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Differential proteomic profiles from distinct *Toxoplasma gondii* strains revealed by 2D-difference gel electrophoresis

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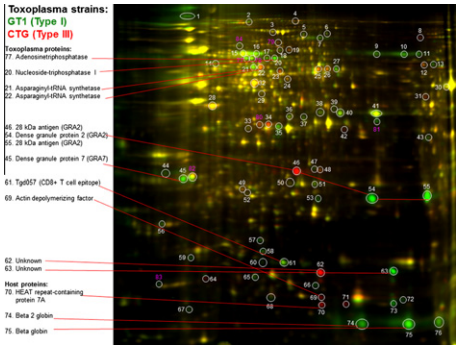
HIGHLIGHTS

- The differential proteomic profiles of 3 distinct *T. gondii* strains were shown by 2D-DIGE and MS.
- 84 protein spots were differentially expressed by greater than 1.5-fold in relative abundance.
- 13 protein spots with greater than 2-folds in relative abundance were successfully identified.
- Ten differentially expressed proteins were identified to reveal 7 *T. gondii* proteins in database.
- GT1 and PTG strains have similar proteomic profiles and both are different from that of CTG strain.

GRAPHICAL ABSTRACT



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ABSTRACT

Toxoplasma gondii is an obligate intracellular protozoan that infects mammals and birds. Human infection during pregnancy may cause severe damage to the fetus. Reactivation of latent infection in immunocompromised patients can cause life-threatening encephalitis. *T. gondii* strains are highly diverse but only a few lineages (Type I, II and III) are widely spread. In mouse model, Type I strains are highly virulent, whereas Type II and III strains are intermediately or non virulent. It is not clear how much quantitative difference exists in proteomic profiles among these distinct *T. gondii* lineages. In the present study, the proteomic profiles of *T. gondii* tachyzoites from these lineages were investigated by two dimensional fluorescence difference gel electrophoresis (2D-DIGE) and mass spectrometry (MS) technologies. A total of 2321 protein spots were detected. Overall, the GT1 strain of Type I lineage and the strain PTG of Type II lineage have highly similar proteomic profiles and both are different from that of the CTG strain of Type III lineage. Eighty-four protein spots were differentially expressed by greater than 1.5-fold in relative abundance and 10 of them were identified to 7 *T. gondii* proteins in existing database. Investigation of the quantitative differences in proteomics among distinct *T. gondii* strains should facilitate our understanding of difference in biological processes and pathogenesis of distinct *T. gondii* genotypes, which will provide basic information to determine treatment regimen for different manifestation of toxoplasmosis.

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1. Introduction

Toxoplasma gondii is an obligate intracellular protozoan parasite that infects virtually all warm-blooded mammals and birds (Dubey, 2009). In particular, *T. gondii* infections can cause ocular toxoplasmosis in healthy individuals, and infection acquired during pregnancy may spread and cause severe congenital toxoplasmosis. In immunocompromised patients, reactivation of latent infection can cause life-threatening encephalitis (Montoya and Liesenfeld, 2004; Miller et al., 2009). *T. gondii* infection in livestock is also a threat to public health from food-borne outbreaks and causes a great economic loss (Dawson, 2005). Currently, treatments of toxoplasmosis depend primarily on chemotherapy, but available drugs have many side effects, and reactivation may occur at any time. Even though there is a vaccine available for livestock, which is able to limit the incidence of abortion in sheep (Buxton et al., 1991), no *T. gondii* vaccine is available for humans and there is a need for vaccine development to control toxoplasmosis in humans (Bout et al., 2002).

Toxoplasma gondii consists of six major clusters with a large number of different genotypes based on multilocus genotyping study (Su et al., 2012). A few major genotypes, particularly the Type I, II and III strains, have a global distribution. These three genotypes differ in their virulence and/or pathogenicity in mouse model of infection (Sibley and Boothroyd, 1992; Howe and Sibley, 1995). The Type I strains are highly pathogenic in mice, Type II strains are intermediately virulent ($LD_{50} = 10^3$–10^4) and Type III strains are non-virulent ($LD_{50} \geqslant 10^5$) (Howe and Sibley, 1995; Dardé, 1996). Though phenotypically different, data on protein expression among these *T. gondii* lineages is still very limited.

