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Original Article

Standardization of Sample Preparation for Two-Dimensional Electrophoresis of *Cryptosporidium parvum* Sporozoites

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***Cryptosporidium* is an important parasite of human and animals that belongs to the group Apicomplexa. However, the organism diverges from other Apicomplexa in several important aspects such as its unusual host location, atypical developmental biology, unique metabolism and recently described relict mitochondria. All or some of these unique features may be responsible for the lack of effective therapeutic agents against this parasite. The recent completion of genome sequence projects for *C. parvum* and *C. hominis* facilitated post-genomic investigations including proteomic analysis. Sample preparation is the first important step for any proteomic analysis. In this study we have attempted to develop the suitable sample preparation protocol for successful two-dimensional electrophoresis (2-DE) of *Cryptosporidium* sporozoite proteins prior to mass spectrometry. The 2-DE gels were analysed by automated image analysis software and number of protein spots were used as the indicator for maximum protein extraction from *Cryptosporidium* sporozoite samples.**

Keywords: Proteomics, *Cryptosporidium*, Two-dimensional electrophoresis (2-DE), Solubilization

Introduction

Cryptosporidium parvum is an important zoonotic protozoan that has been found in human and animal populations throughout the world[1-3]. It has a predilection for epithelial cells in the digestive tracts of a wide variety of hosts which includes humans, livestock, companion animals, wildlife, birds, reptiles and fishes. Recent release of complete genome sequences for *C. parvum* and *C. hominis* has facilitated further studies of this interesting opportunistic pathogen[4-5]. The post-genomic investigations of *Cryptosporidium* sp. is now underway in different laboratories, while no suitable protocol has not yet developed for maximum extraction of protein from the sporozoite sample[6]. Therefore, the study was undertaken to assess different sample preparation protocol that will be significant for a successful global or organellar proteomic analysis of *Cryptosporidium* sp.

Sample preparation is the first important step of every proteome analysis and especially for two-dimensional electrophoresis (2-DE)[7-8]. The aim of sample preparation for 2-DE is to convert the native sample into a suitable physicochemical state for first dimension isoelectric focusing (IEF) while preserving the native charge and molecular weight (Mr) of the protein constituent. This generally includes protein solubilization, disaggregation, denaturation and reduction. However, the sample preparation method for any given sample is variable and depends on the nature of the sample and aim of the separation[7]. As the protein expression of each cell is highly sensitive to changes in external parameters such as pH, hypoxia or drug administration, all possible experimental parameters should be held constant to achieve a better description of the individual proteome state[9]. Changes during sample preparation may result in quantitative and qualitative variations in protein patterns on 2-DE gels.

Several proteomic analyses of apicomplexan parasites have been published with considerable variations in the composition of the lysis and rehydration buffers used in 2-DE separations[10-18]. These illustrate the variations in sample preparation methods which have been determined empirically for each organism. A good extraction buffer will generally contain a combination of chaotropes (urea or thiourea) and zwitterionic detergents (CHAPS, Triton-X 100 or SB3-10), which solubilize proteins from a wide range of organisms[19-20]. The use of different sample preparations can produce quite variable results. For example, four different methods have been tested for *Leishmania*[11]. Here the use of NP-40 buffer provided the best result in terms of quality, reproducibility and quantity of detectable spots. Again, lysis buffer was omitted from the sample preparation step in some experiments for *Eimeria*[10], *Plasmodium*[13] and *Trypanosoma*[16]. Similarly, rehydration buffer was not used in some experiments for *Trypanosoma*[17-18]. As a general rule urea, CHAPS and Tris are the most common ingredient of most lysis and rehydration buffers, while freeze-thaw followed by vortexing contributes to effective lysis of most parasitic protozoa.

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In this study, a comparison of four different protocols for the extraction and solubilization of proteins from *Cryptosporidium parvum* sporozoite was made. The protocols were based on previously published protocol for protein extraction from *Toxoplasma gondii*[15], which uses a combination of urea, CHAPS and Tris. Protein extracts were separated on 2-DE gels and compared using three criteria: the resolution of the protein spots, the number of the protein spots detected and the distribution of protein spots at low/ high molecular weight/isoelectric point (pI).

