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Production of a conjugate between the rK346 antigen from *Leishmania infantum* and the horseradish peroxidase C for the detection of rK346 antibodies.

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Key words: diagnostic leishmaniasis; proteins conjugation; reporter system.

Abstract. It was designed and characterized a reporter system to be captured by antibodies bound to ELISA plates. The system was designed with the rK346 from *Leishmania infantum*, a highly antigenic and specific protein. The rK346 was coupled to the horseradish peroxidase C (HRPc) from *Armoracia rusticana* using glutaraldehyde or sulfo-SMCC. Glutaraldehyde conjugation was performed in two steps. Separation of conjugates was carried out using a Sepharose S-200 in size exclusion chromatography (SEC); fractions were analyzed via HRPc activity and through ELISA plates sensitized with polyclonal anti-rK346 IgG purified from rabbit serum. A heterogeneous population of conjugates rK346-HRPc was obtained with molecular weights ranging between 109.7 ± 16.5 to 67.6 ± 10.1 kDa; with rK346-HRPc stoichiometries of 1:2; 2:1; 3:1; and 2:2. Conjugation using sulfo-SMCC was carried out first by introducing -SH groups onto the HRPc using the SATA reagent and the antigen was modified with sulfo-SMCC during 45 min. Separation and analysis of conjugates was performed similarly as with glutaraldehyde, resulting in a heterogeneous population of conjugates rK346-HRPc with molecular weights between 150.5 ± 22.6 to 80.0 ± 12.0 kDa; with rK346-HRPC stoichiometries of 2:1; 1:2; 2:2; and 1:3, with an increased conjugation efficiency in comparison with glutaraldehyde. This enables sulfo-SMCC to be used as a potential reagent for coupling the antigen to the HRPc, to design an economic, specific and easy method to apply as a reporter system, available to assess individuals at risk and/or at early and late stages of visceral leishmaniasis.

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Producción de un conjugado entre el antígeno rK346 de *Leishmania infantum* y la peroxidasa C de rábano picante para la detección de anticuerpos rK346.

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Palabras Clave: diagnostico de leishmaniasis; conjugación de proteínas; sistema reportero.

Resumen. Se diseñó y caracterizó un sistema reportero para ser capturado por anticuerpos enlazados a placas de ELISA. El sistema fue diseñado con una proteína altamente antigénica y específica, la rK346 de *Leishmania infantum*. La rK346 fue acoplada a la peroxidasa C de rábano picante (HRPc) de *Armoracia rusticana* usando glutaraldehido o sulfo-SMCC. La conjugación con glutaraldehido fue realizada en dos pasos. La separación de los conjugados fue llevada a cabo a través de una cromatografía de exclusión molecular sefarosa S-200 (CES), las fracciones fueron analizadas midiendo la actividad HRPc y por placas ELISA sensibilizadas con inmunoglobulina G policlonal anti-rK346, purificada desde suero de conejo. Se obtuvo una población heterogénea de conjugados rK346-HRPc en un rango de pesos moleculares entre 109,7 ± 16,5 a 67,6 ± 10,1 kDa; con estequiometria rK346-HRPc de 1:2; 2:1; 3:1; y 2:2. La conjugación usando sulfo-SMCC se llevó a cabo primero introduciendo grupos -SH en la HRPc usando el reactivo SATA; el antígeno se modificó con sulfo-SMCC. La separación y el análisis de los conjugados se realizaron de forma similar que con el glutaraldehido, resultando en una población heterogénea de conjugados rK346-HRPc con un rango de pesos moleculares entre 150,5 ± 22,6 a 80,0 ± 12,0 kDa; con estequiometria rK346-HRPC de 2:1; 1:2; 2:2 y 1:3, y con una eficiencia de conjugación incrementada en comparación con glutaraldehido. De esta forma, se habilitó al sulfo-SMCC como un reactivo potencial para acoplar antígenos a la HRPc, como método para el diseño de un sistema reportero económico, especifico y fácil de aplicar, útil en la evaluación de individuos en riesgo y/o en estados tempranos o avanzados de leishmaniasis visceral.

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INTRODUCTION

Modification of proteins, nucleic acids and other biomolecules through chemical reagents has been an active field for several decades, becoming a part of biotechnology that functionally revolutionizes genomics, proteomics and glycomics (1-3). Nowadays, proteins are chemically modified for experimental approaches allowing a better understanding of their function in the cell (4), or even more, tests with clinical and biomedical applicability, such as drug transporting protein systems, proteins-compatible with biomaterials, therapies, diagnosis tests and biosensors (2). The last ones represent diagnostic methods used for quality control to the detection and quantification of specific molecules for a biological system. One of these methods is the bioconjugation, in which a stable conjugate is generated by two or more biomolecules, to act as a functional detector system (*in vitro* or *in vivo*) of other biomolecules. This method ex-

ploits, in one hand, the reporting property of one of the two components and, in the other hand, their specificity, resulting in a novel system that combines the properties of each protein to operate at more efficient levels(5).

The use of biotechnology has achieved innovative advances in the diagnostic field; allowing many diseases, that mainly affect tropical countries, to be detected and treated at an early stage as in the case of Visceral Leishmaniasis (VL). VL is a systemic disease, with injuries that are not visible and that can be fatal if not treated on time, unlike other leishmaniasis manifestations (cutaneous and mucocutaneous). The worldwide distribution of VL is extensive and endemic in Latin American, African and Mediterranean countries (6). In Venezuela, the most associated Leishmania species is *Leishmania infantum* whose reservoirs are mammals. Cases of VL are mainly distributed in the coast and Venezuelan eastern regions with annual incidence rates reported from 0.08 (1990) and 0.22 (2005) per 100.000 inhabitants (7). Nowadays, the two most important parameters counting in routine serological and molecular diagnosis tests for VL are sensitivity and specificity, whose quantitative values should exceed 95% to ensure suitable diagnosis. However, the methods being currently applied are still yielding values below this percentage due to the use of crude parasite extracts as antigens (8,9). Furthermore, al-though early diagnostic practices do not necessarily assure the patient survival, rapid diagnosis and a higher sensitivity, allow doctors to prescribe treatments with fewer side effects, representing a greater life expectancy.

