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Synthesis of β-D-Galactofuranosides and Their Behavior toward β-Galactosidase

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After ethanolysis of galactose with Dowex-50 as catalyst, ethyl β-D-galactofuranoside, ethyl β-D-galactopyranoside and ethyl α-D-galactopyranoside were isolated by cellulose column chromatography. *m*-Tolyl and guaiacol β-D-galactofuranoside were also prepared.

Ethyl, phenyl, *m*-tolyl and guaiacol β-D-galactofuranoside were hydrolyzed by almond emulsin β-galactosidase, but not hydrolyzed by *E.coli* K 12 and bovine liver β-galactosidase. The kinetics of the hydrolysis of β-D-galactofuranoside by almond emulsin β-galactosidase were investigated. The pH optimum of the β-D-galactofuranoside is pH 5.0 in phosphate–citrate buffer. The Michaelis constant of phenyl β-D-galactofuranoside is 5.6×10^{-2} M.

The kinetics of the hydrolysis of corresponding β-D-galactopyranoside were also investigated. The pH optimum is pH 4.5—5.0 for almond emulsin β-galactosidase, pH 5.5 for bovine liver β-galactosidase and pH 7.0—7.5 for *E. coli* K 12 β-galactosidase. The Michaelis constant of phenyl β-D-galactopyranoside for almond emulsin β-galactosidase is 3.2×10^{-3} M. The Michaelis constant of phenyl β-D-galactopyranoside is smaller than that of corresponding furanoside.

Many reports have already been made on the specificity of β-galactosidase to β-D-galactopyranoside. Wallenfels has made a detailed study of the specificity of *E. coli* ML 309-[2] and calf intestine β-galactosidase[3] to β-D-galactopyranosides. On the other hand, little attention has been paid to the action of β-galactosidase on β-D-galactofuranoside. There was only report by Wallenfels in 1960, which showed that calf intestine β-galactosidase does not hydrolyze ethyl β-D-galactofuranoside.[3]

In the preceding papers, it was clarified that rabbit liver β-glucuronidase hydrolyzes β-D-glucofuranosiduronic acid[4] and that almond emulsin β-glucosidase hydrolyzes β-D-glucofuranoside.[5] In the light of these facts it is of interest to make reexamination of the specificity of β-galactosidase to β-D-galactofuranoside by synthesizing appropriate chromogenic substrates.

In this paper will be described the synthesis of β-D-galactofuranoside and the hydrolytic actions of almond emulsin, bovine liver and *E.coli* K 12 β-galactosidases to the furanoside and its corresponding pyranoside.

Experimental

Materials

The substrates used in this study were prepared by the methods referred in Table except the following compounds.

Ethyl β-D-Galactofuranoside and Ethyl β-D-Galactopyranoside——Ten grams of D-galactose and 10 g of dry Dowex-50 were refluxed with 50 ml of EtOH for 1 hr. The ion exchange resin was filtered off from

1) Location: *a*) *Tamagawa-cho, Takamiya, Fukuoka*; *b*) *Katakasu, Fukuoka.*
2) K. Wallenfels, J. Lehmann, and O.P. Malhotra, *Biochem. Z.*, 333, 209 (1960).
3) K. Wallenfels and J. Fischer, *Z. Physiol. Chem.*, 321, 223 (1960).
4) K. Kato, K. Yoshida, and H. Tsukamoto, *Chem. Pharm. Bull.* (Tokyo), 12, 670 (1964).
5) K. Yoshida, T. Miyawaki, N. Harada, and K. Kato, *Chem. Pharm. Bull.* (Tokyo), 14, 583 (1966).

the reaction mixture and washed with several portions of EtOH. The filtrate and the washings were combined and concentrated to about 15 ml under reduced pressure at 40°. The solution was chromatographed on a 4.5×80 cm column containing Whatman CF 11 cellulose powder, using AcOEt–*n*-PrOH–H_2O (5:3:2) as developer. The optical rotation of the effluent was measured and the elution curve is shown in Fig. 1. The effluent was collected in three fractions as indicated in Fig. 1, and the solvent was removed from each fraction under reduced pressure at 40°. The first fraction was dissolved in acetone and upon refrigeration crystalline ethyl β-D-galactofuranoside was obtained. Crude ethyl β-D-galactofuranoside was recrystallized from acetone, mp 88°, $[\alpha]_D^{15}$ −104°(c=0.25, l=1, H_2O). Yield 1.1 g. The second and third fractions were dissolved separately in acetone to yield ethyl α-D-galactopyranoside; total yield 1.9 g. Crude ethyl α-D-galactopyranoside was recrystallized from EtOH, mp 140°, $[\alpha]_D^{15}$ +180° (c=0.25, l=1, H_2O). Yield 1.5 g. Ethyl β-D-galactopyranoside was separated from mother liquor of the third chromatographic fraction: yield 0.3 g. Additional yield of 0.2 g was obtained after concentration of the mother liquor. The combined crude ethyl β-D-galactopyranoside was recrystallized from EtOH, mp 161°, $[\alpha]_D^{15}$ −6° (c=0.25, l=1, H_2O). Yield 0.3 g.