Materials and Methods

Apparatus and chemicals

Unless otherwise stated, all equipments and reagents for isoelectric focusing (IEF) and sodium dodecyl sulphate (SDS) electrophoresis (IPGphor, Ettan DALT*twelve*® electrophoresis unit, EPS 3500 XL power supply, Multitemp II thermostatic circular, ImageMaster 2D elite V3.01 software, Labscan® scanner, Immoboline drystrip kit, IPG buffers, TEMED, CHAPS, DTT, APS, urea, glycine, Tris, Protein Silver staining kit) were purchased from Amersham Biosciences Ltd (Bucks, UK). The EDTA, coomassie brilliant blue G250 were purchased from Sigma Aldrich Ltd (Poole, UK). The 30% polyacrylamide solution was purchased from BioRad Ltd (Hertfordshire, UK) and the BCA protein assay kit® was purchased from Pierce Biotechnology Inc.(Rockford, IL, USA).

Source and purification of parasite materials

Oocysts of *Cryptosporidium parvum* passaged in lambs (IOWA strain) were purchased from Moredun Research Institute (MRI, Edinburgh, Scotland). This strain was continually passaged in sheep by MRI. Oocysts were concentrated by sucrose density centrifugation, washed and resuspended in phosphate-buffered saline (PBS, pH 7.2). The parasite suspension was stored at 4°C in the presence of 1,000 U/ml penicillin and 1,000 µg/ml streptomycin.

Excystation of Cryptosporidium parvum oocysts

Excystation is an essential step that frees the sporozoites from their protective shell and allows subsequent protein extraction. The excystation procedure for this study was as described by Campbell *et al.*[21]. Briefly, 10 µl of sodium deoxycholate solution (1% sodium deoxycholate in Hanks minimal essential medium) and 10 µl of sodium hydrogen carbonate (2.2% sodium hydrogen carbonate in HBSS) were added to 100 µl of purified oocyst suspension. The oocyst and the excystation medium were mixed thoroughly prior to incubation at 37°C and thereafter every 15 min. Various incubation time lengths were compared, 1 , 2 and 3 h, all incubated at 37°C with the same excystation medium. Excysted oocysts were counted as those with burst membranes whereas non-excysted oocysts were counted as those with intact membranes. It was found that the excystation efficiencies varied between different time lengths and almost 80% excystation happened within 1 h (data not shown). Although, longer incubation times usually increase the excystation percentage, prolonged incubation times in this study revealed that some sporozoites began to degrade after extended incubation in excystation media.

Sporozoite count and excystation rate

For enumeration of excystation percentages and sporozoite ratios, 10 µl aliquots of excystation suspensions were viewed under a microscope (x400) with Nomarski filters and the proportions of partially excysted oocysts and non-excysted oocysts were determined. Free sporozoites were also counted using haemocytometer and the excystation ratio of oocysts was calculated. In each case, 100 oocysts were counted and the percentage excystation calculated as follows:

$$\frac{\textit{No. of excysted oocysts + No. of partially excysted oocysts}}{\textit{Total No. of oocysts}} \textit{ x 100}$$

The sporozoite ratios were calculated according to following formula:

$$\frac{\textit{No. of free sporozoites}}{\textit{No. of excysted oocysts}}$$

Sporozoites were either separated from the intact oocysts and empty oocyst walls by passaging the excystation mixture through a membrane filter unit (5 µm) or used directly as a mixture (of sporozoites, oocysts and oocyst walls) for subsequent sample preparation. Where whole oocyst proteins were taken for 2-DE, the excystation mixtures were centrifuged @ 10,000x *g* for 3 min. The supernatant was discarded from the Eppendorf tube and replaced by 1 ml of PBS. After dissolving the pellet in PBS, the mixture was washed by centrifuging @ 13,000x *g* for 3 min. Sporozoites were then given a final wash in PBS, pelleted by centrifugation at 13,000x *g* for 3 min at 4°C and stored in batches of 5×10^7 at –80°C.

