A RAPID PROCEDURE FOR DERIVATIZING AGAROSE WITH A VARIETY OF CARBOHYDRATES: ITS USE FOR AFFINITY CHROMATOGRAPHY OF LECTINS

Robert BLOCH and Max M. BURGER

Biochemistry Department, Biocenter of the University of Basel, Klingelbergstrasse 70, CH 4056 Basel, Switzerland

Received 22 May 1974

1. Introduction

The purification of lectins [1] by affinity chromatography [2–7] has been limited by difficulties in derivatizing sugars for covalent attachment to cyanogen bromide-activated agarose [8,9]. We present here a rapid method for derivatizing agarose with a variety of sugars by reduction of their *p*-nitrophenyl glycosides and subsequent coupling with activated resin. The derivatives obtained have a high capacity for lectins of the appropriate specificity and can be used to purify agglutinins from crude preparations. As many such glycosides are commercially available, this method should be generally useful in studies of carbohydrate-specific proteins.

2. Materials and methods

The *p*-nitrophenyl derivatives of α-L-fucose, α-D-mannose, and *N*-acetyl-β-D-galactosamine were obtained from Sigma; those of β-D-galactose and *N*-acetyl-β-D-glucosamine were from Serva. L-Fucose, α-methyl-D-glucoside and lactose were purchased from Merck. Wheat germ agglutinin [10], chitin hydrolyzate [10] and the agglutination assay [11] have been described previously. Concanavalin A was purified by the method of Agrawal and Goldstein [12]. Ammonium sulfate precipitates from extracts of *Lotus tetragonolobus* (Haubensack, 4103 Bottmingen, Switzerland) and untoasted soybean flour (Central Soya, Chicago) were prepared according to Blumberg et al. [5] and Gordon et al. [6], respectively. Protein was quentitated from the absorbance at 280 nm. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate [13] followed the method of Weber and Osborn [14]. Quantitation of the gels was done by densitometry with a Joyce-Loebl Chromoscan.

Coupling of sugars to agarose was as follows. 10 mg of the *p*-nitrophenyl glycoside was dissolved in 5 ml 0.5 M $NaHCO_3$ containing 0.1 M $Na_2S_2O_4$ and mixed vigorously for two or more hours at room temperature. Control samples subjected to alkaline hydrolysis after this treatment showed no yellow color, indicating complete reduction of the *p*-nitro group. 5 ml Sepharose 4B (Pharmacia) was activated with 1.25 g CNBr according to Cuatrecasas [9]. The moist resin was then scraped into the tube containing the glycoside; the coupling proceeded overnight with vigorous shaking at 4°C. For the control, the resin was treated identically but reacted in the absence of glycoside. The resins were then poured into columns and washed extensively with 0.9% saline or phosphate-buffered saline. Coupled resins were stored and used at 4°C.

The amount of *p*-aminophenyl glycoside coupled to the resin was measured indirectly by determining the amount of material left in solution after the coupling reaction. To 0.2 ml of the supernatant 1.8 ml 1% Na_2CO_3 were added and incubation was performed at 37°C until the control sample with no glycoside reached an optical density of less than 0.02 at 292 nm (30–60 min). This treatment destroyed the remaining dithionite, which would otherwise interfere with absorbance measurements. The optical density of the solution at 292 nm was determined and converted into concentration using a molar extinction coefficient of 1.31×10^3. This value was obtained by reducing known amounts of the glycosides used, as described above. The final concentration of ligand was between 0.7 and 1.4 μmole/ml moist Sepharose. Amino acid analysis

North-Holland Publishing Company – Amsterdam

[15] after acid hydrolysis (5 N HCl, 4 hr, 105°C) of the resin coupled with *N*-acetyl-glucosamine gave similar results (0.5–0.7 μmole/ml compared to 0.7 μmole/ml from absorbance measurements). The capacity of the coupled resin for the appropriate agglutinin was determined for the α-mannoside and *N*-acetyl-β-D-glucosaminide derivatives using concanavalin A (Con A) and wheat germ agglutinin (WGA) respectively, and a method already described [10]. Elution of the bound lectin by hapten and quantitation of protein [16,17] indicated that the resins bind about 3 mg Con A/ml and 8 mg WGA/ml. This corresponds to 0.04 mole Con A/mole attached mannoside and 0.3 mole WGA/mole attached *N*-acetyl-glucosaminide.

