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CHARACTERIZATION OF IMMOBILIZED β-GLUCURONIDASE IN AQUEOUS AND MIXED SOLVENT SYSTEMS

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β-Glucuronidase (β-D-glucuronide glucuronosohydrolase, EC 3.2.1.31) was covalently attached to alkylamine-controlled pore glass via a glutaraldehyde immobilization scheme. The activity of this immobilized β-glucuronidase was studied with respect to several kinetic parameters in comparison with the behavior of the soluble enzyme. K_m values for *p*-nitrophenyl glucuronide, estriol-3-glucuronide, and estriol-16α-glucuronide were determined. For each substrate the K_m was essentially the same, 0.2 mM, and this value did not change when the enzyme was immobilized. The soluble and immobilized enzyme both displayed a relatively broad pH maximum centered at pH 6.8 for all substrates. Several organic-aqueous mixtures including methanol, ethanol, acetonitrile and ethylene glycol were tested, and their effects on the activity of immobilized β-glucuronidase were similar to those found for the soluble enzyme. Long-term (1 year) storage stability tests of the immobilized enzyme were carried out. The immobilized enzyme retained 40% of its initial activity after 1 year and was very robust towards most of the organic solvents tested.

Introduction

The use of β-glucuronidase (β-D-glucuronide glucuronosohydrolase, EC 3.2.1.31) as a reagent for cleavage of steroid conjugates prior to analysis has been well established [1–3]. Although the advantages of enzymatic hydrolysis are well known, the expense and time involved in this procedure are deterents to its use. The advantages of immobilized enzymes in analytical work have been well documented [4,5]. In particular, the increased stability and usefulness of the immobilized β-glucuronidase may decrease both the cost and the time involved in the analysis through exposure of the sample to greater amounts of activity. Although previous attempts have been made to immobolize β-glucuronidase [6,7], its use in the capacity reported here has had limited success.

The activity of β-glucuronidase towards various steroid substrates is a function of the steroid and the position of conjugation [2,3]. Immobilization has been shown to change the kinetic properties of the enzyme [8–10]. We have compared the activity of the soluble form of the enzyme to its immobilized counterpart for *p*-nitrophenyl glucuronide, estriol-3-glucuronide, and estriol-16α-glucuronide, the latter two being of particular interest to the clinical laboratory. Early reports indicated an enhancement of reaction rate in the presence of aqueous-organic solvent mixtures [11–14]. Fishman and Green, using primarily bovine liver β-glucuronidase, attributed the enhancement due to alcohols to a glucuronyl transfer mechanism [11,12]. Similar effects have been noted for the bacterial enzyme [13,14]. We have compared the effect of various organic additives on the characteristics of the soluble and immobilized enzyme.

Methods

Immobilization of β-glucuronidase. Controlled pore glass was acid washed and activated with 3-ami-

nopropylmethyl dimethoxysilane at 90°C, for 2 h, under aqueous coinditions [15]. 12% glutaraldehyde (w/v) was prepared in 0.1 M pyrophosphate buffer (pH 8.5) (buffer 1) and reacted with the amino-controlled pore glass for 2 h to generate aldehyde functional groups for coupling of the enzyme. Excess reagent was removed by washing with 100 ml each of deionized water and buffer 1. The β-glucuronidase (1.4–74 mg/g support) was coupled to the controlled pore glass in buffer 1. Adsorbed or entrapped enzyme was removed by washing the preparation with 200 ml each of deionized water, 1.0 M NaCl, deionized water and buffer 1.

Determination of coupling efficiency. Total protein assays were performed in the initial enzyme solution in contact with the controlled pore glass (t_0), and the solution in contact with the controlled pore glass at the end of the reaction period by the method of Hartree [16]. The method was calibrated using albumin solutions (Miles Laboratory, Inc.). The amount of bound protein was calculated from the difference in supernatant concentration before and after the reaction.

Measurement of enzyme activity. The soluble enzyme was assayed using 2.1 mM *p*-nitrophenyl-glucuronide in 0.1 M phosphate buffer (pH 6.8) at 37°C. After about 10% of the substrate was consumed, a 0.5-ml aliquot was adjusted to pH 12 with 0.1 M phosphate buffer (pH 12). In order to minimize the effect of alkaline hydrolysis of the ester on the rate measurement the tubes were read as rapidly as possible in conjunction with a reagent blank, consisting of an appropriately diluted solution of the *p*-nitrophenyl glucuronide and alkaline buffer. The free *p*-nitrophenyl was quantitated by absorbance measurements at 400 nm. In experiments involving the steroid conjugates, a procedure similar to that outlined above was used. After alkalinization to quench the enzyme reaction, however, a 20-μl aliquot of the mixture was injected onto a reversed phase HPLC column. Quantitation was by peak height, referenced to standard solutions of the free steroid.

