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PURIFICATION AND PROPERTIES OF β-GALACTOSIDASE FROM *HIBISCUS SABDARIFFA* L. VAR. *ALTISSIMA*

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ABSTRACT

*β-Galactosidase was purified from the seeds of Thai jute (*Hibiscus sabdariffa *L. var.* altissima*) by ammonium sulfate fractionation, DEAE-cellulose column chromatography, Sephadex G-100 column chromatography, Lactosyl-Sepharose chromatography, and a second DEAE-cellulose chromatography step. The final product was purified 868-fold with a yield of 13%, and gave a single major band of molecular weight 66,000 on SDS-PAGE, similar to the native molecular weight of 55,000 determined by gel filtration. The K_m values for various substrates were as follows: 0.80 mM for p-NP-β-D-galactoside, 12.8 mM for o-NP-β-D-galactoside and 84.7 mM for β-lactose, and the relative Vmax values were 100% for p-NP-β-D-galactoside, 26.7% for o-NP-β-D-galactoside and 9.4% for β-lactose. Hydrolysis of p-NP-β-D-galactoside was strongly inhibited by various substances including D-galactal, galactono-1,4-lactone, methyl-α-D-galactoside, methyl-β-D-galactoside, $HgCl_2$ and p-hydroxymercuribenzoate.*

INTRODUCTION

β-Galactosidase (EC 3.2.1.23) is an enzyme that catalyses the hydrolysis of the glycosidic bond formed between a galactose residue and an aglycon or another sugar residue. This enzyme has been extensively studied in numerous organisms, both in terms of function and properties[1-3]. The β-galactosidase in the gram-negative rods of Enterobacteriaceae have been extensively investigated, since tests for the fermentation of lactose play an important role in diagnostic bacteriology[4]. In mammals, intestinal β-galactosidase plays an important role in the hydrolysis and absorption of lactose4, while lysosomal β-galactosidase is now recognized as a key enzyme in the degradation of glycolipids, mucopolysaccharides and glycoproteins and its deficiency has been shown to cause a number of diseases[5]. In plants, β-galactosidase has been found in many species[6-17], including almond, barley, jack bean, pea, apple, peach and tomato. Its activity is mainly found in the seeds and leaves, and may be related to the catabolism of galactolipids or glycogalactans in plant cell walls[3].

β-Galactosidase has been widely used for studies such as in elucidation of the primary structure of glycans and in determination of the anomeric linkage of conjugated

monosaccharides[18-19]. Recently, we have been interested in the reversal of the hydrolytic action of glycohydrolases for the enzymatic synthesis of oligosaccharides[20]. Since β-galactosides are found in most glycoproteins, we have searched for suitable sources of this enzyme from Thai plant seeds. β-Galactosidase is found at moderately high levels in many species in the Malvaceae and Tiliaceae family[20], and synthesis by reversing the action of this enzyme could be observed. Accordingly, further studies were performed on the purification and properties of β-galactosidase from the seeds of Thai Jute (*Hibiscus sabdariffa* L. var. *altissima*) as described in this paper.

MATERIALS AND METHODS

Materials

Thai Jute seeds were kindly provided by the Field Crops Research Institute, Department of Agriculture, Ministry of Agriculture. Nitrophenyl-β-D-glycosides, phenylmethylsulfonyl fluoride (PMSF), D-galactal, D-galactono-1,4-lactone, β-lactose, isopropyl-β-D-galactoside (IPTG), p-hydroxymercuribenzoate (PHMB), molecular weight standards kit for SDS-polyacrylamide gels, polyvinyl-polypyrrolidone (PVPP), and divinyl sulfone were from Sigma Chemical Co., St. Louis, MO, USA. Lactosyl Sepharose 4B was prepared as previously described[21], involving activation of Sepharose 4B with divinyl sulfone, followed by coupling with 10% β-lactose.

Preparation of crude extracts

Imbibed or germinated seeds were homogenized with a blender in ice-cold 0.05 M sodium acetate buffer, pH 5.0 (2 ml per 1 g weight seed), containing 1 mM phenylmethylsulfonylfluoride (PMSF) and 5% (w/v) PVPP. The homogenate was filtered through 6-8 layers of cheesecloth and 2 layers of miracloth, and centrifuged at 10,000 g for 30 min. Activated Dowex 2-X8 (25 g per 100 ml) was added to the filtrate, and the mixture stirred for 1 h. The Dowex 2-X8 was removed by filtration through Whatman No. 4 paper, and the filtrate was centrifuged at 10,000 g for 30 min to yield the crude extract. All steps were performed at 4°C.

Germination studies

Batches of *Hibiscus sabdariffa* L. var. *altissima* seeds (10 g each) were surface-sterilized with 0.1% sodium hypochlorite, and imbibed in water for 1 or 2 days at room temperature. After 2 days of imbibing, some batches were allowed to germinate on 2-3 layers of cheesecloth in transparent boxes at room temperature for 1, 2 or 3 days. After treatment, imbibed or germinated seeds at a similar stage were selected for preparation of crude extracts according to the above procedure, with the percent of seeds at the selected stage being noted.

