# Inhibition Of Cotton β-Galactosidase by a Water-Soluble Component in Crude Cotyledon Extracts

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**Cotton seeds were germinated and seedlings were grown for up to 10 days. During this period, β-*N*-acetylglucosaminidase and α-mannosidase activities increased, but β-galactosidase activity decreased steadily after a brief increase in the early stage of germination, when determined by using crude extracts. However, the parallel purification of β-galactosidase from cotyledons in early period of germination and fully grown cotyledons revealed that the total enzyme activity per cotyledon was higher in fully grown cotyledons than that in young, emerging tissue. Thin layer chromatographic analysis of crude extracts showed that free galactose was not present there. The crude extract from 10-day-old cotyledons also inhibited activities of jack bean meal and *Aspergillus niger* β-galactosidases. The same crude extract inhibited specifically the activity of cotton β-galactosidase, but not *N*-acetylglucosaminidase and α-mannosidase activities. Treatment of crude extract with PVPP eliminated the β-galactosidase inhibition. A fluorescent fraction isolated from crude extract of 10-day-old cotyledons by TLC was found to inhibit cotton β-galactosidase to a degree corresponding to the inhibition by crude extracts. The size of the fluorescent spot on TLC appeared larger with increasing cotyledon age.**

## INTRODUCTION

β-Galactosidases, which cleave the terminal β-galactosidic linkage of oligosaccharides, have been isolated from various plant tissues. Jack bean meal β-galactosidase is one of the better characterized ones; it typically releases a D-galactosyl residue with a β-1→4 bond from substrates such as lactose and larchwood galactan (1). The enzyme has been found to be located in cell wall (2) and membrane (3) fractions. It has been suggested that β-galactosidases of the cell wall in suspension culture were released into growth media after cell expansion (4,5). A soluble, cytoplasmic β-galactosidase also was found in extracts of sugar cane leaves (6).

In cotton plants, a variety of soluble glycosidases including β-galactosidase were detected in dry cotton seeds, and their activities increased following imbibition (7). The content of β-*N*-acetylglucosaminidase in cotyledons increased as the leafy tissue grew (8). During isolation of various glycosidases from mature cotton cotyledons, we found that the subsequent purification of crude extracts resulted in a recovery greater than 100% of β-galactosidase. Phenolic compounds in plant tissues are well known to inhibit many enzyme activities (9, 10). Such an inhibition of glycosidases, in crude extracts has not been reported. In this paper, therefore, we attempt to clarify the inhibition phenomenon and to better characterize the inhibiting substance.

## MATERIALS AND METHODS

### Plant Material

Seeds of *Gossypium hirsutum* L. cv. IM 216 were obtained from Dr. W.M. Johnson, Oklahoma State University, Stillwater, Oklahoma. Seeds were planted in sterile vermiculite in plastic pans. Seedlings were grown with ample supply of water in a growth chamber with 16-hr photoperiod at 27 °C and 8-hr dark period at 21 °C. For seeds germinated for 2 days or less, seeds were removed from the vermiculite, washed and divested of seed coats. For seedlings germinated from 3 to 10 days, the emerging

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cotyledons were removed with a sharp razor blade, the remainder of the seedling being discarded. The 0-2-day-treated seeds and 3-10-day-old cotyledons were washed with cold distilled water and immediately frozen at -4 °C.

**Chemicals**

Commercial β-galactosidases from jack bean meal and from *Aspergillus niger* were obtained from Sigma Chemical Co. Contaminating glycosidases were removed by gel filtration by using Sephadex G-200 column, 1.5 x 90 cm. CM-Sephadex C-50, DEAE-Sephadex A50, and other chemicals, unless otherwise stated, were obtained from Sigma Chemical Co.

**Enzyme extraction**

For the determination of β-galactosidase and other glycosidase activities, 10 seeds or 20 cotyledons from 10 seedlings were homogenized with a prechilled mortar and pestle by using 10 mL of 50 mM Na acetate buffer, pH 5.0, per g fresh weight. The resultant slurry was squeezed through four layers of cheesecloth and centrifuged at 10 x $10^3$ g for 20 min. The supernatant, which is called the crude extract in the present study, was used for enzyme assay and protein content determination. All extractions were performed at 4 °C.

