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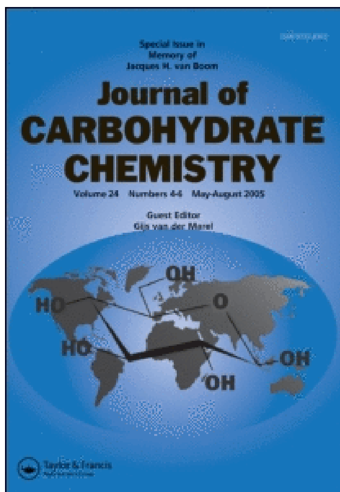

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APPLICATION OF PERIODATE OXIDIZED GLUCANS TO BIOCHEMICAL REACTIONS*,[1]

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ABSTRACT

Periodate oxidation of glucans afforded a dialdehyde structure, which was highly reactive with various compounds containing amino groups. A covalent Schiff base linkage was readily formed at the alkaline pH of 8-9 and cyclodextrin dialdehyde gave both positively and negatively charged derivatives upon incubation with hexamethylenediamine and ε-aminocaproic acid, respectively. Binding of the amino group containing a fluorescent probe of ethylenediaminonaphthalene yielded fluorescent glycogen, which was hydrolyzed with Taka-amylase A. By gel filtration with a Bio-Gel P-2 column, hydrolyzed oligosaccharides containing a fluorescent probe were strongly retained to the column. Dextran dialdehyde was useful in producing a covalent linkage with trypsin under very mild conditions, and the enzyme-dextran complex formed was recovered in a high-molecular weight and active form. Thus, various glucan dialdehydes may serve as useful cross-linking reagents for enzymes.

INTRODUCTION

Various aspects of covalent linkage formation between aldehyde groups of sugars and amino acids have been studied.[2] In the case of reactions with reducing sugars and bovine serum albumin (BSA), complex formation proceeded very slowly and lasted over 200 h.[3] Taking advantage of the reactive dialdehyde obtained by the periodate oxidation of

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various glucans, we have already reported on some practical uses of dialdehyde derivatives.[4-7] In the present paper, a fluorescent labeled glycogen as a substrate of α-amylase reaction and the covalent attachment of dextran chains to the trypsin molecule are described as two examples of applicable uses of glucan dialdehyde.

RESULTS

Reactivity of oxidized glucans. Optimum pH for the reaction between dialdehyde groups and amino groups of BSA was examined. As shown in Fig. 1, complex formation between cellulose dialdehyde and BSA proceeded much better at pH 8.0 and 9.0 than at pH 7.0 and 10.0. In this experiment, the amount of free BSA was measured after centrifugation, which precipitated the BSA bound to the insoluble cellulose dialdehyde (periodate oxidized cellulose powder).

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High reactivity of dialdehyde derivatives of carbohydrate molecules was illustrated, for example, by the complex formation of α-cyclodextrin dialdehyde with alkyl amines. The coupling reaction with hexamethylenediamine and ε-aminocaproic acid gave positively and negatively charged α-cyclodextrin derivatives, respectively. These reaction products were clearly separated from the unreacted compounds by the use of column chromatography with Bio-Gel P-2 (Fig. 2a, b). In the case of a negatively charged complex of ε-aminocaproic acid - α-cyclodextrin, the derivative was eluted at the void volume of the column, and in contrast the positively charged complex of hexamethylenediamine - α-cyclodextrin was eluted after the peak of unreacted α-cyclodextrin. Thus, it is interesting to note that the Bio-Gel P-2 resin has some particular interaction with these charged cyclodextrin derivatives.

