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Comprehensive Proteomic Analysis of *Trypanosoma cruzi* Epimastigote Cell Surface Proteins by Two Complementary Methods

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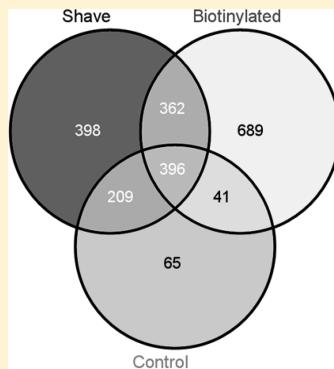
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Supporting Information

ABSTRACT: *Trypanosoma cruzi* is a protozoan that causes Chagas' disease, a neglected infectious illness that affects millions of people, mostly in Latin America. Here, the cell surface subproteome of the *T. cruzi* epimastigote life form was characterized. In order to prepare samples enriched in epimastigote plasma membrane protein, two distinct methodologies were optimized and evaluated. The first methodology was based on cell surface trypsinization (Shave) of intact living cells while the second approach used biotinylation of cell surface proteins followed by streptavidin affinity chromatography isolation of the labeled proteins. Both *T. cruzi* subproteomes were analyzed by LC-MS/MS. The results showed that the methodologies offered comprehensive and complementary information about the parasite's plasma membrane subproteome.



KEYWORDS: *Trypanosoma cruzi*, epimastigotes, subproteome, plasma membrane, cell surface trypsinization, biotinylation

INTRODUCTION

Plasma membrane (PM) associated proteins carry out vital functions for cell activities, including metabolism, cell–cell interactions, response to environmental stimuli, and transport of ions and solutes. The characterization of the cell surface proteomes has profound importance for the understanding of cell differentiation and undoubtedly relevance to modern medical research, since PM proteins are the target of about 70% of approved pharmaceutical agents.¹

The subset of PM proteins encompasses integral membrane proteins, which span the lipid bilayer with up to 15 transmembrane domains, and peripheral membrane proteins that are associated with the PM by interaction with integral membrane proteins or the lipid bilayer itself.² Despite the enormous advances in mass spectrometry (MS) technologies, accessing the PM proteome remains a challenging task due to the heterogeneity and the amphiphilic nature of membrane proteins as well as their overall low relative abundance.^{2,3} Probably, the proteomic characterization of this subcellular fraction as any proteome will never be complete, since cells and their organelles are dynamic structures, constantly shuffling proteins between compartments and undergoing chemical and structural modifications.⁴ Moreover, cell surface proteins share similar properties with those of other organellar membranes, making difficult their purification prior to proteomic analysis.

Ultracentrifugation can be regarded as the workhorse for subcellular fractionation. However, protocols for cell surface membrane protein isolation normally make use of other tools

besides ultracentrifugation in order to improve protein yield and increase specificity for PM proteins.⁵ Therefore, other techniques, such as chemical capture of cell surface glycoproteins, cell surface biotinylation, cationic colloidal silica-bead coating, and membrane shaving, are also used (reviewed in refs 5 and 6). As expected, all these techniques have drawbacks and advantages that should be taken into account, especially if there is interest in assessing protein post-translational modifications (PTM).⁷

In the present work we carried out the analysis of the cell surface subproteome of *Trypanosoma cruzi*, the protozoan parasite that causes Chagas' disease. According to the World Health Organization, Chagas' disease is a neglected infectious disease with an estimated ten million cases worldwide in 2009, and more than 10,000 deaths annually, mostly in Latin America, where the disease is endemic.⁸ The drugs currently used for its chemoprophylactic treatment are highly toxic and present variable efficacy while vaccines against the parasite are not available yet.⁹ This scenario reflects the need for greater understanding of the biology of *T. cruzi*.

T. cruzi is a single cell parasite which has four main developmental stages, with two being in the triatomine bug vector (epimastigote and metacyclic tryomastigote forms) and two in the mammalian hosts (bloodstream tryomastigote and amastigote forms).¹⁰

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To our knowledge, only two studies have been reported on the analysis of the *T. cruzi* membrane subproteome. The first one used hydrophobic/hydrophilic phase partitioning to obtain fractions enriched in GPI-anchored and other biomembrane proteins from the insect developmental forms (epimastigote and metacyclic trypomastigote) followed by 2D-LC-MS/MS.¹¹ This approach permitted the demonstration of stage specific biomembrane protein profiles among both life stages but was not specific for the analysis of PM proteins. The same group in a second work reported a large-scale *in silico* study of *T. cruzi* GPI-anchored proteins predicted from genome data and also a mass spectrometric analysis of GPI anchors from epimastigote cells.¹²

Here, we describe two complementary strategies to study *T. cruzi* PM, (a) trypsin cleavage of cell surface proteins (Shave) and (b) biotinylation of cell surface proteins followed by streptavidin affinity chromatography purification and, after each enrichment, the identification of the proteins by mass spectrometry.

The Shave technique has the advantage of being relatively straightforward, as the only major concern is to maintain cellular integrity (e.g., cell friendly pH and mild centrifugations) during the enzymatic digestion in order to avoid contamination with cytosolic proteins due to cell lysis. A Control sample was used to assess potential identifications that are not derived from the PM protein digests, e.g. secreted proteins. On the other hand, cell surface biotinylation followed by streptavidin affinity chromatography has potential use in PM phosphoproteomics, as the complete primary structure of the proteins is maintained with this method and PTM on both sides of the membrane can be observed. The integrity from the proteins obtained by this approach also enables immunoblotting to be used to validate the enrichment.

