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Two-dimensional gel electrophoresis and immunoblot analysis of *Neospora caninum* tachyzoites

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Identification of expressed protein profiles and antigenic determination are some of the most challenging aspects of proteomics. Two-dimensional gel electrophoresis (2-DE) combined with immunoblot analysis were employed to study the *N. caninum* proteome. Protein sample preparation was carried out by first conducting sonication, followed by adding lysis buffer containing 7M urea plus 2M thiourea to the purified tachyzoites in order to complete disruption. A total of 335 differentially expressed protein spots were detected using pH 4-7 IPG strip (7 cm) that were run in a 56 kVh isoelectric focusing (IEF) system. Of the spots analyzed, 64 were identified as antigenic spots on immunoblot profile. Major antigenic spots appeared at 65 kDa (pI 5.2-5.3), 51 kDa (pI 5.5), 38 kDa (pI 5.1), 33 kDa (pI 4.4), 29 kDa (pI 5.6) and 15.5 kDa (pI 5.0) were observed to be significantly distinct compared to the rest of the antigenic spots. The results indicate that combination of 2-DE and immunoblotting methods were thought as very useful tools in defining both proteins and antigens of *N. caninum* tachyzoites. Additionally, present 2-DE profiles may be valuable in further proteomic approaches and study of the pathogen.

Kewords: *Neospora caninum*, two-dimensional gel electrophoresis (2-DE), immunoblot

Introduction

Neospora caninum (*N. caninum*) is an obligate cyst-forming intracellular protozoan (Apicomplexa) parasite of animals [10,11]. It was usually misdiagnosed as *Toxoplasma gondii* (*T. gondii*) because of their morphological and biological similarities up to the middle of 1980s. But it was then distinguished due to its distinct morphology of forming cysts in tissue and their antigenic differences [4,5,10]. In recent years, *N. caninum* has been identified as a major causative agent of abortion or stillbirths in both dairy and beef cattle worldwide, including Korea [1,12,19,22,24]. The economic losses due to infections it causes have encouraged a general investigation of the pathogen, and many reports were published concerning its pathogenicity. However, no study of the expressed proteins from the whole organism of *N. caninum* was performed.

The global analysis of protein expression profiles might be invaluable for obtaining a more complete understanding of biological events, such as, development, evolution, and pathogenicity of this organism [21,36]. Two-dimensional electrophoresis (2-DE) which is considered as a powerful and widely used method for analyzing complex protein mixtures extracted from cells, tissues, or other biological samples. This technique was originally described by Klose [27] and O'Farrell [34], which involves separation of cellular proteins according to their isoelectric points (p*I*) and relative molecular masses (M_r). With this method, a protein can only be visualized and analyzed if it can be brought and kept in solution during the entire 2-DE separation process. The cells or tissues must be efficiently disrupted and cells contents must be solubilized completely [30]. This is one of the most important points to consider in 2-DE. Over the last few yea rs, 2-DE with immobilized pH gradients (IPGs) has been improved to its superior resolution and reproducibility [14,15,37]. Moreover, its combination with immunoblotting assay is allowed to find out many and distinct antigens compared with conventional SDS-PAGE and its immunoblotting analysis. These approaches prove a highly successful in characterization of the expressed proteins of some parasitic organisms such as *T. gondii* [8,9,13], *Fasciola hepatica* [20], *Schistosoma japonicum* [25], *Ascaris suum* [26], and even comparison of 2-DE profiles between *N. caninum* and *T. go*ndii [17].

In this study, 2-DE profiles were employed for the analysis of expressed proteins of *N. caninum* tachyzoites.

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Proper optimization of sample preparation were known to play a key role in obtaining suitable images of 2-DE. In addition, two-dimensional antigen profiles were observed with the use of rabbit anti-sera specific for *N. caninum* tachyzoites (KBA-2).

