0273-2289/92/32(1/3)—0127$02.00

Rapid Determination of Immobilized ConA Lectin Activity

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Received November 5, 1991; Accepted November 17, 1991

ABSTRACT

ConA was immobilized on an epoxy-activated copolymer of 2-hydroxyethyl-methacrylate and ethylene-dimethacrylate and commercially available high-pressure liquid chromatography (HPLC) sorbents Separon HEMA 1000 EL, Separon HEMA 1000 E, and Separon HEMA 1000 EH (Tessek, Prague, CSFR Denmark). Specific, sensitive, and rapid method for determination of immobilized ConA lectin activity was developed. β-Galactosidase from *Aspergilus oryzae* oligomannosyl residues was used as specific affinant. After separation of bound and unbound β-galactosidase, enzyme activity was measured in supernatant and thus immobilized ConA lectin activity was calculated easily. The use of the method for evaluating the properties of immobilized ConA, efficiency of immobilization, specific activity, and thermostability is shown. The method developed could be generalized by using artificially glycosylated enzyme for any lectin.

Index Entries: Lectin; ConA; immobilization; β-galactosidase; HPLC.

INTRODUCTION

Lectins are sugar-binding proteins that agglutinate cells or precipitate glycoconjugates (*1*). Each lectin binds specifically to a certain sugar sequence in oligosaccharides and glycoconjugates. The wide-spread interest

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on lectins is attributed to the possibility of using them as tools in the study of cell architecture, malignant transformation, clinical diagnosis, isolation, characterization of glycoconjugates, and so on (2). For analytical and preparative purposes, it is important to know how the immobilized lectin activity is affected by the immobilization process, the stability of the immobilized lectin in time, and its temperature dependence.

Although the activity of immobilized enzymes can be simply determined by conversion of substrate to the product, precise determination of lectin activity is much more difficult. The common test for detecting the presence of a lectin in a given extract is hemagglutination assay (3). The assay based on specific binding of the radiolabeled high-mannose-type oligosaccharide to immobilized ConA lectin was developed for glycosidases determination (4,5). Modified, immobilized lectin column chromatography could be used for immobilized lectin activity evaluation. However, this procedure is handicapped by the fact that it cannot be used for a large number of samples and is time consuming.

The aim of our work was to describe a simple, rapid method for the determination of immobilized ConA or other mannozo-specific lectin activity by use of oligomannozyl β-galactosidase *Aspergilus oryzae* residues as affinants and the enzyme activity as an amplifier of the binding signal.

MATERIALS AND METHODS

Chemicals

ConA-Sepharose was obtained from Pharmacia (Uppsala, Sweden). Concentration of ConA on the sorbent is declared by the producer as 10 mg/mL of the wet gel. As a control gel, we used Sepharose 4B (Pharmacia). Separon HEMA 1000 EL, Separon HEMA 1000 E, and Separon HEMA 1000 EH (epoxy-activated HPLC sorbents, 10 μm in three degrees of activation), and EL = 100–200, E = 700–900, and EH > 1400 μeq, epoxy groups per g of dry sorbent, were kindly given by TESSEK. β-Galactosidase (β-*D*-galactosidase galactohydrolase, EC 3.2.1.23.) from *Aspergilus oryzae* (mol wt cca 100,000, 12–14 U/mg) and *p*-nitrofenyl-β-*D*-galactopyranoside were purchased from Serva (Heidelberg, Germany). ConA was purchased from the Laboratory for Producing and Control of Lectin Preparatives (Charles University Prague, CSFR). Other chemicals of pa quality were obtained from Lachema (CSFR).

