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Studies on the Immobilization of Glucuronidase (Part 1)

Covalent Immobilization on Various Carriers (A Comparison)

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ABSTRACT

β-Glucuronidase (EC 3.2.1.31) was immobilized on various organic and inorganic carriers by different methods. Optimum coupling conditions have been worked out. The immobilization were characterized and compared to each other. Parameters resulting in most stable preparations with high activities are discussed.

Index Entries: Immobilized β-glucuronidase; immobilization techniques; glutaraldehyde.

INTRODUCTION

β-Glucaronidase (β-D-Glucuronid-glucuron hydrolase EC 3.2.1.31) splits naturally occurring and artificial glucuronides into glucuronic acid and the aglycones. The enzyme is a tetramer with a mol wt of 220,000 dalton. It can dissociate into inactive subunits that can be reactivated under certain conditions (*1,2*).

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Dissociation and association depend on the pH of the medium, the concentration of the enzyme, and the presence of NaCl or bovine serum albumin (BSA) (*3–5*). The enzyme, a glycoproteide, tends to self-degradation digesting its own carbohydrate moiety (*6*). The glucuronidase is a hysteretic enzyme (*7*). It has a stable pH between 4 and 7 (*8*), has an optimum pH between 4.5 and 6, and an isoelectric point between 5 and 6 p*I*. Also, ligand-induced conformational changes are referred in the literature (*9*). The properties of the enzyme (dissociation into subunits, conformational changes, and so on) makes it more difficult to obtain optimal active and stable immobilizates. A comparison of various types of immobilized glucuronidase is given in this article.

MATERIALS

β-Glucuronidase (β-D-Glucuronid-glucuron hydrolase EC 3.2.1.31) from Helix pomatia, controlled pore glass (CPG-240-200), 2,4,6-trinitrobenzene sulfonic acid (TNBS), 4-nitro-phenyl-glucuronide, 4-nitrophenyl-glucoside, 3-aminopropyl-triethoxy silane, cyanogen bromide, 4-nitrophenol, pyridine-4-aldehyde, dithiothreitol, bovine serum albumin (BSA), and dialysis sacs were obtained from Sigma, St. Louis; ninhydrin, sodium borohydride, inorganic acids, organic solvents, hydrazine hydrate, 2,4-dinitrophenyl hydrazine, and all buffer substances were purchased from Merck, Darmstadt; Gulsenit was a gift of Magindag, Vienna; glutaraldehyde (25%) was from Fluka, Buchs; Sephadex G-200 was purchased from Pharmacia, Uppsala; Biogel P-60 was from Bio Rad, Richmond, CA.

METHODS

Purification of Glucuronidase

The commercially available glucuronidase ($M = 220,000$) preparations were separated from arylsulfatase ($M = 55,000$) via gel filtration with Sephadex G-200.

Immobilizates

Polyacrylamide–Hydrazide Activated with Glutaraldehyde

Polyacrylhydrazide (Fig. 1) was prepared according to Inman and Dintzis (*10*). Dry Biogel P-60 (10 g) was allowed to swell overnight in water at rt (Type 1). It was then filtered, suspended in 75 mL of water, heated to 47°C, and then treated with 68 mL of 90% hydrazine hydrate. The mixture was kept at 47°C and after 20 min the gel was removed from the reaction mixture, filtered, and washed with 0.1*M* NaCl. The hydrazide gel was activated with an excess of 10% aq. glutaraldehyde solution for 4 h at rt.