Protein estimation

Protein assay was performed using a bicinchoninic acid (BCA) assay (Pierce Ltd, UK).

Two-dimensional gel electrophoresis

Sample preparation for 2DE: Purified sporozoites (5×10^7) were dissolved in 40 µl lysis buffer (8 *M* urea, 4% (w/v) CHAPS and 40 m*M* Tris), disrupted several times by a freeze-thaw cycle in liquid nitrogen, and then sonicated at low power for 5 min in a water bath at room temperature (Ultrasonics Ltd). The sporozoites lysate was then incubated on ice for 1 h for improved solubilization with subsequent addition of rehydration buffer containing 8 *M* urea, 2% (w/v) CHAPS, 22 m*M* DTT (added fresh), 0.5% (v/v) immobilised pH gradient buffer and 0.002% bromophenol blue, to a final sample volume of 350 µl (for 24 cm large gels). Insoluble material was removed by centrifugation at 13,000x *g* for 3 min at 4°C before loading onto immobilized pH gradient (IPG) strip.

First dimension separation by IEF

Proteins were separated in the first dimension using the IPGPhor® isoelectric focusing (IEF) system employing immobilised pH gradient (IPG) strips. The required numbers of strip holders were placed on to the IPGPhor® and 125 µl of sample containing

rehydration solution was evenly pipetted in the strip holder groove, a few centimetres from the electrode. The IPG strips were lowered, gel side down on to the rehydration solution without trapping air bubbles. To minimize evaporation and urea crystallization, this strip was then overlayed with DryStrip Cover Fluid® (Amersham) before the plastic cover was applied. The proteins were then focused to their isoelectric points using 7 cm Immobiline DryStrips® according to the programmed conditions in Table 1. Rehydration and isoelectric focusing were performed at this integrated system (Ettan IPGPhor® II) at 20°C. Following isoelectric focusing, the proteins were reduced and bound to sodium dodecyl sulphate (SDS) by equilibrating each strip for 15 min in 10 ml of SDS equilibration buffer (50 m*M* Tris–HCl, 6 *M* urea, 30% v/v glycerol, 2% w/v SDS) containing 100 mg dithiothreitol (added fresh before use). A second equilibration step in SDS equilibration buffer containing 250 mg alpha-iodoacetamide (added fresh before use) instead of dithiothreitol was performed in order to prevent protein re-oxidation and to minimise reactions of cysteine residues.

Table 1. *Isoelectric focusing steps used to focus 3-10 non-linear IPG strips*

Step	Voltage	Duration (h)	Gradient type
Rehydration	-	12	-
1	300	1	Step-n-hold
2	1,000	0.5	Gradient
3	5,000	2*	Step-n-hold

Running conditions: Temperature 20 °C; Current 50 µA per strip. These steps were extended as necessary until the final cumulative volt-hour (Vh) reached a minimum of 5000 Vh for 7 cm strip.

Second dimension separation by SDS-PAGE

After equilibration, the immobilised pH gradient strips were rinsed in electrophoresis buffer before being embedded on to a 12.5% (w/v) homogeneous polyacrylamide gel prepared earlier. The IPG strips were inserted between the glass plates with a spacer and brought in close contact with the upper edge of the SDS gel. Then the area was sealed with hot 0.5% (w/v) agarose. After the agarose has set, the SDS-PAGE was run in BioRad electrophoresis tank at 120 Volt for 3 h. When the bromophenol blue front had completely migrated out of the SDS gel, the gel was ready to stain.

Gel staining

The gels were stained using colloidal coomassie staining technique[6]. The coomassie stock (5% Coomassie brilliant blue G-250) and the colloidal stock (50 g ammonium sulphate, 6 ml phosphoric acid, 10 ml coomassie stock and ddH_2O up to 500 ml) were made up before while the actual stain was made fresh each time. The actual stain was prepared by adding 1 part methanol and 4 parts of colloidal stock. For colloidal staining, the SDS gels were fixed for 2 h in a fixative solution containing 40% ethanol and 10% acetic acid. The gel was then washed twice in ddH_2O for 10 min each. After wash, the gel was immersed in actual stain and incubated for 1-7 days. The destaining was done by multiple washes in ddH_2O until the background is clear.