Methods

ConA immobilization was performed in 0.1*M* carbonate buffer pH 9.5 containing 1*M* NaCl, 1 m*M* $CaCl_2$, 1 m*M* $MgCl_2$, and 0.02% natrium azide (buffer A). For immobilized ConA determination, 0.1*M* acetate buffer pH 6.0 (buffer B) containing the same salts as a buffer A was used. Immobili-

zation was performed according to the method recommended by the producer and modified as follows. Epoxy-activated sorbent (1 g) was washed on a sintered glass filter. Gel suspension containing 1 g of the gel was added to 5 mL ConA solution (1 mg/mL) in buffer A. Immobilization was carried out at room temperature for 12 h using shaker. Samples of 200 μL of the suspension were taken out at different time intervals of the immobilization and centrifuged at 3000 rpm for 10 min. Protein concentration in the supernatant was determined by the method of Sedmak and Grosberg (1977) as well as by measuring absorption at 280 nm (*6*). Unreacted epoxy groups were blocked with 10 μL 0.1*M* ethanolamine in 0.1*M* borate buffer pH 9.0 overnight. Finally, the suspension was washed with buffer B.

Determination of Immobilized Lectin Activity

A solution of β-galactosidase (0.05 mg/mL) in buffer B was mixed with gel of immobilized ConA (ConA-Sepharose and ConA-Separon HEMA 1000 EH) and incubated in a shaker for 2 h at 37°C. The mixture was centrifuged at 3000 rpm for 10 min. The activity of β-galactosidase was measured in supernatant (*F*) at appropriate time intervals. ConA-Sepharose (Pharmacia) was used for comparison. The binding of β-galactosidase (B = 100 –F) expressed in percentages reflects lectin activity of immobilized ConA.

Determination of β-galactosidase Activity

Supernatant with β-galactosidase (100 μL) and 0.5 mL of substrate solution (4-nitrofenyl-β-*D*-galactopyranoside, 6 mg/5 mL in buffer B) were incubated for 30 min at room temperature. The reaction was stopped by a 2 mL Na_2CO_3 solution (4% w/v in water), and the absorbance of the liberated *p*-nitrophenol was measured immediately at 405 nm.

Thermostability

Thermostability of *Immobilized ConA* was studied at 30, 50, and 70°C during 24 h on ConA-Separon HEMA 1000 EH. The gel suspension (4 mL) was incubated in water bath and shaked. At appropriate time intervals, 100 μL of the suspension was taken out, and the activity of immobilized ConA was determined by the method described above.

RESULTS AND DISCUSSION

Various methods for the coupling of proteins to insoluble sorbents can be used for preparing immobilized lectins. However, since some lectins are unstable at coupling conditions, careful examination of these conditions is always necessary to know actual lectin binding properties. The derivatives prepared by the epoxy method using bisoxirane (*7*) or epichlorhydrin (*8*) are free of charge (*9*) and quite stable under alkaline conditions.

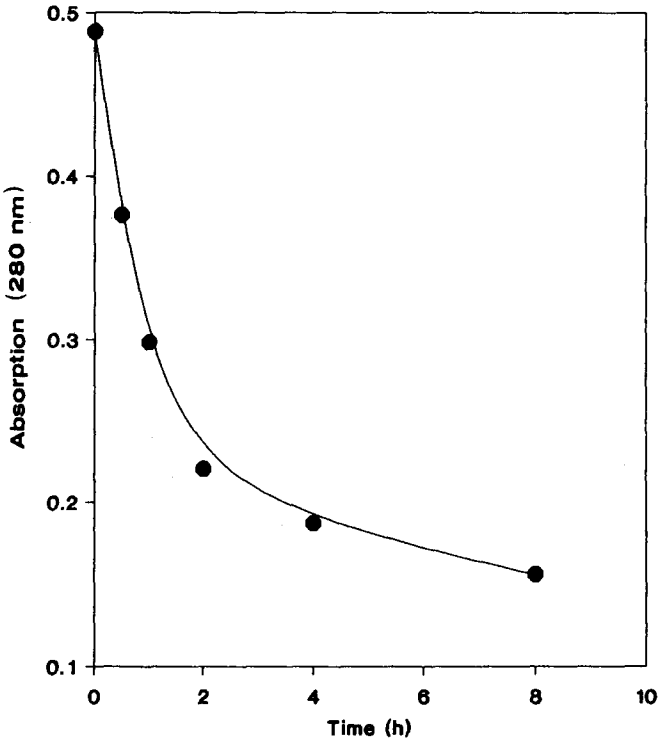

Fig. 1. Time dependence of ConA immobilization to Separon HEMA 1000 EH. Sorbent (1 g) was washed on a sintered glass filter. The gel suspension containing 1 g of the gel was mixed with 5 mL ConA solution (1 mg/mL) in buffer A. Samples (200 μL) were centrifuged at 3000 rpm for 10 min, and absorbance of the undiluted supernatant was measured at 280 nm.