Activity measurements with the immobilized β-glucuronidase were made using a gradientless recirculation reactor similar to that described by Ford et al. [17]. Fig. 1 illustrates the reduction in rate-limiting mass transfer effects at high flow rates and small percentage conversion on each pass through the reactor for the assay system used. In order to assure that external mass transfer was not influencing the enzyme characteristics, all rate measurements were made at flow rates of 16 ml/min or greater. For experiments involving *p*-nitrophenyl glucuronide the conversion of substrate was monitored continuously at 400 nm with reaction conditions of pH 6.8 and 37°C. Initial substrate concentration was 2 mM. Reaction rates were calculated by comparing the absorbance values in the experimental run to absorbance values for *p*-nitrophenol standards at the same pH. This corrected for any absorptivity change due to pH. Measurement of the absorbance spectra indicated that the organic solvents had no effect on the molar absorptivity of the *p*-nitrophenol.

Conversion of estriol glucuronides by the immobilized species was monitored by removing 200-μl aliquots of solution from the reactor system at various times during operation (0,10,20 min). The aliquot was then injected into a reversed phase HPLC system using a mobile phase composition of 55% methanol and 45% water. Estriol, having been separated from its conjugate, was quantified by peak height and peak area, relative to standard solutions of estriol, using fluorescence detection.

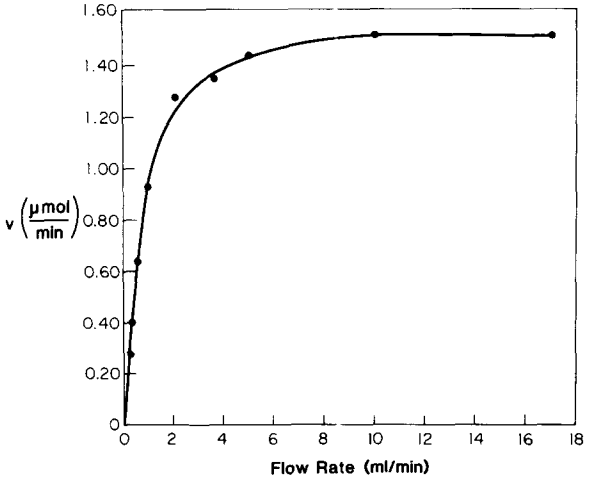

Fig. 1. Velocity of the enzyme reaction as a function of flow rate in a gradientless reactor. All subsequent runs were made at flow rates greater than 16 ml/min to insure independence from external mass transfer effects.

Materials

Instrumentation. All spectrophotometric measurements were made either on a Sargent-Welch SP-6-450 photometer on a Beckman Model 25 spectrophotometer. Flow in the immobilized enzyme reactor (5 X 1 cm) was maintained by a Rainin Rabbit peristaltic pump. 1–5 mg immobilized enzyme (dry weight) were used in the reactor. All tubing was washed with solvent mixtures prior to use. Temperature control was provided by a Haake Model FE circulating water bath.

Chromatographic measurements were obtained with a Perkin Elmer Series 3 Liquid Chromatograph employing either a Perkin Elmer LC-15 spectrophotometer or a Kratos SF-970 fluorimeter for detection. Data were recorded on a Perkin Elmer Sigma 10 Data Station. All separations were performed on a Whatman PXS 10/25 ODS column.

Reagents. All chemicals were reagent grade and were used without further purification. The Folin reagent was obtained from Fisher Scientific. The solvents were obtained form Burdick and Jackson except for *t*-butanol which was distilled at 82°C from 99.5% alcohol (Aldrich) immediately prior to use. β-Glucuronidase (from *Escherichia coli* types VA and IX), *p*-nitrophenyl glucuronide, phenolphthalein glucuronide, estriol, estriol-3-glucuronide, estriol-16α-glucuronide, and glutaraldehyde were purchased from Sigma. The albumin standard solution was a product of Miles Laboratories. The 3-aminopropylmethyl dimethoxysilane was obtained from Petrarch Fine Chemicals. Controlled porosity glass (530 Å pores, 200–400 mesh) was obtained from ElectroNucleonics or Pierce Chemical Company.