Image analysis

Images of gels were acquired at 300 dots per inch (dpi) using Labscan v3.0 software (Amersham Pharmacia Biotech, UK) on a Umax flatbed scanner (OD maximum 3.4) with integrated transparency adapter. The tiff images generated were analysed using ImageMaster® 2D Platinum® 6.0 software (Amersham). The authenticity and outline of each spot were validated by visual inspection and edited where necessary.

Results

Sample preparation is critical for good SDS-PAGE analysis. Ideally this process includes complete solubilization, disaggregation, denaturation and reduction of the proteins in the sample. Four different sample extraction and solubilization protocols were compared using 5 x 10^7 sporozoites of *Cryptosporidium parvum* in each case. A summary of the four protocols is given in Table 2. In addition, 12% SDS-PAGE mini gels were also run to assess the relative concentration and quality of protein extracted using each protocol (Figure 1). Subtle difference were observed between the protein extracts separated on the SDS-PAGE gel although protocol P3 gave a higher number of protein bands with an especially good representation of those at high molecular weight. The protein quantitation assay also indicates a maximum amount of protein extracted through protocol P3 (Table 3).

Table 2. *Summary of protocol P1, P2, P3 and P4*

Reagent/Condition	Protocol 1	Protocol 2	Protocol 3	Protocol 4
Lysis buffer	40 µl	125 µl	40 µl	40 µl
Rehydration buffer	80 µl	-	85 µl	75 µl
Protease inhibitor cocktail	5 µl	-	-	10 µl
Freeze/thaw/sonication	Yes	Yes	Yes	No

The volumes given in the table correspond to the volumes of reagents used for the extraction of proteins from 5 x 10^7 sporozoites of *Cryptosporidium parvum* loaded on to a 7 cm 2-DE gel.

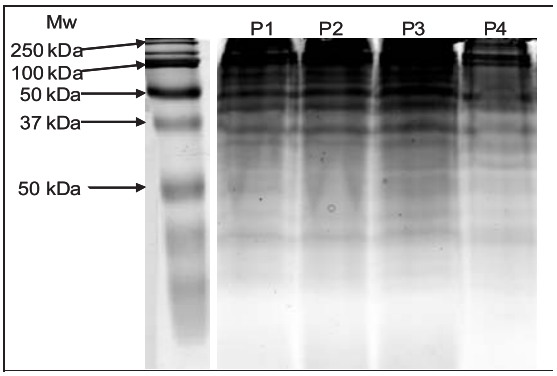

Figure 1. *SDS-PAGE analysis of proteins extracted using protocols P1, P2, P3 and P4. Proteins from 5 x 10^7 sporozoites of Cryptosporidium parvum were extracted. The extracts were run on a 12% SDS-PAGE gel to assess the quantity and quality of the extracts. The gels were stained with colloidal Coomassie stain. Lane 1: broad range molecular weight marker (Catalogue # 161-0374, Bio-Rad).*

Table 3. *Summary of quantitative analysis to assess the different protein extraction and solubilization protocol*

Protocol	Protein (in μg) per 5 x 10^7 sporozoites (approximately)
P1	75
P2	77
P3	92
P4	70

Proteins from 5 x 10^7 sporozoites of *Cryptosporidium parvum* were extracted and quantitation assays were performed using the PlusOne 2D Quant Kit®.

In order to assess further the quality of the proteins extracted using the four protocols, the extracts were separated on 7 cm pH 3-10 non linear IPG strips in the first dimension followed by 12.5% SDS gels in the second dimension. The gels were stained with colloidal Coomassie, scanned and analysed using the ImageMaster 2D Elite® software (Figure 2). It was found that the maximum number of spots (250) were detected on the 2-DE gels run with protein extracted using protocol P3 (Figure 2C), whilst 204, 190 and 100 protein spots were detected using protocols P1, P2 and P4 respectively (Figure 2A, 2B and 2D).

