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Extraction of β-galactosidase and β-glucosidase from the seeds of *Tamarindus indica*

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Abstract

The enzymes β–galactosidase and β–glucosidase were extracted from the tamarind seeds using different buffers at different pH. Highest activity was obtained with 10 mM sodium acetate buffer, pH 5.6 and 10 mM tris buffer, pH 7.4. The effect of NaCl and Triton X–100 at different concentrations on the extraction of the enzymes indicated 10 mM sodium acetate buffer, pH 5.6 containing 1 M NaCl as a better extractant of the enzyme. The enzyme assay was carried out using p–nitrophenyl–β–D–galactoside and p–nitrophenyl–β–D–glucoside as substrates. Highest enzyme activities were observed on 6th and 24th day of germination. The protein content gradually decreased upto 5th day of germination and suddenly increased on 6th day. However, on subsequent days of germination, the protein content greatly decreased upto 11th day. During the latter period of germination (18th day onwards) the content remained almost constant. The kinetic parameters varied for both β–galactosidase and β–glucosidase. The activity of β–galactosidase was show to have an optimal operating condition at pH 5.5 and a temperature of 50°C. The thermostability of the enzyme was in the range of 40°C – 70°C with the ^{pH} stability in the range of 5.0 – 7.0. The Km and Vmax values for pNPGal were determined as 66μM and 2.27nmolesmin^{-1}. In contrast the activity of β–glucosidase was shown to have an optimal operating condition at pH 5.0 and a temperature of 30°C. The thermostability of the enzyme was in the range of 27°C – 35°C with the pH stability in the range of 4.0 – 7.0. The Km and Vmax values for pNPGlu were determined as 121μM and 5.26nmolesmin^{-1}. The presented study is a preliminary work carried out for the standardization of protocols. The purification and characterization of β–galactosidase and β–glucosidase is under progress.

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Introduction

The tamarind tree (*Tamarindus indica* L.) belonging to family Caesalpinaceae is found in both tropical and subtropical regions of the world. It is grown extensively in the dry tracts of Central and South Indian States for its sour fruit pulp, which is used extensively in the local confectionary industry and is a common article of trade in India. The seed comprises 20 – 30 % seed coat or testa and 70 – 75 % kernel or endosperm (Coronel, R.E. (1991); (Shankaracharya, N.B. (1998)). The kernel contains 55 – 70 % polysaccharide, 16 – 20 % proteins, 5 – 7 % oils, and minerals (Shlini and Siddalinga Murthy, 2011)).

The post-germinative mobilization of seed xyloglucans has been studied both ultrastructurally and biochemically (Buckeridge *et al*., 1992; Edwards *et al*., 1985; Reid *et al*., 1987). Nasturtium xyloglucan mobilization involves the simultaneous action of four enzymes, a xyloglucan-specific endo-(1→4)-β-D-glucanase or xyloglucan endo-transglycosylase (XET) (Edwards *et al*., 1986; Fanutti *et al*., 1993, 1996; Farkas *et al*., 1992; de Silva *et al*., 1993), a β-galactosidase with action both on polymeric xyloglucan and its subunit oligosaccharides (Edwards *et al*., 1988; Reid *et al*., 1988), a xyloglucan oligosaccharide-specific α-xylosidase or oligoxyloglucan exo-xylohydrolase (Chengappa *et al*., 1993; Fanutti *et al*., 1991), and a β-glucosidase (Edwards *et al*., 1985).

β-Galactosidases (EC 3.2.1.23), a widespread family of glycosyl hydrolases, are characterized by their ability to hydrolyze terminal, non-reducing β-D-galactosyl residues from β-D-galactosides. Exoglycosidases are widely distributed in many species, including microorganisms, animals and plants. These enzymes are useful in determining the anomeric configuration and sequence of glycoconjugates. β-D-Galactose is commonly found in glycoproteins and glycolipids. Most β-Galactosidases isolated so far, are from microbial sources (Erick and Steers, 1970; Steers *et. al*,. 1971; Glasgow *et al*., 1977; Hirata *et al*., 1984; Tanaka *et al*., 1988; Nagano *et al*., 1992; Shigeta *et al*., 1983). Plant galactosidases have been characterized from jack bean (Li *et al*., 1975), and from wheat grain (Carratu *et al*., 1985; Papet *et al*., 1992). α and β-mannosidase, and β-galactosidase were identified from green onion (Kim *et al*., 1991b).

