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STUDIES ON ALMOND EMULSIN β-D-GLUCOSIDASE

II. KINETIC EVIDENCE FOR INDEPENDENT GLUCOSIDASE AND GALACTOSIDASE SITES

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Summary

A purified β-D-glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21) isozyme isolated from almond emulsin was found to catalyze hydrolysis of β-D-glucopyranosides and β-D-galactopyranosides but not the corresponding α-D-derivatives. Hydrolysis of the corresponding β-D-thioglycopyranosides at rates 10^3—10^4 times lower than those for the hydrolysis of the β-D-glycopyranosides was also noted. The enzyme does not exhibit any transferolytic activity using D-glucose or D-galactose as acceptors. D-glucose, *p*-nitrothiophenyl-β-D-glucopyranoside, 5-deoxy-5-thio-D-glucose and D-glucono-δ-lactone are shown to exert mainly competitive inhibition on β-D-glucopyranoside hydrolysis and non-competitive inhibition on β-D-galactopyranoside hydrolysis. D-galactose, *p*-nitrothiophenyl-β-D-galactopyranside and methylthio-β-D-galactopyranoside are shown to inhibit the glucopyranoside hydrolysis mainly non-competitively and to exert competitive inhibition on galactopyranoside hydrolysis. The inhibition caused by the antibiotic Nojirimycin (5-amino-5-deoxy-D-glucose) is shown to be more complex. Analysis of the kinetic data indicates that the catalytic site of the enzyme responsible for the β-D-glucosidase activity is kinetically distinct from the β-D-galactosidase site.

Introduction

Glycosidases with different stereospecificity for the substitutents at the C-4 position of the pyranose ring have been examined by various workers. Almond emulsin glucosidase preparations have been demonstrated to hydrolyze β-D-glucopyranosides as well as β-D-galactopyranosides [1,2,3]. However, *Escherichia coli* β-D-galactosidase [4,5] and yeast β-D-glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21) [6] are stereospecific for substituents at the C-4

position. Several authors have implied that the dual activity of the almond emulsin enzyme is due to the same protein [1—3,7—9]. We have established the bifunctional * nature of a β-D-glucosidase isozyme, isolated in high purity from almond emulsin, in the accompanying communication [10].

The dual activity of the purified enzyme may be due to the existence of one non-stereospecific site for both functions or due to two independent sites on the same protein — one responsible for the glucosidase activity and the other for the galactosidase activity. In this communication we present evidence distinguishing between the two possibilities. Inhibition kinetics of the purified almond emulsin β-D-glucosidase isozyme by a variety of inhibitors are reported. Analysis of the results of the inhibition studies reveal that β-D-glucosidase activity and β-D-galactosidase activity occur at two different sites, kinetically distinct from one another.

Materials and Methods

Materials

Almond emulsin, *p*-nitrophenyl-β-D-glucopyranoside, *p*-nitrophenyl-β-D-galactopyranoside, D-glucono-δ-lactone, methyl-thio-β-D-galactopyranoside, α-D-bromoglucose tetraacetate and α-D-bromogalactose tetraacetate were purchased from Sigma Chemical Company, and 5-deoxy-5-thio-D-glucose was obtained from Aldrich Chemical Company, Inc. Cellobiose was purchased from Eastman Kodak Company and *p*-nitrothiophenol and Rhodamine 6G from K and K Laboratories. Liquifluor and uniformly ^{14}C-labelled D-glucose (0.021 mol/Ci) and D-galactose (0.016 mol/Ci) were obtained from New England Nuclear of Canada. The cellulose derivatives, Whatman CM 52 and Whatman DE 32 were obtained from Reeve-Angel. Tri-Sil was purchased from Pierce Chemical Company. Nojirimycin sulfate (5-amino-5-deoxy-D-glucopyranose sulfate) was a generous gift from Dr. S. Inouye of Meiji Seika Kaisha, Ltd., Japan. D-Talose was a generous gift of Professor R.U. Lemieux, Chemistry Department, University of Alberta and Dr. B. Coxon, National Bureau of Standards, Washington, D.C. All other chemicals were purchased from standard commercial sources.

Enzyme preparation and assays

β-D-Glucosidase from almond emulsin was purified approximately 30-fold. The purification and quaternary structure of the enzyme are described in the accompanying communication [10].

The enzyme activity was measured by monitoring the increase in absorbance at 400 nm due to hydrolysis of *p*-nitrophenyl-β-D-glycopyranosides. All assays were carried out in the presence of 0.05 M sodium acetate pH 6.0 buffer, at room temperature. The glycosides and the various inhibitors employed in the various assays are specified in the Results. The assay solutions, containing the required concentrations of the substrates and the effectors, were prepared 16—18 h in advance. Exceptions have been specified in the Results. The

* A glycosidase capable of catalyzing the hydrolysis of substrates bearing C4-axial and C4-equatorial hydroxyl groups is referred to as bifunctional. It does not imply the two activities occur at spatially distinct active sites.

enzyme concentration in these assays was adjusted so that the reaction velocity remained unaltered for 5 min, if no effectors were present in the reaction mixtures. The definition and the method of determination of enzyme units from an assay are given in the previous paper [10].

