



Cloning and Characterization of the Gene Encoding the Heat Shock Protein HSP83 from *Trypanosoma cruzi*

Clonación y caracterización del gen codificante de la proteína de choque térmico HSP83 de Trypanosoma cruzi

Clonagem e caracterização do gene que codifica a proteína de choque térmico HSP83 do Trypanosoma cruzi

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Abstract

[Objective] *Trypanosoma cruzi*, a causal agent of Chagas' disease, is a parasite whose life cycle alternates between an invertebrate (triatomine) and a vertebrate (mammal) host. Various studies have shown that in *T. cruzi*, HSP83 (a homolog of HSP90) is essential for cell division and control of the response to thermal stress. This investigation focused on studying the cloning, bioinformatic characterization, and expression of the heat shock protein HSP83 *T. cruzi* gene for further cell signaling studies. **[Methodology]** RNA was extracted from *T. cruzi* epimastigotes (EPm6 clone, MHOM/VE/2007/ 6c) using a commercial kit. The cDNA encoding HSP83 was determined using RT-PCR on extracted mRNA, for which the primers were designed based on the HSP83 sequence of *T. cruzi* strain CL Brener. Cloning was performed using pGEM[®]T-Easy and subcloned into the expression vector pQE30. Sequence and bioinformatic characterization were performed. The gene was expressed, and the recombinant protein was purified using affinity chromatography and identified through immunoblotting. **[Results]** Sequence analysis showed similarity to the gene encoding HSP83 from *Trypanosoma cruzi*, and HSP domains and B epitopes in the sequence were also observed. After 3 hours of induction with IPTG, a recombinant protein with an approximate weight of 83 kDa was obtained. The immunoblotting reaction with hyperimmune anti-*T. cruzi* epimastigote serum helped detect a single band with a molecular weight of nearly 83 kDa. **[Conclusions]** All results indicate that the cloning and characterization of HSP83 from *Trypanosoma cruzi* was achieved.

Keywords: *Trypanosoma cruzi*; heat shock protein; HSP83; cloning; expression.

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Resumen

[Objetivo] *Trypanosoma cruzi*, un agente causal de la enfermedad de Chagas, es un parásito que presenta una alternancia en su ciclo de vida entre un hospedador invertebrado (triatomino) y uno vertebrado (mamífero). Varios estudios han demostrado que en *T. cruzi*, HSP83 (homólogo de HSP90) es esencial para la división celular y el control de la respuesta al estrés térmico. Esta investigación se concentró en el estudio de la clonación, la caracterización bioinformática y la expresión del gen de la proteína de choque térmico HSP83 de *T. cruzi* para estudios posteriores sobre señalización. **[Metodología]** El ARN fue extraído de epimastigotes *T. cruzi* (clon EPm6/MOHM/VE/2007/ 6c), utilizando un kit comercial. El ADNc que codifica HSP83 se obtuvo utilizando RT-PCR, a partir del ARNm extraído, para lo cual se diseñaron los cebadores con base en la secuencia HSP83 de la cepa *T. cruzi* CL Brener. La clonación se realizó usando pGEM®T-Easy y se subclonó en el vector de expresión pQE30. Se efectuó la caracterización bioinformática y de secuencias. El gen se expresó y la proteína recombinante se purificó por cromatografía de afinidad, igual que se identificó por inmunotransferencia. **[Resultados]** El análisis de secuencia mostró similitud con el gen HSP83 de *Trypanosoma cruzi* y se observaron dominios HSP, así como epítopos B en la secuencia. Después de 3 horas de inducción con IPTG, se obtuvo una proteína recombinante con un peso aproximado de 83 kDa. La reacción de inmunotransferencia con suero hiperinmune anti-epimastigote de *T. cruzi* permitió la detección de una sola banda con un peso molecular de aproximadamente 83 kDa. **[Conclusiones]** Todos los resultados indican que se logró la clonación y caracterización de HSP83 de *Trypanosoma cruzi*.

Keywords: *Trypanosoma cruzi*; proteína de choque térmico; HSP83; clonación; expresión.

