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The conversion of group B red blood cells into group O by an α-D-galactosidase from taro (*Colocasia esculenta*)*

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ABSTRACT

An α-D-galactosidase (EC 3.2.1.21), capable of converting group B into group O red cells, was isolated from the stem portion of taro. It was purified about 3000 fold by gel filtration and ion-exchange chromatography. The blood group-converting activity was demonstrated by hemolysis and hemagglutination studies. This activity is comparable to that of α-D-galactosidase isolated from coffee beans. Taro α-D-galactosidase also hydrolyzes (1→4)- and (1→6)-linked α-D-galactopyranosyl groups from D-galactose-containing glycoconjugates. Taro α-D-galactosidase has a low K_m value (0.28mM), a low molecular weight (40 000), and a neutral optimal pH (6.0). At a final enzyme concentration of 30 units/mL in the incubation mixture, the conversion of group B into group O activity was completed within two hours, without apparent changes in the shape of the red cells.

INTRODUCTION

α-D-Galactosidase (EC 3,2,1.21) has been isolated from various sources, such as coffee beans[1,2], *Aspergillus niger*[3], figs[4], *Clostridium sporogenes*[5], *Bacillus cereus*[6], and *Trichomonas foetus*[7,8]. Of those isolated, *B. cereus*, *T. foetus*, and coffee bean α-D-galactosidases have been shown to be able to hydrolyze the (1→3)-linked α-D-galactopyranosyl group from blood group B antigen and has been used to convert human group B to group O red blood cells.

Since 25.8% of the Chinese population possess blood group B type and there is a continual shortage of group O red blood cells in transfusion services, the possibility of converting group B to group O red blood cells is of great importance. Although coffee bean α-D-galactosidase is commercially available, its use is not economical. In addition the purification of the coffee bean α-D-galactosidase is very difficult, and the green coffee beans are not available in this country. We describe herein the isolation of α-D-galactosidase from taro and its hydrolyzing activity of the terminal (1→3)-linked

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α-D-galactopyranosyl end-group from the carbohydrate chains of human blood group B antigen.

EXPERIMENTAL

Materials. — The Stem portion of taro plant, *Colocasia esculenta*, (potato-like and rich in starch) was purchased locally. The synthetic substrates, 4-nitrophenyl glycopyranosides, coffee bean α-D-galactosidase (lot 16F-0678), galactose dehydrogenase, β-nicotinamide adenine dinucleotide, and bovine serum albumin were purchased from Sigma Chemical Co. (St. Louis, MO). Rabbit complement (Terasaki HLA-ABC locus; lot 4/87) was purchased from Terasaki Co. (CA). Lactosylceramide and $GbOse_3$-Cer were purchased from Glycolipid Biochemical Co. (Birmingham, AL). CPDA-1 group B blood was obtained from Taipei Blood Donation Center. Anti-B antiserum was purchased from BCA Cooper Biomedical Co., (Malvern, PA). Anti-H lectin was extracted from *Ulex europaeus*. Anti-P1 antiserum was purchased from Ortho Diagnostic System Inc. (Raritan, NJ). 50mM Citrate–phosphate buffer, pH 6.0, was prepared from 0.05M Na_2HPO_4 and 0.015M citric acid.

Methods. — Protein content was determined by the Lowry *et al.* method[9] with bovine serum albumin (Sigma) as standard. The enzymic assay for α-D-galactosidase activity was carried out by a modification of the standard method[10]; one unit of enzyme was defined as that amount of enzyme which hydrolyzes 1μ mol of 4-nitrophenyl α-D-galactopyranoside/min at 37°; specific activity was defined as unit/mg of protein. The molecular weight of the purified α-D-galactosidase was estimated by the slab-gel electrophoresis technique.

Slab-gel electrophoresis was carried out under dissociating conditions in a 12.5% poly(acrylamide) gel of 0.75-mm thickness, according to the procedure of Hames and Rickwood[11]. Analytical thin-layer chromatography for glycosphingolipids was performed according to Svennerholm *et al.*[12].