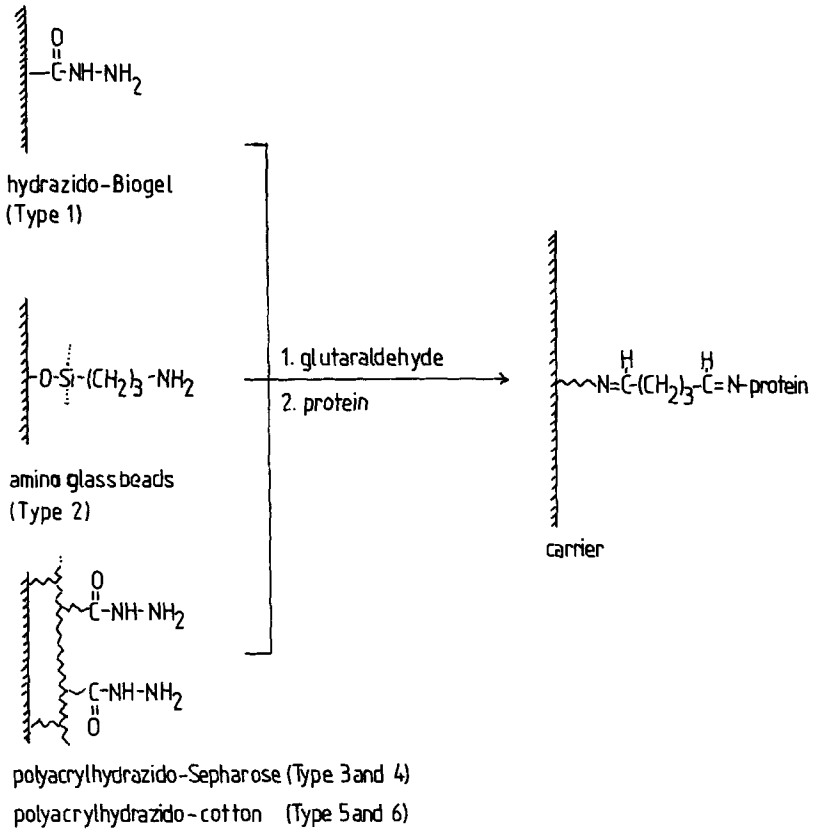

Fig. 1. Immobilization of β-glucuronidase to glutaraldehyde activated amino-carriers (Types 1–6).

The gel was then filtered and washed with ice cold water until the filtrate showed no reaction with 2,4-dinitrophenyl hydrazine. The completion of the coupling reaction was indicated by a negative TNBS test.

For coupling of glucuronidase, the gel was shaken for 24 h at 25°C with a solution of enzyme (25 mg/g packed gel) in 0.1*M* citrate buffer, pH 6.7. The filtered gel was washed with buffer followed by 0.1*M* aq. NaCl solution until no protein could be found in the filtrate with ninhydrin. The gel was stored in 0.1*M* NaCl solution at 4°C.

Amino Glass Beads Activated with Glutaraldehyde

Controlled pore glass beads CPG-240-200 (Type 2) were treated for 1 h with 3% HNO_3 at 90°C, rinsed with water until the filtrate was neutral, and kept for several days in distilled water (*11*). The carriers (10 g) were then mixed with 50 mL 10% aq. 3-aminopropyl triethoxy silane and the pH adjusted to 3.5 with 6*M* HCl. The suspension was gently agitated for 2 h on a shaker. The coated glass beads were filtered, rinsed with water,

and dried overnight at 115°C. The activation of the aminocarrier with glutaraldehyde and the coupling of the enzyme was the same as mentioned with Type 1.

Polyacrylhydrazido–Sepharose Prepared via Periodate Oxidation and Activated with Glutaraldehyde

Polyacrylhydrazido-Sepharose (Types 3 and 4) was prepared via periodate oxidation according to Miron and Wilchek (*12*): Sepharose 4B (10 g) was suspended in freshly prepared 0.25*M* sodium periodate (30 mL). The suspension was stirred at 24°C for 3 h in the dark. The oxidized Sepharose was washed with cold water and suspended in three vol of a 0.5% polyacrylhydrazide solution in water under stirring in the dark overnight. The conjugate was washed with 0.1*M* NaCl until the washings showed no color reaction with TNBS and then treated with sodium borohydride. Activation of the carrier with glutaraldehyde and coupling of the enzyme was carried out as described with Type 1 immobilizates.

Polyacrylhydrazido–Sepharose Activated with Glutaraldehyde

Polyacrylhydrazido-Sepharose (preparation via cyanogen bromide treatment) was prepared according to Miron et al. (*13*): packed Sepharose CL-4B (10 g) was washed with water, suspended in 20 mL 2*M* K_2CO_3 solution, and activated at 0°C with 1 mL cyanogen bromide solution in *N,N*-dimethyl formamide (DMF) (1 g/mL) for 90 s. The gel was then filtered and washed with 50% aq. DMF followed by ice water. The activated gel was suspended immediately in 30 mL 0.2*M* aqueous $NaHCO_3$ solution, containing 0.5% water soluble polyacrylhydrazide (*13*), and the mixture was stirred for 16 h at 4°C. The gel was then filtered and washed with 0.1*M* NaCl solution until samples of the washings showed no color when tested with TNBS. Activation of the carrier with glutaraldehyde and coupling of the enzyme was carried out as described with Type 1 immobilizates.