Results and Discussion

Enzyme immobilization

β-Glucuronidase was immobilized from solutions containing 75–400 mg/ml of packaged material. By the Lowry technique, actual protein concentrations were in the range of 1.4–71.4 mg protein/ml buffer. The amount of β-glucuronidase protein bound averaged 60%, independent of the total amount of protein in contact with the support. The recovery of enzyme activity was about 90% for light enzyme loadings based on the assumption that full recovery would yield an enzyme of equivalent specific activity. This near 100% recovery of activity indicates that the enzyme characteristics are free of both internal and external mass transfer effects assuming the enzyme is not activated upon immobilization. Enzyme loadings of 43 mg/g glass and 500 I.U./g glass were routinely obtained.

The storage stability of the enzyme was very good with 48% of the initial immobilized activity present after 8 months of storage, as a wet cake at refrigerated temperatures. Subsequent measurements have indicated that 40% of the activity remains after 1 year of storage at 4°C. We have successfully operated an immobilized β-glucuronidase reactor at 25°C for several weeks at low flow rates (1 ml/min) with no apparent decrease in activity, although the effects of excess enzyme and diffusion control may mask the actual rate of activity loss.

Effect of immobilization on enzyme characteristics

The effects on the K_m of the enzyme are not significant for the three substrates studied. The K_m for *p*-nitrophenyl glucuronide, estriol-3-glucuronide and estriol-16α-glucuronide was found to be 0.2 mM using both Lineweaver-Burk plots and non-linear least-squares curve fitting. This is in good agreement with the reported values [18]. The K_m values obtained for the immobilized species were statistically indistinguishable from those calculated for the soluble enzyme. It is apparent that immobilization does not significantly modify the kinetic properties of the enzyme.

The effect of the immobilization process on the

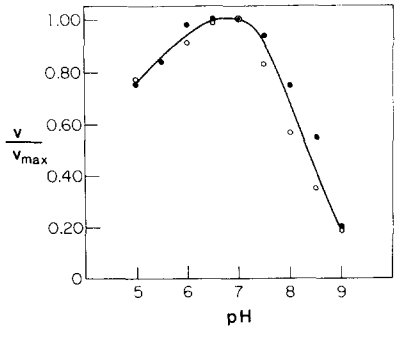

Fig. 2. Relative enzyme activity as a function of pH with *p*-nitrophenyl glucuronide substrate. The data points represent soluble (○) and immobilized (●) enzyme respectively.

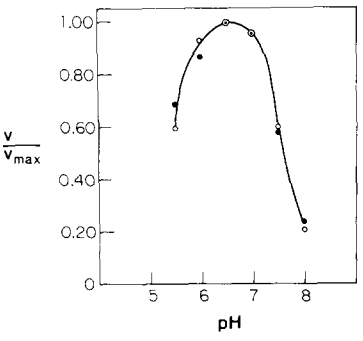

Fig. 3. Relative enzyme reaction rate as a function of pH with either estriol-3-glucuronide (○) or estriol-16α-glucuronide (●) for immobilized β-glucuronidase.

pH optimum of the enzyme was also studied. As shown in Figs. 2 and 3, no significant difference in the pH optimum for either the soluble or immobilized enzyme was observed for *p*-nitrophenyl glucuronide, estriol-3-glucuronide or estriol-16α-glucuronide.