Proteome analyses by 2-DE and MS in combination with database searching made it possible to differentiate and identify complex protein mixtures extracted from cells, tissues, or other biological samples (Görg et al., 2002; Liska and Shevchenko, 2003; Xiong et al., 2003). A systematic analysis of the proteome of *T. gondii* is important not only for understanding this parasite's biology, but also for the discovery of potential drug targets and new preventative and therapeutic strategies. So these proteomic approaches would be valuable for obtaining a comprehensive understanding on several aspects of biological function, diversity and pathogenicity of *T. gondii.*

Attempts have been made in characterizing the proteome of *T. gondii*, which have helped in elucidating the specialized invasion organelles and their composition, as well as proteins associated with the cytoskeleton (Cohen et al., 2002; Dlugonska et al., 2001; Xia et al., 2008; Che et al., 2011). For example, Cohen et al. (2002) characterized a reproducible 2-DE map of *T. gondii* tachyzoites of RH strain and resolved over 1000 polypeptides and analyzed 71 of these proteins by MALDI MS and MALDI post-source decay analysis. Another early proteomics effort was that of Dlugonska et al. (2001) who identified 13 excretory antigens from tachyzoites on a standardized 2-DE map. Xia et al. (2008) characterized the proteome of 3 strains of *T. gondii* (ME49, GT1, and VEG) by 2-DE, gel liquid chromatography-linked tandem mass spectrometry, and multidimensional protein identification technology (Mud-PIT) and identified 2252 proteins with 2477 intron-spanning peptides. Moreover, Che et al. (2011) analyzed the membrane proteome of *T. gondii* tachyzoites of RH strain by one-dimensional gel electrophoresis liquid chromatography–tandem mass spectrometry (1D gel LC–MS/MS), biotin labeling in conjunction with 1D gel LC–MS/MS analysis, and three-layer "Sandwich" Gel Electrophoresis (TLSGE) with MudPIT, identified 2241 proteins which grouped into 841 sequentially non-redundant protein clusters.

At present, there is still a lack of quantitative comparison of proteome map among major lineages of *T. gondii*. The 2D-DIGE technology, using a mixed-sample internal standard is now recognized as an accurate method to determine and quantify proteins, reducing inter-gel variability and simplifying gel analysis. In the present study, we employed 2D-DIGE technology in combination with mass spectrometry to compare proteomic profiles and identified a few differentially expressed proteins among Type I, II and III *T. gondii* lineages. The results also showed that Type I and II strains had a very similar quantitative proteomic profiles and they are different from the Type III strain.

2. Materials and methods

2.1. Growth of T. gondii in vitro and isolation of tachyzoites

Tachyzoites for proteome analysis were prepared from 3 distinct strains of *T. gondii* (Type I strain GT1, Type II strain PTG and Type III strain CTG). The parasites were expanded in Human foreskin fibroblasts (HFF). Cells and parasites were grown in 25 cm^2 vented flasks in Dulbecco's modified eagle medium (DMEM, pH 7.4) supplemented with 10% (v/v) fetal calf serum 100 μg/ml of gentamicin, and incubated at 37 °C in a 5% CO_2 humidified incubator. The parasites were harvested 3 days later by scraping infected HFFs into growth medium, isolated from HFF by passage through with a 25-gauge needle followed by filtration through 3 μm pore size membranes (Osmonics Inc. Minnetonka, MN). Filtered tachyzoites were washed twice in phosphate buffered saline (PBS), of pH 7.4 by centrifugation at 1500*g* for 10 min at room temperature. Approximately 1×10^7 tachyzoites (counted using a hemocytometer) were purified from infected cells and stored at −80 °C until required.

