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Evaluation of the Performance of Khramkova-Abelev's Immunodiffusion Method as Used for Determining Lectin Concentrations in Solution

Yu. A. Kalinin, Yu. S. Tatarinov, and A. A. Terent'ev

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The immunodiffusion method proposed by N. I. Khramkova and G. I. Abelev, which is a modification of Ouchterlony's test, is intended for semiquantitative determination of protein antigens. It is characterized by a relatively high sensitivity and absolute specificity and permits objective recording of the results without separating the test antigens from the protein mixtures. In addition, it allows the content of immunoreactive molecules to be determined in purified protein preparations and can thus be used as a reference method in evaluating the nativity of the proteins obtained.

In this comparative study, protein preparations of animal and plant origin were analyzed using spectrophotometric techniques and the immunodiffusion method to determine protein concentrations in solution.

MATERIALS AND METHODS

Preparations of the following proteins were used: human serum albumin (HSA), bovine serum albumin (BSA) (both from Serva, Germany); horseradish peroxidase (HRP) (PZ=3.0), pea seed lectin (PSL), peanut seed lectin (PNSL), and black elderberry bark lectin (BEBL) (all from *Lektinotest*, Lvov) [1,2].

Antisera to the peroxidase and lectins were obtained by immunizing random-bred gray rabbits, with each rabbit receiving subcutaneously 15 mg of the appropriate protein dissolved in 1 ml of

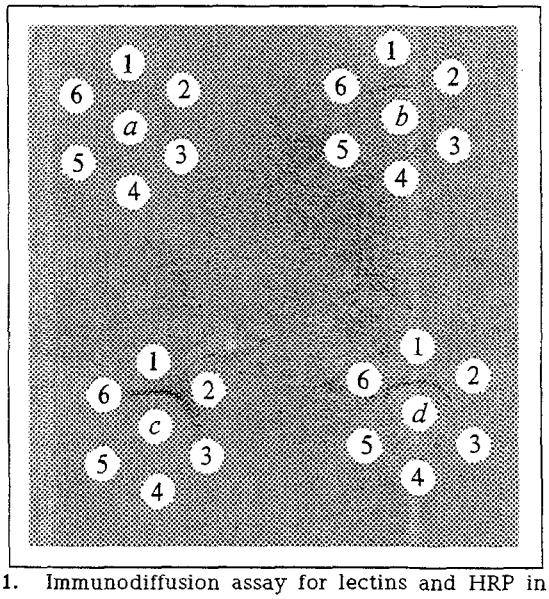

Fig. 1. Immunodiffusion assay for lectins and HRP in agar gel. Dilutions of antiserum: *a)* 1:4 to HRP; *b)* 1:12 to PSL; *c)* 1:16 to PNSL; *d)* 1:24 to BEBL. Dilutions of respective antigens (μg/ml): *1)* 32; *2)* 16; *3)* 8; *4)* 4; *5)* 2. *6)* physiological saline.

Department of Biochemistry and Laboratory for Immunochemistry of Malignant and Embryonic Tissues, Russian State Medical University, Moscow

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Freund's complete adjuvant. The immunization cycle consisted of five injections performed at 5-day intervals; 90 days after the last immunization, the rabbits were reimmunized with the same amount of protein without adjuvant. On days 7, 10, and 13 after the reimmunization, blood was taken from a marginal vein of the ear.

The immunodiffusion assay was run in a 1.5% Difco agar gel (USA) prepared in 50 mmol/liter Tris-veronal buffer supplemented with 2.5% sodium chloride.

The purity of the test protein preparations was assayed immunochemically by estimating the number and titers of the antigenic components detected with the corresponding antisera.

Protein concentrations were determined by three different techniques: Lowry's method [5,6]; staining with Coomassie P-250 [3]; and direct scanning of unstained solutions at 275 nm. HSA and BSA of 95% purity were used as standards for the construction of calibration curves.

RESULTS

The construction of calibration curves using various weighted portions of the standard proteins (HSA and BSA) and the three above-mentioned methods of protein determination indicated that the most sensitive of these was staining of the standard solutions with Coomassie P-250 followed by spectrophotometry at 595 nm. The calibration curve obtained was then used as a basis for further work.