Purification of *Hibiscus sabdariffa* L. var. *altissima* β-Galactosidase

All steps in purification were carried out at 4 C. Crude extracts were prepared from 50 g of *Hibiscus sabdariffa* L. var. *altissima* seeds, which had been imbibed for 2 days, according to the procedure described above. The crude extracts were fractionated by ammonium sulfate at 35-70% saturation, and the precipitate obtained was redissolved in a small volume

of 10 mM potassium phosphate buffer, pH 7.0 and dialyzed in the same buffer. The solubilized and dialyzed precipitate was applied to a DEAE-cellulose column (2.0 x 15 cm), equilibrated with the same buffer. The column was washed with 10 mM potassium phosphate buffer, pH 7.0 (2 column volumes), followed by a gradient of 0 - 0.2 M NaCl in the same buffer (3 + 3 column volumes) and 1.0 M NaCl in the same buffer (3 column volumes). Flow rate was 30 ml per h and fractions of 3 ml were taken, and analyzed for A_{280}, β-galactosidase activity and α-mannosidase activity. Unbound fractions with β-galactosidase activity were pooled, and concentrated by Amicon ultrafiltration.

The concentrated DEAE-cellulose pool was further fractionated on a Sephadex G-100 column (1.5 x 80 cm) , equilibrated and eluted with 10 mM potassium phosphate buffer, pH 7.0 containing 50 mM NaCl. Flow rate was 15 ml/h and fractions of 1.0 ml were collected and analyzed for A_{280}, β-galactosidase activity and α-galactosidase activity. Two pools were taken, one containing β-galactosidase free of α-galactosidase and the other containing both enzymes. Both fractions were desalted and concentrated by Amicon ultrafiltration. Each fraction was separately loaded to a Lactosyl Sepharose 4B column (1.2 x 12 cm), equilibrated with 10 mM sodium acetate buffer, pH 4.0, and percolated through the column at 15 ml/h for 1 h. The column was washed with starting buffer until A_{280} was zero, and then was eluted with 2 column volumes of 0.1 M sodium acetate buffer, pH 4.0. Fractions of 1 ml were collected and analyzed for A_{280}, β-galactosidase activity and β-galactosidase activity. Fractions containing β-galactosidase activity were pooled and subjected to a repeat chromatography on a DEAE-cellulose column (1 x 9 cm), pH 7.0, equilibrated with 10 mM potassium phosphate buffer, pH 7.0. The column was washed with 10 mM potassium phosphate buffer, pH 7.0 (2 volumes), 0.1 M potassium phosphate buffer, pH 7.0 (2 volumes) and 1 M NaCl in starting buffer (2 volumes). The flow rate was 50 ml/h and fractions of 1 ml were collected and analyzed for A_{280}, β-galactosidase, and α-galactosidase activity. The purified β-galactosidase so obtained was stored in saturated ammonium sulfate at 4°C until required.

Determination of β-Galactosidase Activity

The standard reaction mixture contained 50 μl diluted enzyme, 100 μl 0.01 M p-NP-β-D-galactoside, 850 μl 0.1 M sodium acetate buffer, pH 4.0 (adapted from Montreuil[22]). The reaction was incubated at 30°C for 10 min before being stopped by addition of 2 M Na_2CO_3. The liberated p-nitrophenol was quantitated spectrophotometrically at 400 nm. One unit (U) of enzyme is the amount of enzyme that will release one (mole of p-nitrophenol per minute. The pH optimum of purified enzyme was studied in the same manner except that 0.1 M McIlvaine buffer at varying pHs from 2.5 to 7.5 was used as incubation buffer. Optimum temperature of purified enzyme was determined in the same manner except that reactions were incubated at varying temperatures ranging from 10°C to 80°C. The ability to hydrolyze other substrates was tested by performing the standard reaction with various p-NP-glycosides (final concentration of 1 mM). The inhibitory effect of various compounds on p-NP-β-D-galactoside hydrolysis was tested by preincubating 0.05 units of purified enzyme in 0.1 M sodium acetate buffer, pH 4.0 with various substances at 30 °C for 30 min, before assaying with the standard methods.

Protein Determination

The protein content of solutions was routinely determined by the Coomassie blue protein assay[23] using bovine serum albumin as standard. In the case of chromatographic separations, protein concentration was followed by measuring A_{280}.

Molecular Weight Determination

The molecular weight of β-galactosidase in the denatured state was determined by SDS-polyacrylamide gel electrophoresis on a 10 % slab gel[24], using rabbit muscle myosin (205 kD), *E. coli* β-galactosidase (116 kD), rabbit muscle phosphorylase b (97.4 kD), bovine serum albumin (66 kD), ovalbumin (45 kD), and carbonic anhydrase (29 kD) as standards. The native molecular weight of the enzyme was determined by gel filtration on a Sephadex G-200 column (1.8 x 90 cm) in 0.2 M NaCl, 10 mM potassium phosphate buffer, pH 7.0, using β-amylase (200 kD), bovine serum albumin (66 kD), ovalbumin (45 kD) and cytochrome C (12.4 kD) as standards.

Kinetic Studies with p-NP-β-D-Galactoside, o-NP-β-D-Galactoside and β-Lactose

With p-NP-β-D-galactoside, about 0.01 U of purified enzyme was reacted with 0.1 mM to 10.0 mM substrate in 0.1 M sodium acetate buffer, pH 4.0 (final volume 1.0 ml) for 5 min at 30°C before stopping the reaction with 2 M Na_2CO_3. With o-NP-β-D-galactoside, experiments were performed similarly except that 0.8 mM to 40.0 mM substrate were used, and released o-nitrophenol was monitored at 410 nm. With β-lactose, 0.03 U of purified enzyme was incubated with 10 mM to 150 mM β-lactose in 0.1M sodium acetate buffer, pH 4.0 (final volume 0.1 ml) at 30°C for 20 min before being heated to stop the reaction. Released glucose was quantitated with a glucose oxidase-peroxidase kit (B.M. Labs, Bangkok). K_m and V_{max} values of the β-D-galactosidase with the three substrates were determined by Michaelis-Menten plots and/or Lineweaver-Burk plots using the Enzfitter computer program (Elsevier Biosoft, Cambridge, U.K.).