2.2. Preparation of total proteins from T. gondii for 2D-DIGE

Frozen parasite pellets were disrupted in lysis buffer composed of 8 M urea, 4% (w/v) CHAPS, 0.5% (v/v) immobilized pH gradient buffer, 1% mM dithiothreitol (added fresh), 10 mM phenylmethanesulfonyl fluoride (PMSF), 0.3 mg/ml EDTA, 0.7 μg/ml pepstatin and 0.5 μg/ml leupeptin on ice for 30 min, followed by sonication on ice using a sonicator (80 W, 20 s duration, 3 times, with 10 s intervals). The samples were added into 5 μg/ml RNAase and 20 μg/ml DNase at 4 °C for 30 min and centrifuged at 15,000*g* for 30 min at 4 °C. Then the proteins were concentrated using 5 K MWCO spin column (VIVA Biosciences) to exchange the buffer to 2D lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris–HCl, pH 8.8). Protein concentration was measured using Bio-Rad protein assay method.

2.3. Protein labeling with CyDye DIGE fluors

For each sample, 30 μg of protein was mixed with 1.0 μl of diluted CyDye (1:5 diluted with DMF from 1 nmol/ul stock), and kept in dark on ice for 30 min. Samples from each pair were labeled with Cy3 and Cy5, respectively while the same amount of the pool standard that contained equal quantities of all the samples was labeled with Cy2. The labeling reaction was stopped by adding 1.0 μl of 10 mM lysine to each sample, and incubating in dark on ice for additional 15 min. The labeled samples were then mixed together. The 2 × 2D Sample buffer (8 M urea, 4% CHAPS, 20 mg/ml DTT, 2% pharmalytes and trace amount of bromophenol blue), 100 μl destreak solution and rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 20 mg/ml DTT, 1% pharmalytes and trace amount of bromophenol blue) were added to the labeling mix to make the total volume of 250 μl. The labeled samples were well mixed and spun before loading into the strip holder.

2.3.1. 2D-DIGE

After loading the labeled samples to pH 3–10 linear IPG strips (GE healthcare), the IEF was run with 12 h rehydration at 20 °C, followed by 500 V for 1000 VHr, 1000 V for 2000 VHr, and 8000 V for 24000 VHr. Upon finishing the IEF, the IPG strips were incubated in the freshly made equilibration buffer-1 (50 mM Tris–HCl, pH 8.8, containing 6 M urea, 30% glycerol, 2% SDS, trace amount of bromophenol blue and 10 mg/ml DTT) for 15 min with gentle shaking. Then the strips were rinsed in the freshly made equilibration buffer-2 (50 mM Tris–HCl, pH 8.8, containing 6 M urea, 30% glycerol, 2% SDS, trace amount of bromophenol blue and 45 mg/ml Iodacetamide (Sigma, St Louis) for 10 min with gentle shaking. Next the IPG strips were rinsed in the SDS-gel running buffer before transferring into 12% SDS-gels (18 × 16 cm) followed by sealing with 0.5% agarose (Bio-Rad, CA) in SDS–PAGE running buffer. The SDS-gels were run at 15 °C until the dye front running out of the gels.

2.4. Analysis of gels

Gel images were scanned immediately following the SDS–PAGE using Typhoon TRIO (Amersham BioSciences). The scanned images were then analyzed by Image Quant software (version 6.0, Amersham BioSciences), followed by in-gel analysis using DeCyder software version 6.0 (Amersham BioSciences) containing ANOVA test and *t*-test. The fold change of the protein expression levels was obtained from in-gel DeCyder analysis. Criteria for differential protein expression were false discovery rate (FDR)<0.05.

2.5. Protein enzymolysis

The spots of interest were picked up by Ettan Spot Picker (Amersham BioSciences) based on the in-gel analysis and spot picking design by DeCyder software. The gel spots were washed a few times then digested in-gel with modified porcine trypsin protease (Trypsin Gold, Promega). The digested tryptic peptides were desalted by Zip-tip C18 (Millipore Corp., MA, USA). Peptides were eluted from the Zip-tip with 0.5 μl of matrix solution α-cyano-4-hydroxycinnamic acid (5 mg/ml in 50% acetonitrile, 0.1% trifluoroacetic acid, 25 mM ammonium bicarbonate) and spotted on the MALDI plate (model ABI 01-192-6-AB).

