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Properties of β-Glucuronidase Bound to *p*-Aminophenyl 1-Thio-β-D-glucopyranosiduronic Acid–CH-Sepharose 4B

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β-Glucuronidase [EC 3.2.1.31] from bovine liver was bound to the adsorbent prepared by coupling *p*-aminophenyl 1-thio-β-D-glucopyranosiduronic acid with CH-Sepharose 4B. The properties of the immobilized β-glucuronidase were studied in comparison with those of the soluble enzyme. The inhibition of immobilized β-glucuronidase by saccharo-1,4-lactone is slightly lower than that of the soluble enzyme. The pH–activity profile for the immobilized enzyme is similar to that for the soluble enzyme. The K_m values of the soluble enzyme for *p*-nitrophenyl β-D-glucopyranosiduronic acid, estriol-3-β-D-glucopyranosiduronic acid and estriol-16α-β-D-glucopyranosiduronic acid were 0.94, 0.25 and 6.6 mM, respectively, and these values did not change significantly in the immobilized form. The immobilized enzyme retained 66% of its initial activity after storage for 6 months.

Keywords——immobilized enzyme; β-glucuronidase; physical property; kinetic property; bovine liver; *p*-aminophenyl 1-thio-β-D-glucopyranosiduronic acid

In the previous work, we partially purified β-glucuronidase [EC 3.2.1.31] from rabbit liver with the adsorbent prepared by coupling *p*-aminophenyl 1-thio-β-D-glucopyranosiduronic acid (PAPTGA) with CH-Sepharose 4B.[1] β-Glucuronidase was adsorbed at pH 7.8, and the adsorbed enzyme was eluted by NaCl solution or basic buffer, but not by glucuronic acid or glucuronolactone. Moreover, other glycosidases such as *N*-acetyl-β-D-glucosaminidase were also adsorbed slightly on the adsorbent. These results suggest that the interaction between the adsorbent and β-glucuronidase does not involve the active sites of the enzyme.

The analytical advantages of immobilized enzymes have been well documented.[2] In recent years, the use of immobilized β-glucuronidases bound on a polyvinylchloride membrane[3] or a controlled pore glass[4] for hydrolysis of steroid glucuronides in clinical analysis has been reported.

In this paper, we present a comparison of the properties of soluble β-glucuronidase from bovine liver (which is commercially obtainable) and those of the enzyme bound to PAPTGA–CH-Sepharose 4B.

Experimental

Materials——*p*-Nitrophenyl β-D-glucopyranosiduronic acid was purchased from E. Merck. Estriol-3-β-D-glucopyranosiduronic acid, estriol-16α-β-D-glucopyranosiduronic acid and estriol were purchased from Sigma Chemical Co. Bovine liver β-glucuronidase was purchased from Sigma Chemical Co. PAPTGA was prepared according to the method of Iino.[5] PAPTGA–CH-Sepharose 4B was prepared as described in the preceding paper.[1]

Immobilization of β-Glucuronidase——First, 1544 units of β-glucuronidase in 7.5 ml of 5 mM Tris acetate buffer (pH 7.8) was added to 1 ml of the adsorbent (PAPTGA–CH-Sepharose 4B). After shaking for 1 h, the adsorbent was filtered on a glass filter, and washed with 5 mM Tris acetate buffer (pH 7.8), followed by 5 mM Tris acetate buffer (pH 5.0). In this method, 1472 units of the enzyme was adsorbed, and the activity of the immobilized enzyme was 2 units

per mg of the adsorbent. The immobilized enzyme was stored at 5 °C in 5 mM Tris acetate buffer (pH 5.0).

Assay for Soluble β-Glucuronidase——A mixture of 0.5 ml of the sample, 1 ml of 0.2 M acetate buffer (pH 5.0) and 0.5 ml of 0.1 M *p*-nitrophenyl β-D-glucopyranosiduronic acid was incubated at 37 °C for 10—30 min. After the incubation, 3 ml of 0.5 M glycine buffer (pH 10.4) was added. The absorbance of the mixture was measured at 400 nm. One unit of the enzyme is the quantity required to release 1 nmol of *p*-nitrophenol per min under the conditions described above.

