Chemistry of Natural Compounds, Vol. 36, No. 6, 2000

CHEMICAL MODIFICATION OF COMPONENTS OF THE COTTON CYTOKININ HORMONE—RECEPTOR COMPLEX FOR CREATION OF PESTICIDE BIOSENSORS

V. V. Uzbekov, O. N. Veshkurova, N. Zh. Sagdiev, and Sh. I. Salikhov

UDC 547.962+577.33+573.6

Highly specific polyclonal antibodies to the cotton cytokinin receptor were isolated and labeled with fluoresceinisothiocyanate to give a conjugate of the natural phytohormone zeatinriboside with bovine serum albumin. The possible use of cotton cytokinin receptor as a biosensor to analyze pesticides, phenylurea derivatives, was investigated.

Key words: phytohormone receptors, pesticides, immunochemistry.

Progress on environmental research is due largely to the creation of highly sensitive rapid methods for monitoring pesticides and their metabolites.

Measurement methods that are based on the use of biosensors, i.e., any biological systems (enzyme—substrate, hormone—receptor), are currently being extensively developed. The competing receptor probe (CRP) method that was previously developed by Hock is used to analyze xenobiotics that possess estrogen activity [1].

Cotton phytohormone receptors that we isolated earlier [2, 3] are promising as biosensors for analyzing several pesticides.

Certain compounds [aquodiaceto(N-hydroxymethylthiourea), copper hemihydrate (XTC-1), diacetamide of zinc chloride (DAZC), and a growth stimulator based on the thyroid hormone T-3] that are structurally different from natural auxins were demonstrated to inhibit binding of ^{3}H-IAA (indole acetic acid) to a preparation of soluble auxin-binding proteins. Their inhibiting concentrations were the same as for unlabeled IAA [4].

Analogous investigations were carried out with the cytokinin receptor. Despite the significant structural differences among natural cytokinins, which are derivatives of adenine (isopentyladenine, kinetin), and pesticides, which are derivatives of phenylurea (thidiazuron), the common –N–C–N– group was demonstrated to create a competitive displacement by the last of ^{3}H-BAP (6-benzylaminopyrine) from the binding sites of the receptor [5]. The order of competitive binding of pesticides to the active center of cytokinin and auxin receptors correlates with their physiological activity.

The CRP method proposed by us consists of the determination of the amount of pesticide in a sample using the degree of competitive displacement by pesticide of the cytokinin receptor from its complex with zeatin immobilized on a solid phase.

The amount of bound receptor is determined from the fluorescence of the ternary complex zeatin—receptor—fluorescent-labeled antibody as a control.

Displacement by pesticide (phenylurea derivative) of a certain amount of receptor from the zeatin—receptor complex decreases proportionately the amount of bound antibody. Therefore, the fluorescence intensity also decreases.

The test system is created by the following steps: Rabbits are immunized with purified cotton cytokinin receptor. Antibodies to the cytokinin receptor are isolated from blood serum. The immunoglobulins are labeled with fluoresceinisothiocyanate (FITC). A conjugate of the natural phytohormone zeatin—riboside with bovine serum albumin (BSA) are synthesized to improve the adsorptivity of the phytohormone. The optimal relationships between the CRP components are determined.

A. S. Sadykov Institute of Bioorganic Chemistry, Academy of Sciences of the Republic of Uzbekistan, 700143 Tashkent, ul. Kh. Abdullaeva, 83, tel. 162 35 40, fax (99871) 162 70 71, e-mail: root@ibc.edu.uz. Translated from Khimiya Prirodnykh Soedinenii, No. 6, pp. 490-493, November-December, 2000. Original article submitted December 11, 2000.

