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The diagnostic performance of recombinant *Trypanosoma cruzi* ribosomal P2β protein is influenced by its expression system

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Abstract

In the present work, we have determined the effect of expression vectors and their corresponding host bacteria on the antigenic performance of *Trypanosoma cruzi* P2β (TcP2β) full-length recombinant protein. The gene encoding the TcP2β ribosomal protein was cloned in pMAL-c2 and pET-32a vectors that allow the expression of high levels of soluble fusion proteins. A panel of 32 positive and 32 negative sera was assayed with the purified proteins expressed using pMal-c2 (TcP2β–MBP) and pET-32a (TcP2β–TRX) vectors and with MBP and TRX purified from pMAL-c2 and pET-32a vectors, respectively. The antigenic behavior of each TcP2β recombinant protein differed in the diagnostic performance in terms of DI(+) (93.7 for TcP2β–MBP vs 100% for TcP2β–TRX), in DI(−) (90.5 for TcP2β–MBP vs 100% for TcP2β–TRX) and in cross-reaction with negative sera. To determine if the higher reactivity of expressed pMAL-c2 protein was due to folding during protein expression or to a steric effect related to the protein adsorption at the titration plate, the reactivity of sera against soluble proteins was assessed by ELISA inhibition assays. As each soluble protein preserved its level of reactivity, we concluded that differences in reactivity were due to intrinsic characteristics of the proteins and not to differences in patterns of adsorption to the plates.

Chagas' disease is one of the main public health problems in Latin America, where 16–20 million people are infected with *Trypanosoma cruzi* [1]. The infection is characterized by an acute phase, which appears shortly after the infection, and by a chronic phase, which develops after several years of an asymptomatic period where 27% of the cases develop cardiac lesions, 6% digestive damage, and 3% peripheral nervous involvement [2]. The direct parasitologic assay is highly sensitive to diagnose acute cases but not for patients with chronic disease. In these cases, the most important diagnostic method used consists of indirect protocols for the detection of antibodies against parasitic antigens that are mainly enzymo-immunoassays, indirect hemagglutination, and immunofluorescence [3]. The antigens most frequently used may be classified into two different groups: undefined antigens, which consist of whole extracts or semi-purified fractions of *T. cruzi* proteins [4], and defined antigens, which consist of purified [5–8] or recombinant ones (for a review see [9]). Most of commercially available diagnosis systems rely on the use of undefined antigens that are relatively inexpensive. However, recombinant antigens are replacing them in some diagnostics kits due to their high diagnostic sensitivity (detectability index of negatives) and reproducibility. In the recent history of recombinant antigenic protein selection, the most common procedure used has been to construct cDNA libraries in phages and perform an immunoscreening on them, using human positive sera or experimental immunized animal sera as probes [10–12]. Recently, based on the increasing offer of new commercially available vectors, several expression systems for *Escherichia coli* have been used [13–16] to obtain *T. cruzi* recombinant proteins. When the

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performance of recombinant antigenic proteins was analyzed for diagnostic purposes, authors generally described the characteristics of the cloned sequences but did not emphasize the criteria for the selection of expression systems with regard to the antigenic activity of the molecule. To obtain comparable results, some authors used the same vector to study different proteins [14], but did not analyze the influence of a substitution in vector and/or bacterial host on the antigenic activity of the protein.

To compare the influence of the expression system on the diagnosis profile, we choose an antigenic protein as a model. In this regard, the complete *T. cruzi* P2β ribosomal protein was considered as an interesting protein because this polypeptide shows high reactivity levels with human sera from chagasic patients and low reactivity with non-infected human sera (although it shows some cross-reactivity with Lupus human sera [17]). Commercial plasmids pMALc2 (New England Biolabs) and pET 32a (Novagen) were used as expression vectors. In both cases, the fused proteins were expressed in its soluble form, so denaturation–renaturation processes were not necessary and did not influence the antigenic pattern. In the present work, the reactivities of proteins expressed using both systems against chagasic and non-chagasic human sera were compared. The obtained results showed different reactivity patterns for each fusion protein.

