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82. Kazuo Yoshida, Takako Kamada, Nobuko Harada,*1 and Keitaro Kato*2 : Hydrolysis of Aryl β-D-Glucofuranoside by Almond Emulsin β-Glucosidase.

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Many reports have been made on the specificity of almond emulsin β-glucosidase for β-D-glucopyranoside[1,2] and it has been concluded that β-D-glucopyranosides are substrated for this enzyme. Its specificity for β-D-glucofuranosides was observed as early as 1914 and 1932 by Fischer[3] and Haworth[4] respectively. They reported that both methyl β-D-glucofuranoside and ethyl β-D-glucofuranoside did not undergo hydrolysis by emulsin. Since then, it has been considered that it is not β-D-glucofuranoside but β-D-glucopyranoside that is a substrate for almond emulsin β-glucosidase. The authors have previously studied the specificity of β-glucuronidase for 2-naphthyl β-D-glucofuranosiduronic acid and clarified that β-D-glucofuranosiduronic acid as well as β-D-glucopyranosiduronic acid is a substrate for β-glucuronidase.[5]

In view of the above mentioned facts for β-glucuronidase, it appeared of interest to reinvestigate the specificity of almond emulsin β-glucosidase for β-D-glucofuranosides. This paper deals with the synthesis of several new β-D-glucofuranoside derivatives and their reactions with almond emulsin β-glucosidase.

Materials and Methods

Materials——The substrates used in this study were prepared by the methods referred in Table unless otherwise specified. Aryl β-D-glucofuranosides were prepared according to the method of Kato, *et al.*[6] as follows : aryl di-O-acetyl-β-D-glucofuranosidurono-γ-lactone are yielded by fusing phenols with tri-O-acetyl-β-D-glucofururono-γ-lactone in presence of *p*-toluensulfonic acid and converted to aryl β-D-glucofuranoside by reduction with lithium aluminum hydride.

Phenyl β-D-glucofuranoside, m.p. 78°; $[\alpha]_D^{15}$ −145.2° (c=0.25, H_2O). *Anal.* Calcd. for $C_{12}H_{16}O_6$: C, 56.25; H, 6.25. Found : C, 56.35; H, 6.36.

Guaiacol β-D-glucofuranoside, m.p. 122°; $[\alpha]_D^{15}$ −132.8° (c=0.25, H_2O). *Anal.* Calcd. for $C_{13}H_{18}O_7$: C, 54.55; H, 6.29. Found : C, 54.50; H, 6.38.

m-Cresyl β-D-glucofuranoside, m.p. 100°; $[\alpha]_D^{15}$ −143.6° (c=0.25, H_2O). *Anal.* Calcd. for $C_{13}H_{18}O_6$: C, 57.78; H, 6.66. Found : C, 57.61; H, 6.50.

The mixed melting point determinations of these aryl β-D-glucofuranosides with corresponding pyranoside derivatives showed a marked depression of melting point and the IR spectra of these furanoside derivatives were not identical with those of corresponding pyranoside derivatives. Ring structure of these aryl β-D-glucofuranosides were confirmed by periodate oxidation according to preceding papers.[6,7]

Enzyme Preparation——Almond emulsin was obtained from Sigma Chemical Co. Nitrogen content of this preparation was 14.6%.

Methods——After enzymic hydrolysis of the substrates at appropriate pH and temperature, the liberated aglycon was determined as follows. 2-Naphthol was determined according to the method of Goldbarg, *et al.* in which liberated aglycon was converted to a blue azo dye by reaction with tetrazotized *o*-dianisidine.[8]

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1) B. Helferich : Ergeben. Enzymforsh., **2**, 74 (1933).
2) W. W. Pigman : Adv. Enzymol., **4**, 41 (1946).
3) E. Fischer : Ber., **47**, 1980 (1914).
4) W. N. Haworth, C. R. Porter, A. C. Waine : J. Chem. Soc., **1932**, 2254.
5) K. Kato, K. Yoshida, H, Tsukamoto : This Bulletin, **12**, 670 (1964).
6) *Idem* : *Ibid.*, **12**, 664 (1964).
7) *Idem* : *Ibid.*, **10**, 1242 (1292).
8) J. A. Goldbarg, *et al.* : Gastroenterology, **36**, 193 (1959).

