The Interaction of *N*-Acetylhexosaminidase with Insolubilized Concanavalin A

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The specific interaction between human *N*-acetylhexosaminidase and concanavalin A was evaluated with respect to temperature, time, pH and concentration of specific ligand in incubation mixtures containing the enzyme and insolubilized lectin. Elution of the enzyme from insolubilized concanavalin A is dependent on both temperature and concentration of α -methyl mannoside. Conditions for a high yield of the enzyme from chromatography on insolubilized concanavalin A are described.

It has been suggested that chromatography with concanavalin A might be a generally useful procedure in the purification of lysosomal hydrolases (Phillips *et al.*, 1976). The binding of human *N*-acetylhexosaminidase, an enzyme which is deficient in Tay–Sachs disease (Okada & O'Brien, 1969), to concanavalin A has been reported (Hickman *et al.*, 1974). Cohen *et al.* (1976) have reported the partial purification of the human placental enzyme eluted from insolubilized concanavalin A with 0.1M- α -methyl mannoside at neutral pH; however, we are not aware of any detailed description of the interaction between human hexosaminidase and insolubilized concanavalin A.

In the present paper we describe the interaction between concanavalin A covalently bound to Sepharose and hexosaminidase from human tissues. We also describe conditions for the chromatography of hexosaminidase on concanavalin A–Sepharose which permit a high yield of the enzyme.

Materials and Methods

Human tissues were obtained either at surgery or at autopsy, transported to the laboratory in cold Joklik's tissue-culture medium (Grand Island Biological Co., Grand Island, NY, U.S.A.), stripped of connective tissue and fat, and stored at -20°C . Tissue was thawed in 0.9% NaCl containing 0.1% Triton X-100 (5 ml/g of tissue) and minced to 1–2 mm pieces with scissors. The minced tissue was homogenized for 15 min at 25000 rev./min in an Osterizer blender followed by sonication for 2 min with a Bronwill Biosonic IV apparatus at 80% of the low-energy setting. The resulting homogenate was centrifuged at 17000g for 10 min. The supernatant was assayed for

hexosaminidase activity by the spectrophotometric determination of *p*-nitrophenol released from *p*-nitrophenyl *N*-acetyl- β -D-glucosaminide (Sigma Chemical Co., St. Louis, MO, U.S.A.) or *p*-nitrophenyl *N*-acetyl- β -D-galactosaminide (Sigma). Assay mixtures contained, in addition to enzyme, 4 μmol of *p*-nitrophenyl *N*-acetyl- β -D-glucosaminide (or 50 nmol of *p*-nitrophenyl *N*-acetyl- β -D-galactosaminide) and 50 μmol of sodium citrate buffer (pH 4.5) in a total volume of 1 ml. The reaction was terminated by the addition of 1 ml of 0.4M-glycine buffer (adjusted to pH 10.5 with NaOH), and the amount of *p*-nitrophenol released from the substrate was determined spectrophotometrically at 410 nm. One unit of activity is defined as the release of 1 nmol of *p*-nitrophenol from *p*-nitrophenyl *N*-acetyl- β -D-glucosaminide/min at 37°C . Protein was determined by a modification of the method of Lowry (Hartree, 1972). The ratios of the A and B forms of hexosaminidase in the supernatants obtained from the various homogenates were determined by isoelectric focusing with Ampholine buffers (pH 3.5–10.0) in a 110 ml LKB apparatus according to the manufacturer's instructions.

The supernatants from homogenates of tissue (containing 60–90 units of hexosaminidase) were diluted 1:5 (v/v) with either 0.1M-sodium phosphate buffer (pH 7.0) or 0.1M-sodium citrate buffer (pH 4.5) and added to concanavalin A–Sepharose (3 mg of protein) previously equilibrated with the appropriate buffer [concanavalin A–Sepharose (10 mg of protein/ml) was obtained from Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A.]. These mixtures were incubated at various temperatures and the extent of the binding of hexosaminidase to concanavalin A–Sepharose was determined by the assay of a portion of the supernatant (properly diluted with the appropriate buffer) at various times during the

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incubation. Concanavalin A-Sepharose and the enzyme bound to it were recovered after centrifugation for 10 min at 97*g* and removal of the supernatant. After the addition of various concentrations of α -methyl mannoside in either phosphate or citrate buffer (the final volume was equal to that of the original supernatant), the elution of hexosaminidase from concanavalin A-Sepharose was monitored as described above. The decrease in volume resulting from the removal of samples of enzyme was insignificant compared with the volume of the original supernatant.