Furthermore, the macroporous structure and hydrophylic nature of the copolymer of 2-hydroxyethyl-methacrylate and ethylene-dimethacrylate is suitable to prepare powerful affinity matrices for chromatography of biomolecules. Immobilization was performed according to the method recommended by the producer. Protein concentration during immobilization was evaluated by the method of Sedmak and Grosberg (1977) as well as by measuring of absorption at 280 nm (*6*). Comparable results were obtained by both methods. Thus, only the last method was applied in further experiments. Approximately the same time dependence was recorded for three different sorbents in three different degrees of activation (Separon HEMA 1000 EL, E, EH). The result for Separon HEMA EH is shown in Fig. 1.

Determination of the lectin activity of immobilized ConA was based on ConA affinity to oligomannosyl residues as a natural glycosidic part of β-galactosidase from *Aspergilus oryzae* described by Nakao et al. (1987). The authors showed that carbohydrate parts of β-galactosidase were composed of two types of sugar chains. The long sugar chains were composed

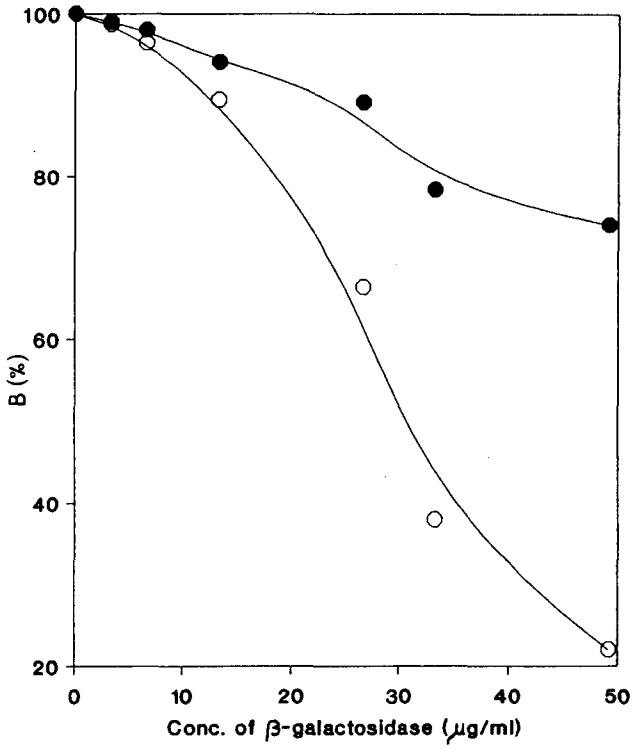

Fig. 2. Activity of β-galactosidase (B) bound to ConA-Sepharose (-●-●-) and ConA-Separon HEMA 1000 EH (-○-○-). Constant amounts of gels (5 μL) were mixed with increased amounts of β-galactosidase (0–200 μL), and buffer B was added to a constant vol of the mixture (300 μL). The mixture was incubated for 1 h at 37°C; then immobilized ConA activity was determined.

of galactomannan-type oligosaccharides. The short sugar chains of the second fraction corresponding to 96% of the total carbohydrate chains are a high mannose type oligosaccharides with about 91% of the structure ManGlcNAc. Therefore, a strong affinity of β-galactosidase to ConA-Separon is expected.