Polyacrylhydrazido–Cotton Tissue Activated with BrCN

The immobilizate was prepared according to the procedure described with Type 4 immobilizate, using 1 g cotton tissue instead of Sepharose.

Polyacrylhydrazido–Cotton Tissue Periodate Activated

The immobilizate was prepared following the procedure described with Type 3, using cotton tissue (1 g) instead of Sepharose.

Pyridino–Gulsenit and Pyridino Glass Beads Activated with BrCN

Gulsenit (Fig. 2, Type 7) or controlled pore glass beads CPG-240-200 (for Type 8) were treated with 3-aminopropyltriethoxysilane as described with Type 2. The coated carriers (10 g) were treated with 250 mL 2.5% pyridine-4-aldehyde in 0.25*M* phosphate buffer, pH 7, stirred at rt for 1 h, washed carefully with water, and dried in a desiccator over NaOH. The completion of the reaction was indicated by a negative test with TNBS. The particles (1 g) were moistened with dry dioxane, degassed, and treated

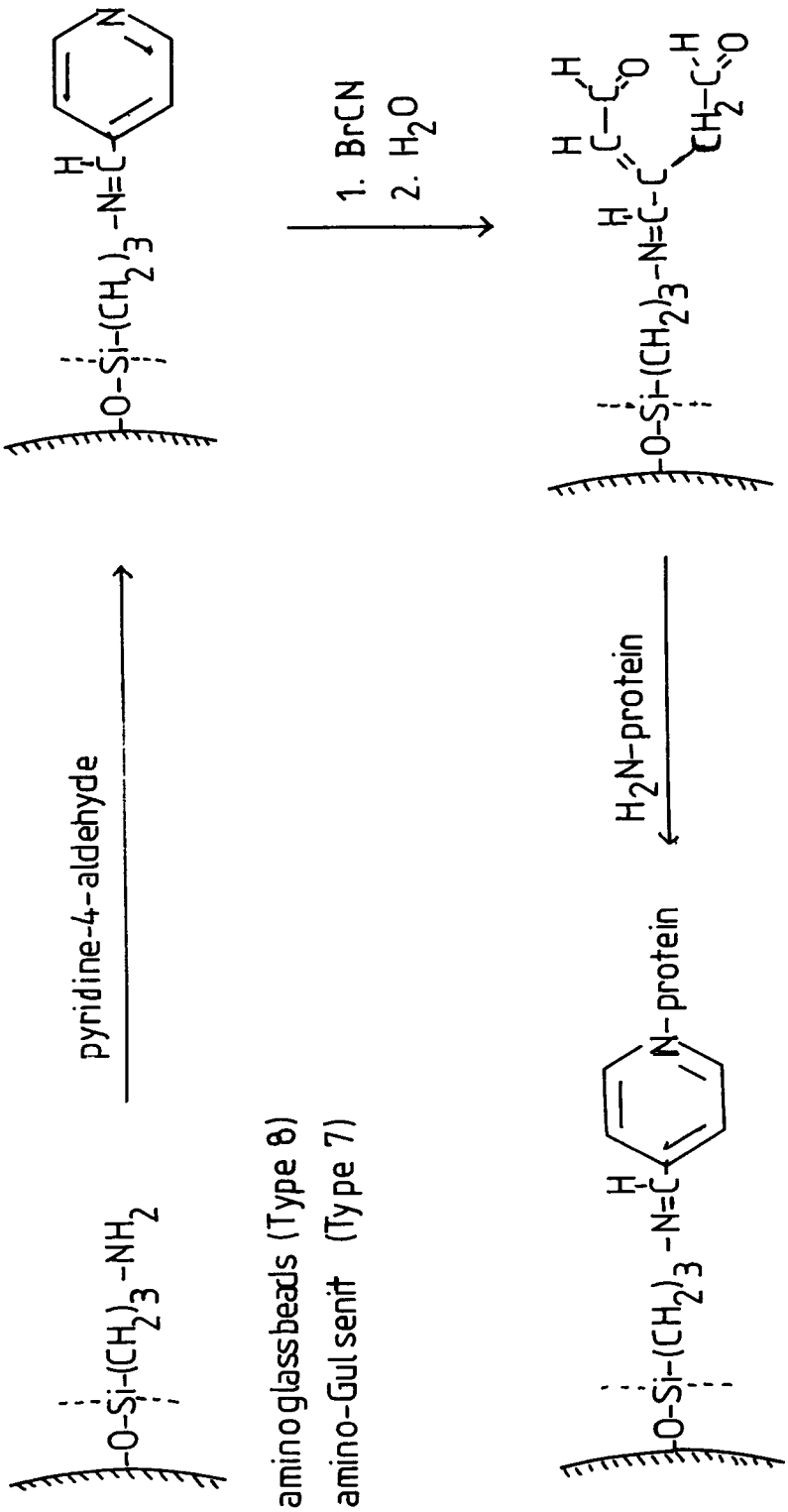

Fig. 2. Immobilization of β-glucuronidase to pyridino-carriers (Types 7 and 8).

with cyanogen bromide (1 g/mL absolute dioxane). After 5 min of activation, 20 mL of 0.2*M* borate buffer, pH 9, were added, and the mixture stirred at rt for an additional 20 min, while the pH was kept constant by manual titration with 2*M* NaOH. The carriers were then filtered as quickly as possible and washed with ice cold water. The coupling procedure to the protein was the same as for Type 1 immobilizates.

Enzyme Bound to Controlled Pore Glass via Glutaraldehyde Crosslinking