Resumo

[Objetivo] O *Trypanosoma cruzi*, agente causador da doença de Chagas, é um parasita que apresenta uma alternância em seu ciclo de vida entre um hospedeiro invertebrado (triatomíneo) e um vertebrado (mamífero). Vários estudos demonstraram que, no *T. cruzi*, a HSP83 (homóloga da HSP90) é essencial para a divisão celular e o controle da resposta ao estresse térmico. Esta pesquisa concentrou-se no estudo da clonagem, caracterização bioinformática e expressão do gene da proteína de choque térmico HSP83 do *T. cruzi* para estudos adicionais de sinalização. **[Metodologia]** O RNA foi extraído de epimastigotas de *T. cruzi* (clone EPm6/MOHM/VE/2007/ 6c) usando um kit comercial. O cDNA que codifica a HSP83 foi obtido por meio de RT-PCR a partir do mRNA extraído, para o qual os primers foram projetados com base na sequência da HSP83 da cepa *T. cruzi* CL Brener. A clonagem foi realizada com o pGEM®T-Easy e subclonada no vetor de expressão pQE30. Foi realizada a caracterização bioinformática e de sequência. O gene foi expresso e a proteína recombinante foi purificada por cromatografia de afinidade e identificada por imunotransferência. **[Resultados]** A análise da sequência mostrou similaridade com o gene HSP83 do *Trypanosoma cruzi* e os domínios HSP, bem como os epítopos B, foram observados na sequência. Após 3 horas de indução com IPTG, foi obtida uma proteína recombinante com peso aproximado de 83 kDa. A imunotransferência com soro hiperinmune anti-epimastigota de *T. cruzi* permitiu a detecção de uma única banda com peso molecular de aproximadamente 83 kDa. **[Conclusões]** Todos os resultados indicam que a clonagem e a caracterização da HSP83 do *Trypanosoma cruzi* foram alcançadas.

Palavras-chave: *Trypanosoma cruzi*; proteína de choque térmico; HSP83; clonagem; expressão.



Introduction

Trypanosoma cruzi, a causal agent of Chagas' disease, is a parasite that presents alternation in its life cycle between an invertebrate (triatomine) and vertebrate (mammal) host. Individuals with Chagas' disease may present cardiological, digestive, and neurological symptoms and might die. Currently, 6 to 7 million people are affected by the disease, and 25 million are at risk of infection (WHO, 2023). In Venezuela, increases in prevalence and oral transmission outbreaks have been described in several states (Alarcón de Noya *et al.*, 2015; Añez *et al.*, 2020).

The parasite response is essential for viability in the heat and osmotic stress during the transition from the insect to the mammalian host, as well as when invading mammalian cells. The contribution of heat shock proteins in morphological and functional changes during stress situations has been implicated. This class of proteins is divided into several conserved families according to molecular weight and broad function, among which the five major families are HSP104, HSP90, HSP70, chaperonins, and small HSPs (Folgueira & Requena, 2007; Urményi *et al.*, 2014). They participate in protein folding and cell signaling processes and play an important role in the host's immune response. They have been identified as dominant antigens in diseases caused by protozoa (De Andrade *et al.*, 1992; Requena *et al.*, 1993; Angel *et al.*, 1996; Menezes-Souza *et al.*, 2014) and helminths (Rothstein *et al.*, 1989; Moser *et al.*, 1990; Colebrook & Lightowers, 1997; Ferrer *et al.*, 2005).

There is evidence that indicates that in *T. cruzi* Hsp are constitutively expressed and induced by different types of stress.

In particular, 90 kDa heat shock proteins (HSP90) are involved in numerous intracellular processes in eukaryotic cells. Studies have shown that in *T. cruzi* HSP83 (homolog of HSP90) is essential for cell division and control of the response to thermal stress (Jhonson, 2012). The overexpression and the participation of HSP83 in the differentiation processes of *T. cruzi* have been suggested, and the inhibition of HSP83 of *T. cruzi* produces arrest in proliferation, demonstrating the importance in the control of the cell cycle (Graefe *et al.*, 2002).

To date, there are merely two old reports about cloning this molecule (Dragon *et al.*, 1987; Nadeau *et al.*, 1992), and only its genomic structure and ATPase activity have been studied. This study aimed to perform the cloning, bioinformatics characterization, and expression of the heat shock protein HSP83 of *T. cruzi* (EPm6 clon, MHOM/VE/2007/Elpidio Padrón 6c) coding gene. The study would enable subsequent studies on the adaptation processes of the parasite to different microenvironments, the identification of new therapeutic or diagnostic targets, and the study of host-parasite interaction in Chagas disease.

Methodology

Parasite Material

T. cruzi epimastigotes obtained from a human Chagas disease case (EPm6 clon, MHOM/VE/2007/Elpidio Padrón 6c) were cultivated at 27 °C in liver infusion tryptose broth (LITB) (Contreras *et al.*, 1994). Parasites were counted employing a Neubauer chamber and phase contrast microscope (Nikkon, Optiphot). They were adjusted to 1.5×10^9 epimastigotes/mL. Subsequently, the parasites were pelleted by centrifugation



at 8,000 rpm for 15 minutes at four °C (IEC Centrifuge, B22M) and washed twice in 0.15 M phosphate saline buffer (PBS) pH 7.2 (57 mM Na₂HP0₄, 18 mM KH₂P0₄, 76.9 mMNaCl) to eliminate the remains of the culture medium. Finally, they were stored at -80 °C until their use.