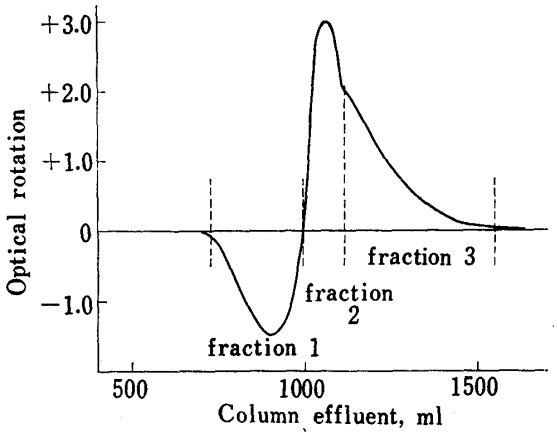

Fig. 1. Chromatographic Separation of Ethyl Galactoside

***m*-Tolyl β-D-Galactofuranoside**——A mixture of 19 g of pentaacetylgalactofuranose,[6] 20 g of *m*-cresol and 0.2 g of *p*-toluensulfonic acid was fused *in vacuo* for 30 min at 115—120°. The reaction mixture was extracted with 200 ml of benzene. The benzene extract was washed with 10% NaOH solution and H_2O. The benzene solution was then dried over anhydrous magnesium sulphate, and evaporated to dryness under reduced pressure. The residue was dissolved in 300 ml of anhydrous MeOH, and 30 ml of 1% CH_3ONa was added to the solution. After standing 4 hr, the solution was evaporated to dryness under reduced pressure. The residue was dissolved in 70 ml of H_2O, and deionized with IR-120 and IR-45. The deionized solution was evaporated to dryness under reduced pressure. The residue was dissolved in AcOEt, and crystallized by addition of $CHCl_3$. The crude product was recrystallized from AcOEt and $CHCl_3$ several times. Pure *m*-tolyl β-D-galactofuranoside melt at 69—71°, $[\alpha]_D^{15}$ −168° (c=0.25, l=1, H_2O). Yield 0.8 g. *Anal.* Calcd. for $C_{13}H_{18}O_6$: C, 57.78; H, 6.66. Found: C, 57.41; H, 6.63.

Guaiacol β-D-Galactofuranoside——It was prepared as described above for *m*-tolyl β-D-galactofuranoside; mp 82°, $[\alpha]_D^{15}$ −152° (c=0.25, l=1, H_2O). *Anal.* Calcd. for $C_{13}H_{18}O_7 \cdot 1/2H_2O$: C, 52.86; H, 6.48. Found: C, 53.04; H, 6.32.

The mixed melting point determination of these aryl β-D-galactofuranosides with corresponding pyranosides[7,8] showed a marked depression of melting point and the IR spectra of these furanosides were not identical with those of corresponding pyranosides. Ring structure of these aryl β-D-galactofuranosides was confirmed by periodate oxidation according to the preceding papers.[9,10]

Enzyme Preparation——Almond emulsin and bovine liver β-galactosidase and alcohol dehydrogenase were obtained from Sigma Chemical Co. *E. coli* K 12 β-galactosidase was prepared by the method of Craven, *et al.*[11]

Methods

After enzymic hydrolysis of the substrates at appropriate pH and temperature, the liberated aglycon was determined as follows.

Determination of Phenol, *m*-Cresol and Guaiacol——Liberated phenol, *m*-cresol and guaiacol were extracted from incubation mixtures with 10 ml of benzene, and extracted again from the benzene solution with 2 ml of 1% NaOH solution respectively. To the extract was added 5 ml of Folin reagent,[12] followed by 15 ml of 20% Na_2CO_3 solution and 8 ml of H_2O. The mixture was allowed to stand at room temperature for 20 min, and the absorbancy at 520 mμ was measured in Hitachi spectrophotometer Model EPU 2A.