0009-3130/00/3606-0611$25.00

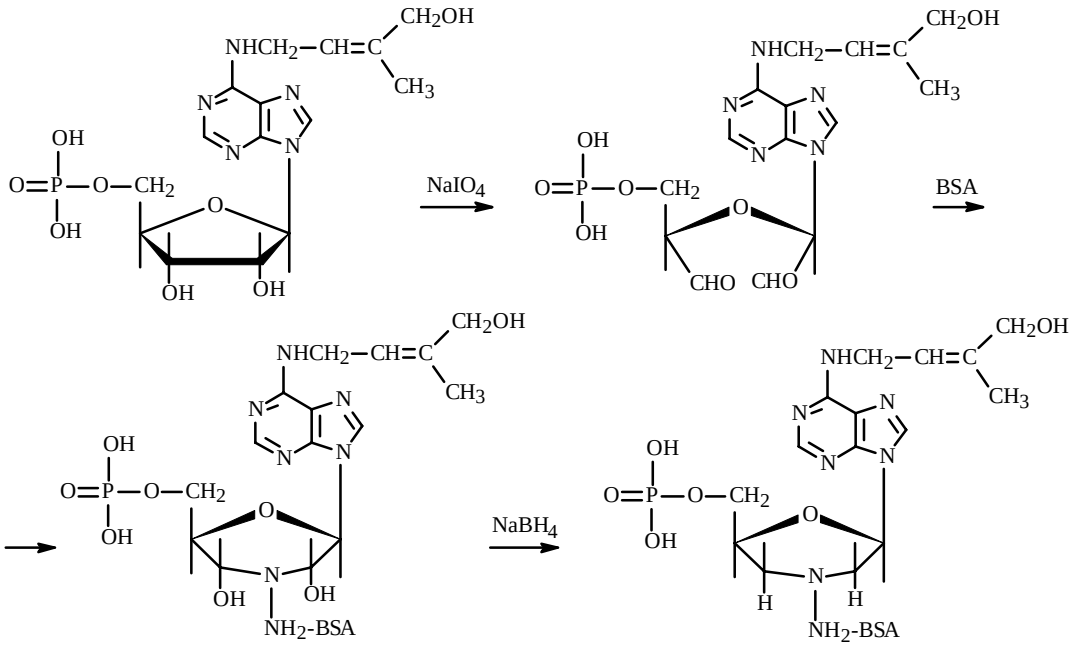

Fig. 1. Conjugation of natural phytohormone zeatin—riboside to BSA by periodate carbohydrate oxidation.

Rabbits of average mass and age were selected for immunization. The antigen was injected subcutaneously as an emulsion consisting of equal volumes of solutions of total Freund adjuvant and CBP solution in physiological buffer. The serum content of specific antibodies was monitored by the Ouchterlony method [6].

The entire fraction of immunoglobulin G (IgG), which was separated by precipitation with 33% ammonium sulfate, was labeled with FITC for quantitative determination of antibodies bound during the analysis. Conjugates with a degree of substitution close to unity were obtained.

Zeatin—riboside was conjugated to the BSA amino group according to the scheme shown in Fig. 1 in order to strengthen the sorption of the phytohormone to the solid phase.

The levels of nonspecific binding of components, the optimum concentration of labeled antibodies, and the correlation between the added phytohormone or pesticide and amount of bound labeled antibodies were then determined from the change of fluorescence intensity.

OPTIMAL LABELED ANTIBODY CONCENTRATION

The initial concentration of FITC—IgG was diluted from 500 to 1 μg/mL (in protein). The gradual change of the amount of labeled antibodies affected the fluorescence. The exponential nature and leveling off of the change indicates that the sorption sites are saturated (Table 1). The range of working concentrations of labeled IgG is 1/64-1/16 (8-30 μg/mL).

NONSPECIFIC SORPTION OF CRP COMPONENTS

Three series of experiments were performed. One of the components was missing in each of these, except for labeled IgG, which is a marker.

We present data for the levels of nonspecific sorption of labeled antibodies and receptor.

Expt. No.	Added components*	Fluorescence level, counts/sec
1	FITC–IgG	760±36
2	Zeatin–BSA + FITC–IgG	545±45
3	Empty cuvette	1370±50

*FITC–IgG concentration 15 μg/mL.

TABLE 1. Fluorescence Intensity as a Function of Amount of Added Labeled Antibody in a Model Experiment

Dilution of serum	FITC–IgG concentration, µg/mL	Fluorescence, counts/sec
1:1	500	8680
1:2	250	8540
1:4	125	8260
1:8	62.5	7880
1:16	31.25	5120
1:32	15.63	2500
1:64	7.81	150
1:128	3.91	110
1:256	1.95	116
1:512	0.98	100

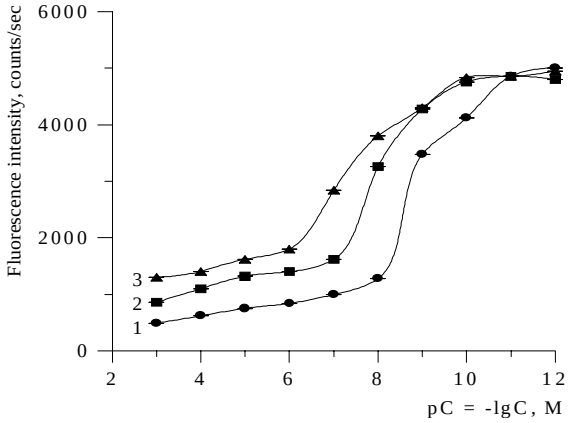

Fig. 2. Change of fluorescence intensity by various concentrations of added cytokinin-like compounds: zeatin (1), diuron (2), thidiazuron (3).

