Rat Liver ß-Glucuronidase; Its Purification and Inhibition Studies

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ß-Glucuronidase (EC 3.2.1.31) which hydrolizes D-glucuronate from ß-D-glucuronide was purified from rat liver, using ammonium sulfate fractionation, DEAE-cellulose chromatography, Concanavalin-A Sepharose 4B chromatography and gel filtration on Sephadex G-200. This enzyme has the molecular weight of 280,000 daltons by gel filtration and 75,000 daltons by SDS-polyacrylamide gel electrophoresis. As its funtion is reverse of detoxification in the liver, the inhibition of the enzyme was tested with extracts of several food products and medicinal herbs, some are known as anti-cancer agents. Among them, *Panax ginseng* and *Cortnellus shiiake* inhibited the enzyme competitively and the K_i values were 9.22×10^{-2} and 0.102 mg/*ml*, respectively. These inhibitors strongly bound to DEAE-cellulose. The negatively charged amino acids, *L*-aspartate and *L*-glutamate, inhibited the enzyme, and *K_i value of L-aspartate was 0.80 mM*. The interaction between ß-glucuronidase and p-nitropheny;-ß-D-glucuronide was found to involve ionic forces by the effect of ionic strength on the kinetic constant, Vmax/Km. It was inferred from these findings that cationic group at the active center of the enzyme is probably involved in attacking the substrate.

Introduction

ß-Glucuronidase (EC 3.2.1.31) is a hydrolytic enzyme which catalyzes ß-D-glucuronide + H_2O ⟶ Alcohol + D-glucuronate. It is distributed in animal tissues, plants and bacteria, and has been purified and characterized from human liver,[1] bovine liver,[2] mouse kidney,[3] ox spleen,[4] rabbit,[5] rat liver,[6-8] and rat uterus.[9].

It was reported that ß-glucuronidase is complexed into different subcellular structures, the lysosomes and microsomes,[10] which is derived from the same structural gene, *Gus*, on chromosome 5 and the two forms arised by post-translational processing.[11] Though they are heterogeneous after isoelectric focusing over the range PI 5.4-6.0,[7] the two forms differ only a little in both carbohydrate and amino acid compositions.[8]

But there is also increasing interest on the role of glucuronidase in the destruction of host material at the invading edge of many tumors.[12,13] Benzo(a)pyrene is a carcinogenic hydrocarbon commonly found in the environment and its metabolic formation of a diol epoxide is highly mutagenic[14-16] and binds very actively to nucleic acids.[17,18] The water soluble conjugates of benzo(a)pyrene, such as the glucuronides, have been generally viewed as detoxification products.[19] The banzo(a)pyrene glucuronides are not entirely detoxification product but converted by glucuronidase to carcinogens at the sites distal to their formation.[20] (Kinoshita & Gelboin, 1978).

In this study, we found that *Panax ginseng* and *Cortnellus shiiake* contained some inhibitors of ß-glucuronidase. Previously Saccharo-1,4-lactone was known to be powerful competitive inhibitor of ß-glucuronidase and inhibitor was isolated from porcine sublingual gland (Sakamoto, 1973).[21]

Materials and Methods

Materials. Rats (300-500g) were obtained from animal breeding center of Seoul National University. p-Nitrophenyl-ß-D-Glucuronide, α-methyl-D-mannoside, *L*-arginine and all marker proteins for molecular weight determination were purchased from Sigma Chemical CO. DEAE-Cellulose was from Whatman Ltd., Sephadex G-200 and Concanavalin-A Sepharose 4B were obtained from Pharmacia Fine Chemicals. *Panax ginseng* was obtained from the Office of Monopoly and other herbs were purchased at the market. *L*-Cysteine and *L*-glutamate were purchased from Merck and other amino acids from Eastman Kodak Co.

All chemicals were of the highest purity available.