**Purification of cotton β-galactosidase**

1. Increase in β-Galactosidase Activity (results in Table 1).

Emerging cotyledons taken from 200 germinating seeds after 3 days of germination and cotyledons taken from 200 seedlings after 6 and 10 days of germination were homogenized with 50 mM Na acetate buffer (pH 5.0) in a Waring blender. After centrifugation as before, the supernatants were applied to CM-Sephadex columns (2.4 x 40 cm) that has been pre-equilibrated with the same buffer. Glycosidases were eluted by a continuous salt gradient (0.0 M to 0.5 M NaCl) (Fig. 2).

2. Purified Glycosidases (results in Table 3).

Cotyledons (15-day-old) were homogenized, and the subsequent purification of β-galactosidase including ammonium sulfate fractionation and CM-Sephadex and DEAE-Sephadex chromatography was performed as described by Yi (8). The enzyme preparation after DEAE-Sephadex chromatography (Fig. 3) was not contaminated by α-galactosidase and other glycosidases, and was used for studying enzyme properties and inhibition.

**Enzyme assay**

The activity of β -galactosidases, including those purified from jack bean meal and *A. niger*, was measured by using *p*-nitrophenyl β -D-galactopyranoside as substrate. Activities of β-galactosidase, α-mannosidase and β-*N*-acetylglucosaminidase were determined by using the respective *p*-nitrophenyl glucoside. All enzyme activities were measured at pH 5.0 (8).

When larchwood galactan was used as substrate, β -galactosidase activity was assayed by the release of reducing sugar at 37 °C (11). The reaction mixture contained 1 unit of cotton β-galactosidase and substrate (10 mg/mL 0.1 M Na acetate buffer, pH 5.0). Hydrolysis of lactose by cotton β-galactosidase was followed by TLC. In this assay 1% lactose solution containing 1 unit of β -galactosidase in the same buffer was incubated for 24 hr at 25 °C. After ter-

TABLE 1. Changes in glycosidase activities during germination of cotton seeds.

| Purification Step | Germination (Days) | Enzyme Activity (units/100 cotyledons) | | |
|---|---|---|---|---|
| | | β-*N*-Acetyl-glucosaminidase | β-Galactosidase | α-Mannosidase |
| Crude extract | 3 | 2 | 5 | 13 |
| | 6 | 5 | 4 | 23 |
| | 10 | 8 | 3 | 31 |
| CM-Sephadex chromatography | 3 | 2 | 8 | 15 |
| | 6 | 4 | 10 | 26 |
| | 10 | 13 | 17 | 53 |

One unit is defined as the amount of enzyme that releases 1 μmole of *p*-nitrophenol per min at 37 °C in 0.1 M Na acetate buffer (pH 5.0).

minating the reaction by heating, 50 μL of the aliquot was applied to a Whatman LK5 thin-layer plate.

For studying the inhibition, the reaction mixture contained 0.1 mL of purified β-galactosidase from cotton cotyledons, jack bean meal, or *A. niger,* 0.1 mL of crude extract and 0.1 mL of 2mM *p*-nitrophenyl substrate in 0.1 M Na acetate buffer, pH 5.0. The absorbance change was read against the blank containing zero-time mixture with crude extracts.

Aqueous polyvinylpolypyrrolidone (PVPP, w/v), 1-5%, was added to 10 mL of crude extract that had been used for β-galactosidase assay. After PVPP was removed by centrifugation at 10 x $10^3$ g for 10 minutes, the supernatant was used for determinaion of β-galactosidase activity and the enzyme activity was compared with that of crude extract not treated with PVPP.

**Thin layer chromatography (TLC)**

Samples for carbohydrate analysis of crude extracts were prepared according to the method described by Elbein et al. (12). The concentrated residue was dissolved in a minimum amount of water and then applied to preabsorbent strips of Whatman LK5 plates which had been impregnated with 0.2 M $NaH_2PO_4$. TLC plates were developed for 4 hr with a solvent system of 1-propanol/methanol/water (70:15:15, v/v) or 1-propanol/acetic acid/water (3:3:2, v/v) (13). The carbohydrate was detected by spraying *p*-anisidine reagent or Seliwanoff's reagent (7).

