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SHORT COMMUNICATION

Proteomic map of *Trypanosoma cruzi* CL Brener: the reference strain of the genome project

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Abstract In this work two-dimensional gel electrophoresis combined with mass spectrometry was carried out in order to start the construction of a map of soluble proteins from epimastigote form of *Trypanosoma cruzi* CL Brener. This strain is a hybrid organism derived from two genotypes, *T. cruzi* I and *T. cruzi* II and was chosen for genome sequencing. The two-dimensional gel electrophoresis showed that most of proteins focused at 4–7 pH range. The identification demonstrated that several proteins were in multiple isoforms, such as tubulin and heat shock proteins. Potential targets for development of chemotherapeutic agents like arginine kinase, an enzyme absent from mammalian tissues that is involved in the energy supply of the parasite, were also detected.

Keywords Proteome · *Trypanosoma cruzi* · Epimastigote · CL Brener strain

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Introduction

In terms of public health and economic impact, Chagas disease is the most important parasitic infection in Latin America. There are 18–20 million people infected with *Trypanosoma cruzi* (*T. cruzi*) and another 40 million people are at risk of acquiring the disease. Recent surveys indicate that there are ~200,000 new cases and 21,000 deaths associated with this condition every year (WHO 2002).

In distinct geographical regions, the prevalence of clinical forms and morbidity of Chagas disease are different. In Brazil, the asymptomatic or indeterminate form is the most common (60–70%), followed by the cardiac and digestive forms (20–30% and 8–10%, respectively). However, in Central Brazil and Chile, the latter presentation predominates, while it is practically non-existent in Venezuela and Central America (Miles 1997; WHO 2002). These observations associated with the variable response to treatment and diverse biological behaviour in mammal and triatomine bugs have led to the assumption that *T. cruzi* might not be a single entity, but a heterogeneous complex of organisms. Specific variations among *T. cruzi* have been observed based on their genetic and biological characteristics and their behaviour in the vertebrate host. Although genetic studies are important to clarify the intra-specific heterogeneity of the parasite, the study of the biological behaviour and the host-parasite relationships could clarify the importance of different strains, in the determination of clinic-pathological manifestations of Chagas disease. Indeed, *T. cruzi* strains represent a complex being nominated like that (Devera et al. 2003) due to the diversity of intrinsic characteristics such as antigenic composition, morphology, susceptibility to chemotherapy, isoenzyme patterns, genomic profiles of kinetoplast DNA and several nuclear genes and chromosomal profiles, as well as in the host-parasite

relationship. *T. cruzi* strains typed by several molecular methods were finally classified into two major groups named *T. cruzi* I and II, as well as additional groups yet to receive designations. The former is mostly associated with the sylvatic transmission cycle and infection of marsupials (Clark and Pung 1994) while *T. cruzi* II consists of five related subgroups: 2a, 2b, 2c, 2d and 2e (Brisse et al. 2000) and is associated with the domestic transmission cycle and infection of placental mammals (Briones et al. 1999).

The morpho-biological diversity of *T. cruzi* may suggest different patterns of protein expression dependent upon growth phase, metabolic status or life cycle stage. Some stage-specific proteins could play major roles in infectivity and survival therefore providing rational targets for drug design. The *T. cruzi* genome needs, therefore, to be linked to the characterization of the parasite proteome to provide information about the biological phenotype and processes of the parasite. Several gene expression levels do not accurately predict protein levels due to the existence of other control mechanisms, including post-transcriptional regulatory ones. Some *T. cruzi* proteomic studies have been reported (Paba et al. 2004; Parodi-Talice et al. 2004; Atwood et al. 2005, 2006).

In this study, a combination of two-dimensional gel electrophoresis (2-DE) analysis with mass spectrometry (MS) protein identification was carried out in order to construct a map of soluble proteins from the epimastigote forms of *T. cruzi* CL Brener. This strain is a member of the subgroup IIe and was chosen for genome sequencing (El-Sayed et al. 2005) because it is well characterized experimentally (Zingales et al. 1997). Data from several studies (Brisse et al. 1998; Machado and Ayala 2001; Gaunt et al. 2003; Westenberger et al. 2005) are consistent with CL Brener being a hybrid due to the frequency of heterozygosity.