Glucuronidase (162.5 mg) and bovine serum albumin (487.5 mg) were dissolved in 30 mL 1*M* acetate buffer, pH 4.5, containing 5 mg *p*-nitrophenyl glucuronide and 2 mg dithiothreitol and kept at rt for 10 min. Then 6 mL 2.5% glutaraldehyde (Fig. 3) solution were added slowly under stirring. Controlled pore glass beads (5 g) were degassed in acetate buffer, pH 4.5, and quickly added as wet particles to the protein solution containing the glutaraldehyde (Type 9). The reaction mixture was agitated for 2 min. Then the wet carrier was removed by filtration and kept in a covered petri dish for 3 h at rt and for an additional 48 h at 4°C to allow copolymerization of the proteins. The protein coated glass beads were washed with 0.1*M* NaCl solution until no protein could be found in the filtrate with ninhydrin and stored in 0.1*M* NaCl at 4°C. Excess aldehyde groups were removed by treatment with $NaBH_4$.

Enzyme Bound to Cotton Tissue via Glutaraldehyde Crosslinking and in the Presence of BSA

Enzymes were prepared in the same manner as Type 9, using cotton tissues (1 g) instead of controlled pore glass beads. The Type 10 immobilizate contained only crosslinked glucuronidase, whereas Type 11 was a coimmobilizate of glucuronidase and bovine serum albumin.

Enzyme–BSA Gel Obtained by Cocrosslinking of the Proteins with Glutaraldehyde

Glucuronidase (162.5 mg) and bovine serum albumine (487.5 g) were crosslinked with glutaraldehyde as described with Type 9 in absence of a carrier (Type 12). The mixture was allowed to form a gel by storing at 4°C for 48 h. Then the protein gel was cut with a blender and homogenized by dispersing in excess buffer through the needle of a syringe. The particles were washed with buffer and 0.1*M* NaCl solution until no reaction with ninhydrin in the filtrate could be detected.

Determination of Protein Content

The quantitative determination of immobilized protein was carried out on an amino acid analyzer after acid hydrolysis with 6*M* HCl in a sealed tube for 22 h at 110°C or according to Jacobs (*14*).