One of the most advanced, economic and reproducible techniques currently applied in the immunodiagnostic field is the ELISA (Enzyme-Linked Immuno-Sorbent Assay) which is characterized by having sensitivity and specificity values near 100%. However, we know that these values can vary because of both, the antigen and the geographical origin of the sera. To apply this technique, we continue to rely on the conjugation between a reporter enzyme and a commercial secondary antibody, which will be used in the primary antibody immunocapture. In order to modify this procedure, this study applies bioconjugation techniques to design a reporter system comprising the recombinant antigen rK346 from *L. infantum* and the reporter horseradish peroxidase C. This modification aims to contribute to the development of a new, ELISA system more efficient and based on the increased number of HRPc molecules coupled to the antigen; this last one demonstrated 100% of specificity. This no our detection system was stable, more sensitive and economic because no commercial secondary antibody was needed to measure the signals.

MATERIALS AND METHODS

Reagents and proteins

The rK346 recombinant protein from *L. infantum* and the corresponding hyperimmune rabbit serum were both obtained from the Laboratorio de Enzimología de Parasitos (Universidad de Los Andes). The HRPc, Reinheitszah value (RZ) ≥ 3 was purified from horseradish roots. The Laboratorio de Inmunoparasitología of the Instituto de Biomedicina (Universidad Central de Venezuela) donated positive-VL human serum. Goat secondary antibodies conjugated to HRP (anti-rabbit IgG and anti-human IgG) were purchased from Sigma Aldrich (USA). The stock of glutaraldehyde (GA) at 25% was obtained from Scharlau (Spain) and SATA, sulfo-SMCC, and hydroxylamine-HCl reagents were obtained from Thermo Scientific (USA). The separation of conjugates by size exclusion chromatography (SEC) was performed with an S-200 sepharose resin (Sigma Aldrich) and a closed-system column coupled to a peristaltic pump to regulate the elution flow.

Purification of the detection system components

rK346 antigen from *L. infantum.* An aliquot of an *E. coli* culture (25 mL) was used. Bacteria were transformed with the pET-K346 plasmid, and finally grown in autoinduction medium. Cells were disrupted by sonication and the antigen was purified by an IMAC (immobilized metal ion affinity chromatography) previously equilibrated with 20 mM NaH_2PO_4; 20 mM Na_2HPO_4 at pH 7.8 following the manufacturer recommendations (Invitrogen, USA). Purification steps were visualized by a polyacrylamide electrophoresis under denaturing conditions (SDS-PAGE) at 15%. The antigen was concentrated by salting-out with ammonium sulfate at 80% saturation and further dialyzed in conjugation buffer for GA (100 mM phosphate buffered saline (PBS), 100 mM NaCl, 1 mM $CaCl_2$) pH 6.8 or sulfo-SMCC (50 mM PBS, 50 mM NaCl) pH 7.8.

HRPc. The HRP purification protocols cited in previous studies were applied to a sample of horseradish roots (10,11); the enzymatic activity (EA) was determined following methodologies previously suggested (12-15) and RZ values through the optic densities (OD) ratios at 403 nm and 280 nm(16). HRPc purification steps were visualized by SDS-PAGE at 12% and then concentrated and dialyzed as cited above in conjugation buffer for GA pH 6.8 or sulfo-SMCC pH 7.5.

Total IgG. Applying methodologies previously described (17-19), a volume of 1.15 mL of rabbit serum was used to purify total IgG by using 10 mM Tris-HCl buffer, pH 8.0 and 40 mM NaCl for their elution. The IgG concentration in each well during the capture assays was determined by an indirect ELISA performing serial dilutions of IgG (1/1000 - 1/18000) and a 1/10000 dilution for the HRP-coupled commercial secondary antibody. The rabbit-IgG capture capacity was verified by a sandwich ELISA where a 1/50 dilution of human serum positive for VL was used as well as 58 µg of anti-Pgr24 IgG (*Trypanosoma cruzi* marker) as cross-reaction control and a dilution of 1/10000 corresponding to an anti-human IgG coupled to HRP as secondary antibody.

Conjugation of the rK346 antigen from *L. infantum* to the HRPc using GA

Considering conjugation methodologies used by other authors (20-22), the reaction was carried out in two steps via a pre-standardization of optimum reaction conditions (temperature, pH, one or two-step reaction and reaction time). In the first step 0.5 mg of HRPc ($RZ \geq 3$) were incubated with GA at a final concentration of 1.25% during 18 hours, at 4°C in darkness and gentle stirring. In the second step 1.2 mg of the rK346 antigen were added in order to obtain a 6-fold molar excess relative to that from HRPc, under the same conditions.

Conjugation of the rK346 antigen from *L. Infantum* to the HRPc using sulfo-SMCC

According to the reported bioconjugation protocols based in heterobifunctional cross-linkers (23-26), a prior standardization of the stability and conjugation reaction conditions was performed (temperature, pH, molar ratio, reaction time and buffer).

Putative –SH groups added on HRPc: 1.40 mg of HRPc ($RZ \geq 3$) were incubated with the SATA reagent (considering a minimum of 10-fold molar excess) during 30 min at room temperature (RT), in darkness and gentle stirring using a PBS at 50 mM, 50 mM NaCl, 5 mM EDTA, pH 7.5. The reagent excess was removed by passing the sample through a PD-10 dialysis column (GE Healthcare Biosciences) previously equilibrated with the same buffer. The sulfhydryl groups were further activated by adding a solution of 0.1 M hydroxylamine•HCl in 50 mM PBS, 50 mM NaCl, 25 mM EDTA, pH 7.5

and then incubated 2 hours at RT, in darkness and gentle stirring. The sample was dialyzed in a PD-10 column previously equilibrated with the same buffer (pH 7.2) supplemented with 10 mM EDTA verifying the HRPc enzymatic activity (EA) in the collected fractions.