MATERIALS AND METHODS

Fetal bovine serum (FBS) was purchased from Soral Biotecnologia (Campo Grande, Brazil). The MicroBCA kit, Sulfo-NHS-LC-Biotin, and the prepacked 1 mL immobilized streptavidin columns were from Pierce (Rockford, IL, USA). GeLoader tips were from Eppendorf (Hamburg, Germany). Amicon filter units with a 3 kDa cut off membrane were from Millipore (Billerica, MA, USA). Modified trypsin was from Promega (Madison, WI, USA). TPCK treated porcine trypsin, Dulbecco's modified eagle medium (DMEM), and all others reagents were purchased from Sigma/Aldrich (St. Louis, MO, USA), unless stated otherwise.

Epimastigote *In Vitro* Culture

Epimastigotes were obtained as described elsewhere.¹³ Briefly, epimastigote forms of *T. cruzi* Berenice strain were grown at 28 °C in liver infusion tryptose (LIT) medium supplemented with 5.0% inactivated FBS.

Optimization of Protocol for Cell Surface Trypsinization (Shave)

Prior to shave experiments, the maximum time in which epimastigote cells remained intact upon incubation in trypsin solution was determined. For that, 1 × 10⁹ cells were incubated in 1 mL of a trypsin solution at 20 µg/mL in Tris buffered saline pH 7.4 (TBS), at 28 °C, the optimal temperature for epimastigote cells. Aliquots of living cells were taken every 15 min and observed by optical microscopy. The maximum incubation time was considered to be the one in which all cells maintained their motility.

The effects of cell incubation in TBS or DMEM (both adjusted to pH 7.4, because this was found to be the optimal pH to maintain cell integrity) were also tested. Therefore, the epimastigote cells were washed three times with each buffer, resuspended in the same buffer (1 × 10⁹ cells/mL) at 28 °C, and submitted to harvest of supernatant by centrifugation under two conditions: (A) immediately after resuspension of washed cells; (B) after incubation of washed parasites at 28 °C for 30 min.

Conditions A and B were also tested using a concentration of 2.5 × 10⁸ parasites/mL in DMEM.

Cell Surface Trypsinization (Shave)

Two ×10⁹ epimastigote cells per biological replicate were washed 3 times with 4 mL of DMEM, resuspended in 8 mL of DMEM preheated at 28 °C at a final concentration of 2.5 × 10⁸ parasites/mL, and equally divided into two tubes. Twenty micrograms of TPCK treated trypsin was added to one of the tubes (Shave sample), and the other sample was used as a control to assess the content of secreted proteins and proteins leaked from the cells. Both samples were incubated at 28 °C for 30 min. To remove cells after the incubation time, the tubes were centrifuged for 5 min at room temperature in three rounds to ensure complete removal of cells and avoid mechanical cell lysis: first at 2000g, then at 4000g, and the last at 6000g. The supernatants were transferred to new tubes after each centrifugation. Then, 20 µg of TPCK treated trypsin was added to the Control sample and incubated at 28 °C for 30 min. Both Control and Shave samples were vacuum-dried and stored at -20 °C.

Cell Surface Biotinylation and Streptavidin Affinity Chromatography

The procedure was based on the manufacturer's instructions and modified as follows:

First Protocol for Cell Surface Biotinylation and Streptavidin Affinity Chromatography. Parasites (5 × 10⁹) were washed 3 times with phosphate buffered saline pH 8.0 (PBS containing 80 g/L NaCl, 2 g/L KCl, 26.8 g/L Na₂HPO₄·7H₂O, and 2.4 g/L KH₂PO₄) and resuspended in 5 mL of 250 µg/mL Sulfo-NHS-LC-Biotin in PBS for 10 min at 28 °C. Afterward cells were washed once with TBS, to quench the reaction, and lysed with 2 mL of 2% Triton X-100 with a complete cocktail of protease and phosphatase inhibitors (Roche), followed by three freeze–thawing cycles with liquid N₂. The sample was centrifuged, and the supernatant was stored at -20 °C. The extract containing biotinylated proteins was passed through a prepacked 1 mL immobilized Streptavidin column (PIERCE) for 1 h at room temperature. The column was washed with 15 mL of 1% Triton X-100 in TBS and then 10 mL of 1% Triton X-100 in 1 M NaCl to remove nonspecifically bound material.

Biotinylated proteins were first eluted with 4 mL of a solution of 5 mM biotin in 1% Triton X-100, followed by elution with 5 mL of 8 M guanidine-hydrochloride, pH 1.5. Afterward the guanidine-HCl eluted fraction was neutralized and diluted with 5 mL of 250 mM Tris pH 7.0, in order to make the solution appropriate for ultrafiltration with 3 kDa Amicon filters. The neutralized diluted samples were concentrated to a volume of approximately 300 µL and subsequently precipitated with TCA. These samples, as well as aliquots from the loading flow-through (unbound fraction), both column washes, and a sample of epimastigote water-

soluble proteins obtained by freeze–thawing in 100 mM Tris, pH 7.4, were submitted to 8–15% SDS-PAGE.

