RESEARCHREPORT
PORK SAFETY

Title: Identification of diagnostic proteins of *Toxoplasma gondii* by targeted proteomic analysis **NPB# 00-025**

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I. Abstract:

Humans become infected with the ubiquitous protozoan parasite, *Toxoplasma gondii*, by congenital transmission from mother to fetus, through ingestion of tissue cysts (cyst structure found in animal tissues which contains the bradyzoite [=slow] form) in under cooked or uncooked meat, or by ingesting food or water contaminated with sporulated oocysts (cyst structure found in cat feces which contains the sporozoite [=reproductive] form) from infected cat feces. Pigs become infected by the same routes, resulting in meat products containing tissue cysts which could infect consumers. Reduction of risk of human and swine infection with *T. gondii* is hampered by a number of factors, making epidemiological studies which could lead to the development of strategies to reduce infection in humans and pigs difficult. Though experimentally infected pigs elicit a strong antibody response to the tachyzoite (rapidly dividing, non-encysted tissue form) stage of the parasite, available serological assays which utilize a crude tachyzoite extract as the antigen failed to detect nearly 30% of naturally exposed pigs killed at a commercial abattoir in which tissue cysts were later detected. In addition, there is a lack of epidemiological data documenting the predominant routes of infection (oocyst versus tissue cyst consumption) in horizontally transmitted toxoplasmosis. Existing serological assays can determine previous exposure to the parasite, but there are no tests which can differentiate between oocyst ingestion versus tissue cyst ingestion as the infection route. In this study, we have used surface enhanced laser desorption/ionization time of flight-mass spectrometry (SELDI-TOF-MS) in combination with 1 and 2 dimensional electrophoresis to identify stage specific proteins in *T. gondii* sporulated oocysts, tachyzoites, and bradyzoites. Specific, reproducible, mass spectra protein profiles were produced for each sample. Western blots of 2-D gels of the *T. gondii* oocyst proteins using pooled sera from 10 pigs with acute oocyst-induced *T. gondii* infection were used to select a single 18.3 kDa protein which matched the mass of one of the stage specific peaks identified in the SELDI spectral analysis. One dimensional Western blots revealed a single protein band of Mr 18,350 which was

recognized by sera from pigs infected orally with *T. gondii* oocysts and not by sera from pigs infected orally with *T. gondii* tissue cysts. The protein was also recognized by chronically infected pigs (pigs infected with oocysts 1 year earlier), indicating the persistence of antibodies to the 18.3kDa oocyst-specific protein and suggesting that this protein could be useful even in chronic, long standing infections to determine the original infection route. A serological test based upon this 18.3 kDa antigen may provide epidemiological evidence of the predominant infection route in humans and swine, and may make the development of control strategies for *Toxoplasma* infection feasible. Proteins that appeared to be specific for tachyzoites and bradyzoites by SELDI analysis were isolated from 2D gels and are currently being analyzed for amino acid sequence information. These proteins will be cloned and tested in ELISA assays for sensitivity and specificity for detection of chronic *T. gondii* infection in pigs.

II. Introduction: Explain why this project is important to producers. The introduction to your research proposal is probably a good guide.

Toxoplasmosis, caused by the protozoan parasite *Toxoplasma gondii*, is one of the most common parasitic infections of man and other warm-blooded animals. It has been found worldwide from Alaska to Australia. Nearly one-third of humanity has been exposed to this parasite. In most adults it does not cause serious illness, but it can cause blindness and mental retardation in congenitally infected children and devastating disease in immunocompromised individuals [1]. Humans become infected by congenital transmission from mother to fetus, through ingestion of tissue cysts in under cooked or uncooked meat, or by ingesting food or water contaminated with sporulated oocysts from infected cat feces. Food animals, such as pigs, become infected by the same routes, resulting in meat products containing tissue cysts which could infect consumers. Detection of infected pigs using serological assays is hampered by the lack of specificity and sensitivity of the currently available ELISA, which uses a crude tachyzoite lysate to detect latently infected pigs, which harbor only the bradyzoite stage of the parasite. There are no tests which can differentiate between oocyst ingestion versus tissue cyst ingestion as the infection route, making epidemiological studies which could lead to the development of strategies to reduce infection in humans and pigs difficult. Here we have used surface enhanced laser desorption/ionization time of flight-mass spectrometry (SELDI-TOF-MS) to compare the protein profiles of *T. gondii* oocysts, tachyzoites, and bradyzoites in order to identify stage specific proteins which might be useful in serologic assays to identify latently infected pigs and the route of infection in humans and pigs. Delivery of a wholesome, safe, and high quality product is essential for pork to remain a competitive commodity due to heightened awareness of the consumer to issues concerning contaminated foods. Accurate ELISA assays to detect *T. gondii* cysts in pigs and to identify the routes of infection would contribute significantly to the prevention and control of *T. gondii*, and in conjunction with implementation of farm management practices to reduce exposure of pigs to *T. gondii*, could form the basis for the development of a quality assurance program.