Materials and methods

Patients' sera

Positive sera were obtained from chronically infected chagasic individuals ($n = 32$) from an endemic region located in northeast Argentina. Negative sera were obtained from healthy blood donors ($n = 32$) of the same region. The *T. cruzi* infection status was determined by using two conventional tests: commercial ELISA and IHA based on parasite homogenate antigens. Positive sera for both reactions were considered as true positives. Negative sera for both reactions were considered as true negatives.

Expression screening

The protein used for this work was cloned in the context of a screening program to establish a bank of proteins with diagnosis perspectives. Briefly: a *T. cruzi* trypomastigote cDNA library constructed in λgt11 phage was kindly provided by Dr. Mariano Levin (INGEBI—University of Buenos Aires). Immunoreactive proteins were screened from approximately 10^4 PFU. Petri dishes were plated and incubated at 42 °C until plaques formed. Nitrocellulose filters, pre-wetted with 10 mM isopropyl-β-D thiogalactopyranoside (IPTG),[1] were placed on the plates and then incubated for 3 h at 37 °C. The filters were then washed three times with phosphate buffered saline (0.16 M NaCl; 0.0027 M KCl; 0.01 M Na_2HPO_4; and 0.0018 M KH_2PO_4; pH 7.2) containing 0.05% Tween 20 (PBST), blocked with 5% low-fat milk in PBS, and washed three times with PBST. The filters were then incubated for 1 h with λgt11-*E. coli* absorbed pool sera of 140 chronic patients, washed three times with PBST, and incubated with goat anti-human immunoglobulin G peroxidase conjugate. The filters were finally washed three times with PBST and developed with 0.4% hydrogen peroxide and 3,3′-diaminobenzidine tetrahydrochloride in PBS. Reactive plaques were then excised and purified in second and third steps. The plaques showing the highest reactivity were selected and the DNA contained in these clones was purified. The inserts were amplified by PCR using λgt11 specific primers forward and reverse (Sigma). The PCRs were carried out with purified λgt11 DNA using a standard protocol in the presence of 1.25 mM $MgCl_2$. The program used in all cases was: denaturation, 30 s at 95 °C; annealing, 30 s at 50 °C; and extension, 2 min at 72 °C. The obtained amplicons were subcloned in a pGEM-T easy vector (Promega). After *E. coli* transformation, the plasmidic DNA was obtained and inserts were sequenced using *Promega Kit fmol DNA sequencing System*. The sequences corresponding to the cloned genes were compared by local alignment with the GenBank database using the Blast program. Among this protein bank, the clone containing the full length ORF coding for the ribosomal *T. cruzi* protein P2β was selected for this study.

Subcloning of recombinant proteins

A *T. cruzi* trypomastigote cDNA clone encoding a 12 kDa protein (ribosomal P2β protein) was purified and subcloned in the *Eco*RI site of vector pET32a (Novagen) and pMALc2 (New England Biolab). The ligation mixtures were use to transform BL21 (DE3) and DH5α competent cells, respectively.

Expression and purification of recombinant proteins

Escherichia coli cells, containing the plasmids pET or pMAL as well as the same plasmids that have been ligated to the gene encoding the *T. cruzi* P2β protein, were

[1] *Abbreviations used:* IPTG, isopropyl-β-D thiogalactopyranoside; PBS, phosphate buffered saline; PBST, phosphate buffered saline plus Tween; H_2O_2, hydrogen peroxide; TMB, tetramethylbenzidine; TcP2β, *Trypanosoma cruzi* P2β ribosomal protein; MBP, maltose binding protein; TRX, thioredoxin; LB, Luria–Bertani; GST, glutathione *S*-transferase; Ni–NTA, Ni^{2+}–nitrilotriacetic acid; EDTA, ethylenediaminetetraacetic acid.