Measurement of hemolysis was carried out according to Grundbacher[13]. Hemagglutination tests were performed according to the standard tube method of the American Association of Blood Banks[14]. The D-galactose dehydrogenase assay was used to determine the released D-galactose from red cells which had been prefixed with glutaraldehyde.

Isolation of α-D-galactosidase from taro by column chromatography. — Unless otherwise indicated, all operations for the isolation were carried out at 4°. Taro (3 kg) was homogenized with 0.05M citrate–phosphate buffer, pH 6.0 (5 L), and the suspension was filtered. Solid $(NH_4)_2SO_4$ was added to the clear buffer extract to give a 80% saturation. The precipitate was collected and dissolved in 0.05M citrate–phosphate buffer, pH 6.0.

A Sephadex G-100 column (5 × 90 cm) was preequilibrated with 0.05M citrate–phosphate buffer, pH 6.0. The crude enzyme solution (3.4 g of protein in 50 mL) was applied to the column and eluted with the same buffer. Fractions 42–68 containing α-D-galactosidase (Fig. 1) were pooled and concentrated by reverse dialysis with saturated $(NH_4)_2SO_4$.

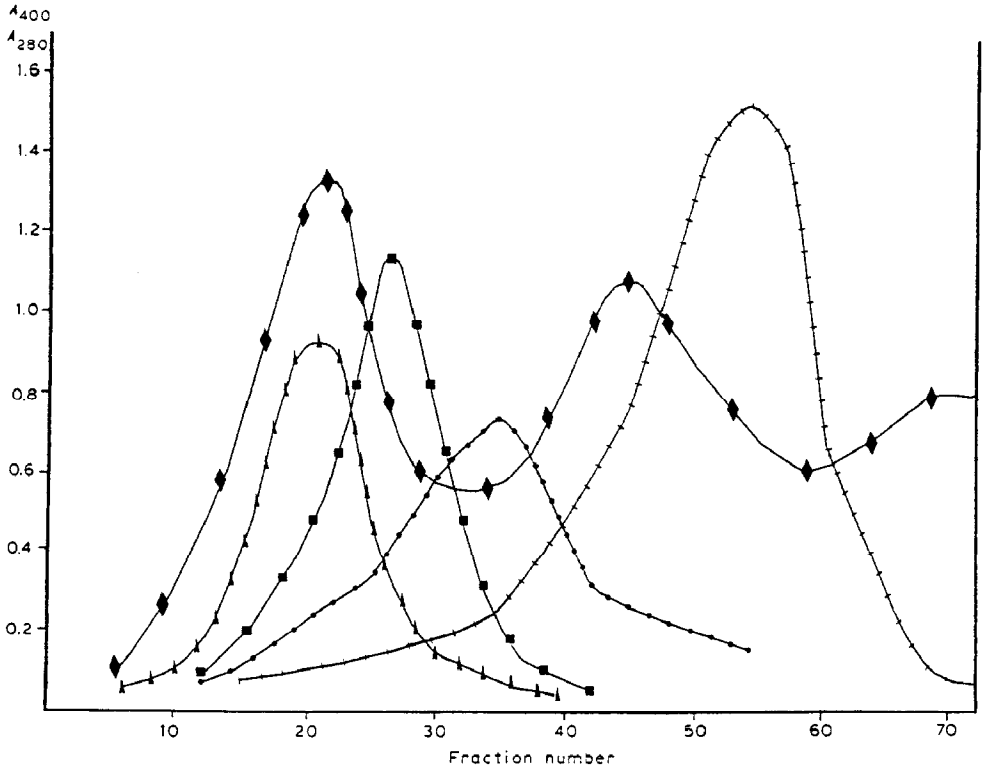

Fig. 1. Gel filtration of α-D-galactosidase on Sephadex G-100 column at a flow rate of 60 mL h. An aliquot (25 μL) of a 15-mL fraction was added to 4mM 4-nitrophenyl glycosides (50 μL) for measuring the enzyme activity. The incubation time for all the enzymes was 15 min, except for β-D-galactosidase which was incubated for 60 min: (+ + +) α-D-galactosidase activity; (●–●–●–●) β-D-galactosidase activity, (–■–■–■–) *N*-acetyl-β-D-glucosaminidase activity, (–▲–▲–▲–▲–) α-D-mannosidase activity measured by A_{400}; and (–◆–◆–◆–◆–) A_{280}.

