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Molecular cloning, sequencing, and expression of *Eimeria tenella* HSP70 partial gene

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Genet. Mol. Res. 16 (1): gmr16018964
Received July 13, 2016
Accepted February 20, 2017
Published March 15, 2017
DOI http://dx.doi.org/10.4238/gmr16018964

ABSTRACT. Members of the *Eimeria* genus are protozoan parasites of the subphylum Apicomplexa (Eimeriidae family), and belong to the coccidia group. *Eimeria tenella* is one of the most pathogenic species owing to its ability to penetrate the mucosa, and cause inflammation and damage. It is an obligate intracellular parasite that causes disease by destroying the host cells during multiplication. Heat shock protein 70 (HSP70) is a molecular chaperone that prevents cellular stress. The objective of this study was to clone, sequence, and express *E. tenella* HSP70 protein. After selecting the region of highest hydrophilicity in the *hsp70* gene, we cloned complementary DNA (cDNA) into a pTrcHis2-TOPO vector and transformed it into TOP10 *Escherichia coli* cells; after induction, the bacteria expressed a 23-kDa protein with insoluble expression levels of approximately 5 mg/L. In summary, the partial *hsp70* gene was successfully expressed in *E. coli*, producing a 23-kDa protein under insoluble conditions, and the antigen characteristics predicted by hydrophilicity analysis suggest the development of a vaccine for use in avian coccidiosis.

Key words: Coccidiosis; Eimeriosis; Molecular biology; Cloning; pTrcHis2-TOPO; Hydropathy analysis

INTRODUCTION

Avian coccidiosis is caused by protozoan parasites of the genus *Eimeria*, which cause economic global losses of more than three billion dollars per year in the poultry industry (Shirley et al., 2004; Zhang et al., 2012). Control of poultry coccidiosis is based on the use of prophylactic coccidiostat drugs. However, new means of control are necessary owing to the emergence of resistance to anticoccidial products and the regulation of these drugs in the production of broiler chickens (Williams, 2002; Jang et al., 2011).

Recombinant protein vaccines derived from *Eimeria* spp may induce protective immunity against coccidiosis from a specific antigen, especially if associated with adjuvants (Lillehoj et al., 2005; Jang et al., 2011), presenting great potential for their use as an alternative to anticoccidial agents.

Many antigens - such as organelles from the apical complex (micronemes, rhoptries, and dense granules), structural elements (conoid, polar, and microtubules ring), and membrane proteins (Ma et al., 2011) - have been studied because they have the potential to interact with the host's immune system. Reviews by Ahmad et al. (2016) and Meunier et al. (2016) describe several types of antigens that have been evaluated as subunit vaccines against avian coccidiosis. In this context, heat shock protein 70 (HSP70) has emerged as an immunostimulatory component for the development of subunit vaccines (Zhang et al., 2012). A comparison of heat shock proteins from different organisms, from bacteria to humans, has identified highly conserved domains (Del Cacho et al., 2008). The role of these proteins is to promote the maintenance of homeostasis and cellular functions through protection from the deleterious effects caused by cellular stress from both the host and the environment (Del Cacho et al., 2008; Zhang et al., 2012).

The purpose of this study was to clone the regions of greatest hydrophilicity in HSP70 from *Eimeria tenella* and express them in *Escherichia coli.*

MATERIAL AND METHODS

Eimeria tenella strain

Eimeria oocysts were multiplied in day-old, coccidian-free Cobb broiler chicks housed in wire cages; food and water were supplied *ad libitum*. The experiment received ethics approval according the norms of the Ethics Committee on Animal Experimentation of Universidade Estadual de Londrina (ECAE/UEL No. 96/09). *E. tenella* sporozoites were purified according to the method described by Garcia et al. (2008). Briefly, the oocysts were sterilized with sodium hypochlorite and sporulated in 2.5% potassium dichromate. After sporulation, the oocysts were washed with phosphate-buffered saline (PBS; pH 7.5) to remove potassium dichromate, and the sporulated oocyst walls were disrupted by shaking with sterile 3-mm glass beads in a vortex for 5 min. The obtained sporocysts were resuspended in 50-mL tubes containing 0.25% trypsin (v/v) (Sigma-Aldrich, St. Louis, MO, USA) and 4% taurodeoxycholate acid (w/v) (Sigma-Aldrich, St. Louis, MO) in PBS, and placed in a shaking water bath at 41°C for 45 min. Subsequently, the excystated sporozoites were centrifuged at 700 *g* for 20 min at room temperature, and washed three times with PBS. The suspension was adjusted to a concentration of 10^7 sporozoites/mL.