Results and Discussion

The tissue homogenates included in this study were human liver, normal colon, colonic carcinoma and metastases to the liver from a primary colonic carcinoma. Homogenates from both normal liver and colon were found to contain more hexosaminidase A than B activity (ratio of total activities of A/B = 3:2), but the homogenates from the two colonic carcinomas contained less hexosaminidase A than B activity (ratio of total activity of A/B = 2:3). The binding capacity of concanavalin A-Sepharose (in 0.1M-phosphate buffer, pH 7.0) was 80 units of liver hexosaminidase after overnight incubation of 100–200 units of the enzyme with 1 mg of insolubilized protein at 25°C. The binding capacity was essentially unaltered by incubation in 0.1M-citrate buffer, pH 4.5. Overnight incubation at 4°C resulted in a slight decrease (less than 8 units/mg of immobilized protein) in the binding capacity, whereas incubation at 37°C resulted in an increase of approx. 15% in the binding capacity at 25°C. The binding capacity of the insolubilized lectin was not found to depend on the tissue from which the enzyme was obtained.

All subsequent experiments were performed with excess concanavalin A-Sepharose (20–30 units of hexosaminidase added/mg of insolubilized protein) to allow for the complete binding of the enzyme. With increasing temperature, the rate of hexosaminidase binding by concanavalin A-Sepharose at pH 7.0 increased; however, after 7 h incubation little difference was observed in the amount of enzyme bound by concanavalin A-Sepharose incubated at the indicated temperatures (Fig. 1). When incubations were performed in citrate buffer (pH 4.5) at 37°C the rate of binding was more similar to that shown for incubation at 25°C in phosphate buffer, pH 7.0 (Fig. 1). The extent of binding observed after 7 h was not affected by incubation of concanavalin A-Sepharose and hexosaminidase at pH 4.5 or the tissue from which the enzyme was obtained.

The release of hexosaminidase from concanavalin A-Sepharose was dependent on the concentration of α -methyl mannoside used for elution (Fig. 2). A second elution of concanavalin A-bound enzyme at

Fig. 1. Binding of hexosaminidase from normal liver to concanavalin A-Sepharose at pH 7.0

The percentage of bound hexosaminidase is expressed as a function of time at 4°C (●), 25°C (○) and 37°C (▲). Details for the determination of bound enzyme are given in the Materials and Methods section.

Fig. 2. Release of hexosaminidase from concanavalin A-Sepharose as a function of the concentration of α -methyl mannoside used for elution

The enzyme was incubated with concanavalin A-Sepharose for 4 h at 25°C. Enzyme bound to concanavalin A-Sepharose was recovered by centrifugation and eluted from the lectin by the indicated concentrations of α -methyl mannoside in a volume equal to that of the original supernatant. After incubation at 37°C for 2 h, samples were removed from the supernatant containing the ligand, diluted with the appropriate buffer, and assayed as described in the Materials and Methods section. Although the presence of α -methyl mannoside inhibited hexosaminidase activity, the concentration of the ligand present in diluted samples was negligible with regard to inhibition.

4°C after an initial elution with 0.1M- or 0.5M- α -methyl mannoside yielded approx. 30 or 20% respectively, as much enzyme as was eluted in the

Fig. 3. Elution of hexosaminidase (from liver metastases) from concanavalin A-Sepharose at pH4.5

The percentage of hexosaminidase eluted is expressed as a function of time after the addition of 0.5M- α -methyl mannoside at 4°C (●), 25°C (○) and 37°C (▲). Details for the determination of enzyme are given in the Materials and Methods section.

Table 1. Effect of temperature on the elution of hexosaminidase from concanavalin A-Sepharose

Enzyme was permitted to bind to concanavalin A-Sepharose (50units/mg of protein) for 16h at 22°C. Enzyme bound to concanavalin A-Sepharose (40 units/mg of protein) was recovered by centrifugation and eluted by various concentrations of α -methyl mannoside (0.05–0.6M) at 4, 22 and 37°C. After incubation for 16h at the appropriate temperature the amount of enzyme eluted was determined as described for Fig. 2. The amounts of enzyme eluted at infinite concentration of α -methyl mannoside and *K* values were determined from plots of 1/(units of enzyme eluted/mol of concanavalin A) versus 1/[α -methyl mannoside] for each temperature as described in the text.