Enzyme Assay. ß-Glucuronidase assays were performed by the method of Harris *et al.*[22] with some modification. The assay mixture consisted of 0.5 *ml* of 0.2 *mM* p-nitrophenyl-ß-D-glucuronide in 0.2 *M* sodium acetate buffer, pH 4.5, and 0.1 *ml* of enzyme solution. The mixture was incubated for 1 hr at 37°C. The reaction was terminated by the addition of 0.5 *ml* of 2.0 N sodium hydroxide solution. The color developed was measured by reading the absorbance at 400 nm. The amount of p-nitrophenol formed was determined from standard curve. One unit of the enzyme activity was defined as the amount of enzyme that release 1 μ mole of p-nitrophenol from p-nitrophenyl-ß-D-glucuronide per hour under the condition specified above.

The protein was determined by the method of Lowry *et al.*[23] with crystallized bovine serum albumin as standard.

Enzyme Purification

Step I, Preparation of Crude Extract. About 10g of rat liver was minced with razor and homogenized with prestle in 2 volume (w/v) of 50 *mM* Tris-HCl buffer, pH 7.2, for 10 min. The pellet was resuspended in 2 volumes of the buffer, and homogenization and centrifugation were repeated. The supernatants were pooled.

Step II, $(NH_4)_2SO_4$ Fractionation. The crude extract was fractionated by adding solid ammonium sulphate to 70% saturation over a period of 20 min with constant stirring with magnetic stirrer. After 20 min setting the mixture was centrifuged at 30,000 g for 10 min and the pellet was dissolved in 30 m*l* of 50 mM Tris-HCl buffer, pH 7.2, and dialyzed against 2 l of the same buffer with three changes.

Step III, DEAE-Cellulose Chromatography. The dialyzed sample was loaded on DEAE-Cellulose column (3×10 cm)

pre-equilibriated with 50 mM Tris-HCl buffer, pH 7.2. The column was washed with 150 m*l* of the same buffer and eluted with 500 ml of the same buffer and eluted with 500 ml of a linear gradient of 0-0.5 M NaCl in the buffer at the rate of 30 m*l*/hr, and 3 m*l* fractions were collected. The enzyme active fractions were pooled and concentrated to 10 ml with Dioflo PM 10 filter.

Step IV, Concanavalin-A Chromatography. The concentrated sample was applied to a column of Concanavalin-A Sepharose 4B (1.5×7 cm) pre-equilibriated with 50 m*M* $CaCl_2$, 0.4 M NaCl. The column was washed with 100 m*l* of the above buffer and the enzyme activities were eluted with 100 m*l* of 0-0.6 M linear gradient α-methyl-D-mannoside in 50 mM Tris-HCl buffer, pH 7.2, at the rate of 5 m*l*/hr. β-Glucuronidase active fractions were pooled and concentrated to 5 m*l* by Diaflo PM 10 filter.

Step V, Sephadex G-200 Chromatography. The pooled sample from the previous step was applied to a column of Sephadex G-200 (2.6×30 cm) pre-equilibriated with 50 m*M* Tris-HCl buffer, pH 7.2. The column was eluted with this buffer at the rate of 2.5 m*l*/hr and the void volume was measured by loading Blue Dextran 2000 as marker. β-Glucuronidase active fractions were pooled and used to inhibition studies.

Inhibition Studies. Panax sinseng extracts was diluted with 10 fold (w/v) with 50 mM Tris-HCl buffer, pH 7.2. *Cortnellus shiiake* was homogenated with prestle in the same buffer, after boiling for 5 min, centrifuged 4,000 g×5 min (Kokusan) and the supernatant was used. The inhibitor was mixed with 0.9 m*l* of 0.2 M sodium acetate buffer, pH 4.5 and 0.1 m*l* of purified enzyme. After the pre-incubation at 37°C for 10 min, 0.5 m*l* of 0.2 m*M* of p-nitrophenyl-β-D-glucuronide in the same buffer was added to the reaction tube. Enzyme reaction was carried out for 1 hr until 0.5 m*l* of 2.0 N NaOH was added as terminator. The color developed was detected at 400 nm. For determination of K_i values, the concentrations of substrate 4.0, 2.0, 1.0 and 0.5 m*M* were used and the incubation time was 5 min.