RNA Extraction and the Complementary DNA (cDNA) Obtaining

RNA extraction was carried out through the FavorPrep™ Viral Nucleic Acid Extraction Kit (Favorgene), where the parasite masses were previously treated with lysis buffer supplemented with Proteinase K (0.45 mg/mL) and RNase (80 U/μL). Then, the extraction continued with the instructions described in the commercial kit. The complementary DNA (cDNA) was obtained by implementing the Reverse Transcription-Polymerase Chain Reaction technique and using the Access RT-PCR System (Promega) commercial kit. Specific primers were designed manually using BioEdit® Software v7.1.11 (Hall, 2013) and, as a template sequence, the hsp83 of a reference strain of *T. cruzi* of 2115 bp (*Trypanosoma cruzi* strain CL Brener hsp83) obtained from GenBank (Accession No. XM_809799) strain used in the *T. cruzi* genome project (El-Sayed *et al.*, 2005). The primers used were TcHSP83-D (5'- ATGACCGAGACATTCGCA -3') and TcHSP83-R (5'- CTAGTCAACCTGCTC-CAT -3'). The reaction mixture consisted of AMV/Tfl reaction buffer, deoxynucleotide triphosphates (0.2 mM), forward and reverse primers (1 μM) each, MgSO₄ (1 mM), AMV Reverse Transcriptase (0.1 U /μL), *Thermus flavus* (Tfl) DNA polymerase (0.1 U / μL), 10 μL of the extracted RNA sample, resulting in a final reaction volume of 50 μL.

The amplification reaction was carried out in an automatic C1000™ Thermal Cycler (BioRad) using the amplification program. The reverse transcription was conducted at 45 °C x 45 min; the initial denaturation, at 94 °C x 2 minutes, 30 cycles (denaturation at 94 °C x 1 min, hybridization at 60 °C x 1 min, extension at 68 °C x 2 min); and the final extension, at 68 °C x 7 min.

Electrophoresis

The amplification products of RT-PCR reactions were detected by electrophoresis (2% agarose gel, stained with 0.5 μg/mL ethidium bromide). The DNA migration was conducted in a horizontal electrophoresis chamber (Minicell * EC 370M), with a constant 60-100 V voltage and TAE buffer (40 mM Tris-acetic acid, 0.5 M EDTA, pH 8). DNA bands were visualized on a photodocumentation system, Gel Doc 1000 (Bio-Rad®), using the Multi-Analyst Program (Bio-Rad®); the size of the amplification bands was compared with a molecular marker of 1 kb DNA Ladder (Bioneer).

cDNA Purification and Cloning

The cDNA samples to be purified were fractionated on agarose gels and prepared in the TAE buffer, and the bands were visualized on the ultraviolet light transilluminator. The fraction of interest was cut with a sterile scalpel, and the DNA was eluted with the commercial Wizard®SV Gel and PCR Clean-Up System kit (Promega), according to the manufacturer's instructions. For the ligation reaction of the purified PCR products and the commercial vector pGEM®-T Easy, 1 U of the T4 DNA ligase enzyme was used. A plasmid-insert molar ratio 1: 5 was used in [30 mM Tris-HCl (pH 7.8), ten mM MgCl₂, ten mM DTT, one mM ATP] buffer



and incubated at four °C for 12 h. After confirming the hsp83 into pGEM®-T Easy, this recombinant molecule was digested, and the purified cDNA was subcloned in the expression plasmid pQE30 (Qiagen, Valencia, CA).

cDNA Sequencing and Bioinformatic Analysis

Sequencing of cloned cDNA was achieved at the Sequencing Service of the National Center for Microbiology, Instituto de Salud Carlos III, Madrid, Spain, using 373A system, Model 377, Applied Biosystem, following the protocol Big-Dye Terminator Cycle Sequencing Ready Reaction Kit (ABI-PRISM, PE Biosystems). The cDNA sequence obtained was compared with the sequences included in the databases (nucleic acid and proteins) (GenBank, EMBL) and analyzed by bioinformatics programs. Multiple alignments were done using the Clustal W. The alignment was revised using BioEdit Sequence Alignment Editor, version 7.0.5.3 (Hall, 2013). Nucleotide and amino acid sequences were analyzed using the EditSeq program of DNASTar (Lasergene®, Madison, USA). Identity and similarities were looked for in the databases (protein and nucleic acid) (EMBL, GenBank) by BLAST (Boratyn *et al.*, 2019). Other analyses were performed using CDD-Search of the NCBI (National Center for Biotechnology Information) (Lu *et al.*, 2020), Interpro of the EBI (European Bioinformatics Institute) (Mitchell *et al.*, 2019), Motif scan, and ExPASy (Expert Protein Analysis System) of the SIB (Swiss Institute of Bioinformatics) (Proteomics Server) (Artimo *et al.*, 2012). Epitopes B prediction was performed using the Protean program from DNASTar (Lasergene®, Madison, USA). In addition, the program

BcePred (Prediction of continuous B-cell epitope in antigenic sequences using physico-chemical properties) (Saha *et al.*, 2005) was employed.