Synthesis of p-nitrothiophenyl-β-D-glycopyranosides

Synthesis of *p*-nitrothiophenyl-β-D-glucopyranoside (VIII) and *p*-nitrothiophenyl-β-D-galactopyranoside (III) was carried out by the method of Janaki et al. [11] who prepared *o*-nitrothiophenyl-β-D-galactopyranoside except that an excess of mercaptan was used in the reaction. α-D-Bromoglucose tetraacetate (25 g) was dissolved in chloroform (100 ml) and filtered through Whatman No. 1 paper. To the filtrate 100 ml of petroleum ether (30—60° C) was added while swirling. Needle-like crystals appeared on overnight storage at —20° C. The crystals were separated by filtration and washed with 200 ml of the petroleum ether (m.p. 85—86° C). In 150 ml of dry methanol, 44.5 mmol of KOH were dissolved while bubbling nitrogen through the solution and 62.5 mmol of *p*-nitrothiophenol was added under nitrogen. After the mercaptan had dissolved, 41.6 mmol of the freshly crystallized α-bromoglucose tetraacetate, dissolved in 75 ml chloroform, was slowly added. The contents were sealed under nitrogen. The reaction mixture changed colour from orange to yellow in 12—18 h and was then poured over 100 g of crushed ice. The chloroform layer was separated after shaking. The aqueous layer was reextracted 7 times with 20 ml of chloroform. The chloroform layers were pooled and washed three times with 1% $NaHCO_3$ (500 ml) and then three times with water (500 ml). The washed chloroform layer was concentrated by rotoevaporation under vacuum to a yellow powder which was redissolved in 300 ml of dry chloroform. Anhydrous ether (500 ml) was added to obtain a light yellow precipitate. The chloroform-ether precipitation was repeated and the powder again suspended in 300 ml of chloroform and crystallized by addition of 300 ml of anhydrous ether and storage at —20° C. The light yellow crystals of *p*-nitrothiophenyl-β-D-glucopyranoside tetraacetate were separated by filtration, washed with 200 ml anhydrous ether and dried under vacuum (m.p. 177—178° C). The yield was 30.9 mmol (74%). The crystals were suspended in 1 l of dry methanol and 3.2 mmoles of sodium methoxide was added. The suspension was shaken for 16 h, and then shaken again with 21 g of Dowex 50 WX-8 (H^+) for 15 min. The contents were filtered and the residue washed with 100 ml dry methanol. The pooled filtrate was concentrated and rotoevaporated to dryness. To the powder, 100 ml of water was added and removed by rotoevaporation. Addition and removal of water were repeated. The solids were crystallized from ethanol, thus obtaining 25.2 mmol (79%) of *p*-nitrothiophenyl-β-D-glucopyranoside, VIII, m.p. 148—149° C. VIII and Tri-Sil were mixed under anhydrous conditions. After 10—15 min of the reaction the reaction mixture was lyophilized. This trimethylsilyl derivative of VIII was used for mass-spectrometry, *m/e* 361, 100%; 217, 48%; 147, 41%; 103, 32%; and 73, 81%. Calculated for $C_{12}H_{15}O_7NS$: C, 45.88%; H, 4.77%; N, 4.41%; and S, 10.09%. Found *: C, 45.59%; H, 4.55%; N, 4.40%; and S, 10.16%.

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Synthesis of the corresponding *galacto* derivative was carried out in a procedure analogous to the one described for *p*-nitrothiophenyl-β-D-glucopyranoside. The resulting *p*-nitrothiophenyl-β-D-galactopyranoside tetraacetate (yield 67%, m.p. 150—151°C) was deacetylated to obtain *p*-nitrothiophenyl-β-D-galactopyranoside, III, in 87% yield, m.p. 158—159°C. Trimethylsilyl derivative of III was prepared and used for mass spectrometry in the same manner as for that of VIII. *m/e* 361, 29%; 217, 39%; 147, 37%; 129, 37%; 103, 40%; and 73, 100%. Calculated for $C_{12}H_{15}O_7NS$: C, 45.88%; H, 4.77%; N, 4.41%; and S, 10.09%. Found: C, 45.49%; H, 4.48%; N, 4.41%; and S, 10.07%.

NMR data (^1H and ^{13}C) were consistent with the two structures III and VIII.

Detection of transferolytic activity of β-D-glucosidase

The following reaction mixtures, each in 70 μl of 0.05 M sodium acetate pH 6.0 buffer, were prepared: (a) *p*-nitrophenyl-β-D-glucopyranoside 1.5 μmol, D-glucose 0.5 μmol, and D-[^{14}C]glucose, 1 μCi; (b) *p*-nitrophenyl-β-D-glucopyranoside, 1.5 μmol, D-galactose, 0.5 μmol, and D-[^{14}C]galactose, 1 μCi; (c) *p*-nitrophenyl-β-D-galactopyranoside, 1.5 μmol, D-Glc, 0.5 μmol, D-[^{14}C]glucose, 1 μCi; and (d) *p*-nitrophenyl-β-D-galactopyranoside, 1.5 μmol, D-galactose, 0.5 μmol, D-[^{14}C]galactose, 1 μCi. Almond emulsin solution (10 μl) containing 10 mg/ml of the emulsin was added to each in order to start the reaction. After 54 h of incubation at room temperature, 10 μl (0.125 μCi) of each sample were spotted on Whatman No. 3 paper for chromatography. The chromatograph was developed for 40 h (solvent moved 42—44 cm) in a solvent system containing *n*-butanol, ethanol and water (40 : 11 : 19). The paper was then cut into 1-cm strips and counted in 10 ml of scintillation cocktail (60 g naphthalene, 100 ml methanol, 20 ml ethylene glycol, 42 ml Liquifluor and dioxane to 1 l) using a Beckman LS 200 B Liquid Scintillation Counting System. Standard compounds were also chromatographed under the same conditions. Arylglycosides were detected as fluorescence quenching spots in ultraviolet light, after spraying the paper with 0.005% Rhodamine 6G solution in acetone. Other carbohydrates were detected using a periodate-permanganate spray procedure [12]. Nitrophenol could be detected directly due to its yellow color. The R_f values of the compounds tested were: D-lactose 0.12, D-cellobiose 0.14, D-galactose 0.24, D-glucose 0.27, *p*-nitrophenyl-β-D-galactopyranoside 0.59, *p*-nitrophenyl-β-D-glucopyranoside 0.62 and *p*-nitrophenol 0.92.

Determination of kinetic parameters

The data were fitted by log $(v/(V-v))$ vs. log $[S]$ plots (Hill Plots) where v is the initial reaction velocity employing the substrate concentration $[S]$ and V is the maximal velocity. Similar linear fits were also obtained between log $(v/(V'-v))$ and log $[I]$, where $[I]$ is the inhibitor concentration and V' in this instance is defined as the reaction velocity in the absence of any inhibitor [13]. Least square fits [14] were used in all instances. If the Hill coefficient in these plots approached 1 (−1 in the log $(v/(V'-v))$ vs. log $[I]$ plots) the isotherms were fitted to linear Lineweaver-Burk ($1/v$ vs. $1/[S]$) and Dixon ($1/v$ vs. $[I]$) plots [15], respectively. The data were fitted to these plots by the method of weighted least squares using weights of $1/\sigma^2$, where σ is the standard deviation [16]. The data were fitted to a mixed type inhibition mechanism [17]. The ini-

tial velocity rate equation for this mechanism is: $1/v = ((1 + [I]/K_{IN})/V) + ((K_M/V[S])(1 + [I]/K_{IC}))$ where $K_{IC} = [EI]/[E][I]$ and $K_{IN} = [EIS]/[ES][I]$. Here $[E]$, $[ES]$, $[EI]$ and $[ESI]$ represent the concentrations of free enzyme, enzyme-substrate complex, enzyme-inhibitor complex and enzyme-substrate-inhibitor complex, respectively. Thus, a plot of the intercepts ($1/V_{app}$) on the $1/v$ axis in the Lineweaver-Burk plots vs. $[I]$ yields a plot with slope $1/VK_{IN}$, and a plot of the Lineweaver-Burk plot slopes vs. $[I]$ gives a slope of K_m/VK_{IC}. Similarly, slopes from the Dixon plots, when plotted against $1/[S]$, give a line with the intercept $1/VK_{IN}$ and slope K_m/VK_{IC}. The values of various inhibition constants, for all the inhibitors except Nojirimycin, were estimated from the data by using the above methods and are reliable within error limits of ± 30%.