RESULTS

Germination of *Hibiscus sabdariffa* L. var. *altissima* seeds

Our previous screening of glycohydrolase enzymes identified β-galactosidase, α-mannosidase, and N-acetyl-β-glucosaminidase as the major enzymes present in *Hibiscus sabdariffa* L. var. *altissima*. Accordingly, the levels of these enzymes were followed during the course of germination (Table 1), so as to select the most suitable time of seed treatment to maximize the level of β-galactosidase for further purification. To ensure that data obtained were representative of the each stage, only seeds which appeared to be at a similar state of development were selected at each stage, and the percentage of seeds in the selected group compared to the total seeds were noted. The germination of the seeds appeared to be moderately synchronous, in that at days 1,2 and 3, the percent of seeds at the selected stage were over 95%, while the percent of seeds at the selected stage on days 4 and 5 were still over 70% (Table 1). Growth was slow in the first two days at the imbibing stage, but was much more rapid during the last three days at the time of germination. In terms of enzyme activity (units per gram of original seed), both β-galactosidase and α-mannosidase reached

a maximum after two days of imbibing, and then decreased during germination. Accordingly, two days of imbibing, without subsequent germination, was selected as the most suitable treatment of seed for maximizing the level of β-galactosidase for purification.

Purification of β-Galactosidase from *Hibiscus sabdariffa* L. var. *altissima*

β-Galactosidase was purified from *Hibiscus sabdariffa* L. var. *altissima* seeds using a combination of steps, with typical results being shown in Table 2. Ammonium sulfate fractionation removed a large amount of undesired proteins with good yield of the enzyme. The first DEAE-cellulose column at pH 7.0 (Figure 1) was very effective in separating β-galactosidase, eluted out in the unbound peak from the major contaminating glycohydrolase enzyme, α-mannosidase which bound to the column. In addition, this step was very effective in removing other protein contaminants which bound to the column, so that the specific activity of the β-galactosidase was increased by some 15-fold (Table 2). The Sephadex G-100 filtration step (Figure 2) was useful for separating the major component β-galactosidase from the minor component α-galactosidase, but unfortunately there was some overlap between the two peaks. Accordingly, two pools of β-galactosidase were taken, Pool 2 (tubes 41-50) containing β-galactosidase free of any α-galactosidase, and Pool 1 (tubes 33-40) containing about 4% α-galactosidase in addition to β-galactosidase.

Each of the Sephadex G-100 pools were separately purified further. β-Galactosidase bound to the Lactosyl-Sepharose affinity column in 0.01 M sodium acetate buffer, pH 4.0, with some unbound proteins being removed (profile not shown). Since affinity elution with 1.0 M lactose was not effective in completely releasing the enzyme, β-galactosidase was eluted out with 0.1 M sodium acetate, pH 4.0. In the purification described here, the first DEAE-cellulose column was somewhat overloaded, so a second DEAE-cellulose step (profile not shown) was added after the Lactosyl-Sepharose column to ensure that the isolated β-galactosidase was of the highest purity.

In the summary of the purification shown in Table 2, results are shown for both preparations of β-galactosidase, namely that derived from Sephadex G-100 Pool 2 (free of α-galactosidase) and that derived from Sephadex G-100 Pool 1 (containing some α-galactosidase). The overall purification gave β-galactosidase free of α-galactosidase in a yield of 13% with a purification of 868-fold. In addition, β-galactosidase containing some contamination with α-galactosidase was obtained in a yield of 17% with a purification of 442-fold, and although the level of α-galactosidase contamination was less than 5%, this pool was not used for subsequent studies.

Properties of *Hibiscus sabdariffa* L. var. *altissima* β-Galactosidase

All the studies described below only utilized the purified β-galactosidase that was free of any α-galactosidase contamination. SDS-polyacrylamide gel electrophoresis showed a single protein band (Figure 3), confirming that the isolated β-galactosidase was of high purity. The molecular weight determined for the denatured β-galactosidase on SDS-polyacrylamide gels was 66,000. This is of similar magnitude to the 55 kD determined for the molecular weight of the native enzyme using gel filtration on Sephadex G-200 filtration, suggesting that β-galactosidase from *Hibiscus sabdariffa* L. var. *altissima* consisted of a single polypeptide chain.

Table 1. Effect of imbibing and germination on the content of β-galactosidase and α-mannosidase from the seeds of *Hibiscus sabdariffa* L. var. *altissima*

Day of Treatment	% Seed selected*	Morphology of germinating seed selected	Activity (U) β-gal per g seed**	Specific activity U β-gal/mg	Activity (U) α-man per g seed**	Specjfic activity U α-man/mg	Weight (g) of, seed, root, plantlet per g seed**
Imbibing							
1	97	root 1 mm	1.15	0.034	1.04	0.03	2.15
2	98	root 2 mm	1.79	0.037	1.35	0.028	2.47
Germination							
1	98	root 5-10 mm	0.74	0.026	0.84	0.031	3.1
2	76	root 10-15 mm, plant 20-30 mm	0.74	0.026	0.91	0.032	4.66
3	71	root 15-20 mm, plant 50-100 mm	0.41	0.011	0.83	0.029	8.92

* % Seed selected was calculated from the number of seeds with the specified morphology, used for crude extract preparation compared to the number of seeds at the start.

