Identification of immunodominant proteins of the microalgae *Prototheca* by proteomic analysis

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Abstract

Prototheca zopfii associated with bovine mastitis and human protothecosis exists as two genotypes, of which genotype 1 is considered as non-infectious and genotype 2 as infectious. The mechanism of infection has not yet been described. The present study was aimed to identify genotype 2-specific immunodominant proteins. *Prototheca* proteins were separated using two-dimensional gel electrophoresis. Subsequent western blotting with rabbit hyperimmune serum revealed 28 protein spots. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry analysis resulted in the identification of 15 proteins including malate dehydrogenase, elongation factor 1-alpha, heat shock protein 70, and 14-3-3 protein, which were previously described as immunogenic proteins of other eukaryotic pathogens.

Keywords: Bovine mastitis, immunodominant proteins, MALDI-TOF MS, proteomics, *Prototheca*, western blotting
Original Submission: 17 July 2014; **Revised Submission:** 18 September 2014; **Accepted:** 16 October 2014
Available online 8 November 2014

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Protothecosis refers to a rare infection caused by achlorophyllous unicellular algae of the genus *Prototheca*, which are reported to be saprophytic, ubiquitously distributed in the environment and closely related to *Chlorella* species [1,2]. Currently, among the six generally accepted species of *Prototheca*, *Prototheca zopfii*, *Prototheca wickerhamii*, *Prototheca blaschkeae* and *Prototheca cutis* have been associated with infections in cows, dogs, cats and, rarely, in humans. *P. zopfii* and *P. blaschkeae* are associated with antimicrobial-resistant bovine mastitis, which leads to heavy economic loss while *P. zopfii* appears to be frequently reported [3,4]. On the basis of biochemical, serological and phylogenetic analysis, *P. zopfii* has been divided into two genotypes, of which genotype 1 is considered to be non-pathogenic and genotype 2 is associated with bovine mastitis and human protothecosis [5–8]. Recently, differences at the proteomic level between these two genotypes of *P. zopfii* have been described [9–11]; however, the mechanism of infection has still not been described. Hence, the present study was carried out to identify the immunogenic proteins of *P. zopfii* genotype 2 using two-dimensional (2D) gel electrophoresis–western blotting with hyperimmune serum from experimentally immunized rabbits.

The type strain *P. zopfii* genotype 2 (SAG 2021), isolated from bovine mastitis [7], was utilized for extraction of protein [9]. In brief, cells cultured overnight were harvested by centrifugation at 1000 g for 5 minutes, reconstituted in lysis buffer (20 mM HEPES, pH 7.4, 10% glycerol, 1% Triton X-100, 1 mM EDTA, 0.5% benzonase and 0.5% protease inhibitor cocktail tablet), sonicated and the supernatant was collected for further study. The specific rabbit primary polyclonal antibodies were raised in New Zealand White rabbits as described previously [2]. In brief, rabbits of body weight 2.5–3.5 kg were intradermally injected with 1.0 mL of emulsion containing equal volume of phosphate-buffered saline containing 10^7 cells/mL and Freund incomplete adjuvant (Sigma-Aldrich, Steinheim, Germany). Following 3 weeks of priming, the rabbits were boosted intravenously, biweekly three times with 10^7 viable cells of the homologous strain. Seven days after the last booster

http://dx.doi.org/10.1016/j.nmni.2014.10.004

application and 10 weeks after priming, the rabbits were bled, serum was collected and stored at −80°C until further use. The immunization experiments with the rabbits were performed according to the German law on animal welfare and as approved by the German authorities (Regierungspräsidium Leipzig, Permission No. V 4/04).

Western blotting was carried out following the 2D separation of *Prototheca* proteins on 12% polyacrylamide gel after rehydration and isoelectrofocusing of 250 µg of whole cell lysate on an immobilized pH gradient strip of 7 cm and pI 3–10 (non-linear) (Immobilian drystrip; GE Healthcare, Munich, Germany). The separated proteins were then transferred to a nitrocellulose membrane (Trans Blot trans medium pure nitrocellulose membrane; Bio-Rad, Munich, Germany) using semi-dry transfer unit (80 mA per gel for 90 min) (GE Healthcare). Electrophoresis and blotting quality were checked by staining the membrane for 30 s in Ponceau S (1% Ponceau S, 0.1% acetic acid) followed by short destaining with Tris-buffered saline with Tween-20 (TBST). The membrane was incubated overnight in 1% skimmed milk powder (Carl Roth, Karlsruhe, Germany) in TBST, washed and incubated for 90 min with 1 : 100 diluted rabbit hyperimmune serum. The membrane was briefly rinsed with TBST and incubated for another 90 min with 1 : 2500 diluted goat anti-rabbit horseradish-peroxidase conjugated IgG-h-l (Biomol, Hamburg, Germany). Detection was carried out using 3,3′,5,5′-tetramethylbenzidine kit (Sigma Aldrich Chemie, Steinheim, Germany). The respective protein spots on a 2D gel performed in parallel and stained with Coomassie Brilliant Blue were identified after overlaying of the western blot image using Delta2D software version 4.0 (Decodon, Greifswald, Germany) [12].