2.6. Protein identification by mass spectrometry

MALDI-TOF MS and TOF/TOF tandem MS/MS were performed on an ABI 4700 mass spectrometer (Applied Biosystems, Framingham, MA). MALDI-TOF mass spectra were acquired in reflectron positive ion mode, averaging 4000 laser shots per spectrum. TOF/TOF tandem MS fragmentation spectra were acquired for each sample, averaging 4000 laser shots per fragmentation spectrum on each of the 10 most abundant ions present in each sample (excluding trypsin autolytic peptides and other known background ions).

2.7. Database search and bioinformatics analysis

Both of the resulting peptide mass and the associated fragmentation spectra were submitted to GPS Explorer workstation equipped with MASCOT search engine (Matrix science) to search the database of National Center for Biotechnology Information non-redundant (NCBInr) and ToxoDB (www.toxodb.org/toxo/). Searches were performed without constraining protein molecular weight or isoelectric point, with variable carbamidomethylation of cysteine and oxidation of methionine residues, and with one missed cleavage also allowed in the search parameters. Candidates with either protein score C.I.% or Ion C.I.% greater than 95 were considered significant.

3. Results

3.1. Comparative proteome analysis by 2D-DIGE

Protein expression profiles of *Toxoplasma* tachyzoites from the GT1, PTG and CTG strains were obtained by 2D-DIGE. Proteins with MW ranging from 14 to 150 kDa and pI between 4 and 9 were separated well. A total of 2321 spots were detected. Spots exhibiting over 1.5-fold difference in their relative abundance with a *p*-value less than 0.05 in *t*-test between both samples were considered as differentially expressed (Fig. 1). A total of 84 differentially expressed protein spots were identified after analysis of the 2D gel using DeCyder software (Fig. 1). Based on this set of data, Type I and II strains have highly similar proteomic profiles and both are different from that of the Type III strain (Fig. 2).

3.2. Identification of T. gondii tachyzoite proteins

A total of 19 spots with at least 2-fold changes (increase or decrease) in expression levels were trypsin digested and analyzed by MALDI-TOF MS and TOF/TOF tandem MS/MS. Fifteen of these protein spots were successfully sequenced, and 10 of them were identified to 7 *T. gondii* proteins in existing database (Table 1).

The spot 45, highly expressed in the GT1 and PTG strains, was identified as the dense granule protein 7 (GRA7). The spots 54 and 55, highly expressed in GT1 and PTG strains, were identified as the 28 kDa antigen GRA2, while spot 46 highly expressed by CTG strain was also identified as GRA2. The spots 20, 21, 61 and 77, which were highly expressed in the GT1 and PTG strains, were identified as nucleoside-triphosphatase I, putative asparaginyl-tRNA synthetase, tgd057 and adenosinetriphosphatase, respectively. The spots 22 and 69, highly expressed in CTG strain, were identified as putative asparaginyl-tRNA synthetase and actin depolymerizing factor, respectively. Spots 70, 74, and 75 were identified as HEAT repeat-containing protein 7A, beta 2 globin and truncated beta-globin possibly from host cells, respectively. Two additional spots, the 62 and 63, did not match to any annotated proteins in NCBInr and ToxoDB database.

Typical differentially expressed protein spots were revealed in the 3D view corresponding to the pixel volume distribution for the Cy5/Cy3 ratios (Fig. 3). Typical differentially expressed protein spots were revealed in the 3D view corresponding to the pixel volume distribution for the Cy5/Cy3 ratios.

4. Discussion

In the present work, we investigated proteomic profiles from tachyzoites of three *T. gondii* lineages using 2D-DIGE coupled with MS approach. Comparison of the differentially expressed protein spots indicated a high degree of similarity between the GT1 strain of Type I and PTG strain of Type II lineages, and both with more difference from the CTG strain of Type III lineage (Fig. 2). Ten differentially expressed proteins were identified to reveal 7 *T. gondii* proteins (Table 1), including GRA2, GRA7, tgd057, actin depolymerizing factor (ADF), nucleoside-triphosphatase I (NTPase), putative asparaginyl-tRNA synthetase and adenosinetriphosphatase.