In plants, β-glucosidases (EC 3.2.1.21) and related glycosidases play roles in many biological processes, including defence, lignifications, phytohormone activation and cell-wall modification (Esen, 1993). Their physiological function depends upon their location and substrate-specificity. Most plant β-glucosidases belong to glycosyl hydrolase family 1 (GH1), which also includes myrosinases (thio-β-glucosidases), β-mannosidases. β-galactosidases, phospho-β-glucosidases and phospho- β-galactosidases (David *et al*., 2000).

Glucosidases and galactosidases are distributed widely in soils and are important enzymes in the soil carbon cycle. Although some research has been done on β-glucosidase in some soil types or ecosystems, few have been conducted in paddy soils.

Numerous studies have shown that β-galactosidases catalyse the hydrolysis of terminal galactosyl residues from carbohydrates, glycoproteins and galactolipids (David *et al*., 2000). β-Galactosidases action has been proposed to release stored energy for rapid growth (lactose hydrolysis in mammals and bacteria, xyloglucan mobilization in cotyledons), release free Gal during normal metabolic recycling of galactolipids, glycoproteins, and cell wall components, and degrade cell wall components during senescence (Lo *et al*., 1979; Bhalla and Dalling, 1984; Maley *et al*., 1989; Raghothama *et al*., 1991; De Veau *et al*., 1993; Ross *et al*., 1993; Buckeridge and Reid, 1994; Hall, 1998). Many β-galactosidases have specific biosynthetic activities such as transglycosylation and reverse hydrolysis under favorable thermodynamic *in vitro*

conditions (Bonnin *et al*., 1995; Yoon and Ajisaka, 1996).

Much attention has been focused on the enzyme β-galactosidase, which is involved in the bacterial metabolism of lactose. In addition to normal hydrolysis of the β-D-galactoside linkage in lactose, some β-D-galactosidase enzyme may catalyze the formation of galactooligosaccharides through transfer of one or more D-galactosyl units onto the D-galactose moiety of lactose. This transgalactosylation reaction (Huber *et al*., 1976) has been shown to be a characteristic of β-galactosidase enzyme from a great variety of bacterial and fungal species (Dumortier *et al*., 1994; Nakao *et al*., 1994; Onishi *et al*., 1995; Yoon *et al*., 1996.).

The present investigation is aimed at extracting β-galactosidases and β-glucosidases from Tamarind seeds during germination. These enzymes from other sources are unsuitable because of high cost. The enzyme β-galactosidases and β-glucosidases obtained from agricultural product such as the seeds of Tamarind is a heat stable enzyme and can be commercially exploited for the production of the enzyme. In the current study, β-galactosidases and β-glucosidases of *Tamarindus indica* seeds has been examined as a new source for producing these enzymes.

Material and methods

Plant material

The seeds of *Tamarindus indica* were collected using random sampling technique (RST) from local areas of Bangalore district, Karnataka State, India. After dehulling the fruits, equal samples of seeds were combined to give one bulk population sample from which sub samples were taken for test. Collected seed samples were dried in the sunlight for 24 hrs. After removing immature and damaged seeds, the dried matured seeds were washed under tap water, dried and stored in plastic containers or refrigerator until further use.

Enzyme extraction

All procedure were carried out at 4° C. Unless otherwise specified, endosperm tissue was homogenized (1g/10ml) with 0.05 M sodium acetate buffer, pH 5.6 and 0.01 M Tris-HCl buffer, pH 7.4 and also with the same buffers containing different concentrations of NaCl (0.2, 0.5, 1.0, 2.0, and 4.0 M NaCl) and 0.5 % Triton X-100, respectively. The homogenate was centrifuged at 6000g for 20 mins and the supernatant was used for further assay.

Protein assay

Protein content was determined according to the method of Lowry *et al*. (1951) with bovine serum albumin as standard.