Fluorescent labeling of oxidized glucans. Based on the high reactivity of dialdehyde derivatives of glucans, the preparation of fluorescent labeled α-glucans, such as glycogen and clinical dextran, was examined. As candidates

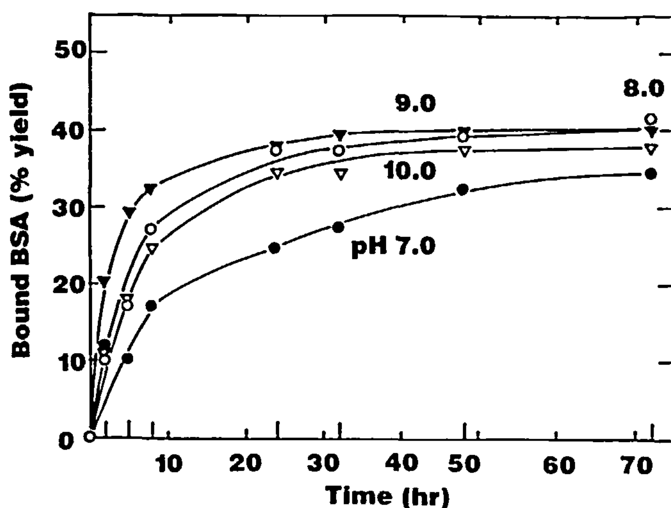

FIG. 1. Effect of pH on the reaction of cellulose dialdehyde with BSA. Triethanolamine-HCl buffer 100 mM adjusted to pH 7.0 (●), 8.0 (○), 9.0 (▼), and 10.0 (▽) was used for the reaction.

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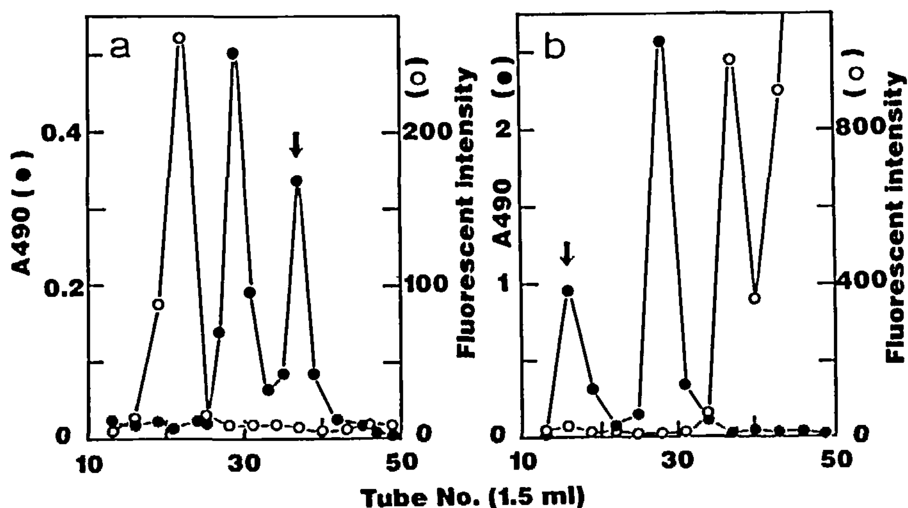

FIG. 2. Elution patterns of the α-cyclodextrin dialdehyde-hexamethylenediamine (a) and ε-aminocaproic acid adducts (b). Total sugar (●) and amino group (○) were determined by the phenol-sulfuric acid method and o-phthalaldehyde method, respectively. Peaks with arrow were ascribed to the adducts.

for fluorescent markers, 2-aminopyridine (2AP) and ethylene-diaminonaphthalene (EDAN) were compared with respect to fluorescent intensity and stability of the complex. Although both 2AP complexes of glycogen and dextran dialdehydes were readily formed, the fluorescent intensity of these complexes were much lower than that of dextran dialdehyde-EDAN complex (Fig. 3). EDAN complex of glycogen dialdehyde gave almost comparable fluorescence to the EDAN-dextran complex. None of these fluorescent glucan complexes showed any significant loss of fluorescent intensity when the solutions were kept at room temperature for 1 month in the dark. Because of the relatively higher fluorescent intensity of EDAN complexes, fluorescent EDAN labeled glycogen was subjected to further characterization.

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Action of TAA on EDAN-glycogen complex. Since the detection limit of EDAN-glycogen by a fluorospectrophotometer was about 1000-fold higher than that of the total sugar measured by the phenol-sulfuric acid method, the use of fluorescent EDAN-glycogen enabled a very sensitive and convenient assay system of α-amylase. As shown in Fig. 4, Taka-amylase A (TAA), a typical α-amylase of fungal origin, hydrolyzed the substrate of EDAN-glycogen and released fluorescent-low molecular weight products. The enzyme concentrations used in this experiment were within a 35-fold range whereas time-dependent hydrolysis of EDAN-glycogen within all ranges of the enzyme concentration was observed. In the cases of the two lower TAA concentrations, the reducing sugar assay method exhibited no clear hydrolysis patterns because of low sensitivity.