Modification of the rK346 antigen with sulfo-SMCC: parallel to the previous experience and based on reported conjugation protocols (27-31); 3.25 mg of the antigen were incubated with sulfo-SMCC at 10-fold molar excess or higher. The reagent was previously dissolved in the same buffer at pH 7.8; during 45 min at RT, in darkness and gentle stirring. The excess reagent was removed following the methodology described in paragraph 2.4.1.

Conjugation of HRPc-SH to the rK346-maleimido antigen: the two modified proteins were incubated at a molar ratio of 1:6 (rK346:HRPc) during 30 min at RT, in darkness and gentle stirring.

Conjugate analysis

The conjugation mixture was charged in a sepharose S-200 in order to separate conjugates by SEC. The resin was previously equilibrated with conjugation buffer pH 6.8 (for GA) or pH 7.2 (for sulfo-SMCC) and adapted to a flow of 38 mL/h. Fractions of 1.2 mL were collected and then each one quantified by their OD280 nm and their EA following reported methodologies (12-15). Those fractions with higher RZ and EA were analyzed by a direct ELISA and further visualized on a SDS-PAGE at 10% and by Western-blot (WB) to verify the antigenic component in conjugates. In each sub-population of conjugates, the molecular weight was quantified as well as their proportion. Finally, to examine the feasibility and efficiency of conjugation between these proteins, all experiments were performed twice in order to assign their corresponding statistical value and reproducibility.

ELISA

Each assay was analyzed by a direct ELISA in which plates (COSTAR model) with 350 ng of anti-rK346 IgG were sensitized using a phosphate buffer pH 7.2 (1.5 mM KH_2PO_4, 130 mM NaCl, 5 mM Na_2HPO_4). Each well was blocked overnight with a solution of 2% casein and 0.05% Tween 20 dissolved in the same buffer. One hundred µL of each fraction were added and further incubated during 1 h at 37°C subsequently, carrying out five washes of 200 µL each one with the same buffer and 0.05% Tween 20. The development was performed during 10 min by adding 90 µL of a solution of TMB (Chagas disease diagnosis kit CruziELISA from the Laboratorio de Enzimología de Parásitos). At the same time, a test to verify the anti-rK346 IgG capture capacity was performed. The colorimetric reaction was stopped with 45 µL of HCl 0.5 N and then, the signal intensity at 450 nm was quantified with a Microplate Reader (CPD-212 model).

SDS-PAGE and WB

Samples prepared from each fraction with higher EA (Enzyme Activity) and ELISA signals, as well as a pool formed with the EA peak, were both visualized with Coomassie blue by a SDS-PAGE at 10% made according to the established methodology (32). The antigenic component in conjugates was verified through a WB for which, conjugates separated on SDS-PAGE were actively transferred onto a PVDF membrane (Pierce) for 80 min at 65 mA. Subsequently, the membrane was blocked during 3 hours at RT with 5% casein and 0.1% Tween 20 using the reported methodology(33). After, the membrane was incubated during 1 hour with the primary antibody (anti-rK346 IgG) diluted at 2000-fold at RT and gentle stirring. The same procedure was applied with the secondary antibody (anti-rabbit IgG) diluted at 5000-fold. The development was performed by adding 20 mL of a solution 100 mM PBS supplemented with 0.05% DAB (Dia-

minobenzidine) 100 mM NaCl, 0.003% $CoCl_2$ pH 7.2 initiating the reaction with 20 µL of 3% H_2O_2 and stopping it with distilled H_2O.

RESULTS

Silva-Barrios, (36) evaluated the antigenicity of the rK346 antigen, a kinesin of approximately 17.2 kDa whose primary sequence is principally composed of tandem repeats conferring its acidic character (isoelectric point: 4.6). It has been demonstrated for rK346, a specificity of 99.43% by ELISA against VL positive and negative human sera. However, its sensitivity varies from 59.3% to 87.5% respectively in ELISA and MABA representing low values compared to other molecular markers used in diagnosis methods for VL (34, 35, 6, 36).

To increase the sensitivity of rK346-based immunoassays, several chemical crosslinkers were used to couple the HRPc reporter enzyme to the antigen in order to capture bioconjugates directly by the anti-rK346 IgG antibodies immobilized on ELISA plates. The use of such crosslinkers involved a previous research on the primary sequence (using the ProtParam program available on http://web.expasy.org/protparam/) for both HRPc and rK346 proteins to determine the target residues that might be enough reactive to generate a stable bond without affecting the integrity and functionality of proteins during bioconjugation. These analyses revealed respectively 13.6% and 9.7% on basic residues allowing (i) a greater reactivity of both proteins for conjugation and (ii) a better control over the reaction given these low proportions. In the case of the crosslinkers selection, we considered their length, water solubility, target residues, stability after bioconjugation, pH, and reaction conditions. We used GA as homobifunctional crosslinker given its versatility during chemical reactions and standardization of conjugation between proteins, and finally the sulfo-SMCC was used as heterobifunctional crosslinker according to its specificity for target residues.

Purification of the detection system components

rK346

The rK346 antigen was purified to homogeneity using the Invitrogen "ProBond™ Purification System: For purification of polyhistidine-containing recombinant proteins" and imidazole at 100 mM with a purification yield of 1.91 mg from a culture of 25 mL after total precipitation with ammonium sulfate and dialyzed in conjugation buffer for GA or sulfo-SMCC (Fig. 1A).

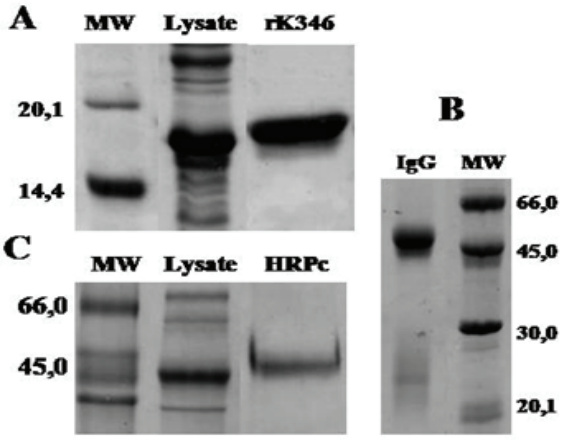

Fig. 1. Purification of the detection system components. A) Purification of the recombinant protein rK346 by IMAC. Elution with imidazole 100 mM. B) Purification of total IgG by salting-out with $(NH_4)_2SO_4$ and ion exchange chromatography (DEAE-Cellulose). Elution with TRIS•HCl pH 8.0 + NaCl 40 mM. C) Purification of the HRPc by affinity chromatography (Sepharose 4B - Concanavalin A). Elution with a linear gradient of methyl α-D-mannopyranoside.