Materials and Methods

Maintenance and purification of *N. caninum* tachyzoites

N. caninum tachyzoites, KBA-2 isolate [23], was used at the present study. The tachyzoites were maintained in Vero cell monolayer. For purification, tachyzoites were harvested by scraping the infected Vero cell monolayer into growth medium. Suspension of tachyzoites including cellular debris were done through centrifugation at 1,000 × g for 10 min. Pellet was resuspended in phosphate-buffered saline (PBS, pH 7.4). Tachyzoites were released from infected cells by pulling and pushing a syringe fitted with a 23-guage and 27-guage needle, respectively. Subsequently, suspensions were loaded on 30%, 50% and 80% osmotic percoll gradient (Amersham Bioscience, Sweden), and centrifuged at 2,000 × g for 30 min. The viable tachyzoites band formed between 50% and 80% osmotic percoll gradients were collected and washed three times with PBS. Generally, a total of approximately 1×10^8 tachyzoites as counted by the use of hemocytometer were purified from infected monolayer using four pieces of 175 cm^2 size culture flasks. The purified tachyzoites were stored at −70°C until further use.

Comparison of sample preparation for 2-DE

In order to optimize sample preparation for 2-DE, purified tachyzoites were lysed in three different methods. First, tachyzoites dissolved in 40 mM Tris-base (pH 9.6) were disrupted by rapid freezing and thawing 3 times using liquid nitrogen, and continually disrupted using lysis buffer containing 9.5 M urea, 40 mM Tris-base, 4% (w/v) CHAPS, 1% (w/v) DTT, 1 mM PMSF, and 0.5% (v/v) IPG-buffer pH 3-10 (Amersham Bioscience, Sweden). Secondly, after successive freezing and thawing, the intact tachyzoites remained were sonicated (XL-2020, Misonix, USA) at a low power for 1 min in ice slurry and continually disrupted using 9.5 M urea lysis buffer. Finally, the same procedures employed in second method were followed except that the lysis buffer which is composed of 7 M urea and 2 M thiourea. The lysates were maintained for 1 h in ice slurry and then centrifuged at 16,000 × g for 30 min at 4°C. Subsequently, the supernatants were transferred to micro centrifuge tube and stored at −70°C. The total protein concentrations were estimated at approximately 0.5 mg/mL based on the Bradford protein assay method [7] using bovine serum albumin as standard.

2-DE and comparison of two different IEF conditions

Isoelectric focusing (IEF) was carried out by using IPGphor system (Amersham Bioscience, Sweden) according to Görg *et al.* [15] and utilizing Immobilized pH gradient (IPG) strips (Immobiline DryStrip, pH 3-10 and pH 4-7, 0.5 × 3 × 70 mm, Amersham Bioscience, Sweden). The tachyzoite samples separately prepared were mixed with rehydration buffer (8 M urea or 2 M thiourea/6 M urea, 4% (w/v) CHAPS, 65 mM DTT, 0.5% IPG buffer, 0.002% (w/v) bromophenol blue) and then loaded on the ceramic strip holders by in gel rehydration method. The absorbed proteins in strips were focused in an automated run at 20°C. After IEF, IPG strips were equilibrated with 10 mg/mL DTT in equilibration buffer (6 M urea, 2% (w/v) SDS, 30% (v/v) glycerol, 0.002% (w/v) bromophenol blue, 50 mM Tris-HCl, pH 8.8) for 15 min and further incubated in the same buffer for another 15 min replacing DTT by 4 mg/mL iodoacetamide. After equilibration, the IPG strips were placed onto either 12.5% or 10% SDS-polyacrilamide gels (80 × 80 × 1 mm) and sealed with 0.5% (w/v) agarose. SDS-PAGE was run at 5 mA/gel for 15 min as initial migration and increased to 10 mA/gel for separation until front dye reached the bottom of the gel. In order to establish an accurate IEF result, two different conditions were compared using pH 3-10 IPG strip (7 cm). First sample (4 μg/IPG strip) was focused in an automated run by programming 12 hrs in gel rehydration (without current), 1 h at 500 V, 1 h at 1000 V, and 10 hrs at 4500 V, having a total of 46.5 Kilovolt-hour (kVh). On the other hand, same sample conditions were rehydrated at 14 hrs (7 hrs at 0 V and 7 hrs at 30 V), 2 hrs at 200 V, 1 h at 500 V and 1000 V, 2 hrs at 2000 V and 10 hrs at 4500 V reaching a total of 56.1 kVh.

