A Rapid and Sensitive Assay for Detection of Nanogram Quantities of Castor-Bean (*Ricinus communis*) Lectins

By Mridul GHOSH, Bimal K. BACHHAWAT and Avadhesh SUROLIA
Indian Institute of Experimental Medicine, Calcutta-700032, India

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Inhibition of lysozyme conjugated with *p*-aminophenyl β -D-galactopyranoside by galactose-specific lectins from castor beans (*Ricinus communis*) has been utilized for assaying these lectins in the nanogram range.

Lectins, carbohydrate-binding proteins, have found widespread use in analytical biochemistry, cell and molecular biology (Lis & Sharon, 1973; Rapin & Burger, 1974). Because of widespread usage of lectins, a quantitative assay for them has been sought. The most commonly used assay to quantify lectin involves agglutination of erythrocytes. Though the agglutination assay of lectins is rapid, it is at best only semiquantitative and subjective (Lis & Sharon, 1973; Rapin & Burger, 1974). The presence of a variety of carbohydrate determinants on the cell surface and more than one lectin differing in sugar specificity in the same seed extract may further complicate their quantification by the agglutination assay (Lis & Sharon, 1973; Matsumoto & Osawa, 1969, 1970). In addition, for many lectins either the cells have to be treated with enzymes such as trypsin or sialidase, or cells from different animal species have to be tested for their sensitivity to a particular lectin (Lis & Sharon, 1973; Rapin & Burger, 1974). A desirable assay for lectins should not only be rapid and quantitative but also be highly specific and capable of measuring minute quantities of lectins in crude extracts as well as in the purified preparations. The present paper reports a novel and quantitative assay for lectins, a procedure that depends exclusively on the sugar specificity of lectins together with advantages inherent in an enzyme-multiplied technique used for immunoassay of drugs (Rubenstein *et al.*, 1972; Rowley *et al.*, 1975). In the present method, lectin-specific sugar is conjugated to the lysozyme near its active site. When the sugar-substituted lysozyme is incubated with the lectin, the enzyme is inhibited, owing to binding of lectin to the sugar. The inhibition of sugar-substituted-enzyme activity occurs presumably because of steric hindrance to the enzyme-substrate interaction, and the inhibition is proportional to the concentration of lectin used. Lysozyme appeared to be an ideal enzyme, since its natural substrate, bacterial peptidoglycan, is a high polymer that should be amenable

to steric effects, and, also, the lysine residue (97) near the active site (Phillips, 1966) can be modified without seriously impairing the enzyme activity (Spector & Parker, 1970). The assay can detect as little as 60 ng of binding proteins and can, in principle, be applied for all lectins.

Materials and Methods

Hen's-egg-white lysozyme (EC 3.2.1.17; 50000 units/mg), dried cells of *Micrococcus luteus*, *p*-nitrophenyl β -D-galactopyranoside and dimethyl suberimide were obtained from Sigma. All the other chemicals used were of analytical grade. Both univalent and bivalent lectins from castor bean (*Ricinus communis*) (Podder *et al.*, 1974; Olsnes *et al.*, 1974) were prepared as described by Appukuttan *et al.* (1977). *Bandeiraea simplicifolia* lectin was a gift from Professor I. J. Goldstein, University of Michigan at Ann Arbor, MI, U.S.A.

Enzyme unit

A unit of enzyme was that amount causing a decrease in A_{450} of 0.001/min at 25°C at pH 6.24.

Determination of neutral sugar and proteins

Sugar determinations were carried out as described by Dubois *et al.* (1965). Protein was determined by A_{280} ; a molar absorption coefficient of 35000 litre·mol⁻¹·cm⁻¹ for lysozyme was used throughout the experiment (Chipman *et al.*, 1967). Concentrations of lectins were determined from the specific absorption coefficient at 280 nm, by using $A_{1\%}^{1\text{cm}}$ values of 14 for castor-bean lectin I (Podder *et al.*, 1974) and 11.8 for castor-bean lectin II (ricin) (Olsnes *et al.*, 1974).