3. Results and discussion

Our agarose derivatives are capable of binding pure lectins stereospecifically. Con A binds quantitatively to a column of Sepharose-O-(*p*-aminophenyl)-α-D-mannoside and is eluted by its hapten, α-methyl-glucoside (fig. 1A). It does not bind to Sepharose coupled with *p*-aminophenyl-β-D-galactoside (fig. 1A) or to activated but underivatized resin (not shown). Recovery of Con A from the mannoside column after elution with hapten is complete. WGA also binds quantitatively to the appropriate resin, Sepharose-2-acetamido-O-(*p*-aminophenyl)-2-deoxy-β-D-glucoside, and is eluted with a 1% (w/v) solution of chitin hydrolyzate (fig. 1B). 0.1 M *N*-acetylglucosamine does not readily elute bound WGA. Unlike Con A, however, WGA weakly interacts with β-galactoside–Sepharose (fig. 1B) and with activated but underivatized resin (not shown); this interaction is inhibited by chitin hydrolyzate. About 75% of the WGA applied to the columns can be recovered, as previously found with other ligand matrices [2,10]. The fact that both Con A and WGA bind quantitatively and reversibly to resin coupled with their respective haptens shows that the derivatization procedure can yield good matrices for affinity chromatography of lectins. We have therefore examined its use for the purification of two agglutinins.

When a sample obtained from a soya extract is applied to a column of Sepharose-2-acetamido-O-(*p*-aminophenyl)-2-deoxy-β-D-galactoside, most of the material absorbing light at 280 nm fails to bind. In contrast, most or all of the agglutinating activity in the

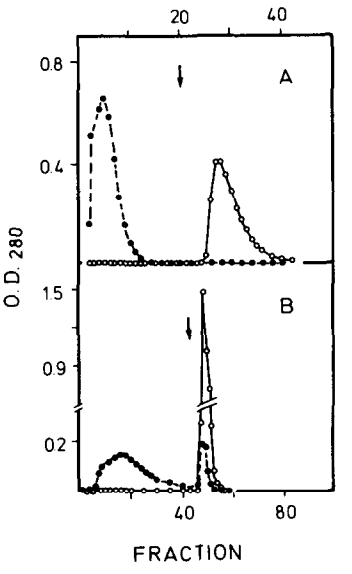

Fig. 1. Chromatography of pure lectins on Sepharose coupled with hapten (–○–) or with β-D-galactoside (–●–). A. Con A; the hapten is α-D-mannoside, and elution, was effected with 0.2 M α-methyl-D-glucoside. B. WGA; the hapten is *N*-acetyl-β-D-glucosaminide, and elution was with 1% chitin hydrolyzate. The buffer was phosphate-buffered saline, pH 7.4. 0.4–0.6 ml fractions were collected from 1.3 ml columns. The arrow indicates the addition of the sugar solution.

extract is retained by the column and is eluted upon addition of 0.2 M lactose (fig. 2A). The agglutinin is purified approximately 15-fold, as judged by recovery of optical density. Examination of the purified product by gel electrophoresis in the presence of sodium dodecyl sulfate [13,14] shows that its main (> 90%) constituent is a polypeptide chain with an apparent mol. wt. of 3.0×10^4, in agreement with a previous report [1]. Similarly, a crude preparation of the fucoside-binding lectins from *Lotus tetragonolobus* was purified 8-fold using Sepharose-O-(*p*-aminophenyl)-α-L-fucoside as the chromatographic matrix (fig. 2B). The product is composed largely (> 80%) of a single polypeptide chain with an apparent mol. wt. of 2.8×10^4, in good agreement with previous reports of one binding site for L-fucose per $2.8–3.2 \times 10^4$ [5,18,19].

The potential usefulness of the *p*-aminophenyl glycosides for lectin purification has previously been suggested by the effectiveness of the fucoside derivative in the preparation by immunoprecipitation of agglu-