Toxoplasma gondii dense granule proteins may participate in the modification of the parasitophorous vacuole (PV) for the maintenance of intracellular parasitism in nucleated host cells (Nam, 2009). Previous studies have shown that GRA proteins are involved in parasite survival and virulence (Mercier et al., 2005) and they are major components of both the parasitophorous vacuole in which tachyzoites multiply and the cyst wall surrounding the more slowly dividing bradyzoites (Craver and Knoll, 2007). In the present study, both GRA2 and GRA7 are differentially expressed among

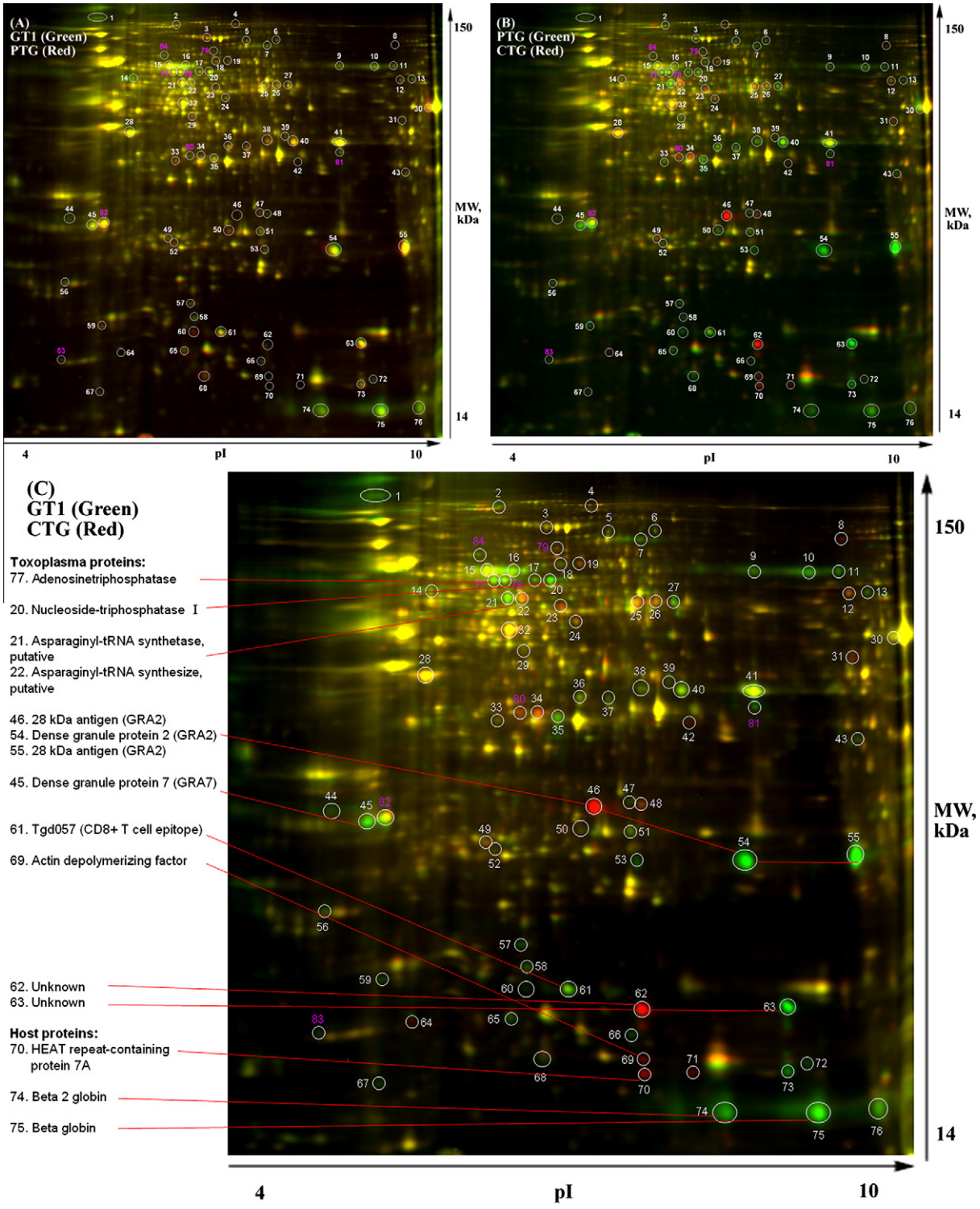

Fig. 1. 2D-DIGE of *T. gondii* tachyzoite proteins extracted from Type I, II and III strains GT1, PTG and CTG, respectively. Proteins were separated in the first dimension at pH 4.0–9.0 and on a 12% SDS–PAGE gel in the second dimension. **A**. Pair-wise comparison of GT1 (green) and PTG (red) strains. Proteomic profiles of these two strains are very similar. **B**. Pair-wise comparison of PTG (green) and CTG (red) strains. **C**. Pair-wise comparison of GT1 (green) and CTG (red) strains. Differentially expressed proteins are shown as green or red spots. Protein spots that showed $\geqslant$1.5-folds difference in abundance are circled and numbered. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the three *T. gondii* strains, but the consequence of such difference is not clear.

Tgd057 (spot 61) was originally characterized as a highly abundant expressed sequence tag (EST) in *T. gondii* strain RH tachyzoites (Wan et al., 2004). The tgd057 is located within the parasite's cytoskeleton. DNA sequences amongst Type I, II and III strains of *T. gondii* are identical (<www.toxodb.org/toxo/>) and encode characterized CD8(+) T cell epitopes, which elicit corresponding antigen-specific CD8(+) T cell populations associated with control of infection.

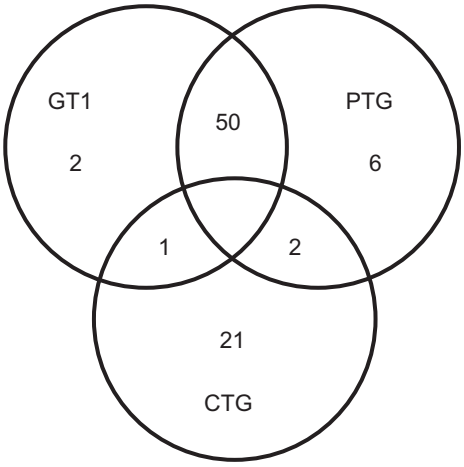

Fig. 2. Venn diagram of the distribution of protein spots exhibiting ⩾1.5-fold changes among the Type I (GT1), Type II (PTG), and Type III (CTG) strains of *T. gondii*. GT1 and PTG had similar protein expression patterns and they were different from that of CTG.