Based on these results, protocol P3, combining lysis and rehydration buffer with freeze/thaw lysis was the most efficient sample preparation for extraction and analysis of *C. parvum* sporozoite proteins. Extreme care was taken to prevent any protein degradation by keeping the sample at low temperature and minimising processing time. Therefore, the variation of protein expressions in 2-DE gels might be solely due to the efficiency of lysis protocols. The addition of a protease inhibitor cocktail was found ineffective during lysis of parasite materials. This was evident by apparently better resolution of protein spots in 2-DE gels by protocol P2 and P3 (Figure 2B, 2C), both of which are devoid of protease inhibitors. Therefore, protocol P3 was found to be most effective solubilizing agent as it gives better resolution and maximum extraction of protein.

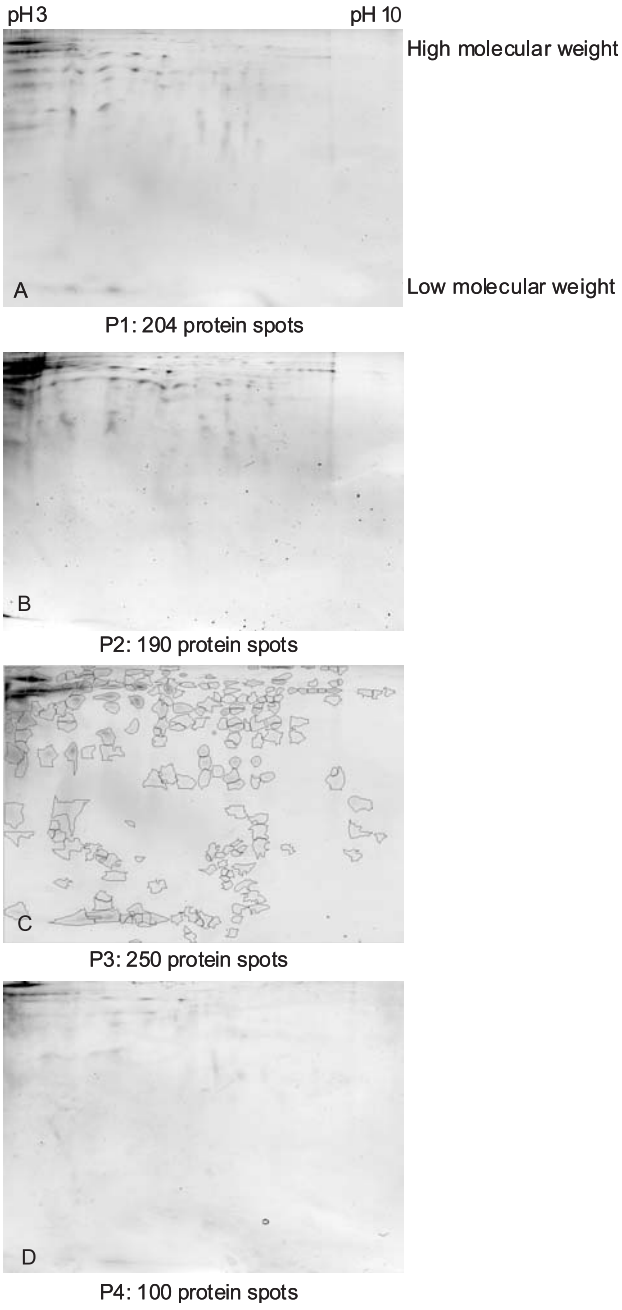

Figure 2. *2-DE separation of proteins extracted using protocol P1, P2, P3 and P4. Proteins were extracted from 5 x 10^7 sporozoites of Cryptosporidium parvum separated on 7 cm Drystrip® and broad pH IEF (pH 3-10NL) 2D gels and stained with colloidal Coomassie stain. The gels were analysed using Image Master 2D Elite® software to determine the number of protein spots separated (as shown in Figure 2C with outline).*