III. Objectives: From your research proposal.

1.) To determine if the sensitivity of ELISA for detecting tachyzoite induced antibodies can be improved by using specific antigenic components rather than a crude lysate, as presently used.

2.) To determine if the bradyzoite stage elicits specific antibody production in the pig and if the use of bradyzoite specific antigens in an ELISA assay would increase our ability to detect the presence of tissue cysts.

3.) To determine if pigs infected via the oocyst route develop specific anti-sporozoite antibodies, whether these antibodies persist during the acute and chronic phases of the infection, and whether the presence or absence of these antibodies could be used to distinguish the source of infection.

IV. Procedures:

Collection of parasite stages

Toxoplasma gondii (VEG strain) oocysts were collected by sucrose floatation from feces of cats fed tissues of mice experimentally infected with *T. gondii*; these procedures have been previously described [2,3]. Collected oocysts were sporulated in 2% H_2SO_4 while shaking for 7 days at rt. Sporulated oocysts were washed in water by centrifugation, treated for 5 min in 0.525% sodium hypochlorite and washed in Hanks Balanced Salt Solution (HBSS) until the wash solution pH was neutral. Oocyst proteins were extracted in 5M urea, 2M thiourea, 2% 3-3-chol-amidopropyldimethyl-ammonio-1-propanesulfonate (CHAPS), 2% capryly sulfobetaine (SB 3-10), and 40mM Tris, 2% tributylphosphine (TBP) while vortexing with 0.5mm glass beads. Protein concentrations were determined using a modified Bradford protein assay (BioRad, Hercules, CA). Extracted proteins were frozen at -80^{o}C until used. *Toxoplasma gondii* tachyzoites were produced in the HCT-8 cell line (ATCC #CCL-244). T-75 flasks with a 75% confluent cell layer were seeded with 1 x 10^5 *T. gondii* bradyzoites and maintained at 37^{o}C and 10% CO^2 in high glucose Dulbeccos Modified Eagles Medium (DMEM) containing 3% fetal calf serum (FCS), 50 U/ml penicillin, 50 □g/ml streptomycin, 2mM glutamine, 100mM HEPES, 1x MEM non-essential amino acids, and 1mM sodium pyruvate. After 7-10 days of culture, as tachyzoites began to emerge from cells, the media was switched to HBSS for 2-3 hrs each day, and emerged tachyzoites were collected from the supernatant by centrifugation at 1200 x g for 7 min daily until the cell monolayer was completely disrupted. Collected parasites were washed 2-3 times in HBSS, and forced through a 26 gauge needle to disrupt any collected cells containing parasites. The parasite preparation was then pushed through a 5□m Millex-SV syringe filter (Millipore, Bedford, MA) to remove cell debris, washed twice in HBSS, centrifuged as above, and immediately frozen at -80^{o} C until used. All tissue culture supplies, unless otherwise noted, were purchased from Sigma Chemical Co., St. Louis, MO. Frozen pellets containing parasites (50-100 x 10^6 tachyzoites) were thawed and sonicated for 30 sec, and protein extractions and concentration determinations were carried out as described above. Control preparations of the uninfected HCT-8 cell line were treated identically to parasite material and used to identify host cell and tissue culture protein contaminants in tachyzoite preparations. *Toxoplasma gondii* tissue cysts containing bradyzoites were collected from the brains of Swiss-Webster mice inoculated s.c. with isolated bradyzoites 6-8 weeks previously by the method of Petersen [4]. Isolated tissue cysts were treated with 0.25% trypsin for 5 min, and released bradyzoites were collected and washed by centrifugation in 0.85% saline, and extracted as described above.