The electrophoretically determined purity of the proteins we used was no less than 95%, as specified in the manufacturer's catalogs. Our aim was to evaluate the purity of these proteins immunochemically, i.e., to determine the number and titers of the antigenic components constituting the lectin and peroxidase preparations. To this end, double radial immunodiffusion in agar gel was used. The initial dilution of each protein was 10 mg/ml. With the use of paired protein dilutions, the proteins were found to be of high purity, with a virtual absence of impurities (<1%) and with a predominance of the major antigenic component (Table 1).

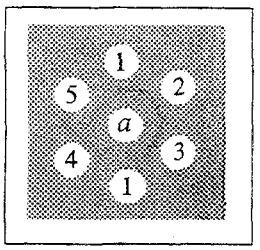

Fig. 2. Immunodiffusion analysis of PSL in agar gel using the monospecific test system. *a)* antiserum to PSL, 1:12 dilution. Concentrations of PSL antigen (μg/ml): *1*) 10; *2*) 4; *3*) 2; *4*) 1. *5*) physiological saline.

After the immunochemical assay of the proteins for purity, the percentage protein contents of the test lectins and peroxidase were determined. For this purpose, 100 μg of each protein was dissolved in 1 ml of 0.1 mol/liter phosphate buffer, pH 7.2, and the protein concentrations were determined in aliquots using the calibration curve; from the results obtained, the percentage contents of the proteins were calculated and found to constitute 93% in HPR, 92% in PSL, 92% in PNSL, and 91% in BEBL (Table 2).

To evaluate the detection limits of the precipitation assay in agar, twofold dilutions of the test antigens in concentrations from 32 to 1 μg/ml were prepared and added to wells 2 mm in diameter made with a standard gel well puncher. The appropriate diluted antiserum was added to the central well. Dilutions of the antisera were determined by means of a double radial immunodiffusion assay with antigen in a concentration of 20 μg/ml. The optimal dilution was taken to be the last dilution at which a distinct precipitation line was produced. The antiserum to HRP was used in a dilution of 1:4, that to PSL in 1:12, that to PNSL in 1:16, and that to BEBL in 1:24. After a 4-hour incubation at 20°C, the formation of a precipitation line was noted visually in scattered light. The last antigen dilution producing a clearly visible precipitation line was a protein concentration of 8 μg/ml for both HRP and the three lectins (Fig. 1).

Based on the results of the immunochemical and spectrophotometric assays for purity of the protein preparations under study, the sensitivity (lower detection limit) of the precipitation test was considered to be 7.2-7.4 μg/ml. It should be noted that we did not observe any differences in sensitivity with antigens differing in molecular weight.

TABLE 1. Immunochemical Assay for Purity of Test Proteins

Protein	Mol. weight, kD	Subunit structure	Number and titer of antigenic components			Degree of purity, %
			1	2	3	
Horseradish peroxidase	44	Monomer	–	1/4	1/1024	99
Pea seed lectin	48	Tetramer	–	–	1/1024	99
Peanut seed lectin	110	Tetramer	–	1/2	1/1024	99
Black elderberry bark lectin	140	Tetramer	1/2	1/2	1/1024	99

TABLE 2. Protein Concentrations and Percentage Contents in Test Preparations (Means±SEM)

Protein	μg/ml	Protein concentration (μg/ml) as measured by			% protein
		Lowry's method	Coumassie P-250 stain	direct scanning at 275 nm	
Horseradish peroxidase	100	85±8.5	93±4.5	84±12.6	93
Pea seed lectin	100	82±8.2	92±4.6	78±11.7	92
Peanut seed lectin	100	83±8.3	90±4.5	81±12.2	92
Black elderberry bark lectin	100	80±8.0	91±4.6	81±12.2	91

In an attempt to increase the sensitivity of the method, a monospecific test system for one of the lectins (PSL) was used, consisting of antigen in a dilution of 10 μg/ml and antiserum in a 1:12 dilution. Paired PSL dilutions from 4 to 1 μg/ml were examined. The lower detection limit of the method was then found to have dropped to 4 μg/ml (the precipitation line became more arched) and even to about 3.6 μg/ml when the actual percentage protein content in the initial preparation was taken into account (Fig. 2).

In summary, this study with highly purified lectins and horseradish peroxidase indicated that the lower detection limit of the precipitation assay in agar is about 7.2 μg/ml using simple titration and can be lowered to 3.6 μg/ml by the use of a monospecific test system with optimal dilutions of the antigen and antiserum. These results are in full agreement with those reported by Khramkova and Abelev [4], who determined the detection limits of their immunodiffusion method for two proteins of animal origin, namely rabbit γ globulin and rabbit albumin.

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