Evaluation of Fluorescence Polarization Method for Binding Study in Carbohydrate-Lectin Interaction

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The fluorescence polarization (FP) technique was evaluated to determine molecular interaction between plant lectins and polysaccharides, yeast cells and glycopeptide after labeling the lectins with fluorescein isothiocyanate. Use of *Lycoris radiata* agglutinin allowed determination of the molecular interactions with large biomolecules containing mannose oligomers and polymers. Another example using a fluorescein-labeled glycopeptide also indicated that use of the FP method would allow easy observation of the molecular interactions on the quantitative base. The present technique is highly sensitive and facile because it does not require any washing procedures before measurement.

Key words fluorescence polarization; lectin; yeast; mannan; soybean agglutinin

We have developed a fluorometric assay of molecular interaction between lectin and yeast cells based on the determination of fluorescent intensity of fluorescein-labeled lectins attached to the cells.¹⁾ The method includes a washing procedure to remove excess labeled lectin in the reaction media. Other binding assay methods using lectins labeled with an isotope such as I^{125} have also been widely used, but are restricted due to environmental pollution²⁾. The hemagglutination assay using lectin solutions diluted in a stepwise manner sometimes lacks stoichiometry.³⁾

Fluorescence polarization (FP) is a useful technique for measuring molecular interactions and allows observation of the interaction in solution in a quantitative base without any pre-washing procedures. FP also enables observation of the true equilibrium state, because the measurement is done in solution without solid support. In the present paper, we evaluate the usefulness and limit of the FP method in the observation of the molecular interactions between plant lectins and some samples of polysaccharide, cells and a glycopeptide sample.

MATERIALS AND METHODS

Lycoris radiata agglutinin (LRA), *Tulipa gesneriana* lectin (TGL) and *Crocus sativum* lectin (CSL) were obtained from the bulbs of *L. radiata*, *T. gesneriana* and *C. sativum*, respectively, as described previously.^{2,4,5)} Concanavalin A (Con A), *Lens curinalis* agglutinin (LCA), wheat germ agglutinin (WGA) and *Pisum sativum* agglutinin (PSA) were obtained from Seikagaku Kogyo (Chuo-ku, Tokyo, Japan). Samples of *N*-acetylglucosamine (GlcNAc) and mannan from *Saccharomyces cerevisiae* were obtained from Sigma-Aldrich Japan (Chuo-ku, Tokyo). Yeast cells used in the present study were from the Institution for Fermentation (Yodogawa-ku, Osaka, Japan). Fluorescein isothiocyanate (FITC) was obtained from Dojin-do (Kumamoto, Japan). FP was measured with a Beacon 2000 Valuable Temperature Fluorescence Polarization System (Pan Vera Corp., Madison, WI, U.S.A., and available from Takara Shuzo in Japan) using disposable glass test tubes (12×75 mm). A sample of glycopeptide from soybean agglutinin was prepared from soybean flour (Sigma) according to the procedures described by Dorland *et al.* and refer-

ences therein.⁶⁾ Labeling of the glycopeptide was performed in a manner similar to that described below.

Labeling of lectins with FITC A sample of lectin (1.0 mg) was dissolved in one ml of 50 mM sodium bicarbonate buffer (pH 9.5) containing 0.9% sodium chloride. A solution (5 μ l) of FITC in dimethylformamide (1 mg/50 μ l) was added to the solution, and the mixture was kept at 4°C overnight. The solution was separated on a column (1×30 cm) of Sephadex G-25 using phosphate-buffered saline (PBS) as the eluent. The eluent was monitored with a fluorometer at 520 nm by irradiating a 490 nm light. Fluorescent fractions eluted earlier were collected and diluted to the appropriate volume with the same buffer. All the lectin solutions thus prepared showed almost the same fluorescent intensities as that of the solution of 10 μ M fluorescein sodium.

Procedure for binding assay A portion (100 μ l) of the solution of FITC-labeled lectin prepared as described above was previously incubated at 37°C in a thermostated water bath. The sample tube was set in the apparatus of which cell chamber was maintained at 37°C. FP of the solution (100 μ l) was measured using the static mode with the initial parameters installed in the equipment. In the binding study, a solution (10 μ l) of mannan (10 mg/ml) or a suspension (10 μ l) of yeast cells (10^5 cells/ml) was added to the solution of the labeled lectin in PBS, and FP of the mixture was measured after 2 min-incubation at 37°C.

RESULTS AND DISCUSSION

FP has been used for the purpose of clinical diagnosis for more than a decade, but its use has been limited in the research area probably due to lack of a sensitive and versatile instrument. However, FP is a useful technique for direct determination of molecular interaction in solution, because the method does not require any pre-washing procedure. The principle of FP is briefly as follows. A small molecule labeled with a fluorescent probe moves and rotates freely and rapidly in solution, and is isotropic. However, when the labeled molecule is bound to a large molecule that is slow in rotation and tumbling, the fluorescent probe attached to the small molecule becomes anisotropic. The extent of polariza-

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Table 1. Change in FP in the Binding Study between Fluorescein-Labeled Plant Lectins and Yeast Mannan

Lectin	Molecular weight (kD)	Binding specificity	Concentration (nM) ^{a)}	FP (mP) ^{b)}		
				Before binding	After binding	Difference with S.D.
Con A	104	Glc/Man	48	118	139	21±1.5
WGA	21.6	GlcNAc	231	114	115	1±1.0
LCA	46	Glc/Man	108	153	151	-2±1.8
CSL	48	Manno-oligomer	104	83	84	1±1.5
PSA	50	Glc/Man	100	162	167	5±1.5
TGL	68	Manno-oligomer	74	157	168	11±7.0
LRA	45	Manno-oligomer	111	127	160	33±2.5

a) Concentrations of each lectin solution were adjusted to show similar fluorescent intensities (see Materials and Methods). b) The number of measurement was 5.