Materials and methods

Cell culture and protein preparation

Epimastigotes were grown in liver infusion tryptose (LIT) medium supplemented with 10% fetal bovine serum at 28°C. Cells (1×10^9) from 7-day-old cultures were centrifuged and washed three times with phosphate buffered saline (PBS). Parasites were submitted to four cycles of alternate freezing (2 min in liquid nitrogen)–thawing (37°C water bath) in presence of PBS and protease inhibitor cocktail (1 mM PMSF, 10 μg/mL pepstatin A, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 100 μM E-64, 1 mM *o*-phenanthroline). The samples were centrifuged at 14,000*g* for 20 min at 4°C and proteins in the resulting supernatant were precipitated by 17% tricholoracetic acid (TCA) and then solubilized in 2% CHAPS, 8 M urea, 20 mM DTT and stored at −70°C. Protein concentration was determined by the RC DC method (Bio-Rad), using bovine serum albumin as standard protein. This assay is based on the Lowry assay (Lowry et al. 1951) but has been modified to be reducing agent compatible (RC) as well as detergent compatible (DC).

Two dimensional gel electrophoresis and gel analysis

A total of 700 μg of proteins were applied to 17 cm immobilized pH gradient (IPG) gel strips (Bio-Rad) with a linear separation pH range by in gel sample rehydration. After 11 h of active rehydration at 50 V at 20°C using a Protean isoelectric focusing system (IEF) (Bio-Rad), proteins were separated using the following protocol: 250 V, 20 min; 10,000 V, 2 h 30 min; 10,000 V, 90,000 VH. Gels (12% SDS-PAGE) were stained with Coomassie Blue G-250 (Merck, Whitehouse Station, NJ, USA), and gel images were captured with a densitometer (GS-800, Bio-Rad), digitalized and analyzed by PDQuest™ software (Bio-Rad).

Protein digestion and identification

Several spots were manually excised from the gel and destained with 100 μL 25 mM NH_4HCO_3 pH 8.0 in 50% acetonitrile for 15 min. After three washings, gel pieces were dehydrated with 100% (v/v) acetonitrile and then dried using a Speed Vac evaporator (Savant, Farmingdale, NY, USA). Proteolytic in-gel digestion was performed using 10 μL of ice-cold sequence grade modified trypsin (Promega) solution (20 ng/μL in 25 mM NH_4HCO_3 pH 8.0) at 37°C, overnight, followed by peptide extraction from the gel with 50% acetonitrile in 5% trifluoroacetic acid in a sonicator for 30 min. The extracts were dried under vacuum, and then solubilized in 50% (v/v) acetonitrile in 0.1% trifluoroacetic acid. For mass spectrometry analysis the peptides were co-crystallized with 0.3 μL of 10 mg/ml α-cyano-4-hydroxycinnamic acid solution in 0.1% (w/v) trifluoroacetic acid, 50% (v/v) acetonitrile directly onto a MALDI target plate. Raw data for protein identification were obtained on the 4700 Proteomics Analyzer (Applied Biosystems, Foster City, CA). Both MS and MS/MS data were acquired in positive and reflectron mode using a neodymium-doped yttrium aluminum garnet (Nd:YAG) laser with a 200-Hz repetition rate. Typically, 1,600 shots were accumulated for spectra in MS mode while 2,400 shots were accumulated for spectra in MS/MS mode. Up to five of the most intense ion signals with signal to noise ratio above 30 were selected as precursors for MS/MS acquisition excluding common trypsin autolysis peaks and matrix ion signals. External calibration in MS mode was performed