A column (2.5 × 30 cm) of DEAE-Sephadex A-50 was preequilibrated with 0.01M phosphate buffer, pH 7.0. The concentrated sample from fractions 42–68 containing 2 g of protein in 50 mL (Fig. 1) was dialyzed against the same buffer, and then applied to the column. α-D-Galactosidase was eluted by the same buffer in fractions 45–75 (Fig. 2), which were pooled and concentrated by reverse dialysis with saturated $(NH_4)_2SO_4$.

An aliquot from this concentrated solution (100 mg of protein in 7 mL), was applied to a Sephadex G-100 column (2.6 × 90 cm), which had been preequilibrated with 0.05M citrate–phosphate buffer, pH 6.0. The α-D-galactosidase-active fractions (45–62, Fig. 3) were pooled and concentrated by reverse dialysis with saturated $(NH_4)_2SO_4$.

Agglutination of group B red blood cells with anti-B serum and anti-H lectin after treatment with taro α-D-galactosidase. — The packed group B red blood cells were treated, for various lengths of time, with an equal volume of α-D-galactosidase in 0.01M citrate–phosphate buffer, pH 6.0, containing 0.09% NaCl and 3% glycerol (v/v) at a

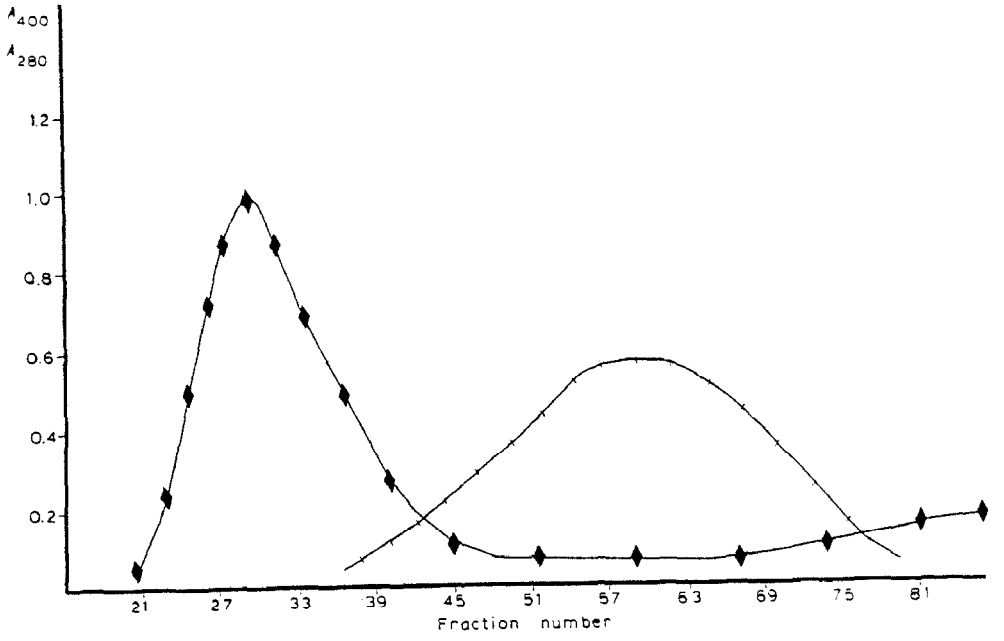

Fig. 2. Purification of α-D-galactosidase by chromatography on a DEAE-Sephadex A-50 column at a flow rate of 50 mL/h and 7-mL fractions were collected: (+ + +) α-D-galactosidase activity measured at A_{400}; (–◆–◆–) protein content measured at A_{280}.

