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# Cloning *Dictyostelium* Paracaspase Protein in the *Acanthamoeba* Expression Plasmid

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**Abstract:** *Acanthamoeba castellanii* and the slime mold *Dictyostelium discoideum* are members of Amoebozoa. Both microorganisms were applied as model organisms in different biological studies. *A. castellanii* metacaspase (Acmcp) and *D. discoideum* paracaspase (Ddpcp) proteins have been recently discovered. Determining the function of Acmcp and Ddpcp provides a valuable information about their role in more complex organisms. This study aims to clone the Ddpcp gene in the *Acanthamoeba* expression plasmids. The Ddpcp gene was inserted in a plasmids containing the TBP Promoter Binding Factor (TPBF) gene promoters from *A. castellanii* and enhanced green fluorescent protein (EGFP) as the reporter gene. The promoters for *Acanthamoeba* TPBF gene was used to drive constitutive expression of EGFP protein in stably transfected *Acanthamoeba*. Recombinant TPBF is able to bind DNA and activate transcription as the natural *Acanthamoeba* TPBF. The results showed a sucssesful constract of production of recombinant Ddpcp gene in pTPBf plasmid that will be able to use later to provide a valuable information about the function of Ddpcp in the *Acanthamoeba* parasite.

**Key words:** Paracaspase, *Acanthamoeba*, pTPBf-EGFP plasmid

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## Introduction

*Acanthamoeba* is an opportunistic protozoan parasite that lives in soil. It causes granulomatous amoebic encephalitis and it can also cause amoebic keratitis (1). *A. castellanii* has the ability to survive in various environments (2) including public water supplies, swimming pools, contact lenses, skin lesions, and cerebrospinal fluid (3, 4, 5). It has a two-stage life cycle: trophozoite and an encysted form (6). Trophozoites undergo movement and phagocytosis. Under unfavorable conditions, trophozoite can then become a cyst via the encystation process (7, 8). The cyst has a rigid double-layered wall that protects the parasite against the host's immune response, and many biocides have failed to destroy the very resistant cysts (7, 9). A better understanding of the encystation process and the important proteins that participate in this process would aid in the search for more effective treatments for the diseases caused by this dangerous parasite. *D. discoideum* is a non-pathogenic unicellular eukaryotic microorganism (10). This amoeboid protozoan is a powerful system to study gene function through genetic and functional analysis (11). Recently, *A.*

*castellanii m*etacaspases (Acmcp) and *Dictyostelium discoidium* paracaspases (Ddpcp) have been identified (12, 13). A current study creates a cell line of *Dictyostelium* that express the full length of Acmcp and Ddpcp using GFP-tagged protein. This study determined that Acmcp and Ddpcp were associated with both localization and the functioning of the contractile vacuolar system (14, 15). Finding that Acmcp and Ddpcp share the same location and function within the cell provides new understanding into the possibility that the caspase-like proteins in the protozoan amoeba may share similar functions. Thus, Acmcp and Ddpcp could be a candidate drug target. For that reason, this study aimed to insert Ddpcp in a plasmid that can express in *A. castellanii* as a primary step to investigate Ddpcp function in *A. castellanii.*

## Material and Methods

### Design the primer and Cloning *pcp*

The sequence of *D. discoideum* paracaspase was obtained from the online database http://dictybase.org/gene/DDB_G0293196 (Dictybase.org) and NCBI Gene Bank: AF316600). *Dictyostelium* genomic DNA was isolated using the Qiagen Dnaesy kit. Paracaspase gene was used as a template for PCR to amplify the gene. Based on this sequence, PCR primers were designed with addition of restriction sites *Xho*I to the forward and reverse primers. The primer sequences (**CTCGAG** ATG GCA TAC CCC TAC GGA G) as a forward primer and (**CTCGAG**TTA CAT GAT GAA CTG GGC G) as a reverse primer. PCR was performed with a hot start at 94°C for 2 minutes. This was followed by 30 cycles consisting of a denaturing step of 94°C for 2 minutes, an annealing temperature of 55°C for 1.45 minutes and an extension temperature of 72°C for 1.45 minutes. A final extension at 75°C was performed for 10 minutes. The resulting PCR product was subjected to agarose gel electrophoresis analysis to verify the size of the resulting PCR product (Figure 1) (16).