** g seed refers to the weight of original seed corresponding to the selected group.

TABLE 2. Purification of (-galactosidase from *Hibiscus sabdariffa* L. var. *altissima* seeds

Purification Steps	Total activity	Total protein unit (U)	Specific activity mg	Purification Fold U/mg	Yield (%)
Crude extract	70.2	2430	0.029	1	100
35-70 % $(NH_4)_2SO_4$ fraction	58.8	414	0.14	4.8	84
DEAE-cellulose chromatography	43.9	20.2	2.17	75	63
Sephadex G-100 filtration					
Pool 1 (with α-gal)	25.7	10.6	2.42	83	37
Pool 2 (without α-gal)	14.1	6.96	2.02	70	20
Affinity chromatography					
from Pool 1 (with α-gal)	19.3	6.66	2.91	100	27
from Pool 2 (without α-gal)	13.9	2.39	5.82	201	20
DEAE-cellulose chromatography					
from Pool 1 (with α-gal)	12.14	0.947	12.8	442	17
from Pool 2 (without α-gal)	8.88	0.353	25.2	868	13

TABLE 3. Kinetic properties of *Hibiscus sabdariffa* var. *altissima* β-galactosidase

Substrate	K_m (mM)	V_{max} (nmol/min/μg protein)	V_{max}/K_m (nmol/min/μg/mM)
p-NP-β-D-Galactoside	0.80±0.02	53.3±0.3	66.1
o-NP-β-D-Galactoside	12.8±0.51	14.2±0.3	1.11
β-Lactose	84.7±1.28	5.0±0.3	0.060

Reactions were performed in 0.1 M sodium acetate buffer, pH 4.0 at 30°C ; values represent mean ±S.D, determined by regression analysis.

TABLE 4. Effect of various substances on the activity of β–galactosidase from *Hibiscus sabdariffa* L. var. *altissima*

Substance	Concentration (mM)	Relative Activity (%)
Control	-	100
$MgCl_2$	1	111
KCN	1	110
NiOAc	1	105
$CuSO_4$	1	108
CaCl2	1	106
NaF	1	105
LiOH	1	103
CdOAc	1	95
$ZnSO_4$	1	93
EDTA	1	96
$HgCl_2$	1	1
Iodoacetic acid (IAA)	1	71
p-Hydroxymercuribenzoate (PHMB)	1	2
Isopropyl-thio-β-galactoside (IPTG)	1	95
Methyl-β-galactoside	5	9
Methyl-α-galactoside	5	2
p-NP-β-galactoside	5	26
p-Aminophenyl-thio-β-galactoside	5	105
p-Aminophenyl-β-galactoside	5	92
Phenyl-β-galactoside	5	53
Galactono-1,4-lactone	5	5
Glucono-1,5-lactone	5	85
D-Galactal	5	9
D-Galactose	5	27
β-Lactose	50	84

Enzyme was preincubated with each substance for 30 min and then assayed with 1 mM pNP-β-D-galactopyranoside in 0.1 M sodium acetate buffer, pH 4.0 at 30°C.