Effect of Amino Acid. The enzyme was pre-incubated with 1.0 and 10 m*M* of each amino acid in standard assay mixture without the substrate for 10 min at 37°C. Then added 0.2 m*M* substrate, the reaction mixture was incubated for 1 hr. For *L*-aspartate, the K_i value was calculated as in the case of inhibition studies.

Effect of Salt. Enzyme reactions were carried out in 10, 50 and 100 m*M* of NaCl, KCl, $CaCl_2$ and $MgSO_4$ to check the ionic strength effect on enzyme activity. To test the suppression by salt, 80 μg/ml of *ginseng* extract was added to the assay mixture. After incubation for 1 hr at 37°C, the activities were compared to the control which didn't contain any additional salt but inhibitor.

Results and Discussion

Purification of β-Glucuronidase. Overall purification was 430 fold with 36% recovery of the enzyme. The entire purification scheme is summarized in Table 1.

DEAE-cellulose column provided 10.3 fold increase in β-glucuronidase activity and showed double peaks at about 0.1 *M* NaCl with a little tailing. When the DEAE-Sephacyl was used, there were double peaks and tailing, too, but a little lower efficiency. The profile of DEAE-cellulose chromatography is shown in Figure 1.

The double peaks of the DEAE-cellulose chromatography means there are a few different types of β-glucuronidase. Plapp & Cole[24] showed multiple peaks of the DEAE-cellulose chromatography and descrived that the pattern was due to the difference in amount of some material bound to the protein. Now it is known that the six intracellular forms of the enzyme is *L* forms in the lysosomal fraction, a *X* form and four *M* forms in microsome, and the M forms are complexes containing 1, 2, 3, or 4 molecules of egasyn for each β-glucuronidase *X* tetramer.[11, 25]

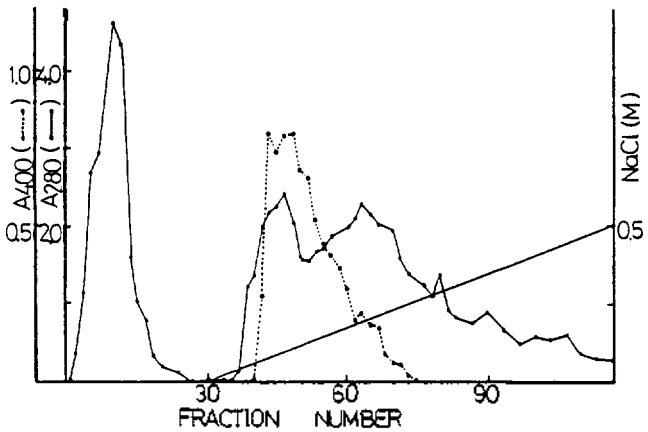

Figure 1. DEAE-Cellulose Chromatography. The column was eluted with 50 m*M* Tris-HCl buffer, pH 7.2 and developed with linear gradient of 0-0.5 *M* NaCl. Its dimension was 3×10 cm and 3 m*l* fractions were collected. ●—● ; protein. O----O; β-glucuronidase.