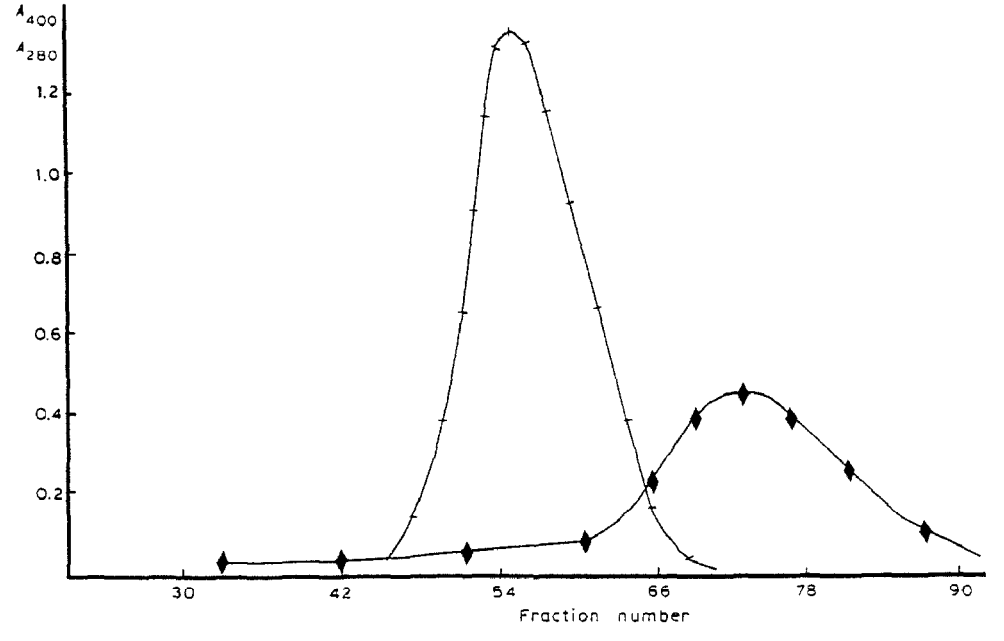

Fig. 3. Second gel filtration on a Sephadex G-100 column at a flow rate of 20 mL/h and fractions (4 mL) were collected. An aliquot (10 μL) from each fraction was used for measuring enzyme activity for 10 min: (+ + +) α-D-galactosidase acitivity at A_{400}; (–◆–◆–◆–) protein content measured at A_{280}.

final concentration of 30 units/mL of the mixture. After the incubation, the cells were washed with phosphate-buffered saline solution (PBS) containing 3% glycerol and resuspended in the same buffer (20 vols.). A control experiment was carried out under the same conditions without the addition of the enzyme. The agglutination test was carried out as follows. The α-D-galactosidase-treated B red blood cells (50 μL) were added to various dilutions of anti-B or anti-H antiserum (50 μL). After 5 min, the cells were lightly centrifuged and the agglutination of the cells was observed by eyes.

Hemolysis of group B red blood cells by anti-B after treatment with taro α-D-galactosidase. — Group B red cells were treated with taro α-D-galactosidase as just described. A control experiment was carried out under the same condition without the enzyme. Analysis of hemolysis was carried out as follows. A mixture of enzyme-treated cells (100 μL), anti-B antiserum (200 μL), and complement (100 μL) was incubated with shaking at 37° for 2 h. The nonhemolyzed cells were removed by centrifugation, the volume of the supernatant was completed to 1 mL with PBS, and the absorbance at 540 nm was measured. The degree of hemolysis was calculated as the ratio of the readings between the tested samples and that of the same amount of red cells hemolyzed by the addition of distilled water (0.9 mL). All tests were performed in duplicate.