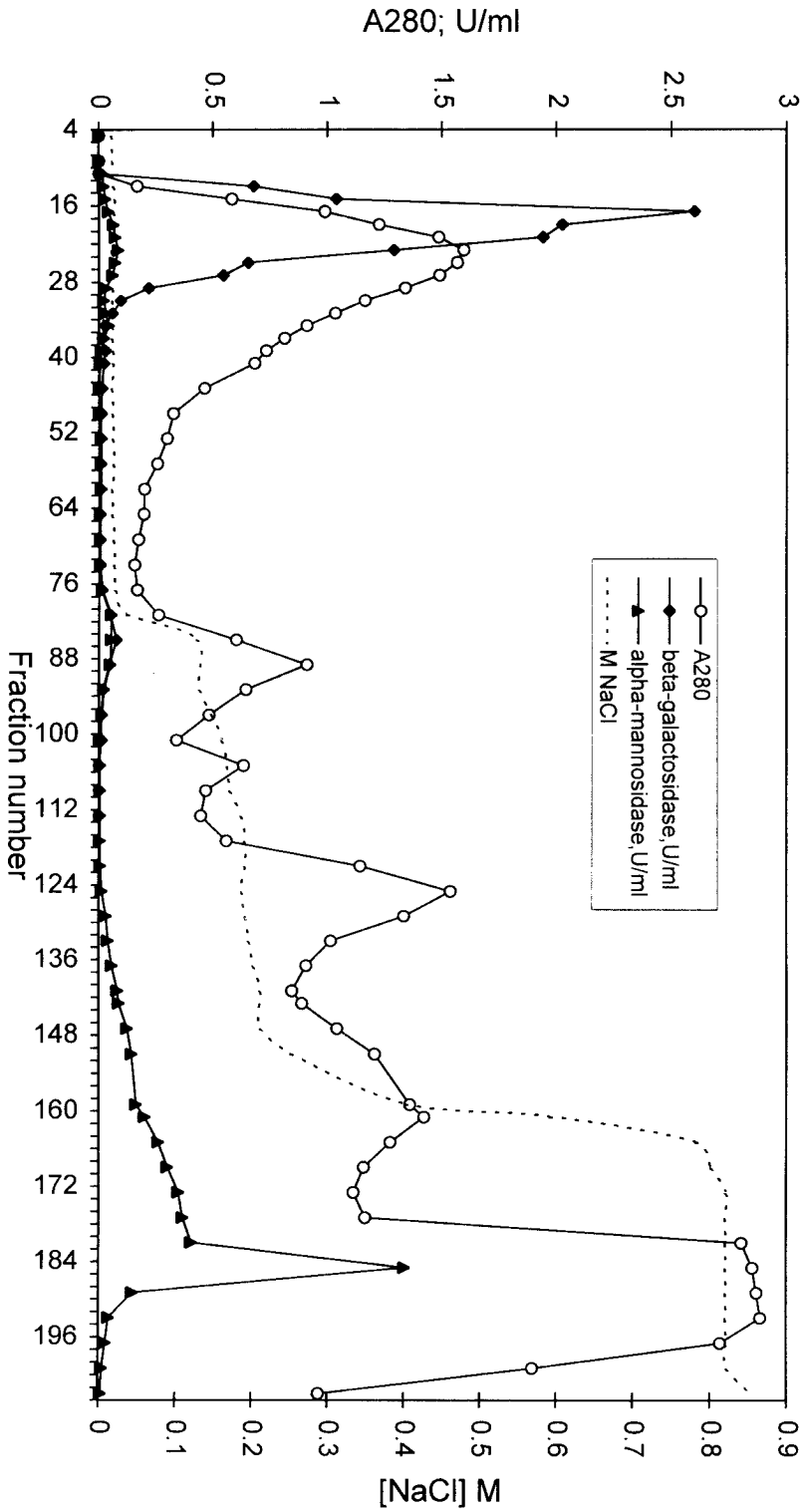

Fig.1. DEAE-cellulose chromatography of the dialyzed 35-70% ammonium sulfate fraction from Thai Jute seeds. The column (2.0 x 15 cm) was washed with 10 mM potassium phosphate buffer, pH 7.0, followed by a gradient of 0 - 0.2 M NaCl in the same buffer (starting at tube 50), and then by 1.0 M NaCl in the same buffer. - ○ - A_{280}; - ◆ - β galactosidase activity; - ▲ - α-mannosidase activity; ----- [NaCl], M.