grown overnight in a shaker at 37 °C in 10 ml Luria–Bertani [18] liquid medium (LB) supplemented with 0.1 mg/ml ampicillin. An aliquot of this culture was diluted 100 times in LB medium and cells were grown under the same conditions until an OD_{600} of 0.5 was reached. TcP2β–MBP protein and MBP were purified with amylose resin (New England Biolab) following the manufacturer's instructions. TcP2β–TRX and TRX were purified using the Ni–NTA resin (Quiagen) according to the manufacturer's specifications. Briefly, cultures were induced to protein expression for 3 h with 1 mM IPTG, and then sonicated and centrifuged for 30 min at 4500*g* and 4 °C. The supernatants were passed through an amylose column, washed with 200 mM NaCl, 1 mM EDTA buffer and then eluted with the same buffer plus 0,2% maltose. The Ni–NTA column was washed with 50 mM NaH_2PO_4 (pH 8), 300 mM NaCl, and 50 and 100 mM imidazol buffer and then eluted with the same buffer plus 250 mM imidazol. The purity of the recombinant protein was analyzed by 12% sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis [19], followed by Coomassie blue staining. Protein quantification was performed with the Bradford assay [20].

ELISA with recombinant proteins

The optimum concentrations for sera, antigens, and conjugate were determined by a chessboard titration. The optimum concentrations of antigen were determined by checking amounts of 50, 100, 500, and 1000 ng/well for each protein and analyzing the best discrimination between two positive and two negative sera. Polystyrene microplates (Costar, USA) were then sensitized with 0.5 μg protein/well. Microplates were incubated with a 1/100 dilution of human sera in PBS-1% low-fat milk. After washing, the second antibody was added. All incubations were performed at 37 °C for 60 min. The reaction was developed with trimethylbenzidine in H_2O_2 and absorbance was read at 450 nm.

In inhibition assays, microplates were sensitized with TcP2β–MBP and sera were first incubated for 1 h at 37 °C with decreasing amounts (5, 0.5, 0.05, and 0.005 μg/ml) of TcP2β–MBP and TcP2β–TRX proteins.

Data analysis

The detectability index of positives, DI(+), is defined as the ability of the test to give a positive result for positive samples:

$$\mathrm{DI}(+) = \mathrm{TP}/(\mathrm{TP} + \mathrm{FN}) \times 100$$

and the detectability index of negatives, DI(−), is defined as the ability to give a negative finding for negative samples:

$$\mathrm{DI}(-) = \mathrm{TN}/(\mathrm{TN} + \mathrm{FP}) \times 100$$

TP and TN being the true positive and true negative samples, respectively, and the FP and FN being the false positive and false negative results obtained with the antigens that are being evaluated [21].

The results recorded as optical densities at 450 nm were distributed by using a computer scatter graphics software. The cut-off value for ELISA was calculated as the mean OD_{450} of the true negative sera plus 2 standard deviations (SD). Degrees of significance were assessed by *t* test. The κ indices [22] were calculated for the ELISA results according to the formula:

$$\kappa = (P_o - P_e)/(1 - P_e),$$

where P_o is the observed agreement and P_e is the expected agreement by chance. The normality was tested by Kolmogorov–Smirnov test and the comparisons were done with *t* test using GraphPad Prism Software.

Results

Antigen selection

Along the immunoscreening, a highly reactive λgt11 phage clone containing a cDNA coding for the TcP2β was purified. The insert was amplified by PCR as established in Materials and methods, subcloned into the plasmid pGEM-T-Easy^R for sequencing, and subcloned into the *Eco*RI sites of pMAL and pET polylinker site for expression. Using this protocol recombinant plasmids were obtained for TcP2β–MBP and TcP2β–TRX fusion proteins (Fig. 1). The transformations were carried out in DH5α and BL21 (DE3) strains of *E. coli*, respectively.