Results

Reactions catalyzed by the enzyme

The crude and the purified enzymes hydrolyze *p*-nitrophenyl-β-D-glucopyranoside (VII) as well as *p*-nitrophenyl-β-D-galactopyranoside (II). Both substrates reveal hyperbolic kinetics as demonstrated by linear Lineweaver-Burk plots (Figs. 1 and 2, lower curves) and by the Hill coefficient, which was 1 ± 0.003 in each instance. Hill coefficients and values of K_m and V are listed in Table I. The K_m for the glucopyranoside is lower by an order of magnitude

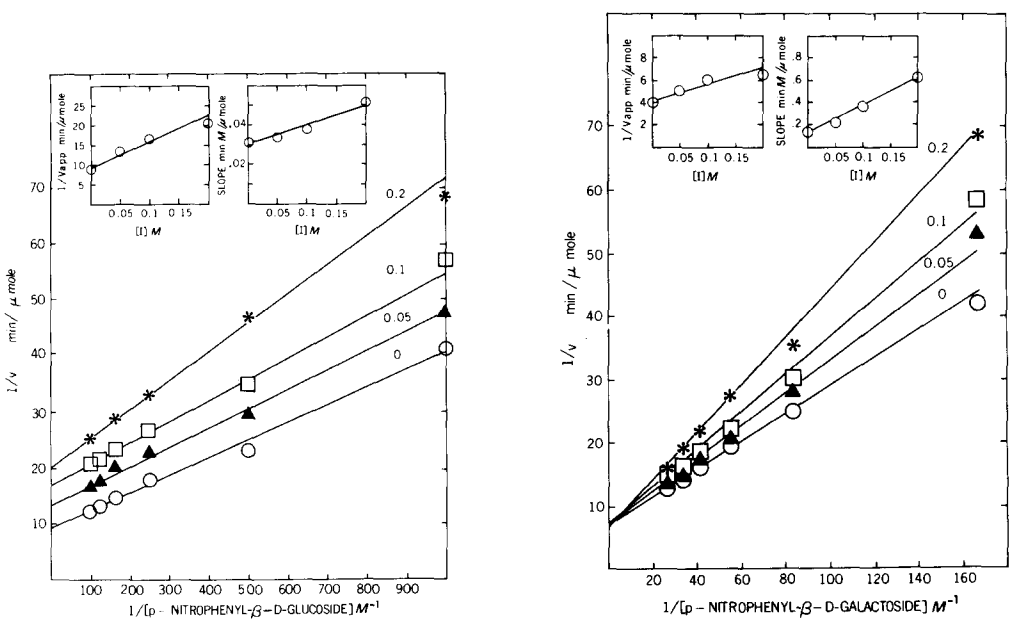


Fig. 1. Inhibition of *p*-nitrophenyl-β-D-glucopyranoside (VII) hydrolysis by thiomethyl-β-D-galactopyranoside (IV). Numbers along the curves indicate the various thiomethyl-β-D-galactopyranoside concentrations. Intercepts ($1/V_{app}$) and slopes of these curves have been plotted against the thiomethyl-β-D-galactopyranoside concentrations in the inserts.

Fig. 2. Inhibition of *p*-nitrophenyl-β-D-galactopyranoside (II) hydrolysis by thiomethyl-β-D-galactopyranoside (IV). See Fig. 1 legend for explanation.

TABLE I

COMPARISON OF HYDROLYSIS OF *p*-NITROPHENYL-β-D-GLYCOSIDES AND *p*-NITROPHENYL-THIO-β-D-GLYCOSIDES BY β-D-GLUCOSIDASE

Substrate	$K_m \cdot 10^3$ (M)	V (s^{-1})	Hill coefficient	Activation energy (kcal/mol)
p-Nitrophenyl-β-D-gluco-pyranoside (VII)	2.5 ± 0.5	290 ± 23 *	1.000 ± 0.003	16.1 ± 1.2
p-Nitrophenyl-β-D-galacto-pyranoside (II)	27.0 ± 3.0	165 ± 13 *	1.000 ± 0.003	15.2 ± 1.0
p-Nitrothiophenyl-β-D-glucopyraniside (VIII) **	5.2 ± 1.3	0.075 ± 0.025	1.00 ± 0.03	16.1 ± 1.0
p-Nitrothiophenyl-β-D-galactopyranoside (III) **	20.3 ± 5.0	0.0085 ± 0.0025	1.00 ± 0.03	15.3 ± 1.0

* Turnover rate of the enzyme in mol substrate hydrolyzed/s/mol enzyme. The V data are based on various enzyme preparations.

** Based on $\epsilon_{400\ nm}$ of $8.6 \cdot 10^3\ M^{-1} \cdot cm^{-1}$ for *p*-nitrothiophenol in 0.05 M sodium acetate pH 6.0.

than the K_m of the galactopyranoside. The turnover rate for the galactopyranoside (II) hydrolysis is only 57% of that of the glucopyranoside hydrolysis (VII). The activation energy for the enzyme catalyzed hydrolysis of the glucopyranoside (VII) and the galactopyranoside (II) were 16.1 and 15.2 kcal/mol, respectively.

The enzyme can also hydrolyze *p*-nitrothiophenyl-β-D-glucopyranoside (VIII) as well as *p*-nitrothiophenyl-β-D-galactopyranoside (III). However the rate of hydrolysis of the two arylthioglycosides is very low. Thus the turnover rate for the hydrolysis of the thioglucoside (VIII) is 0.025% of the turnover rate for the corresponding glucoside (VII, see Table I). Similarly *p*-nitrothiophenyl-β-D-galactopyranoside (III) hydrolysis is carried out at a turnover rate 0.005% of the rate for the corresponding galactoside (II). The K_m values for the thioarylglucoside (VIII) and thioarylgalactoside (III) are 5.2 and 20.3 mM, respectively. The two thioglycosides also reveal hyperbolic kinetics as demonstrated by the Hill coefficient which was 1.00 ± 0.03 in each instance (Table I). The activation energies for the hydrolysis of the two thioglycosides, VIII and III, were 16.1 and 15.3 kcal/mol respectively.

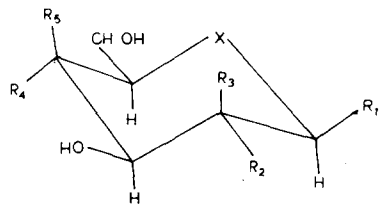
The almond emulsin glucosidase was also tested for a transferolytic activity using *p*-nitrophenyl-β-D-glucopyranoside (VII) or *p*-nitrophenyl-β-D-galactopyranosides (II) employed as substrates and D-glucose or D-galactose as acceptors as described in the Materials and Methods. Although the main ^{14}C-label peaks corresponding to D-glucose (VI) or D-galactose (I) contained as many as $2 \cdot 10^5$ cpm, no ^{14}C label could be detected at R_f values corresponding to the disaccharide standards or *p*-nitrophenyl-β-D-glycoside (II,VII) standards. Thus the enzyme does not carry out a detectable transferolytic reaction using either of the hexoses as acceptors.