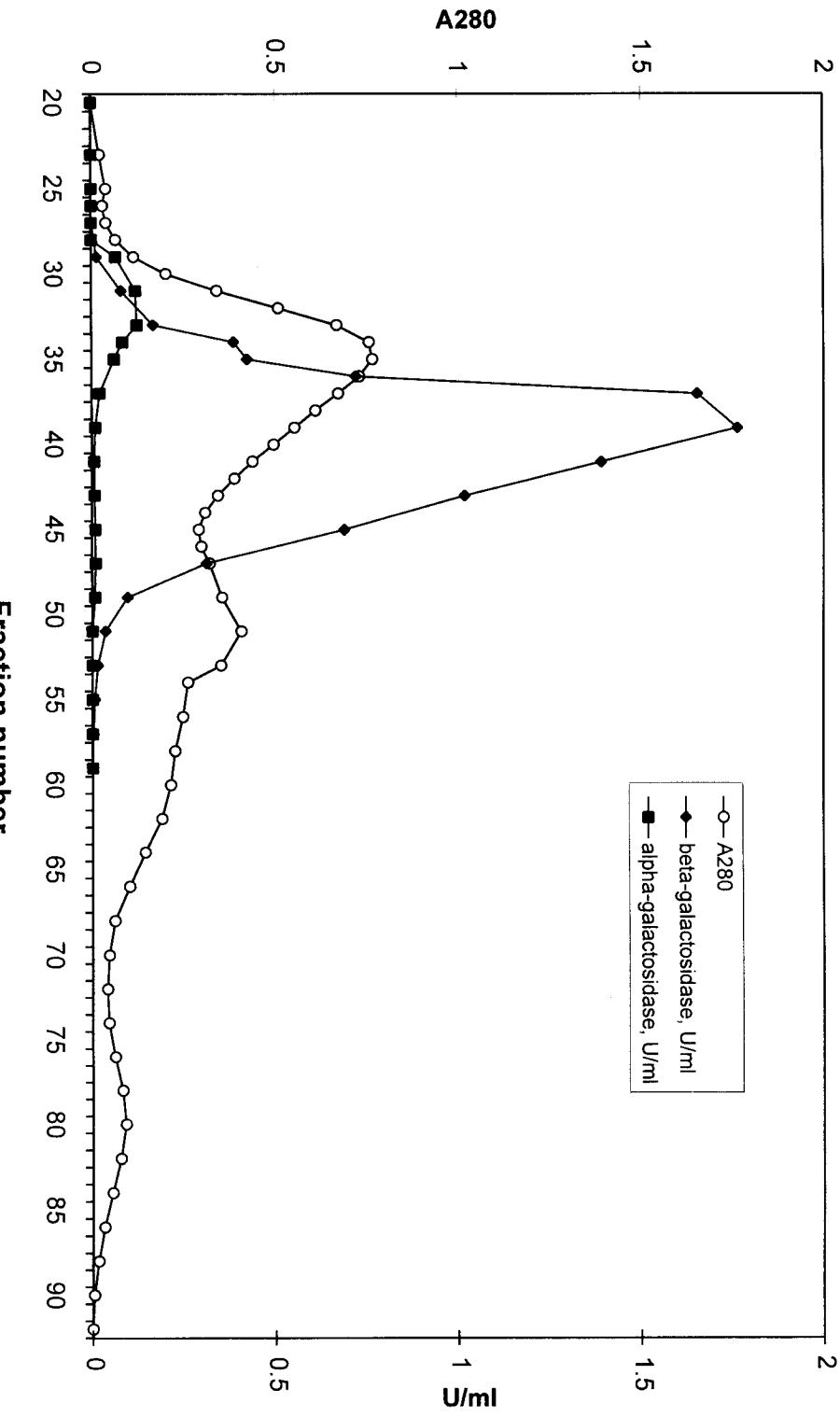

Fig.2. Sephadex G-100 chromatography of the unbound DEAE-cellulose fraction from Fig. 1. The column (1.5 x 80 cm) was eluted with 10 mM potassium phosphate buffer, pH 7.0 containing 50 mM NaCl. -○- A_{280}; -◆- β-galactosidase activity; -■- α-mannosidase activity.

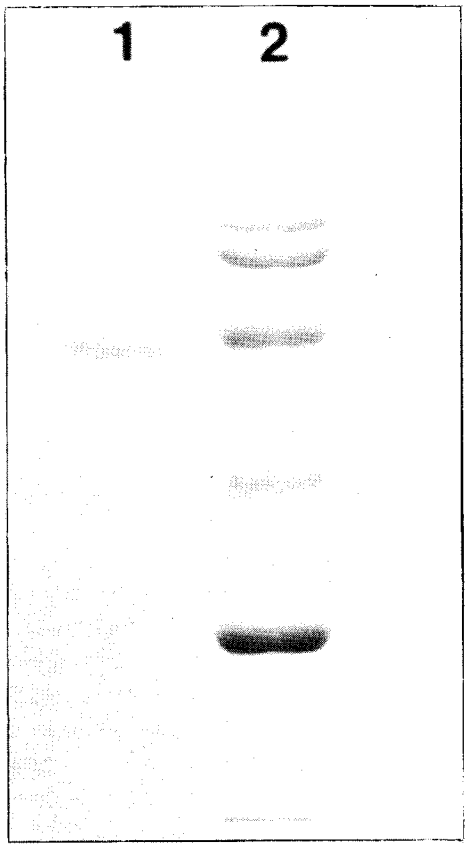

Fig.3. SDS-polyacrylamide gel electrophoresis of purified *Hibiscus sabdariffa* L. var. *altissima* β-galactosidase on a 10% slab gel. *Lane 1*: purified β-galactosidase (5 μg); *Lane 2*: high molecular weight markers (205 kD, 116 kD, 97 kD, 66kD, 45 kD and 29 kD).

With p-NP-β-D-galactoside as substrate, purified Thai Jute β-galactosidase showed the optimum pH of 4.0 and optimum temperature of 55°C. Compared to p-NP-β-D-galactoside as substrate, the enzyme showed 6% activity towards p-NP-α-L-arabinoside and 1 % activity towards p-NP-(-D-glucoside, but no detectable activity towards p-NP-α-D-galactoside, p-NP-α-D-glucoside, p-NP-α-D-mannoside, p-NP-β-D-mannoside, p-NP-α-L-fucoside, p-NP-β-L-fucoside, p-NP-β-D-xyloside, p-NP-β-D-N-acetylglucosamine, and p-NP-β-D-N-acetylgalactosamine (at 1 mM concentration of each substance). The K_m and V_{max} values of the enzyme (Table 3) could be determined using non-linear regression for p-NP-β-D-galactoside and o-NP-β-D-galactoside, but required linear regression for β-lactose because it was not possible to use high enough concentrations of β-lactose due to some product inhibition at higher concentrations. The V_{max}:K_m ratios clearly indicate that p-NP-β-D-galactoside was the preferred substrate, followed by o-NP-β-D-galactoside, while β-lactose was a rather poor substrate.

The inhibitory effects of metal ions, sugar derivatives and other compounds on the hydrolysis of p-NP-β-D-galactoside was studied (Table 4). The results showed 99% inhibition with 1 mM $HgCl_2$, 98% inhibition with 1 mM p-hydroxymercuribenzoate and 29% inhibition by 1 mM iodoacetate, but other metal ions and the chelating agent EDTA did not show significant inhibition. Of the sugar derivatives at 5 mM concentration, D-galactal, galactono-1,4-lactone, methyl-α-D-galactoside and methyl-β-D-galactoside showed more than 90% inhibition. Less inhibition was observed with 5 mM D-galactose, 5 mM p-NP-α-D-galactoside, and 5 mM phenyl-β-D-galactoside. Very slight inhibition was observed with 50mM β-lactose or 5 mM D-glucono-1,5-lactone.