SELDI TOF-MS

SELDI TOF-MS was performed using cation exchange (WCX2) ProteinChip array slides (Ciphergen Biosystems, Palo Alto, CA). The WCX2 slides were equilibrated in 10mM HCl for 10 min, and washed 3 times for 5 min with water. Extracted proteins from

oocysts, tachyzoites, and bradyzoites were adjusted to 10□g/50□l in 50mM NaAc binding buffer, pH. 4.5. The samples were diluted 1:4 in the NaAc binding buffer, and 200□l of each sample was applied directly to a single spot on a WCX2 array slide. The slides were incubated at rt for 1 hr, then washed 3 times for 5 min in the NaAc binding buffer, followed by a water wash. The slides were air dried for 10 min, and a saturated solution of sinapinic acid in 50% acetonitrile and 0.5% trifluoroacetic acid was crystallized onto each spot. Mass analysis was accomplished by 80 averaged laser shots in a Ciphergen SELDI Protein Biology System (PBS 2). Each sample was analyzed in triplicate to assess reproducibility.

2-Dimensional Western Blots of T. gondii oocyst, tachyzoite, and bradyzoite proteins

Immobilized pH gradient gel strips (11cm, pH 3-10 gradient) were rehydrated in buffer (8M urea, 2% CHAPS, 2mM TBP, 0.2% Bio-Lytes 3/10, and 0.001% bromophenol blue) containing 125□g of *T. gondii* solubilized oocyst, tachyzoite, or bradyzoite proteins from the extraction procedures described above at 50V for 12 hrs, followed by focusing at 5000V for 35,000 Vh using the BioRad IPG focusing cell. Strips containing focused proteins were soaked at room temperature in equilibration buffer (6M urea, 0.375 M Tris-HCl, pH 8.8, 2% SDS, 20% glycerol, 2% dithiothreitol (DTT)) for 30 min and electrophoresed in the 2nd dimension on 4-12% gradient Bis-Tris gels (Invitrogen, Carlsbad, CA) in 50mM MOPS-SDS running buffer (50mM 3-N-morpholino propane sulfonic acid, 50mM Tris base, 3.5mM SDS, 1mM EDTA, pH 7.7) at 150V for 75 min. All 2-D gel supplies, including 2-D analysis software, unless otherwise noted, were purchased from BioRad Laboratories (Hercules, CA). Electroblotting was carried out on unfixed gels by transfer of proteins onto Immobilon (PVDF) nylon blotting membrane (Millipore, Bedford, MA) using a Novex gel transfer apparatus (Novex, San Diego, CA) set at 40V for 80 min in 25mM Bicine, 25mM Bis-Tris, 1mM EDTA, 20% methanol, pH 7.2 blotting buffer. For Western blotting, the membranes were rinsed in 50 mM Tris buffered, 0.85% saline (TBS) and unbound sites on the membrane were saturated with Detector Block solution (Kirkegaard and Perry, Gaithersburg, MD). The membranes were incubated in pools of porcine sera (diluted 1:500) from 10 pigs with acute oocyst-induced *T. gondii* infection (~1000 oocyst per os; positive MAT titer of ≥1:400; pool prepared from sera taken from week 4 through week 12 post infection), 10 pigs that were experimentally infected with *T. gondii* via oral inoculation of tissue cysts in mouse brain tissue (~5000 tissue cyst per os; positive MAT titer of ≥1:100; pool prepared from sera taken from week 6 through week 10 post infection), or sera from chronically infected pigs harboring tissue cysts (sera collected 12 months after initial infection with oocysts as described above). Horseradish peroxidase-conjugated goat anti-pig IgG (Sigma Chemical. St. Louis, MO) was used as the 2nd step antibody at a dilution of 1:800. Oocyst, tachyzoite, and bradyzoite proteins recognized by porcine anti-*Toxoplasma* antibodies were visualized using the 4CN membrane developer kit (Kirkegaard and Perry, Gaithersburg, MD). Western blot images were captured using the ProExpress proteomics image acquisition system (Perkin Elmer, Boston, MA); spot matching and image analysis of the 2-dimensional Western blot images was accomplished using the PDQuest software system. Identical 2-D gels containing oocyst proteins were stained with Reversible Copper stain (BioRad, Hercules, CA), and the 2-dimensional images were analyzed for spot matching and comparisons to the Western blot images using the PD Quest software. Proteins which were recognized by anti-*Toxoplasma* antibodies in the Western blots and matched the masses identified in the SELDI analysis as specific to the oocyst, tachyzoite, or bradyzoite profile were excised from the Copper stained gels, washed in water, emulsified in extraction buffer, and applied to 1D PAGE gels. Four to 12% gradient Bis-Tris gels containing the specific oocyst, tachyzoite, or bradyzoite proteins were electrophoresed in 50mM MOPS-SDS running