Second Protocol for Cell Surface Biotinylation and Streptavidin Affinity Chromatography. Parasites (5×10^9) were washed three times with PBS and resuspended in 5 mL of 250 $\mu\text{g}/\text{mL}$ Sulfo-NHS-LC-Biotin in PBS for 10 min at 28 °C. Subsequently, cells were washed once with TBS, to quench the reaction, and lysed in 1 mL of Milli-Q water containing a complete cocktail of protease and phosphatase inhibitors, through three cycles of freeze–thawing in liquid N₂. The biotinylated lysate and supernatant were centrifuged at 15000g for 20 min, and the pellet was washed once with Milli-Q water and centrifuged again (15000g, 20 min) to remove excess water–soluble proteins. The pellet was incubated in 2% Triton X-100 for 1 h in ice with occasional vortexing and then centrifuged at 10000g for 20 min at 4 °C to remove debris. The extract containing biotinylated proteins was subjected to streptavidin affinity purification as described above. The chromatographic fractions were neutralized and concentrated to a volume of about 300 μL on a 3 kDa Amicon filter unit, and quantified by MicroBCA. Then an aliquot was precipitated with TCA and submitted to SDS-PAGE 8–15%, as were aliquots of the unbound fraction, the washes, and a soluble fraction.

Immunoblotting

Protein samples were subjected to 12% SDS-PAGE under reducing conditions. Proteins were electroblotted onto a nitrocellulose membrane (Hybond-C Extra, GE Healthcare Pittsburgh, PA, USA). The membrane was blocked by incubation with 5% (w/v) nonfat milk in PBS overnight at 4 °C. The blot was incubated for 2 h with polyclonal antiserum against TcMTAP (methylthioadenosine phosphorylase of *T. cruzi*) diluted to 1:400 in 1% nonfat milk/PBS. After three washes of 5 min each with PBS, the membrane was incubated for 1 h with alkaline phosphatase-conjugated goat antirabbit IgG, diluted to 1:1000, and washed as above. Immunocomplexes were revealed with the alkaline-phosphatase substrate 5-bromo-4-chloro-3-indolyl-1-phosphate/Nitro Blue Tetrazolium (BCIP/NBT - Promega).

Sample Preparation for LC-MS/MS

Protein samples were first quantified using amino acid analysis using a Biochrom 30 amino acid analyzer (Biochrom, Cambridge, U.K.), following the protocol from the manufacturer.

Shave and Control samples were resuspended in 20 mM triethylammonium bicarbonate (TEAB), reduced with 20 mM DTT at 56 °C for 45 min, alkylated with 40 mM IAA at room temperature in the dark for 60 min, and further digested overnight at 37 °C with 1 μg of modified trypsin (Promega). After digestion, the samples were acidified to 0.1% TFA final concentration and desalted on home-made microcolumns of Poros Oligo R3 resin (PerSeptive Biosystems) packed (1 cm long) in p200 tips (adapted from ref 14). To remove the pH indicator, which is included in the DMEM, the desalted samples were resuspended in 90% ACN/0.1% TFA and passed through ZIC-HILIC resin (10 μm particle size and 100 Å, SeQuant), packed into GeLoader tips, and eluted with 0.1% TFA. To ensure minimal sample loss due to overloading, the flow-through was passed through another ZIC HILIC microcolumn, and both eluates were combined before being vacuum-dried.

Biotinylated cell surface proteins were submitted to acetone/ethanol precipitation to remove guanidine-HCl and traces of Triton X-100. Briefly, the dried sample was resuspended in 100

μL of 20 mM TEAB, diluted 4 times with ice cold ethanol, and vortexed. Then the same volume of ice cold acetone was added, vortexed vigorously, and incubated overnight at –20 °C. After incubation, the material was centrifuged at 20000g at 4 °C for 15 min and the supernatant was discarded. The pellet was washed another three times with ice cold 40% ethanol/40% acetone solution. Finally, the sample was resuspended in 20 mM TEAB, reduced with 20 mM DTT at 56 °C for 45 min, alkylated with 40 mM IAA at room temperature in the dark for 60 min, and digested overnight at 37 °C with 2 μg of modified trypsin. After digestion, the sample was acidified to 0.1% TFA final concentration and desalted on home-made microcolumns of Poros Oligo R3 resin (PerSeptive Biosystems, Framingham, MA, USA) packed (1 cm long) in p200 tips (adapted from ref 14).