The absorbancy at 560 mμ was measured in Hitachi photoelectric spectrophotometer. Phenol, guaiacol and *m*-cresol were determined with Folin-Ciocalteu reagent.[9] Liberated phenol, guaiacol and *m*-cresol were extracted with 10 ml. of benzene, and the benzene extract was extracted with 2 ml. of 1% NaOH. To the extract was added 5 ml. of Folin reagent, followed by 15 ml. of 20% Na_2CO_3 and 8 ml. of H_2O. The mixture was allowed to stand at room temperature for 20 min., and then the absorbancy at 520 mμ was measured in Hitachi photoelectric spectrophotometer.

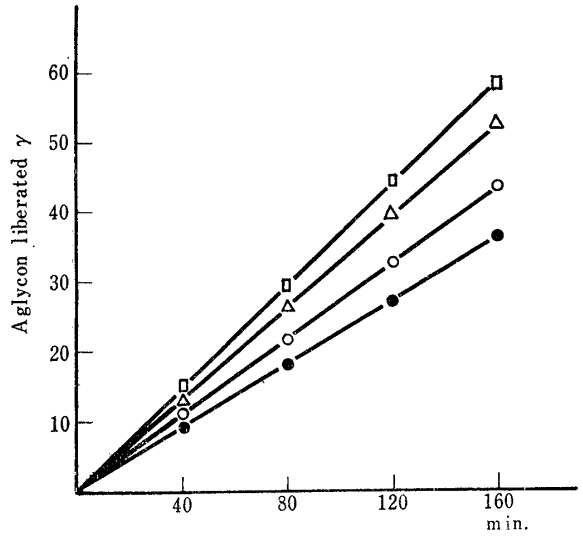

Fig. 1. Course of Enzymic Hydrolysis of Aryl β-D-Glucofuranoside in Relation to Time

The system consisted of 1 ml. of 0.2*M* phosphate −0.1*M* citrate buffer, pH 5.0, 0.5 ml. of 0.004*M* aryl β-D-glucofuranoside and 0.5 ml. of enzyme solution. The incubation was carried out at 30°.

—○— 2-Naphthyl β-D-glucofuranoside
—●— Phenyl β-D-glucofuranoside
—△— Guaiacol β-D-glucofuranoside
—□— *m*-Cresyl β-D-glucofuranoside

Results

Hydrolysis of Aryl β-D-Glucofuranoside by Almond Emulsin β-Glucosidase——Table I shows that aryl β-D-glucofuranoside can be hydrolyzed by almond emulsin β-glucosidase and the hydrolysis is inhibited by glucono-1, 4-lactone and glucono-1,5-lactone. Inhibitory effect of glucono-1,5-lactone is stronger than that of glucono-1,4-lactone.

Time Course of Hydrolysis——In an experiment to determine the course of hydrolysis with time, 0.001 *M* aryl β-D-glucofuranoside in phosphate-citrate buffer, pH 5.0, was hydrolyzed by almond emulsin β-glucosidase for the periods of time varying from 40 minutes to 160 minutes. The graph shown in Fig. 1 demonstrates that during this period of time the velocity of hydrolysis is constant, and linearity is maintained.

pH Optimum——The pH optimum for the hydrolysis of aryl β-D-glucofuranoside and corresponding glucopyranoside was determined in 0.2 *M* phosphate-0.1 *M* citrate buffer

Table I. Hydrolysis of 0.001*M* Aryl β-D-Glucofuranoside by Almond Emulsin β-Glucosidase and Inhibition of the Hydrolysis by 0.0001*M* Gluconolactone

Substrate	Inhibition	Aglycon liberated (γ)	Inhibition (%)
2-Naphthyl β-D-glucofuranoside[6]	none	45.5	
〃	glucono-1,4-lactone[a]	36.6	19.6
〃	glucono-1,5-lactone[b]	19.8	56.5
Phenyl β-D-glucofuranoside	none	74.0	
〃	glucono-1,4-lactone	53.0	28.9
〃	glucono-1,5-lactone	26.0	65.1
Guaiacol β-D-glucofuranoside	none	65.5	
〃	glucono-1,4-lactone	43.5	33.6
〃	glucono-1,5-lactone	17.5	73.3
m-Cresyl β-D-glucofuranoside	none	58.0	
〃	glucono-1,4-lactone	42.5	26.7
〃	glucono-1,5-lactone	15.0	74.1

Incubation mixture contained 1 ml. of 0.2*M* phosphate−0.1*M* citrate buffer, pH 5.0, 0.5 ml. of 0.004*M* substrate solution and 0.5 ml. of enzyme solution. To inhibition test of the hydrolysis, 1 ml. of buffer contained gluconolactone in a final concentration of 0.0001*M* was employed. Incubation was carried out for 1 hr. at 30°.

a) Glucono-1,4-lactone was prepared as described by Hedenburg.[10]
b) Glucono-1,5-lactone was purchased from Tokyo Kasei Co.