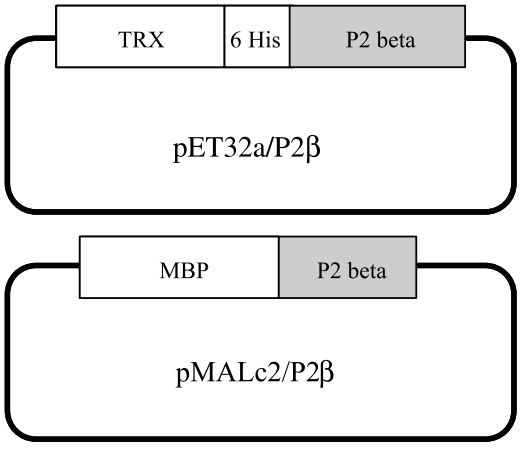

Fig. 1. Schematic representation of the constructions used in this work. The white boxes indicate sequences of the vectors. Shaded boxes indicate coding regions for the expressed proteins.

Expression and purification of recombinant proteins

Recombinant TcP2β–MBP and TcP2β–TRX were expressed. MBP and TRX plasmid proteins were also expressed by inducing the gene expression in the BL21 (DE3) *E. coli* carrying pET plasmid and the DH5α carrying pMAL plasmid, respectively. The purification yield was 30 mg/L of culture for pET expressed protein and 5 mg/L for pMAL expressed protein. The yield was similar for pET fusion protein TRX and pMAL fusion protein MBP. The protein purity was evaluated by SDS–PAGE. A single band was detected by Coomassie blue staining when 10 μg protein/well was used (Fig. 2).

Diagnostic performance evaluation

Antigenic evaluation was performed by enzyme immune assay. The values for detectability index of positives DI(+) and detectability index of negatives DI(−) were established. The wells were coated with 500 ng of the corresponding protein and the sera were diluted 1/100. Thirty-two positive and 32 negative sera were assayed for each protein. The antigenic behavior of each TcP2β recombinant protein was different in terms of DI(+) and DI(−). Thirty out of 32 true positive, sera were shown to be reactive for TcP2β–MBP (DI(+) = 93.7%) while all 32 true positive were reactive with TcP2β TRX (DI(+) = 100%). Twenty-nine out of 32 true negative sera resulted as non-reactive with TcP2β–MBP (DI(−) = 90.5%) while all of the true negative sera in the panel were negative for the assay using TcP2β TRX as antigen (DI(−) = 100%). These differences were quantified using the κ indices (observed agreements against chance-expected agreement by chance), which are 0.84 and 1.00 for TcP2β–MBP and TcP2β–TRX, respectively. These values corresponded to very good and total agreement, respectively [23]. Dispersion results for the different assays are shown in Fig. 3 and summarized in Table 1. In the dispersion graph, an important factor in the protein's behavior for diagnostic use can be observed: the non-specific reactivity of the sera with TcP2β–MBP protein is higher than with TcP2β–TRX protein. As the absorbance distribution was Gaussian for positive and negative sera, reactivity to each protein was compared by unpaired *t* test (Table 1). The reactivity of negative sera is higher when confronted to pMAL expressed protein than confronted to pET expressed protein (OD = 0.165 vs 0.086). The difference was significant giving a *p* value $<10^{-4}$. The dispersion for positive sera is similar when confronted to both proteins and, although the mean for the assay with pMAL expressed protein is slightly higher (OD = 0.803 vs 0.711), the difference is of low significance ($p = 0.207$).

On the other hand, when non-specific reactivity of the negative sera with both fusion proteins is analyzed, the reactivity with TcP2β–MBP is higher than the reactivity with MBP alone (OD = 0.165 vs 0.077) with a significant difference ($p < 10^{-4}$ for *t* test). We can see here that this reactivity is related to the conformation of the pMAL expressed protein and not to the fusion protein MBP. If the same assay is performed for negative sera with pET expressed protein, an OD of 0.086 is observed when confronted to TcP2β–TRX and an OD of 0.077 for the same sera with TRX. The difference is not significant ($p = 0.406$), indicating a similar reactivity against the complete fusion protein and against TRX alone. When the reactivities of positive and negative sera were compared against MBP, a small significant difference was