LC-MS/MS and Data Analysis

Samples were analyzed using an EASY-nano LC system (Proxeon Biosystems) coupled online with an LTQ-Orbitrap Velos mass spectrometer (Thermo Scientific). Three micrograms of each sample were loaded onto a 18 cm fused silica emitter (75 μm inner diameter) packed in-house with reverse phase capillary column ResiproSil-Pur C18-AQ 3 μm resin (Dr. Maisch GmbH, Germany) and eluted using a gradient from 100% phase A (0.1% formic acid) to 26% phase B (0.1% formic acid, 95% acetonitrile) for 180 min, 26% to 100% phase B for 5 min, and 100% phase B for 8 min (a total of 193 min at 200 nL/min). After each run, the column was washed with 90% phase B and re-equilibrated with phase A. Mass spectra were acquired in positive ion mode by applying a data-dependent automatic survey MS scan and tandem mass spectra (MS/MS) acquisition mode. Each MS scan in the Orbitrap analyzer (mass range of *m/z* 350–1800 and resolution 100000) was followed by MS/MS of the fifteen most intense ions in the LTQ. Fragmentation in the LTQ was performed by collision-induced dissociation, and selected sequenced ions were dynamically excluded for 30 s. Raw data were viewed in Xcalibur v.2.1 (Thermo Scientific), and data processing was performed using Proteome Discoverer v.1.3 beta (Thermo Scientific). The raw files were submitted to searching using Proteome Discoverer with an in-house Mascot v.2.3 algorithm against a *Trypanosoma cruzi* database downloaded using the Database on Demand tool¹⁵ containing the proteins of the parasite found in UniProt/SWISS-PROT and UniProt/TrEMBL. Contaminant proteins (several types of human keratins, BSA, and porcine trypsin) were also added to the database, and all contaminant proteins identified were manually removed from the result lists. The searches were performed with the following parameters: MS accuracy 10 ppm, MS/MS accuracy 0.5 Da, 2 missed cleavage sites allowed, carbamidomethylation of cysteine as fixed modification and oxidation of methionine and protein N-terminal acetylation as variable modifications. For the biotinylated sample search peptide N-terminus NHS-LC-biotinylation and NHS-LC-biotinylated lysine were also added as variable modifications (increment of 339.16 Da). Number of proteins, protein groups, and number of peptides were filtered for False Discovery Rates (FRD) less than 1%, and only peptides with rank 1 and minimal of 2 peptides per proteins were accepted for identification using Proteome Discoverer. ProteinCenter software (Thermo Scientific) was used to interpret the results at protein level, e.g., statistical comparison of Gene Ontology terms between data sets and number of proteins with transmembrane domains. Better

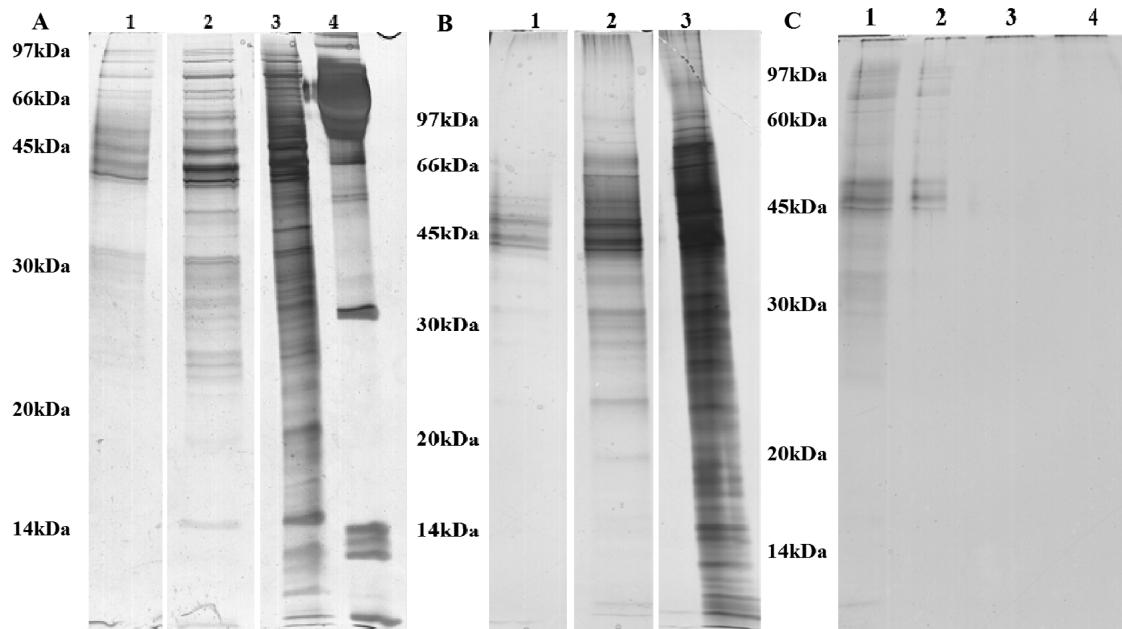


Figure 1. Effect of different media and cell concentrations on epimastigote released proteins. Samples were subjected to 12% SDS-PAGE and silver-stained. (A) TBS incubation: (1) supernatant harvested immediately after resuspension of washed cells, (2) after incubation of washed parasites (1×10^9 cells/mL) at 28 °C for 30 min, (3) water-soluble extract, and (4) LIT medium with 5% FBS. (B) DMEM incubation: (1) supernatant harvested immediately after resuspension of washed cells, (2) after incubation of washed parasites (1×10^9 cells/mL) at 28 °C for 30 min, (3) water-soluble extract. (C) DMEM incubation: supernatant harvested immediately after resuspension of washed cells at (1) 1×10^9 cells/mL and at (3) 2.5×10^8 cells/mL and after incubation at 28 °C for 30 min of washed parasites at (2) 1×10^9 cells/mL and at (4) 2.5×10^9 cells/mL.

annotation from subsets of the identified proteins and KEGG pathway analysis were acquired using Blast2GO software (<http://www.blast2go.com/b2gome>) using default parameters.

RESULTS AND DISCUSSION

Incubation Conditions for Cell Surface Trypsinization

Since the present work aimed at analyzing *T. cruzi* surface membrane subproteome obtained by living cell incubation with trypsin, and to avert contamination due to cell lysis, several incubation conditions were tested. Systematic optical microscopy observation of epimastigote samples incubated with trypsin solution revealed that all cells maintained their motility at 30 min of incubation and even some replicating events could be noticed. However, at 45 min of incubation some parasites were motionless and after 60 min all were immobile (data not shown).

