A Modified, Highly Sensitive Enzyme-linked Immunosorbent Assay (ELISA) for *Rhizobium meliloti* Strain Identification

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An evaluation of the ELISA technique was made in order to obtain a very specific and sensitive method for strain identification of *Rhizobium meliloti*. Antisera against an *R. meliloti* strain were produced by using as antigen either whole cells or purified lipopolysaccharides from the cell wall. The use of whole cells as antigen gave rise to antibodies which were more sensitive and strain specific than those produced from purified lipopolysaccharides. A further evaluation of the ELISA method was the use of a new type of conjugate consisting of a purified antibody linked to β -galactosidase by using as coupling reagent a hetero-bifunctional reagent, *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP), which contains two reactive groups directed towards different functional groups. Substrate for this type of conjugate consisted of *o*-nitrophenyl- β -D-galactopyranoside. This improved technique makes it possible to detect bacteria in samples containing 1×10^3 cells ml⁻¹ whilst retaining strain specificity. Furthermore, preparations of nodules formed by different *R. meliloti* strains containing 1×10^5 cells ml⁻¹ could be analysed whilst retaining strain specificity. The technique presented thus offers advantages in higher sensitivity and reliability than conventional ELISA techniques.

INTRODUCTION

The production of effective inoculants for agricultural use presumes a sensitive method for detection of bacteria used as inoculant. Otherwise, it will be impossible to answer such questions as: what is the competitive ability of the introduced strain against indigenous rhizobia and other soil organisms; how often is it necessary to re-inoculate; and is it possible by soil management to encourage or discourage the development of an effective symbiosis?

Until recently the agglutination and immunodiffusion tests (Vincent, 1970) have been the ones most commonly used for *Rhizobium* strain identification. However, these methods require isolation and subculture if, for instance, nodule-forming bacteria are to be tested. The specificity is low and the readout of the tests is not fully objective. Other marker techniques for recognizing rhizobia are the use of genetic markers by antibiotic resistance (Schwinghamer & Dudman, 1971), and auxotrophy (Johnston & Beringer, 1975). The uncertainty associated with the genetic stability makes these techniques doubtful.

With the introduction of enzyme-labelled antibodies (Nakane & Pierce, 1966; Wicker & Avrameas, 1969) for the detection of virus antigens in tissue culture, a possibility for detecting different strains of micro-organisms became available. The quantitative use of enzyme-labelled antibodies was reported by Engvall & Perlmann (1971); Engvall & Perlmann (1972) claimed

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Abbreviations: KDO, 2-ketodeoxyoctonate; LPS, lipopolysaccharide; SPDP, *N*-succinimidyl-3-(2-pyridyldithio)propionate.

that the enzyme-linked immunosorbent assays had sensitivities comparable to radioimmunoassays and offered several advantages over them, for instance the elimination of health risks due to radiation, ability to store the conjugates for long periods of time and less expensive equipment for analysis. The ELISA method has been extended from the medical research field to applications in the biological sciences. Plant viruses were detected by Clark & Adams (1977) using a 'double antibody sandwich' as presented by Voller *et al.* (1976). The use of ELISA for identification of *Rhizobium* was reported by Kishinevsky & Bar-Joseph (1978), investigating nodules of *Arachis hypogaea*. Berger *et al.* (1979) avoided the difficulties in obtaining specific antibodies due to the nature of the cell wall of *Rhizobium* by using sheep anti-rabbit globulin for conjugation with alkaline phosphatase. A further modification was the use of fluorescent substrate to improve the sensitivity of the test (Morley & Jones, 1980). In the present study, a further improvement of the sensitivity of the ELISA test is described. Modifications have been made by using β -galactosidase for conjugation, the enzyme being coupled to the purified gamma-globulin by a hetero-bifunctional reagent.

METHODS

Preparation of antigen. *Rhizobium meliloti* strain 29 (Leguminous Plant Laboratory, Swedish University of Agricultural Sciences) was grown on a defined medium (Bishop *et al.*, 1976) in a 4-l fermenter containing 3.5 l substrate at 28 °C, supplied with 0.3 l air (l substrate)⁻¹ min⁻¹; the pH was kept at 7.0 by automatic titration with KOH. After 30 h growth the cells were harvested. One litre of the cell suspension was cooled to 2 °C and centrifuged at 5000 g for 15 min at 2 °C, washed twice with 2 × 200 ml 0.02 M-sodium phosphate buffer/0.02 M-NaCl, pH 7.0 and stored frozen at -70 °C for later use for immunization with whole cells.