Two-dimensional gel electrophoresis western blots from hyperimmune serum from two rabbits revealed 28 signals following analysis with Decodon software (Fig. 1). The corresponding protein spots were excised from the 2D gel and digested with trypsin. Protein identification was performed using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (Ultraflex II TOF/TOF; Bruker Daltonics, Bremen, Germany), as described previously [9]. Protein identification was considered to be valid if more than two peptides matched and the MASCOT score was greater than or equal to the significance threshold ($p < 0.05$). As a result, 15 proteins were successfully identified (Table 1). The identified proteins were found to be enzymes of energy metabolism and proteins involved in cellular transport and cell signalling including stress response.

Among the identified proteins, malate dehydrogenase, elongation factor 1-alpha (EF-1α), heat shock protein 70 (Hsp70) and 14-3-3 protein have been described as immunoreactive proteins in serological studies with other eukaryotic pathogens [13–15]. The negative control serum appeared to possess antibodies against *S*-adenosyl-L-homocysteine hydrolase and EF-1α; however, the latter was earlier identified as an immunoreactive protein of *Prototheca* [16]. On the other hand, four proteins (malate dehydrogenase, *S*-adenosyl-L-homocysteine hydrolase, EF-1α, and Hsp70) were previously reported to be differentially expressed in *P. zopfii* genotype 2 [9]. Hence *P. zopfii* genotype 2 hyperimmune serum appeared to possess specific antibodies against two of the proteins, malate

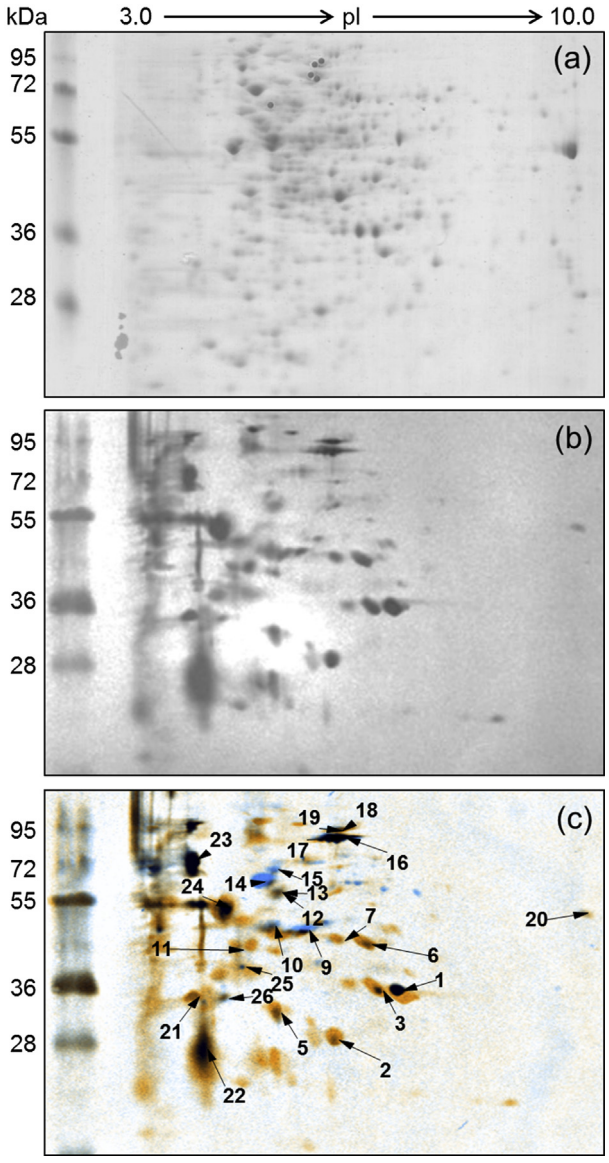

FIG. 1. Two-dimensional gel electrophoresis (2DE) and western blot image of *Prototheca* cell lysate. (a) A representative 2DE (pH 3–10, non-linear, 7 cm) image showing the separation of *Prototheca zopfii* genotype 2 cell lysate. (b) A representative image of a western blot with serum from a rabbit intradermally challenged with live *P. zopfii* genotype 2 (SAG 2021). (c) Overlaid image of the two 2DE blots for identification of corresponding spots. The spots were numbered randomly.