Silver staining

Silver staining was performed according to Mortz *et al.* [33] with slight modification. Briefly, the gels after 2-DE were fixed in 50% (v/v) methanol, 12% (v/v) acetic acid and 0.05% (v/v) formalin for 2 hrs and then washed 3 times with 50% (v/v) ethanol for 20 min. The gels were then sensitized with 0.01% (w/v) sodium thiosulfate for 1 min and washed with distilled water 3 times for 20 sec. The washed gels were incubated in 0.2% (w/v) silver nitrate containing 0.076% (v/v) formalin for 30 min. After incubation, it was rinsed with distilled water followed by adding the developing solution which contained 3% (w/v) sodium carbonate with 0.05% (v/v) formalin until intensity desired. Development was terminated by adding 50% (v/v) methanol and 12% (v/v) acetic acid.

Production of rabbit anti-sera specific *N. caninum* tachyzoites

Anti-*N. caninum* polyclonal antibodies were raised by immunizing rabbit (New Zealand White, 1.5 kg). Briefly, rabbit was immunized subcutaneously with 1×10^7 live tachyzoites (KBA-2) mixed with Freunds complete (first

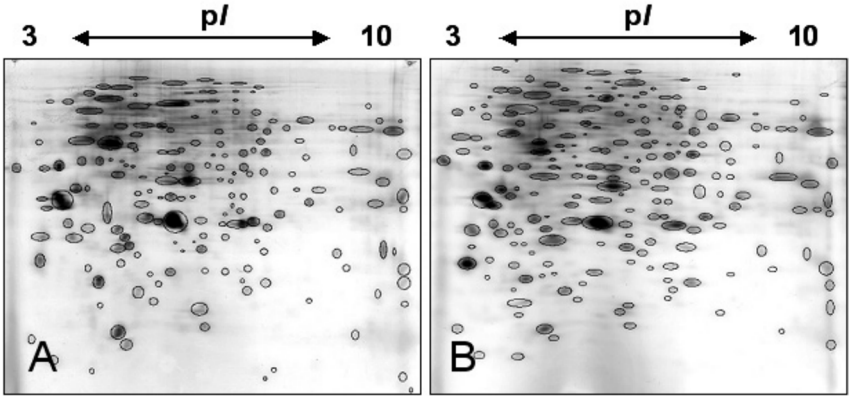

Fig. 1. Comparison of two IEF conditions. IEF was performed using pH 3-10 IPG strips (7 cm) with 8 μg of lysate from *N. caninum* tachyzoites. SDS-PAGE was performed using 12% gels which were then stained with silver nitrate. (A) In gel rehydration were done at 0 V for 12 hrs, 500 V for 1 h, 1,000 V for 1 h and 4,500 V for 10 hrs, having a total of 46,5 kVh, (B) In gel rehydration conditions were at 0 V for 7 hrs followed by 30 V for 7 hrs, 200 V for 2 hrs, 500 V for 1 h, 1,000 V for 1 h, 2,000 V for 2 hrs and 4,500 V for 10 hrs, having a total of 56,1 kVh.

immunization) and incomplete adjuvant (second and third immunization) at 2-weeks-interval. Booster dose was given a week after. Sera were collected 7 days after booster injection and stored at −70°C until use.

Immunoblot analysis

The gels containing spots separated with 2-DE were transferred to nitrocellulose membrane (Immobilon-NC, 0.45 μm, Millipore, USA). The blotted membranes were rinsed with TBS-T buffer (20 mM Tris, 500 mM NaCl, 0.05% v/v tween 20, pH 7.4) and then blocked with blocking buffer (5% w/v skim milk in TBS-T buffer) overnight at 4°C. The membranes were incubated with an anti-*N. caninum* rabbit antisera diluted in a ratio of 1 : 200 in blocking buffer for 2 h and then washed 3 times with TBS-T buffer for 20 min. Further incubation were done using the goat anti-rabbit IgG antibody conjugated with horseradish peroxidase (Santa Cruz Biotechnology, USA) with 1 : 2,000 dilution in blocking buffer for 1 h. After washing five times with TBS-T buffer, the membranes were treated with ECL solution (Amersham Bioscience, Sweden) for 1 min and then exposed to X-ray film for 5-30 sec.