RESULTS AND DISCUSSION

Our purification scheme is summarized in Table I. Starting with 3 kg of taro, we were able to recover about 26% of purified α-D-galactosidase which showed a major

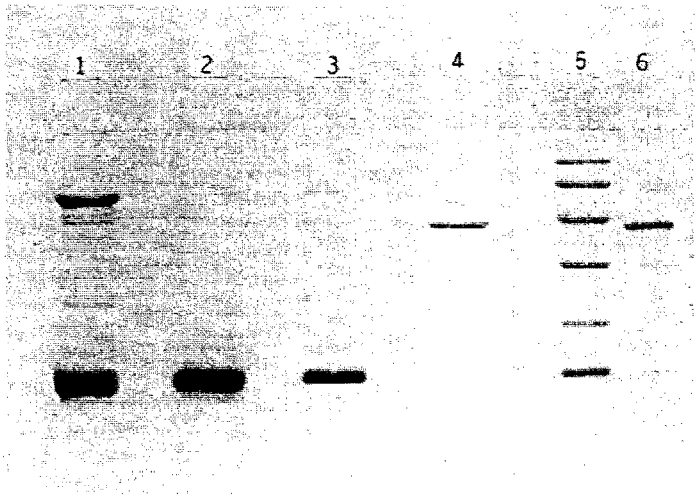

Fig. 4. Slab-gel electrophoresis of enzyme proteins obtained from different steps of purification. A 12.5% poly(acrylamide) gel, 0.75-mm thickness, was prepared in Tris–glycine buffer, pH 8.4, and the experiment was performed at a constant current at 30 mA for 2 h. Then the gel was stained with 0.5% Coomassie Blue in 5:5:2(v/v) water–methanol–glacial acetic acid, and destained with the same solution without the dye: Lane 1, crude extract; lane 2, fractions 42–68 of Fig. 1; lane 3, fractions 45–75 of Fig. 2; lanes 4 and 6, purified α-D-galactosidase from fractions 45–62 of Fig. 3; and lane 5, standard proteins (from top): phosphorylase b, 94 000; BSA, 67 000; ovalbumin, 43 000; carbonic anhydrase, 30 000; soy bean trypsin inhibitor, 20 100; and α-lactalbumin, 14 400.

protein band and a faint, fast-moving contaminant band in gel electrophoresis (Fig. 4, lanes 4 and 6). The enzyme was free of protease activity. After purification, the specific activity, was increased ~ 3000-fold. From the result of poly(acrylamide) gel electrophoresis, the mol.wt. was found to be ~40 000. The isoelectric point (pI), as determined by

TABLE I

Purification of α-D-galactosidase from taro[a]

Steps	*Protein* (*mg*)	*Activity* (*units*)	*Recovery* (%)	*Specific activity* (*units/mg*)	*Purity* (*fold*)
Crude extract	7629	263	100	0.034	1.00
Ammonium sulfate ppt.	3624	213	81	0.059	1.73
Sephadex G-100	1596	189	71	0.118	5.47
DEAE-Sephadex A-50	8.6	95	35	10.988	323
Sephadex G-100	0.7	68	26	97.143	2857

[a] 3 kg.