Thus, it was found that the levels of nonspecific sorption are comparable with the background of an empty cuvette and have practically no effect on the analytical result (see expt. No. 1). Various types of carriers with different compositions and polymer adsorption properties can be used in the experiments. Therefore, the nonspecific binding and background should be measured on going to each new batch of plastic cuvettes. The value obtained should be subtracted from the total number of fluorescence intensity counts.

SENSITIVITY LIMITS FOR ZEATIN AND PESTICIDES

The constant components in the experiment are FITC—IgG at 1/32 dilution, zeatin—BSA conjugate at 1 mg/mL, and CBP at 0.1 mg/mL in protein. Dilution of all compounds were prepared starting from 10^{-3} M. Each subsequent point is the dilution of the previous one by 10 times.

Zeatin at a concentration of 10^{-3}-10^{-7} M practically completely suppressed sorption of antibodies at the receptor. Concentrations $<10^{-9}$ had little effect on the binding between immobilized hormone and receptor. The sensitivity limit of the system for zeatin is 10^{-9} M for a 5-sec scan.

Pesticides were analyzed analogously. The amounts of diuron ([N-3,4-dichlorophenyl-N′,N′-dimethylurea]) and dropp (thidiazuron, [N-(1,2,3-thiadiazolyl-5)-N′-phenylurea]) (Table 2, Fig. 2) were measured.

The experimental results showed that the threshhold of the determined concentrations in the laboratory is 10^{-6}-10^{-7} M. This sensitivity is comparable with that of chromatography (pesticide with a molecular weight of the order of 250 is determined at a concentration of 1 ppm or 1 µg/L).

TABLE 2. Determination of CRP Sensitivity Limits to Zeatin (Control) and Certain Pesticides

Studied substance	$pC_{substance}$, M									
	12	11	10	9	8	7	6	5	4	3
	Fluorescence, counts/sec									
Zeatin	5006	4860	4120	3480	1276	994	840	752	620	490
Diuron	4800	4860	4760	4280	3260	1616	1400	1320	1100	860
Dropp	4950	4860	4840	4300	3800	2840	1798	1620	1400	1300

Thus, we modified chemically components of the CRP, cotton cytokinin receptor and antibodies to it. A test system for analyzing pesticides, phenylurea derivatives, based on CRP is developed on the laboratory scale.

EXPERIMENTAL

The defoliants diuron and dropp (Schering, Germany), natural phytohromones zeatin and zeatin—riboside (Serva, Germany), and fluorochrome (fluoresceinisothiocyanate, Sigma, USA) were used.

The sorbents were Sephadex G-25 medium (Pharmacia, Sweden) and Toyopearl DEAE-TSK 650M (Toyosoda, Japan).

UV absorption of the eluates was measured at 277 nm in a Uvicord S-II (LKB, Sweden) transmission UV spectrometer linked to a standard low-pressure chromatography system from the same company.

Fluorescence intensity was determined on an automated microcuvette photometer/fluorimeter (Wallac, Finland) by varying as necessary the scan time, which was usually 5 sec. The emission intensity of the microcuvettes was measured in relative units at 535 nm.

We used polystyrene immunological microcuvettes (Corning) with 96 wells of 250 µL.

Synthesis of Zeatin—Riboside—BSA Conjugate. Zeatin—riboside (5 mg) was conjugated to BSA (10 mg) by periodate oxidation using the literature method [7].

Preparation, Purification, and Fluorochromation of IgG. Two rabbits (chincilla, 2-2.5 kg, 2-3 months) were immunized. Antigen (200-250 µl of an emulsion consisting of equal volumes of Freund adjuvant and CBP solution in 0.15 M NaCl at 0.5 mg/mL) was injected subcutaneously at 4-5 points during the first week. Then, immunization was performed without Freund adjuvant 4-5 times until an acceptable level of specific antibody in blood serum was attained. The antibody content was monitored by the Ouchterlony method [6]. The final antibody titer at an antigen concentration (in protein) of 1 mg/mL was 1:64.