The rest of the cell mass was used for phenol/water extraction of the cell wall lipopolysaccharide (LPS) (Westphal & Jann, 1965), with the following modifications involving the removal of nucleic acids in the crude LPS extract. After dialysis, the LPS-containing material (185 ml) was passed through a 10 ml bed of Dowex AG 1 × 1 in 1 M-acetic acid (Bio-Rad) and evaporated at room temperature to 130 ml. The concentrated LPS solution was made 10 mM with respect to MgSO₄ and 50 mM with respect to Tris/HCl, and adjusted to pH 7.1. RNAase A (2 mg, 60 kUnits mg⁻¹, EC 3.1.27.5, Sigma) and 2 mg DNAase I (2 mg, 2150 kUnits mg⁻¹, EC 3.1.22.1, Sigma) was added and the solution was stirred overnight at 5 °C and later lyophilized. The lyophilized LPS-containing preparation was dissolved in 25 ml of a buffer containing 20 mM-imidazole-HCl/100 mM-NaCl, pH 7.1, and chromatographed on a DEAE-Sepharose CL-6B (Pharmacia) chromatography column (2.5 × 40 cm), all fractions being analysed for 2-ketodeoxyoctonate (KDO) by the thiobarbituric acid method by Weissbach & Hurwitz (1958). KDO-positive fractions were lyophilized and dissolved in 12.5 ml of a buffer containing 10 mM-EDTA and 300 mM-triethylamine, pH 7.1, and chromatographed on a Sepharose 4B chromatography column (2.5 × 40 cm, Pharmacia); the eluted KDO-positive fractions containing LPS were lyophilized.

Production of antisera. A 1 ml portion of a bacterial suspension (10⁹ cells ml⁻¹) and 1 ml of an LPS saline (0.85%) solution containing 50 µg of purified LPS ml⁻¹ was mixed with Freund's complete adjuvant (1:1) and injected through the marginal ear vein of rabbits. After two and four weeks the injections were repeated; a month later a booster dose was given consisting of 1 ml bacteria or LPS concentrations as earlier described mixed with Freund's incomplete adjuvant (1:1). One week after the booster was given the rabbits were bled, and the blood was allowed to clot for 4 h at room temperature. The serum was collected and 3 ml fractions were stored frozen (-70 °C) before use.

Antibody purification. To samples of 2 × 3 ml of the respective antisera 1 ml 0.02 M-sodium phosphate buffer, pH 7.4, was added. Drop by drop, 5 ml saturated ammonium sulphate (pH 6.6 by titration with ammonium hydroxide) was added with vigorous stirring, the precipitate being stirred for 15 min and then allowed to settle for 7 h at room temperature. The precipitate was centrifuged (70000 g) for 2 h at 5 °C. The supernatant was pipetted off and the gamma-globulin was dissolved in 5 ml 0.02 M-sodium phosphate buffer, pH 7.2. Then 20 ml 0.1 M-NaCl, pH 7.1, was added and the solution was concentrated to 5 ml by ultrafiltration, using an Amicon ultrafilter cell equipped with a PM30 membrane. Then 20 ml 0.1 M-sodium phosphate/0.1 M-NaCl, pH 7.1, was added to the filter cell and the antibody solution was concentrated to 6 ml and pipetted off and set apart. A further 2 ml of 0.1 M-sodium phosphate/0.1 M-NaCl, pH 7.1, was added to the ultrafilter cell and allowed to stand for 5 min; it was then added to the gamma-globulin concentrate and diluted by adding 24 ml distilled water. Separation of active and inactive gamma-globulins in the solutions was made by column chromatography, using a DEAE-Sephadex A-50 column (Pharmacia, 1.8 × 10 cm). The column was equilibrated with 0.02 M-sodium phosphate/0.02 M-NaCl, pH 6.5; the gamma-globulin suspension was added to the column and a NaCl gradient was formed using 50 ml 0.02 M-sodium phosphate/0.02 M-NaCl, pH 6.5, as starting buffer and 50 ml 0.02 M-sodium phosphate/0.5 M-NaCl, pH 6.5, as final buffer. Fractions containing active immunoglobulin G (IgG) were concentrated to 5 ml by

ultrafiltration as before. Then 25 ml 0.1 M-sodium phosphate/0.1 M-NaCl, pH 6.5, was added to the filter cell, concentrated to 5 ml and pipetted off. Again 2 ml buffer was added to the filter cell and pipetted off after 5 min; the total volume of active IgG was 7 ml, which was divided into 330 μ l samples and stored at -70°C .