buffer followed by Western blotting, using conditions as described above and pooled sera from the *T. gondii* infected pigs described above.

V. Results: Explain your research results by objective and include a summary of the knowledge that is of immediate or future benefit to pork producers.

SELDI TOF-MS analysis of *Toxoplasma* oocyst, tachyzoite, and bradyzoite proteins bound to chemically defined WCX2 protein chips is shown in Figure 1; both a chromatographic mass:charge map and a converted spectral gel display are shown for each sample. Specific, reproducible protein profiles were produced in each sample analysis display for *Toxoplasma* oocysts, tachyzoites, and bradyzoites. Enhanced spectra of 20 kDa spans of the analyzed proteins revealed several stage specific proteins in the tachyzoite, bradyzoite, and oocyst preparations (Figure 2; Table 1). Molecular mass analysis of the 2-D Western blots of the *T. gondii* oocyst, tachyzoite, and bradyzoite proteins recognized by pooled sera from the *T. gondii* infected pigs with acute oocyst-induced *T. gondii* infection revealed a single protein in the 18 kDa range which could be cleanly excised from Copper stained gels and matched the mass of one of the stage specific peaks identified in the SELDI TOF-MS spectral analysis (Figure 3). Extraction of the 18.3kDa protein from the 2D gels and electrophoresis of the protein on 1 D gels followed by Western blotting revealed a single protein band of M_r 18,350 which was recognized by sera from pigs with acute Toxoplasmosis infected with oocysts and not by sera from pigs infected with tissue cysts (Figure 4). In addition, sera from chronically infected pigs (infected 1 year previously by oral inoculation with oocysts) recognized the 18.3kDa oocyst specific protein, indicating that antibodies to the protein persist for at least 1 year, and that this protein may be useful in epidemiological studies to determine the infection route even in chronic *Toxoplasma* infection.

Human and animal infection with *T. gondii* can result from ingestion of sporulated oocysts or by ingestion of tissue cysts contained in undercooked meat [1, 5, 6, 7]. Available evidence for the oocyst infection route in humans is based entirely upon epidemiological surveys. In certain areas of Brazil, approximately 60% of 6-8 year old children have antibodies to *T. gondii* linked to the ingestion of oocysts from an environment heavily contaminated with *T. gondii* oocysts [8]. The largest recorded outbreak of clinical toxoplasmosis in humans was epidemiologically-linked to drinking water from a municipal water reservoir in British Columbia, Canada [9]. This water reservoir was thought to be contaminated with *T. gondii* oocysts excreted by cougars (*Felis concolor*) [10, 11]. Serological assays of food animals revealed significant but variable level of *T. gondii* infection [7]; in the United States and Europe pigs are generally thought to be the most common source of tissue cyst acquired *Toxoplasma* infection in humans. In one study, viable *T. gondii* was isolated from 17% of 1,000 adult pigs (sows) from a slaughter plant in Iowa [12]. Consumption of undercooked sheep and goat meat and the consumption of raw goats milk have also been implicated in the transmission of *T. gondii* to humans, but are considered an insignificant source of *T. gondii* infections in humans in the U.S. [13]. Recently, free range chickens from several counties in São Paulo state, Brazil were found to have high levels of *T. gondii* infection [14]. *Toxoplasma gondii* infection is also prevalent in game animals, including white-tailed deer [15]. *Toxoplasma gondii* in tissue cysts can survive in food animals for years, and potentially for the life of the host. There are no tests which can discriminate between oocyst ingestion versus tissue cyst ingestion as the infection route in humans or food animals, confounding attempts to develop strategies to reduce or eliminate the risk of *T. gondii* infection for humans through environmental contamination or the consumption of tissue cyst-laden meat products. Serological methods currently in use