Image analysis

Either stained or immunoblotted spots were digitalized by using Agfa Arcus 1200™ image scanner (Agfa-Gevaert, Belgium), and the acquired images were analyzed by using Phoretix™ 2D software (Ver. 5.01, NonLinear Dynamics, UK).

SDS-PAGE and Immunoblot analysis

In order to accurately compare between 2-DE and 1-DE profiles, purified tachyzoites of *N. caninum* were disrupted by freezing and thawing, sonicated as previously described, mixed with the same volume of SDS sample buffer (Sigma, USA) and boiled at 95°C for 5 min. The lysates were centrifuged at 10,000 g for 5 min at 4°C and the supernatants were stored at −70°C. The sample was separated by SDS-PAGE using 12.5% slab gels with a 4% stacking gel. The protein bands were stained with Coomassie blillant blue (CBB) G-250. The separated proteins were transferred to nitrocellulose membrane and underwent immunoblotting as previously described. Images of stained gel and film were digitalized by using Agfa Arcus 1200™ image scanner. Acquired images were analyzed using Quantity One® software (Ver. 4.2, Bio-Rad, USA).

Results

Comparison between two IEF conditions

2-DE profiles stained with silver nitrate showed a significant differences in spot numbers depending on IEF conditions. The spot numbers were analyzed by automatic spot detection mode using Phoretix 2D software. Approximately 182 spots were detected on 2-DE profile focused on IPG strip that were run in 46.5 kVh. On the other hand, approximately 212 spots were observed on the profile at 56 kVh (Fig. 1).

Comparison of 2-DE sample preparation

Comparison of 2-DE sample preparations were carried out by determining the number of resolved spots detected on silver stained gels. The lysates separately prepared were focused at 56.1 kVh using IPG strips of pH 3-10 and pH 4-7 and performed SDS-PAGE with 12.5% gel. Most *N. caninum* tachyzoites protein spots were located between pH 4 and pH 8, and between 25 and 87 kDa. A total of 172 spots (A) were observed in pH 3-10 IPG strip, on the contrary, approximately 243 spots (D) were observed in pH 4-7 IPG strip when samples were prepared by freezing and thawing.

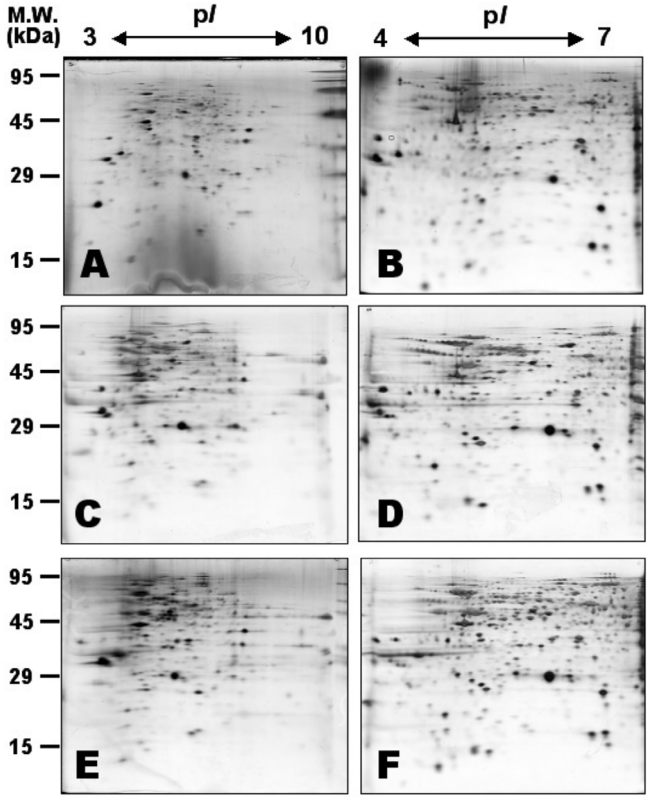

Fig. 2. Comparison of 2-DE profiles with three different sample preparations. 2-DE profiles of *N. caninum* tachyzoites were conducted employing the three different sample preparation process at the same IEF conditions such as freezing and thawing (A and D), sonication (B and E) followed by adding lysis buffer containing 9.5 M urea and sonication. After which, 7M urea plus 2 M thiourea (C and F) were again added. IEF was performed at a total of 56.1 kVh using pH 3-10 and pH 4-7 IPG strips (7 cm). SDS-PAGE was performed in 12% gels which were then stained with silver nitrate. A total of 172 (A), 251 (C) and 256 (E) spots were observed with pH 3-10 IPG strips. On the contrary, approximately 194 (B), 243 (D) and 332 (F) spots were observed with pH 4-7 IPG strips.