cDNA Expression

Recombinant His-fusion proteins were expressed in *Escherichia coli* M15 (Qiagen, Valencia, CA) by induction with isopropyl-β-D-thiogalactopyranoside (IPTG, one mM) (Sigma, Madrid, Spain). The cultures were lysed using sonication and examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the recombinant proteins were purified by affinity chromatography with nickel agarose as described by the manufacturer (Qiagen, Valencia, CA). The protein concentration was determined by the Bradford technique (Bradford, 1976). The purity of the proteins was analyzed by SDS polyacrylamide gel electrophoresis (SDS-PAGE), done under denaturing conditions according to Laemmli (1970), on 10% separation gel and 4.5% packing gel at 15 mA. After separating the sample, the gel was stained with Comassie Blue-Silver (De Moreno *et al.*, 1985) for 45 min, then placed in the bleaching solution until the sharp bands were visible. The molecular weight (MW) of the expressed proteins was compared with the Broad Range Protein Molecular Weigh Markers (Promega).

Western Blot (Immunoblotting)

After the proteins were separated by electrophoresis, they were transferred to a nitrocellulose membrane, according to Towbin *et al.* (1979), using a wet system and the Mini Trans-Blot® Cell (BioRad) equipment. For this, two sponges and two



filter papers the size of the gel, impregnated in transfer buffer and the nitrocellulose membrane, were placed, and the gel was placed on the membrane. Then, two filter papers and a sponge equally impregnated with the buffer were placed in the same way and transferred at 100 mA for one hour at four °C. After the transfer had elapsed, the nitrocellulose membrane was incubated in blocking solution (0.15M PBS pH 7.2, 7.5% skim milk) overnight at four °C. Subsequently, it was incubated with anti-Epi rabbit hyperimmune serum (against *T. cruzi* epimastigotes) in a 1/1500 dilution in reaction solution (0.15 M PBS pH 7.2, 150 mM NaCl, ten mM Tris-HCl pH 7.4) for 90 min at 37 °C. Then, eight washes of 5 min each were performed with the wash solution (0.15 M PBS pH 7.2, 0.1% Tween 20) and subsequently incubated with the conjugate, anti-rabbit IgG coupled to peroxidase at a dilution of 1/3000 in reaction solution (PBS 0.15 M pH 7.2, NaCl 150 mM, Tris-HCl 10 mM pH 7.4) and under the same conditions described above; then, the washes were repeated. The visualization of the immune complexes was carried out by chemiluminescence incubating the membrane with the mixture provided by the commercial Super Signal® West Pico kit (Pierce) for one minute in the dark and developing using a photographic plate according to the manufacturer's instructions with an exposure time of three minutes.

Analysis and Results

By RT-PCR, the cDNA was obtained with an approximate size of 2000 bp, which corresponds approximately to the expected size of the hsp83 molecule of *T. cruzi*. Figure 1A shows the bands obtained

by RT-PCR from the RNA samples extracted with the different commercial kits. For gene expression, M15 transformed cells were induced with IPTG, control aliquots were taken from uninduced cells and post-1, 2, and 3 hours of induction, and analyzed on Coomassie-Silver stained acrylamide gel. The expected size of the recombinant protein, according to the deduced amino acid sequence, is 80.7 kDa + 1 kDa of the histidine tail; so, a molecular weight of almost 83 kDa would be expected. Figure 1B shows the discrete expression of a protein of nearly 83 kDa in sample two at the second hour of incubation and slightly higher at the third hour. Additionally, bands that are not present in the uninduced control are observed and appear at the second and third hour of post-induction (Figure 1B); perhaps they are truncated forms of the protein, which the bacteria tend to eliminate.

The recombinant protein was purified by affinity chromatography from the cultures induced at 3 hours with IPTG. Each fraction obtained by chromatography and the sediment and the supernatant before and after passing through the Ni-NTA nickel column were analyzed using a polyacrylamide gel. For the identification of the recombinant protein, the fractions obtained from the purification of the recombinant protein HSP83 from *T. cruzi* were electrophoresis (SDS-PAGE 10%) and, subsequently, electroblotting on a nitrocellulose membrane. The nitrocellulose membrane was incubated with hyperimmune rabbit serum anti-Epimastigotes of *T. cruzi*; after development with luminol, a specific reaction with the recombinant protein is evidenced (Figure 1C).