for diagnosis or epidemiological surveys of toxoplasmosis in humans and animals utilize whole, fixed tachyzoites, solubilized native tachyzoite proteins, or recombinant tachyzoite proteins [16, 17, 18, 19]. These assays, though partially effective for detection of exposure to *Toxoplasma*, are not useful for determining infection route. No serological assays currently exist which utilize oocyst proteins as a source of diagnostic antigens, though stage specific antigens have been previously described from tachyzoites [20, 21, 22, 23], bradyzoites, and sporozoites [24, 25, 26].

Development of a serological assay useful for both clinical and research studies to determine the predominant routes of infection in humans and food animals would be useful for designing programs to reduce or eliminate exposure to infectious stages of the parasite. In this study, we have utilized SELDI TOF-MS fingerprinting in combination with 1 and 2 dimensional Western blot analysis to identify an oocyst specific 18.3kDa protein that can differentiate between oocyst and tissue cyst induced *T. gondii* infection in pigs. A number of stage specific proteins were identified in the mass spectra produced in the SELDI TOF-MS analysis of *T. gondii* stages. The apparent masses of these protein peaks are similar to those which have been described previously [25, 26]. SELDI TOF-MS has been utilized to identify biomarkers in serum proteins, prostate tumor biopsies, and diseased tissues [27, 28]. Results presented in this study were generated from one set of cation bait substrate and wash conditions, hence only a subset of the entire repertoire of *T. gondii* cellular proteins are represented. However, within this subset of toxoplasmal proteins, significant differences were seen between life cycle stages which could be exploited to differentiate oocyst induced versus tissue cyst induced infections in Western blot assays. The complexity of each of the protein samples as evidenced by 2D gel electrophoresis images makes identification of useful biomarkers difficult, and is complicated by the fact that only a small subset of the expressed proteins in each of the life cycle stages is serologically recognized by an infected host. Numerous protein capture surfaces and wash conditions are available which could be used to generate a more expansive and detailed protein fingerprint for each of the life cycle stages and identify additional biomarkers which could be of use; these studies are currently underway.

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Table 1:

Measured masses of unique protein peaks identified in spectra of *Toxoplasma gondii* life cycle stages.

	Measured masses from each life cycle stage		
	Oz	Tz	Bz
1	4,955	4,957	6,572
2	5,649	5383	9,003
3	5,959	5,959	12,863
4	7,822		15,026
5	8,779		
6	9,003		
7	10,574		
8	10,888		
9	11,645		
10	12,703	11,150	
11	13,186		
12	16,751		
13	16,827		
14	18,319*		
15	19,213	14,645	
16	22,445		
17	23,754		
18	24,467		
19	34,231		