Inhibition of β-D-glycopyranoside hydrolysis by galacto compounds

D-Galactose (I) and thiomethyl-β-D-galactopyranoside (IV) were studied for their influence on the two activities of the enzyme. D-Galactose is a very poor inhibitor. It inhibits the glucopyranoside hydrolysis mainly in a non-competi-

tive fashion (K_{IN} = 1.5 M, K_{IC} = 6.1 M) while it inhibits the galactopyranoside hydrolysis mainly competitively (K_{IC} = 1.4 M, K_{IN} = 4.1 M). Thiomethyl-β-D-galactopyranoside which is a much stronger inhibitor (see Table II), also exhibits a similar inhibition pattern. The Hill coefficients, obtained from the log (v/

SCHEME I



I D-galactose*: $R_1=R_2=R_5=OH$; $R_3=R_4=H$; X=O

II *p*-nitrophenyl-β-D-galactopyranoside: $R_1=-O-C_6H_4-NO_2$; $R_2=R_5=OH$; $R_3=R_4=H$; X=O

III *p*-nitrothiophenyl-β-D-galactopyranoside: $R_1=-S-C_6H_4-NO_2$; $R_2=R_5=OH$; $R_3=R_4=H$; X=O

IV thiomethyl-β-D-galactopyranoside: $R_1=SCH_3$; $R_2=R_5=OH$; $R_3=R_4=H$; X=O

V D-talose*: $R_1=R_3=R_5=OH$; $R_2=R_4=H$, X=O

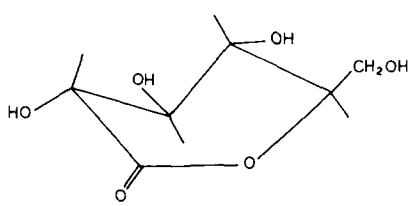
VI D-glucose*: $R_1=R_2=R_4=OH$; $R_3=R_5=H$; X=O

VII *p*-nitrophenyl-β-D-glucopyranoside: $R_1=-O-C_6H_4-NO_2$; $R_2=R_4=OH$; $R_3=R_5=H$; X=O

VIII *p*-nitrothiophenyl-β-D-glucopyranoside: $R_1=-S-C_6H_4-NO_2$; $R_2=R_4=OH$; $R_3=R_5=H$; X=O

IX 5-deoxy-5-thio-D-glucose*: $R_1=R_2=R_4=OH$; $R_3=R_5=H$; X=S

X 5-amino-5-deoxy-D-glucose*: $R_1=R_2=R_4=OH$; $R_3=R_5=H$; X=N



XI D-glucono-δ-lactone

*Only the β-form is shown

(V'—v)) vs. log [I] plots using various concentrations of *p*-nitrophenyl-β-D-galactopyranoside (II) as substrate, were 1.0 ± 0.1. Thus, the inhibitor reveals hyperbolic kinetics. In Fig. 1, the influence of thiomethyl-β-D-galactopyranoside on the enzyme-catalyzed *p*-nitrophenyl-β-D-glucopyranoside (VII) hydrolysis is depicted. The inhibition appears to be mainly non-competitive (K_{IN} = 0.13 M, K_{IC} = 0.3 M). Fig. 2 reveals that the inhibitor thiomethyl-β-D-galactopyranoside (IV) is mainly competitive with respect to the substrate *p*-nitro-

TABLE II

INHIBITION OF β-D-GLUCOSIDASE AND β-D-GALACTOSIDASE ACTIVITIES

Substrate:	p-Nitrophenyl-β-D-glucopyranoside		p-Nitrophenyl-β-D-galactopyranoside	
Inhibitor:	K_{IC} *	K_{IN}	K_{IC}	K_{IN}
D-galactose (I)	6.1	1.5	1.4	4.1
p-Nitrothiophenyl-β-D-galactopyranoside (III) **	—	$17.2 \cdot 10^{-3}$	$18.3 \cdot 10^{-3}$	—
Thiomethyl-β-D-galactopyranoside (IX)	0.3	0.13	0.11	0.4
D-Talose (V)	1.3	0.4	—	—
D-Glucose (VI)	0.20	1.5	2.1	0.41
p-Nitrothiophenyl-β-D glucopyranoside (VIII) **	$4.9 \cdot 10^{-3}$	—	—	$3.6 \cdot 10^{-3}$
5-Deoxy-5-thio-D-glucose (IX)	0.13	1.11	1.07	0.29
5-Amino-5-deoxy-D-glucose (X)	$2.6 \cdot 10^{-3}$ ***	$8.2 \cdot 10^{-3}$	$7.8 \cdot 10^{-6}$	$3.7 \cdot 10^{-6}$
(Nojirimycin)	$1.5 \cdot 10^{-5}$ ***	$6 \cdot 10^{-5}$	$6.5 \cdot 10^{-5}$	$4.0 \cdot 10^{-5}$
D-glucono-δ-lactone (XI)	$3 \cdot 10^{-4}$	$1.3 \cdot 10^{-3}$	$1.1 \cdot 10^{-3}$	$4 \cdot 10^{-4}$

* K_{IC} and K_{IN} are the molar inhibition constants for competitive and non-competitive inhibition, respectively. See Materials and Methods for details.

** These inhibitors had very low solubility in water. The inhibition isotherms obtained in the available concentration range did not permit computation of the constants due to inhibition at the weaker sites.

*** Nojirimycin inhibition patterns were non-hyperbolic, therefore, two inhibition constants of each type were calculated, one for loose binding and another for tight binding.

phenyl-β-D-galactopyranoside (K_{IC} = 0.11 M, K_{IN} = 0.4 M). The inhibition data from Figs. 1 and 2 were also fitted to straight lines in the form of Dixon plots ($1/v$ vs. $[I]$) by the method of least squares. These plots showed coefficients of variation in the range of 0.02—0.1. Slopes of these plots vs. reciprocal substrate concentration are plotted in Fig. 3. The intercept of the plot ($1/VK_{IN}$) is very