9) O. Folin, V. Ciocalteu : J. Biol. Chem., **73**, 629 (1927).
10) O. F. Hedenburg : J. Am. Chem. Soc., **37**, 345 (1915).

between pH 4.0 and 6.0. The pH optimum of 2-naphthyl, phenyl, guaiacol and *m*-cresyl β-D-glucofuranoside is between pH 5.0 and 5.25. The pH-activity curves of corresponding glucopyranosides resemble very closely those obtained for glucofuranosides. The pH-activity curves are given in Fig. 2.

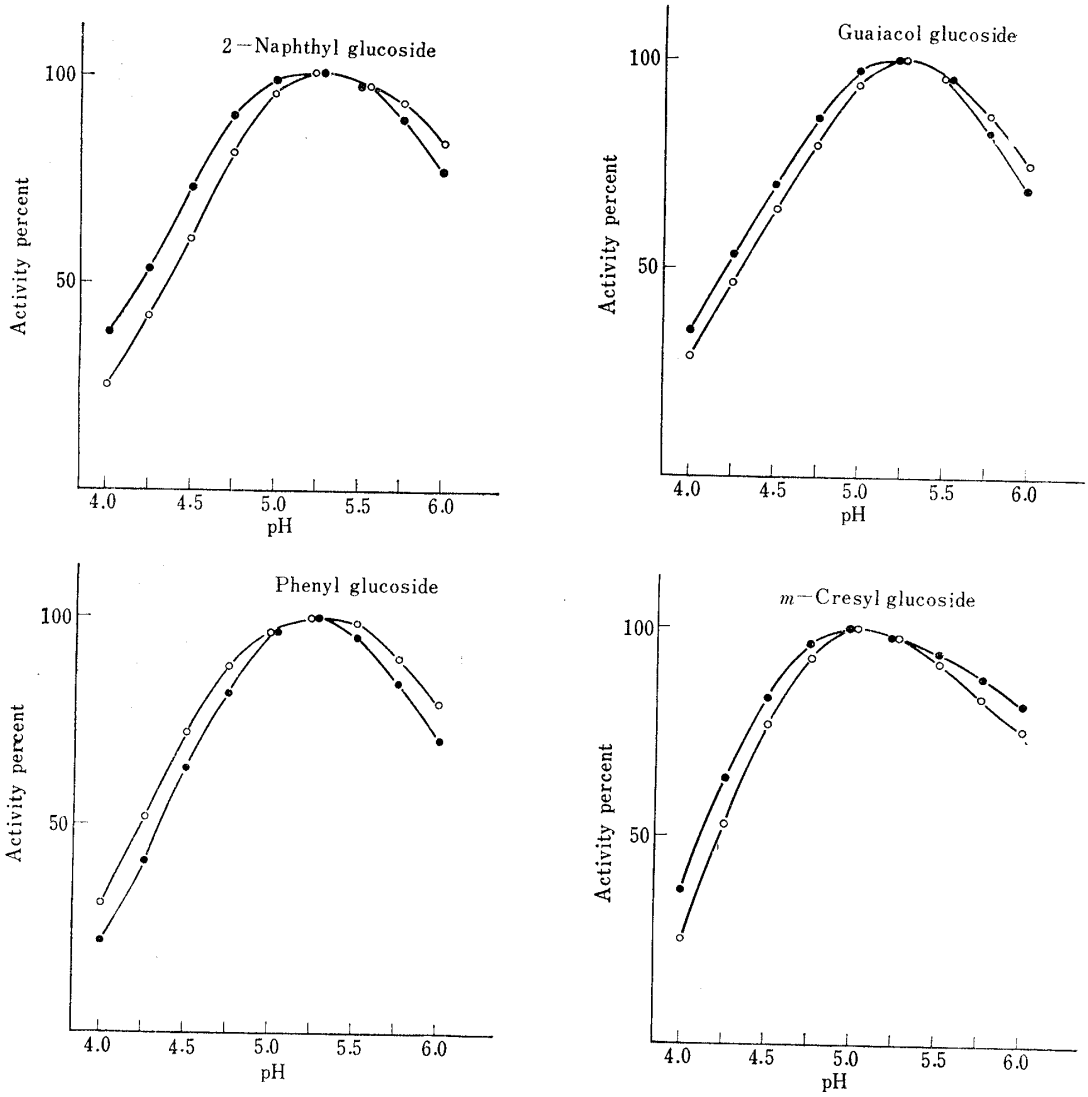

Fig. 2. Effect of pH on the Hydrolysis of 0.001*M* Phenyl, Guaiacol and *m*-Cresyl β-D-Glucoside and 0.0008*M* 2-Naphthyl β-D-Glucoside in 0.2*M* Phosphate-0.1*M* Citrate Buffer