Assay for Immobilized β-Glucuronidase——A mixture of 6 mg of the immobilized enzyme, 1 ml of 0.2 M acetate buffer (pH 5.0), 0.5 ml of water and 0.5 ml of 0.1 M *p*-nitrophenyl β-D-glucopyranosiduronic acid was incubated at 37 °C for 10 min with shaking. After the incubation, 3 ml of 0.5 M glycine buffer (pH 10.4) was added, the immobilized enzyme was removed by centrifugation, and the absorbance of the solution at 400 nm was determined. The activity unit of the immobilized enzyme is the same as that of the soluble enzyme. To confirm that the immobilized enzyme did not leach off into the solution during incubation, the following experiment was performed. A mixture of 12 mg of the immobilized enzyme, 2 ml of 0.02 or 0.2 M acetate buffer (pH 3.5—6.0), 1 ml of water and 1 ml of 0.1 M *p*-nitrophenyl β-D-glucopyranosiduronic acid was incubated at 37 °C for 30 min. After incubation, the mixture was filtered on a glass filter, then 3 ml of 0.5 M glycine buffer (pH 10.4) and 1 ml of water were added to 1 ml of the filtrate. Another 1 ml of the filtrate was further incubated at 37 °C for 10 min, then 3 ml of 0.5 M glycine buffer and 1 ml of water were added to the incubation mixture. The absorbances of the two alkaline mixtures were measured at 400 nm, but no difference between the two was observed.

In experiments involving steroid glucuronides, a mixture of 0.4 ml of 0.02 M acetate buffer, 0.05 ml of 0.01 M steroid glucuronide solution and 0.05 ml of the enzyme solution was incubated at 37 °C. In the case of the immobilized enzyme, 6 mg of the immobilized enzyme and 0.05 ml of water were used instead of 0.05 ml of the soluble enzyme. After the incubation, 0.5 ml of 0.2 M Na_2CO_3 was added to the mixture, and the mixture was extracted with 1.5 ml of ether twice. The ether extract was evaporated under reduced pressure and the residue was dissolved in 0.3 ml of methanol. A 25 μl aliquot of the methanol solution was injected onto a μBondapack C18 high performance liquid chromatography (HPLC) column with methanol–tetrahydrofuran–H_2O (1 : 1 : 3) as the mobile phase. Quantitation was based on peak height, referenced to standard solutions of estriol.

Results and Discussion

pH–Activity Profiles of Soluble and Immobilized Forms of β-Glucuronidase

The effect of pH on the enzyme activity of th soluble and immobilized forms of β-glucuronidase was studied in 0.02 M acetate buffer. The pH–activity curves are given in Fig. 1.

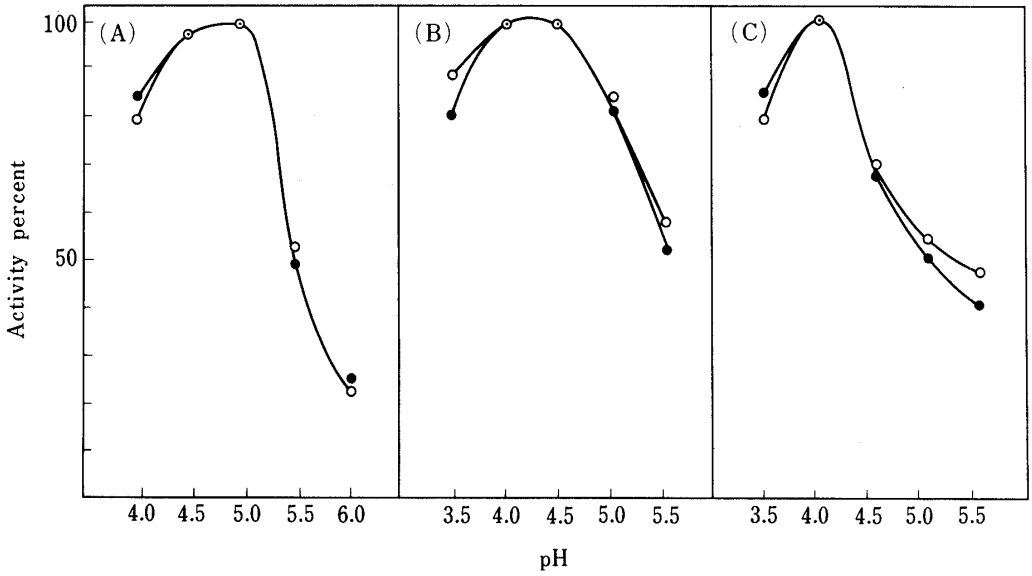

Fig. 1. pH–Activity Curve of Soluble and Immobilized Forms of β-Glucuronidase for Various β-D-Glucopyranosiduronic Acids