The ADF plays an important role in remodeling the actin cytoskeleton which contributes much to the invasion of host cells by the apicomplexan parasite. The ADF/cofilin family of proteins is highly conserved and is essential for increasing actin filament turnover in higher eukaryotes (Lappalainen and Drubin, 1997; Chen et al., 2000). *T. gondii* have one or two ADF/cofilin protein (Allen et al., 1997), whereas multicellular organisms have several ADF/cofilin isoforms. The dynamics of the apicomplexan microfilament system differs fundamentally from the well-understood regulated turnover of actin in mammalian cells (Morrissette and Sibley, 2002).

NTPase is abundantly released from dense granules of *T. gondii* (Sibley et al., 1994), and accumulates as a soluble protein in the vacuolar space and becomes partially associated with the parasitophorous vacuolar membrane (Bermudes et al., 1994). The NTPase genes include three tandemly repeated NTP genes: NTP1, NTP2 and NTP3. NTP1 encodes NTPase-II isoform, NTP3 encodes NTPase-I isoform and NTP2 does not encode protein (Bermudes et al., 1994). Interestingly, the gene encoding NTPase-II has been found in all strains of *T. gondii*, whereas the gene encoding NTPase-I is restricted to virulent strains (Asai et al., 1995). Our results show that NTPase-I (spot 20) was highly expressed in the GT1 and PTG strains, but not in the CTG strain. Spot 77 was adenosinetriphosphatase which is a type of NTPases. Both proteins may play a role in virulence of *T. gondii* tachyzoites.

Asparaginyl-tRNA synthetase (AsnRS) is one of the class-II aminoacyl-tRNA synthetases (AARS), and is responsible for catalyzing the specific aminoacylation of tRNA(Asn) with asparagine (Beaulande et al., 1998). AARS are a family of enzymes that exhibit primary and various secondary functions in different species. Although AARS are extremely diverse in sequence, size, and domain structure, they perform the same function in protein synthesis. Each AARS specifically binds ATP, an isoacceptor tRNA and a particular amino acid, in order to correctly aminoacylate the tRNA. This process is essential for the organisms to maintain the stability. Our results show that AsnRS (spots 21 and 22) differentially express in the GT1, PTG and CTG strains.