### Ligation of Ddpcp into the PCR2.1 (TA) plasmid

Ligation of Ddpcp into the PCR2.1(TA) plasmid from the TA Cloning Kit (Invitrogen) was performed by overnight ligation at 4°C using T4-ligase enzyme. The ligation mixture was then transformed into the provided chemically competent One Shot *E. coli* cells (Invitrogen). This step was done to obtain many copies of Ddpcp -PCR2.1 vector. The transformed *E. coli* were spread on plates with LB media (Bacto-tryptone, yeast extract, NaCl) containing 100 mg/ml ampicillin to select for resistant transformed cells. Colonies were selected, cultured, pelleted and subjected to classic DNA miniprep purification using Qiagen Miniprep Kit. To confirm the successful ligation, DNA from the cultures whose band appeared to contain the Ddpcp insert in the plasmid DNA was subjected to conformational restriction digestion with *Eco*RI restriction enzyme. The resulting of restriction digestion product was subjected to agarose gel electrophoresis analysis to verify the size of the gene. Several of successful colonies were regrown in fresh liquid LB media and DNA was isolated using the Qiagen Miniprep Kit for follow-up sequencing in University

of Arkansas for Medical Sciences (UAMS), Arkansas, USA. DNA sequencing was then performed for the final confirmation of successful ligation by alignment using ClustalX 1.83 program.

**Ligation of Ddpcp into the pTPBf-EGFP plasmid**

After obtaining DNA of Ddpcp in large quantities using the Qiagen Miniprep Kit, the *Xho*I containing PCR insert was then ligated in frame with the EGFP gene in the pTPBf-EGFP plasmid (the destination vector that can expresed in *Acanthamoeba*) (17). Restriction digestion of both the pCR2.1 Ddpcp and pTPBf-EGFP plasmids (5682bp) with *Xho*I were then performed separately and the resulting products were subjected to agarose gel electrophoresis analysis. The resulting linearized vector and insert DNA were extracted from the gel using Qiaquick gel extraction kit and ligated at 4°C overnight using T4-ligase enzyme. The ligation mixture was then transformed into One Shot *E. coli* cells (Invitrogen) and grown on agarose plates containing LB media and ampicillin. Resistant colonies were then selected, cultured and subjected to classic miniprep and agarose gel electrophoresis analysis. Bands corresponding to the appropriate size of pTPBf-EGFP plasmid plus Ddpcp insert were selected and subjected to restriction digestion with *Xho*I for conformation. The cells containing the suspected vector insert combination were subjected to DNA isolation using the Qiagen Miniprep Kit follow-up sequencing in University of Arkansas for Medical Sciences (UAMS), Arkansas, USA.

DNA sequencing was then performed for the final confirmation of successful ligation by alignment using ClustalX 1.83 program.

## Results

**Cloning of *pcp***

In order to explore the theory that Ddpcp and Acmcp share the same function in the parasitic protozoan, we need to express Ddpcp gene in *A. castellanii*. The sequence of *D. discoideum* paracaspase was obtained from Dictybase.org and NCBI Gene Bank: AF316600). The Ddpcp DNA PCR product was approximately 1221 base pairs as shown in (Figure 1).

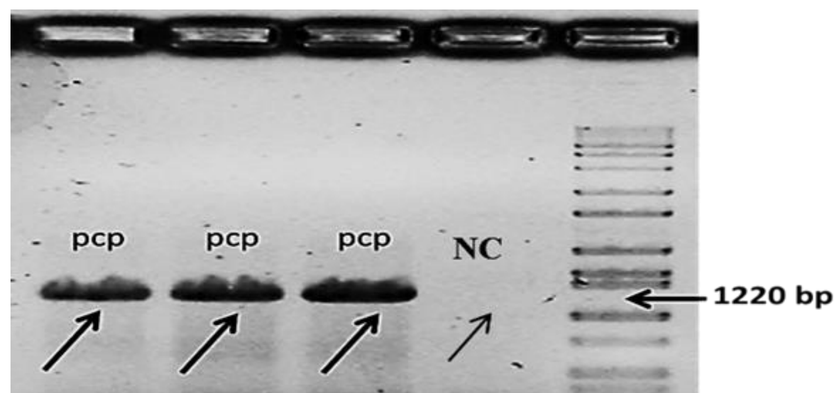


**Figure (1): Conformational agarose gel electrophoresis photographs for PCR visualizing the paracaspase gene (1220 bp) (NC: negative control).**

**Cloning Ddpcp gene into the PCR2.1 plasmid**

Agarose gel electrophoresis showed the separated Ddpcp insert from the PCR2.1 (TA) plasmid (3.9 kb) which confirms the presence of pcp gene in the plasmid (Figure 2).Verification of the success of PCR was performed by the alignment of the newly sequenced DNA with the DNA template used for PCR using ClustalX 1.83 program.