The introduction of an extremely hydrophobic naphthalene group into the high-molecular weight, water soluble glycogen molecules caused an increase of solubility in ethanol. Moreover, hydrophobicity was also illustrated by the elution pattern of TAA hydrolyzate of EADN-glycogen from a Bio-Gel P-2 column (Fig. 5). When the hydrolyzate was subjected to the P-2 column equilibrated with 0.2 M

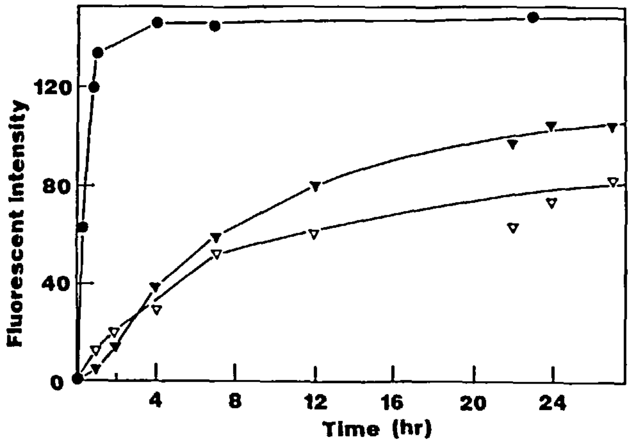

FIG. 3. Course of glycogen and dextran dialdehydes reaction with 2AP and EDAN. Glycogen dialdehyde with 2AP (▽), dextran dialdehyde, with 2AP (▼) and EDAN (●).

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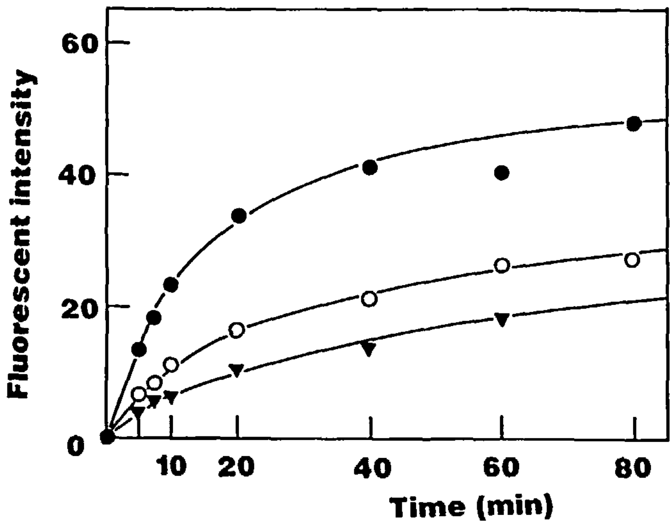

FIG. 4. Hydrolysis of glycogen dialdehyde-EDAN complex with TAA. The enzyme concentrations were 0.027 (●), 0.0039 (○), and 0.0008 (▼) units, respectively.

ammonium bicarbonate, there was a large discrepancy between the patterns measured by the phenol-sulfuric acid method (total sugar) and fluorescence. Except for the high-molecular weight fragment eluted at the void volume (Vo), most of the low-molecular weight fragments were strongly retained to the column and eluted appreciably after the internal volume (Vi). Therefore, the elution volume of EDAN-modified maltose, for example, was much larger than that of free maltose. Moreover, EDAN-substituted oligosaccharides bound tightly to the cation exchange column, CM-Sephadex, whereas oligosaccharides having no EDAN substituents were recovered at the breakthrough fraction.

Oxidized dextran-trypsin complex. An alternative application of the oxidized glucans was a chemical modification of the enzyme molecule. Binding of the dialdehyde of oxidized dextran to an enzyme, such as trypsin, *via* ε-amino groups located at the surface of the enzyme molecule was readily attained under very simple and mild conditions. The resulting Schiff base was reduced by $NaBH_4$ into a stable covalent, alkyl amine.