Toxoplasma proteomics studies of differential protein expression have utilized separation of proteins by 2-DE along with MS (Cohen et al., 2002; Dlugonska et al., 2001; Xia et al., 2008; Che et al., 2011) however, in their 2-DE experiments, technical problems limit sensitivity and reliability for detection of quantitative differences in protein expression levels. Thus, the 2D-DIGE method, a proteomic technology using CyDye DIGE fluors which are spectrally resolvable, size and charge-matched fluorescent dyes, has been considered the most refined technology for the accuracy of proteomics (Marouga et al., 2005; Unlü et al., 1997; Minden et al., 2009).

The 2D-DIGE was originally developed by Minden and co-workers in 1997 (Unlü et al., 1997). As the control and experimental protein samples were resolved in the same gel together with an internal reference, the reproducibility of protein separation is better compared to conventional 2D gel. Moreover, DIGE technique enables the incorporation of the same internal standard on every 2D gel, which is a pool of all the samples within the experiment. The internal standard is used to match the protein patterns across gels, thereby negating the problem of intergel variation, a common problem with standard 2D assays. Therefore, 2D-DIGE technique is very useful for applications requiring accurate quantification and direct differences with high reproducibility and reliability (Seike et al., 2005; Alfonso et al., 2005; Sharma et al., 2005). Particularly in the present study, spots 46, 54, and 55 were identified as the same GRA2 protein, although they showed different molecular weight and isoelectric points on 2D gels. This might arise as a consequence of post-translational modification. Post-translational modifications, such as glycosylation and phosphorylation can

Table 1
T. gondii tachyzoite proteins identified by MS and/or MS/MS.

Spot No.	Protein name	Accession No.	Gene ID from ToxoDB	MW (Da)	Protein pI	Number of matched peptides	Protein score	Protein score C. I. %	Total ion score	Total ion C. I. %	Highly expressed in infection
20	Nucleoside-triphosphatase I	gi|237842327	TGVEG_027890	69112.2	5.9	17	293	100	148	100	Type I, II
21	Asparaginyl-tRNA synthetase, putative	gi|221488174	TGGT1_109050	74871	8.3	20	430	100	295	100	Type I, II
22	Asparaginyl-tRNA synthetase, putative	gi|237832839	TGME49_270510	74957	8.2	15	268	100	187	100	Type III
45	Dense granule protein 7 (GRA7)	gi|3023908	TGVEG_027890	25841.9	5.1	10	359	100	269	100	Type I, II
46	28 kDa antigen (GRA2)	gi|221508380	TGVEG_068530	19831.3	7.9	6	196	100	131	100	Type III
54	Dense granule protein 2 (GRA2)	gi|2506908	TGGT1_083030	19830.3	9.0	7	178	100	109	100	Type I, II
55	28 kDa antigen (GRA2)	gi|237834195	TGME49_227620	19792.3	9.3	7	261	100	193	100	Type I, II
61	Tgd057 (CD8 + T cell epitope)	gi|35187725	NCLIV_060160	19970.3	7.0	11	409	100	269	100	Type I, II
69	Actin depolymerizing factor (ADF)	gi|237844535	TGME49_220400	12913.5	7.6	6	105	100	59	98.6	Type III
77	Adenosinetriphosphatase	gi|833757	TGGT1_001160	69571.3	5.8	26	552	100	298	100	Type I, II