Primer design and antigenic peptide prediction

Specific primers were designed based on the *hsp70* gene from sequence Z46965.1 deposited in GenBank using online software Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). The primer sequences were as follows: *hsp70*-F (5'-GAGAAAGAAGACGGACGCAC-3') and *hsp70*-R (5'-CTGATACACCTTGATGCCCAC-3'). The selected region met two criteria: correspondence with an open reading frame (ORF) to obtain the coding sequence (CDS); and potential hydrophilic regions of the protein identified using the Kyte and Doolittle (1982) algorithm (http://web.expasy.org/ProtScale).

hsp70 cDNA and construction of cloning vector pTrcHis2-TOPO-*hsp70* gene

The total RNA was isolated from sporozoites using TRIzol® (Invitrogen, Carlsbad, CA, USA) according to the manufacturer instructions. The cDNA sequence encoding *E. tenella* HSP70 was obtained by reverse transcription of 1 mg of the total RNA using a ProtoScript® M-MuLV kit (New England Biolabs, Beverly, MA, USA). The polymerase chain reaction (PCR) was carried out in a total volume (25 μL) containing 50 ng *E. tenella hsp70* cDNA template, 1 μL each of the primers at 20 pmol, 200 μM each deoxynucleoside triphosphate, 0.5 μL 50 mM magnesium sulfate, and 1 U Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA). PCR was carried out by denaturing the DNA at 94°C for 5 min, followed by amplification for 35 cycles, secondary denaturation at 94°C for 1 min, annealing at 60°C for 45 s, and extension at 72°C for 1 min, with a final extension at 72°C for 15 min. The PCR product was analyzed by electrophoresis on 1.5% agarose gel stained with SYBR Green (Invitrogen), and the gel image was documented. A 100-bp size ladder (New England BioLabs Inc., Beverly, MA, USA), was used as a marker to indicate the sizes of the amplicons. The fragments containing *hsp70* were purified using an QIAquick Gel Extraction kit (Qiagen Biotechnology Brazil, Ltd., São Paulo, SP, Brazil), ligated into vector pTrcHis2-TOPO® (Invitrogen), and transformed into chemically competent TOP10 One-Shot *E. coli* cells (Invitrogen) according to the manual instructions.

Sequencing and alignment

Insertion of the correct sequence into the expression vector was confirmed by sequencing the plasmid. Briefly, after identifying the colony that expressed the protein, PCR was carried out with the specific primers provide by the pTrcHis2-TOPO kit (Invitrogen), following the manual instructions. In sequence, the DNA was purified from agarose gel using a QIAquick Gel Extraction Kit (Qiagen Biotechnology Brazil Ltd.). Sequencing reactions were performed using a fluorescent dye-labeled dideoxynucleotide system (BigDye Terminator V3.1 Cycle Sequencing Kit; Applied Biosystems, Foster City, CA, USA) with the same primers mentioned above in pTrcHis2-TOPO to confirm the maintenance of the *hsp70* ORF, as described by Igarashi et al. (2010), prior to carrying out protein expression on a larger scale. After careful examination of each electropherogram, the consensus sequences were constructed from alignment data using ClustalX (Thompson et al., 1997) and edited by visual inspection using the BioEdit program.

Identification of expression by the recombinant plasmid and protein purification

We initially carried out a protein expression pilot as a preliminary study to identify HSP70-expressing colonies by inoculating a single colony into test tubes containing Luria-Bertani (LB) broth culture medium (3 mL). Once the colonies had been identified, they were cultured in larger volumes of LB medium.

The protein was expressed in *E. coli* strain TOP10 One shot® (Invitrogen). Briefly, cells were grown in LB broth culture with 100 µg/mL ampicillin at 37°C to an optical density at wavelength 600 nm (OD_{600}) of 0.5-0.7, measured on an iMark™ Absorbance Microplate Reader spectrophotometer (Bio-Rad, Hercules, CA, USA). rHSP70 protein expression was induced in LB medium containing 1 mM isopropyl-thio-D-galactoside for 4 h at 37°C, agitated at 200 rpm. The HSP70 protein was purified by Ni-NTA nickel resin affinity chromatography (Qiagen Biotechnology Brazil Ltd.) under denaturing conditions, according to the manual instructions, whereby guanidine was used to lyse and bind the proteins in the resin and 8 M urea was used for washing and elution. The final purified solution was dialyzed in PBS and subjected to 12% polyacrylamide gel electrophoresis. The protein concentration was determined using a Pierce® BCA Protein Assay Kit (Thermo Scientific, San Jose, CA, USA).