Isolation of IgG Fraction. Rabbit antiserum (5 mL) was diluted with an equal volume of sodium phosphate buffer (0.01 M, SPB) at pH 7.5 containing 0.15 M NaCl. This mixture was treated dropwise with an equal volume of saturated (3.2 M) ammonium sulfate solution (final concentration 1.6 M corresponds to 33% saturation in the cold). The mixture was carefully stirred, left for 30 min, and then centrifuged for 20 min × 5000g at 4°C. The precipitate was dissolved in SPB + 0.15 M NaCl (5 mL) and again precipitated in the same way. The precipitate was again dissolved in SPB (2.5 mL) and dialyzed against it in the cold. The dialyzate was placed on a DEAE-TSK 650F column equilibrated with 0.0175 M SPB calculated for 1 mL of moist gel per 1 mL of serum. Elution began with the initial buffer, collecting approximately one half of a column volume. Then the initial buffer was replaced by a mixture of it with 0.05 M NaCl. A second peak was collected that contained "acidic" IgG. The fractions were combined, made isotonic, concentrated to 5-10 mg/mL, and used for fluorochromation.

Fluorochromation of IgG fraction was carried out according to the literature method [8].

Protein concentration and fluorochrome/protein ratio were determined from the optical density of the solution at 280 and 495 nm. These wavelengths in the visible spectrum correspond to the maximum absorption (Cebra, Goldstein, 1965; Amante, Giuriani, 1969; Amante et al., 1972). Subtractions were made according to the following formulas, in which the absorption spectrum of fluorochrome (absorption maximum of FITC is 495 nm) is considered.

Protein concentration = $[A_{280} - (0.35A_{295})]/1.4$, mg/mL. Number of FITC moles per mole of IgG = $(2.87A_{495})/[A_{280} - (0.35A_{495})]$, mol/mol.

The resulting FITC—antibody solutions were made isotonic by dialysis against SPB and were stored in the cold. From

IgG (10 mg) and FITC (100 μg), a solution of FITC—immunoglobulin (12 mL) with A_{280} = 0.68 and A_{495} = 0.20 (in a 1-cm cuvette) was prepared.

The protein concentration was 440 μg/mL. The degree of substitution was 0.94 mol/mol.

Determination of Optimal CRP Component Ratio and Determination of Amount of Pesticide. The analysis was performed as follows. Conjugate (concentration in protein 1 mg/mL, 150 μL) was placed in cuvettes and incubated overnight at 4°C. The excess was removed by washing three times with 0.02 M SPB at pH 7.5 and drying. The hormone—receptor complex was formed in the cuvettes by adding CBP solution (150 μL, C = 0.1 mg/mL) and incubating for 2 h. The unbound part was rinsed away. The analysed solution (100 μL) or zeatin was added and incubated for 30 min (enough time for competitive displacement of part of the previously bound receptor into solution). Then, the released CBP complex with the cytokinin-like compound was washed away by rinsing three times with SPB. The amount of pesticide in the sample was determined using a calibration curve in coordinates of concentration and decrease of fluorescence relative to a control (a sample without pesticide). The calibration curve was constructed using solutions with known concentrations in the range 10^{-4}-10^{-8} M. The amount of immobilized receptor was determined by placing more or less diluted FITC—IgG (100 μL) in the microcuvettes and incubating for 2 h. The unbound part was rinsed away with SPB. Before the measurement, $MgCl_2$ (4 M, 150 μL) was added to desorb the label into solution and the fluorescence intensity was measured.

REFERENCES

1. M. Seifert, S. Haindl, and B. Hock, in: *Second European Technical Meeting Biosensors for Environmental Monitoring* and *Fifth International Scientific Workshop on Biosensors and Biosensing Devices*, Berlin, Germany, September 16-18, 1998.
2. A. A. Takanaev, O. N. Veshkurova, Kh. K. Avlyanov, Ya. S. Ziyaev, and Sh. I. Salikhov, *Khim. Prir. Soedin.*, 129 (1995).
3. O. N. Veshkurova, A. A. Takanaev, Kh. K. Avlyanov, and Sh. I. Salikhov, *Khim. Prir. Soedin.*, 702 (1992).
4. I. A. Arzanova, A. K. Tonkikh, and Sh. I. Salikhov, *Khim. Prir. Soedin.*, 457 (1995).
5. V. V. Uzbekov, O. N. Veshkurova, Yu. S. Mangutova, N. Zh. Sagdiev, and Sh. I. Salikhov, *Khim. Prir. Soedin.*, 507 (1999).
6. L. A. Osterman, *Investigation of Biological Macromolecules by Electrofocusing, Immunoelectrophoresis, and Radioisotope Methods* [in Russian], Nauka, Moscow (1983).
7. Ya. Turkova, *Affinity Chromatography*, Elsevier, Amsterdam (1978).
8. I. Lefkovits and B. Pernis, eds., *Immunological Methods*, Academic Press, New York, NY (1979).