TABLE 1: Purification of Rat Liver ß-glucuronidase. A unit of Enzyme Activity was Defined as the Amount that Release 1 μmole of p-nitrophenol per hour at 37°C under the Assay Condition Described in the text

Procedure	Total Protein (mg)	Total Activity (units)	Specific Activity (unit/mg protein)	Yield (%)	Purification Ratio
Homogenate	4020	76.8	0.0191	100	1
30,000 g Supernatant	2370	70.7	0.0298	92	1.56
70% $(NH_4)_2SO_4$ Fractionation	1680	53.2	0.0317	69	1.66
DEAE-Cellulose	141	46.2	0.328	60	17.2
Concanavalin-A Sepharose 4B	21.4	37.5	1.75	49	109
Sephadex G-200	3.30	27.5	8.33	36	436

With Concanavalin-A Sepharose affinity chromatography, the enzyme activity was eluted as a single peak at about 0.3 M α-methyl-D-mannoside. It provided 6.3 fold increase in activity and the pattern of the column is shown in Figure 2.

Gel filtration on Sephadex G-200 provided 4.0 fold increase in activity. The profile of this step is shown in Figure 3. After the gel filtration on Sephadex G-200, the enzyme showed one band on polyacrylamide gel, so we could set about the inhibition studies.

Inhibition Studies. Among the several food products and medicinal herbs, known as anti-cancer agent, *Panax ginseng* and *Cortnellus shiiake* showed more inhibition which are shown in Figure 4 and 5. The Km value of β-glucuronidase was 0.735 m*M*.

In the case of inhibition by *Panax ginseng*, as the protein concentration increased 32, 80, 160 μg/m*l*, so the Km values 1.07, 1.50 and 2.10 m*M*, respectively. Each lines of Lineweaver-Burk plot had the common Vmax value. So we concluded that the inhibition by *Panax ginseng* is competitive.

The inhibition by *Cortnellus shiiake* showed the same pattern. When the protein concentrations were 38, 75 and 190 μg/m*l*, the Km values were 1.25, 1.44 and 2.10 m*M*, respectively. Each lines of Lineweaver-Burk plot had the common Vmax value, too, so the inhibition by *Cortnellus shiiake* is deduced to be competitive.

Since the inhibitors bind to the catalytic site of the enzyme or to other potential sites for a reaction to take place, in either case a dead-end complex (EI) would be formed.

The K_I values of *P. ginseng* and *C. shiiake* extracts were 9.22×10^{-2} and 0.102 mg/m*l*, respectively (Figure 6). But this result doesn't show that the former is more effective inhibitor than the latter. As we didn't use the purified materials, merely using the extracts of putative inhibitors, there might be any possibility that the inhibitor proteins from the two sources are the same and there is more quantity in *P. ginseng* components than in *C. shiiake*.

When the extract of *P. ginseng* was loaded on DEAE-cellulose column, the inhibitors were strongly bound to DEAE-cellulose, indicating that they have strong anionic charges. So it is anticipated that the inhibitions are resulting from interaction between the anionic inhibitors and cationic group at the active center of β-glucuronidase. That this cationic group of

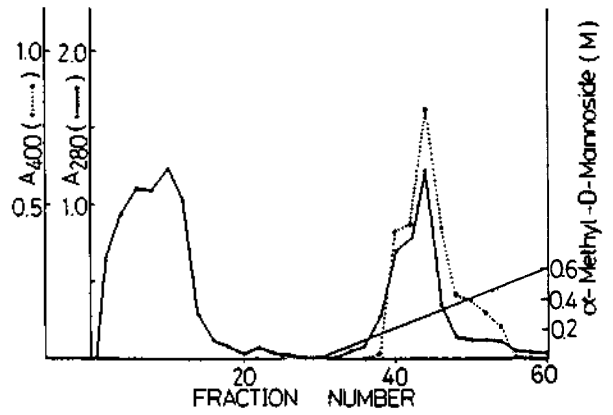

Figure 2. Concanavalin-A Sepharose 4B Affinity Chromatography. The column was eluted with 50 m*M* Tris-HCl buffer, pH 7.2 and developed with linear gradient of 0-0.6 M of α-methyl-D-mannoside in 50 m*M* Tris-HCl buffer, pH 7.2 containing 0.4 *M* NaCl. The column size was 1.5×7.0 cm. ●——●; protein. O----O; β-glucuronidase.