Grand average of hydropathy (GRAVY) analysis of rHSP70 protein

The GRAVY index is the sum of the amino acid hydropathy values divided by the number of residues in the sequence, normalized according to the protein length; hydrophobic proteins have positive GRAVY values and hydrophilic proteins have negative values (Kyte and Doolittle, 1982). This index was calculated using free software available at ExPASy server (http://web.expasy.org/cgi-bin/protscale/protscale.pl).

RESULTS

Molecular characterization of the partial sequence of rHSP70

Figure 1 is a hydropathic index plot of the predicted partial amino acid sequence encoded by the *hsp70* gene obtained by the Kyte and Doolittle method. The observed GRAVY value was -0.089. The primers selected to cover that area were able to amplify the 768-bp fragments from the cDNA and plasmid (pTrcHis2/HSP70), as shown in Figure 2. The new cDNA sequence obtained in this study was deposited in GenBank (accession No: KT347312); the sequence had 100% identity with the unique *E. tenella* HSP70 sequence in GenBank (Figure 3).

Expression and purification of rHSP70

The inclusion of a C-terminal polyhistidine-tag on the HSP70 fragment resulted in a total of 289 amino acids with a theoretical molecular mass of 31.34 kDa, and a dominant protein band at approximately 23 kDa was observed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (12% polyacrylamide gel) when the insoluble fraction was analyzed (Figure 4). The expression level of this recombinant protein in *E. coli* One Shot® TOP10 cells (Invitrogen) was approximately 5.0 mg/L.

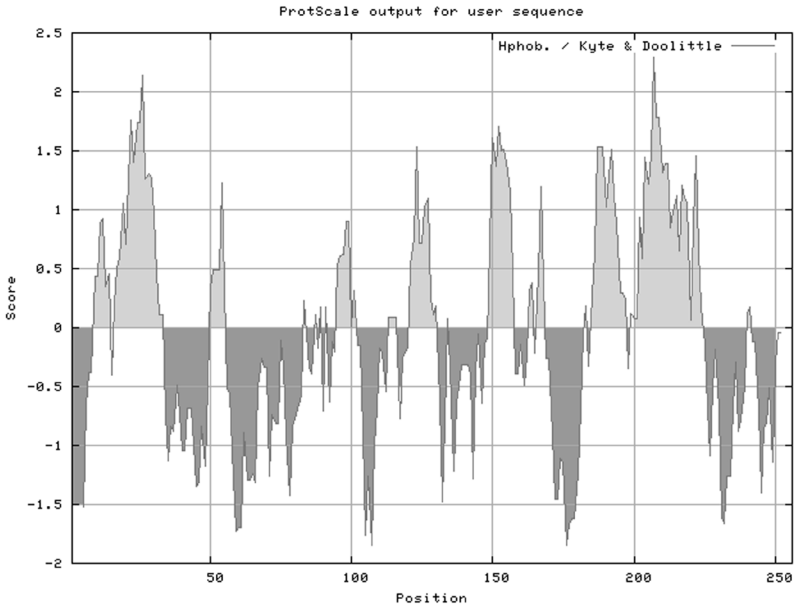

Figure 1. Hydropathic index plot of the partial amino acids sequence of the *hsp70* gene obtained by the Kyte and Doolittle method. The x-axis represents the amino acid position and the y-axis represents the hydropathic score. Hydrophobic amino acids have scores above zero (light gray areas) and hydrophilic amino acids have scores below zero (dark gray areas). The grand average of hydropathy (GRAVY) value of the rHSP70 protein was -0.089.