Enzyme assay

β-glucosidase assay is based on the measurement of the amount of p-nitrophenol formed. The enzyme reaction was initiated by adding 0.25 ml of the extract to 0.75 ml of 1.2 mM p-nitrophenyl-β-D-glucoside (PNPG) in 10Mm acetate buffer, pH 5.6 and incubated at 37° C for 30 mins. The reaction was stopped by adding 4.0 ml of 0.1 M sodium hydroxide. The amount of p-nitrophenol liberated is measued at 440 nm. One enzyme unit corresponds to 0.5µmoles of ρ-nitrophenol/min.

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Kinetic parameters

The optimum incubation period was determined by measuring enzyme activity between 00-30mins.The

optimum pH was determined in reactions carried out at pH values ranging from 2.0 - 9.0 using 0.2 M buffers – Acetate (pH 2.0, 2.5, 3.0, 3.5, 4.0 and 5.0); sodium phosphate (pH 5.2, 6.0 and 7.0); Tris-HCl (pH 7.0, 8.0 and 9.0). The pH stability was determined by preincubating the enzyme in the above buffers for 30mins and assaying the enzyme at pH 5.6. Optimum temperature was determined by assaying the enzyme at different temperatures (7°C – 95°C). Temperature stability was determined by preincubating the enzyme for 30 mins at different temperatures and assaying the enzyme at 37°C. Michaelis-Menten constants were determined using different substrate concentration (0.03-0.15µmoles/ml).

Germination studies

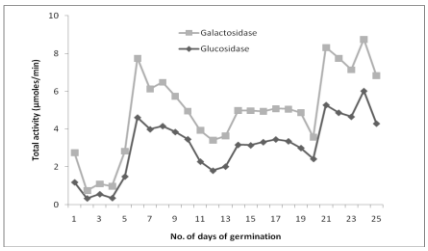
Fifty healthy Tamarind seeds were soaked in cooled 50% H_2SO_4 for 60mins and then dispensed into the soil (1:1 ratio of acid washed sand and cocco peat). Germination of seed was monitored. Cotyledons were collected everyday starting from 1st day till 25th day. Proteins were extracted as mentioned above and analysed on PAGE. The samples were checked for enzyme activity.

Polyacrylamide Gel Electrophoresis (PAGE)

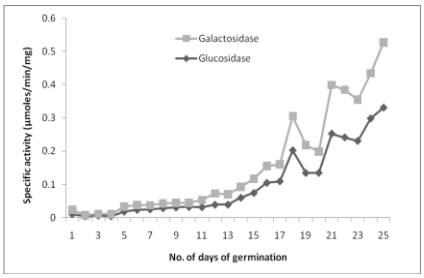
Native-PAGE of 7.5% resolving was performed as described by *Flurkey*. Duplicate samples were run for determination of protein bands. The gel was stained with 0.1% Coomassie Brilliant BlueR-250, destained and visualized the bands.

Results and discussion

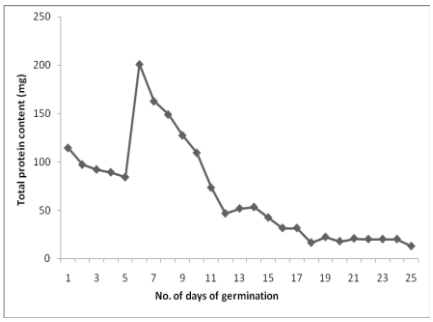
β-galactosidase are known to increase during germination. The activity of some glycosidases in germinating mung bean seeds increased to a high level between days 4-6. The enzymes which increased during germination in mung bean are β-N-acetylglucosidase, β-galactosidase and α-mannosidase. The increase of β-galactosidase activity during seed germination was also reported in plants such as pinto beans (Agrawal and Bahl, 1968), castor beans (Harley and Beevers, 1985), nasturtium (Edwards *et al*., 1988), barley (Giannakouros *et al*., 1991) and lupin (Buckeridge and Reid, 1994). The increase of β-galactosidase activity during the germination of barley seeds seem to be very moderate when compared with the many fold increase in the activity of the same enzyme observed in the seeds of dicotyledon plants (Giannakouros *et al*., 1991). Thus, the developmental regulation of plant β-galactosidase during germination and growth has become evident. However, much less is known about the mechanism underlying this regulation.