using a mixture of four peptides: des-Arg1-Bradykinin (m/z = 904.47), angiotensin I (m/z = 1,296.69), Glu1-fibrinopeptide B (m/z = 1,570.68), and ACTH (18–39) (m/z = 2,465.20). MS/MS spectra were externally calibrated using known fragment ion masses observed in the MS/MS spectrum of angiotensin I. MS/MS database searching was against the NCBInr databases using the Mascot software (www.matrixscience.com). The search parameters were as follows: one tryptic miss cleavages allowed, non-fixed modifications of methionine (oxidation), cysteine (carbamidomethylation) and pyroglutamate formation at N-terminal glutamine of peptides. The pps, pmf and ppw files were generated from the raw (or native) MS data according to the following parameters using the Data Explorer Software (Applied Biosystems). For MS1: mass range: 500–4,000, peak density: 15 peaks per 200 Da, Signal-to-noise ratio: 5, minimum area: 10, maximum peaks per spot: 10. For MS2: mass range: 60 and precursor 20, peak density: 55 peaks per 200 Da, Signal-to-noise ratio: 2, minimum area: 10 and maximum peaks per precursor: 200.

Results and discussion

In general, due to the protein modifications and alterations that often reflect functional states in the cell, analysis of the mRNA may not correspond to proteome data and may not provide results of the dynamic cell state (Anderson and Seihammer 1997; Gygi et al. 1999; Chen et al. 2002). Therefore, highly resolving and sensitive techniques must be applied to enable differentiation of a great number of these proteins. In this context, two-dimensional gel electrophoresis in combination with high-sensitivity mass spectrometry is one of the most chosen approach (Gygi et al. 2000; Wittmann-Liebold et al. 2006). The protocols used herein, for protein preparation and for two-dimensional gel electrophoresis provided good reproducibility and quality of detectable spots on the gel. As shown in Fig. 1a, most of the spots focused in the middle of the gel (selected area) when a pH 3–10 range was used. According to the PDQuest program (Bio-Rad software) analysis, approximately 256 spots were clearly resolved on the gels stained with Coomassie Blue G-250. However, when a