Subsequently, the effect of cell washes as well as incubation times with different buffers without trypsin was also tested. The SDS-PAGE analysis of the culture supernatants of all conditions tested in TBS without trypsin versus the LIT medium presented distinct protein profiles.

Figure 1A shows that the cell washes before incubation in PBS were sufficient to remove the highly abundant proteins from the LIT medium. However, the protein profiles resembled substantially the parasite water-soluble protein extract, indicating significant percentage of cell lysis upon incubation with TBS even when cells were removed almost immediately after resuspension, as in condition A (see Materials and Methods). To test the hypothesis that the lysis was due to nutritional depletion, the experiment was repeated in DMEM without FBS in conditions A and B (Figure 1B), which showed the same soluble extract resembling profile. During this

experiment, the pH indicator present in the DMEM evinced fast acidification of the medium within 60 min (data not shown), suggesting a high metabolic activity of the parasites and a consequent nutritional depletion of the medium. To avoid any metabolic stress, parasites were thereafter submitted to incubations in DMEM under the same conditions, but with cellular concentration 4 times more diluted. Protein samples from the experiments with 1×10^9 cells/mL and 2.5×10^8 cells/mL, in both conditions A and B, were submitted to SDS-PAGE and silver-stained (Figure 1C). The gel showed no detectable protein bands at the lowest cell concentration in either of the conditions.

On the basis of the results above, in order to minimize cell lysis and/or metabolic stress in shaving experiments, cells at a concentration of 2.5×10^8 cells/mL in DMEM were incubated with trypsin for 30 min before protein identification by mass spectrometric analysis.

Cell Surface Biotinylation and Streptavidin Affinity Chromatography

In order to enrich the intact PM proteins with their transmembrane domain or anchor, we performed affinity chromatography of biotinylated PM proteins on a streptavidin column. To try to maintain the intact structure of the PM proteins, we tested mild elution of biotinylated proteins by competition with free biotin molecules. The SDS-PAGE profile (Figure 2A) demonstrates that the mild elution with biotin was not sufficient to elute all proteins bound to the streptavidin compared to the elution with 8 M guanidine–HCl solution. Indeed the SDS-PAGE profile with the guanidine–HCl elution displays more eluted proteins and thus was more efficient (Figure 2A).

The first protocol of biotinylation and streptavidin affinity chromatography, based on the manufacturer's instructions, provided chromatographic fractions that were analyzed by SDS-

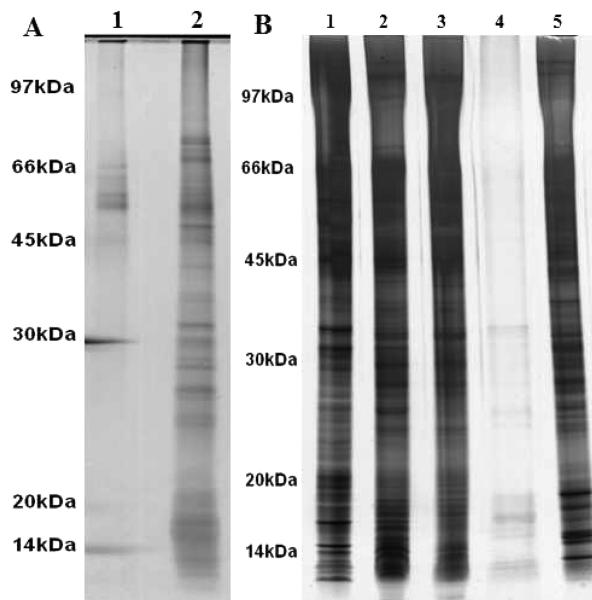


Figure 2. Streptavidin affinity chromatography fractions submitted to SDS-PAGE 8–15%. (A) Streptavidin bound epimastigote proteins eluted with (1) 5 mM biotin in 1% (v/v) Triton X-100 and with (2) 8 M guanidine–HCl. (B) SDS-PAGE 8–15% of (1) water-soluble epimastigote proteins, (2) streptavidin column unbound fraction, (3) wash fraction with 1% (v/v) Triton X-100 in TBS, (4) wash with 1 M NaCl in 1% (v/v) Triton X-100, and (5) 8 M guanidine–HCl eluted fraction.

PAGE. The electrophoretic profiles showed fairly distinct protein patterns between the water-soluble extract and those of the guanidine–HCl elution and the unbound fraction, especially above 45 kDa. However, no considerable differences were observed between the eluted and unbound fractions below 45 kDa (data not shown). Assuming that the low specificity in the chromatography was due to the high complexity of the sample and a low proportion of biotinylated proteins in the lysate and having in mind that the plasma membrane proteins might have low solubility in water, we developed a second protocol of biotinylation and streptavidin affinity chromatography. In this protocol a lysis step in water with protease and phosphatase inhibitors followed by two washes with water to remove the high amount of water-soluble proteins was added. The guanidine–HCl eluted fraction obtained by this protocol has a distinct profile from both soluble extract and unbound fraction (Figure 2B), as well from the washes. Also worthy of notice is that the presence of protein bands in the second wash line demonstrates the relevance of the extensive washes. The yield obtained by this method from 5×10^9 parasites was an average mass of 340 µg of protein, as quantified by the MicroBCA kit.