TABLE 1. List of identified proteins

Spot ID	NCBI accession no.	Uni-Prot no.	Protein	Organism	MOWSE score	Molecular mass (Da)	pI	No. of peptides	Function
1	gi|317033255		Malate dehydrogenase	*Aspergillus niger*	105	32 405	7.71	5	Energy metabolism
2	gi|499658212	Q3IXY7	Serine recombinase	*Rhodobacter sphaeroides*	87	57 111	7.12	14	Site-specific recombinase
4	gi|294486349	D6LCZ5	Triosephosphate isomerase	*Fusobacterium nucleatum*	110	27 607	5.51	7	Energy metabolism (glycolysis)
6	gi|491508197		Radical SAM protein	*(Eubacterium) yurii*	105	36 228	6.36	13	Enzyme superfamily, heterogeneous metabolic functions
7	gi|307102699	E1ZTB0	Hypothetical protein CHLNCDRAFT_59826	*Chlorella variabilis*	92	38 692	6.28	3	
10	gi|308475003	E3MYY5	Hypothetical protein	*Caenorhabditis remanei*	94	62 721	9.21	17	
11	gi|219117017	B7FXA2	Beta chain succinyl-coa synthetase synthetase	*Phaeodactylum tricornutum*	102	48 047	5.06	7	Energy metabolism (citric acid cycle)
12	gi|546319275	R7QHD1	S-adenosyl-L-homocysteine hydrolase, partial	*Chondrus crispus*	113	54 526	5.31	7	Enzyme of metabolism pathway
15	gi|145346182	A4RWG3	Heat shock protein 70	*Ostreococcus lucimarinus*	93	71 918	5.37	4	Stress response, cell signalling
18	gi|547216904	R5LKR2	Sigma-70 region 2	*Eubacterium* sp.	86	18 444	5.97	10	Ribosome biogenesis
19	gi|507081301		Protein BolA	*Citrobacter*	89	12 043	6.18	6	Perhaps cell proliferation and cell cycle regulation (--> signalling)
20	gi|224593225	C0LL61	Translation elongation factor-like protein (EF-1α)	*Parachlorella kessleri*	159	34 932	7.88	10	Protein biosynthesis
22	gi|74272601	Q1WLZ5	14-3-3 protein	*Chlamydomonas incerta*	112	29 683	4.9	4	Diverse cell signalling
23	gi|116750623	A0LN73	Porphobilinogen deaminase	*Syntrophobacter fumaroxidans* MPOB	92	33 376	7.04	8	For synthase of porphyrin--> metabolism
24	gi|82793401		ATP synthase subunit β	*Plasmodium yoelii yoelii*	192	58 109	5.93	7	Energy metabolism

The MOWSE score (MOlecular Weight SEarch score) is calculated by $-10 \log (P)$, where P is the probability that the observed match is a random event. The identification is considered to be valid if the MOWSE score is greater than or equal to the significance threshold value ($p < 0.05$) and more than two peptides match. The molecular mass was calculated from the identified protein sequence, and the pI (isoelectric point) was calculated from the identified protein sequence.

dehydrogenase and Hsp70, indicating a role of these two proteins in the immune reaction.

Hsp70 comprises a highly conserved protein family with housekeeping functions and stress-inducible chaperone functions when located intracellularly [17]. Extracellular Hsp70 in contrast, acts as a cytokine and activates immune cells. We can only speculate about the role of Hsp70 during infection. It should be noted that most of the identified proteins appear to be intracellular in nature, which supports the earlier speculations that the dead *Prototheca* cells induce a local immune response [18].

The absence of *Prototheca*-specific information in the public repositories resulted in the identification of proteins that are best described in other organisms [9]. *De novo* sequencing of the proteins might provide additional clues on the immunogenicity and virulence nature of *Prototheca*. The present study utilized serum from experimentally challenged rabbits; however, *Prototheca* infection in rabbit has not been reported yet. Hence, for a comprehensive description of genotype-specific antigens, which might serve as virulence factors, western blotting using serum of naturally infected animals should be carried out.