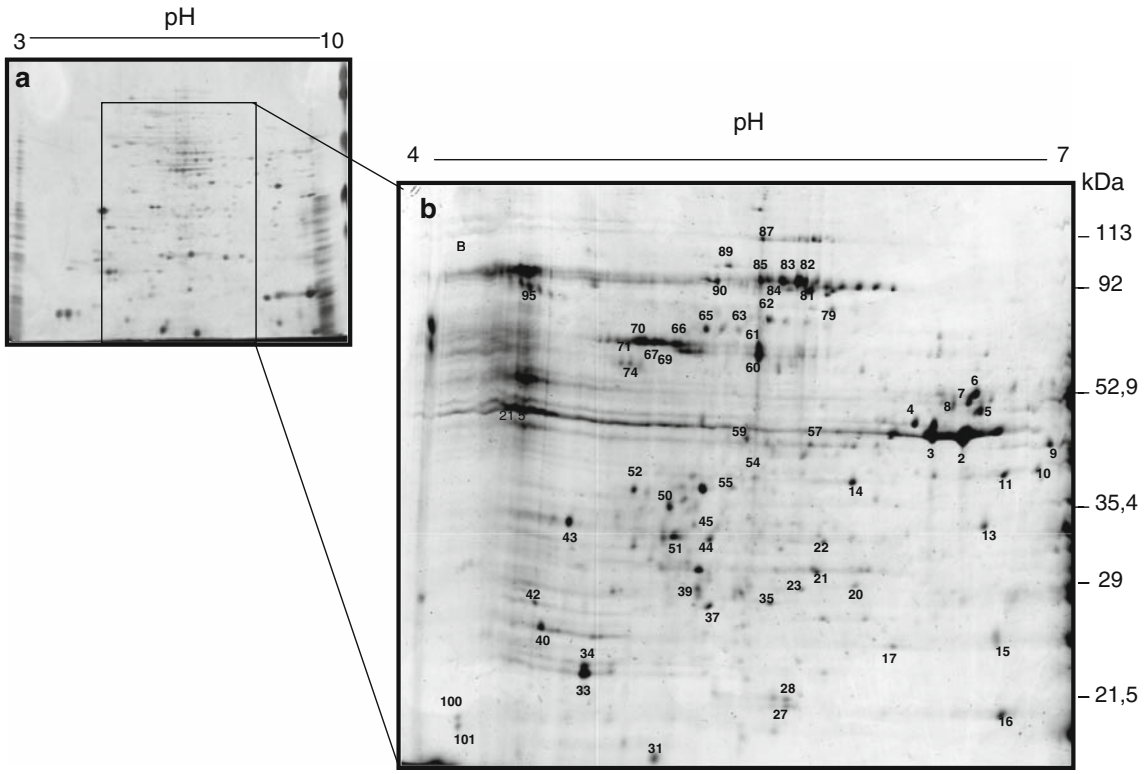

Fig. 1 Two-dimensional gel electrophoresis of soluble proteins from CL Brener epimastigotes. Soluble proteins (700 μg) from parasites were applied to IPG gel strips (Bio-Rad) with linear separation range and separated by IEF using Protean IEF system (Bio-Rad). After first dimension, strips were subjected to reduction by 130 mM DTT in an equilibrium buffer (6 M urea, 2% SDS, 0,375 M Tris-HCl pH 8.8, 20% glycerol) during 10 min and then alkylation by 135 mM iodoacetamide in the same buffer. SDS-PAGE was performed on 12% polyacrylamide gels run on a Protean III system (Bio-Rad). Proteins were visualized by Coomassie Blue G-250 staining. **a** pH range 3–10; **b** pH range 4–7; numbered spots were identified and listed in Table 1

Table 1 Soluble CL Brener proteins identified by MALDI TOF-TOF

Spot	Identification	Accession number	Peptide matching protein (MW)	Theoretical MW (Da)	Theoretical p*I*	Gene ID (according to the TSK-TSC Consortium)
2,3,21,39	Dehydrogenase (old yellow enzyme)/prostaglandin F2alpha synthase	gi61741938/ gi71659766	GGLIFLQLIHAGR (1394.8) FIANPDLVER (1173.6) AQHNWPLNEPRPETYYTR (2272.2) ETYGVPEELTDDEVR (1751.8) YDFEEADQQIR (1413.6)	42,211	6.03	6829.t00023 7739.t00008
33, 34	Putative eukaryotic initiation factor 5a, putative	gi70886939	VSIVATDIFTGNK (1364.8) TSTYSVLDIQEDR (1526.7)	18,004	4.82	7143.t00012
5, 7, 8	Putative glutamamyl carboxypeptidase	gi71402529	GTSHFWVR (989.5) AEFEDAEIVITPR (1489.7)	43,585	6.24	7420.t00002
16	Putative peptide methionine sulfoxide reductase	gi71405176	SAIFYHDDQQLK (1464.7)	20,078	6.12	8524.t00001
11	Cytosolic malate dehydrogenase, putative	gi71405868	NAAIFSEHGR (1101.6) NCIIWGNHSGTQVPDVNSATVR (2425.3)	35,567	6.20	7146.t00001
27, 28, 45, 60, 61	Alpha-tubulin	gi71397525	AVFLDLEPTVVDEIR (1715.9) EIVDLCLDR (1132.5) FDGALNVDLTEFQTNLVPYPR (2409.1)	49,767	4.94	11788.t00001
31	Putative 40S ribosomal protein S12	gi71403039	EELAEWAGLQK (1273.6) ALASQANIDFVEVESR (1748.8) QGEEPTVTQQFDVEPENLR (2216.0)	15,887	5.07	7775.t00002
4	Tyrosine aminotransferase	gi71659497	NLVVPGWR (940.5) EAVATWWR (1018.5) LLIVTNPSNPCGSNFSR (1875.9)	46,123	5.82	8328.t00005
13	Elongation factor 2, putative	gi71415388	DSFVAAWQWATR (1437.7) AYLPVAESFGFTADLR (1756.9) GVIIGEENRPGTPIYNVR (1984.1)	94,130	5.67	7622.t00002
9	Putative arginine kinase	gi70870147	TFLVWVNEEDHLR (1657.7) FLQAAHACEFWPTGR (1790.7) LGFLTFCPTNLGTTIR (1810.8)	40,172	6.29	7265.t00003
14	Putative activated protein kinase C receptor	gi70882944	GAHTDWVSCVR (1287.6) DVLSVTFSPDNR (1349.7) NSEECEYGFPER (1516.6)	35,009	5.73	8621.t00013