6) R.K. Ness, H.G. Fletcher, and C.S. Hudson, *J. Am. Chem. Soc.*, **73**, 3742 (1951).
7) B. Helferich and W. Göller, *Z. Physiol. Chem.*, **247**, 220 (1937).
8) K. Nishizawa, *Bull. Chem. Soc. Japan*, **16**, 155 (1941).
9) K. Kato, K. Yoshida and H. Tsukamoto, *Chem. Pharm. Bull.* (Tokyo), **10**, 1242 (1962).
10) K. Kato, K. Yoshida and H. Tsukamoto, *Chem. Pharm. Bull.* (Tokyo), **12**, 664 (1964).
11) G.R. Craven, E. Steers, and C.B. Anfinsen, *J. Biol. Chem.*, **240**, 2468 (1965).
12) O. Folin and V. Ciocalteu, *J. Biol. Chem.*, **73**, 629 (1927).

Determination of Ethanol——After addition of 0.5 ml of 13.6% $HClO_4$, incubation mixture was centrifuged for 4 min in refrigerated centrifuge at $7000 \times g$. To 3.8 ml of 0.1M pyrophosphate buffer, pH 8.6, were added 1 ml of the supernatant solution, 0.1 ml of 0.02M NAD and 0.1 ml of alcohol dehydrogenase solution. The reaction mixture was incubated for 1 hr at 25°, and the absorbancy at 340 mμ was measured.

Results and Discussion

Synthesis of Substrates——Since Cadotte, *et al.* reported that cation exchange resin, especially those possessing sulfonic acid groups, can be used to catalyze the formation of glycoside,[13] ethyl β-D-galactofuranoside and ethyl β-D-galactopyranoside were prepared by glycosidation with ethanol and cation exchange resin, and ethyl β-D-galactofuranoside, ethyl β-D-galactopyranoside and ethyl α-D-galactopyranoside were isolated by the cellulose column chromatography. Ethyl α-D-galactofuranoside, however, was not obtained. Aryl β-D-galactofuranoside is a substrate advantageous in studying enzymic reaction, as it is possible to determine, by colorimetric method, the aglycon liberated by enzymic reaction. *m*-Tolyl β-D-galactofuranoside and guaiacol β-D-galactofuranoside were synthesized according to the method of Jerkman, *et al.*, who synthesized phenyl β-D-galactofuranoside.[14]

pH Optimum for Hydrolysis of β-D-Galactopyranoside by β-Galactosidase——The pH optimum for the hydrolysis of β-D-galactopyranoside was determined in 0.2 M phosphate —0.1 M citrate buffer between pH 3.5 and 8.0. The pH optimum of β-D-galactopyranoside is between pH 4.0 and 4.5 for almond emulsin β-galactosidase, pH 5.5 for bovine liver β-galactosidase and between pH 7.0 and 7.5 for *E. coli* K 12 β-galactosidase.

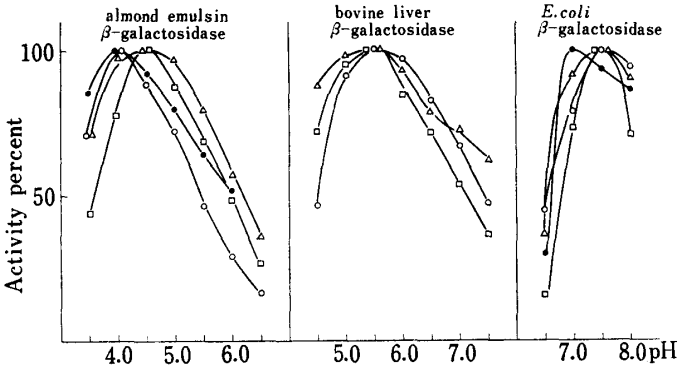

Fig. 2. Effect of pH on the Hydrolysis of Various β-D-Galactopyranosides

Incubation mixture contained 1 ml of 0.2M phosphate–0.1M citrate buffer, 0.5 ml of 0.004M substrate solution and 0.5 ml of enzyme solution. Incubation was carried out for 1 hr at 40°.
—○—: phenyl β-D-galactopyranoside
—△—: *m*-tolyl β-D-galactopyranoside
—□—: guaiacol β-D-galactopyranoside
—●—: ethyl β-D-galactopyranoside

Michaelis Constants (K_m) of Hydrolysis of β-D-Galactopyranoside with β-Galactosidase——Table I shows the values of K_m of the three β-galactosidases for β-D-galactopyranosides.