To further validate the removal of the excess of cytosolic proteins from the enriched sample by this method, the water-soluble extract and guanidine–HCl eluted and unbound fractions were submitted to Western blotting with antibody against TcMTAP, a *T. cruzi* cytosolic water-soluble protein¹⁶ (Figure 3). It showed no marked bands in the eluted fraction, confirming the ability of the method to remove cytosolic proteins.

Epimastigote Cell Surface Proteome

A total of 1488 protein groups could be identified from the epimastigote life form using biotinylation followed by

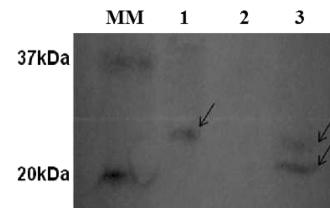


Figure 3. Immunoblotting with antisera against TcMTAP. (1) Epimastigote water-soluble extract, (2) 8 M guanidine–HCl elution, and (3) streptavidin column unbound fraction.

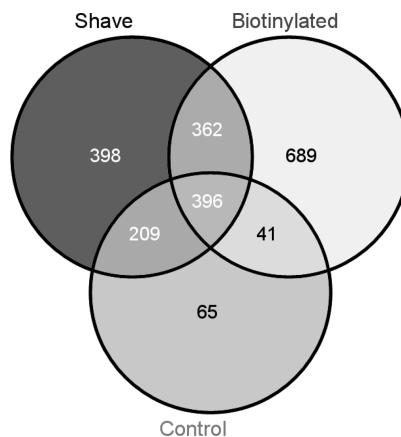


Figure 4. Venn diagram showing the number of common identified protein groups in each of the epimastigote sample sets.

streptavidin affinity chromatography (Supporting Information (SI) Table 1). 1365 protein groups were identified in the Shave experiments (SI Table 1) and 711 in the Control sample (SI Table 1). Figure 4 depicts the overlaps between samples. It is worthy of mention that the sensibility toward trypsin digestion of the epimastigote cell surface proteins is not unexpected, as the triatomines use cathepsins for the digestion of blood proteins, instead of trypsin.¹⁷

Data analysis using ProteinCenter software showed that only the GO Cellular Component terms *membrane*, *membrane part*, *integral to membrane*, and *intrinsic to membrane* are statistically over-represented in the Shave sample compared to the Control sample (Table 1) and no GO term related to membranar components are underrepresented (Table 2), which evinces the method efficiency to enrich membrane proteins. Furthermore, the software detected 643, 550, and 239 proteins with predicted transmembrane domains in the Biotinylated, Shave, and Control samples, respectively.

The high proportion (around 33%) of transmembrane proteins found in the Control sample might be in part explained by microvesicular release of so-called exosomes. In the Control, we could find more than half of the proteins most commonly associated to exosomes.¹⁸ These proteins (*Clathrin heavy chain*, *enolase*, *Rab7*, *ubiquitin*, *glyceraldehyde-3-phosphate dehydrogenase*, *hsp70*, and *90, 14–3–3 proteins*, *cyclophilin A*, *pyruvate kinase*, *tubulin α and β*, and *histones H2A, H2B, H3, and H4*) were also identified in the secretome of the *Trypanosoma brucei* bloodstream form.¹⁹ However, it is worth noting that the proportion of secreted proteins with predicted transmembrane proteins found in *T. brucei* was only about 5%, which is in agreement with lower microvesicle exocytosis observed for *T. brucei* than for *T. cruzi*. Recently, a manuscript regarding the secretome of *T. cruzi* epimastigotes and metacyclic trypomas-

Table 1. Over-represented GO Terms in Epimastigote Shave Compared to Control

	description	count ^a	ref count ^b	raw p-value ^c	FDR p-value ^d
cellular components	membrane	514	197	1.62×10^{-8}	8.83×10^{-6}
	membrane part	508	196	3.63×10^{-8}	8.83×10^{-6}
	integral to membrane	495	190	4.68×10^{-8}	8.83×10^{-6}
	intrinsic to membrane	495	190	4.68×10^{-8}	8.83×10^{-6}
biological processes	-	-	-	-	-
molecular functions	protein binding	199	68	1.21×10^{-4}	1.82×10^{-2}

^aThe number of times this feature occurs in the analysis data set. ^bThe number of times this feature occurs in the reference data set. ^cThe significance of this difference in feature occurrence between the data sets. ^dThe FDR corrected version of the raw p-value.