Fig. 1. Binding Reaction between *Lycoris radiata* Agglutinin and Yeast Cells

●, *P. anomala*; ■, *P. capsulate*. Procedures for binding assay are described in Materials and Methods.

tion is a function of the binding, and the measurement of polarization will lead to determination of the molecular interaction.⁷⁾

Terpetschnig *et al.* reported a good guideline for the applicability of FP to the observation of molecular interaction between fluorescent ligands and large biomolecules.⁸⁾ When a protein sample having large molecular mass is labeled with a fluorescent probe such as fluorescein having a short lifetime of about 4 ns, the protein is believed to show a slight anisotropy change causing difficulty in the measurement of molecular interaction. However, molecular shape or three-dimensional structure (not the molecular mass) is thought to affect the motion of the fluorescent group attached to the protein. Because the FP method is obviously dependent on molecular motion of the fluorescent group attached to the protein molecule, the method must be evaluated in studies on the molecular interaction between lectin and carbohydrate chains to access the molecular masses of the fluorescent-labeled molecules.

We examined the usefulness of this method by observing the binding between FITC-labeled plant lectins and mannan from baker's yeast. The results are summarized in the Table.

WGA having a molecular mass of 21.6 kDa, which shows affinity to GlcNAc, showed no affinity to yeast mannan.⁹⁾

Con A which shows high affinity to mannose and glucose gave a clear difference of 21 ± 1.5 mP, though Con A is a tetrameric protein having molecular mass of 104 kDa. LCA, a protein having a half of the molecular mass (46 kDa) of Con A, did not bind to conjugate mannan molecules, though LCA is considered to show similar binding behavior with Con A. LRA showed a clear FP difference (33 mP) between before and after binding reactions. As shown in the table, there was no obvious relationship between difference of FP in binding reaction and the molecular mass of lectins. We found that some FITC-lectins showed obvious FP change but others did not on the binding reaction with yeast mannan. The following speculations may provide reasons for these different FP changes in the lectins examined. The positions, in which FITC groups are introduced in lectin molecules, vary with the lectins. It may be true that some lectins like LRA have FITC groups near the sites where the binding reaction takes place. FITC groups present in such a microenvironment will show clear FP change because the binding reaction will cause restriction of the FITC molecule.

When LRA, which showed the obvious FP response (see the table), was used in the binding study with cells of *Pichia* (*P.*) *capsulate* and *P. anomala*, the binding was successfully observed on a quantitative basis as shown in Fig. 1. LRA did not interact with the cell surface of *P. capsulate*, but showed high affinity to *P. anomala*. The results obtained in the present study were in good accordance with those obtained earlier.¹⁾

When a small molecule such as an FITC-labeled glycopeptide (molecular mass of carbohydrate portion, 1872) was used as a ligand, the interaction was more clearly observed. An example is shown in Fig. 2. Molecular interaction of Con A was measured with an FITC-labeled glycopeptide obtained from soybean agglutinin, which contains a homogeneous carbohydrate chain as shown in Fig. 3. The glycopeptide was obtained by exhaustive digestion of soybean agglutinin and then labeled with FITC. The expression of the binding reaction by Scatchard plot is also shown in the panel of Fig. 2, and the binding constant could be easily calculated as $K_a = 9.7 \times 10^5 \text{ M}^{-1}$. The results obtained by the present technique were in good agreement with those obtained by microcalorimetry measurements.¹⁰⁾ When Con A labeled with fluorescein was used in the binding study with this glycopeptide, difference in FP was not significant (data not shown). Formation of the conjugation between the labeled Con A and the glycopeptide had no obvious effect on the molecular motion of the labeled Con A, and no distinct change of the FP

Fig. 2. Binding Reactions between Con A and Glycopeptide Obtained from Soybean Agglutinin

value was observed. On the other hand, when the glycopeptide of low molecular mass was labeled with FITC, the difference of FP was quite obvious. This indicated that restriction of the molecular motion of the fluorescent group was the major determinant for application of the FP technique, though the molecular mass should also be considered.

In conclusion, we evaluated the usefulness and limit of the FP method using some plant lectins in quantitative determination of molecular interaction. Though FP observation of molecular interaction was generally difficult when using fluorescent-labeled biomolecules having molecular masses of

Fig. 3. High-Mannose Type Oligosaccharide Obtained from Soybean Agglutinin

20 kDa or higher, a few lectins such as LRA were still available. This was probably due to the rapidity of molecular motions of the fluorescent groups near the binding sites. Modification of labeling reagents and labeling methods will improve the present technique and allow wider range of applications.

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