Table I. Michaelis Constants (K_m) of Almond Emulsin, Bovine Liver and *E.coli* K 12 β-Galactosidases for Various Aryl β-D-Galactopyranosides

	Phenyl β-D-galactopyranoside[15] (*M*)	*m*-Tolyl β-D-galactopyranoside[7] (*M*)	Guaiacol β-D-galactopyranoside[8] (*M*)
Almond emulsin β-galactosidase	3.2×10^{-3}	2.5×10^{-2}	3.5×10^{-3}
Bovine Liver β-galactosidase	7.7×10^{-3}	1.6×10^{-2}	4.5×10^{-3}
E.coli K12 β-galactosidase	2.0×10^{-3}	5.0×10^{-3}	5.0×10^{-3}

Action of β-Galactosidase on β-D-Galactofuranoside——Table II shows that β-D-galactofuranoside can be hydrolyzed by almond emulsin β-galactosidase but can not be hydrolyzed by bovine liver and *E.coli* K 12 β-galactosidase. The hydrolysis of aryl β-D-galactofuranoside

13) J.E. Cadotte, F. Smith, and D. Spriestersbach, *J. Am. Chem. Soc.*, **74**, 1501 (1952).
14) P. Jerkman and B. Lindberg, *Acta. Chem. Scand.*, **17**, 1709 (1963).
15) B. Helferich, S. Demant, J. Goerdeler, and R. Bosse, *Z. Physiol. Chem.*, **283**, 179 (1948).

was inhibited by galactono 1,4-lactone, which inhibits the hydrolysis of *o*-nitrophenyl β-D-galactopyranoside by β-galactosidase of rumen micro-organism,[16] but the hydrolysis of ethyl β-D-galactofuranoside was not inhibited by the same inhibitor.

Table II. Action of Almond Emulsin, Bovine Liver and *E.coli* K 12 β-Galactosidase on the Various β-D-Galactofuranosides

Source of enzyme	Substrate β-D-galactofuranoside	Inhibitor	Aglycon liberated μg	Inhibition (%)
Almond emulsin	phenyl	none	154	
	phenyl	galactono 1,4-lactone	37	76.0
	m-tolyl	none	208	
	m-tolyl	galactono 1,4-lactone	106	49.0
	guaiacol	none	105	
	guaiacol	galactono 1,4-lactone	32	69.7
	ethyl	none	32	
	ethyl	galactono 1,4-lactone	32	0
Bovine liver	phenyl	none	0	
	m-tolyl	none	0	
	guaiacol	none	0	
	ethyl	none	0	
E.coli K 12	phenyl	none	0	
	m-tolyl	none	0	
	guaiacol	none	0	
	ethyl	none	0	

Incubation mixture contained 1 ml of 0.2M phosphate–0.1M citrate buffer, pH 4.5 for almond emulsin, pH 5.5 for bovine liver and pH 7.5 for *E. coli* K12 β-galactosidase, 0.5 ml of 0.04M substrate solution and 0.5 ml of enzyme solution. To inhibtion test of the hydrolysis, 1 ml of buffer contained galactono 1,4-lactone in a final concentration of 0.01M was employed. Incubation was carried out for 10 hr at 20°.

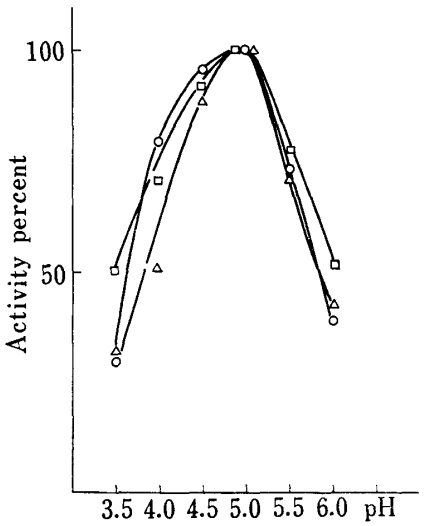

Fig. 3. Effect of pH on the Hydrolysis of Aryl β-D-Galactofuranosides

Incubation mixture contained 1 ml of 0.2M phosphate–0.1M citrate buffer, 0.5 ml of 0.04M substrate solution and 0.5 ml of enzyme solution. Incubation was carried out for 10 hr at 20°.
—○—: phenyl β-D-galactofuranoside
—△—: *m*-tolyl β-D-galactofuranoside
—□—: guaiacol β-D-galactofuranoside

Influence of pH on the Hydrolysis of Aryl β-D-Galactofuranoside by Almond Emulsin β-Galactosidase——The effect of pH on the hydrolysis of aryl β-D-galactofuranoside by almond emulsin β-galactosidase is shown in Fig. 3. The pH optimum for the hydrolysis of aryl β-D-galactofuranoside is pH 5.0. There is little difference between the pH optimum for β-D-galactofuranoside and β-D-galactopyranoside, that is, pH 4.0 for phenyl β-D-galactopyranoside and pH 5.0 for phenyl β-D-galactofuranoside.