Temperature of elution (°C)	Hexosaminidase eluted (units/mg of concanavalin A) at infinite [α -methyl mannoside]	<i>K</i>
37	42	22.7
22	23	21.3
4	18	19.9

initial step. When α -methyl mannoside was added to concanavalin A-Sepharose at the same time as the addition of the enzyme, both concentrations of the ligand were equally effective in blocking the binding of the enzyme to the insolubilized lectin (greater than 95% inhibition relative to binding in the absence of the ligand).

The elution of hexosaminidase from concanavalin A-Sepharose at pH4.5 (Fig. 3) was more dependent on temperature than was the binding of enzyme (Fig.

1). Elution at 37°C was approximately twice as effective with respect to enzyme activity recovered as elution at 25°C, and four times as effective as elution at 4°C. Similar results were obtained at pH7.0, or when the source of enzyme was from tissues other than liver metastases. The temperature-dependence of the elution of another lysosomal enzyme, β -galactosidase, has been reported (Norden & O'Brien, 1974).

The temperature-dependence of the concanavalin A-hexosaminidase interaction was investigated in further detail by studying the effect of temperature on the elution of the enzyme at various concentrations of α -methyl mannoside (Table 1). If all enzyme-occupied binding sites on concanavalin A are considered identical, the amount of bound enzyme eluted as a result of the addition of α -methyl mannoside is representative of the extent of α -methyl mannoside binding, and the interactions between hexosaminidase and concanavalin A can be roughly approximated by methods utilized in the treatment of the binding of small molecules to proteins (Klotz, 1953). From data such as those shown in Fig. 2 one can plot 1/ $\bar{v}$ (where $\bar{v}$ = units of enzyme eluted/mol of concanavalin A) against 1/*C* (where *C* = concentration of α -methyl mannoside added). In this system the intercept at 1/*C* = 0 will be equal to 1/*n* (where *n* = amount of enzyme eluted at infinite α -methyl mannoside concentration) and the slope = 1/*nK*, with *K* approximating to the equilibrium constant for elution.

When bound enzyme was eluted from concanavalin A by various concentrations of α -methyl mannoside at 4, 22 and 37°C and the data were analysed by double-reciprocal plots as described above, it was found that both *K* for elution and the maximum amount of enzyme eluted increased with increasing temperature (Table 1). Straight lines were obtained from the double-reciprocal plots, indicating no interactions between concanavalin A-binding sites. The determination of *K* as a function of temperature permits the calculation of other thermodynamic parameters; however, it should be made clear that the absolute values obtained would not be accurate for the hexosaminidase-concanavalin A interaction, since the *K* values were obtained from an impure preparation of enzyme.

The data shown in Fig. 1 suggest that there is little, if any, significant temperature-dependence for the binding of hexosaminidase to concanavalin A. A glycoprotein such as hexosaminidase has multi-valent binding sites for concanavalin A. Therefore measurements of eluted and bound enzyme by determination of the amount of enzyme in free solution differ in one critical aspect. In the measurement of binding, one is essentially determining only the initial binding site; secondary binding is not measured. The reverse process, i.e. elution, cannot be detected

until the material on the secondary binding sites has been displaced by α -methyl mannoside. Consequently, at low concentrations of enzyme added to a constant amount of concanavalin A (in excess), we found no temperature-dependence for the equilibrium constant of binding (0–50 units of enzyme bound/mg of concanavalin A). Although a temperature-dependence was not observed at these low concentrations, it does not indicate that the extent of secondary binding of the enzyme to concanavalin A was not temperature-dependent. Elution, on the other hand, was temperature-dependent over the same range of bound enzyme (i.e. 40 units of enzyme from 1 mg of concanavalin A–Sephadex protein).