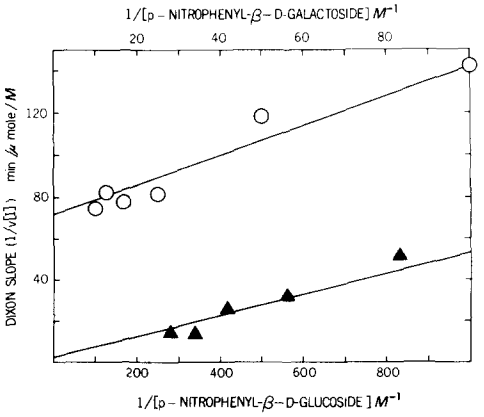


Fig. 3. Enzyme inhibition by thiomethyl-β-D-galactopyranoside (IV). Data from Figs. 1 and 2 were plotted in the form of Dixon plots ($1/v$ vs. $[I]$ at various substrate concentrations. The slopes of these plots are plotted here against $1/[S]$. Top curve (○) was obtained using *p*-nitrophenyl-β-D-glucopyranoside (VII) as substrate and bottom curve (▲) with *p*-nitrophenyl-β-D-galactoside (II) as substrate.

small when *p*-nitrophenyl-β-D-galactopyranoside is used as a substrate, but not when the corresponding glucopyranoside is used. The slope of this plot (K_m/VK_{IC}) however, is higher when the galactopyranoside substrate is employed than when the glucopyranoside is used as a substrate. Thus, thiomethyl-β-D-galactopyranoside inhibition of *p*-nitrophenyl-β-D-galactopyranoside (II) hydrolysis occurs mainly competitively and the hydrolysis of *p*-nitrophenyl-β-D-glucopyranoside (VII) is inhibited mainly non-competitively.

Inhibition patterns of *p*-nitrothiophenyl-β-D-galactopyranoside (III) for the two activities are also very similar, a competitive inhibition towards hydrolysis of the *p*-nitrophenyl-β-D-galactopyranoside ($K_{IC} = 17.2 \cdot 10^{-3}$ M) and a non-competitive inhibition towards *p*-nitrophenyl-β-D-glucopyranoside hydrolysis ($K_{IC} = 18.3 \cdot 10^{-3}$ M).

D-Talose (V), a rare sugar, also inhibits the enzyme in a manner analogous to D-galactose, and probably more strongly. The inhibition constants using D-talose were obtained from two different inhibitor concentrations, each at a different *p*-nitrophenyl-β-D-glucopyranoside concentration. Control experiments in the absence of D-talose at the same substrate concentrations were also conducted. The two inhibitor constants for D-talose listed in Table II were obtained from these four data points. Therefore, the error in determination of these constants may be higher than in other instances.

Inhibition of β-D-glycopyranoside hydrolysis by gluco compounds

The various *gluco* compounds studied for their influence on the enzyme catalyzed glycopyranoside hydrolysis were D-glucose (VI), *p*-nitrothiophenyl-β-D-glucopyranoside (VIII), 5-deoxy-5-thio-D-glucose (IX), 5-amino-5-deoxy-D-glucose (Nojirimycin, X), and D-glucono-δ-lactone (XI). All these compounds inhibit the enzyme and the inhibition appears to be mainly competitive with respect to the glucopyranoside substrate and non-competitive with respect to the galactopyranoside substrate (Table II). D-Glucose (VI) inhibits the *p*-nitrophenyl-β-D-glucopyranoside (VII) hydrolysis with $K_{IC} = 0.2$ M and $K_{IN} = 1.5$ M, and inhibits the *p*-nitrophenyl-β-D-galactopyranoside (II) hydrolysis with $K_{IC} = 2.1$ M and $K_{IN} = 0.41$ M. 5-Deoxy-5-thio-D-glucose, IX, is a somewhat stronger inhibitor and reveals inhibition isotherms similar to those of D-glucose. IX inhibits the *p*-nitrophenyl-β-D-glucopyranoside hydrolysis mainly competitively ($K_{IC} = 0.13$ M, $K_{IN} = 1.11$ M), and hydrolysis of the corresponding galactopyranoside predominantly non-competitively ($K_{IC} = 1.07$ M, $K_{IN} = 0.29$ M). *p*-Nitrothiophenyl-β-D-glucopyranoside (VIII) and D-glucono-δ-lactone (XI) are much stronger inhibitors and again show inhibition properties similar to D-glucose (VI). The various inhibition constants are listed in Table II. Another characteristic common to these inhibitors is a Hill coefficient (from log $(v/(V'-v))$ vs. log $[I]$ plots) of 1.0 ± 0.1.

The inhibition isotherms of Nojirimycin, X, do not resemble those of any other inhibitor studied here. When an aliquot of the enzyme is added to a reaction mixture containing Nojirimycin and *p*-nitrophenyl-β-D-glycopyranoside (*gluco* or *galacto*), the reaction proceeds rapidly for a duration of a minute or less, after which a lower, but constant, reaction velocity is obtained. It is this second, lower but constant, reaction velocity that has been used in the present experiments. A plot of log $(v/(V'-v))$ vs. log $[I]$ using *p*-nitrophenyl-β-D-gluco-

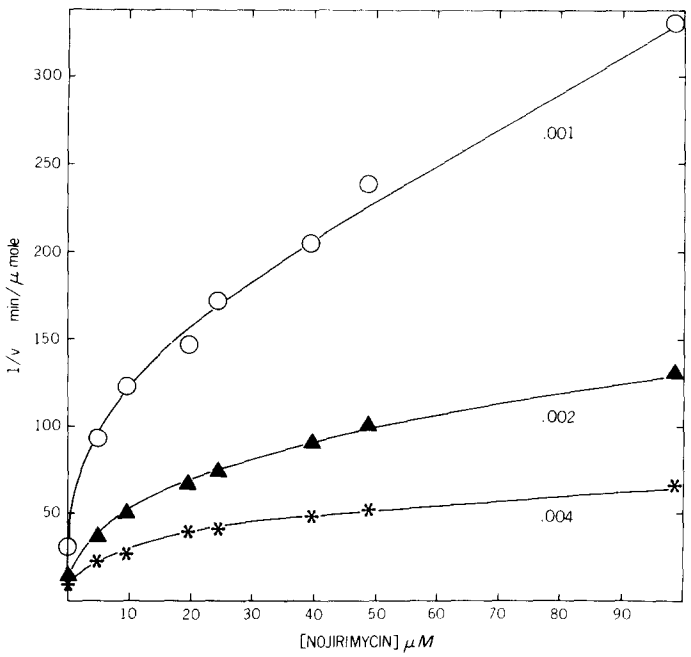


Fig. 4. Dixon plots of inhibition of *p*-nitrophenyl-β-D-glucopyranoside (VII) hydrolysis by Nojirimycin (2-amino-5-deoxy-D-glucose, (X)). Numbers along the curves are the various substrate concentrations.