Activity was measured in 0.02 M acetate buffer at the indicated pH values at 37 °C using *p*-nitrophenyl β-D-glucopyranosiduronic acid (A), estriol-16α-β-D-glucopyranosiduronic acid (B) and estriol-3-β-D-glucopyranosiduronic acid (C) as substrates.
—○—, immobilized enzyme; —●—, soluble enzyme.

The optimum pH values for *p*-nitrophenyl β-D-glucopyranosiduronic acid, estriol-16α-β-D-glucopyranosiduronic acid and estriol-3-β-D-glucopyranosiduronic acid are 5.0, 4.0—4.5 and 4.0, respectively. It is known that the pH optimum and pH–activity profile are sometimes changed by immobilization. The pH optima of jack bean α-D-mannosidase[6] and buckwheat β-glucosidase[7] were shifted slightly to lower pH values by immobilization. In the cases of bacterial β-glucosidase[8] and gastropod *N*-acetyl β-hexosaminidase,[9] bimodal pH–activity curves were observed after immobilization. However, the pH profiles of *E. coli* β-glucuronidase immobilized on controlled pore glass[4] and bovine liver β-glucuronidase immobilized on a polyvinylchloride membrane[3] were almost the same as those of the soluble enzyme. No significant difference in the pH–activity curves between the soluble and immobilized forms of the enzyme was observed in this study, too.

Inhibition of Soluble and Immobilized Forms of β-Glucuronidase by Saccharo-1,4-lactone

Table I shows the inhibition of the soluble and immobilized forms of β-glucuronidase by saccharo-1,4-lactone, which is an inhibitor of β-glucuronidase. In the inhibition tests, 0.2 M acetate buffer solutions contained saccharo-1,4-lactone at the final concentrations shown in Table I were used. The inhibition of the immobilized enzyme by the inhibitor is slightly lower than that of the soluble enzyme.

Effect of Substrate Concentration

The effect of substrate concentration on the reaction rate was studied with various amounts of *p*-nitrophenyl β-D-glucopyranosiduronic acid, estriol-3-β-D-glucopyranosiduronic acid and estriol-16α-β-D-glucopyranosiduronic acid. The K_m values of soluble and immobilized forms of β-glucuronidase were obtained from Lineweaver–Burk plots. As shown in Table II, immobilization does not significantly modify the kinetic properties of the

TABLE I. Inhibition by Saccharo-1,4-lactone of the Hydrolysis of 0.01 M *p*-Nitrophenyl β-D-Glucopyranosiduronic Acid by the Soluble and Immobilized Forms of β-Glucuronidase in 0.2 M Acetate Buffer (pH 5.0)

Enzyme	Inhibitor	Inhibition (%)
Soluble enzyme	0.025 mM Saccharo-1,4-lactone	37
Soluble enzyme	0.125 mM Saccharo-1,4-lactone	72
Soluble enzyme	2.5 mM Saccharo-1,4-lactone	97
Immobilized enzyme	0.025 mM Saccharo-1,4-lactone	31
Immobilized enzyme	0.125 mM Saccharo-1,4-lactone	66
Immobilized enzyme	2.5 mM Saccharo-1,4-lactone	96

TABLE II. K_m Values of the Soluble and Immobilized Forms of β-Glucuronidase for Various β-D-Glucopyranosiduronic Acids

	K_m (mM)		
	p-Nitrophenyl β-D-glucopyranosiduronic acid	Estriol-3-β-D-glucopyranosiduronic acid	Estriol-16α-β-D-glucopyranosiduronic acid
Soluble enzyme	0.94	0.25	6.6
Immobilized enzyme	0.83	0.31	5.0

Assays were conducted as described in the experimental section, at pH 5.0 for *p*-nitrophenyl β-D-glucopyranosiduronic acid, and at pH 4.0 for both estriol β-D-glucopyranosiduronic acids.