Conversion of *p*-nitrophenyl β -D-galactopyranoside into *p*-aminophenyl β -D-galactopyranoside

Reduction of *p*-nitrophenyl β -D-galactopyranoside to *p*-aminophenyl β -D-galactopyranoside was carried

out as described by Bloch & Burger (1974). *p*-Nitrophenyl β -D-galactopyranoside (20mg) was dissolved in 10ml of 0.5M-NaHCO₃ containing 0.1M-Na₂S₂O₃ and stirred vigorously for 3h at room temperature. It was then dried under vacuum and extracted thrice with 10ml of methanol each time. The total methanol extracts were then dried under vacuum. Reduction of the nitro group to an amino group and the purity of the *p*-aminophenyl β -D-galactopyranoside was ascertained by the ratio of amino groups to the neutral sugar determined by diazotization and neutral-sugar (Dubois *et al.*, 1965) measurements respectively. Moreover, an *R_F* value of 0.305 was obtained for *p*-aminophenyl β -D-galactopyranoside, in contrast with a value of 0.528 for *p*-nitrophenyl β -D-galactopyranoside when t.l.c. with chloroform/methanol (4:1, v/v) as solvent was carried out and the spots detected by exposure to I₂ vapour.

Coupling of *p*-aminophenyl β -D-galactopyranoside to lysozyme

Lysozyme (7.5mg) was mixed with 10mg of *p*-aminophenyl β -D-galactopyranoside (contained in 1ml of solution) and 40mg of dimethyl suberimidate in 1.5ml of 0.2M-triethanolamine/HCl buffer, pH 8.5, for 2.5h at room temperature. Uncoupled glycoside and suberimidate were removed by dialysis against 0.2M-triethanolamine/HCl buffer, pH 8.5, for 4–5h. To minimize the hydrolysis of the sugar-lysozyme conjugate, it was kept in the above buffer before the enzyme assay. The sugar-substituted lysozyme has been designated as ' β -D-galactopyranosyl-lysozyme'.

Enzyme activity

The enzyme was assayed as described by Shugar (1952) in which the rate of decrease in *A*₄₅₀ due to the lysis of *M. luteus* was measured in a total volume of 1.5ml, with potassium phosphate buffer, pH 6.2 (0.1M final concn.). Initially the activity of β -D-galactopyranosyl-lysozyme was determined to obtain an enzyme-concentration curve. The effect of lectin concentration on the activity of sugar-substituted lysozymes was monitored by preincubating the enzyme with various concentrations of lectin in a total volume of 0.2ml for 15min before assaying its activity. For a demonstration of the sugar-specific inhibition of enzyme activity by the lectin, the lectin was inactivated by preincubating it for 10min with 0.3M-lactose in a total volume of 0.15ml and then with the enzyme for 15min before the assay of enzyme activity. For determining the amount of lectin in crude extract, preincubation of the substituted enzyme with crude extract was carried out for 15min before assaying its activity. In addition, known amounts of lectin (80, 120 and 880ng) were added in the crude extract samples as internal standards, and the enzyme assayed as described above.

Results and Discussion

Lysozyme conjugated with *p*-aminophenyl β -D-galactopyranoside with dimethyl suberimidate retained all of its activity. This was not surprising, since amidination preserves the charge of lysine residues that are essential for the activity of lysozyme when *M. luteus* cells are used as substrate (Davies & Neuberger, 1969). On determination of the neutral-sugar-to-protein ratio, it was observed that six amino groups of lysozyme were substituted with *p*-aminophenyl β -D-galactopyranoside. In the absence of lectin, β -D-galactopyranosyl-lysozyme activity was proportional to enzyme concentration (Fig. 1). However, when an excess of sugar-specific lectin such as castor-bean lectin I (60 μ g) over the sugar-lysozyme was present in the assay system, a loss in the activity of enzyme of about 80–85% was observed. To establish that the resulting changes in enzyme activity were due only to lectin-sugar interactions, the following observations were made. *M. luteus* cells with castor-bean lectins in the absence of enzyme did not show any change in absorbance, thereby demonstrating that lectin does not react with the cells. Secondly, when albumin replaced the lectin in the

