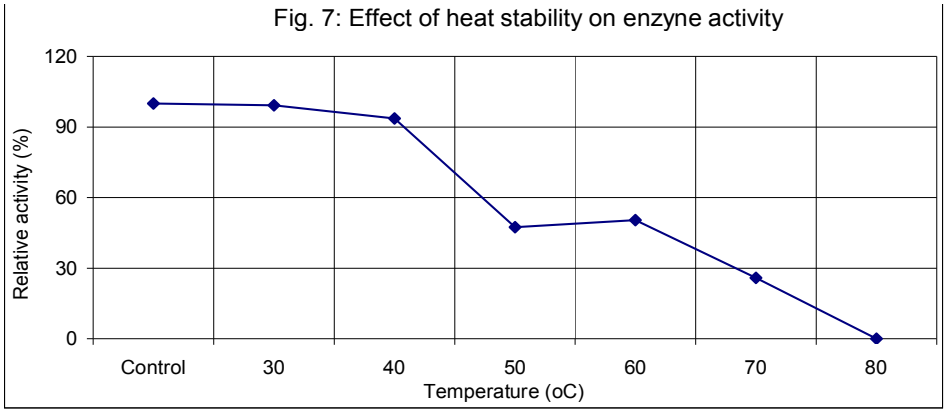
Michaelis–Menten constant:

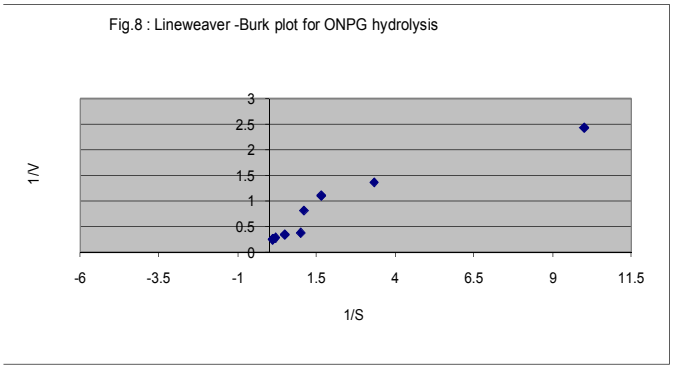
The K_m of purified enzyme was approximately 5.5 *mM and the* V_{max} of the reactions was 0.9 µmoles/mg when ONPG used as a substrate (Fig. 8). Similarly Km values for β-galactosidase from *Mucor humicolus (*Moharam M.,1991), and psychrotrophic entrobacterium *Buttiaauxella agrestis* (strain NC4) were reported to be 11 µ moles and 85 µ moles/mg protein for K_m and $V_{ma,}$ 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 bandsbeing predominant after SDS PAGE.







Effect of metal ions:

The purified enzyme was assayed with 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 (Table 2).

The enzyme thus behaves similar to the enzyme from *Leuconostoc citrovorum* (Singh *et al.,*1997).

Effect of β-gal on milk lactose content and ice milk:

Table (3) represents the data recorded as a response for adding different levels of β-gal to fresh pasteurized milk. Also, these data reveal that the higher the amount of β-gal TIII (33.6 Units/ml), the more hydrolyzed lactose (3.67 %). Some references showed that increasing enzyme concentration increased the rate of hydrolysis as reported by Ismail *et al*. (1997) and El-Hofi (2003).

Data in Table 3 show too the specific gravity, weight per gallon and overrun. The results indicated that both specific gravity and weight per gallon increased with the Increase of β-gal units. It could also be noticed that ice milk mixes had higher values of specific gravity and weight per gallon than that of the resultant ice milk. It was due to incorporation of air by whipping during the freezing process. In addition Table 3 showed that increasing β-gal decreased the overrun values. This can be attributed to the decrease in the amount of incorporation air. This is in full agreement with that of Ismail *et al*. (1997)**.**

Organoleptic properties:

The results indicated that the average score points of TII and TIII decreased as the level of β-gal increased except TI (Table 4). The highest value is recorded with TI this may be due to the increase in the sweet taste based on the hydrolysis of lactose by the β-gal, such as taste may be more preferable by the Egyptian consumer. The average score points of body and texture decrease by increasing the level of added β-gal, being 32.0, 26.6 and 25.0 points for treatments containing 12.3, 24.6 and 36.9 units of β-gal /ml pasteurized milk, respectively, while it was 30.0 for the control (Table 4). This mainly due to the increase in the values of viscosity, also to the decrease in the overrun for all β-gal treatments (El-Tanboly, E. 2004).

Table 3. Characters of ice milk treated with β-gal

Treatment	Lactose content (%)	Hydrolyzedlactosecontent (%)	Hydrolysisrate (%)	Specificgravity	Weight/gallon (Ib)	Overrun (%)
Control	4.87	0	0	0.552	4.312	77.68
I	3.53	1.27	24.85	0.676	5.600	69.85
II	2.62	2.47	52.22	0.783	6.217	57.11
III	1.55	3.67	68.86	0.877	7.501	45.06

Control: ice cream with no enzyme; TI: ice cream with added β-gal extracts (12.3units/ml); TII ice cream with added β-gal extracts (24.6 units/ml); TIII: ice cream with added β-gal extracts (36.9 units/ml)

Table 4. Effect of different β-gal levels on sensory scores of ice cream

Treatment	Appearance (10)	Flavor (50)	Body & texture (40)	Total (100)
Control	7.0	40.5	30.0	77.5
I	8.8	44.6	32.0	85.4
II	5.5	34.8	26.6	66.9
III	7.0	30.4	25.0	62.4

Concluisson

The results of this study indicate that Durban seeds *(Dour zibethinus* produces 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 ice milk.

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

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