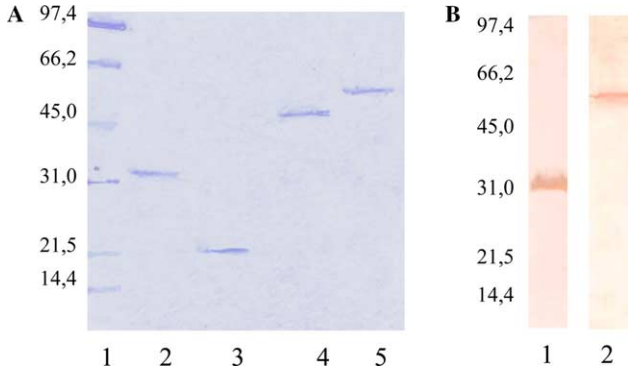

Fig. 2. Purification of proteins on Ni–NTA and amylose resins. (A) Coomassie blue staining of the affinity-purified recombinant proteins separated by SDS–PAGE. Lanes: 1, molecular mass protein marker; 2, TcP2β–TRX; 3, TRX; 4, MBP; and 5, TcP2β–MBP. (B) Western blot analysis of purified recombinant proteins. Purified recombinant proteins were electrophoresed on a 12% polyacrylamide gel and blotted to a nitrocellulose membrane. The membrane was developed using a pool of reactive sera and peroxidase conjugate anti-human IgG. Lanes: 1, TcP2β–TRX; 2, TcP2β–MBP.

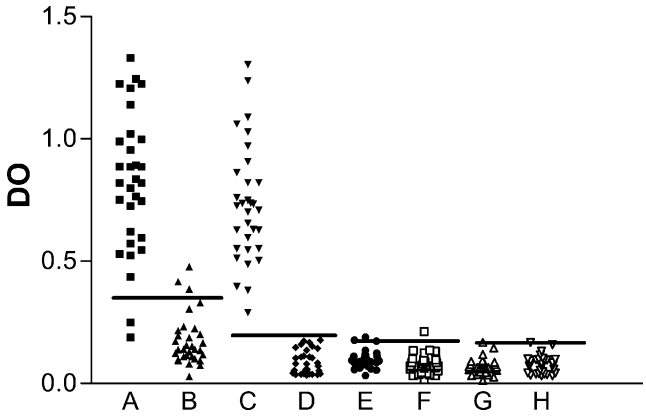

Fig. 3. IgG antibodies against *Trypanosoma cruzi* proteins in sera of patients as detected in ELISAs with each protein. Thirty-two positive sera (A, C, E, and G) and 32 negative sera (B, D, F, and H) were tested with TcP2β–MBP (A and B), TcP2β–TRX (C and D), MBP (E and F), and TRX (G and H). Horizontal bars represent the cut-off values (mean + 2SD of the negative sera) for each protein.

Table 1
Evaluation of serological reactivity for each group of sera in relation with the different antigens

Protein tested	Status of each group sample	Mean of each group sample	Standard deviation	Significance of the difference	Interpretation
TcP2β–MBP	(+)	0.803	0.281	$p = 0.207$	Positive sera show the same reactivity against
TcP2β–TRX	(+)	0.711	0.239		TcP2β–MBP and TcP2β–TRX
TcP2β–MBP	(−)	0.165	0.104	$p < 10^{-4}$	Negative sera show higher reactivity against
TcP2β–TRX	(−)	0.086	0.048		TcP2β–MBP than against TcP2β–TRX
TcP2β–MBP	(−)	0.165	0.104	$p < 10^{-4}$	Cross-reactivity against TcP2β–MBP is higher
MBP	(−)	0.077	0.039		than against MBP.
TcP2β–TRX	(−)	0.086	0.048	$p = 0.406$	There is no difference between reactivity against
TRX	(−)	0.077	0.035		TRX and TcP2β–TRX.
MBP	(+)	0.103	0.034	$p = 0.0229$	Positive sera hardly recognized MBP peptide.
MBP	(−)	0.077	0.039		
TRX	(+)	0.071	0.031	$p = 0.366$	Positive and negative sera display the same
TRX	(−)	0.077	0.035		reactivity against TRX peptide