For study of the inhibiting substance, a portion of each extract (100 μL) that had not been treated with PVPP was applied to preparative TLC plates (Whatman PLK5) and another portion of 10 μL to analytical plates (LK5). Preparative and analytical plates were developed in the solvent system of 1-propanol/methanol/water (70:15:15, v/v) for 4 hr. The fluorescent spots were detected under a short-wavelength ultraviolet lamp (14). The fluorescent spots were collected separately from preparative plates and dissolved in 1-propanol. After evaporation of the solvent, the brown residue was dissolved in 1.0 mL of 50 mM Na acetate buffer, pH 5.0. A tenth of the solution was added into the reaction mixture of β-galactosidase assay containing *p*-nitrophenyl β-D-galactopyranoside as substrate and 0.1 mL of purified cotton β-galactosidase in a total volume of 0.3 mL.

All experiments were repeated at least three times. Similar results and identical trends were obtained each time. The data reported here are the average of three individual experiments.

## RESULTS

**Changes in glycosidase activities during growth of cotton cotyledons**

The growth of cotton cotyledons, when determined on the basis of fresh weight, was most active during the first 10-day period of germination, and then it slowed, followed by senescence after 15 days (8). During this period, most glycosidase activities in the cotyledons were found in α-mannosidase, β-*N*-acetylglucosaminidase, and β-galactosidase. β-Galactosidase activity in crude extracts increased during the first two days of germination and then started to decrease (Fig. 1). The activity of β-galactosidase remained about the same between five and ten days of germination. In contrast, the activities of both β-*N*-acetylglucosaminidase and α-mannosidase continued to increase during the same period.

The reducing sugar content equivalent to that of D-glucose per seed increased during the first two days of germination and then decreased during the next two days. The reducing sugar content in cotyledons increased rapidly as the emerging cotyledons started to become green after five days of germination. When analyzed for carbohydrate by using TLC, crude extracts from fully grown (10-day old) cotyledons gave spots corresponding to $R_f$ values of lactose, sucrose, fructose, ribose, and a fast-moving unknown. No spot corresponding to the $R_f$ value of D-galactose was ever detected by TLC in the extracts from 1-day-, 3-day-, or 6-day-old cotyledons.

**CM-Sephadex chromatography of crude extracts**

The elution pattern of glycosidases in the chromatography of crude extracts was found to be the same for both 3-day- and 6-day-old cotyledons (Fig. 2). The total activity of β-galactosidase shown by chromatography of crude extract of 6-day-old cotyledons was higher than that of 3-day-old

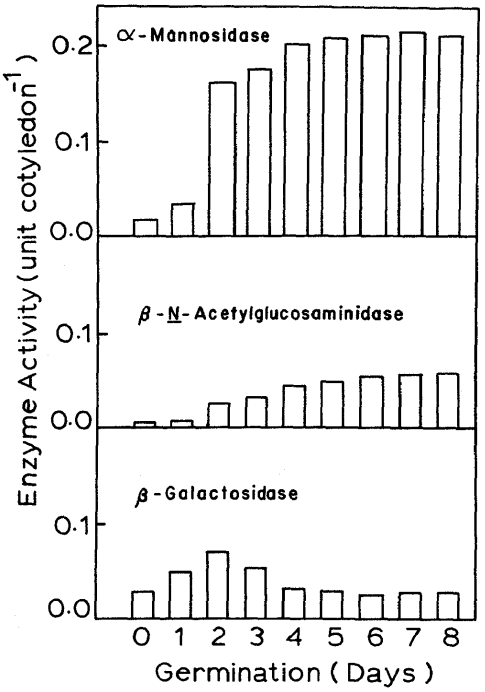


FIGURE 1. Distribution of α-mannosidase, β-*N*-acetylglucosaminidase, and β-galactosidase activities in cotyledons during germination of cotton seeds. Enzyme activities were determined by using crude extracts. The 0 day value indicates crude extract from dry seeds, 1 to 2 day from germinating seeds, and 3 to 10 day from emerging and growing cotyledons.

