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COMPARISON OF EFFECTIVENESS OF THREE METHODS FOR COUPLING OF PEROXIDASE WITH IMMUNOGLOBULINES USED IN THE ELISA TEST

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In Poland, wide application of the ELISA test - particularly for big-series detection of viruses in plants - is hindered by the low availability and the relatively high price of imported alkaline phosphatase. The use of peroxidase is limited by the shortcomings of its substrates. Recently, because of application of tetramethylbenzidine as a substrate of peroxidase [5] and owing to initiation of Polish production of peroxidase characterized by fairly high activity, this enzyme grows increasingly interesting. Three methods for coupling of proteins with peroxidase [8] are known; the aim of the present studies was to select the procedure most suitable for preparation of conjugates for the ELISA test.

MATERIAL AND METHODS

In the experiments broad bean stain virus (BBSV) and the corresponding antiserum with 1:4096 titer [9] were used. γ-globulins were prepared accorging to Kaniewski and Skotland [6] . Purified immunoglobulines were coupled - by the three procedures compared - with horse-radish peroxidase manufactured by Sigma (type VI, activity 330 U/mg). For confirmation of the suitability of horse-radish peroxidase produced by Biomed, Cracow (activity 281 U/mg), a conjugate using this enzyme was simultaneously prepared and coupled with γ-globulins by the periodate method.

To compare the effectiveness of coupling of γ-globulins with peroxidase, the following three procedures were applied [7] :

1) one-step glutaraldehyde method [1-3] involving direct reaction of γ-globulins with peroxidase, as a result of activation by glutaraldehyde;

2) two-step glutaraldehyde method [2] consisting of activation of peroxidase by glutaraldehyde, followed by coupling of the enzyme with γ-globulins;

3) periodate method [10] comparising activation of peroxidase by periodate and coupling with γ-globulins, followed by reduction of the resulting conjugate with sodium borohydride.

The conjugates yielded by these three procedures were made up to 2 cm^3 with PBS, to obtain in each preparation a final γ-globulins concentration of 1 mg/cm^3. The preparations were compared by application in the ELISA test [6] for the determination of two BBSV isolates. In all cases, the dilutions of γ-globulins, conjugates and virus derived from test plants, as well as incubation times were identical. Tetramethylbenzidine was used as substrate [5], and the reaction was developed for 20 min in the dark. The enzymic reaction was stopped by addition of 50 mm^3 of 3 N H_2SO_4, whereupon the absorption was measured at 450 nm.

RESULTS AND DISCUSSION

In preliminary tests aimed at the determination of the enzymic activity of the conjugates compared, the dilutions of γ-globulins and conjugates amounted to 1:1000, and those of plant homogenates - to 1:50. In all cases, the colour reaction was too strong and was accompanied by formation of a precipitate hindering absorption measurements and comparison.

For comparative purposes, the dilutions of γ-globulins and conjugates at a ratio of 1:2000, and those of plant homogenates amounting to 1:150 proved to be suitable. The results of comparative analyses of broad bean plants infected with BBSV isolates G and $R_2$76, obtained upon use of the above dilutions, are presented in Table 1. Absorption measurements were performed on a Beckman DU-8 spectrophotometer at 450 nm, in 1-cm cuvettes, against a substrate solution.

All three methods afforded conjugates of an adequate enzymic activity permitting their effective utilization in the ELISA test. The conjugates obtained by the two-step glutaraldehyde method and periodate method gave in the ELISA test very high readings being statis-

Table 1

Absorption readings at 450 nm, obtained in the determination of two BBSV isolates by the ELISA test, upon use of conjugates prepared by the three methods compared: one-step glutaraldehyde method, two-step glutaraldehyde method, periodate method

No.	Plant					
	I		II		III	
	healthy	infected	healthy	infected	healthy	infected
	Isolate G					
1	1.035	0.019	2.210	0.049	2.372	0.013
2	1.207	0.033	2.254	0.015	2.342	0.007
3	1.103	0.009	2.249	0.035	2.215	0.003
4	1.233	0.037	2.072	0.019	2.160	-0.007
5	1.206	0.033	2.213	0.029	2.013	-0.005
6	1.247	0.013	2.183	0.013	2.051	0.005
$\bar{x}$	1.172	0.024	2.197	0.027	2.192	0.003
s^2	0.0071	0.000140	0.0044	0.000191	0.0217	0.000057
s	0.0843	0.0118	0.0663	0.0138	0.1473	0.0075
	Isolate $R_2$76					
1	1.057	-0.007	2.560	0.019	2.306	-0.003
2	0.748	-0.003	2.621	0.033	2.254	0.013
3	1.300	0.013	2.499	0.041	2.054	-0.001
4	0.938	-0.001	2.044	0.011	1.992	-0.007
5	1.097	0.009	2.174	0.023	1.990	0.001
6	1.054	-0.005	2.169	0.039	2.222	0,009
$\bar{x}$	1.032	0.001	2.345	0.028	2.136	0.002
s^2	0.0333	0.000066	0.0594	0.000122	0.0198	0.000057
s	0.1825	0.0081	0.2437	0.0110	0.1407	0.0075

I-III - Methods.

tically undistinguishable (Table 2). On the other hand, the conjugate prepared by the one-step glutaraldehyde method gave significantly lower results (Table 2). In the analysis of healthy plants, the absorption values obtained upon use of the three methods compared were sufficiently low, though in four cases the differences between methods in their results (Table 2) were significant. In case of all three methods there were no significant differences in the results between both BBSV isolates. Both isolates proved to be statistically undistinguishable in the ELISA test.