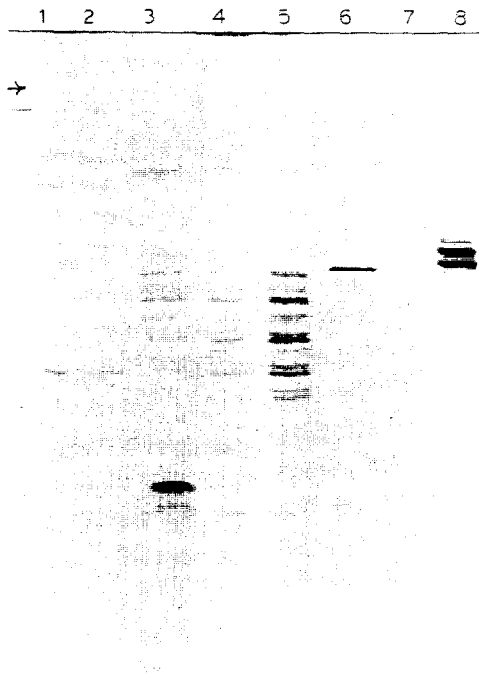

Fig. 5. Isoelectric focusing on agarose gel. The isoelectric point (pI) was measured on ultrathin (0.5-mm thickness) agarose gel containing ampholyte pH 3–10. Standard protein solutions containing 1–2 μg of each protein (pH 3.5–9.3) were used. The experiment was performed at 1800 V h for 1.5 h at 9° with M NaOH as cathode solution and 0.01M H_2SO_4 as anode solution: Lanes 1, 2, and 7, standards with known pI (Pharmacia); lane 3, crude taro extract; lane 4, sample after first gel filtration on Sephadex G-100; lane 5, sample after chromatography on DEAE-cellulose; lane 6, sample after second gel filtration on Sephadex G-100; and lane 8, human hemoglobins (pI 6.8). Arrow indicates the origin.

isoelectric focusing on agarose gel, was 6.7 (Fig. 5). A separate lane of the same gel containing the purified enzyme was incubated with 4-nitrophenyl α-D-galactopyranoside and the enzyme activity corresponded with the protein band. Other lanes of the same gel were incubated overnight with each of the following 4-nitrophenyl glycosides: β-D-galactopyranoside, α-D-mannopyranoside, β-D-mannopyranoside, 2-acetamido-2-deoxy-α-D-galactopyranoside, and 2-acetamido-2-deoxy-β-D-glucopyranoside; no glycosidase activity was found in these incubations. With 4-nitrophenyl α-D-galactopyranoside as substrate, the optimal pH for the purified enzyme was 6 (Fig. 6). The enzyme activity at an acidic pH between 4 and 5 was greater than that at an alkaline pH between 7 and 7.5. The enzyme activity was stable at the acidic side of the optimal pH. The enzyme only lost ~10% of its activity when it was heated at 40° for 100 min. The Michaelis–Menten constant, K_m, was determined to be 0.28mM by use of 4-nitrophenyl α-D-galactopyranoside as substrate, whereas the K_m values for α-D-galactosidases from other sources were 0.43mM for both bean sprouts[15] and *Mortierella vinacea*[16], 0.5mM for fig, and 1.0mM for coffee bean α-D-galactosidase[17].

α-D-Galactosidase isolated from *Aspergillus niger*[3] only cleaves (1→2)-linked α-D-galactopyranosyl nonreducing end groups of oligosaccharide chains. Fig[4] and