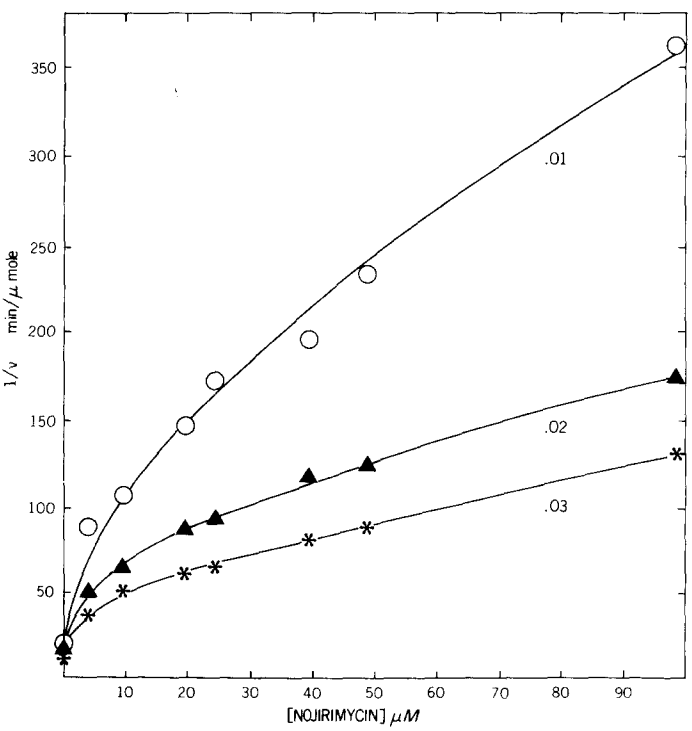
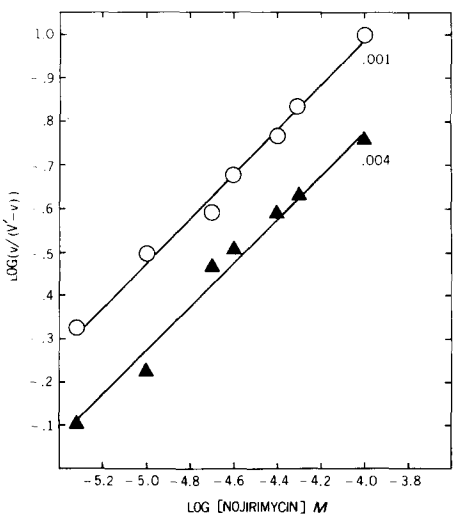


Fig. 5. Dixon plots of inhibition of *p*-nitrophenyl-β-D-galactopyranoside (II) hydrolysis by Nojirimycin (5-amino-5-deoxy-D-glucose, (X)). Numbers along the curves are the various substrate concentrations.

pyranoside (VII) as a substrate is shown in Fig. 6. The slopes of the two lines, each at a different substrate concentration, are 0.51 ± 0.01. Similarly, (Fig. 7 demonstrates that the Hill coefficient is 0.51 ± 0.01 when Nojirimycin is used as an inhibitor and *p*-nitrophenyl-β-D-galactopyranoside (II) as substrate. Thus, Nojirimycin inhibition of the two glycosidase activities is negatively cooperative. This is also revealed in the non-linear Dixon plots using Nojirimycin as inhibitor, and *p*-nitrophenyl-β-D-glucopyranoside (Fig. 4) and *p*-nitrophenyl-β-D-galactopyranoside (Fig. 5) as substrates. In both instances, at low inhibitor concentrations the plots show higher slopes while the slopes are lower at high inhibitor concentrations. Again, the negative co-operatively is independent of the glycopyranoside used as substrate or its concentration. Initial slopes of the Dixon plots ($[I] < 5$ μM) were determined, and a set of inhibition constants computed from them by plotting these slopes against $1/[S]$. The resulting K_I values would be referred to as inhibition constants corresponding to tight binding of Nojirimycin to the enzyme. Similarly, another set of inhibition constants for loose binding of Nojirimycin to the enzyme, were computed from slopes of the Dixon plots at high inhibitor concentrations ($[I] > 20$ μM), in a procedure analogous to the inhibition constants for the tight binding. The various inhibition constants, for tight and for loose binding, are listed in Table II.

Further experiments were conducted in order to investigate the mechanism of inhibition by Nojirimycin. In one experiment, incubation mixtures containing a constant amount of enzyme and varying concentrations of Nojirimycin, were prepared and incubated at room temperature for 60 min. They were then assayed using 20 mM *p*-nitrophenyl-β-D-glucopyranoside. The concentra-



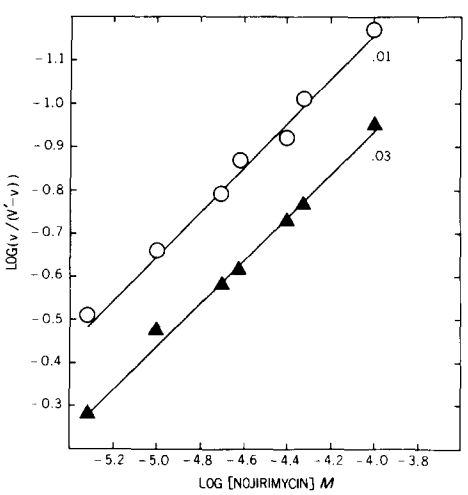


Fig. 6. Hill plots of Nojirimycin (5-amino-5-deoxy-D-glucose, (X), inhibition of *p*-nitrophenyl-β-D-glucopyranoside (VII) hydrolysis. The data were taken from Fig. 4. Numbers along the curves are the substrate concentrations. The value of maximal velocity (V') corresponds to the initial velocity at the specified substrate concentration, in the absence of any inhibitor.

Fig. 7. Hill plots of Nojirimycin (5-amino-5-deoxy-D-glucose, (X)) inhibition of *p*-nitrophenyl-β-D-galactopyranoside (II) hydrolysis (Data from Fig. 5). See Fig. 6 for explanation.

tions of the enzyme and Nojirimycin in the assay solutions were 0.5% of those in the corresponding incubation mixtures. The reaction velocity was initially very low and attained a constant, higher level after a few minutes. In another experiment, similar incubation mixtures, containing fixed enzyme concentration and varying Nojirimycin concentration, were incubated at room temperature for 45 minutes and then dialyzed overnight against 0.05 M sodium acetate pH 6.0 buffer. The initial Nojirimycin concentrations varied from 1.2 μM to 300 μM but after dialysis the concentration was 1.2 μM, in all instances. A further dilution of 200-fold was involved when a small aliquot of the enzyme was added to the assay solution containing 20 mM *p*-nitrophenyl-β-D-glucopyranoside. Thus, the final concentration of Nojirimycin in the assay mixtures was $6 \cdot 10^{-3}$ μM; a concentration at which Nojirimycin exerts no observable inhibitory effect on the enzyme. Again, a short lag phase followed by a constant velocity phase, was observed. The data for each assay were fitted to an exponential curve by plotting $\ln(v_{eq} - v)$ vs. t, where v_{eq} is the constant velocity and v is the velocity at time t. Estimates of the reaction velocity at zero times (v_0) and the exponential decay time constant (τ) were obtained from intercepts and slopes, respectively, of such plots. The data indicate that v_{eq} is independent of the Nojirimycin concentration in the incubation mixtures and τ has a value of 1.25 ± 0.05 min in all cases. However, v_0 decreases with increasing concentration, with K_i = 20 μM. In yet another experiment, the enzyme was equilibrated with Nojirimycin in the presence of D-glucose, D-galactose or thiomethyl-β-D-galactopyranoside in order to determine if these compounds could alter the above influence of Nojirimycin. The experimental procedure was similar to the one described in the previous experiment. The values of τ, v_{eq} and v_0 were not significantly different from those obtained in the presence of Nojirimycin alone.