HRPc

The HRPc was obtained from an extract of horseradish roots (*Armoracia rusticana*) prepared in the Laboratorio de Enzimología de Parásitos and containing 4000 TEU. The enzyme was purified to homogeneity yielding 2710 Total Enzymatic Units (TEU) and an RZ ≥ 3 by applying an affinity chromatography (Concanavalin A) and a further elution gradient using a gradient of methyl α-D-mannopyranoside. The band corresponding to the enzyme (Fig. 1C) coincides with the 45 kDa molecular marker indicating its purity. 4.30 mg of total enzyme were obtained by applying the same procedure cited above.

Total IgG

For the anti-rK346 IgGs, the capture molecules for conjugates, we used 1.15 mL of hyperimmune rabbit serum achieving their purification to homogeneity (Fig. 1B) with a yield of 6.6 mg and titred at 1/6000. From these results, it was decided to use a dilution of 1/1000 (350 ng) to sensitize ELISA wells until complete saturation for conjugates analyzes. The capture capacity of antibodies to the antigen was evaluated by a sandwich ELISA (Fig. 2) obtaining values for absorbances at 450 nm, six times higher in the test A (test of immunocapture for the rK346 antigen) than those obtained for tests B, C, and D, indicating that IgGs were functional and therefore capable to capture the rK346 protein which was not recognized by antibodies from Chagas disease patients.

Conjugation of the rK346 antigen to the HRPc using GA

In order to obtain a high efficiency of conjugation with GA, a prior standardization of reaction conditions through pilot tests was performed, including one or two-step reactions, molar ratios between proteins, pH, temperature and reaction time. It was found that a two-step reaction, modifying the HRPc with GA during 18 hours at 4ºC, pH 6.8 and with a HPRc:rK346 molar ratio of 6, provided the best results in terms of HRPc-rK346 conjugation which were also analyzed by direct ELISA with 350 ng of IgG. The consensus elution profile (Fig. 3A) corresponding to the separation of conjugates by SEC showed two overlapping peaks (1 and 2) represented by asterisks and with HRPc EA (indicated in red) between 315 and 384 mL of elution, near to the peak of the non-conjugated HRPc EA (indicated in blue). Two additional peaks with absorbances at 280 nm were detected, one coinciding with those from conjugated HRPc EA peaks (Peak 3) and another one between 400 and 500 mL of elution (Peak 4).

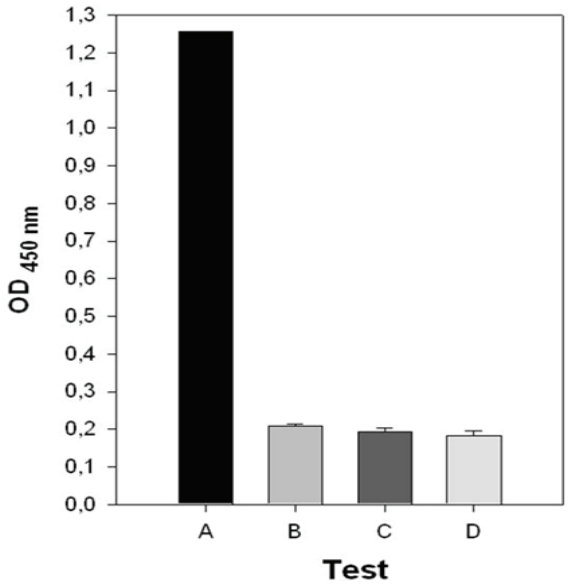

Fig. 2. Immunocapture assay by the IgG in sandwich ELISA: (A) rK346 antigen immunocapture (B) Cross-reaction control with Pgr24 antigen from *T. cruzi*. (C) Primary antibody control (D) Secondary antibody control.

Conjugation of the rK346 antigen to the HRPc using Sulfo-SMCC

Prior standardization of reaction conditions were conducted through pilot tests. In this case, all reactions were performed in two steps and at a molar ratio of 6.0. The temperature, reaction time and the modification of HRPc or rK346 antigen with SATA or sulfo-SMCC was varied. The greatest conjugation efficiency (through direct ELISA with 820 ng of anti-rK346 IgG) was obtained incubating the HRPc with SATA during 30 min (with subsequent activation of sulfhydryl groups with hydroxylamine-HCl) and during 45 min for the rK346 antigen with sulfo-SMCC, in both cases at RT (as in the same case of the HRPc-rK346 conjugation reaction). The consensus elution profile (Fig. 4A) by SEC showed (as previously in conjugation with GA)