DISCUSSION

In this paper, we have reported the purification of a β-galactosidase from Thai Jute (*Hibiscus sabdariffa* L. var. *altissima*). Several steps were required, partly because the Lactosyl Sepharose column did not appear to be as effective as reported for other β-galactosidases. Nevertheless, highly purified β-galactosidase, free of α-galactosidase contamination was obtained with 868-fold purification in a yield of 13% (Table 2). The final product had a specific activity of 25.2 U/mg protein (equivalent to 420 nkat/mg protein) and gave a single major protein band on SDS-polyacrylamide gel electrophoresis. This compares favorably with the results of (-galactosidase purification from radish seeds[16] and jack bean[25], which gave fold-purification, % yield and final specific activity of 281-fold, 13.6% and 5.62 U/mg for radish and 600-fold, 10% yield, and 18.6 U/mg for jack bean respectively. Moreover, our preparation, in addition, gave 17% yield of α-galactosidase of specific activity 12.8 U/mg, which showed less than 5% contamination with (-galactosidase. The latter material may be usable for certain applications, and may also be recycled on a Sephadex column to yield more β-galactosidase free of α-galactosidase contamination.

In the present studies, we have deliberately used only the β-galactosidase fraction, which was free of α-galactosidase, so as to provide an accurate profile of the properties of β-galactosidase from *Hibiscus sabdariffa* L. var. *altissima*. The enzyme appears to consist of a single subunit of molecular weight in the range of 55,000 (by gel filtration) to 66,000 (by SDS-polyacrylamide gel electrophoresis), similar to many other plant β-galactosidases, which generally have molecular weights in the range 45,000 to 80,000[10,12-13,15-17]. The pH optimum of Thai Jute β-galactosidase was 4.0, similar to the 3.1 to 5.0 found for other plant β-galactosidases[8-17], but different from bacterial β-galactosidases, which have higher pH optima in the range pH 6-7[26-28].

In terms of substrate specificity, Thai Jute β-galactosidase was unable to hydrolyze other p-NP-glycosides, except for p-NP-β-D-glucose and p-NP-α-L-arabinoside, which were hydrolyzed at 1% and 6% of the rate of p-NP-β-galactoside. This is not entirely surprising since galactose differs from glucose only in the configuration of the 4'-OH, and from arabinose only in the replacement of the C_6 -CH_2OH group by -H. The K_m value of Thai Jute β-galactosidase for p-NP-β-galactoside (0.80 mM) is in the same range (0.3 mM to 3 mM) as that observed with most β-galactosidases[10-16]. However, the K_m value for o-NP-β-galactoside (12.8 mM) is higher than those (generally 0.5 mM to 3.0 mM) of many other β-galactosidases[9-10,15-17], but higher Km's of 8.4 mM and 12.5 mM have also been observed with the enzymes

from wheat germ[17] and *Saccharomyces lactis*[29]. Moreover, the K_m value for β-lactose (84.7 mM) appears to be somewhat higher than the values (13-39 mM) observed for the enzyme from barley, corn, jack bean, rye, tobacco, spinach and wheatgerm[17], but is similar to that (85 mM) observed with the *Aspergillus niger* enzyme[30].

Various substances were found to decrease the ability of *Hibiscus sabdariffa* L. var. *altissima* β-galactosidase to hydrolyze p-NP-β-D-galactoside. These included the mercuric compounds $HgCl_2$ or p-hydroxymercuribenzoate, which can react with thiols and have also been reported to chelate active site acidic amino acids[31]. Other inhibitors included D-glucono-1,4-lactone and D-galactal, and inhibition by the former may indicate that the reaction proceeds by means of a lactone intermediate, as has been reported for some other glycohydrolases31. Inhibition of p-NP-β-D-galactoside hydrolysis by methyl-β-D-galactoside and phenyl-β-D-galactoside may result from the fact that these compounds may act as alternative substrates, while D-galactose may inhibit as a product of the reaction. On the other hand, methyl-α-galactoside and phenyl-α-galactoside may show their inhibitory action by binding to the active site, while not becoming hydrolyzed, because they have the incorrect anomeric structure. The lower inhibition by β-lactose, another alternative substrate, correlates with the very high K_m observed for this compound, and explains the rather poor elution of the Thai Jute enzyme from the Lactosyl Sepharose column when 1.0 M lactose was used.

In conclusion, we have purified β-galactosidase from a local plant, Thai Jute (*Hibiscus sabdariffa* L. var. *altissima*), and studied various properties of the enzyme, which show both similarities and differences from β-galactosidases from other plants. Further studies are being carried out on the use of this enzyme for oligosaccharide synthesis by reversal of the hydrolytic reaction.

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REFERENCES

1. Wallenfels, K. and Malhotra, O.P. (1961) In *Adv. Carbohydr. Chem.* (Wolform, M.L. and Tipson, R.S. eds.), vol 16, Academic Press, New York, pp. 239-298.
2. Steers, E., Jr. and Cuatrecasas, P. (1974) *Methods Enzymol.*. **34**, 350-358.
3. Dey, P.M. (1984) *Adv. Enzymol.* **56**, 141-249.
4. Wallenfels, K. and Weil, R. (1972) In The Enzymes (Boyer, P.D., ed.), 3rd ed., Academic Press, New York, pp. 617-663
5. Neufeld, E.F. (1991) *Ann. Rev. Biochem.* **60**, 257-280.
6. Palmiano, E.P. and Juliano, B.O. (1973) *Plant Physiol.* **52**, 274-277.
7. Bartley, I.M. (1974) *Phytochem.* **13**, 2107-2111.
8. Pressey, R. (1983) *Plant Physiol.* **71**, 132-135.
9. Komp, M. and Hess, D. (1981) *Phytochem.* **20**, 973-976.
10. Kundu, R.K., Kundu, P.D. and Banerjee, A.C. (1990) *Phytochem.* **29**, 2079-2082.
11. Dey, P.M. and Dixon, M. (1974) *Biochim. Biophys. Acta* **370**, 269-275.