*Oocyst specific protein excised from 2D copper stained gels

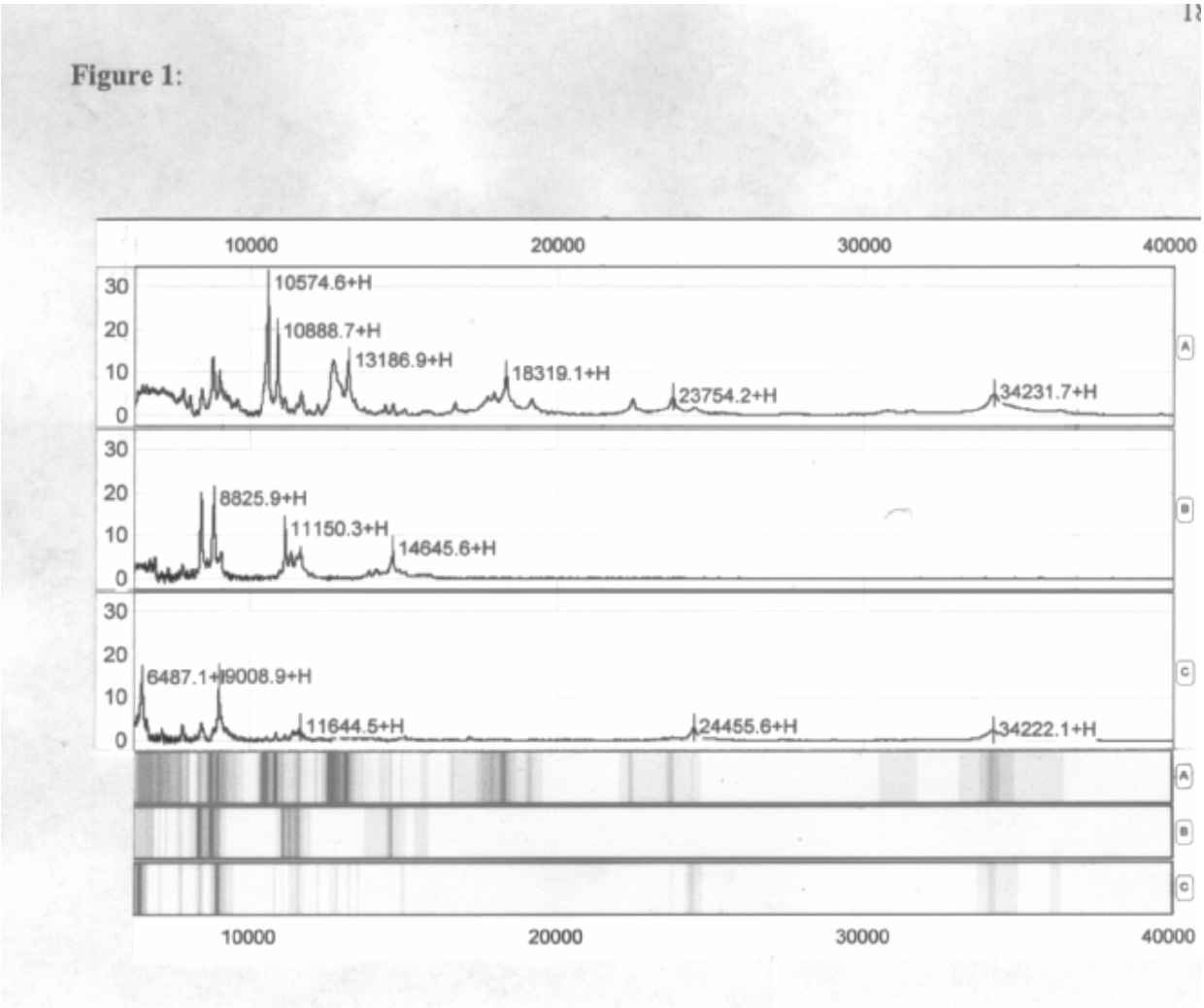

Figure 1:
SELDI TOF-MS analysis of *Toxoplasma gondii* oocyst, tachyzoite, and bradyzoite proteins and transformed gel images of same. Proteins bound to cation exchange WCX biochip arrays. Panel A (top and bottom) shows the chromatographic spectrum (top) and transformed gel image (bottom) for *T. gondii* extracted oocyst proteins. Thirteen major peaks are observed; 8 of which appear to be specific to the oocyst preparation. Panel B (top and bottom) shows the chromatographic spectrum (top) and transformed gel image (bottom) for *T. gondii* extracted tachyzoite proteins. Five major peaks are observed; 3 of which appear to be specific to the tachyzoite preparation. Panel C (top and bottom) shows the chromatographic spectrum (top) and transformed gel image (bottom) for *T. gondii* extracted bradyzoite proteins. Eleven major peaks are observed; 4 of which appear to be specific to the bradyzoite preparation. Spectrum range shows mass to charge ratio from 0 to 40,000; Y axis shows % laser intensity.