Incubation mixture contained 1 ml. of 0.2*M* phosphate −0.1*M* citrate buffer, 0.5 ml. of 0.004*M* substrate solution for phenyl, guaiacol, and *m*-cresyl β-D-glucoside and that of 0.0032*M* substrate solution for 2-naphthyl β-D-glucoside and 0.5 ml. of enzyme solution. Incubation was carried out for 1 hr. at 30°.

—●— Glucofuranoside —○— Glucopyranoside

Influence of Substrate Concentration on Activity of Almond Emulsin β-Glucosidase ——Fig. 3 shows the effect on the activity of the enzyme at pH 5.0 varying 2-naphthyl β-D-glucofuranoside concentration. The results were analyzed by the graphical method of Lineweaver and Burk, plotting $1/S$ against $1/V$.[11] The results in Fig. 3 gave a value of 2×10^{-3} *M* for *K*m, the dissociation constant of active enzyme-substrate complex

11) H. Lineweaver, D. Burk: J. Am. Chem. Soc., **56**, 658 (1934).

(Fig. 4). Phenyl, guaiacol and *m*-cresyl β-D-glucofuranoside were also tested in the same method as above. Table II shows the values of *K*m for almond emulsin β-glucosidase and aryl β-D-glucoside. From comparison of *K*m values shown in Table II, it is considered that the aryl β-D-glucofuranoside have a lower affinity for almond emulsin β-glucosidase than the corresponding glucopyranoside. The ratio of relative hydrolytic velocity of pyranoside to furanoside by almond emulsin β-glucosidase at pH 5.0 was 15.6:1 in 2-naphthyl β-D-glucoside, 9.2:1 in phenyl β-D-glucoside, 67.8:1 in guaiacol β-D-glucoside and 19.2:1 in *m*-cresyl β-D-glucoside, using 0.0025 *M* 2-naphthyl β-D-glucoside and 0.01 *M* phenyl, guaiacol and *m*-cresyl β-D-glucoside.

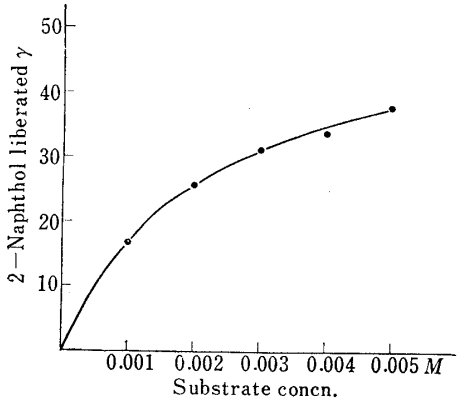

Fig. 3. Effect of Substrate Concentration on the Rate of Enzymic Hydrolysis of 2-Naphthyl β-D-Glucofuranoside

The system consisted of 1 ml. of 0.2*M* phosphate −0.1*M* citrate buffer, pH 5.0, 0.5 ml. of the glucofuranoside, and 0.5 ml. of the enzyme. The incubation was carried out for 1 hr. at 30°.

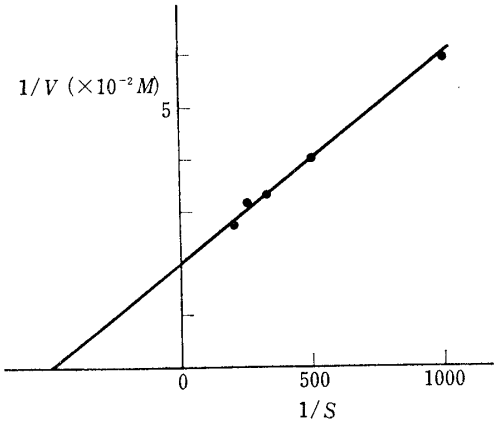

Fig. 4. Effect of Substrate Concentration on the Rate of Enzymic Hydrolysis of 2-Naphthyl β-D-Glucofuranoside

Data of Fig. 3 plotted according to Lineweaver and Burk. The Michaelis-Menten constant was $2\times10^{-3}M$.