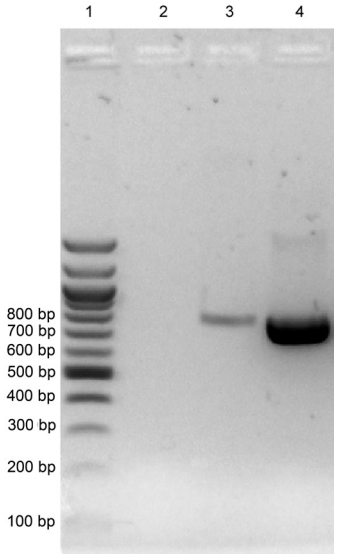

Figure 2. Amplified polymerase chain reaction (PCR) products. *Lane 1*: molecular weight standard 100 pb (NEB, New England); *lane 2*: negative control; *lane 3*: cDNA amplicon; and *lane 4*: plasmid pTrc2His/HSP70 amplicon from the bacterial colony that expressed the rHSP70 protein. After carrying out PCR with the HSP70-F and -R primers, all samples were subjected to electrophoresis on 1.5% agarose gel and stained with SYBR Green®. The presence of the insert was confirmed using *hsp70* primers for cDNA and the pTrc2His/HSP70 plasmid resulting in a 768-pb fragment.

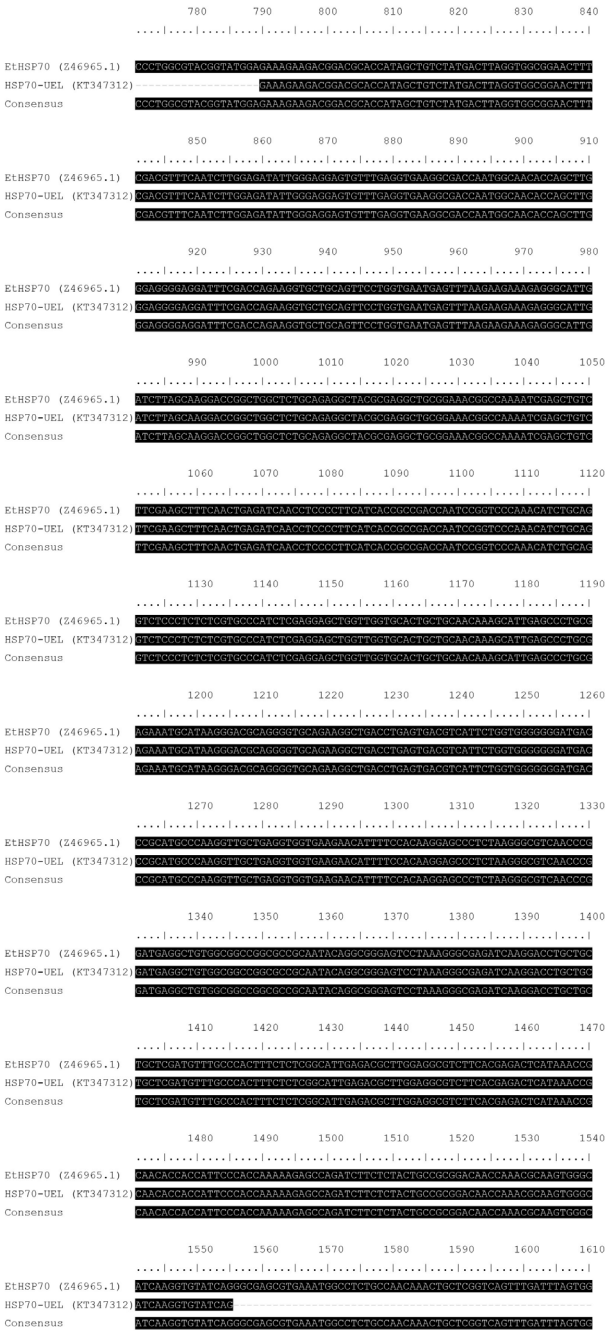

Figure 3. Alignment of the *hsp70* gene. EtHSP70 (Z46965.1): sequence deposited in GenBank (accession number Z46965.1). HSP70-UEL (KT347312): sequencing of the cloned cDNA fragment. Consensus: consensus sequences of EtHSP70 (Z46965.1) and HSP70-UEL (KT347312). The sequence alignment revealed 100% nucleotide identity.