Table 1 continued

Spot	Identification	Accession number	Peptide matching protein (MW)	Theoretical MW (Da)	Theoretical p*I*	Gene ID (according to the TSK-TSC Consortium)
40, 42	Putative IgE-dependent histamine-releasing factor	gi71408210	GSYIEVGGEDYGIAANVDEDAGEGAK (2586.2) VVDVVHNNR (1051.5) WDGEVPYFYYWK (1652.7)	19,660	4.48	8291.t00003
37	Peroxiredoxin (or tryparedoxin peroxidase, putative)	gi71408703	HITVNDLPVGR (1220.6) DYGVLIEEQGISLR (1591.7)	25,490	7.61	8115.t00003
15	Tryparedoxin peroxidase	gi71413207	QITVNDLPVGR (1211.7) SYGVLKEEDGVAYR (1585.8)	22,390	5.69	6413.t00010
20, 23	Adenylate kinase	gi71667254	GWLLDGFPR (1060.5) FGVVHISSGDLLR (1399.8)	29,374	5.65	7118.t00018
10	Putative cytosolic malate dehydrogenase	gi71411668	NAAIFSEHGR (1101.5) VGYALLPLIAGGR (1299.7)	35,486	6.26	5923.t00004
95	Conserved hypothetical protein	gi71651556	AVESQLQVYSEVIGLR (1791.1)	60,535	4.63	6207.t00017
100	Putative 60S acidic ribosomal protein P2	gi71414227	NTLPVIFAR (1030.6)	11,440	4.24	5886.t00004
57	Elongation factor 2	gi71413833	VSEPVVSFR (1019.6) AYLPVAESFGFTADLR (1757.0)	94,129	5.73	8556.t00009
67	Putative 2,3-bisphosphoglycerate-independent phosphoglycerate mutase	gi71666782	LHVLYDGR (972.5) YDGDLGIPNNFLVPPPR (1884.0)	60,327	5.59	6869.t00033
54	60S acidic ribosomal protein P0	gi71668474	TLLGASVATEYEFDEFDGK (2092.1) LDISPFYYQVEVQSVWDR (2244.3)	34,909	5.02	7695.t00026
89	Putative glucose-regulated protein 78 (or HSP70)	gi71415505	SDIHEIVLVGGSTR (1482.8) NAVVTVPAYFNDAQR (1664.9)	71,272	5.09	7009.t00004
51, 81	Putative heat shock 70 kDa protein mitochondrial precursor	gi71407386	SQTFSTAADNQTQVGIK (1795.9) GVNPDEAVALGAATLGGVLR (1880.1)	30,783	5.22	8771.t00001
43	Putative nascent polypeptide associated complex subunit	gi71422974	AVSGVVPETEPR (1240.7) HGSLSFLVNQPELYR (1760.0) KHGSLSFLVNQPELYR (1888.0) FPGTNTFLVFGEAQLGDTAMEAQEAAAR + Oxidation (M) (2958.9)	19,564	4.66	8342.t00006