TABLE II. Dissociation Constants (*K*m) of the Activity Enzyme-Substrate Complex for Almond Emulsin β-Glucosidase and Various Aryl β-D-Glucosides

Compound	*K*m (*M*)	Compound	*K*m (*M*)
2-Naphthyl β-D-glucofuranoside	2.0×10^{-3}	2-Naphthyl β-D-glucopyranoside[12]	1.4×10^{-3}
Phenyl β-D-glucofuranoside	6.2×10^{-2}	Phenyl β-D-glucopyranoside[12]	4.0×10^{-2}
Guaiacol β-D-glucofuranoside	5.5×10^{-2}	Guaiacol β-D-glucopyranoside[12]	3.2×10^{-2}
m-Cresyl β-D-glucofuranoside	5.0×10^{-2}	*m*-Cresyl β-D-glucopyranoside[12]	2.2×10^{-2}

Discussion

Fischer applied emulsin to syrupy methyl β-D-glucofuranoside, but the solution did not reduce Fehling solution. On the other hand, Haworth did not write his experimental method. However, hydrolysis of methyl α-D-glucofuranoside by 0.01 *N* hydrochloric acid was described in the report, in which the polarimetric change was measured. So it is likely that the same method was used in his experiment in which ethyl β-D-glucofuranoside was applied to emulsin. If aryl β-D-glucofuranoside was used as a substrate, it is possible to determine aglycon liberated by enzymic hydrolysis as low as 10 γ and to study the kinetics.

12) E. M. Montogomery, N. K. Richtmeyer, C. S. Hudson : J. Am. Chem. Soc., **64**, 690 (1942).

Ezaki found that glucono-1,4-lactone was an inhibitor of almond emulsin β-glucosidase,[13] but he did not test glucono-1,5-lactone. Conchie and Levvy[14] reported that both glucono-1,4-lactone and glucono-1,5-lactone were strong inhibitors of β-glucosidase from rumen and limpet. In our experiment, almond emulsin β-glucosidase was inhibited by glucono-1,5-lactone as well as by glucono-1,4-lactone. The inhibitory effect of glucono-1,5-lactone was stronger than that of glucono-1,4-lactone. The pH-activity curves of aryl β-D-glucofuranosides as a whole resemble very closely those obtained for the hydrolysis of corresponding glucopyranoside, with optima at pH 5.0~5.25. Aryl β-D-glucopyranosides had higher affinities for almond emulsin β-glucosidase than corresponding glucofuranosides, while in the case of 2-naphthyl β-D-glucofuranoside, the affinity was higher than that of phenyl β-D-glucopyranoside. From these experiments it may be concluded that almond emulsin β-glucosidase hydrolyses glucofuranoside.

Summary

1. β-D-Glucofuranosides of 2-naphthol, phenol, guaiacol and *m*-cresol were hydrolyzed by almond emulsin, and the hydrolysis was inhibited by glucono-1,4-lactone and glucono-1,5-lactone.

2. The kinetics of the hydrolysis of aryl β-D-glucofuranoside by almond emulsin β-glucosidase was investigated. The pH optimum of the β-D-glucofuranoside is between 5.0 and 5.25 in phosphate-citrate buffer at 30°. The Michaelis-Menten constant is : 2-naphthyl β-D-glucofuranoside, 2.0×10^{-3} *M*; phenyl β-D-glucofuranoside, 6.2×10^{-2} *M*; guaiacol β-D-glucofuranoside, 5.5×10^{-2} *M*; *m*-cresyl β-D-glucofuranoside, 5.0×10^{-2} *M*. The kinetics of the hydrolysis of the corresponding glucopyranoside by the enzyme was also investigated. The pH optimum is approximately the same as furanoside. The Michaelis-Menten constant is : 2-naphthyl β-D-glucopyranoside, 1.4×10^{-3} *M*; phenyl β-D-glucopyranoside, 4.0×10^{-2} *M*; guaiacol β-D-glucopyranoside, 3.2×10^{-2} *M*; *m*-cresyl β-D-glucopyranoside, 2.2×10^{-2} *M*.

3. In view of the above facts, it was concluded that β-D-glucofuranoside could be a substrate for almond emulsin β-glucosidase.

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13) S. Ezaki : J. Biochem., **32**, 107 (1940).
14) J. Conchie, G. A. Levvy : Biochem. J., **65**, 389 (1957).