Table 2. Under-represented GO Terms in Epimastigote Shave Compared to Control

	description	count ^a	ref count ^b	raw p-value ^c	FDR p-value ^d
cellular components	ribosome	84	85	1.93×10^{-5}	1.98×10^{-3}
	cytoplasm	251	193	2.49×10^{-5}	2.07×10^{-3}
	ribonucleoprotein complex	89	87	3.62×10^{-5}	2.48×10^{-3}
	metabolic process	642	457	3.63×10^{-13}	2.98×10^{-10}
biological processes	biological_process	755	493	2.86×10^{-9}	1.18×10^{-6}
	primary metabolic process	506	356	1.65×10^{-7}	4.52×10^{-5}
	cellular metabolic process	481	336	1.33×10^{-6}	2.18×10^{-4}
	protein metabolic process	293	220	1.52×10^{-5}	1.79×10^{-3}
	biosynthetic process	238	185	2.52×10^{-5}	2.07×10^{-3}
	cellular biosynthetic process	223	173	5.78×10^{-5}	3.44×10^{-3}
	cellular protein metabolic process	241	184	5.86×10^{-5}	3.44×10^{-3}
	translation	140	120	6.40×10^{-5}	3.51×10^{-3}
	cellular macromolecule biosynthetic process	158	130	1.11×10^{-4}	5.05×10^{-3}
	macromolecule biosynthetic process	158	130	1.11×10^{-4}	5.05×10^{-3}
	cellular process	581	372	2.32×10^{-4}	9.63×10^{-3}
	macromolecule metabolic process	344	240	2.34×10^{-4}	9.63×10^{-3}
	gene expression	162	129	3.27×10^{-4}	1.28×10^{-2}
molecular functions	molecular_function	967	575	6.74×10^{-7}	1.38×10^{-4}
	catalytic activity	538	364	3.49×10^{-6}	4.78×10^{-4}
	structural constituent of ribosome	79	80	3.45×10^{-5}	2.48×10^{-3}
	structural molecule activity	88	84	8.85×10^{-5}	4.55×10^{-3}

^aThe number of times this feature occurs in the analysis data set. ^bThe number of times this feature occurs in the reference data set. ^cThe significance of this difference in feature occurrence between the data sets. ^dThe FDR corrected version of the raw p-value.

tigotes²⁰ showed a high proportion of secreted proteins with transmembrane domains after 6 h of incubation and detected the release of a diverse population of vesicles by electron microscopy, which supports our argument that early vesicle secretion is the source of membrane proteins in the control.

It is known that *T. cruzi* epimastigotes have programmed cell death and presentation of autophagic components induced by serum deprivation.²¹ The presence of histones indicates that apoptotic vesicles might have been released, even under conditions that maintained cell integrity within the 30 min of incubation in DMEM. Moreover, protein quantification by amino acid analysis revealed that the amount of sample in Shave samples was four times higher than that in Control samples in the two biological replicates.

Altogether, the results show the need to carry out both experiments (Shave and Control) in parallel in order to have a *bona fide* cell surface proteome composition, though we cannot exclude the possibility that the proteins identified in both samples are indeed part of the cell surface protein repertoire. A good example is enolase. This protein is annotated as cytosolic and involved in carbohydrate metabolism, but it has been found on the cell surface of several prokaryotic and eukaryotic organisms,^{22–25} in the secretome/exosome of pathogenic organisms,^{19,20,26–32} where it plays a role in virulence and

possibly also in tripanosomatid parasites (reviewed in ref 33). This protein has no secretion signal peptide or anchor region but might be secreted by a nonclassical secretory pathway.¹⁸

A large number of proteins identified in the Biotinylated sample were not found in the Shave sample and *vice versa* (Figure 4). Consequently, carrying out both methods to access the cell surface proteins offers a much broader view of this subproteome. The overlap between Biotinylated and Control, in our opinion, strengthens the argument that some proteins identified in the Control are also in the plasma membrane or strongly associated to its proteins. Since in the Biotinylated sample cells were washed after biotin labeling, most water-soluble unlabeled proteins were removed after lysis and labeled proteins bound to the streptavidin column were extensively washed also with high ionic strength solution (1 M NaCl). Since several wash steps were included to remove proteins that bind weakly to biotin-tagged proteins as well as to remove proteins bound nonspecifically to the column itself, we employed Western blotting using a specific antiserum against TcMTAP. The absence of anti-TcMTAP cross-reactive proteins in the purified fraction validated the procedure (Figure 3).

We identified different histone proteins in the Biotinylated and Shave samples, but the role these proteins might play on the epimastigote cell surface is yet unknown. A previous work

with *Leishmania donovani* used the histone H2B relative expression level between two conditions, using SILAC, to tell apart the protein set that would be considered, in that case, secreted out of those due to cell lysis, as they considered the presence of this protein is due to lysis.²⁸ However, histone H2B was already detected in the cell surface proteome of metastatic human melanoma cell lines and had its surface localization confirmed by immunocytochemical analysis.³⁴

When comparing Biotinylated to Shave samples using ProteinCenter, only the terms “molecular function” and “binding” were statistically under-represented in the Biotinylated compared to the Shave samples and both samples share 85 KEGG pathways (SI Table 2) identified with Blast2GO, but only three (*phosphonate and phosphinate metabolism, biotin metabolism, and porphyrin and chlorophyll metabolism*) and five (*ether lipid metabolism, vitamin B6 metabolism, peptidoglycan biosynthesis, riboflavin metabolism, and other glycan degradation*) pathways were exclusively found in the Shave and the Biotinylated samples, respectively. Despite the low overlap of identifications between both methods, we verified that they do not show any trend toward a specific functional group of proteins.

A known feature of this insect hosted life form is the preference of glucose and amino acids (mainly L-proline) as carbon source,^{35–38} and we can notice that the membrane and membrane-associated proteins detected here reflect this feature, as glycolysis and gluconeogenesis and amino acid metabolism are among the pathways with the greater number of elements identified in the Shave and Biotinylated samples (SI Table 2). In the Shave we detected 27 proteins with 18 different enzyme activities related to the glycolysis/gluconeogenesis pathway and 22 proteins with 16 activities related to the arginine and proline metabolism pathway, and in the Biotinylated sample, there were 31 and 12 proteins with 17 and 11 different activities related to glycolysis/gluconeogenesis and arginine and proline pathways, respectively.