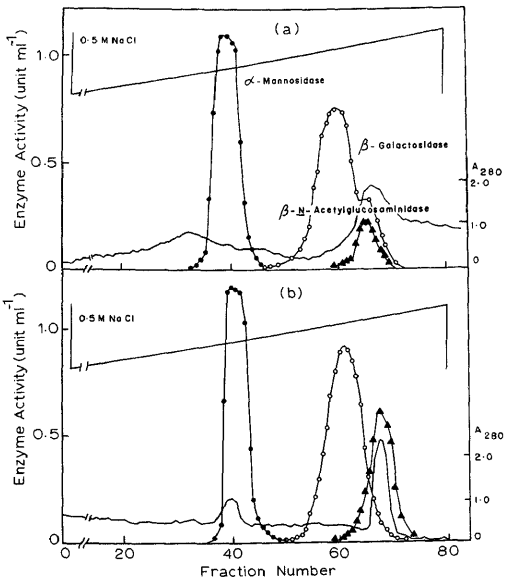


FIGURE 2. Elution profiles of crude glycosidases from 3-day- (a) and 6-day-old (b) cotyledons on CM-Sephadex C-50 chromatography. Five-mL fractions were collected at 30 mL/hour in each chromatography. Symbols refer to protein (——) α-mannosidase (–●–●–), β-galactosidase (–o–o–), and β-*N*-acetylglucosaminidase (–▲–▲–).

cotyledons. By using the same procedure, the recovery of enzyme activity from the 10-day-old cotyledons was even higher than those of 3-day- and 6-day-old cotyledons.

The glycosidase activity based on equal numbers of cotyledons used for each enzyme extraction is shown in Table 1. It clearly indicates that the activity of β-galactosidase along with other glycosidases increases during germination of cotton seeds and subsequent growth of cotyledons.

This same effect was observed when the crude extracts were subjected to ammonium sulfate precipitation; activity of β-galactosidase collected between 35% and 75% saturation was highest in 10-day-old cotyledons and lowest in 3-day-old cotyledons after dialysis against distilled water.

**Properties of β-galactosidase**

Combined fractions of glycosidases from CM-Sephadex chromatography were further purified by DEAE-Sephadex chromatography. The resultant β-galactosidase that was separated from other glycosidases (Fig. 3) was used for the study of enzyme properties.

The maximum activity of cotton β-galactosidase in 0.1 M citrate buffers from pH 3.0 to 10.0 was between pH 4.0 and 4.4. The use of Na acetate buffer between pH 3.0 and 6.0 resulted in the same activity profile.

Cotton β-galactosidase was capable of hydrolyzing larchwood galactan, which contains a β-1→4 bond at the terminal galactosyl residue (6), since reducing sugars appeared as a result of enzyme action. The action of β-galactosidase on lactose (gal-1→4 glu) produced galactose and glucose as shown by TLC. Melibiose, stachyose, raffinose, and *p*-nitrophenyl α-D-galactopyranoside, however, were not hydrolyzed by this enzyme.

**Inhibition of β-galactosidase**

When determined with β-galactosidases purified from jack bean meal and *A. niger,* the combined activity of purified enzyme and the same enzyme activity contained in crude extracts was found to be lower than

expected (Table 2). Instead of a cumulative effect by two enzyme reactions, a subsequent reduction in absorbance change between the reaction and blank occurred from the first day through 10th day samples. In either case, inhibition of β-galactosidase activity became greater as the growth of cotyledons progressed.

α-Mannosidase, β-*N*-acetylglucosaminidase, and β-galactosidase obtained from DEAE-Sephadex chromatography were incubated with crude extracts from 10-day-old cotyledons. The crude extracts portion inhibited cotton β-galactosidase activity to a considerable extent, but not α-mannosidase and β-*N*-acetylglucosaminidase at all (Table 3). It was also observed that the inhibition of cotton β-galactosidase activity became severe as the cotyledons used for extraction became older, as shown in jack bean meal and *A. niger* β-galactosidases.

The absorption of phenolic compounds by PVPP in crude extract resulted in an enhancement of β-galactosidase activity in the subsequent enzyme assay (Table 4). When PVPP (5%) was present in the crude extract, the inhibitory substance was eliminated. The resultant 200% increase in β-galactosidase activity is more than that expected from data shown in Table 2. This may be the result of a direct reduction by PVPP in the blank reading for the enzyme assay. PVPP treatment, however, did not affect absorbance changes for α-mannosidase and β-*N*-acetylglucosaminidase activities.