Figure 2:

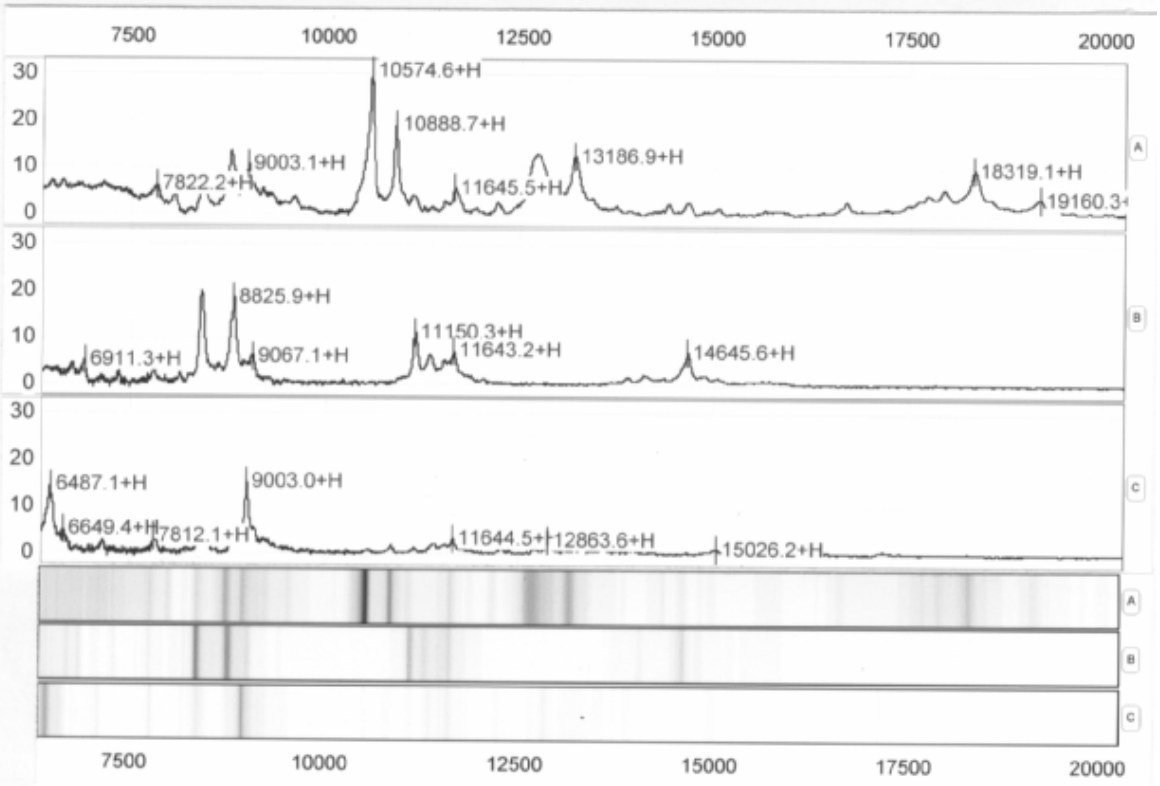

Figure 2:
Enhanced spectrum in 20,000 mass range, as in Figure 1. Panel A (top and bottom) shows oocyst protein spectrum with 18.3 kDa protein shown.