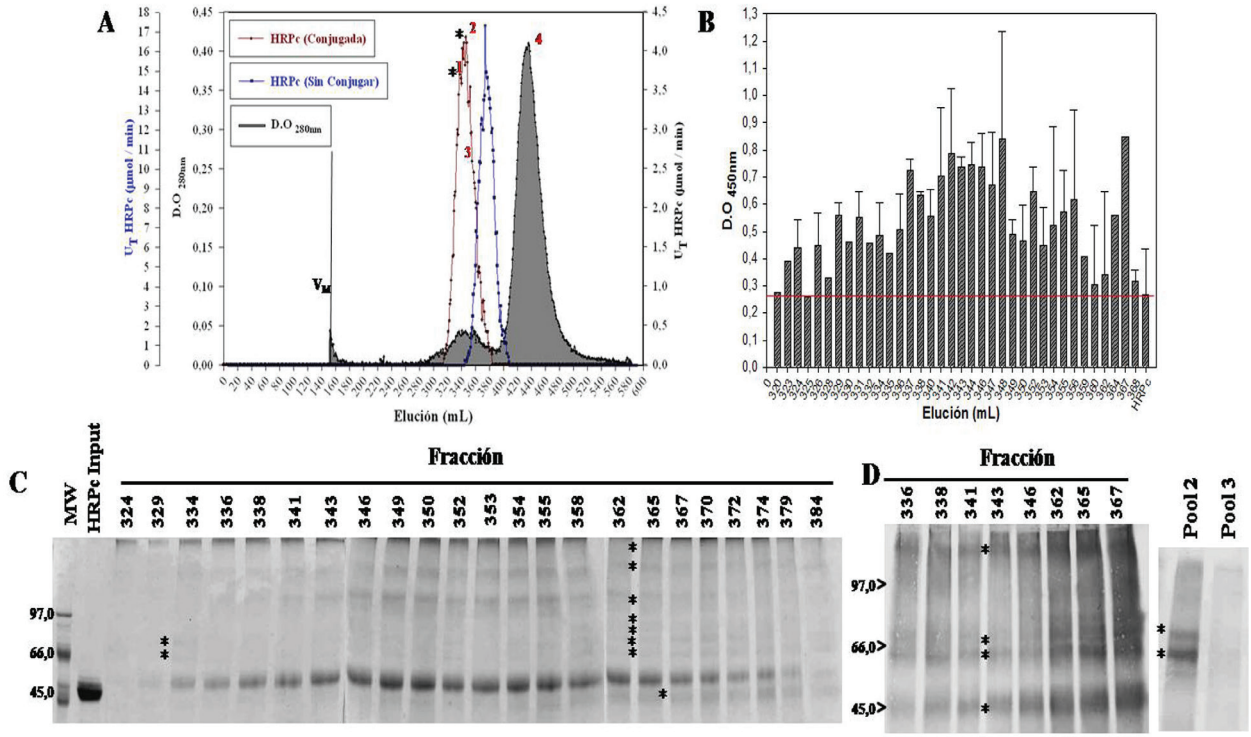

Fig. 3. A) Elution profile consensus at 280 nm and 414 nm corresponding to the separation of HRPc-rK346 conjugates by SEC (Sepharose S-200). Conjugation was performed with GA 1.25%. Profiles at 414 nm shown in red and blue correspond to the determination of EA of the conjugated and non-conjugated HRPc respectively. Asterisks indicate the two peaks with HRPc activity measured at 414 nm (1 and 2). Maximal absorbance at 280 nm (3 and 4) were was also determined. B) Direct ELISA corresponding to fractions with the greatest conjugated-HRPc activity. The SD for two replicates is shown as well as the basal detection limit (red line). C) Visualization of HRPc-rK346 conjugates by SDS-PAGE at 10% and D) by Western-blot. Each lane in Fig. C shows those fractions evaluated by direct ELISA, corresponding to the peak with conjugated-HRPc activity. Conjugates are indicated by asterisks. Pool 2: Fractions with the most HRPc activity (323-388 mL of elution). Pool 3: (413-452 mL of elution).