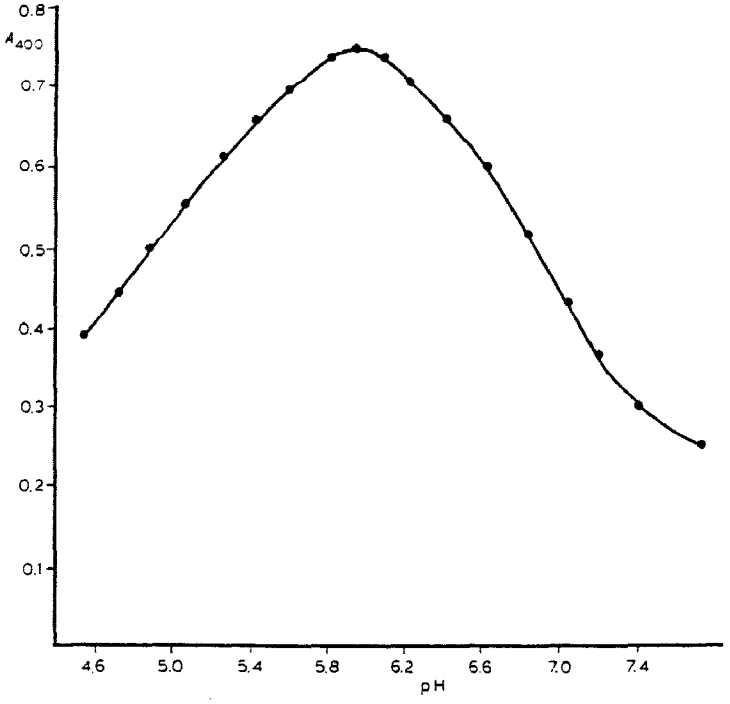

Fig. 6. Optimum pH for taro α-D-galactosidase. The enzyme activity was measured at various pH ranges, for pH 4.6–6.0, 0.1M citrate–phosphate buffer, and for pH 6.1–7.6, 0.1M phosphate buffer. The substrate (25 μL of 8 mM 4-nitrophenyl α-D-galactopyranoside) in water was added to each buffer (25 μL), and then the enzyme solution (0.005 unit, 10 μL) in 0.05 M citrate–phosphate, pH 6.0, was added. After 10 min at 37°, the reaction was terminated by the addition of 0.2M Na_2CO_3, pH 9.8 (1 mL). The absorbance was measured at 400 nm.

Mortierella vinacea[16] α-D-galactosidases are able to cleave (1→4)-linked α-D-galactopyranosyl groups. However, the *Mortierella vinacea* enzyme has limitations in that it is unable to hydrolyze D-galactosyl groups from glycolipids, such as globotriaosylceramide ($GbOse_3Cer$), as reported by Suzuki *et al.*[16]. In 1956, Iseki *et al.*[5] reported the presence, in *Bacillus cereus*, of an α-D-galactosidase that can destroy blood group B substances. Subsequently, Watkins[7] and Yates *et al.*[3] purified three α-D-galactosidases from *Trichomonas foetus*; one of them was the blood group B destroying enzyme. However, until now, the only enzyme used to hydrolyze the (1→3)-linked α-D-galactosyl group from human blood group B antigen is coffee bean α-D-galactosidase[1,2].

Fig (1→4)-α-D-galactosidase has been reported to convert $GbOse_3Cer$ into lactosylceramide[18]. The taro enzyme also hydrolyzed the (1→4)-linked α-D-galactosyl group from $GbOse_3Cer$ and completely converted it to lactosylceramide (Fig. 7, lane 3). Treatment of the red blood cell P1 antigen, which contains a (1→4)-linked α-D-galactosyl end group, with taro α-D-galactosidase in 0.01 M citrate–phosphate buffer, pH 6.0, at 37° for 1 h, eliminated the P1 activity. In the Chinese population, 32% have the P1 and 68% the P2 phenotype[19]. Coffee bean α-D-galactosidase also hydrolyzes ~50% of $GbOse_3Cer$ under the same conditions (Fig. 7, lane 1).

Besides its α-D-(1→4)-hydrolyzing activity, taro α-D-galactosidase also hydrolyzed (1→6)-linked-α-D-galactosyl groups, such as melibiose and raffinose. However, under the same incubation conditions, the rate of hydrolysis of melibiose was greater