Influence of Concentration of Almond Emulsin β-Galactosidase for Hydrolysis of β-D-Galactofuranoside——The hydrolysis of β-D-galactofuranoside by varying the concentration of almond emulsin β-galactosidase is shown in Fig. 4. The reaction velocity is propotional to the enzyme concentration.

Influence of Substrate Concentration on Activity of Almond Emulsin β-Galactosidase——The effect on the activity of the enzyme at pH 5.0 varying the concentration of phenyl β-D-galactofuranoside was determined from the amount of phenol liberated in 1 hr. The results were analyzed by the double reciprocal method of Lineweaver and Burk. The Michaelis cons-

16) J. Conchie and G.A. Levvy, *Biochem. J.*, **65**, 389 (1957).

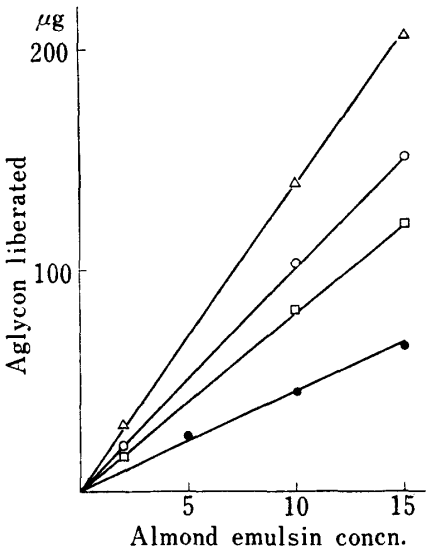

Fig. 4. Effect of Enzyme Concentration on the Hydrolysis of Various β-D-Galactofuranosides

Incubation mixture contained 1 ml of 0.2M phosphate–0.1M citrate buffer, pH 5.0, 0.5 ml of 0.04M substrate solution and 0.5 ml of various dilutions of almond emulsin. Incubation was carried out for 24 hr at 20°.
—○— : phenyl β-D-galactofuranoside
—△— : *m*-tolyl β-D-galactofuranoside
—□— : guaiacol β-D-galactofuranoside
—●— : ethyl β-D-galactofuranoside

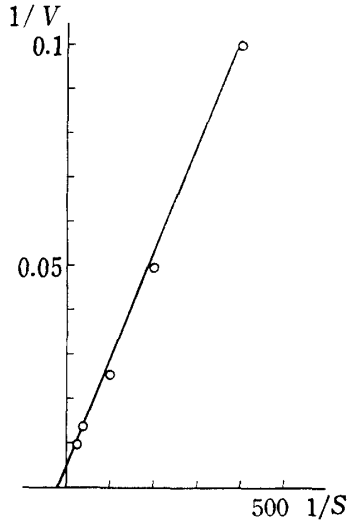

Fig. 5. Effect of Substrate Concentration on the Enzymic Hydrolysis of phenyl β-D-Galactofuranoside

The incubation mixture contained 1 ml of 0.2M phosphate–0.1M citrate buffer, pH 5.0, 0.5 ml of the substrate solution and 0.5 ml of enzyme solution. Incubation was carried out for 10 hr at 20°. Results of the reaction are plotted according to Lineweaver and Burk. The Michaelis constant was 5.6×10^{-2}M.

tant was 5.6×10^{-2} M. From comparison of K_m values of phenyl β-D-galactofuranoside and corresponding pyranoside, it was considered that the β-D-galactofuranoside has a lower affinity for almond emulsin β-galactosidase than the β-D-galactopyranoside. The ratio of relative hydrolytic velocity of phenyl β-D-galactofuranoside to corresponding pyranoside by almond emulsin β-galactosidase at pH 5.0 was 1:332. In the preceding papers[4,5] it was observed that the ratio of the relative hydrolytic velocity in the case of 2-naphthyl β-D-glucofuranosiduronic acid and its corresponding pyranoside by rabbit liver β-glucuronidase is 1:48 and in phenyl β-D-glucofuranoside and its corresponding pyranoside by almond emulsin β-glucosidase is 1:9.2. It may, therfore, be concluded that the hydrolytic action of almond emulsin β-galactosidase to β-D-galactofuranoside is fairly weak.