Portions of crude extracts from 1-through 10-day-old samples were applied to TLC plates for separating inhibitors of cotton β-galactosidase. As shown in Fig. 4, fluorescent compounds were contained in all the preparations of crude extracts. However, only one fraction with an $R_f$ value of 0.64 was effective in inhibiting cotton β-galactosidase This fluorescent compound was not present in the crude extract from 1-day-old cotyledons, but was contained in all crude extracts after 1-day germination. However, the fluorescent spot containing the inhibition of β-galactosidase was larger in extracts from older cotyledons.

For quantitative estimation of β-galactosidase inhibition by the fluorescent fraction, the inhibiting phenolic compound was extracted from preparative TLC plates and a one-tenth portion of the TLC fraction was

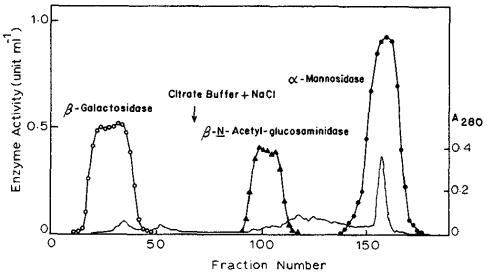


FIGURE 3. Chromatography of partially purified glycosidases on DEAE-Sephadex A-50. Glycosidases were obtained from CM-Sephadex chromatography. Fractions (5 ml each) were collected as the column was developed, starting with buffer and followed by the elution with 50 mM Na citrate buffer (pH 6.0) containing 50 mM NaCl. Fractions representing major peaks of β-galactosidase (–o–o–), β-*N*-acetylglucosaminidase (–▲–▲–), and α-mannosidase (–●–●–), were pooled and then concentrated. (——) refers to protein.

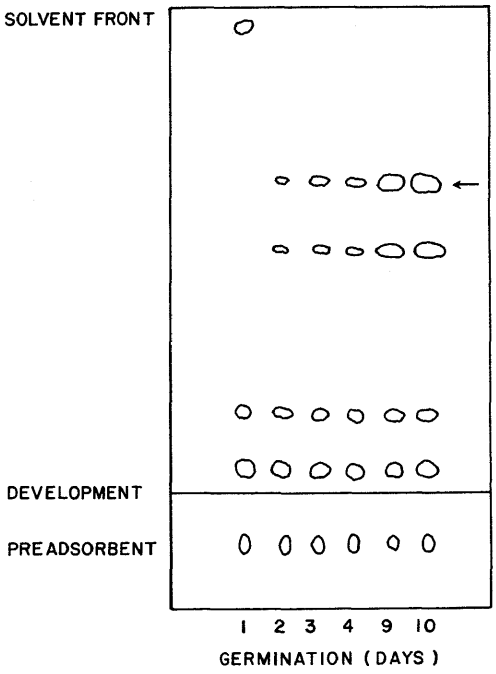


FIGURE 4. Thin layer chromatographic examination of fluorescent compounds from cotton seeds at various stages of germination. Aliquots of 10 μL from crude extracts were chromatographed on a LK5 plate for 4 hr in the solvent system of 1-propanol/methanol/water (70:15:15, v/v). Arrow on the right refers to the fraction containing the compound inhibitory for β-galactosidase.

TABLE 2. Effect of crude extracts on jack bean and *A. niger* β-galactosidase activities.

| Reaction mixture | Germination (Days) | Absorbance change at 410 nm[a] | | |
|---|---|---|---|---|
| | | β-Galactosidase in crude extract | +Jack bean β-galactosidase | +*A. niger* β-galactosidase |
| No crude extract | | | 0.400 | 0.620 |
| With crude extract from plants of different ages | 1 | 0.167 | 0.426 | 0.561 |
| | 3 | 0.133 | 0.343 | 0.546 |
| | 6 | 0.103 | 0.326 | 0.499 |
| | 8 | 0.099 | 0.314 | 0.492 |
| | 10 | 0.097 | 0.298 | 0.474 |

[a]Indicates the net absorbance change for enzymatic reaction for 15 min at 37 °C by using *p*-nitrophenyl β-D-galactospyranoside as substrate. The blank was zero-time reaction.