The lectin activity of immobilized ConA to Separon HEMA 1000 EH was determined and compared with that of ConA-Sepharose using two approaches. First, a constant amount of gels (5μL), an increased amount of β-galactosidase (0–200 μL) and a decreased amount of buffer B to constant vol of the mixture (300 μL) were used (Fig. 2). Then immobilized ConA lectin activity was determined as previously described. In a second experiment, a constant amount of β-galactosidase (200 μL) and an increased amount of the gels (0–100 μL) were added; immobilized ConA lectin activity was determined as described above (Fig. 3). As shown in the figures, at the same amount of gels, ConA-Separon HEMA 1000 EH showed approx 3–4 times lower activity of immobilized ConA than ConA-Sepharose.

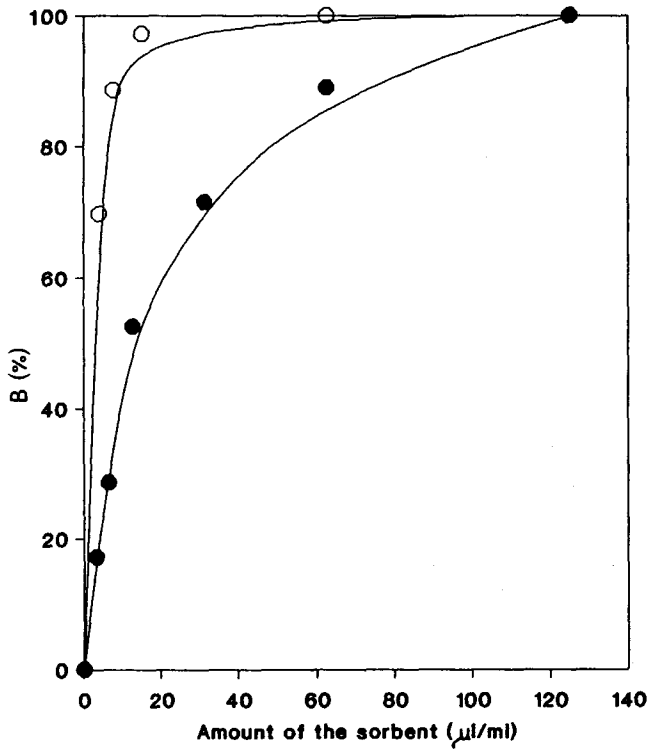

Fig. 3. Dependence of β-galactosidase activity bound (B) to immobilized ConA at various concentrations of ConA-Sepharose (-●-●-) and ConA-Separon HEMA 1000 EH (-○-○-) (0–100 μL). A constant amount of β-galactosidase (200 μL) and increased amounts of gels (0–100 μL) were mixed, and immobilized ConA determined after incubation at 37°C for 1 h.

Further, the effect of ionic strength and storage on the activity and stability of the immobilized ConA were studied. One part of gel Separon HEMA 1000 EH with immobolized ConA was washed by 0.02*M* TRIS buffer pH 7.4 with 0.15*M* NaCl and 0.02% sodium azide; the change of the immobilized ConA activity was measured immediately and compared with that of the acetate buffer containing 1*M* NaCl. Both gels were stored at room temperature for 1 mo and the change of immobilized ConA activity was determined. No detectable change was observed when the gel was stored in buffer B (high ionic strength) for 1 mo (not shown). Small changes were observed in immobilized ConA activity of the gel in TRIS buffer of low ionic strength after rewash and storage. Results are shown in Fig. 4. Thermal stability of immobilized ConA was measured at 37, 50, and 70°C for 24 h. Linear relationship between decreasing immobilized ConA activity and time was detected in semilogarithmic scale (Fig. 5).