Table 2

Evaluation of the significance of the differences in the results between the three methods compared, using the t test of Student; $t_{tab.}(P = 95\%; f = 10) = 2.23$

	Isolate					
	G			$R_2$76		
Plant	I - II	I - III	II - III	I - II	I - III	II - III
Infected	23.41	14.75	0.08	10.56	11.73	1.82
Healthy	0.40	3.66	3.73	4.83	0.22	4.76

I-III - Methods.

Although the one-step glutaraldehyde method for coupling of proteins with peroxidase afforded a conjugate of an evidently lower activity in the ELISA test, it is recommended for its simplicity and rapid obtained of the conjugate.

The two-step glutaraldehyde method affords - in the opinion of its authors [4] - homogeneous conjugates of low molecular weight and with an equimolar ratio of enzyme to antibodies, but with a small coupling yield. According to the latter authors, the antibodies labelled by this method are well suited for immunochemical studies and for experiments requiring penetration of the conjugate into the tissue; upon application in the ELISA test, they usually give worse absorption readings than the conjugates obtained by both remaining me-

thods. The present experiments testified not only to full suitability of this method for preparation of a conjugate for the detection of BBSV by the ELISA test, but even to its superiority over the one-step method.

The periodate method for protein coupling completely differs from the glutaraldehyde methods. This technique involves obtainment - by oxidation of the carbohydrate residues of the enzyme - of active aldehyde groups reacting with the amino groups of proteins to yield Schiff bases. These bases are unstable, but are stabilized by reduction with sodium borohydride. The resulting conjugates resemble in their properties those prepared by the one-step glutaraldehyde method; they are made up of high-molecular weight molecules, and their composition is nonuniform. The authors of this method state that it is characterized by the highest yield of coupling with peroxidase, and that it provides conjugates very well suited for different variants of the ELISA test. In the present studies, this method afforded a satisfactory conjugate being much superior to that obtained by the one-step glutaraldehyde method. The efficiency of coupling by this method is all the more convincing, since it allows for the use of the smallest amount of peroxidase for labelling of the same quantity of γ-globulins.

As well known, each of the three compared methods yields a mixture of conjugates and nonreacted γ-globulins. They can be separated on chromatographic columns packed with a gel acting as a molecular sieve, e.g. Sepharose 6 B or Ultragel 22. In case of preparation of conjugates to be used in the ELISA test, in most instances it is not worthwhile to separate the non-reacted γ-globulins. Since the present experiments yielded conjugates of very high activities, we did not compare the activity levels after separation of uncoupled γ-globulins.

Application of peroxidase of Polish make for coupling afforded a conjugate only slightly departing from that obtained - by the same method - using peroxidase of Sigma Co. make. The background was similarly low and stable, and the absorption readings of virus preparations were sufficiently clearcut. The enzymic activity of our preparation, as compared with that containing Sigma peroxidase, was somewhat lower, doubtless owing to the lower activity of the enzyme of Polish make. The differences between both preparations in the en-

zymic activity were small and did not warrant the use of higher concentrations of the conjugates containing Polish-make peroxidase in the ELISA test.

The use conjugates containing peroxidase of Polish make in the ELISA test lowers the cost of analysis. If it is assumed that 1 mg of the enzyme is required for performing, on the average, 10 000 analyses, then the cost of Polish peroxidase amounts to only 0.7 grosz per one analysis. Therefore, the application of Polish peroxidase for big-series Elisa tests is recommended.

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PORÓWNANIE EFEKTYWNOŚCI TRZECH METOD SPRZĘGANIA PEROKSYDAZY Z IMMUNOGLOBULINAMI STOSOWANYMI W TEŚCIE ELISA

S t r e s z c z e n i e

Sprawdzono przydatność trzech metod sprzęgania γ-globulin z peroksydazą dla uzyskania koniugatów do wykrywania wirusa plamistości bobiku (BBSV) testem ELISA. Wszystkie metody nadają się do tego celu. Każda z nich ma określone zalety i wady omówione w pracy. Do sprzęgania celem uzyskania koniugatów do masowych testów najkorzystniejsza okazała się metoda nadjodanowa. Oceniano przydatność peroksydazy z chrzanu produkcji krajowej do wykrywania testem ELISA, podkreślając wynikłe stąd korzyści ekonomiczne.

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СРАВНЕНИЕ ЭФФЕКТИВНОСТИ ТРЁХ МЕТОДОВ СОПРЯЖЕНИЯ ПЕРОКСИДАЗЫ С ИММУНОГЛОБУЛИНАМИ ПРИМЕНЯЕМЫМИ В ТЕСТЕ ELISA

Р е з ю м е

Проверена была пригодность трёх методов сопряжения γ-глобулинов с пероксидазой с целью получения конъюгатов для выявления вируса пятнистости конских бобов (BBSV) тестом ELISA. Все эти три метода пригодны для этой цели. Каждый из них обладает своими определёнными достоинствами и недостатками, которые представлены в работе. Для сопряжения с целью получения конъюгатов в массовых тестах наиболее полезным оказался метод иоднокислый. Подвергалась оценке пригодность пероксидазы из хрена отечественного производства, которая применялась для выявления вирусов тестом ELISA учитывая вытекающую отсюда экономическую выгоду.