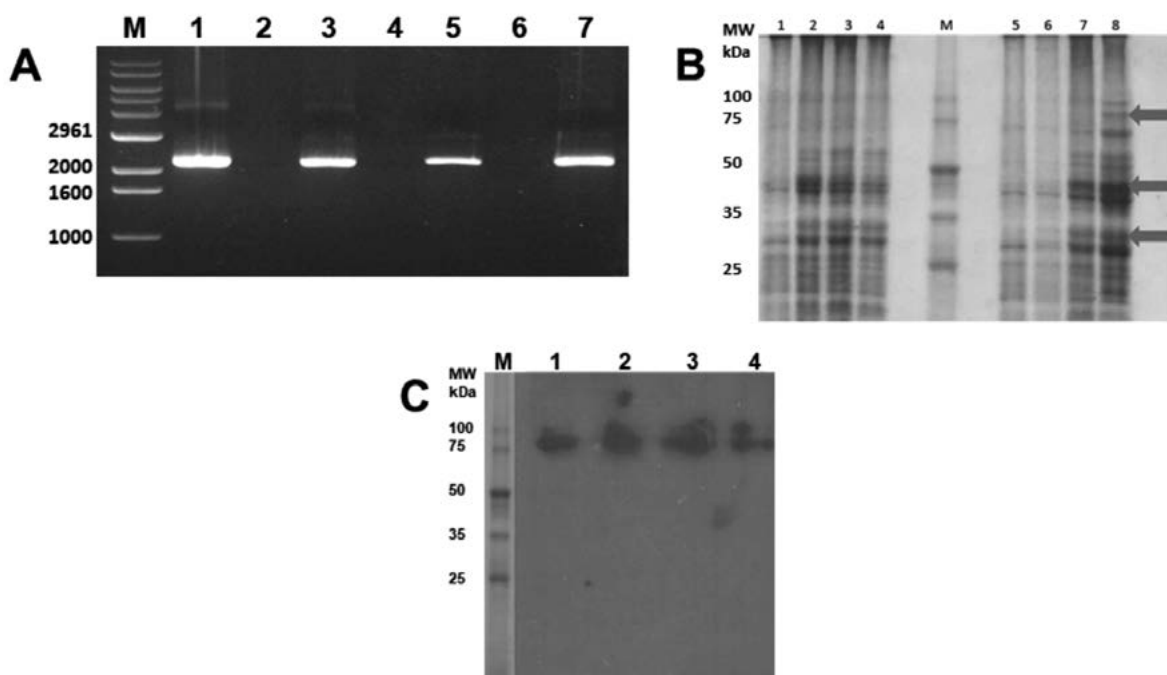


Figure 1. Cloning and expression of HSP83 of *T. cruzi*. A) Bands obtained by RT-PCR from the RNA samples. B) Transformed M15 cells induced with IPTG (1, 5), control aliquots taken from uninduced cells and post-1, 2, and 3 hours of induction (2, 3, 4 and 6, 7, 8, respectively), and analyzed on Coomassie-Silver stained acrylamide gel. C) Immunoblotting using hyperimmune rabbit serum anti-*Epimastigotes* of *T. cruzi* to detect purified recombinant protein.

The complete sequence of hsp83 cDNA was a fragment of 2115 bp corresponding to the open reading frame (ORF). This frame coded for a peptide of 704 amino acids, with a molecular mass of nearly 83 kDa and an isoelectric point of 5.0. The deduced amino acid sequence showed an amidation site (10-13), four possible N-glycosylation sites (48-51, 116-119, 151-154, 452-455), three cAMP and cGMP-dependent protein kinase phosphorylation sites (246-249, 273-276, 329-332), 11 Casein kinase II phosphorylation sites (104-107, 155-158, 164-167, 291-294, 296-299, 306-309, 360-363, 525-528, 531-534, 609-612, 621-624), four N-myristoylation sites (160-164, 190-195, 232-237, 442-447), nine Protein kinase C phosphorylation sites (104-106, 291-293,

332-334, 380-382, 410-412, 531-533, 553-555, 644-646, 685-687), and four Tyrosine kinase phosphorylation sites (57-65, 218-225, 273-281, 616-623). A motif and several domains were found, such as a Chaperone protein htpG [htpG] motif (80-704), a Heat shock hsp90 proteins family signature (103-112), a TonB-dependent receptor proteins signature 1 (1-40), two Bipartite nuclear localization signals (400-414, 554-571), a Glutamic acid-rich region profile (257-327), a HATPase_cHistidine kinase-, DNA gyrase B-, a HSP90-like ATPase domain (105-258), and a HSP90 protein domain (261-704) (InterPro IPR001404 pFam PF00183). With respect to the possible immunogenicity of the complete sequence of hsp83 cDNA, five B epitopes were predicted



in the molecule (²²⁶TKNEE₂₃₀²⁴⁴EEGEKK-
²⁵²KKT<sub>327³³²FEPSKK₃₃₂⁴⁷⁰TGDSKK₄₇₅ and
⁵²⁷EETEEEEKKQR₅₃₆) (Figure 2).</sub>

This sequence showed high identity with heat shock protein 85 [*Trypanosoma cruzi* Dm28c, Venezuela] (ESS64949.1), with Heat shock-like 85 kDa protein [*Trypanosoma cruzi* Dracon, Perú] (P06660.1)

and lower identity with heat shock protein 85, putative [*Trypanosoma cruzi* CL Brener El Sayed, Brazil] (XP_811791.1) (Figure 3).