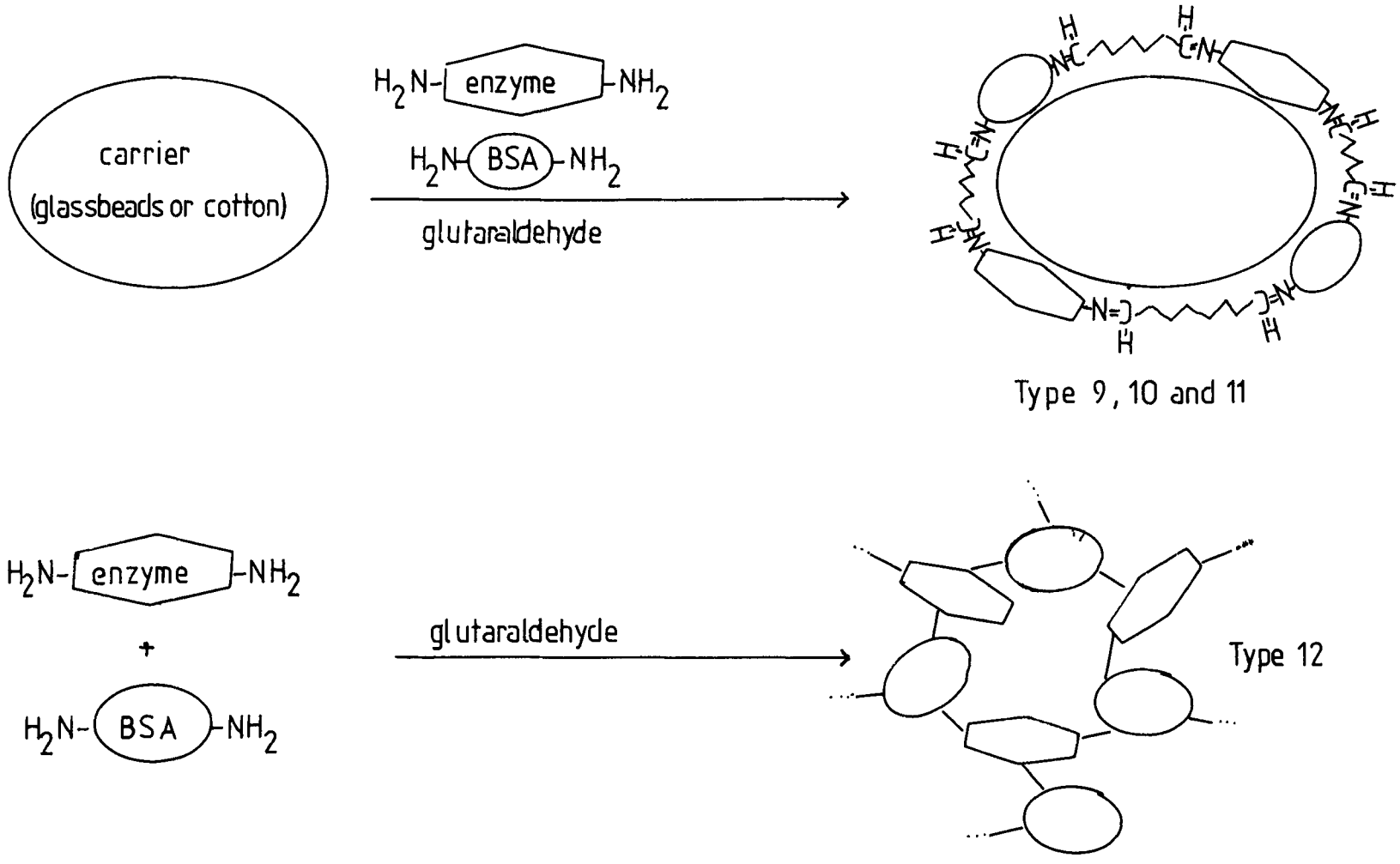

Fig. 3. Crosslinked glucuronidase preparations in the absence and presence of bovine serum albumin (Types 9–12).

Enzyme Assay with Artificial Substrates

A substrate solution containing 50 mg 4-nitrophenyl-glucopyranosiduronic acid in 100 mL of water was prepared. A mixture of 1 mL substrate solution and 4 mL 1*M* acetate buffer, pH 3.5, containing 0.2 mg soluble glucuronidase or 20 mg enzyme immobilizate, was incubated under shaking at 38°C for 30 min, together with a blank without enzyme. Aliquots (2.5 mL) of the incubation mixture were mixed with 5 mL of a 50% aq. triethanolamine solution, and the absorbance was measured spectrophotometrically at 405 nm. A calibration curve with 4-nitrophenol (25–350 nmol/mL) was used for quantitative estimation. If 4-nitrophenyl-β-D-glucopyranoside was used as a substrate an acetate buffer, pH 4.5, had to be employed.