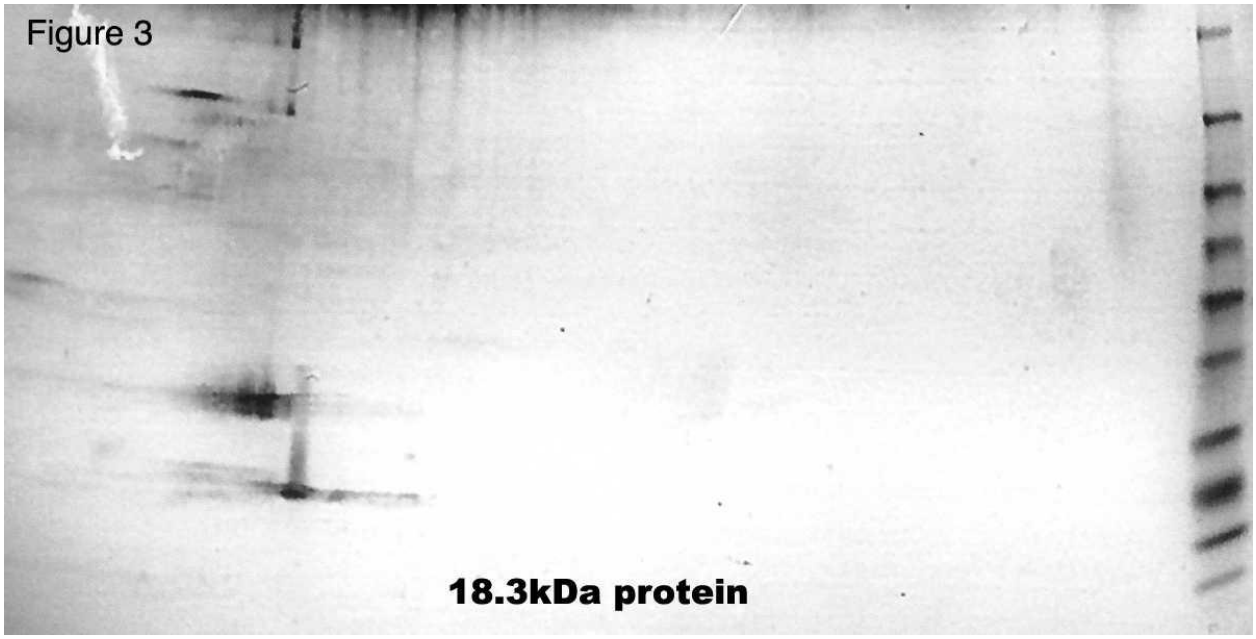

Figure 3:
Western blot of 2-D protein map of VEG strain oocysts of *Toxoplasma gondii*. Immunogenic protein spots were detected using a pool of sera from 10 pigs serologically positive at 1:400 by MAT for oocyst induced *T. gondii* infection. Protein spots visualized in the blots and excised from copper stained gels run in parallel; 18.3 kDa protein indicated by line. PH range of isoelectric points across gel is pH 3 to pH 10 (left to right

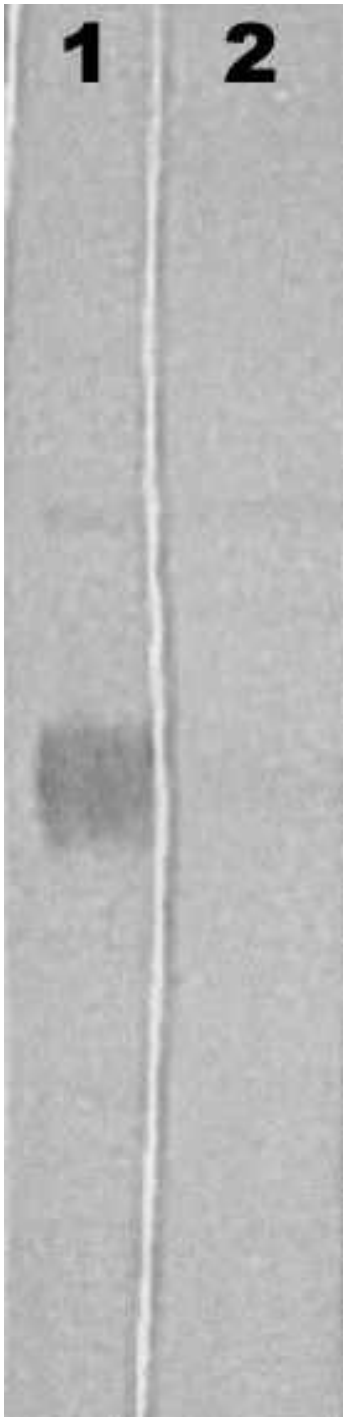

Figure 4:
One dimensional Western blot of 18.3 kDa protein excised from copper stained gel, extracted, and electrophoresed on 4-12% gradient Bis-Tris gels. Blotted protein exposed to pooled sera from 10 pigs infected by oral inoculation of *T. gondii* oocysts (lane 1) and pooled sera from 10 pigs infected by consumption of tissue cysts (lane 2). Oocyst protein specifically recognized by sera from pigs infected with oocysts indicated by line.