Discussion

The cloning of genes and their expression as recombinant proteins facilitates the

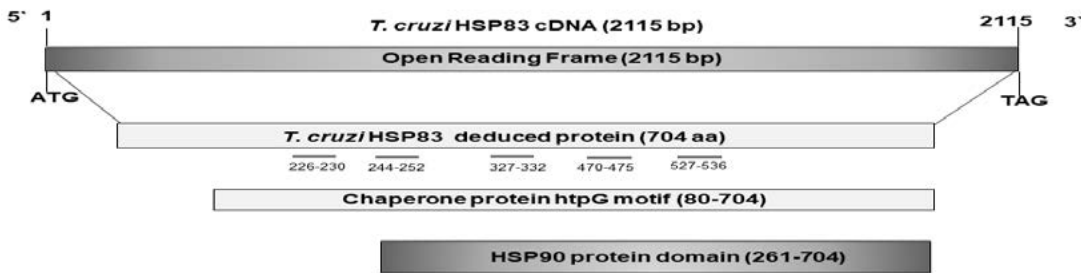


Figure 2. Schematic representation of the cloned cDNAs analyzed. The bars below deduced proteins represent the domains predicted in the sequence. The short grey lines below numbers represent the positions of the B epitopes; bp stands for base pair, and aa means amino acid.

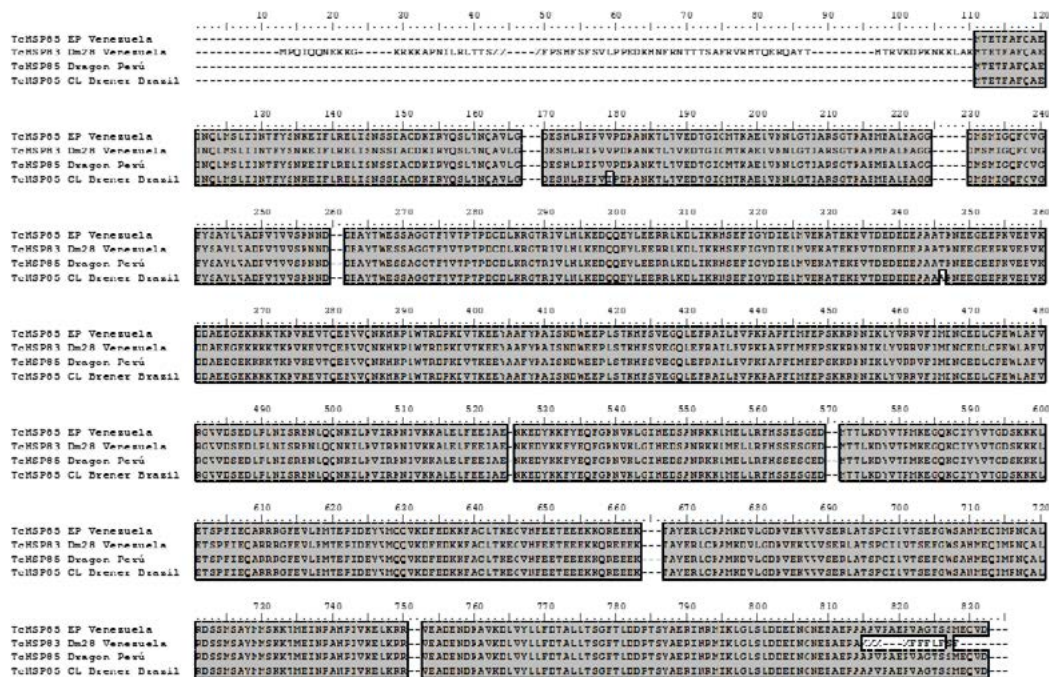


Figure 3. Clustal Alignment with heat shock protein 85 [*Trypanosoma cruzi* Dm28c, Venezuela] (ESS64949.1), Heat shock-like 85 kDa protein [*Trypanosoma cruzi* Dracon, Perú] (P06660.1), and heat shock protein 85, putative [*Trypanosoma cruzi* CL Brener El Sayed, Brazil] (XP_811791.1).



study of both the genes and the proteins they encode, as well as their use as tools in tests that help to elucidate the cellular mechanisms in which they are involved. In the present study, it was possible to clone and express the gene encoding the heat shock protein HSP83 from *T. cruzi*. To date, there are merely two old reports about cloning this molecule (Dragon *et al.*, 1987; Nadeau *et al.*, 1992), and only its genomic structure and ATPase activity have been studied. In addition, cloning and expression of the gene encoding HSP83 has been obtained in other tripanosomatids, such as *Trypanosoma brucei* (Mottram *et al.*, 1989), Hsp83 from *Leishmania mexicana amazonensis* (Shapira & Pinelli, 1989), Hsp83 of *Leishmania infantum* (Angel *et al.*, 1996), and Hsp83 from *Leishmania braziliensis* (Silva *et al.*, 2013; Menezes-Souza *et al.*, 2014).