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To confirm the effectiveness of the dextran dialdehyde coupling method, a model experiment was done with trypsin. After the coupling reaction at 30 °C for 3 h, the reaction mixture was subjected to a gel filtration column of Sepharose 6B (Fig. 6). Of the two protein peaks observed, the first peak corresponded to the peak of dextran and had the enzyme activity, which was determined by the two different procedures. Since the elution volume of the first peak was close to that of cyclodextrin glucanotransferase (*Bacillus subtilis*, M. W. 74,000) and trypsin had a M. W. of 23,000, the dextran dialdehyde-trypsin complex retained enzyme activity and was distinguished from the second peak of inactivated trypsin fragments derived from autolysis.

DISCUSSION

The high reactivity of glucan dialdehyde with amino groups provided several practical applications of polysac-

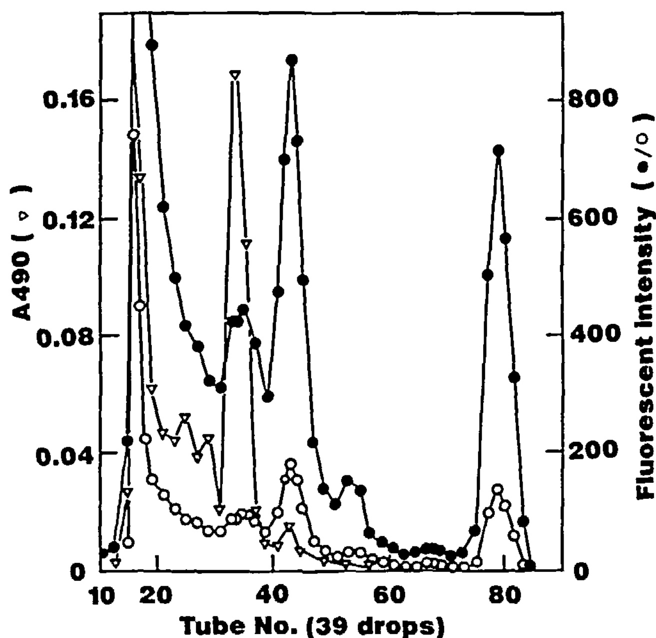

FIG. 5. Elution pattern of glycogen dialdehyde-EDAN complex hydrolyzed with TAA. The Bio-Gel P-2 column was equilibrated and eluted with 0.2 M ammonium bicarbonate. Fluorescent peaks indicated by open circles were reduced to 1/10 scale of closed circles shown on the ordinate.

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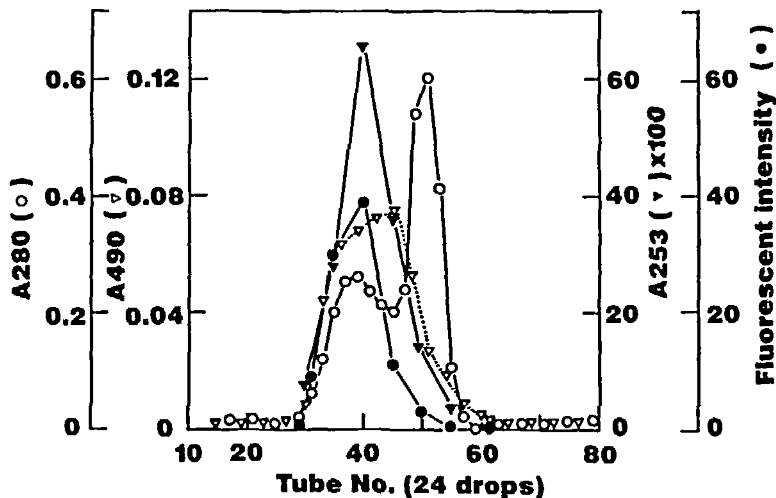

FIG. 6. Elution pattern of dextran dialdehyde-trypsin adduct from a Sepharose 6B column. Trypsin activity was measured by the BAEE method (▼) and the fluorescent method (●) using BSA-EDAN complex as the substrate.

charides. Although the linkage formation between dialdehyde and amino groups was dependent on a rather alkaline pH (Fig. 1), the reaction proceeded rapidly at room temperature, and no particular heating system was required. The most important merit in this procedure was that the coupling reaction proceeded in a water-system, which enabled the glucan dialdehydes to attach to the protein and enzyme molecules.