Preparation of enzyme-conjugate. As a first step the concentration of the hetero-bifunctional reagent *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP, Pharmacia) was estimated. SPDP (10 mg) was dissolved in 10 ml ethanol and 20 μ l was added to 2.5 ml 5 mM-sodium phosphate, pH 7.0. The A_{260} was recorded immediately, after which 0.5 ml 0.3 M- Na_2CO_3 , pH 9.0, was added and the A_{260} read after 10 min. The concentration of *N*-hydroxysuccinimide released from SPDP was calculated by dividing the change in absorbance by the molar extinction coefficient at 260 nm, $8.2 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ (Carlsson *et al.*, 1978). Again 20 μ l of the stock solution of SPDP dissolved in ethanol was taken out and added to 3.0 ml 50 mM-sodium phosphate, pH 7.0. The A_{343} was measured, 100 μ l 50 mM-dithiothreitol was added and the A_{343} was read again after 10 min. The concentration of pyridine-2-thione released from SPDP was calculated by dividing the increase in absorbance by the molar extinction coefficient at 343 nm, $8.08 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ (Stuchbury *et al.*, 1975). The molar concentration of *N*-hydroxysuccinimide was regarded as the concentration of SPDP.

The concentration of IgG was determined as 4.8 mg ml^{-1} by dividing the A_{280} value by 1.4 ($= \text{mg ml}^{-1}$). To 2.16 mg IgG in 450 μ l 0.1 M-sodium phosphate/0.1 M-NaCl, pH 7.1, 80.8 nmol SPDP was rapidly added during stirring and allowed to react for 60 min at 25°C without stirring. The excess reagent and low molecular weight reaction compounds were removed by gel filtration on prepacked disposable columns (PD-10, Pharmacia) packed with Sephadex G-25 and equilibrated with 0.2 M-sodium phosphate/2 mM- MgCl_2 , pH 7.1. The IgG was collected in 1.5 ml 0.2 M-sodium phosphate/2 mM- MgCl_2 , pH 7.1. A 0.3 ml sample was taken out for tests of the protein concentration and degree of substitution. The IgG concentration was measured at 280 nm and the degree of substitution, i.e. mol 2-pyridyl disulphide versus mol protein, was recorded at 343 nm. The number of 2-pyridyl disulphide groups introduced into the IgG was calculated from the amount of pyridine-2-thione released during treatment of the modified IgG with dithiothreitol, which is equivalent to the concentration of 2-pyridyl disulphide residues in the IgG. In the present case it was found to be 2.5.

β -Galactosidase (EC 3.2.1.23, Sigma) (7 mg) was dissolved in 0.5 ml 0.1 M-sodium phosphate/0.1 M-NaCl/30 mM-dithiothreitol, pH 7.1. After 1 h the dithiothreitol was removed by gel filtration on a PD-10 column packed with Sephadex G-25 and equilibrated with 0.2 M-sodium phosphate/2 mM- MgCl_2 , pH 7.1; the enzyme was collected in 1.5 ml. The enzyme concentration was calculated to be 4.2 mg ml^{-1} by dividing the A_{280} value by 2.09 ($= \text{mg ml}^{-1}$).

Thiolated IgG (1.26 mg) was mixed with 1.85 mg β -galactosidase, giving a total volume of 1.64 ml, and kept for 2 h at room temperature and one week at 6°C . Then 0.02% NaN_3 was added and the mixture stored at 6°C before use without further purification.

Electrophoresis of prepared enzyme conjugates. This was done on a polyacrylamide gradient gel, PAA 4/30 (Pharmacia). The buffer used was 0.09 M-Tris/0.08 M-borate/3 mM- Na_2EDTA , pH 8.4, and the electrophoresis was performed at 125 V for 16 h at 15°C . After the electrophoresis was completed, the gel was covered with β -galactosidase substrate (see final ELISA test procedure). The active β -galactosidase could be seen as yellow bands and was photographed. The gel was then stained in 0.7% Amidoblack 10B/7% acetic acid for 30 min and destained in 7% acetic acid for 3 h.