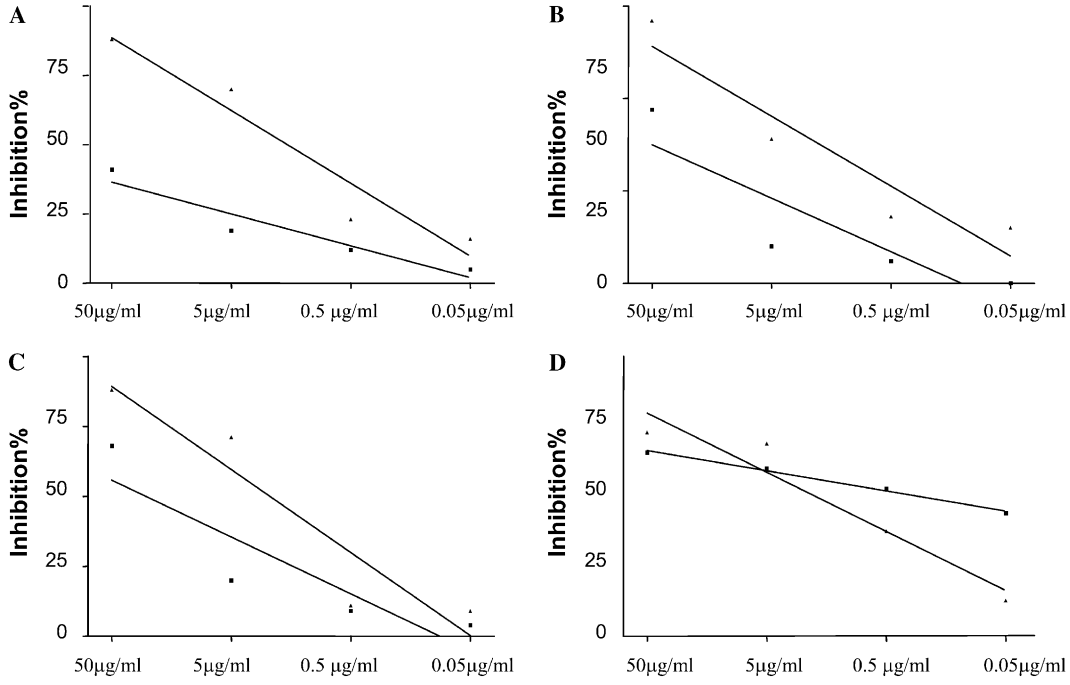

Fig. 4. Inhibition of binding of anti-P2 antibodies in negative sera (A, B, and C) and positive sera (D), showing high difference of reactivity between TcP2β–MBP (▲) and TcP2β–TRX (■). Results show the percentage of inhibition with decreasing amounts of soluble recombinant proteins.

observed ($p = 0.0229$ for t test). No difference was observed in the recognition of both sera populations for TRX ($P = 0.366$ for t analysis).

Determination of the effect of the folding along the expression and adsorption to the plate on the reactivity

Microplate wells were sensitized with TcP2β–MBP. Soluble proteins were evaluated with an inhibition test, by using increasing concentrations of soluble TcP2β–MBP and TcP2β–TRX (Fig. 4). The sera (three negative sera showing the most strong reaction against TcP2β–MBP and one positive serum showing the most strong reaction against TcP2β–MBP) were assayed. A high reactivity was observed with soluble TcP2β–MBP while a low reactivity was observed with TcP2β–TRX.

Discussion

It is well recognized that the optimization of a serological diagnosis assay relies on several factors beyond the choice of antigens, such as the physico-chemical characteristics of the immobilization surface, and blocking and washing conditions [24]. In the present work, we demonstrate that the choice of the expression system used to obtain a recombinant antigen is a major factor to be taken into account for diagnosis purposes.