A series of our studies on reactive dialdehydes began with cyclodextrin and we have demonstrated that dialdehydes of α-, β-, and γ-cyclodextrins were potent inhibitors of various amylases and phosphorylases.[5] Moreover, these dialdehydes served as affinity labeling reagents of phosphorylases.[6,7] In these cases, cyclodextrin dialdehydes served as substrate analogs having potent reactivities with ε-amino groups of Lys residues located in the active site of the enzymes.

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Introduction of a fluorescent reporter group into the glucan dialdehyde could be attained by the use of fluorescent compounds containing a reactive amino group. The fluorescent labeled glycogen served as a substrate of TAA (Fig. 4), where about 1000-fold higher sensitivity was obtained compared with the ordinary colorimetric assay system. Because of the substitution by a hydrophobic naphthalene group, the hydrolyzed oligosaccharides containing EDAN gave a particular elution profile by gel filtration with a Bio-Gel P-2 column (Fig. 5). A unique characteristic of the Bio-Gel P-2 resin was also demonstrated by the elution of positively and negatively charged cyclodextrin dialdehydes as shown in Fig. 2.

The most interesting application of glucan dialdehyde was shown by cross-linking with enzyme molecules. The introduction of dextran dialdehyde into trypsin increased its molecular weight (Fig. 6) and stability against autohydrolysis. We have previously obtained similar results with fungal serine protease.[8] These days, many enzymes are shown to have a glycoprotein structure and in most cases the

carbohydrate chains contribute to the stability of the enzyme. Changes in the tertiary structure of the enzyme molecule by the binding of high-molecular weight polysaccharides may give rise to modifications of enzymatic properties such as substrate specificity, K_m values, and Vmax values.[10] Therefore, glucan dialdehydes may serve as useful cross-linking reagents for various enzymes.

EXPERIMENTAL

Periodate oxidation. Glucan solution (1%) was mixed with 50 mM $NaIO_4$ and oxidized at 4 °C in the dark for 20 h with stirring. Oxidation was stopped by the addition of 100 mM ethylene glycol, and the oxidized polysaccharides were exhaustively dialyzed against water. In the case of cyclodextrin, the reaction mixture was successively treated with Amberlite IR-120 and IRA-410 resins.

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Reaction of glucan dialdehyde with amino groups. Dialdehyde derivatives of various glucans (1%) were mixed with several compounds containing amino group such as BSA (1%), ε-aminocaproic acid (25 mM), hexamethylenediamine (25 mM), 2-AP (0.4%), and EDAN (0.4% in ethanol). Except for the case of BSA, the reactions were done with 100 mM triethanolamine-HCl buffer (pH 8.5) and $NaBH_4$ (50 mg).

Enzymic hydrolysis of fluorescent glycogen. The fluorescent EDAN derivative of glycogen dialdehyde was hydrolyzed with TAA obtained from *Aspergillus oryzae.* Incubation was done at 30 °C and a 50 μL sample was removed and mixed successively with 50 μL of 1 M NaOH and 400 μL of ethanol and centrifuged. The fluorescent intensity of the resulting supernatant was measured at 330 nm for excitation and 430 nm for emission.

Reaction of dextran dialdehyde with trypsin. A lyophilized powder of beef pancreas trypsin (3 mg) was mixed with the clinical dextran dialdehyde (4 mg) in 100 mM triethanolamine-HCl buffer (1 mL), and incubated at 30 °C for 3 h. The reaction mixture was directly applied to a column

of Sepharose 6B (1.0 x 40 cm) and eluted with 20 mM acetate buffer (pH 5.2). Assay of the enzyme activity was done by the BAEE method.[11] Fluorescent determination of trypsin activity was also done with EDAN labeled BSA, where the hydrolyzed low-molecular weight peptides containing EDAN were measured for the supernatant after 5 min boiling treatment and subsequent centrifugation (unpublished data).

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