Discussion

Results obtained from studies with a β-D-glucosidase isozyme purified from almond emulsin indicate that the enzyme catalyzes the hydrolysis of *p*-nitrophenyl-β-D-glucopyranoside (VII), *p*-nitrophenyl-β-D-galactopyranoside (II) and the corresponding β-D-thioarylglycosides (VIII and III) but not the corresponding α-D-glycosides. The rate of hydrolysis of the β-D-thioarylglycosides, however, is extremely low. It does not exhibit any transferolytic activity using the β-D-arylglycosides (II,VII) as substrates and D-glucose (VI) or D-galactose (I) as acceptors. The interaction energy between the catalytic sites is zero for hydrolysis of *p*-nitrophenyl-β-D-glucopyranoside (VII), *p*-nitrothiophenyl-β-D-glucopyranoside (VIII) or *p*-nitrothiophenyl-β-D-galactopyranoside (III). *p*-Nitrophenyl-β-D-galactopyranoside (II), D-talose (V), D-galactose (I), *p*-nitrothiophenyl-β-D-galactopyranoside (III), and thiomethyl-β-D-galactopyranoside (IV) inhibit the glucoside hydrolysis mainly non-competitively and the galactoside hydrolysis predominantly competitively. *Gluco*-type compounds D-glucose (VI), *p*-nitrothiophenyl-β-D-glucopyranoside (VIII), 5-deoxy-5-thio-D-glucose (IX), 5-amino-5-deoxy-D-glucose (Nojirimycin, X) and D-glucono-δ-lactone (XI) inhibit glucopyranoside hydrolysis predominantly competitively and galactopyranoside hydrolysis mainly non-competitively.

β-D-glucosidase from almond emulsin has been reported to hydrolyze β-D-glu-

copyranosides as well as β-D-galactopyranosides [1—3,7—10]. However, the K_m for the β-D-glucopyranoside substrates is lower by a factor of ≈10. Results presented in this communication confirm these observations (Table I). The enzyme can also hydrolyze the corresponding β-D-thioglycosides (VIII,III). The rate of hydrolysis of the latter compounds, however, is lower by three to four orders of magnitude. The affinities of the thioarylglycosides exhibited by their K_m values (*gluco*, VIII, 5.2 mM; *galacto*, III, 20.3 mM), however, are similar to those for the corresponding arylglycosides (*gluco*, VII, 2.5 mM; *galacto*, II, 27.0 mM). The kinetics exhibited by both types of substrates is hyperbolic (Hill coefficients 1.000 ± 0.003) and the activation energies are also similar. The enzymatic hydrolysis of a thioglycoside may thus be carried out at the same kinetic site as the hydrolysis of the corresponding glycoside. The Hill coefficients for the enzyme activity with all the substrates are 1.00 ± 0.03. Thus, the interaction energy between various *p*-nitrophenyl-β-D-glucopyranoside (VII) binding sites or between various *p*-nitrothiophenyl-β-D-glucopyranoside (VIII) binding sites is zero. Similarly interaction energy between various *p*-nitrophenyl-β-D-galactopyranoside (II) binding sites or between various *p*-nitrothiophenyl-β-D-galactopyranoside (III) binding sites is also insignificant. Although the enzyme is a dimer [10] the active sites on the two subunits thus appear to be independent with regard to binding of the substrates.

β-D-glycosidases from a variety of sources have been reported in the literature to catalyze transfer of aglycone moiety of the substrates to a variety of acceptors [5]. The almond emulsin did not exhibit any detectable transferolytic activity when β-D-glycopyranosides (II or VII) were employed as substrates and D-glucose (VI) or D-galactose (I) as acceptors. It does not rule out the possibility of the enzyme catalyzing such a reaction when more suitable acceptors are available. However, it ensures that the kinetics observed with the various inhibitors studied here is purely a study of hydrolytic reactions. Furthermore, since the rates of hydrolysis of the β-D-thioarylglycosides (III, VIII) * are three to four orders of magnitude lower than those of the corresponding β-D-arylglycosides, thioglycosides can be used as inhibitors when β-D-glycosides are used as substrates.

Previous studies by various workers have shown that D-glucose (VI) and D-galactose (I) are very weak inhibitors of the glucosidase activity [1] while D-glucono-δ-lactone (XI) and Nojirimycin (X) inhibit this activity very strongly [18]. The kinetic results presented in Table II confirm these findings. In addition, *p*-nitrothiophenyl-β-D-glucopyranoside (VIII), 5-deoxy-5-thio-D-glucose (IX), *p*-nitrothiophenyl-β-D-galactopyranoside (III) and thiomethyl-β-D-galactopyranoside (IV) have been observed to inhibit the enzyme activity significantly. D-Talose (V) is also found to be a very weak inhibitor.

In the present work, however, the inhibitory effects of various compounds have been studied on the galactosidase as well as on the glucosidase activities of the enzyme. The inhibitors can be classified into *galacto*-(I,III-V) and *gluco*-

* The major interest in the study of hydrolysis of β-D-arylthioglycosides was their potential use as substrate analogue inhibitors for the study of mechanism of the β-D-glucosidase action using ^{13}C-NMR. The NMR studies would employ enzyme concentrations (1—2 mM) four to five orders of magnitude higher than these kinetic studies and would have to be carried out for periods 100 times longer. The extremely small rates of hydrolysis were, thus, of extreme importance.