Final ELISA test procedure for detecting antigen. Polystyrene test tubes (vol. 4 ml) were washed once with 2 ml buffer containing 0.9% NaCl/0.02 M-sodium phosphate/0.25% Tween 20/2 mM- MgCl_2 , pH 7.1. Test sample (200 μ l) was added to each test tube and 100 μ l enzyme conjugate was added and incubated for 4 h, shaken at 225 r.p.m. at room temperature. To the test tube 2 ml of the washing buffer was added, and the mixture was allowed to stand for 10 min and then centrifuged (2000 g) for 10 min at room temperature, after which 1.7 ml was drawn off; this procedure was repeated three times. Then 200 μ l substrate, consisting of *o*-nitrophenyl- β -D-galactopyranoside (Sigma; 3.5 mg ml^{-1} in 0.2 M-sodium phosphate/2 mM- MgCl_2 /20% methanol, pH 7.1), was added and the test tubes were incubated for 1 h. The reaction was stopped by adding 0.7 ml 0.7 M- Na_2CO_3 . The test tubes were finally centrifuged (2000 g) at room temperature for 10 min and the A_{418} of the supernatants was recorded by using a Beckman spectrophotometer equipped with a micro-quartz cuvette with a 1 cm light path.

Optimization of some parameters of the ELISA test. The effect of different washing procedures of the antigen, using *R. meliloti* strain 29 (10^6 cells ml^{-1}), was studied by applying the described ELISA test, using enzyme conjugate derived from antisera prepared by whole-cell immunization, $2 \mu\text{g IgG ml}^{-1}$. The antigen used was washed three times in different buffers before use, namely: (1) distilled water, (2) 0.02 M-sodium phosphate/0.5 M-NaCl, pH 7.1, (3) 0.02 M-sodium phosphate/0.02 M-NaCl, pH 7.1, (4) 0.02 M-sodium phosphate/0.02 M-NaCl/0.5% Tween 20, pH 7.1; and (5) the antigen was boiled for 30 min in 0.02 M-sodium phosphate/0.02 M-NaCl, pH 7.1, and washed in the same buffer.

The effect on the ELISA test of using antigens of different origin was studied using *R. meliloti* strain 29 (1×10^7 cells ml^{-1}) grown on either solid BIII or liquid BIII substrate (Bishop *et al.*, 1976). The enzyme conjugate used in this study derived from antisera produced by whole-cell immunization, $2 \mu\text{g IgG ml}^{-1}$. The test procedure was carried out as described earlier.

The effect on enzyme conjugate activity of dilution in different buffers was studied. A stock culture of enzyme conjugate derived from antisera prepared by whole-cell immunization was diluted, to a final concentration of $2 \mu\text{g IgG ml}^{-1}$, in different buffers, namely: (1) 0.9% NaCl/0.3% dextran/0.5% Tween 20/0.02 M-sodium phosphate, pH 7.1; (2) 0.2 M-sodium phosphate/2 mM-MgCl₂/2 mg human IgG ml⁻¹ (Sigma), pH 7.1; (3) 0.2 M-NaCl/2 mM-MgCl₂/0.1% Tween 20, pH 7.1; (4) 0.9% NaCl/0.3% dextran/0.5% Tween 20/0.02 M-sodium phosphate/2 mM-MgCl₂, pH 7.1. To 200 μl of enzyme conjugate 100 μl substrate (for concentration see description of the final test procedure) was added. After 3 min the enzyme reaction was stopped by adding 0.7 ml 0.7 M-Na₂CO₃ and the A_{418} was recorded.

The effect of enzyme conjugate concentration on the ELISA test was investigated by using *R. meliloti* strain 29 (10^6 cells ml⁻¹) and varying the concentration of enzyme conjugate when applying the ELISA test. The enzyme conjugate investigated was derived from antisera produced by whole-cell immunization.

The effect of the time for which the antigen was incubated with the enzyme conjugate was studied, using *R. meliloti* strain 29 ($1 \times 10^{3.5}$ cells ml⁻¹ and $1 \times 10^{4.5}$ cells ml⁻¹) and enzyme conjugate derived from antisera produced by whole-cell immunization, $2 \mu\text{g IgG ml}^{-1}$. The cell suspensions were incubated with the enzyme conjugate for periods up to 24 h, after which the ELISA test procedure was performed as before.

The linearity of the formation of the product during incubation of the enzyme conjugate with antigen was studied using *R. meliloti* strain 29 (10^8 cells ml⁻¹) and enzyme conjugate derived from antisera produced by whole-cell immunization, $2 \mu\text{g IgG ml}^{-1}$; the test was as previously described, except that the enzyme reaction was stopped at 15 min intervals. This experiment was also used for a study of the absorbance maximum of the product, using the sample being incubated for 60 min and monitoring between 350 nm and 550 nm.