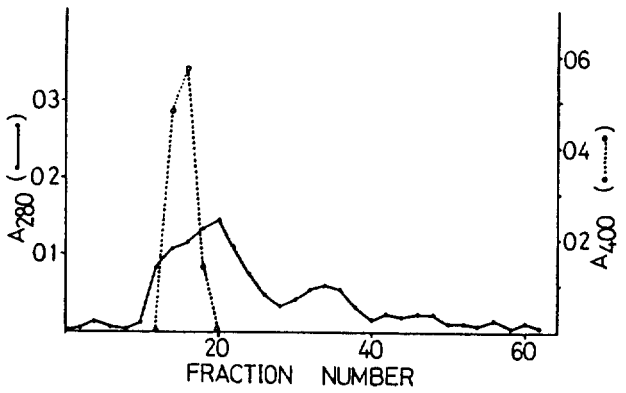

Figure 3. Gel Filtration on Sephadex G-200. The column was eluted with 50 m*M* Tris-HCl buffer, pH 7.2. The dimension of the column was 2.6×30 cm. The void volume was measured by loading Blue Dextran 2000 as marker previous to apply the sample. ●——●; protein. O----O; β-glucuronidase.

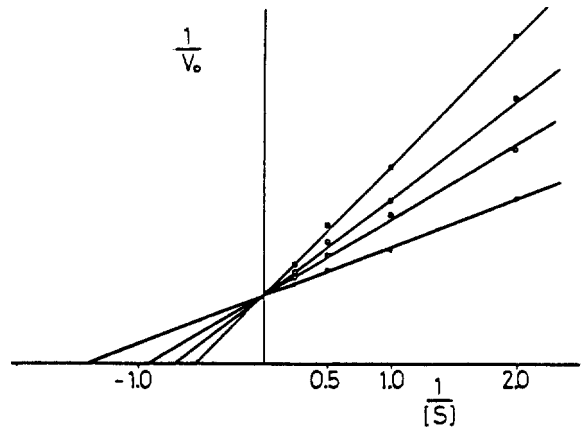

Figure 4. Lineweaver-Burk plot of β-glucuronidase in the presence of *Panax ginseng*. Enzyme activity was measured as described in the text and inhibitor concentrations and *Km* values of each cases are as follows. ●; control Km=0.735 m*M*, □; 32 μg/m*l* Km=1.07 m*M*, O; 80 μg/m*l* Km=1.50 m*M*, ■; 160 μg/m*l* Km=2.10 m*M*.

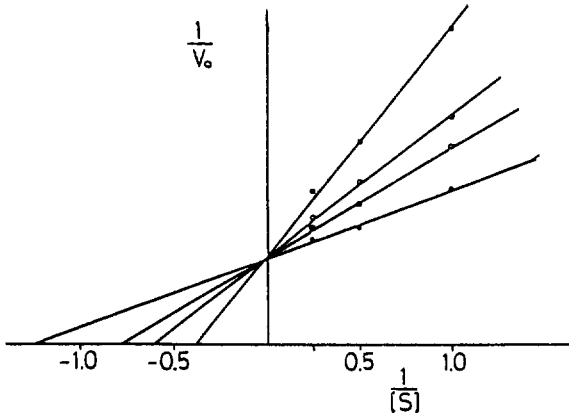

Figure 5. Lineweaver-Burk plot of the enzyme in the presence of *Cortnellus shiiake* extract. Enzyme activities were measured as described in the text. Inhibitor concentrations and Km values of each line are as follows. ●; control Km=0.735 m*M*, □; 38 μg/m*l*. Km=1.25 m*M*, O; 75 μg/m*l* Km=1.44 m*M*, ■; 190 μg/m*l* Km=2.10 m*M*.