A previous work, as already mentioned, has addressed the epimastigote detergent-solubilized membrane subproteome using a lower resolution mass spectrometer, which yielded only 280 epimastigote membrane proteins identified. We used the protein list provided by the authors, translated its GenBank to Uniprot accession numbers as used in our data, and compared the identifications. The 280 proteins were mapped to 267 nonredundant Uniprot identifiers, from which the vast majority (237 identifiers) did not match any protein presented in the enriched PM samples or the Control, as shown in Figure 5. The fact that our work deals with PM proteins while the previous work reported hydrophobic proteins, which include proteins from PM and other membranes, may explain the differences observed.

The first comprehensive proteome analysis of all four major life forms of *T. cruzi* has reported some proteins, such as histidine ammonia-lyase and urocanate hydratase, being more abundant in insect life stages.³⁹ We could detect these enzymes in our enriched samples, but the presence of them also in the Control may suggest that they are possibly contamination, although they may be associated with the plasma membrane or other membrane proteins. This manuscript also reported that no trans-sialidases were detected in epimastigotes, but in our study they were identified in the Biotinylated and Control samples, which suggests that the insect stage expresses low levels of this membrane protein family.

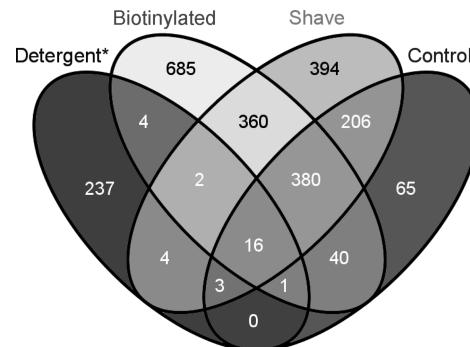


Figure 5. Comparison between protein identifications obtained by the two methodologies (Biotinylated and Shave) optimized here for plasma membrane subproteome analysis versus the experimental Control and the previously published neutral detergent-solubilized membrane proteins (marked by *).¹¹

Little is reported about proteins mediating the interaction between *T. cruzi* epimastigotes and the insect hosts, but it has been observed that, on the anterior midgut surface, parasites were bound through the cell body or flagellum and, on the posterior midgut, only flagellar attachment to perimicrovillar membranes occurred.⁴⁰ This attachment is presumably due to the involvement of glycoinositol phospholipid molecules.^{41–43} When a gene encoding a surface glycoprotein was deleted in those epimastigotes, a significant decrease of the parasite population attached to the triatomine vector *Rhodnius prolixus* was observed.^{44,45} This may suggest the involvement of glycoproteins that are concentrated in the flagellar attachment zone in the process. In our study we could identify several putative uncharacterized proteins that are annotated and described by Blast2GO software as flagellar associated proteins (SI Table 1), which leads us to believe that, by the cell surface trypsinization and biotinylation methodologies employed here to analyze the cell surface proteome, one can detect the proteins that are involved in the parasite–vector interaction.

CONCLUSION

The Shave and Biotinylation methods were able to successfully enrich PM proteins, as demonstrated by protein annotation analysis, and none of them have shown bias toward any specific functional group of proteins. Thus, the two techniques succeeded to access the epimastigote cell surface proteome, and the low overlap of protein identifications demonstrates that their use together provides a complementary view of this subproteome. Furthermore, our results with these techniques provided a great number of epimastigote membrane proteins not previously reported in a proteomic study, expanding the coverage of this subproteome.

Although the optimal incubation conditions established for the Shave experiments seem to have prevented extensive contamination of cytosolic proteins due to lysis and/or metabolic stress, the control still presented quite a number of proteins, of which over a third have predicted transmembrane domains. On the basis of several indications, these proteins, probably released by vesicular secretion of the parasite, could be due to the constitutive release of exosomes or physiological response to serum deprivation, which could not be avoided. By all means, the sample yield in the Shave was significantly higher than that in the Control in both biological replicates and the GO terms related to membrane cellular component were statistically over-represented. Thereafter we have confidence

that the vast majority of the proteins identified in the Shave belongs to the PM or are associated to it, but it is advisable to always perform the Control in parallel and exclude the common identifications if the goal is to identify *bona fide* PM proteins.

The PM protein enrichment by biotinylation preserves the primary structure, which makes the approach recommendable to use in PM phosphoproteomics, but the removal of the detergents and salts, needed for solubilization and elution, led to significant losses of sample. Also worth mention is that this technique requires a larger amount of cells and, therefore, might not be the best option to use with scarce samples.

The methodologies optimized here may be applied to access the plasma membrane subproteome of other *T. cruzi* life forms as well as in cell differentiation and drug response studies.

■ ASSOCIATED CONTENT

Supporting Information

Supplementary tables showing protein groups identified in each protein set and KEGG pathways determined for each protein sample set. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

PM, plasma membrane; GPI, glycosylphosphatidylinositol; FBS, fetal bovine serum; DMEM, Dulbecco's modified eagle medium; LIT, liver infusion triptose; TEAB, triethylammonium bicarbonate; IAA, iodoacetamide; TcMTAP, *T. cruzi* methylthioadenosine phosphorylase

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