Fig. 1. Effect of castor-bean lectin I on the activity of β -D-galactopyranosyl-lysozyme in the presence and absence of lactose

The lytic activities of various concentration of β -D-galactopyranosyl-lysozyme was determined in the presence (Δ) and absence (O) of lectin in 0.1M-potassium phosphate buffer, pH 6.2, in a total volume of 1.5ml. For studying the effect of lectin on the enzyme activity, β -D-galactopyranosyl-lysozyme was preincubated with 60 μ g of castor-bean lectin I for 15min in a total volume of 0.2ml and then assayed. For reversal of castor-bean-lectin-I-induced inhibition of enzyme, the lectin was incubated with 0.3M-lactose for 10min and then β -D-galactopyranosyl-lysozyme was added to it (0.2ml total vol.). After 15min it was assayed for enzyme activity ($\bullet$).

Fig. 2. Lectin-induced inhibition of β -D-galactopyranosyl-lysozyme

A fixed concentration of β -D-galactopyranosyl-lysozyme ($1.9\mu\text{g}$) was incubated in a total volume of 0.2ml with various concentration of castor-bean lectin I (○) or univalent castor-bean lectin II (ricin) (●), for 15min in 0.1M -potassium phosphate buffer, pH 6.2, and assayed for lytic activity of lysozyme on *M. luteus* in 0.1M -potassium phosphate buffer, pH 6.2.

assay system, no change in lysozyme activity was observed. Thirdly, lysozyme unsubstituted with sugar did not show any inhibition with lectin; and finally, when $100\mu\text{g}$ of *Bandeiraea simplicifolia* lectin (Hayes & Goldstein, 1974), which is specific for α -D-galactopyruvate residues, replaced the castor-bean lectin I in the assay system, no change in substituted-lysozyme activity was observed. This indicated that lectin specifically inhibits β -D-galactopyranosyl-substituted enzyme. This was further substantiated by a nearly complete retention of conjugated-lysozyme activity when the lectin inactivated with lactose was added in the assay system (Fig. 1). Moreover the inhibition could be reversed by incubating 0.3M -lactose with the conjugated lysozyme-lectin complex for 15min before the assay of enzyme activity. These observations led us to develop a method for quantifying the lectins. The results of titration of β -D-galactopyranosyl-lysozyme with castor-bean lectin I, a bivalent lectin, are shown in Fig. 2. As expected, the inhibition increased nearly linearly with the amount of lectin in the assay system. As little as 60ng of agglutinin can be detected by using this method. When the amount of conjugated lysozyme was increased from $1.9\mu\text{g}$ to 3.8 and $5.7\mu\text{g}$ in the assay system, the quantities of lectin required to produce the same degree of inhibition increased to two and three times respectively. In addition, it was noteworthy that the univalent lectin, castor-bean lectin II (ricin) also inhibited β -D-galactopyranosyl-lysozyme activity in a concentration-dependent manner (Fig.

2). Thus, in contrast with the erythrocyte-agglutination assay, which cannot be used for the univalent lectin (Olsnes *et al.*, 1974), the enzyme-multiplied lectin assay described here can be used for the detection and determination of univalent lectin as well. This is not surprising, since the enzyme activity could be inhibited by steric hindrance or be due to conformational 'freezing' of enzyme as a consequence of lectin binding (univalent or bivalent) as envisioned by Rubenstein *et al.* (1973) and Rowley *et al.* (1975) because of immunoglobulin G or Fab' fragment binding to hapten-substituted enzyme in their enzyme-multiplied immunoassay technique.