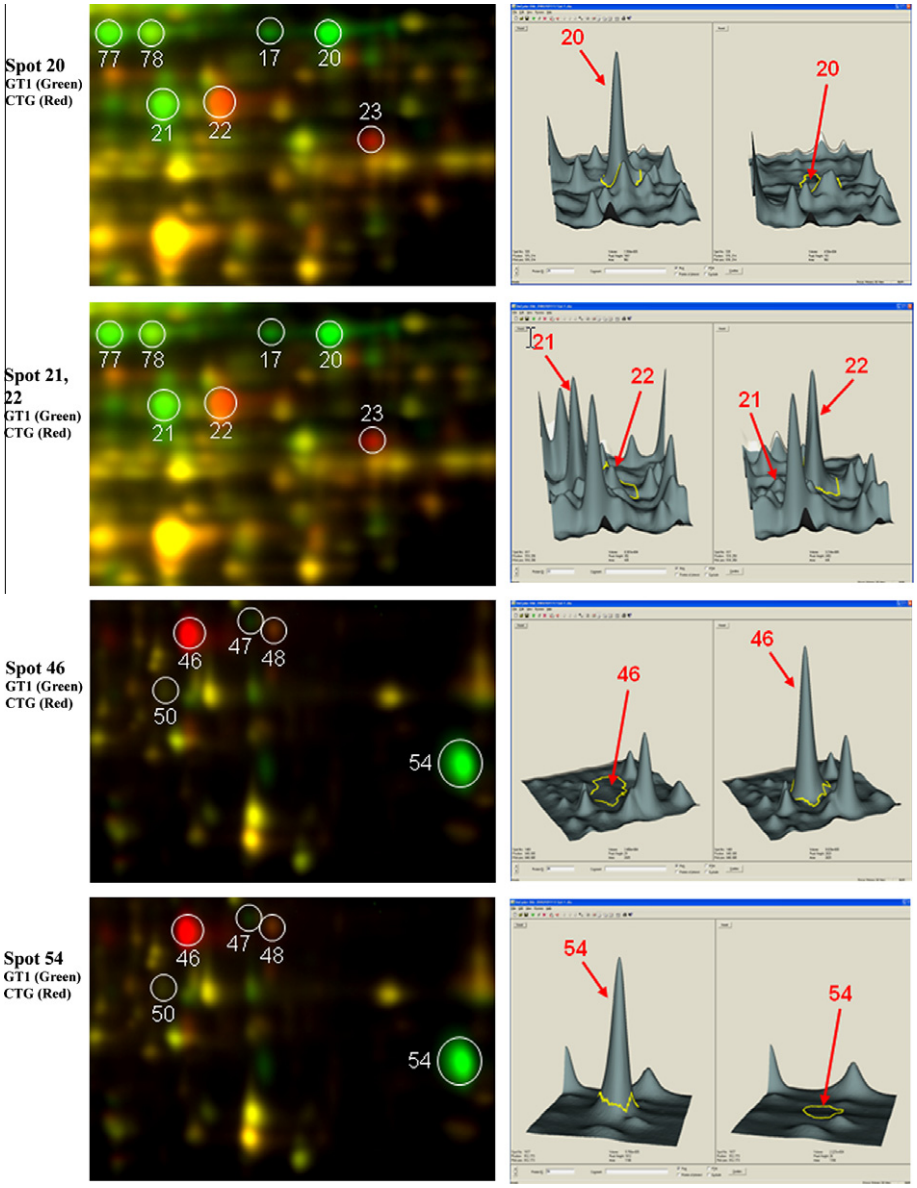

Fig. 3. Examples of differentially expressed protein spots on 3D view. The volume of each spot was calculated using Decyder software and is graphically represented. Then these protein spots were analyzed by mass spectrometry.

change the molecular weight and/or isoelectric points (Jensen, 2004). Further investigation into biological meaning of GRA2 protein post-translational modification may be more interesting and meaningful than protein identifications.

In summary, we report the study of differential proteomic profiling in distinct *T. gondii* strains using 2D-DIGE with MS. Our data showed that Type I (GT1) and II (PTG) strains have similar proteomic profiles, which are more different from that of the Type III (CTG) strain. This result is in parallel with recent finding that, mice infected with Type I and II *T. gondii* strains had similar gene expression profiles and they were different from the mice infected with the Type III strain (Hill et al., 2012). These studies suggest that distinct *Toxoplasma* strains have different protein expression profiles, which in turn may interact with its host differently and lead to specific outcomes in pathogenesis. Future studies on the characterizations and functions of the differentially expressed proteins would be valuable for understanding of biological processes and pathogenesis of *T. gondii* as well as the design of new vaccines or therapeutic compounds against toxoplasmosis.

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