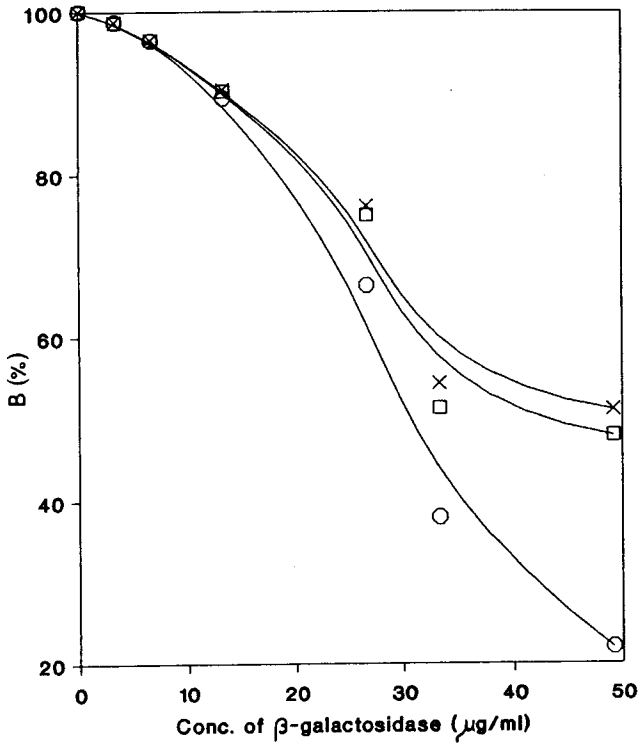

Fig. 4. Effect of ionic strength and storage on immobilized ConA activity. One part of gel in buffer B with immobilized ConA was washed with 0.02*M* TRIS pH 7.4 including 0.15*M* NaCl and 0.02% sodium azide, and the change of immobilized ConA activity (-□-□-) was compared with that of the acetate buffer B containing 1*M* NaCl (-○-○-). Both gels were incubated in the dark at room temperature for 1 mo, and the change in immobilized ConA activity was determined. No detectable change was observed when the gel was stored in buffer B (high ionic strength) for 1 mo (not shown). Small changes were observed in immobilized ConA lectin activity of the gel in TRIS of low ionic strength after storage (-×-×-).

The method presented in this study seems to be very useful for specific, sensitive, and rapid determination of immobilized ConA activity. The method developed can be generalized by using artificially glycosylated enzyme for any natural lectins.

ACKNOWLEDGMENTS

The authors wish to thank Ing. Ivan Vinš CSc. for the gift of carriers.

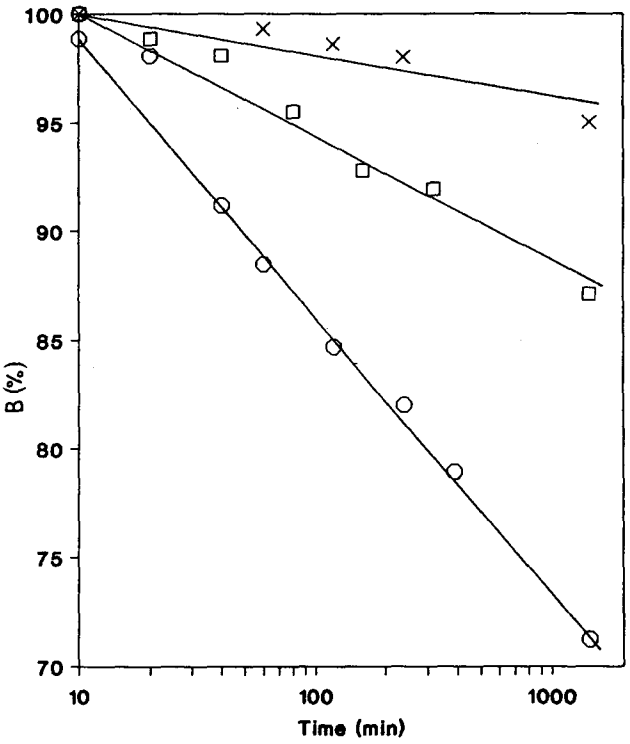

Fig. 5. Thermal stability of immobilized ConA activity on ConA-Separon HEMA 1000 EH at 30 (-×-×-), 50 (-□-□-), and 70°C (-○-○-). The gel suspension (4 mL) was incubated in thermostat water bath and gently shaked. At appropriate time intervals, 100 μL of the suspension was taken out, and the activity of immobilized ConA was determined.

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