(VI,VIII-XI) types. All inhibitors exhibit mixed-type inhibition [15]. Therefore, two types of inhibition constants, K_{IC} and K_{IN}, have been defined (see Materials and Methods), corresponding to a competitive inhibition constant and a non-competitive inhibition constant, respectively. The *galacto* compounds, D-galactose (I), *p*-nitrothiophenyl-β-D-galactopyranoside (III), thiomethyl-β-D-galactopyranoside (IV) and D-talose (V), inhibit *p*-nitrophenyl-β-D-galactopyranoside (II) hydrolysis mainly competitively as indicated by lower values of K_{IC} than K_{IN}. Similarly, *gluco* compounds, D-glucose (VI), *p*-nitrothiophenyl-β-D-glucopyranoside (VIII), 5-deoxy-5-thio-D-glucose (IX) and D-glucono-δ-lactone (XI), inhibit *p*-nitrophenyl-β-D-glucopyranoside (VII) hydrolysis mainly competitively. However, *galacto*-type compounds exert non-competitive inhibition on glucosidase activity of the enzyme, and *gluco*-type compounds exhibit non-competitive inhibition on the galactosidase activity, Thus, a competitive inhibition is observed when both the inhibitor and the substrate have identical C-4 substituents. An inhibitor with a C-4 substituent dissimilar to that of the substrate exerts non-competitive inhibition of the substrate hydrolysis. Another interesting characteristic that emerges from consideration of the inhibition constants in Table II is that K_{IC} for a *gluco*-type inhibitor-glucopyranoside substrate is in the same range as K_{IN} for the same inhibitor using a galactopyranoside substrate. For example, when 5-deoxy-5-thio-D-glucose (IX) is used as inhibitor and *p*-nitrophenyl-β-D-glucopyranoside (VII) as substrate, K_{IC} = 0.13 M and K_{IN} = 1.11 M, and when *p*-nitrophenyl-β-D-galactopyranoside (II) used as a substrate, K_{IC} = 1.07 M and K_{IN} = 0.29 M. Thus, the K_{IC} in the first instance is in the same range as K_{IN} in the second instance. Similarly, the former K_{IN} has a value similar to the latter K_{IC}. Using thiomethyl-β-D-galactopyranoside (IV) as inhibitor, K_{IC} with a galactopyranoside substrate (0.11 M) corresponds to the K_{IN} with a glucopyranoside substrate (0.13 M); and K_{IN} with a galactopyranoside substrate (0.4 M) corresponds to K_{IC} with a glucopyranoside substrate (0.3 M). From these findings, we conclude that the *gluco*- and *galacto*-type compounds bind at kinetically distinct sites on the protein. Binding of an inhibitor at the *gluco*-site results in: (a) a lower turnover rate at the *galacto*-site; (b) competition with the *gluco* substrates. Similarly, an inhibitor bound at the *galacto*-site: (a) lowers the turnover rate at the *gluco* site; (b) competes with *galacto*-substrates. However, what remains to be explained is the finding that the *gluco*-type compounds can also cause a slight competitive inhibition at the *galacto*-site; and a *galacto*-inhibitor exerts a slight competitive inhibition at the *gluco*-site. Weak binding of the *gluco*-compounds at the *galacto*-site, and of *galacto*-compounds at the *gluco*-site would explain such behavior.

The inhibition of glucosidase and galactosidase activities of the enzyme by the *gluco*-analogue, Nojirimycin (X), appears to be more complicated. The Hill coefficient, computed from the log $(v/V'-v)$ vs. log $[I]$ plots, is 0.51 ± 0.01, irrespective of the glucopyranoside used as substrate or the concentration of the glycopyranoside. A Hill coefficient = 0.51 is indicative of a large energy of interaction between the various inhibitor binding sites. Therefore, this inhibition would result from binding of Nojirimycin at various interdependent sites. This is also illustrated by the non-linear Dixon plots using Nojirimycin as inhibitor (Figs. 6 and 7). An attempt was also made to fit this inhibition data to the second order parabola, $1/v, = a + b\ [I] + c\ [I]^2$, where a, b and c are arbi-

trary constants, by the method of least squares [14]. However, the data does not fit the second order equation very well (coefficient of variation >0.1). Therefore, the values of various inhibition constants listed in Table 2 for tight and loose bindings are only limiting values. The actual number of sites involved must be >2. The experiments which comprised incubation of the enzyme with Nojirimycin followed by dialysis to remove the unbound inhibitor, reveal that Nojirimycin can bind the free enzyme 'irreversibly', with K_i = 20 μM. This binding cannot be dissociated by the presence of D-glucose, D-galactose or thiomethyl-β-D-galactopyranoside under the experimental conditions employed. However, when the enzyme-Nojirimycin complex obtained as a dialysate, is added to the assay solutions, the Nojirimycin/substrate ratio is very low ($6 \cdot 10^{-9}$ M/$2 \cdot 10^{-2}$ M) and, under these conditions, the Nojirimycin-enzyme complex can be dissociated with τ = 1.25 min. Thus, the Nojirimycin-enzyme inactive complex dissociation process is very slow. If the complex formation is also a slow process, addition of free enzyme to an assay solution containing substrate and Nojirimycin at μM concentration should result in an initial rapid reaction phase followed by a constant low velocity phase. This is indeed, what was observed in the experiments in which the enzyme was added directly to the assay solutions containing Nojirimycin. Inactivation of this type may be the predominant mode of the inhibition of Nojirimycin at high concentrations. In another experiment, *p*-nitrophenyl-α-D-glucopyranoside and *p*-nitrophenyl-α-D-galactopyranoside were observed to weakly inhibit the glucosidase activity. Thus, α-Nojirimycin may also inhibit the enzyme. This would make the isotherms of inhibition by Nojirimycin very complex and composed of β-Nojirimycin-*gluco*-site binding, α-Nojirimycin-*gluco*-site binding, β-Nojirimycin-*galacto*-site binding, α-Nojirimycin-*galacto*-site binding and slow formation of inactive Nojirimycin-enzyme complex. Using 5-amino-5-deoxy-β-D-glucopyranosides (Nojirimysides) would thus be of interest for delineation of the mechanism of inhibition by Nojirimycin

Conclusion

β-D-Glucosidase isolated from almond emulsin catalyzes the hydrolysis of β-D-glucopyranosides and β-D-galactopyranosides and hydrolyzes the corresponding β-D-thioglycosides although at rates 10^3—10^4 lower than those for the glycosides. Affinities of the β-D-arylglycosides and the corresponding β-D-thioarylglycosides for the enzyme, and the activation energies of their enzymatic hydrolysis, are similar and all the substrates studied exhibit hyperbolic The enzyme, however, does not catalyze hydrolysis of α-D-glycopyranosides or transfer of the aglycone moeity of a β-D-arylglycoside substrate to D-glucose or D-galactose as acceptors. The kinetic studies conducted in this work have revealed that *galacto*-type inhibitors (I,III—V) mainly compete with the galactopyranoside substrate and lower the turnover rate of the hydrolysis of the glucopyranoside substrate. Similarly, *gluco*-type inhibitors (VI, VIII—XI) mainly compete with the glucopyranoside substrate and lower the maximal velocity of hydrolysis of the galactopyranoside site. From these findings we conclude that the glucosidase and galactosidase sites are kinetically distinct and, hence, may be spatially distinct. Nojirimycin inhibition is more complex, yields non-hyper-

bolic kinetics, and may involve a slow formation and dissociation of an enzyme-inhibitor complex.

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