Two different expression vectors with their corresponding *E. coli* strains were used to produce the complete *T. cruzi* ribosomal recombinant protein P2β fused to MBP or TXR. The diagnostic performance was evaluated for both fusion proteins using a well-characterized panel of human sera from infected or not infected people as a standard. For both systems, the one-step affinity purification allowed us to obtain in a straightforward procedure the antigenic protein for diagnostic assays. Different research groups have used this approach and molecular cloning in different vectors to assess different antigens. Even if in some studies researchers cleaved proteins enzymatically [13] and used purified protein fractions, in most cases the complete fusion proteins were used. To evaluate the fusion protein reactivity in the assay, some studies have established the behavior in ELISA of these molecules against each positive and negative serum in evaluation in the study [14,16]. In this regard, for instance, the DI(+) for glutathione *S*-transferase (GST) was established at 4% for chagasic patients [14]. Keeping this in mind, when assessing the DI(+) and DI(−) indices for the recombinant proteins, the fused purification tag added to the antigen of interest by different vectors was not considered to play a role in the protein's diagnostic characteristics. An interesting point that raised along this work is the fact that it was not possible to predict the influence of the peptide used as a purification tag from the analysis of the performance of these peptides isolated. In fact, it was observed that the antigenic characteristics of each fusion are a property of the whole construction and not only a sum of the properties of individual components. Our results are compatible with those obtained by Sepulveda et al. (2000) by expressing the TcP2β using two different vectors. They observed that different constructions gave rise to different immunological responses in mice, confirming that the development of the immune response against different epitopes is a function of the expression system [25].

To optimize the ELISA antigen-conjugate concentrations, the same conditions were chosen for both proteins. The protein expressed in pMAL showed a stronger reactivity against negative sera and this increased dispersion and optical density. Therefore, the cut-off value for the assay also increased. Positive sera had a slightly higher reactivity with pMAL expression protein than for pET expression protein, but the increased value of the cut-off decreased the assay's sensitivity with the first one. Having obtained a higher non-specific reactivity for TcP2β–MBP than for TcP2β–TRX, we asked ourselves if this was due to a different protein (vector-conditioned) folding in the synthesis process or to a conformation change during adsorption to the ELISA wells. To discriminate both situations, an inhibition assay was performed. Three negative sera showing a big difference in background values for both proteins and a positive serum with a big difference in reactivity for the same proteins were selected. In both cases, their reactivity was higher against the soluble protein TcP2β–MBP. These results reveal a non-specific reactivity given by the structure of the fusion protein. The cross-reactivity of the fusion protein is higher than the one that MBP epitopes could contribute to leading to a less specific assay and contributing to false positive results. Compared to the reactivity of negative sera with MBP and TcP2β–MBP, the difference in values is significant (see Table 1 $p < 0.0001$ for t test). Therefore, reactivity is increased when tested against the fusion protein. The inhibition assay also indicates, as described by other authors for similar assays [26], that the avidity of specific antibodies in the sera used is higher for the pET expressed protein than for the pMAL expressed protein. In fact, high dilutions of soluble TcP2β–TRX produce more inhibition than high dilutions of soluble TcP2β–MBP. It is worth mentioning that along the *T. cruzi* infection, the rising of antibodies that display different avidities with respect to different antigens [27] was observed. These antibodies present a strong avidity to bind their ligands, which allow detecting them in high stringency conditions with high sensitivity and specificity. In the particular case of the antigen used in the present work, the selection was based on a comparatively strong signal/noise relationship. The higher level of avidity displayed by the protein, when fused to TRX, is a relevant point that needs to be taken into account since it may help us to diminish unspecific cross-reactivity. In conclusion, when a recombinant protein is produced for antibody determination the influence of the expression system is a major variable to be considered since it may affect the sensitivity and specificity of the serologic assay.

Acknowledgments

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