12. Dey, P.M. (1977) *Phytochem.* **16**, 323-325.
13. Matheson, N.K. and Saini, H.S. (1977) *Carbohydrate Res.* **57**, 103-116.
14. Corchete, M.P. and Guerra, H. (1987) *Phytochem.* **26**, 927-932.
15. Biswas, T.K. (1987) *Phytochem.* **26**, 359-364.
16. Sekimata, M., Ogura, K., Tsumuraya, Y., Hashimoto, Y. and Yamamoto, S. (1989) *Plant Physiol.* **90**, 567-574.
17. Simos, G., Giannakouros, T. and Georgatsos, J.G. (1989) *Phytochem.* **28**, 2587-2592.
18. Kobata, A. (1979) *Anal. Biochem.* **100**, 1-14.
19. Welply, J.K. (1989) *Trends Biotechnol.* **7**, 5-10.
20. Surarit, R. Svasti, M.R. J., Srisomsap, C., Suginta, W., Khunyoshyeng, S., Nilwarangkoon, S., Harnsakul, P. and Benjavongkulchai, E. (1995) Proceedings, 11th Symposium of the Federation of Asian and Oceanian Biochemists and Molecular Biologists. Submitted.
21. Levi, G. and Teichberg, V.I. (1981) *J. Biol. Chem.* **256**, 5735-5740.
22. Montreuil, J., Bouquelet, S., Debray, H., Fournet, B., Spik, G., and Strecker, G. (1986) in Glycoproteins (Chaplin, M.F. and Kennedy, F., eds.) pp. 143-204, IRL Press, Oxford.
23. Bradford, M.M. (1976) *Anal. Biochem.*, **72**, 248-254
24 Laemmli, U. (1970) *Nature* **227**, 680-685.
25. Li, S.C., Mazotta, M.Y., Chien, S.F. and Li, Y.T. (1975 *J. Biol. Chem.* **250**, 6788-6791.
26. Marchesi, S.L., Steers, E. and Shifrin, S. (1968) *Biochim. Biophys. Acta* **181**, 20-34.
27. Miyazaki, Y. (1988) *Agric. Biol. Chem.* **52**, 625-631.
28. Lind, D.L., Daniel, R.M., Cowan, D.A., Morgan, H.W. (1989) *Enzym. Microb. Technol.* **11**, 180-186.
29. Mbuyi-Kalala, A., Schnek, A.G. and Leonis, J. (1988) *Europ. J. Biochem.* **178**, 437-443.
30. Widmer, F. and Leuba, J.L. (1979) β-Galactosidase from *Aspergillus niger*. *Europ. J. Biochem.* **100**, 559-567.
31. Esen, A. (ed.) (1993) *β-Glucosidases: biochemistry and molecular biology*. American Chemical Society, Washington D.C.

บทคัดย่อ

เอนไซม์ เบต้า-ดี-กาแลคโตซิเดสสามารถแยกได้จากเมล็ดปอแก้ว (*Hibiscus sabdariffa* L. var. *altissima*) โดยการตกตะกอนด้วยเกลือแอมโมเนียมซัลเฟต ผ่านคอลัมน์ดีอีเออี เซลลูโลส คอลัมน์เซฟาเดกซ จี-100 คอลัมน์ แลคโตซิล เซฟาโรส และคอลัมน์ ดีอีเออี เซลลูโลสครั้งที่สอง พบว่าผลสุดท้ายสามารถทำเอนไซม์ให้บริสุทธิ์ได้ 868 เท่า ได้ผลลัพธ์ (yield) 13 เปอร์เซนต์ เมื่อทำอิเลคโตรโฟรีซิสแบบ SDS-PAGE ปรากฏเป็นแถบโปรตีนแถบเดียวที่มีขนาดน้ำหนักโมเลกุลเป็น 66,000 ดาลตัน ในขณะที่มีน้ำหนักโมเลกุลเป็น 55,000 ดาลตันในสภาพธรรมชาติเมื่อหาโดยวิธีเจลฟิลเตรชัน เอนไซม์มีค่า K_m ต่อสับสเตรทต่างๆ ดังนี้ 0.08 มิลลิโมลาร์สำหรับ พีเอนพี-เบต้า-ดี-กาแลคโตไซด์ 12.8 มิลลิโมลาร์สำหรับ โอเอนพี-เบต้า-ดี-กาแลคโตไซด์ และ 84.7 มิลลิโมลาร์สำหรับ เบต้า-แลคโตส มีค่าความเร็วสูงสุดสัมพัทธ์เป็น 100 เปอร์เซนต์ สำหรับ พีเอนพี-เบต้า-ดี-กาแลคโตไซด์ 26.7 เปอร์เซนต์ สำหรับ โอเอนพี-เบต้า-ดี-กาแลคโตไซด์ และ 9.4 เปอร์เซนต์ ต์สำหรับ เบต้า-แลคโตส นอกจากนี้ยังพบว่า ดี-กาแลคตัล กาแลคโตโน-1,4-แลคโตน เมทิล-อัลฟา-ดี-กาแลคโตไซด์ เมทิล-เบต้า-ดี-กาแลคโตไซด์ เมอคิวริคคลอไรด์ และ พารา-ไฮดรอกซีเมอคิวริเบนโซเอท เป็นตัวยับยั้งที่รุนแรงสำหรับการย่อยพีเอนพี-เบต้า-ดี-กาแลคโตไซด์