Sample preparation using sonication followed by adding lysis buffer containing 9.5 M Urea shows an increase in spot numbers. Based on the data gathered, approximately 194 (B) and 256 spots (E) were identified with pH 3-10 and pH 4-7 IPG strips, respectively. Sonication and utilization of 2 M thiourea plus 7M urea proved to obatin the highest number of spots, since it was able to detect around 251 (C) and 332 spots (F) using pH 3-10 and pH 4-7 IPG strips, respectively (Fig. 2).

Immunoblot analysis

A total of 335 spots were detected on silver stained gel using pH 4-7 IPG strip based on sample preparation utilizing sonication and lysis buffer (7 M urea plus 2 M thiourea). Among them, 64 spots were identified as antigenic spots on immunoblot image with the used of rabbit antisera specific for KBA-2. Most *N. caninum* antigenic spots were located in between 28 kDa and 97 kDa and between pH 4.4 and pH 6.1 except one antigenic spot having 15.5 kDa (spot 64). A number of large antigenic spots (spot 61 and 64) were also observed. Concurrently, a series of antigen spots having similar molecular weight but different p*I* values (spot 16-19 and 27-34) were also determined in immunoblotting profile. Major antigenic spots were noted as spot 16, 17, 19, 42, 59, 61 and 64 (Fig. 3). Molecular weight and isoelectric point of the respective antigenic spots were shown in Table 1.

Rabbit antiserum specific for *N. caninum* tachyzoites (KBA-2) was able to recognize 20 bands located from 15.5 to 80 kDa, of which thick bands were observed such as 18-15.5, 22-21, 25, 31-30, 39, 44, 49, 65, and 80 kDa. Whereas, minor bands were identified having 28, 33, 35, 37, 55, 74, and 108 kDa. Among them, only few antigenic bands corresponds to antigenic spots on 2-DE profiles using pH 4-7 IPG strips and were determined as 15.5, 28, 33, 35, 37, 39, 44, 55, 65 and 80 kDa antigenic bands (Fig. 4).

Discussion

One of the most important points in proteomic approach is to obtain a reproducible 2-DE gels which primarily depends on sample preparation. The cells or tissue must be efficiently disrupted and solubilized completely in order to obtain a representative protein population through sample lysis methods (sonication, french pressure, grinding and mechanical homogenization) [30]. Three sample preparation methods were compared at the present study. Among them, sonication for disrupting *N. caninum* tachyzoites combined with Urea-thiourea mixture as strong neutral chaotrope were found suitable method since more spots were detected compared with the other methods previously mentioned. It also observed and allowed to detect clear spots. Urea-thiourea mixtures (typically 2 M thiourea and 5-8 M urea) were reported to exhibit superior solubilizing power, especially on membrane proteins [32,37]. Furthermore, the mixtures were able to resolve many high molecular weight proteins [29].

Most *N. caninum* tachyzoites spots were placed at p*I* values below 7 by using a wide-range of IPG gels (pH 3-10) [17]. At present study, most spots were placed between pH 4 and 8, and between molecular weights of 25 and 87 kDa. In addition, many spots were found over pH 7. A few number of 2-DE profile for *N. caninum* tachyzoites were available, but present results were slightly different from Heckeroth *et al.* [17] in terms of pH range probably caused by different sample preparation methods, different IEF conditions and IPG gel sizes. However, most spots observed in acidic part have identical result between the two experiments.