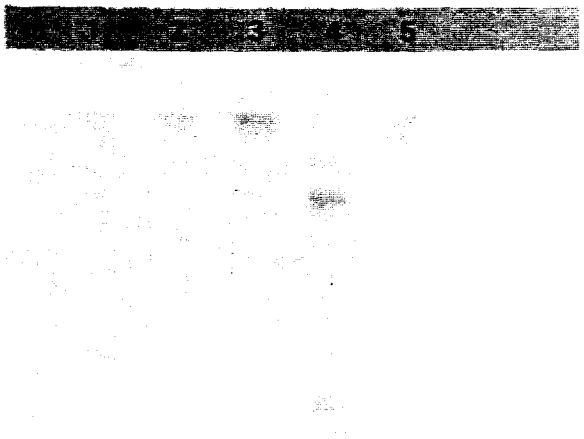

Fig. 7. T.l.c. of the hydrolysis of $GbOse_3Cer$ by taro and coffee bean α-D-galactosidases. $GbOse_3Cer$ (20 μg) was incubated with purified taro or coffee bean α-D-galactosidase (0.3 unit) in 0.05 M citrate–phosphate buffer, pH 6.0 (0.4 mL), containing sodium taurodeoxycholate (250 μg) for 16 h at 37°. After incubation, 2:1 chloroform–methanol (4 vols.) was added. The bottom layer was removed, dried, and applied to the t.l.c. plate which was irrigated with 60:40:9 chloroform–methanol–water. The dried plate was sprayed with 1% orcinol–5% H_2SO_4 and heated at 120° for 15 min: Lane 1, $GbOse_3Cer$ hydrolyzed with coffee bean α-D-galactosidase; lanes 2 and 5, lactosylceramide (standard); lane 3, $GbOse_3Cer$ hydrolyzed with taro α-D-galactosidase; and lane 4, $GbOser_3Cer$ (standard).

TABLE II

Agglutination of taro α-D-galactosidase-treated group B red blood cells with anti-B antiserum and anti-H lectin

Duration of enzyme treatment (min)	*Agglutination with*						
	Anti-B serum			*Anti-H lectin*			
Dilutions	1:10	1:20	1:40	1:1	1:2	1:4	1:8
0	4+	3+	2+	4+	3+	3+	1+
30	2+	2+	1+	4+	4+	4+	4+
60	2+	±	1+	4+	4+	4+	4+
120	±	±	±	4+	4+	4+	4+

than that of raffinose.

Taro galactosidase also showed a good activity in cleaving (1→3)-linked α-D-galactosyl end groups, as shown by the conversion of human blood group B antigen into O. It has been reported that the human group B red blood contains (1→3)-linked α-D-galactosyl end groups[20,21]. The hydrolysis of these groups from group B antigen (conversion to H antigen) was monitored by hemolysis[13] and hemagglutination[14] tests. The terminal groups were hydrolyzed by taro α-D-galactosidase (0.5 unit/mL of RBC) at 37° overnight. The galactose dehydrogenase method[22] indicated that 4 μg of D-galactose/mL of RBC had been released.

The decrease in the agglutinability of group B red blood cells by anti-B antiserum treatment and the concomitant increase in the agglutinability by anti-H antiserum, by taro α-D-galactosidase, treatment (30 U/mL) is shown in Table II. After 30 min, the cells showed partial conversion from B to H activity, and the conversion was completed within 120 min. The hemolysis (by anti-B antiserum) of group B red blood cells treated with 30 U/mL (final concentration) of taro α-D-galactosidase is shown in Table III. After 120 min, the activity had decreased to 0%. A similar result was observed with 30 U/mL of coffee bean α-D-galactosidase.

TABLE III

Hemolysis of group B red blood cells by anti-B serum after taro α-D-galactosidase treatment[a]

Duration of enzyme treatment (min)	*Hemolysis (%) after treatment with α-D-galactosidase from*		
	Taro	*Coffee bean*	
	(30 U/mL)	*(30 U/mL)*	*(60 U/mL)*
0	44–45	44–45	44–45
30	26–27	26–29	5–8
60	2–7	9–10	2–3
120	0	0	0

[a] The results show the ranges of four experiments.

Until now, coffee bean α-D-galactosidase has been one of the few known α-D-galactosidases reported to be capable of converting group B red blood cells into group O. The converted group O red blood cells are able to survive *in vivo* in group A, B, and O individuals, as reported by Goldstein *et al.*[23,24]. Our studies on the *in vivo* survival of taro galactosidase-treated group B red blood will be reported elsewhere.

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