(a)



(b)



(c)

Fig 1. Summary of germination profile. (a) Total activity (b) Specific activity (c) Protein.

On the otherhand, the degradation of the cell wall polysaccharides, primarily β-glucans, is a rate-limiting step in the mobilization of energy reserves in the seed.

These cell wall degradation products may also provide a significant source of carbohydrate for the germinating seedling. The complete hydrolysis of β-glucans is facilitated by several enzyme activities. These include endo-(1-4)-β-glucanase, endo-(1-3)-β-glucanase, and endo-(1-3, 1-4)-β-glucanase capable of hydrolyzing most of the endosperm β-glucans to a mixture of β-linked oligosaccharides. In addition, β-glucosidase activities may be required to further degrade β-linked oligosaccharides to glucose (Stone and Clarke, 1992).

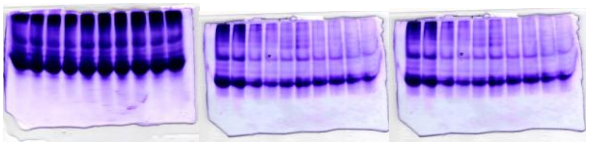
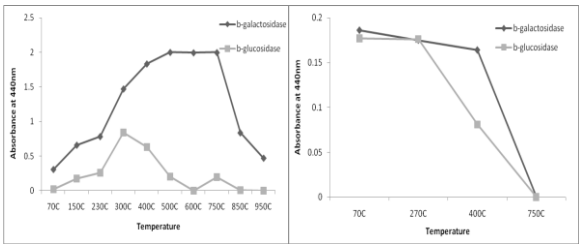


Fig 2. Summary of germination profile. Native – PAGE of protein pattern of germinated seed sample of 1- 30 days.

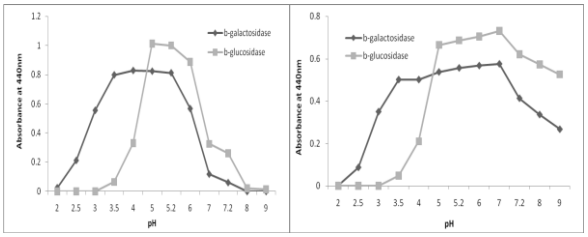
In case of our study, the activity of both enzymes increased during the first and third week of germination (Fig 1 a). The activity of the enzymes were high on 6th day which gradually decreased from 7th to 12th day and almost stable for about 5 days between 13th to 19th day and again the activity was high on 21st to 23rd day of germination. By the end of 25th day, the shoot starts developing with well grown leaves. The seeds during this stage almost dettaches from the shoot and falls down. The size of the seed is reduced on 25th day when compared to the seed during initial stage of germination. The observed variation in β-galactosidase and β-glucosidase activity during germination of tamarind seed indicates that the enzyme produced by the source is utilized for the development of the seeds. Further constant in the graph during the 2nd week of germination indicates that the seed is well nourished with the nutrients supplied by the action of the enzymes. Further as observed, the β-galactosidase and β-glucosidase activity increased in the 3rd week of germination implies the utilization of nutrients for the development of shoot and leaves. In constrast, the specific activity of β-galactosidase gradually increased during the course of germination (Fig. 1b). On contrary, the total protein content gradually decreased after 1st week of germination (Fig. 1c). For further analysis, 20 - 23 days old plants endosperm was used.



(a) (b)

Fig 3. Effect of temperature at pH 5.6 on activity and thermal stability of the enzyme β- galactosidase and β-glucosidase from tamarind seeds. The results are mean values of three determinations.

The protein pattern on PAGE did not show any change in the number of bands (Fig. 2). The profile was same starting from the first day to the twentyfifth day of germination. But the intensity of the bands decreased which indicates that the amount of protein produced by the seed is as compared to the initial days of germination i.e., 12mg/ml to .72mg/ml.



(a) (b)

Fig 4. Effect of pH on activity and pH stability of the enzyme β-galactosidase fromtamarind seeds. The results are mean values of three determinations.