the β-glucuronidase probably belong to an imidazolium group had been reported by Wang (1972). Table 2 shows the effect of ionic strength in the reaction mixture. The double reciprocal plot of enzyme activities in different NaCl concentrations is shown in Figure 7 and the Km and Vmax values are in Table 3. When the inhibition reactions were tested in 10-30 m*M* of salt solution, such as $CaCl_2$, NaCl, KCl and $MgSO_4$, there were not such drastic suppression as Wataru Sakamoto had reported (1973). Instead, the ionic strength affects to enzyme activity as some mixed inhibition. The equation relating the rate of an enzyme reaction at low substrate concentration to the ionic strength of the medium on the basis of the primary kinetic effect is

$$\log(k_2/\mathrm{Km}) = \log(k_2/\mathrm{Km})_0 + 1.02 z_A z_B I$$

where Z_A and Z_B are the charges on the active site and on the interacting substrate, respectively, I is the ionic strength, and $(k_2/\mathrm{Km})_0$ is the second order rate constant at zero ionic strength (Mintel & Westley, 1966). A plot of log (k_2/Km) versus the square root of ionic strength should yield a straigt line with a slope approximately equal to $Z_A Z_B$. We have used Vmax in place of k_2 due to the uncertainty of the enzyme concentration used. From experiments employing sodium chloride and substrate concentrations, log (Vmax/Km) for β-glucuronidase has been plotted against I, giving a straight line with a slope of −1.7 (Figure 8). The magnitude of this value must be regarded as approximate, since the law of Debye-Huckel is strictly valid only at low salt concentrations. The direction of the effects, however, indicates that charge neutralization occurs when glucuronides react with the enzyme.

Another evidence that the active site of β-glucuronidase is charged is the result of amino acid effects. We tested with nine amino acids and found that *L*-aspartate and *L*-glutamate in-

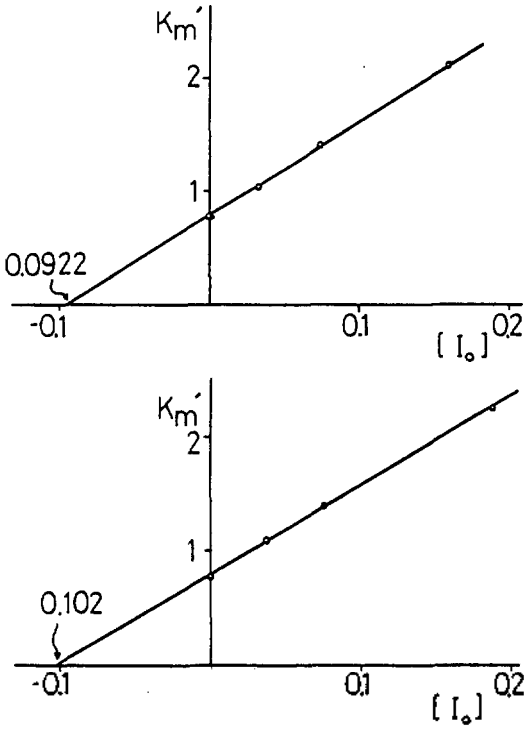

Figure 6. Secondary Plots for Inhibitions. Upper ; Inhibition by *Panax ginseng* extract $K_I = 9.22 \times 10^{-2}$ mg/m*l* Lower; Inhibition by *Cortnellus shiiake* extract KI = 0.102 mg/m*l*.

TABLE 2: Effect on Enzyme Activity and Suppression by the Ionic Strength. Suppression tests were carried out in 80 μg/m*l* of *P. ginseng* extract. Activities are in % Compared to Control not Containing any Additional Salt, Respectively

Salt		Without Inhibitor	With Inhibitor
NaCl	10 m*M*	98	98
	50 m*M*	89	93
	100 m*M*	83	90
Kcl	10 m*M*	97	94
	50 m*M*	91	88
	100 m*M*	83	81
$CaCl_2$	10 m*M*	97	105
	50 m*M*	84	80
	100 m*M*	38	60
$MgSO_4$	10 m*M*	96	121
	50 m*M*	79	104
	100 m*M*	30	75