Transparency declaration

The authors state that they have no conflicts of interest.

Acknowledgements

We would like to acknowledge the assistance of the Bio-MS unit of the Core Facility BioSupraMol supported by the DFG. We would like to thank Michael Kühl for technical assistance.

References

[1] Satoh K, Ooe K, Nagayama H, Makimura K. *Prototheca cutis* sp. nov., a newly discovered pathogen of protothecosis isolated from inflamed human skin. Int J Syst Evol Microbiol 2010;60:1236–40.

[2] Roesler U, Hensel A. Longitudinal analysis of *Prototheca zopfii*-specific immune responses: correlation with disease progression and carriage in dairy cows. J Clin Microbiol 2003;41:1181–6.

[3] Marques S, Silva E, Kraft C, Carvalheira J, Videira A, Huss VAR, et al. Bovine mastitis associated with *Prototheca blaschkeae*. J Clin Microbiol 2008;46:1941–5.

[4] Thompson G, Silva E, Marques S, Müller A, Carvalheira J. Algaemia in a dairy cow by *Prototheca blaschkeae*. Med Mycol 2009;47:527–31.

[5] Jagielski T, Lagneau P. Protothecosis. A pseudofungal infection. J Mycol Med 2007;17:261–70.

[6] Möller A, Truyen U, Roesler U. *Prototheca zopfii* genotype 2—the causative agent of bovine protothecal mastitis? Vet Microbiol 2007;120:370–4.

[7] Roesler U, Möller A, Hensel A, Baumann D, Truyen U. Diversity within the current algal species *Prototheca zopfii*: a proposal for two *Prototheca*

zopfii genotypes and description of a novel species, *Prototheca blaschkeae* sp. nov. Int J Syst Evol Microbiol 2006;56:1419–25.

[8] Kishimoto Y, Kano R, Maruyama H, Onozaki M, Makimura K, Ito T, et al. 26S rDNA-based phylogenetic investigation of Japanese cattle-associated *Prototheca zopfii* isolates. J Vet Med Sci 2010;72:123–6.

[9] Murugaiyan J, Weise C, von Bergen M, Roesler U. Two-dimensional proteome reference map of *Prototheca zopfii* revealed reduced metabolism and enhanced signal transduction as adaptation to an infectious life style. Proteomics 2013;13:2664–9.

[10] von Bergen M, Eidner A, Schmidt F, Murugaiyan J, Wirth H, Binder H, et al. Identification of harmless and pathogenic algae of the genus *Prototheca* by MALDI-MS. Proteomics Clin Appl 2009;3:774–84.

[11] Murugaiyan J, Ahrholdt J, Kowbel V, Roesler U. Establishment of a matrix-assisted laser desorption ionization time-of-flight mass spectrometry database for rapid identification of infectious achlorophyllous green micro-algae of the genus *Prototheca*. Clin Microbiol Infect 2012;18:461–7.

[12] Berth M, Moser FM, Kolbe M, Bernhardt J. The state of the art in the analysis of two-dimensional gel electrophoresis images. Appl Microbiol Biotechnol 2007;76:1223–43.

[13] Ludolf F, Patrocínio PR, Corrêa-Oliveira R, Gazzinelli A, Falcone FH, Teixeira-Ferreira A, et al. Serological screening of the *Schistosoma mansoni* adult worm proteome. PLoS Negl Trop Dis 2014;8:e2745.

[14] Chaturvedi AK, Weintraub ST, Lopez-Ribot JL, Wormley FL. Identification and characterization of *Cryptococcus neoformans* protein fractions that induce protective immune responses. Proteomics 2013;13:3429–41.

[15] Shi L, Li F, Huang M, Lu J, Kong X, Wang S, et al. Immunoproteomics based identification of thioredoxin reductase GliT and novel *Aspergillus fumigatus* antigens for serologic diagnosis of invasive aspergillosis. BMC Microbiol 2012;12:11.

[16] Marques S. Protothecosis: agent characterization and pathogenesis. PhD Thesis. Porto. 2010.

[17] Daugaard M, Rohde M, Jäättelä M. The heat shock protein 70 family: Highly homologous proteins with overlapping and distinct functions. FEBS Lett 2007;581:3702–10.

[18] Pérez J, Ginel PJ, Lucena R, Hervás J, Mozos E. Canine cutaneous protothecosis: an immunohistochemical analysis of the inflammatory cellular infiltrate. J Comp Pathol 1997;117:83–9.