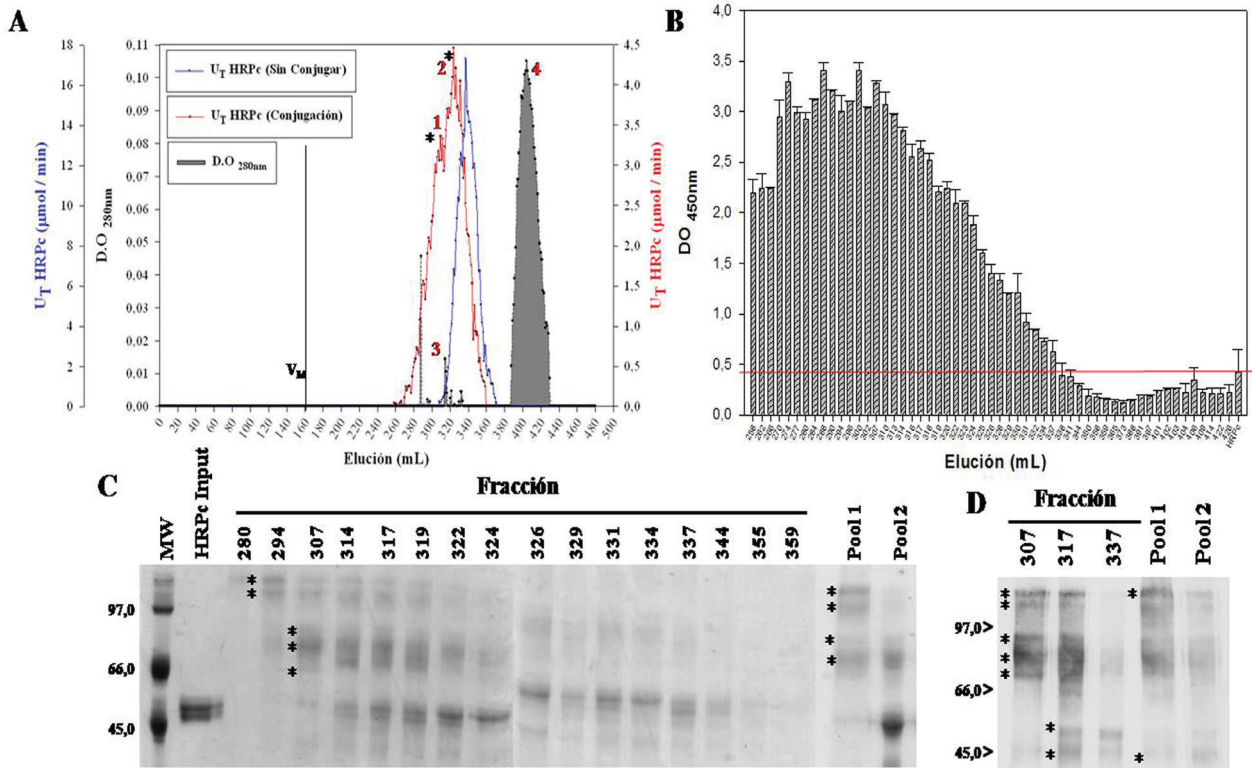

Fig. 4. A) Elution profile consensus at 280 nm and 414 nm corresponding to the separation of HRPC-rK346 conjugates by SEC (Sepharose S-200). Conjugation was performed with SATA (sulfhydryl-adding reagent) and sulfo-SMCC (conjugation reagent). Profiles at 414 nm shown in red and blue correspond to the determination of EA of the conjugated and non-conjugated HRPc respectively. Asterisks indicate the two peaks with HRPc activity measured at 414 nm (1 and 2). Absorbance maxima at 280 nm (3 and 4) were also determined. B) Direct ELISA corresponding to fractions with the greatest conjugated-HRPc activity. The SD for two replicates is shown as well as the basal detection limit (red line). C) Visualization of HRPc-rK346 conjugates by SDS-PAGE at 10% and D) by Western-blot. Each lane in Fig. C shows those fractions evaluated by direct ELISA, corresponding to the peak with conjugated-HRPc activity. Conjugates are indicated by asterisks. Pool 1: (270-317 mL of elution). Pool 2: Fractions with the most HRPc activity (318-334 mL of elution).

two overlapping peaks (represented by asterisks) with HRPc activity (indicated in red) eluting between 270 and 360 mL near to the EA measured for the non-conjugated enzyme (indicated in blue). Two peaks with absorbances at 280 nm were also observed, one coinciding with those from the conjugated HRPc (Peak 3) and another between 390 and 430 mL (Peak 4).

Analysis and visualization of HRPc-rK346 conjugates

GA

Fractions with HRPc activity were analyzed by ELISA using 100 µL of each one on ELISA plates containing 350 ng of IgG. We obtained values ranging from 0.32 ± 0.04 to 0.84 ± 0.39 (Fig. 3B) corresponding to signals from

1.2 to 3.2 times higher than those given by the non-conjugated enzyme (0.27 ± 0.17; see threshold in Fig. 3B), and values between 1.5 and 3.8 times higher than the non-conjugated enzyme (at the corresponding dilution after SEC) when analyzing respectively 100 and 200 µL from the pool formed with these fractions (data not shown), indicating that conjugates possess the antigenic component which gives them the ability to be captured by anti-rK346 IgG.

Quantification of the HRPc EA, RZ and total protein (mg) was also calculated before and after conjugation (Table I) in order to determine the conjugation yield, the results of two replicates were considering as well as the estimated number of HRPc molecules in conjugates. 67.71% of recovery from the initial activity was obtained 67% of total mg and RZ of 0.4 for the peaks 1 and 2. The same fractions were concentrated and visualized on SDS-PAGE (Fig. 3C). Our results show the presence of a heterogeneous population of conjugates (indicated with asterisks) with molecular weights upper to 45 kDa co-eluting with the non-conjugated HRPc

TABLE I
DETERMINATION OF TEUs AND QUANTITATION OF rK346-HRPc CONJUGATES (USING GA) THROUGH TOTAL mg AND RZ.

Sample	U_T	Total mg	RZ
Conjugation Mix	156.70	1.62	0.80
Peak with HRPc EA (Elution 323–388 mL)	106.10	1.09	0.40
Yield of HRPc EA (%)		67.71	
Total yield: mg (%)		67.00	

TABLE II
DETERMINATION OF TEUs AND QUANTITATION OF rK346-HRPc CONJUGATES (USING SULFO-SMCC) VIA TOTAL mg AND RZ.

Sample	U_T	Total mg	RZ
Conjugation Mix	177.62	4.65	0.60
Peak with HRPc EA (Elution 270 – 334 mL)	82.80	1.93	0.20
Yield of HRPc EA (%)		46.62	
Yield total: mg (%)		41.47	

molecules. Using values from the calibration curve (r^2 = 0.93) calculated on the Sepharose S-200, and based on the elution interval where peaks (with or without HRPc activity) were detected, the molecular weight of conjugates was calculated resulting in values from 109.7 ± 16.5 to 67.6 ± 10.1 kDa for both peaks 1 and 2; and between 60.4 ± 9.1 to 29.9 ± 4.5 kDa for the fourth peak where no EA was detected. Some of the previous analyzed fractions were also evaluated by WB with the aim to prove the presence of the antigen in conjugates (Fig. 3D). The most of the resulting bands displayed on SDS-PAGE are also shown in WB (indicated by asterisks) meaning that many of the conjugates are enzymatically active and are captured by anti-rK346 IgG during the ELISA as well.

Sulfo-SMCC

Fractions with HRPc activity were analyzed by ELISA each well containing 820 ng of IgG. The signals showed values between 0.63 ± 0.11 and 3.41 ± 0.07 (Fig. 4B) corresponding to signals between 1.5 and 8.1 times higher than those given by the non-conjugated HRPc (0.42 ± 0.23; see threshold in Fig. 4B), indicating that conjugates contain the rK346 antigen coupled to the enzyme allowing the conjugates to be captured by the anti-rK346 IgGs. Measurements of EA and the quantification of HRPc-rK346 conjugates are shown in Table II. The conjugation yield using sulfo-SMCC resulted in lower values: 41.47% of recovery from the initial EA; 46.62% of total mg and RZ of 0.2 for the peak 1. These fractions were also visualized on SDS-PAGE (Fig. 4C) where it was observed the presence of a heterogeneous population of conjugates (indicated by asterisks) with molecular weights greater than 66 kDa eluting relatively before the non-conjugated HRPc molecules allowing a much more precise separation compared with GA-based conjugation. The molecular weights of complexes resulted in values between 150.5 ± 22.6 and 80.0 ± 12.0 kDa for peaks 1 and 2 and between 64.8 ± 9.7 and 48.9 ± 7.3 kDa for the peak 4.