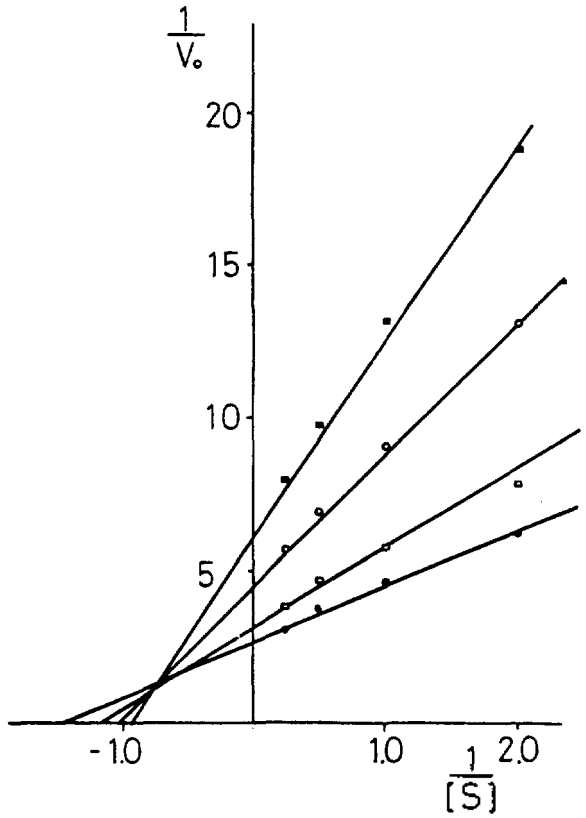

Figure 7. Ionic Strength Effect on Km and Vmax. From 50 m*M* sodium acetate buffer, pH 4.5, the ionic strength was gradually increased by adding NaCl to 0.1, 0.2 and 0.2 *M*, respectively. In each ionic strength, the enzyme activities were measured with the variation of substrate concentrations; 0.5, 1.0, 2.0 and 4.0 m*M*. ●; No NaCl (I = 0.05), □; 0.1 *M* NaCl (I = 0.15), ○; 0.2 *M* NaCl (I = 0.25), ■; 0.3 *M* NaCl (I = 0.35). Km and Vmax values are in Table 3.

TABLE 3: Km and Vmax Values from NaCl Inhibition Reactions. All the Parameters Presented were Obtained from Figure 10

Concentration (*M*)	I	Km (m*M*)	10×Vmax (OD_{400}/min)	$\sqrt{I}$	log (10×Vmax/Km)
0	0.05	0.735	18.6	0.22	1.53
0.1	0.15	0.775	15.2	0.39	1.29
0.2	0.25	0.870	10.6	0.50	1.09
0.3	0.35	0.952	7.7	0.59	0.91

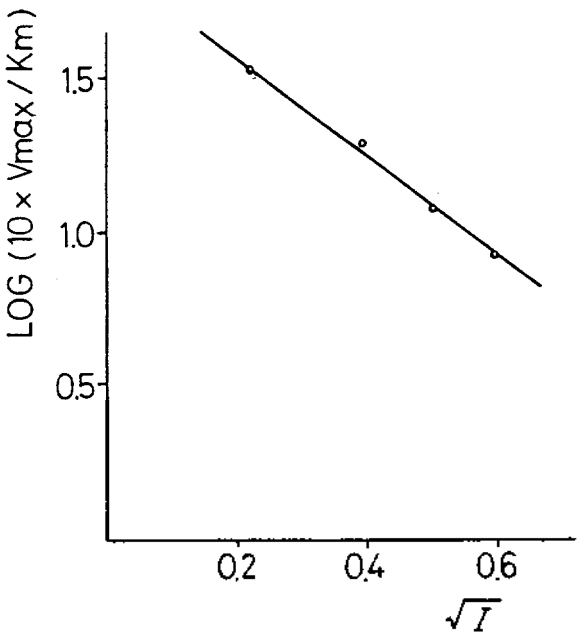

Figure 8. Ionic strength effect. The logarithm of 10 × Vmax/Km was plotted against the square root of ionic strength. This plot is based on Table 3.