Water Soluble Polyacrylhydrazide

This solution was prepared according to Miron et al. (*15*).

Conditions to Cause Dissociation of Glucuronidase into the Subunits

It is known from the literature that glucuronidases can be dissociated into subunits that can be reassociated into a catalytically active holoenzyme (*1,2*). Dissociation and association depend on pH, dilution, and absence or presence of NaCl or bovine serum albumin (*3–5*). To dissociate subunits of the immobilized enzyme, which were not covalently bound to the matrix, 1 g immobilizate was kept in 30 mL 1*M* acetate buffer, pH 3.5, (in the absence of stabilizing agents) at 25°C under constant shaking. The buffer was changed every day until no more leakage of the subunits could be observed. This was the case after 5 d. The remaining protein bound was determined with the help of an amino acid analyzer after acid hydrolysis as previously described.

Reassociation of the Immobilized Holoenzyme

Immobilizate (1 g) containing only covalently bound subunits was suspended in 20 mL 0.1*M* citrate buffer, pH 6.7, 0.1*M* with respect to NaCl, containing 25 mg soluble glucuronidase. The mixture was kept under constant shaking at 25°C until the activity of the immobilizates were fully restored. This was the case within 1 to 3 d.

RESULTS AND DISCUSSION

Stable Preparations with High Activity

Glucuronidase was coupled to several inorganic carriers and organopolymers employing different methods to obtain preparations for applica-

Table 1
Data of Immobilized Glucuronidase[a]

Type	Protein bound/wet carrier (mg/g)	pH optimum	Specific activity, pkat/mg protein	Activity compared to the soluble enzyme,[b]	Remarks
1	10.6	3.5	2.6	1.3	Leakage
2	15.2	3.5	38.8	19.0	Leakage
3	5.0	3.5	5.2	2.6	Leakage
4	10.5	3.5	0.1	0.05	Leakage
5	2.0	3.5	3.0	1.5	
6	1.2	3.5	10.0	5.0	Leakage
7	18.0	3.5	17.4	8.7	Leakage[c]
8	18.2	3.5	196.1	98.0	Leakage[d]
9	8.8	3.5	730	365.0	No leakage
10	2.0	3.5	3.8	1.9	Leakage
11	40.0	3.5	950	475	No leakage
12	75[e]	3.5	500	250	No leakage

[a]Type 1, polyacrylamid-hydrazide activated with glutaraldehyde; Type 2, amino controlled pore glass beads activated with glutaraldehyde; Type 3, polyacrylhydrazido-Sepharose prepared via periodate oxidation, activated with glutaraldehyde; Type 4, polyacrylhydrazido-Sepharose 4B activated with glutaraldehyde; Type 5, polyacrylhydrazido-cotton tissue activated with BrCN; Type 6, polyacrylhydrazido-cotton tissue activated via periodate oxidation; Type 7, pyridino-gulsenit activated with BrCN; Type 8, pyridino controlled pore glass beads activated with BrCN; Type 9, enzyme bound to controlled pore glass via glutaraldehyde crosslinking in the presence of BSA; Type 10, enzyme bound to cotton tissue via glutaraldehyde crosslinking; Type 11, enzyme bound to cotton tissue via glutaraldehyde crosslinking in the presence of BSA; and Type 12, enzyme/BSA gel obtained by mere cocrosslinking of the proteins with glutaraldehyde.

[b]The specific activity of the soluble enzyme under our working conditions was 200 pkat/mg (=100%).

[c]Negative zetapotential of the carrier, positive charges of the pyridinium linkage.

[d]Positive charges of pyridinium linkage.

[e]Containing 25% enzyme protein.

tion in various enzyme reactors. Optimal coupling and working conditions have been worked out. The procedures are described in the Methods section. The immobilizates were tested for their protein content and activities with the artificial substrate *p*-nitrophenyl-glucuronide (*p*-nitrophenyl-glucopyranosiduronic acid). The data are given in Table 1.

All immobilizates were stable for more than 6 mo when stored in the refrigerator and slightly moistened with 0.1*M* NaCl.