Others have reported the partial purification of hexosaminidase by using relatively low concentrations of α -methyl mannoside and low temperatures (Cohen *et al.*, 1976; Bishayee & Bachhawat, 1974). The recoveries were approx. 55% for the enzyme from human placenta (Cohen *et al.*, 1976) and 40% for hexosaminidase from sheep brain (Bishayee & Bachhawat, 1974). We obtained similarly decreased recoveries with similar conditions for the partial purification of liver hexosaminidase. The elution of the enzyme from concanavalin A with 0.1 M- α -methyl mannoside in 0.1 M-phosphate buffer (pH 7.0) at 4°C resulted in the recovery of 49% of the enzymic activity applied to the insolubilized lectin in a volume which was approx. 50 times that of the initial sample volume. Since the experiments described above (Figs. 2 and 3) suggested that better recovery of hexosaminidase could be obtained by elution at 37°C with 0.5 M- α -methyl mannoside, we partially purified the enzyme using these conditions. The partial purification of hexosaminidase in phosphate buffer (pH 7.0) and in citrate buffer (pH 4.5) is summarized in Table 2. Incubation (2 h) of the enzyme at 37°C in 0.1 M-citrate buffer (pH 4.5) before incubation with concanavalin A–Sephadex resulted in approx. a 5-fold increase in specific

activity. After chromatography on concanavalin A–Sephadex, 84% of the hexosaminidase activity added to the lectin was recovered with a 35-fold increase in specific activity relative to the crude extract (in a volume which was approx. 33% of that added to the concanavalin A–Sephadex). Purification in the same manner with 0.1 M-phosphate buffer (pH 7.0) resulted in a final purification of only 16-fold. This was probably due to the fact that incubation at 37°C, at neutral pH, before chromatography, was not as effective in precipitating contaminating protein as incubation at pH 4.5. Similar results were obtained with the enzyme obtained from liver metastases, except that the resulting specific activity and recovery were 60 and 83% of those obtained for the enzyme from normal liver partially purified at pH 4.5.

A wide variety of conditions with respect to pH, temperature and ligands for elution have been used in the chromatography of glycoproteins on insolubilized concanavalin A (Norden & O'Brien, 1974; Bishayee & Bachhawat, 1974; Brattain *et al.*, 1975, 1976a,b; Carrico & Usategui-Gomez, 1975; Harvey & Chu, 1975; Cohen *et al.*, 1976; Davey *et al.*, 1976). The present report shows the feasibility of chromatography on concanavalin A–Sephadex at 37°C and at relatively acidic pH. The ability to perform chromatography of hexosaminidase on concanavalin A–Sephadex at pH 4.5 allows for the partial purification of the enzyme, before incubation with the lectin, without any additional manipulations of the enzyme, such as freeze-drying or dialysis. It has been shown that hexosaminidase is most stable around pH 4.5 (Robinson & Stirling, 1968). Elution at 37°C with 0.5 M- α -methyl mannoside permits greater recovery of the enzyme from concanavalin A–Sephadex than previously reported (Cohen *et al.*, 1976; Bishayee & Bachhawat, 1974). Although the slightly lower specific activity and recovery obtained from the partial purification of hexosaminidase from liver

Table 2. *Partial purification of hexosaminidase on concanavalin A–Sephadex after incubation at 37°C at pH 7.0 or pH 4.5*
Crude extracts were diluted with phosphate (pH 7.0) or citrate (pH 4.5) buffer (final volume 20 ml) and incubated for 2 h at 37°C. The resulting supernatants (20 ml) were allowed to bind to concanavalin A–Sephadex (3 ml) for 16 h at 25°C. The concanavalin A–Sephadex and supernatant were quantitatively transferred to a column 1 cm in diameter; recovery of the supernatant was followed by washing the concanavalin A–Sephadex with 20 ml of the phosphate or citrate buffer. The enzyme was eluted by 0.5 M- α -methyl mannoside in phosphate or citrate buffer at a flow rate of 0.5 ml/min.

Treatment	Recovery (%)	Specific activity (units/mg of protein)	Purification (fold)
A. Sample in 0.1 M-phosphate buffer, pH 7.0	100	5.6	—
(1) 2 h incubation at 37°C	94	9.7	1.7
(2) Chromatography on concanavalin A–Sephadex	63	90.0	16.0
B. Sample in 0.1 M-citrate buffer, pH 4.5	100	5.6	—
(1) 2 h incubation at 37°C	91	27.0	4.9
(2) Chromatography on concanavalin A–Sephadex	76	195.0	35.0

metastases might reflect the different ratios of iso-enzymes in this tissue, we were unable to obtain separation of the A and B forms of hexosaminidase by chromatography on concanavalin A-Sepharose. The lower specific activity and recovery might also have been the result of the enzyme constituting a smaller proportion of the concanavalin A-binding material in liver metastases than in normal liver.

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