In addition, some fractions with the highest EA were also evaluated by WB to prove the antigenic character in conjugates (Fig. 4D). It was observed that most of the bands on the SDS-PAGE are also shown in the blot (indicated by asterisks), meaning that conjugates are enzymatically active and can be captured by the anti-rK346 IgGs. Furthermore, it was also noted that a minority population of rK346-rK346 conjugates corresponded with the 45 kDa molecular marker. Fractions within the peak 1 (in this case represented by the pool 1) possessed the highest proportion of active HRPc-rK346 conjugates since the signal intensities on fractions of the peak 2 (represented by the pool 2) were quite lower.

DISCUSSION

There is a wide difference between the number of cases reported for VL and the real cases due to several factors, including life-style conditions, accessibility and availability of resources for the implementation of inexpensive and effective large-scale diagnostic techniques. Applying the appropriate and well-defined techniques, we should know how many infected people could develop the disease and how many would be diagnosed before they show clinical manifestations (37). VL diagnostic techniques have been mainly divided into three categories: (i) methodologies comprising the direct parasite visualization; (ii) those using DNA as target (e.g. the PCR), resulting in techniques with high values of sensitivity and specificity, but inaccessible in developing countries; and (iii) large-scale serological methods such as IFAT or DAT which are continuously applied as reference techniques (38,39). In the past recent years, the ELISA has been the most used technique for VL diagnosis given its practical procedure but also because it seems to be the most appropriate

standard technique to evaluate several serological samples in parallel. Moreover, the sensitivity and specificity values that ELISA incorporates are both considerably higher if appropriate antigens are used. Some examples are cited: the recombinant proteins rLACK, rP20, rK26 and especially the rK39 (37,38, 40 - 44).

In this work, it was designed and characterized a reporter system of antibodies against one of the most antigenic proteins from *L. infantum*, the recombinant protein rK346. The high yield and purity obtained (1.91 mg) from a culture of 25 mL is quite suitable for requirements in mg that the conjugation reactions need. In the case of IgGs, it was found that such antibodies were able to recognize the rK346 antigen (Fig. 2) without cross-reactions with antigens involved in other diseases caused by kinetoplastides such as Chagas disease, whereby the specificity of our system is assured. Finally, during the purification steps of HRPc, the predominant isoform in horseradish roots (45,46), evidence of purity was obtained (Fig. 1C) with a total yield of 7.69 mg; the total units corresponding to 2710; and the RZ $\geq$ 3 crucial for conjugation reactions. For diagnostic methods, the enzyme is commonly coupled to antibodies and other biomolecules, previously considering an analysis on the presence and reactivity of target residues that in the case of rK346 and HRPc proteins corresponded respectively to values of 13.6% and 9.7 % when only basic residues are analyzed. Among the advantages included in our results, we have (i) a decreasing on the probability of losing the biological function of both antigen and enzyme by steric hindrance, (ii) a decreasing on the probability of formation of non-soluble polymers even though their possible high reactivity and (iii) the adaptability to common protocols using crosslinkers reacting with basic residues. Based on these observations, we used GA as a homobifunctional crosslinker and sulfo-SMCC as a heterobifunctional reagent to compare the functionality of the conjugates given that the chemical modification of lysines and cysteines has been extensively reported.

During the biconjugation tests with GA, several variables as pH, temperature, molecular ratios (MR) between proteins and between the proteins and GA, as well as time and order of incubation with the crosslinker, were considered. The results incubating the antigen with GA and the modified protein with the HRPc at a MR<6, showed relatively low absorbance values (0.15 $\pm$ 0.01 – 0.53 $\pm$ 0.01) perhaps due to the low rate of reaction with GA (data not shown), resulting in a low proportion of HRPc coupled to the antigen, but also due to the competition events between conjugates (rK346-rK346) and those composed of HRPc-rK346 therefore decreasing the signal intensity.

In previous studies (47) the GA has been used for conjugation between the HRP and antibodies in two-step reactions. These have been reported by the presence of non-conjugated molecules after crosslinking, given that the molecular configurations and the accessibility to target amino acid residues in the second step change; therefore, the second reaction is not completely feasible. It could be argued that given the high intrinsic reactivity of the rK346 antigen at the end of the first reaction; a high proportion of rK346-rK346 conjugates are formed leaving a minimal fraction of free molecules to react in a second step with the HRPc. Furthermore, it has been reported that GA based reactions generate polymeric conjugates which are eluted by SEC near the void volume and with no detectable EA (48). For these reasons, it was decided to change the order of chemical reactions by first incubating the HRPc at six times greater than the antigen molecules at lower pH (6.8) resulting in a 5-fold higher signal than those obtained during the first tests. This represents a more efficient conjugation, characterized by a reaction with a lower probability of polymerization. Given the nature of both antigen and GA, one-step reactions are disadvantaged when coupling the en-

zyme to the rK346 protein, even when these reactions are performed for less than 10 hours. In similar works (49,50), conjugation protocols were applied at a pH near to neutrality and with incubation times of 18 hours to allow, at least, a small proportion of modified HRPc molecules to be available for subsequent coupling.

The peak with the highest conjugated-HRPc EA detected between 323 and 388 mL of elution coincided with two bands visualized on Western-blot analysis (Fig. 3A and 3D) meaning that the first peak probably contains conjugates with a high proportion of enzyme, whereas the second peak comprises conjugates wherein the antigen is predominant. Maybe, the detection of two overlapping peaks is due to the ability of the resin to separate a conjugate with a molecular weight relatively similar to that of the HRPc (44 kDa). Its exclusion range is greater than 70 kDa, which prevents somewhat a complete and efficient separation between conjugates and free enzyme molecules. On the other hand, it could also be due to non-specific interactions between the conjugated and the non-conjugated enzyme. Visualizing HRPc-active fractions by SDS-PAGE (Fig. 3C), we observed conjugates of high molecular weights (> 97 kDa) as well as another important proportion co-eluting with non-conjugated HRPc molecules showing molecular weights between 66 and 97 kDa. However, when these values are calculated from the calibration curve on SEC, results on a range from 67.6 ± 10,1 kDa to 109.7 ± 16.5 kDa.