As can be seen from Table 1, the most common immobilization method via glutaraldehyde activation of amino- or hydrazido-carriers before coupling of the enzyme, gave the poorest results. Coupling to BrCN activated pyridino glass beads (Type 8 immobilizates) led to preparations where the enzyme could be immobilized while nearly fully preserving its activity under the native conditions. Maybe the positive charges of the pyridinium linkers stabilize the enzyme. In regard to this suggestion, it was somewhat surprising that the activity with Type 7 immobilizates was so low, though both carriers were silicates and the coupling procedure was the same. Maybe the influence of the high negative zetapotential (*16*) of the Gulsenit surface predominated by far the influence of the positive charges of the pyridinium groups. Since BSA is well known as a stabilizing agent of glucuronidase, it could be safely assumed that immobilization in presence of this agent might result in highly active preparations. Table 1 shows that all immobilizates obtained via crosslinking of the enzyme in presence of BSA (Types 9, 11, and 12) exhibit high specific activities, even exceeding the activity of the soluble unstabilized enzyme from 2.5 to nearly 5-fold.

Stability of the Immobilized Glucuronidase

Long-term experiments showed significant leakage of many types of covalently immobilized glucuronidase. The only exceptions were immobilizates of Types 9, 11, and 12 where crosslinking procedures in presence of BSA were employed. Fortunately, the stable immobilizates were also the most active ones.

The question was, why and under which conditions do such immobilizates leak out? Preliminary experiments showed that distinct leakage could be observed in absence of 0.1*M* NaCl or BSA in acetate buffer, pH 3.5. Beyond that, the amount of leaking enzyme could be further increased to some extent by diluting the incubation mixture with buffer. This process is demonstrated with Type 8 immobilizate as an example (*see* Table 2). When 1 g immobilizate was stored in 50 mL acetate buffer, pH 3.5 at 25°C in the absence of stabilizing agents, the activity continuously decreased to a constant value of 5% within 5 d. Simultaneously, the protein content decreased to 47–55% of the original amount. Making the mixture 0.1*M*, with respect to NaCl, gradually led to a slight recovery of the activity. When soluble enzyme was added according to the coupling conditions, the original protein content of the immobilizate could be fully restored. Also, the specific activity could be regenerated to 98–105%.

Table 2
Change of Protein Content and Activity with Time of a Type 8 Immobilizate Under Leaking Conditions

Incubation time, d	Activity, %	Protein content, %
0	100	100
1	84	
3	41	
5	5	47
7	5	47

Since it is known that the glucuronidase molecule consists of four subunits with similar molecular weight, one can sum up the following facts from these experiments

1. Only about two subunits/enzyme molecule could be covalently immobilized with these types of immobilizates.
2. About 5% of the enzyme protein can dissociate from the matrix owing to pH, composition of the medium, and dilution.
3. The glucuronidase molecule has to be composed of all four subunits for maximum activity.

Since the aim of these studies was, among other things, to obtain stable immobilizates for applications in long-term runs, the immobilization procedure had to be altered: just to be on the safe side, that the enzyme did not partly dissociate into inactive subunits they had to be crosslinked under stabilizing conditions probably conjugated to stabilizing agents. So immobilization via crosslinking with glutaraldehyde, in presence of BSA, turned out to be the optimal procedure. BSA even acted as a potent activator of the enzyme as can be seen in Table 1 with Types 9, 11, and 12 immobilizates. However, it has to be pointed out that oxidation of the sugar moiety of the enzyme with IO_4—followed by crosslinking with a diamine led to completely inactive preparations, even in presence of BSA, though the enzyme was still highly active after periodate oxidation—it even degraded the dialysis sacs when dialyzed against buffer for prolonged periods to remove excess periodate. The crosslinking procedure probably prevented active orientations of the subunits.

Summing up we can say that under the described test conditions with the artificial substrate nitrophenylglucuronide optimal active, covalently bound glucuronidase preparations have to be crosslinked with dialdehyde in presence of the stabilizing protein BSA to prevent leakage and diminuation of activity. Stripping of the immobilizate with 0.1*M* NaCl solution after several hours of incubation in long-term runs removed adsorbed product. Thus, declines in activity owing to product inhibition can be avoided or reversed, leading to increased turnovers.

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