Identification of antigen using the ELISA test. *Rhizobium meliloti* strains were tested with the ELISA test at different cell concentrations and with enzyme conjugate derived from either LPS immunization or whole-cell immunization. Nodules from aseptically grown inoculated lucerne were crushed and suspended in distilled water at 10^5 cells ml⁻¹, and incubated with enzyme conjugate derived from antisera produced by whole-cell immunization, $2 \mu\text{g IgG ml}^{-1}$.

RESULTS AND DISCUSSION

A new method for preparing enzyme conjugate used in ELISA tests is presented. This technique offers several advantages over that suggested by Clark & Adams (1977), the main one being detection and identification of micro-organisms in samples with very low cell concentrations. For instance, in the present study, *Rhizobium meliloti* strains were detected and differentiated in samples containing 1×10^3 cells ml⁻¹ (Table 1). The technique presented will therefore facilitate further studies in this field in samples with low cell concentrations. A beneficial step in preparing the ELISA conjugates was the choice of β -galactosidase as enzyme, which enabled the free -SH groups present on the enzyme to be used in the coupling reaction between the antibody and the enzyme. β -Galactosidase is also easily available, and very stable. In the conventional method (Voller *et al.*, 1976), conjugation is made by glutaraldehyde. The use of a hetero-bifunctional reagent such as SPDP offers several advantages: homopolymerization and intramolecular (versus intermolecular) cross-linking is avoided, the reactions can be performed under mild conditions, and the degree of substitution with protected thiol groups is controllable and can be determined. In the present study, the degree of substitution, i.e. mol disulphide versus mol IgG, was between 2.4 and 2.5. Electrophoresis showed that most of the conjugate consisted of one molecule of β -galactosidase and one IgG, 1:1, and some 1:2 and 1:3. Further purification did not therefore seem necessary.

Several parameters of the ELISA test procedure were investigated in order to evaluate a proper method for routine analysis in the future. Washing of the antigen in distilled water was preferable to other pretreatments (Table 2). This is in contrast to the findings of Kishinevsky & Bar-Joseph (1978), who presented results indicating that higher ELISA values were obtained with preheated antigens. This difference might be explained if thermostable antigens are released, as expected from the lysed cells. Nevertheless, it was decided to wash the cells in distilled water during routine ELISA tests. Slightly higher ELISA values were obtained with antigen from cells grown in liquid culture than with that from cells grown on solid substrate. However, this character was not statistically significant and of no importance for the ELISA values. When the conjugate was diluted in different buffers, differences between the buffers were small, but the buffer containing dextran, Tween 20, MgCl₂ and sodium phosphate in saline

Table 1. Cross-reactivity of four strains of *R. meliloti* using the ELISA test

Enzyme conjugates were prepared from antisera of strain 29 prepared by whole-cell immunization ($2.0 \mu\text{g IgG ml}^{-1}$) and by LPS immunization ($2.3 \mu\text{g IgG ml}^{-1}$). Three replicates were done of each test. The control was distilled water.

Strain no.	log (No. of cells ml^{-1})	Whole-cell immunization			LPS immunization		
		A_{418}	SD	<i>P</i> value*	A_{418}	SD	<i>P</i> value*
Control	0	0.140	0.028	—	0.114	0.021	—
29	7.6	0.734	0.029	0.005	0.394	0.033	0.010
	4.4	0.447	0.012	0.005	0.167	0.032	NS
	3.0	0.287	0.016	0.05	0.114	0.007	NS
	2.5	0.215	0.019	NS	0.117	0.035	NS
4	7.6	0.448	0.015	0.010	0.395	0.052	NS
	4.4	0.212	0.017	0.005	0.174	0.035	NS
	3.0	0.211	0.011	0.05	0.120	0.017	NS
	2.5	0.121	0.018	NS	0.099	0.023	NS
23	7.6	0.553	0.020	0.05	0.481	0.024	NS
	4.4	0.264	0.027	0.05	0.188	0.036	NS
	3.0	0.160	0.005	0.05	0.090	0.005	NS
	2.5	0.130	0.016	NS	0.110	0.009	NS
27	7.6	0.446	0.065	0.05	0.255	0.006	NS
	4.4	0.217	0.049	0.05	0.187	0.025	NS
	3.0	0.158	0.009	0.05	0.107	0.004	NS
	2.5	0.131	0.019	NS	0.093	0.018	NS

* Each strain was compared to strain 29 at the same cell concentration; strain 29 was compared to the control at each cell concentration. NS, Not significant.