Table 1 continued

Spot	Identification	Accession number	Peptide matching protein (MW)	Theoretical MW (Da)	Theoretical p*I*	Gene ID (according to the TSK-TSC Consortium)
59	Conserved hypothetical protein	gi71407656	YTGDWAFGR (1072.6) DGNIVGQGEFR (1191.7) YEGYWQFDR (1263.6) YFYADGGVYEGEWNDGR (1998.0)	40,835	5.28	6192.t00006
63, 65	Heat shock protein 60, mitochondrial precursor	gi71665068	AVGVILQSVAEQSR (1456.8) AAVQEGIVPGGGVALLR (1607.1) ALDSLLGDSSLTADQR (1662.0) VLENNDVTVGYDAQR (1693.0)	59,129	5.38	7414.t00029
82, 83, 84, 85	Heat shock protein 70, putative	gi71661434	FEELCGDLFR (1285.6) GDDKPVIQVQFR (1401.7) TTPSYVAFTDTER (1487.8) AVVTVPAYFNDSQR (1566.8) DCHLLGTFDLSGIPPAPR (1966.1)	73,252	5.42	8621.t00017
44, 50, 52, 55, 61, 66, 69, 70,71	Beta tubulin	gi71656281	YLTASALFR (1041.6) LAVNLVPFPR (1125.6) FPGQLNSDLR (1146.6) INVYFDEATGGR (1341.6) GHYTEGAELIDSVLDVCR (2033.9)	49,668	4.70	6998.t00004
79	Putative proteasome alpha 7 subunit	gi71652599	DVFATPIPGSVLASR (1529.9)	25,725	5.80	8813.t00005
74	Putative Hsc70-interacting protein (Hip)	gi71420437	ALDLNPENVR (1140.5) DLSEAQAVDYDEGIDEILR (2151.0)	48,062	4.83	8318.t00015
81	Putative heat shock 70 kDa protein mitochondrial precursor	gi71407515	VLENTEGFR (1064.5)	70,946	5.75	7180.t00003
87	Heat shock-like 85 kDa protein	gi71652472	AELVNNLGTIAR (1270.7) GVVDSEDLPLNISR (1513.7) YQSLTNQAVLGDESHLR (1930.9)	80,690	5.07	8001.t00014
90	Putative heat shock 70 kDa protein/Putative DNAK protein	gi71420615	TTPSYVAFTETER (1501.9)	30,128	6.23	5334.t00012
		gi71408185	AVITVPAYFNDSQR (1580.8) ATAGDTHLGGEDFDNR (1675.7) NAVENYTFSLR (1313.6) SQTFSTNADNQR (1368.8)	27,131	4.84	5736.t00005
101	60S acidic ribosomal protein P2, putative	gi71402937	NTLPVIFAR (1030.6)	11,405	4.24	4611.t00001

Table 1 continued

Spot	Identification	Accession number	Peptide matching protein (MW)	Theoretical MW (Da)	Theoretical *pI*	Gene ID (according to the TSK-TSC Consortium)
6	Enolase, putative	gi71665461	AGSFNEALR (964.5) AQVVGDDLTVTNVSR (1573.8) SAVPSGASTGIHEACELR (1841.8)	46,415	5.92	4911.t00014
17	co-Chaperone GrpE, putative	gi71402335	TPASSEFPSGHISIVLK (1769.8)	24,292	8.49	7532.t00002
22	Proteasome alpha 7 subunit, putative	gi71399771	NQYDTNTTTWSPTGR (1741.8)	5,381	5.97	7469.t00002
35	hslvu complex proteolytic subunit-like, putative	gi71416273	VGEFPQQLTR (1174.5)	22,867	6.77	5979.t00026

narrower gradient pH 4–7 was applied, more than 338 spots were resolved (Fig. 1b). The experiments were performed in triplicate in order to assess reproducibility. Several spots were manually excised from the gel (Fig. 1b) and proteins were identified by MS (Table 1).

The table summary demonstrates that several proteins were detected in multiple isoforms, such as tubulin and heat shock protein. Different post-translational modifications causing peptides to have diverse biochemical properties could account for such unusual migration in the gel as previously described for *Trypanosoma brucei* (Jones et al. 2006) and *Leishmania* (Drummelsmith et al. 2003).

Interesting proteins such as arginine kinase (spot 9) deserve more commentaries. This enzyme is involved in the energy supply for the parasite since it catalyses the production of phosphoarginine, allowing ATP to be produced rapidly when required. It is absent from mammalian tissues, consisting in a possible target for further development of chemotherapeutic agents (Silber et al. 2005). Likewise, "old yellow enzyme", TCOYE, a prostaglandin F2α synthase that can reduce either trypanocidal agents and is responsible for most of the antioxidant activity in *T. cruzi* (Kubata et al. 2002) could be a good candidate as target for drug design (Parodi-talice et al. 2004).

The fact that gene expression in *T. cruzi* and other trypanosomatids is regulated firstly at post-transcriptional level, and that post-translational modifications play an important role in modulation of protein functions in this parasite, makes proteomic analysis especially attractive for observe changes in protein expression.

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