Effect of immobilization on activity in cosolvent

The activity of β-glucuronidase in both the soluble and insoluble form was studied in the presence of various aqueous-organic solvent mixtures. As shown in Fig. 4, several patterns are observed. Note in the methanol panel that for *p*-nitrophenyl glucuronide, there is no significant effect of immobilization on the kinetic properties of the enzyme. Upon returning the support-bound enzyme from the optimum methanol concentration to aqueous solution, the original activity was observed indicating that no irreversible change in enzyme structure had occurred. At methanol fractions exceeding 15%, different behavior was noted. For example, if the support-bound enzyme were assayed in 20% methanol, the activity would be 80% of that of the same aliquot of enzyme assayed in aqueous media. (see Fig. 4). After extensively washing the enzyme with buffer, the enzyme would have the same activity as it did in 20% methanol indicating an irreversible loss of activity. This loss is probably due to denaturation. Similar observations were made in ethanol/water mixtures. The curve seen with acetonitrile is distinctly different from that of the hydrogen-bonding alcohols. Quite low mol fractions of this solvent denature the enzyme, even in the immobilized state. Ethylene glycol exhibits activity enhancement similar to the other alcohols as shown in Fig. 4. If an aliquot of immobilized enzyme is exposed to a volume fraction of ethylene glycol, sufficient to decrease activity, and is subsequently washed and assayed in aqueous buffer, the original aqueous activity is recovered. This is quite different from the behavior noted for methanol and ethanol. It would thus appear that this solvent acts in a very different mode.

It is interesting to compare these observations with other reported studies of *E. coli* β-glucuronidase behavior in mixed solvent systems. Using phenolphthalein glucuronide as a substrate at pH 6.8, Gauntney et al. [13] noted an increase of activity in the presence of a series of monofunctional alcohols. This series included methanol through *n*-amyl/alcohol, with the latter yielding an 8-fold enhancement. The isolation of glucuronide conjugates of cosolvents in the series from methanol to *n*-butanol, from bacterial enzyme reaction mixtures [19] lends support to the transfer mechanism postulated by Fishman and Green [11,12] for the bovine liver enzyme. More recently, Lovrein and coworkers [20,21] have concluded that monofunctional alcohols have an effect both on the rate of catalysis and the degree of substrate inhibition, observed on the reaction of phenolphthalein glucuronide in pH 5.0 acetate buffer. Greater effects were observed for alcohols with three to five carbon atoms, e.g., *t*-butanol. In the cases where ethylene glycol was studied [19], no enhancement was observed.

In the present study, *p*-nitrophenol glucuronide and two isomeric estriol glucuronides were studied at pH 6.8. As seen in the methanol panel of Fig. 4, significant differences were observed for the two substrate classes. The behavior for *p*-nitrophenol glucuronide is significantly different from that reported for phenolphthalein glucuronide. We attribute this behavior to the higher turnover number for the former substrate and the high degree of water solubility of the *p*-nitrophenol product. It is interesting to note that the dielectric constants of the optimal methanol and ethanol percentages are 74.6 and 76.0, respectively [22]. If the effect of the dielectric constant on the reaction was purely a charge interaction one would expect an optimum at about 10 vol. percent acetonitrile [23]. Clearly this is not the case. This is not surprising in light of the previous data [14]. Also, the activated complex and reactants