TABLE 3. Effects of crude extract on purified glycosidase activities.

| Enzyme | Absorbance change at 410 mn | | |
|---|---|---|---|
| | Crude extract | Purified fraction[a] | Crude extract + purified fraction |
| β-*N*-Acetylglucosaminidase | 0.09 | 0.56 | 0.58 |
| β-Galactosidase | 0.02 | 0.84 | 0.33 |
| α-Mannosidase | 0.50 | 0.37 | 0.76 |

[a]Indicates those from DEAE-Sephadex chromatography.

added to the reaction mixture for cotton β-galactosidase. The fluorescent residue substantially inhibited β-galactosidase activity (Table 5). This compound, however, had no effect on α-mannosidase and β-*N*-acetylglucosaminidase activity. The inhibition of cotton β-galactosidase (Table 5) of about 60% is less than that expected from the results shown in Tables 1 and 4, which may be due to a lower recovery or decomposition during extraction from the TLC plates.

## DISCUSSION

The properties of cotton cotyledon β-galactosidase are similar to those in other plants (2, 4, 6) with respect to optimum pH and substrate specificity. This β-galactosidase activity increased during the first two days of cotton seed germination. As cotyledons grew, however, the crude extracts of cotyledons contained less enzyme activity than that in the early stages of germination, while α-mannosidase and β-N-acetylglucosaminidase activities continued to increase (Fig. 1). The result from the parallel purification of β-galactosidase extracted from emerging cotyledons or fully grown tissues (Fig. 2 and Table 1) suggests that β-galactosidase is inhibited when crude extracts were used for the assay.

Since D-galactose has been shown to be a potent inhibitor of β-galactosidase (1, 6,13), it was considered that the decrease in extractable activity of β-galactosidase and inhibition of cotton enzyme by crude extracts might be attributable to this sugar. However, carbohydrate analysis using TLC indicated that the highly elevated content of reducing sugar in old cotyledons did not include D-galactose (see RESULTS). In addition, the absence of stachyose and raffinose in crude extracts of old cotyledons and the minimal activity of α-galactosidase which could release D-galactose from substrates such as stachyose and raffinose indicate that there was little chance of D-galactose being present in crude extracts to interfere with β-galactosidase.

It is well known that phenolic compounds are present in various plant tissues (9), including cotton cotyledons (14). Most phenolic compounds in crude extracts are adsorbed by added PVPP (10,15). These facts and the evidence shown in this study (Tables 4 and 5) led us to speculate that the

TABLE 4. Effect of polyvinylpolypyrrolidone treatment on β-galactosidase activity in crude extract.

| PVPP Concentration | $\Delta A_{410}$[a] | % Increase |
|---|---|---|
| 0% | 0.19 | 0 |
| 1% | 0.33 | 74 |
| 2% | 0.47 | 147 |
| 3% | 0.51 | 168 |
| 4% | 0.54 | 184 |
| 5% | 0.57 | 200 |

[a]Indicates enzyme activity determined by using the zero-time blank.

TABLE 5. Inhibition of cotton β-galactosidase activity by fluorescent fraction.

| Experiment | | % Inhibition |
|---|---|---|
| No. 1 | Control | 100 |
| | TLC Fraction | 63 |
| No. 2 | Control | 100 |
| | TLC Fraction | 57 |

inhibiting substance(s) might be a related phenolic compound. In the present work, unfortunately, the inhibiting substance could not be identified. Nevertheless, the most striking feature is the fact that the inhibition was specific to β-galactosidase and did not affect the other glycosidases α-mannosidase and β-*N*-acetylglucosaminidase (Table 3). This effect was also evident in assays by using jack bean meal and *A. niger* β-galactosidases (Table 2). This differential effect on glycosidases implies that the use of β-galactosidase, unlike α-mannosidase, as a marker enzyme to determine the purity of cell organelles such as vacuoles (2, 3, 16) may not be warranted, unless levels of inhibitors are assessed.

It is also evident that the inhibition of β-galactosidase became severe as the cotyledon grew (Table 2 and Fig. 4). Such change in inhibitor level for an enzyme following physiological changes has been reported (9,17). However, this relationship between β-galactosidase and an inhibitor in crude extracts, possibly a phenolic compound, has not been recorded. More research is required in order to understand the mechanism of action of inhibitors on β-galactosidase.

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