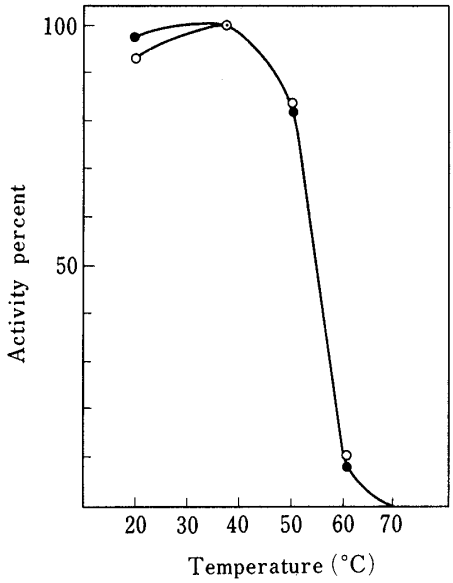

Fig. 2. Thermostability of Soluble and Immobilized Forms of β-Glucuronidase

The soluble and immobilized enzymes were incubated in 0.2 M acetate buffer (pH 5.0) at the indicated temperatures for 5 min. After the incubation, the mixture was cooled and remaining activities was measured at 37 °C using *p*-nitrophenyl β-D-glucopyranosiduronic acid as the substrate.
—○—, immobilized enzyme; —●—, soluble enzyme.

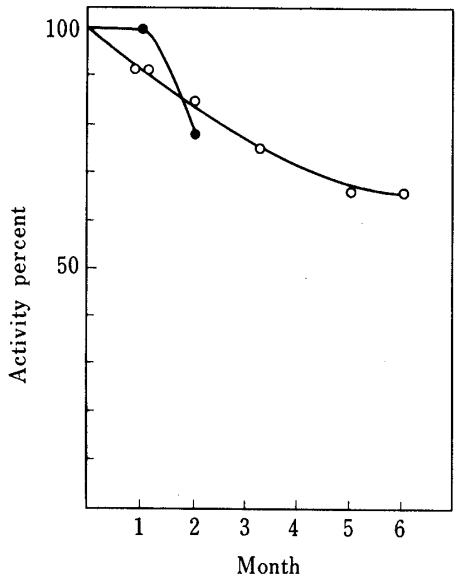

Fig. 3. Storage Stability of Soluble and Immobilized Forms of β-Glucuronidase

—○—, immobilized enzyme; —●—, soluble enzyme.

enzyme. Himeno *et al.* reported that the K_m value of purified β-glucuronidase from bovine liver was 0.33 mM for *p*-nitrophenyl β-D-glucopyranosiduronic acid.[10] Bowers and Johnson found that the K_m values for *p*-nitrophenyl β-D-glucopyranosiduronic acid, estriol-3-β-D-glucopyranosiduronic acid and estriol-16α-β-D-glucopyranosiduronic acid of *E. coli* β-glucuronidase were the same, 0.2 mM, and this value did not change when the enzyme was immobilized.[4]

Thermostability of Soluble and Immobilized Forms of β-Glucuronidase

The activities remaining after incubation for 5 min at the indicated temperatures are given in Fig. 2. The two forms of the enzyme were inactivated within 5 min at temperature above 60 °C. Immobilization of buckwheat β-glucosidase increased the stability of the enzyme to heat,[7] but in our experiment, no difference in the stability to heat of the two forms could be detected.

Stability of Immobilized β-Glucuronidase during Storage

The soluble and immobilized forms of β-glucuronidase were stored in 5 mM Tris acetate buffer (pH 5.0) at 5 °C, and their activities were assayed at suitable time intervals. However, the soluble enzyme solution became moldy after about one month, and so the storage stability of the soluble enzyme in 5 mM Tris acetate buffer (pH 5.0) was not determined after the second month. Bowers and Johnson showed that immobilized β-glucuronidase from *E. coli* retained 40% of its initial activity after one year.[4] In our experiment (Fig. 3), the immobilized enzyme retained 66% of its initial activity after 6 months.

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