Table 2. Effect on the ELISA test of different washing procedures for antigen of *R. meliloti* strain 29

The enzyme conjugate was derived from antiserum prepared by whole-cell immunization, $2 \mu\text{g IgG ml}^{-1}$. Three replicates were done of each test.

Preparation of antigen by washing in:	log (No. of cells ml^{-1})	A_{418}	SD	<i>P</i> value*
Distilled water	4.5	0.547	0.047	—
	0	0.121	0.010	—
0.02 M-Sodium phosphate/0.5 M-NaCl, pH 7.1	4.5	0.292	0.022	0.05
	0	0.127	0.017	NS
0.02 M-Sodium phosphate/0.02 M-NaCl, pH 7.1	4.5	0.417	0.031	0.10
	0	0.139	0.027	NS
0.02 M-Sodium phosphate/0.02 M-NaCl/ 0.5% Tween 20, pH 7.1	4.5	0.463	0.044	NS
	0	0.118	0.039	NS
'Boiled antigen' in 0.02 M-sodium phosphate/ 0.02 M-NaCl, pH 7.1	4.5	0.333	0.001	0.05
	0	0.133	0.017	NS

* Each washing in buffer was compared to washing in distilled water at each cell concentration. NS, Not significant.

gave the best enzyme activity. For routine ELISA, it was decided to use this buffer composition. The relation between the concentration of the enzyme conjugate and the ELISA test (Fig. 1) demonstrated that the optimal reaction between enzyme conjugate and antigen occurred at an enzyme conjugate concentration of $2 \mu\text{g IgG ml}^{-1}$ with respect to background levels. Consequently, this will be the concentration to be used in routine ELISA. The optimum time for incubation of the antigen with the enzyme conjugate was 4 h (Fig. 2). The enzyme reaction was linear for at least 75 min; it was decided to incubate the ELISA samples for 1 h. The maximum absorbance of the product was at 418 nm, and the ELISA test samples will be measured at this

Fig. 1

Fig. 2

Fig. 1. Effect of enzyme conjugate concentration on the ELISA test. The enzyme conjugate was prepared from antisera produced by whole-cell immunization. Three replicates were done of each test. ○, Concentration of *R. meliloti* strain 29 at 1×10^5 cells ml^{-1} ; △, no bacteria (control).

Fig. 2. Effect on the ELISA test of the time of incubation of *R. meliloti* strain 29 with conjugate prepared from antisera produced by whole-cell immunization, $2.0 \mu\text{g IgG ml}^{-1}$. Three replicates were done of each test. Cell concentrations [log (no. of cells ml^{-1})]: △, 4.5; □, 3.5; ○, 0 (control, distilled water).

Table 3. Cross-reactivity of nodule extracts of four strains of *R. meliloti* using the ELISA test

The cell concentration of each strain was 1×10^5 cells ml^{-1} ; the enzyme conjugate used was derived from antisera produced by whole-cell immunization of *R. meliloti* strain 29, $2 \mu\text{g IgG ml}^{-1}$. Three replicates were done of each test. The control was distilled water.

Strain no.	A_{418}	SD	<i>P</i> value*
Control	0.162	0.030	0.010
29	0.640	0.021	—
4	0.284	0.035	0.010
23	0.523	0.027	0.10
27	0.481	0.040	0.05

* Samples compared to strain 29.

wavelength. The results of the testing of different ELISA procedures showed that the presented final ELISA test procedure gave maximum ELISA values. When applying the designed ELISA test with enzyme conjugate derived by whole-cell immunization it was possible to detect and recognize strains of rhizobia in samples containing 1×10^3 cells ml^{-1} (Table 1), a concentration lower than in earlier reports (Kishinevsky & Bar-Joseph, 1978; Berger *et al.*, 1979; Morley & Jones, 1980). Strain specificity of the ELISA test was also obtained in nodule extracts containing 1×10^5 cells ml^{-1} (Table 3).

The enzyme conjugate derived from the antisera produced by LPS immunization was compared to the 'whole-cell immunization' enzyme conjugate and it was concluded that the 'whole-cell' enzyme conjugate was preferable (Table 1). Despite the LPS antibodies not being completely unspecific, future applications will concentrate on the purification of the antibodies obtained by whole-cell immunization.

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