Immunodominant antigens of *N. caninum* were detected as groups comprising molecules of 16/17, 29, 37, 46 kDa

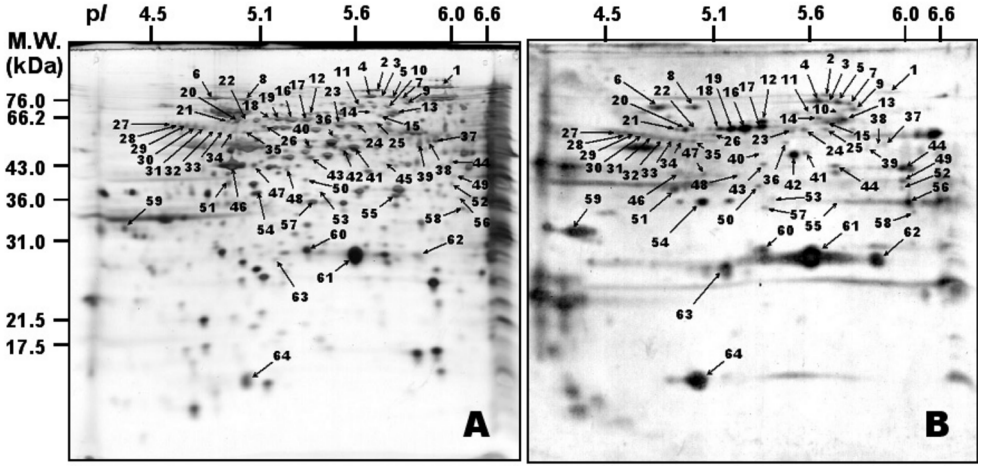

Fig. 3. Analysis of 2-DE and immunoblot profiles of *N. caninum* tachyzoites. (A) A total of 335 spots were detected on the 2-DE profile. Of these, 64 spots were identified as antigenic through comparison with 2-DE immunoblotting profile with the use of Phoretix™ 2D software on (B) 2-DE immunoblotting profile. Separated proteins after 2-DE were transferred to NC membrane and antigenic spots were detected with the use of rabbit anti-serum specific for *N. caninum* tachyzoites.

Table 1. Isoelectric point andMolecular weight of antigenic spots of Fig. 4

Spot No.	p*I* [a)]	M_r [b)] (kDa)	Spot No.	p*I*	M_r (kDa)	Spot No.	p*I*	M_r (kDa)
1	5.93	96.5	23	5.51	62.6	45	5.71	44.7
2	5.68	95.0	24	5.58	62.5	46	4.94	44.3
3	5.69	88.2	25	5.67	62.4	47	5.06	44.8
4	5.65	87.3	26	5.12	61.7	48	5.24	42.4
5	5.73	86.9	27	4.61	63.3	49	5.98	41.1
6	4.85	86.6	28	4.65	61.9	50	5.34	40.3
7	5.77	85.5	29	4.69	61.4	51	4.91	39.6
8	5.02	84.6	30	4.73	61.1	52	5.97	39.3
9	5.79	80.1	31	4.78	60.6	53	5.44	37.8
10	5.70	76.3	32	4.85	59.2	54	5.09	37.8
11	5.62	76.0	33	4.90	58.8	55	5.75	37.0
12	5.36	69.4	34	5.01	58.5	56	6.01	36.8
13	5.74	68.0	35	5.06	58.9	57	5.37	36.0
14	5.65	66.4	36	5.55	56.6	58	6.11	35.3
15	5.68	65.4	37	5.91	55.2	59	4.36	32.6
16	5.27	65.0	38	5.88	54.1	60	5.35	29.4
17	5.34	65.0	39	5.84	52.9	61	5.60	28.7

[a)]Isoelectric point
[b)]Molecular weight

using hyperimmune rabbit anti-*N. caninum* (NC-1) serum [2]. Similar molecular bands, including 17, 29/30, 37 and 46 kDa, were observed from sera of cattle, dogs, sheep, goats, rabbits and pigs [6]. Bands such as 15.5-18, 30/31 and 37 kDa, were also detected using rabbit sera raised against *N. caninum* tachyzoites (KBA-2). In addition, thick bands, for example 22-21, 25, 39, 44, 49, 65 and 80 kDa, were identified which were thought as main antigens of the parasite.