Since HSP83 has been reported to be a protein highly expressed by *T. cruzi*, even under basal conditions (Dragon *et al.*, 1987), and given its importance in the mechanisms of cellular metabolism of the parasite and the need to expand studies in this area, it was decided to address the cloning strategy using the mRNA as a starting sample and the amplification of the coding region by means of the RT-PCR technique. Thanks to the information from the *T. cruzi* Genome project, this sequence was made available to design the cloning strategy.

In all cases, a band with a size of 2139 bp was amplified, the expected size according to the GenBank sequence. The verification of the cloning by colony PCR, using the primers for the coding region of HSP83, helped amplification of a band of 2139 bp in eight of the 19 colonies analyzed. The lack of expression in a high percentage of colonies analyzed could be due to the conditions of the cells used in cloning (which were not as competent at accepting the plasmid with the gene).

Likewise, the cDNA was subcloned into different expression vectors to facilitate its expression. However, despite successful verification of the insert's presence in the recent subcloning, the HSP83 sequence or the entire plasmid disappeared in some cases when trying to express it. This phenomenon suggests that the HSP83-plasmid construct may be unstable within these complex plasmids, and bacteria may degrade the plasmid (with the loss of the HSP83 sequence) or even eliminate it (Maizels *et al.*, 1991).

Subsequently, it was decided to carry out the subcloning process in the vector pQE30, which was simpler and used for the cloning and expression of HSP83 from *Leishmania infantum* (Angel *et al.*, 1996). However, the same result occurred: subcloning was achieved but followed by loss. This reinforces the recurrence of the hypothesis that the recombinant plasmid is expelled from the bacterium, perhaps because the HSP83 sequence, being evolutionarily conserved, can interfere with the cell's metabolism, and the cell tends to eliminate it (Huynh *et al.*, 1985). On the other hand, sometimes fragments of genetic material do not always integrate precisely; this may lead to artifacts that the sera would not recognize.

Considering the high similarity reported between Hsp83 sequences from *T. cruzi* and those from *T. brucei* and Hsp83 from *L. m. amazonensis*—being 95% and 85%, respectively (Rondinelli, 1994)—, along with the reported expression of *L. infantum* Hsp83 in cells transformed with pQE30 (Angel *et al.*, 1996), and thinking that the problem may be the residence time in the cell, a decision was made to clone into the latter vector directly, without waiting for the processes to verify the presence of the insert, that is, a “blind” strategy. Thus, the residence time in the bacterium would be reduced, which



could prevent expulsion before the respective expression. In this case, it was possible to obtain positive results, with the appearance of a nearly 83 kDa protein in chromatography-eluted fractions from lysates of competent cells transformed *E. coli* M15.

Reviewing published articles on cloning genes encoding Hsp90 heat shock proteins in different parasitic models, either from genomic DNA or mRNA, reveals that the expression of proteins from the Hsp90 family of Trypanosomatids was possible through cloning in expression vectors. Examples of these vectors are the Hsp83 from *L. braziliensis* with the vector pET28a-TEV (Menezes-Souza *et al.*, 2014), Hsp90 from *T. evansi* with the vector pRSETA (Pallavi *et al.*, 2010), Hsp83 from *Leishmania braziliensis* from pET28a (Silva *et al.*, 2013), and Hsp83 from *Leishmania infantum* with pQE30 (Angel *et al.*, 1996). Among the expression vectors used in these studies, only pRSETA was more complex and employed in the current investigation. However, the results obtained were unsatisfactory, perhaps because the time lapses between subcloning in the expression vectors and expression were several weeks, and bacteria might have shed the recombinant plasmid due to its instability during that time. Using a direct “blind” subcloning strategy may have yielded better results in these vectors. Alternatively, this could be a particular characteristic of the Hsp83 of *T. cruzi* since it is strange that such an important molecule from a parasite causing serious public health problems has not had more cloning and expression studies, possibly due to the difficulty of its expression in prokaryotic systems.

In this sense, it is striking that during the review of the cloning and expression procedure of *Plasmodium falciparum* Hsp90, it was verified that the researchers

directly cloned into the expression vector pET-23a and obtained the recombinant protein by using competent cells *E. coli* BL21 (Kumar *et al.*, 2003). Although the authors do not mention this aspect, they might have encountered circumstances similar to those discussed in this paper.

Regarding the expression of the recombinant protein, a low expression level and smaller bands were observed, which could be truncated forms of the protein, possibly due to bacterial attempts to degrade the recombinant protein. This degradation might occur due to sequence similarities, interfering with its metabolism (Huynh *et al.*, 1985). This could explain the limited observation of the recombinant protein.