Moreover it is possible to detect or quantify the lectins in crude extract of seeds by the present method. Thus β -D-galactopyranosyl-lysozyme showed an inhibition corresponding to lectin concentration of 60 , 80 and 120ng respectively. When known concentrations of castor-bean lectin I (80 , 120 and 880ng) were added to the above extracts, inhibition of β -D-galactopyranosyl-lysozyme activity corresponding to lectin concentrations equal to 140 , 200 and 1000ng was obtained, and a curve corresponding to a standard curve could be constructed. It is pertinent to mention here that nanogram quantities of the two lectins from jequirty bean (*Abrus precatorius*) (Olsnes *et al.*, 1974) and a lectin from bitter melon (*Momordica charantia*) (Tomita *et al.*, 1972) could also be quantified from the purified preparations as well as from the crude extracts. There is, however, a limitation of the present technique; it cannot be used for lectins that bind to *M. luteus* cells. However, this problem can be obviated by using other enzymes, such as malate dehydrogenase, in place of lysozyme (Rowley *et al.*, 1975). A radioimmunoassay for lectins has been described that requires production of antibodies against each lectin to be quantified (Howard & Shannon, 1977), whereas the method described here does not require production of antibodies and is primarily sugar-specific, sensitive and rapid. Our method of enzyme-multiplied lectin assay obviates the problems inherent in erythrocyte-agglutination assay of lectins. Its rapidity, sensitivity and selectivity may provide a simple procedure for quantification as well as the screening of mono- and poly-valent lectins from the crude extracts.

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References

- Appukuttan, P. S., Surolia, A. & Bachhawat, B. K. (1977) *Indian J. Biochem. Biophys.* **14**, 382-384
- Bloch, R. & Burger, M. M. (1974) *FEBS Lett.* **44**, 286-289

- Chipman, D. M., Grisaro, V. & Sharon, N. (1967) *J. Biol. Chem.* **242**, 4388–4394
- Davies, R. C. & Neuberger, A. (1969) *Biochim. Biophys. Acta* **178**, 306–307
- Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. & Smith, F. (1965) *Anal. Chem.* **28**, 350–356
- Hayes, C. E. & Goldstein, I. J. (1974) *J. Biol. Chem.* **249**, 1904–1914
- Howard, J. & Shannon, L. (1977) *Anal. Biochem.* **79**, 234–239
- Lis, H. & Sharon, N. (1973) *Annu. Rev. Biochem.* **42**, 541–574
- Matsumoto, I. & Osawa, T. (1969) *Biochim. Biophys. Acta* **194**, 180–189
- Matsumoto, I. & Osawa, T. (1970) *Arch. Biochem. Biophys.* **140**, 484–491
- Olsnes, S., Saltvedt, E. & Pihl, A. (1974) *J. Biol. Chem.* **249**, 803–810
- Phillips, D. C. (1966) *Sci. Am.* **215**, 78–90
- Podder, S. K., Surolia, A. & Bachhawat, B. K. (1974) *Eur. J. Biochem.* **44**, 151–160
- Rapin, A. M. C. & Burger, M. M. (1974) *Adv. Cancer Res.* **20**, 1–78
- Rowley, G. L., Rubenstein, K. E., Huisjen, J. & Ullman, E. F. (1975) *J. Biol. Chem.* **250**, 3759–3766
- Rubenstein, K. E., Schneider, R. S. & Ullman, E. F. (1972) *Biochem. Biophys. Res. Commun.* **47**, 846–851
- Shugar, D. (1952) *Biochim. Biophys. Acta* **8**, 302–309
- Spector, S. & Parker, C. W. (1970) *Science* **168**, 1347–1348
- Tomita, T., Kurokawa, T., Onozaki, K., Ichiki, N., Osawa, T. & Ukita, T. (1972) *Experientia* **28**, 84–85