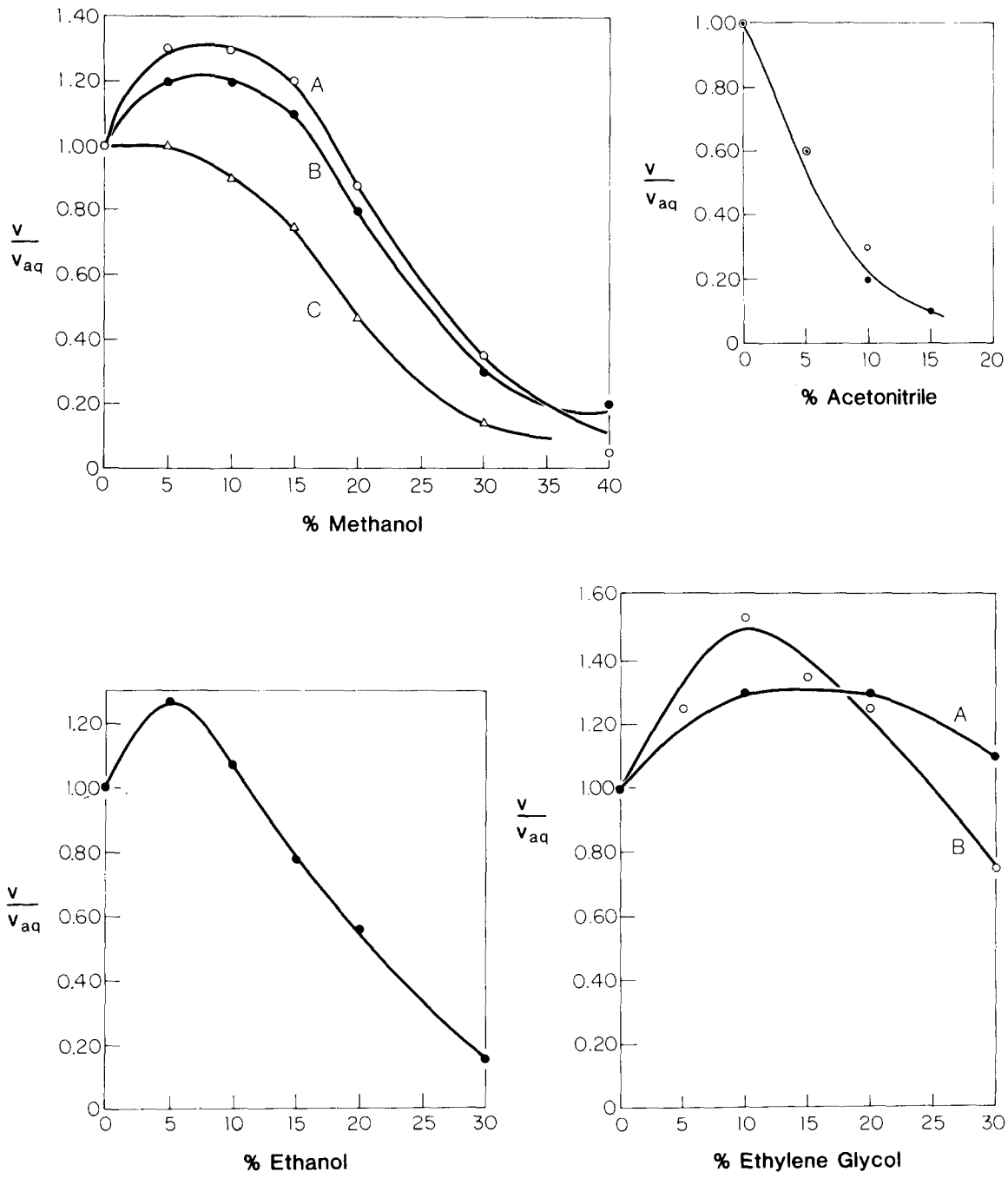

Fig. 4. Normalized reaction rate as a function of vol. percent organic cosolvent. In the methanol panel, curves A and B represent the behavior observed for immobilized and soluble enzyme, respectively, with *p*-nitrophenyl glucuronide substrate, while curve C represents the reaction of estriol-3-glucuronide with immobilized enzyme. The aceonitrile and ethanol panels are the results of *p*-nitrophenyl glucuronide hydrolysis by immobilized β-glucuronidase. The ethylene glycol panel illustrates the difference observed between the immobilized (A) and soluble (B) enzyme with *p*-nitrophenyl glucuronide substrate. In all cases the buffer was 0.1 mol/l phosphate, pH 6.7.

may have identical charge, thereby diminishing any dielectric constant effects [24]. It would appear, then, that specific solvent interactions with substrate, product or enzyme are required for enhancement of reaction rate.

The behavior of the estriol conjugates is expected to be more analogous to the phenolphthalein conjugate based on their more similar structure and water solubility relative to *p*-nitrophenol. The reaction rate relative to *p*-nitrophenol glucuronide also reflects the lower affinity for estriol conjugates. We expected, therefore, that a pattern of activity enhancement similar to that found with the longer chain alcohols would be observed for the estriol glucuronides. Experiments with *t*-butanol resulted in a curve similar to that found for acetonitrile in Fig. 4. These results do not agree with the work reported for soluble β-glucuronidase. One possible explanation is the inability of an immobilized enzyme to make use of micellular organic solvent [13,14], since the microenvironment inside the matrix may be quite different than the bulk of solution [8] and may not, therefore, contain a significant mol fraction of organic solvent. A second possibility is a change in pH during the reaction as a result of the presence of the organic cosolvent or as a result of the use of low buffer concentration, which is mandated by the poor solubility of the buffer in the organic media. A third possibility is that monofunctional alcohols smaller than propanol serve a different function than the larger alcohols which might tend to form micelles. We have noticed what appears to be a significant retention of the steroid product of the enzyme reaction by an immobilized β-glucuronidase packed bed reactor. This binding appears to be enzyme specific. The steroid can be eluted with higher concentrations of organic solvent, an observation which lends support to Pesheck and Lovrein's observation on product inhibition [21].

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