Considering these values and the diminution of the departing RZ by a factor of four by the presence of antigens in conjugates, it could be postulated the formation of a heterogeneous mixture of conjugates composed by HRPc:rK346 ratios of 2:1 (105 kDa); 1:2 (78 kDa); 1:3 (96 kDa); and 2:2 (122 kDa). These ratios support the results obtained during the evaluation of conjugates in ELISA plates (Fig. 3B) in which values were ranging between 1.7 and 1.4 times greater than signals obtained using the first scheme of conjugation. Having a relative higher proportion of HRPc coupled to the antigen, the signal intensity is enhanced resulting in a more efficient system in terms of capture by IgG anti-rK346.

In bioconjugation protocols with sulfo-SMCC, we varied the pH, temperature and the MR between proteins and sulfo-SMCC (and/or between SATA), as well as the time and order of incubation with the corresponding crosslinker. The results for the six tests analyzed by a direct ELISA (data not shown) displayed relatively higher absorbance values (2.01 ± 0.19 to 3.04 ± 0.09) compared to those obtained using GA. In addition to the characteristics of conjugates being captured by antibodies, bioconjugation with sulfo-SMCC involved a more favorable and efficient reaction, where the homopolymerization rate between molecules is considerably decreased whereas the selectivity and the reaction control are both increased. Furthermore, the modification of HRPc with sulfhydryl groups changes its structure but not its enzymatic function (data not shown). Indeed, there are reports where protein modifications with SATA do not significantly affect the protein function (51,25). Based on the chromatogram, on differences in the EA detected between first and second peaks, and on the elution profile of the non-conjugated enzyme (Fig. 4A, highlighted in blue), it could be proposed that the first peak corresponds to the EA of conjugates and the second one to the EA of a minority population of these later but also corresponding to the non-conjugated HRPc. The proximity of these two peaks may be due to the resolution of the resin during SEC already mentioned above. However, in this case, the separation between conjugates and the free enzyme is more marked when using sulfo-SMCC, so with a greater proportion of conjugates eluting between 294 and 319 mL whereas the free enzyme enrichment is displayed between 319 and 344 mL of elution (Fig. 4C). Pooling each one of these elution ranges separately, we founded

that pool 1 is characterized by the presence of HRPc-rK346 conjugates compared to the pool 2 where the intensity of the band corresponding to the free enzyme is increased. This finding was further confirmed in Western-blot analysis (Fig. 4D). In SDS-PAGE analysis (Fig. 4C), we observed conjugates with molecular weights ≤ 97 kDa. The band under the 45.0 kDa molecular marker may be due to the formation of rK346-rK346 polymers due to the reactivity of the antigen but in a lower rate. Given that sulfo-SMCC reacts with sulfhydryl and amine groups on proteins, homoconjugates may form assuming that a small proportion of all cysteines in the antigen (1.4%) are not forming intramolecular disulfide bridges.

Calculating the molecular weight range of peaks with HRPc activity, we obtained values from 150.5 ± 22.6 to 80.0 ± 12.0 kDa. In the fourth peak (Fig. 4A), values were ranging from 64.8 ± 9.7 to 48.9 ± 7.3 kDa. Considering these results, and the decreased RZ by a factor of two (compared to the initial RZ), we can argue the formation of a heterogeneous mixture of conjugates composed by the following HRPc:rK346 ratios: 1:2 (78 kDa); 2:1 (105 kDa); 2:2 (122 kDa); and 3:1 (149 kDa), that might be directly related to the results obtained by ELISA, reporting absorbances between 0.63 ± 0,11 and 3.41 ± 0.07 (Fig. 4B) that correspond to values between 2 and 4 times greater than signals obtained with GA. Again, having a relative higher proportion of HRPc coupled to the antigen, the signal intensity is enhanced resulting in a much more efficient reporter system in terms of complexes IgG-Antigen. Note that despite high signals, the conjugation yield with sulfo-SMCC (Table II) was found to be less than the one obtained with GA (Table I) in terms of total EA and recovered mg. This could be attributed to a relative loss of non-coupled antigen, which could consequently be eluted between 390 and 430 mL during conjugation with sulfo-SMCC (Fig. 4A). Thus, clear differences in stability, yield and performance are detected by conjugating proteins with GA or with sulfo-SMCC. More refined procedures have reported that performance in EA becomes higher when heterobifunctional crosslinkers are used. In these works, values between 79 and 100% based on heterobifunctional cross-linkers and between 52 and 77% based on homobifunctional reagents (51, 52) are reported. The molecular ratios HRP:IgG obtained with these methodologies correspond to values between 1:3 and 1:1 using both crosslinkers whereas such ratios (obtained with our methodologies and for conjugation between HRPc and rK346) are reported to be 2:2 - 2:1 - 1:2 and 1:3 with GA; and 3:1 - 2:2 - 2:1 and 1:2 with sulfo-SMCC indicating that this last is more efficient.

A crucial aspect in these findings corresponds to the preservation of the antigenic specificity of the rK346 protein after both modifications and coupling with the used reagents. This is supported by the fact that in all tests, with our experimental conditions, the capture capacity of IgG anti-rK346 remains active so enabling conjugates to be used in detection techniques using sera samples. Other studies also suggest that using sulfo-SMCC allows for the conjugates to be lyophilized with a longer half-life, given the cyclic nature and stability of this crosslinker (28,53).

Many biomedical and biotechnological advances have been achieved with protocols for coupling biological molecules in which antibodies, antibody fragments, nucleic acids, proteins, hormones, peptides, and other biomolecules participate to generate constructs with defined properties and a broad applicability reflected in drug design as well as in toxins, fluorophores, inhibitors, protein complexes, detection systems, among others. The refinement and applicability of these tests have led to multiple infections caused by microorganisms to be detected. In addition, the progression of any disease in infected individuals can be also monitored with

these advances. Furthermore, the fact of applying a direct ELISA entails independence about using secondary antibodies and hence an important decreasing in cost and time, both required when obtaining polyclonal antibodies. Therefore, the marketing of a specific, sensitive, reproducible method and easy to apply could be provided to ensure the benefit and accessibility to a large population of individuals including those at risk and those with the disease but showing no symptoms.

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