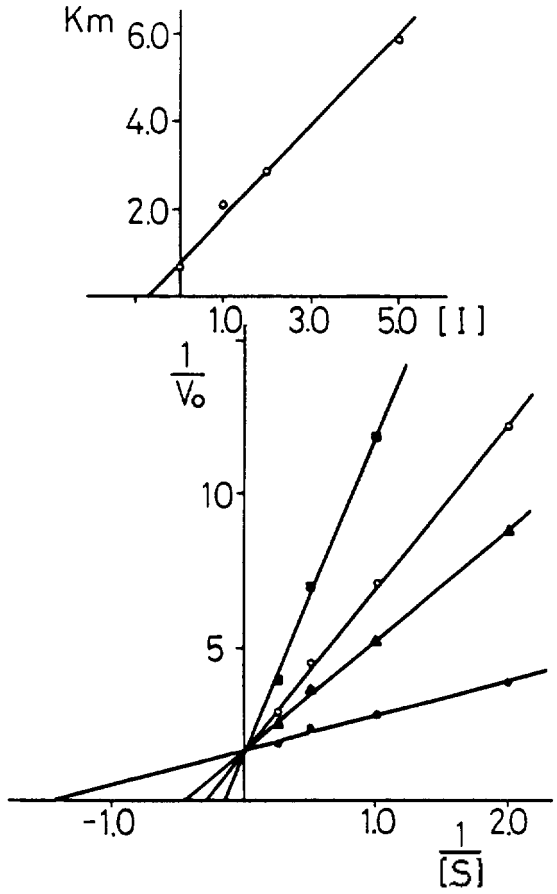

Figure 9. Lineweaver-Burk plots on the enzyme in the presence of *L*-aspartate. Upper; Secondary plot for inhibition to find K_i value. The K_i is 0.80 m*M*. Lower: Enzmye activities were measuredas the same method of the tests to find the Km values. ●; control ▲; 1.0 m*M* L-aspartate. O; 2.0 m*M* ■; 5.0 m*M*. Km and Vmax values are in Table 3.

hibited the enzyme activity (Table 4). When the reaction was performed with 10 mM of *L*-aspartate at 37°C for 1 hr, the remained activity was only 7%. The inhibition pattern of *L*-aspartate was competitive and the K_1 value was 0.80 m*M* (Figure 9). This result means the active site has positive charge and binding seems not exclusively dependent on the charge. *L*-glutamate, larger one carbon than *L*-aspartate, didn't show such drastic inhibition, which means that steric effect is another important effect in binding substrates and active site.

TABLE 4: Amino Acid Effect on the Enzyme Activity.
Measurements were Carried in the Standard Assay Mixture for 1 hr. Activities are in % Compared to no Effector Present

Amino acid	Concentration (mM)	
	1.0	10.0
L-Arginine	100	100
L-Aspartate	38	7
L-Cysteine	97	102
L-Glutamate	93	78
L-Histidine	95	98
L-Leucine	98	102
L-Proline	100	101
L-Threonine	99	100
L-Tyrosine	101	104

In this paper, we showed that *Panax ginseng* and *Cortnellus shiiake* contain a certain inhibitor of β-glucuronidase and L-aspartate is another inhibitor. All the inhibition patterns were competitive and the K_1 values were 9.22×10^{-2}, 0.102 mg/m*l* and 0.80 m*M*, respectively. There was little suppression by salts and the effect of ionic strength was verified. All the inhibitions were seemed to be related to the charges on the active site and the interacting substrates.

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