The expected size of the recombinant protein according to the deduced amino acid sequence is 80.7 kDa + 1 kDa for the histidine tail; so, a molecular weight of nearly 82 kDa would be expected, which was observed in the results. It is important to note that HSP83 or HSP85 was so named since the native protein has a weight of 83-85 kDa. The small difference in size of nearly three kDa may be due to glycosylation of the native protein, as the prediction analysis of possible post-translational modifications of the sequence by Motif Scan of ISB-ISREC (Pagni *et al.*, 2001) evidenced four potential N-glycosylation sites. One of these sites can be considered functional since it is estimated that an N-glycosylation contributes an additional three kDa to the protein's molecular weight (Obregon-Henao *et al.*, 2001).

As expected, problems were also encountered during the protein purification process because it was a large molecule of nearly 83 kDa, and its same weight was difficult to bind to the resin of the column only by a six histidine tail. It may be convenient to use another purification protocol



or subclone the cDNA into a vector capable of binding to a larger fusion molecule that serves as a support and can then be eliminated, such as GST or MBP.

Considering the poor expression of hsp 83 and aiming to confirm that the band of nearly 83 kDa, whose expression increased with induction time, corresponded to the HSP83 protein of the parasite, a rabbit's hyperimmune serum against *T. cruzi* epimastigotes (from the same isolate used in the present work) was used. This serum has been employed in several experimental protocols, with recognition of antigens with approximate molecular weights of 85, 80, 65, 62, 52, 38, 35, 34, 30, and 27 kDa in epimastigotes of the EP strain (Contreras *et al.*, 1998; Graterol *et al.*, 2013).

Immunoblotting analysis of the fractions obtained by nickel Ni-NTA column chromatography using hyperimmune anti-epimastigote serum facilitated the identification of a single band with an approximate molecular weight of 83 kDa, indicating, in this case, a reaction with the recombinant protein obtained in this study. Due to the low concentration of the recombinant protein used in the immunoblotting technique, the researchers decided to use a highly sensitive development system such as luminography, in particular with the Super Signal® West pico system, enabling the detection of immune complexes in picograms. This explains the strong signal of the band observed on the X-ray film used to document the reaction.

Heat shock proteins have been shown to be immunogenic in various parasitic models (Polla, 1991), and most are important antigens recognized by patient sera. In addition, it has been reported that the immune response is directed to the less conserved areas of the protein, ensuring

the specificity of the response (Skeiky *et al.*, 1995; Angel *et al.*, 1996). For example, specific anti-HSP90 antibodies have been documented in schistosomiasis (Johnson *et al.*, 1989), leishmaniasis (de Andrade *et al.*, 1992), and malaria (Zhang *et al.*, 2001). Immunodominant HSP70 antigens have also been reported in patients with hydatidosis (Colebrook & Lightowers, 1997), schistosomiasis (Moser *et al.*, 1990), onchocerciasis (Rothstein *et al.*, 1989), leishmaniasis (de Andrade *et al.*, 1992; Wallace *et al.*, 1992; Skeiky *et al.*, 1995; Quijada *et al.*, 1996), Chagas disease (Engman *et al.*, 1989; Requena *et al.*, 1993) and malaria (Kumar *et al.*, 2003), and HSP60 in the case of leishmaniasis (Rey-Ladino *et al.*, 1997). Regarding low molecular weight HSPs, their diagnostic value in schistosomiasis and cysticercosis has been studied and confirmed (Nene *et al.*, 1986; Ferrer *et al.*, 2005).

It is important to note that no reactivity of the *T. cruzi* anti-epimastigote hyperimmune serum was observed with any other band of any size, only the 83 kDa, which would indicate that the possible truncated molecules lack the epitopes recognized by these antibodies. Using the complete protein to immunize rabbits and obtain hyperimmune sera that allow epitope mapping assays would be interesting.

Even at low concentrations, this recombinant protein HSP83 from *T. cruzi* can be used to elaborate a monospecific polyclonal antibody, which constitutes a very valuable tool to study the participation of this stress protein in the parasite's differentiation processes, such as epimastigogenesis, metacyclogenesis, amastigogenesis, and trypomastigogenesis.



Conclusions

Despite the difficulties in the expression and purification of the recombinant protein, it could be expressed and identified with the hyperimmune serum. Therefore, this cloning and expression strategy is recommended for molecules with difficult cloning and expression.

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Conflict of Interest

The authors declare no competing interests.

Author contribution statement

All the authors declare that the final version of this paper was read and approved.

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Data availability statement

Data sharing is not applicable since no new data was created or analyzed in this study.

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Cloning and Characterization of the Gene Encoding the Heat Shock Protein HSP83 from *Trypanosoma cruzi* (Ana Fernández • Ana Rita De Lima • Elizabeth Ferrer)

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