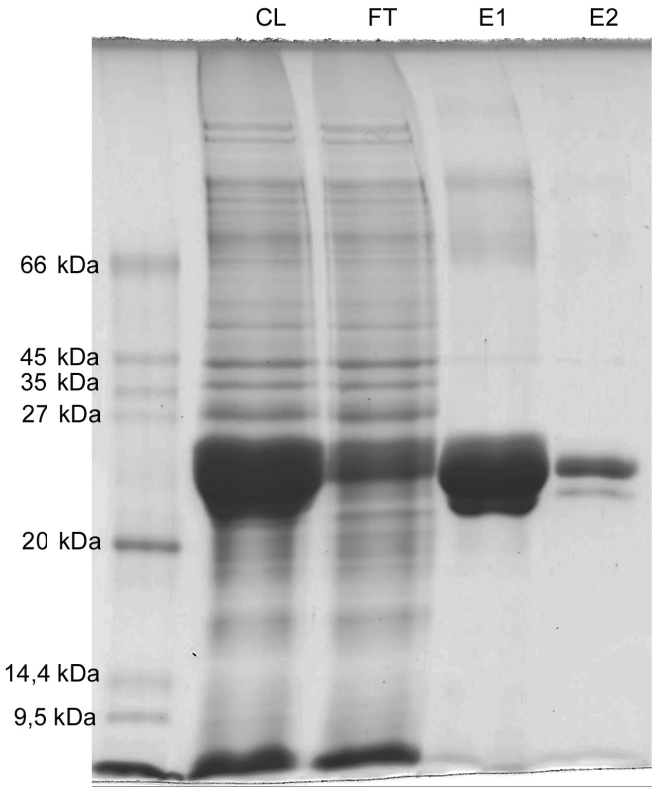

Figure 4. Expression and purification of recombinant protein HSP70. Purified insoluble proteins were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (polyacrylamide gel 12%) and stained with Coomassie Brilliant Blue. *Lane 1*: molecular weight; *lane 2*: crude bacterial lysate; *lane 3*: Ni-NTA column flow-through; *lane 4*: first elution; and *lane 5*: second elution.

DISCUSSION

The rHSP70 obtained in the present work had a negative GRAVY value, indicating a hydrophilic tendency. This condition is important for immunogenicity, and may indicate potential for vaccine development (Mohamed et al., 2003; Zhang et al., 2012) because it facilitates the increased production of IL-12, IL-17, and IFN-γ (Zhang et al., 2012). These cytokines are associated with a strong Th1 response, which is the main immune response against intracellular parasites such as those of the *Eimeria* genus (Dalloul and Lillehoj, 2005). The use of recombinant proteins for assessing the ability to stimulate protective immunity against avian coccidiosis has been studied previously (Dunn et al., 1995; de Venevelles et al., 2004; Ding et al., 2005; Liu et al., 2009; Song et al., 2013), with the aim of producing an alternative to the anticoccidial agents used in poultry feed.

The *hsp70* gene encodes an internal protein (Liu et al., 2009) with a molecular mass of approximately 70 kDa (del Cacho et al., 2001); it is found on the sporozoites of *Eimeria* spp and plays an important role in the formation of sporocysts and sporozoites during sporulation, and the formation of the synaptonemal complex during meiosis (Laurent et al., 1994; Del

Cacho et al., 2008). Furthermore, HSP70 is essential for host cell invasion, working with HSP90 to facilitate the stabilization of transmembrane proteins in cell adhesion (Péroval et al., 2006).

Analysis of the soluble and insoluble fractions revealed that rHSP70 appeared only in the insoluble fraction, as expected, and that most of the contaminant insoluble proteins were removed from the supernatant, representing the flow-through of insoluble proteins not bound to the nickel in the column.

We selected a 489-bp PCR amplicon of the *hsp70* gene in the initial stages of this study. However, a 1092-bp fragment was observed. The difference in size was due to the presence of introns. Therefore, the sequence was deposited in GenBank (KT364872) with the appropriate annotations for introns and exons. Other researchers have evaluated the HSP70 proteins of *Eimeria* spp in terms of sequencing, expression, and antigenic/immunological characterization (Laurent et al., 1994; Bumstead et al., 1995; Dunn et al., 1995; Brown et al., 2000; del Cacho et al., 2001; de Venevelles et al., 2004; del Cacho et al., 2005; Del Cacho et al., 2008; Liu et al., 2009; Zhang et al., 2012). Studies on HSP70 from other species of microorganisms also suggest it has immunogenic potential, as observed by Li et al. (2011) when evaluating the HSP70 of *Mycoplasma ovipneumoniae* in sheep.

In conclusion, we obtained a high yield of insoluble recombinant protein by cloning, expressing, and purifying *E. tenella* rHSP70. However, additional studies are needed to assess and characterize its immunogenicity for vaccine protocols.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

Research supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq; grant #578721/2008-2) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES #AUXPE-1345/11). J.L. Garcia is the recipient of CNPq fellowships.

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