Further the influence of different molarity and percentages of NaCl and Triton-X-100 on enzyme extraction was investigated and the enzyme activity significantly increased with NaCl (Table1). Therefore the different NaCl concentrations on enzyme activity

showed that 1.0M NaCl was apt for the extraction of enzyme with high activity (Table 2 and Table 3).

Further increase in the molarity of NaCl decreased the enzyme activity. The β-galactosidase and β-glucosidase activity was almost same when 0.5 and 2.0M NaCl was used Using 1.0M NaCl indicated a better results in both the cases. The results were similar when buffer of pH7.4 was used.

Table 1. Effect of NaCl and Triton X-100 on β- galactosidase and β-glucosidase extraction from Tamarind seeds.

pH	1.0M NaCl	B-galactosidase activity(µm/min/ml)	B-glucosidase activity(µm/min/ml)	pH	Triton X-100 (%)	B-galactosidase activity(µm/min/ml)	B-glucosidase activity(µm/min/ml)
5.6	0.0	0.0121	.002	5.6	0.5	0.0092	.0013
5.6	1.0	0.0108	.006	5.6	1.0	0.0089	.0004
7.4	0.0	0.0072	.0005	7.4	0.5	0.0090	.0013
7.4	1.0	0.0103	.007	7.4	1.0	0.0081	.0012

Table 2. Effect of NaCl concentration on B-galactosidase extraction from Tamarind seeds.

pH	B-galactosidase activity (µmoles/min/ml) in different concentrations of NaOH				
	0.2	0.5	1.0	2.0	5.0
5.6	0.0100	0.0097	0.0109	0.0097	0.0093
7.4	0.0065	0.0070	0.0086	0.0082	0.0064

Table 3. Effect of NaCl concentration on B-glucosidase extraction from Tamarind seeds.

pH	B-galactosidase activity (µmoles/min/ml) in different concentrations of NaOH				
	0.2	0.5	1.0	2.0	5.0
5.6	0.0026	0.0051	0.0057	0.0052	0.0040
7.4	0.0011	0.0044	0.0046	0.0033	0.0049

The kinetic parameters of the unpurified β-galactosidase and β-glucosidase enzyme are as shown (Fig. 2 and 3). β-galactosidase and β-glucosidase enzyme showed highest activity at 70°C and 30°C. To examine the heat resistance of the enzyme from Tamarind seeds, it was pre-incubated at different temperatures before determination of the activity. The β-galactosidase enzyme was stable at temperatures of upto 40°C and β-glucosidase enzyme was stable at temperatures of upto 35°C (Fig. 2). The examined β-galactosidase enzyme displayed a maximum activity at pH 5.0 and showed stability from pH4.0 to pH 7.0. Whereas β-glucosidase enzyme displayed a maximum activity between pH 4.0 and pH 5.0 and showed stability from pH 4.0 to pH 7.0 (Fig. 3). The kinetic parameters of Tamarind seed β-galactosidase and β-glucosidase for the hydrolysis of p–nitrophenyl–β–D–galactoside and p–nitrophenyl–β–D–glucoside are in similar order of magnitude to those of other plant enzymes (Masayuki Sekimata *et al*., 1989, Yeong Shik Kim *et al*., 1993, Sing-Chung *et al*., 2001, Dong Hoon et al., 2003, Masayuki *et al*., 2000, Hazel *et al*., 1998). The course of conversion of substrate into product determined at an increased substrate concentration indicated that Km value of 66 μM and121 μM and Vmax value as 2.27 nmoles / min and 5.26nmoles/min for β-galactosidase and β-glucosidase enzyme respectively.

Conclusion

Presented results seem to be the first to determine the production, activity and partial purification of the enzyme β-galactosidase and β-glucosidase from Tamarind seeds. Our results suggest that this enzyme is a β-D-galactosidase as it hydrolysis ρ-nitrophenyl-β-D-galactoside and β-D-glucosidase as it hydrolysis ρ-nitrophenyl-β-D-glucoside . Observed thermal stability of the enzyme β-D-galactosidase allows us to achieve a high level of substrate conversion. The further purification and more detailed characterization of the enzyme from Tamarind seed is currently in progress.

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