Most studies were performed based on conventional SDS-PAGE and immunoblotting assay. Although differences of sample preparation were not allowed to compare directly between 2-DE profiles and conventional results, a number of antigen spots on 2-DE using pH 4-7 IPG strip were found and considered as corresponding antigen bands on SDS-PAGE immunoblotting profiles: Spot 9 corresponds to 80 kDa band, spots 15, 16, 17, 18 and 19 to 65 kDa band, spot 37 to 55 kDa band, spot 44 and 46 to 44 kDa band, spot 52 to 39 kDa band, spot 55 and 56 to 37 kDa band, spot 58 to 35 kDa band, spot 59 to 33 kDa band, spot 63 to 28 kDa band, spot 64 to 15.5 kDa band. Spot 42, 54 and 61 exhibited strong signals on 2-DE immunoblotting profile on

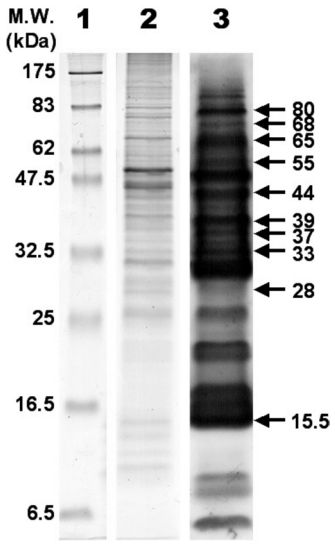

Fig. 4. SDS-PAGE and its immunoblot analysis of the *N. caninum* tachyzoites. SDS-PAGE (line 1) and its immunoblotting profiles (line 2) were resolved under reducing conditions. The antigen bands were detected with rabbit anti-serum specific for *N. caninum* tachyzoites and were expressed in numbers (arrow on the right of immunoblotting image) allowing to find its corresponding antigen spots on 2-DE immunoblotting profiles. Molecular weight markers are indicated on the left side and expressed in kilo Dalton (kDa).

pH 4-7 having no corresponding antigen bands on SDS-PAGE immunoblotting profiles. Differences might be due to 2-DE resolution which able to separate not only molecular weight but also p*I* for each molecule.

Antigen bands 116, 65 and 25 kDa were detected using sera from cows which were confirmed as *Neospora*-induced abortion by immunoblot analysis [3]. At the present study, 65 kDa antigen band was separated into a series of antigenic spots, such as spot 15, 16, 17, 18 and 19, which had similar molecular weight but different p*I* values. Isoform proteins might be separated into chain-like patterns. Each spot of the isoforms might be either originated from different gene or the same gene but occurred usually in post translational modification [31]. Spots from 27 to 34 were also observed possessing a chain-like patterns but displayed very weak signals.

The 29/30 kDa antigens might be associated with dense granules, network and limiting membrane of the parasitophorous vacuole [6]. Spots 60, 61 and 62 were thought to be corresponding spots of 29 kDa antigen band. Among them, spot 61 was suspected as a major antigenic spot in 2-DE antigen profiles. Dense granule associated in 33 kDa protein (NCDG1) was subcloned and identified [28]. On the other hand, affinity purified anti-Nc-p33 antibodies were uniquely recognized against 33 kDa band by immunoblot performed under both reducing and non-reducing conditions [18]. Spot 59 was assumed corresponding to 33 kDa antigen band and need to be confirmed using MALD-TOF MS assay.

Both *N. caninum*-specific sheep and rabbit sera were recognized as antigenic spots, at molecular weight range of 11 to 18 kDa and at p*I* range of 5 to 6. But the two antigenic spots did not react with *T. gondii*-specific anti-sera using 2-DE immunoblotting assay [17]. Spot 64, a large antigenic spot, was detected at 15.5 kDa and at p*I* value of 5.03 on 2-DE immunoblot profile. The spot was distinguished from the other due to its low molecular weight on 2-DE profiles and was suspected as the same spot pointed by Heckeroth *et al.* [17].

This study was conducted by optimization of sample preparation and IEF condition for 2-DE and analysis of 2-DE profiles. Results collected allowed to manifest the usefulness of 2-DE combined with immunoblotting in defining proteins and antigens of *N. caninum* tachyzoites. In addition, 2-DE profiles of *N. caninum* tachyzoites may be useful in further proteomic approaches.

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