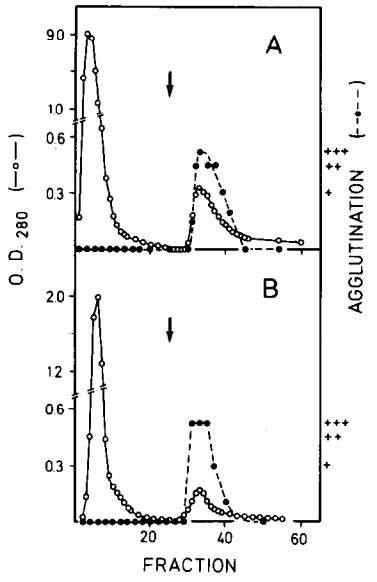

Fig. 2. Affinity chromatography of crude lectin preparations. A. Soybean agglutinin: a 47–80% ammonium sulfate fraction of soybean extract was dialyzed against 0.9% NaCl and a sample was applied to a column containing Sepharose coupled with *N*-acetyl-β-D-galactosaminide. Elution was with 0.2 M lactose in saline. Agglutination assays of 0.05 ml samples were performed on type A+ erythrocytes, B. *Lotus tetragonolobus* agglutinin: a 30–60% ammonium sulfate fraction of *Lotus tetragonolobus* extract was dialyzed against 0.9% NaCl and a sample was applied to a column containing Sepharose coupled with α-L-fucoside. Elution was with 30 mM L-fucose in saline. Agglutination assays of 0.025 ml samples were performed on type O+ erythrocytes. Agglutination tests were performed after removal of sugar by dialysis. The column size was 11 × 0.9 cm, and 2 ml fractions were collected. The arrow indicates the addition of the sugar solution.

tinins from *Lotus tetragonolobus* [19]. The results reported above indicate that the products of the coupling of four different *p*-aminophenyl glycosides to CNBr-activated Sepharose are stereospecific matrices of high capacity for affinity chromatography of lectins. It remains to be determined, however, whether our present method can be used without modification to purify other sugar binding proteins. β-Galactosidase, for example, will not bind to a similar agarose derivative unless a long arm is used to increase the distance between the galactoside and the agarose backbone [20]. Although Sepharose derivatized with *p*-aminophenyl-α-galactoside fails to retain agglutinin from extracts of *Bandeira simplicifica* seeds [7], our results with the *p*-aminophenyl glycosides suggest that similar adaptations [20] may not be necessary for affinity chromatography of many lectins. They are certainly not necessary for studies of the role of carbohydrates in cell surfaces, which are now in progress.

Acknowledgement

We thank our colleagues for their valuable suggestions during the preparation of the manuscript, Pamela Turner for the crude lectin fractions, and Dr. T. Morimasa for the amino acid analyses. This work was supported by a National Institutes of Health postdoctoral fellowship to R.B. (No. 1-Fo2-GM-55, 402-01) and by a grant to M.M.B. from the Swiss National Foundation (No. 3.1330.73).

References

[1] Lis, H. and Sharon, N. (1973) Ann. Rev. Biochem. 42, 541–571.
[2] Lotan, R., Gussin, A. E. S., Lis, H. and Sharon, N. (1973) Biochem. Biophys. Res. Commun. 52, 656–662.
[3] Shaper, J. H., Barker, R. and Hill, R. L. (1973) Anal. Biochem. 53, 564–570.
[4] Rafestin, M. E., Obrenovitch, A., Oblin, A. and Monsigny, M. (1974) FEBS Letters 40, 62–66.
[5] Blumberg, S., Hildersheim, J., Yariv, J. and Wilson, K. J. (1972) Biochim. Biophys. Acta 264, 171–176.
[6] Gordon, J. A., Blumberg, S., Lis, H. and Sharon, N. (1972) FEBS Letters 24, 193–195.
[7] Hayes, C. E. and Goldstein, I. J. (1974) J. Biol. Chem. 249, 1904–1914.
[8] Axen, R., Porath, J. and Ernback, S. (1967) Nature 214, 1302–1304.
[9] Cuatrecasas, P. (1970) J. Biol. Chem. 245, 3059–3065.
[10] Bloch, R. and Burger, M. M. (1974) Biochem. Biophys. Res. Commun., in press.
[11] Nagata, Y. and Burger, M. M. (1972) J. Biol. Chem. 247, 2248–2250.
[12] Agrawal, B. B. L. and Goldstein, I. J. (1967) Biochim. Biophys. Acta 147, 262–271.
[13] Shapiro, A. L., Vinuela, E. and Maizel, J. V. (1967) Biochem. Biophys. Res. Commun. 28, 815–820.
[14] Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406–4412.
[15] Walborg, E. F., Jr., Cobb, B. F., III, Adams-Mayne, M. and Ward, D. N. (1963) Anal. Biochem. 6, 367–373.
[16] Yariv, J., Kalb, A. J. and Levitski, A. (1968) Biochim. Biophys. Acta 165, 303–305.

[17] Nagata, Y. and Burger, M. M. (1974) J. Biol. Chem. 249, in press.
[18] Kalb, A. J. (1968) Biochim. Biophys. Acta 168, 532–536.
[19] Yariv, J., Kalb, A. J. and Katchalski, E. (1967) Nature 215, 890–891.
[20] Steers, E., Jr., Cuatrecasas, P. and Pollard, H. B. (1971) J. Biol. Chem. 246, 196–200.