Discussion

During the study, the oocysts of *Cryptosporidium parvum* were used as starting material to analyse the proteome of sporozoite stage. As it was difficult to separate the sporozoites from the excystation mixture, the protein sample used for 2-DE analysis was essentially a mixture of sporozoites, unexcysted oocysts, partially excysted oocysts and oocyst walls. An attempt to purify the sporozoite material from the excystation mixture has been proved ineffective during this study. The use of filter (5 μm pore size) was found partially successful while more than 50% (data not shown) of the sporozoites were missing (as they remained bound with the sieve). A further improvement of the purification technique is therefore important prerequisite to get a more accurate picture of the sporozoite proteome of *Cryptosporidium.*

Protein degradation or modification must be prevented during the sample preparation step. Cell disintegration may give rise to the release of proteases, which contribute to protein degradation and increases the heterogeneity of the protein mixture. Prevention of protein degradation or modification can be achieved either by disrupting the sample directly into strong denaturants such as 8 *M* urea, 10% TCA or 2% SDS[22-26] or by adding commercially available broad range protease inhibitor "cocktails". Again, proteolysis can often be inhibited by preparing the sample in the

presence of Tris base, sodium carbonate or basic carrier ampholyte mixtures[27].

Precipitation of the proteins in the sample and removal of interfering substances are optional steps. Contaminants like salts, small ionic molecules, ionic detergents, nucleic acids, polysaccharides, lipids and phenolic compounds can interfere with separation and subsequent visualisation of the 2-DE result, if they are not removed[27]. For complete and reproducible sample preparation, a number of additional steps may improve the quality of the final result. However, these additional steps can also result in the unacceptable selective loss of proteins.

Although the use of silver stain instead of colloidal coomassie stain could significantly increase the total visible spots from the same gel, the relative incompatibility of silver staining with mass spectrometer limits their further analysis. Thus it will not be possible to obtain sequence information for those proteins. Although, there are nearly 4,000 proteins predicted by the genome sequencing project of *C. parvum*[4], it is not known how many proteins are expected in the sporozoite stage of *Cryptosporidium.* Some of them will be unique for sporozoites stage while some of them might be commonly expressed in more than one life cycle stages. It can be deduced that, any projected figure is likely to be an underestimate due to the well documented occurrence of multiple protein isoforms. Furthermore, membrane proteins have been shown to be difficult to solubilize which limits their separation by 2-DE[28]. As it appears difficult to define the percentage of non-solubilized proteins of the sporozoite proteome, the number of proteins separated by 2-DE reflects only a fraction of the soluble proteome. Further stage specific comprehensive analysis and use of non-gel based approaches (like multidimensional protein identification technology or MudPIT) could be useful to know the number of predicted proteins in any specific life cycle stage.

Standardization of sample preparation is important for production of reproducible 1D-SDS or 2-DE gels. The sporozoites should be efficiently disrupted and solubilized completely in order to obtain a representative sample through identical lysis method. Four sample lysis methods were compared at the present study to identify a suitable sample preparation protocol for 2-DE analysis. Tris-HCl was found to be crucial for better solubilizing capacity and 40 m*M* Tris-HCl was able to resolve more spots. Different concentrations of Tris base (5-40 m*M*) were used for sample preparation of *Cryptosporidium* sporozoites in our laboratory where 10-20 m*M* of Tris was found more effective in producing better 2-DE map (S. Sanderson, personal communication). However, a maximum of 40 m*M* Tris is recommended for sample preparation depending on the complexity of the cell lysate. Again it should be taken into consideration that the analysis of extremely low (<15 kDa) and high (>150 kDa) molecular weight proteins are difficult using the conventional 2-DE gels. The important issue is that most of the high molecular weight proteins and hydrophobic proteins are not soluble by standard urea lysis and the rehydration methods used here for sample preparation. The ultimate success of any proteome analyses therefore partly relies on effective sample solubilization prior to production of reference 2-DE map. Further comparative study using different concentrations of Tris base as well as other components like urea, detergents and protease inhibitors could potentially improve sample preparation protocol.

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