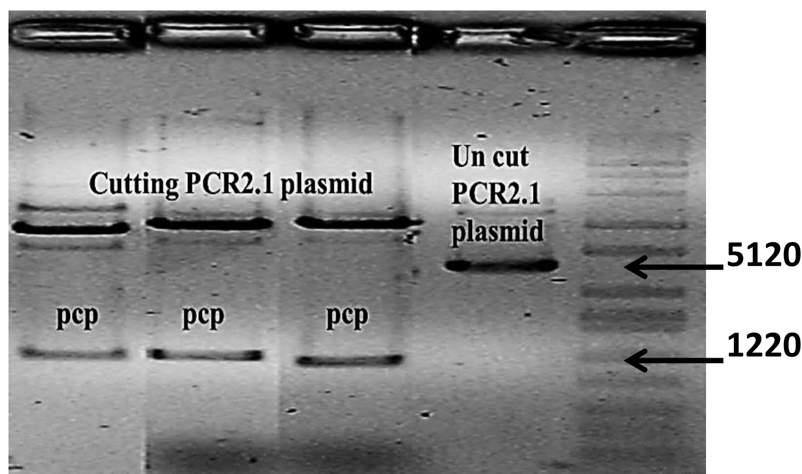


**Figure (2): Conformational agarose gel electrophoresis photographs of *EcoRI* restriction digestion of pcp gene containing PCR2.1 plasmid (5120bp). Pcp was digest with *EcoRI* restriction enzyme to cut out pcp gene to confirm the presence of pcp gene on PCR2.1 plasmid.**

Agarose gel electrophoresis separated the Ddpcp insert from the PCR2.1 plasmid using *XhoI* restriction digestion enzyme (Figure 3). Plasmid pTPBf-EGFP was linearized using *XhoI* restriction digestions.

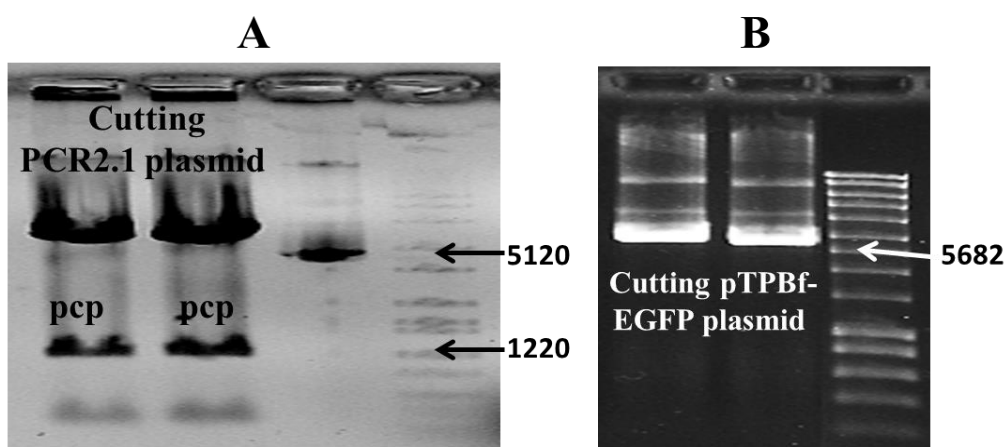


**Figure (3): Agarose gel electrophoresis photographs. A. PCR2.1+Ddpcp (5120bp) plasmid was digested with *Xho*I restriction digestion of pcp to cut out pcp gene for the next step (ligation pcp in the destination plasmid). B) pTPBf-EGFP plasmid (5680bp) was linearized using *Xho*I restriction digestions.**

**Ddpcp gene into the destination pTPBf-EGFP plasmid**

Pcp was then ligated in frame with the EGFP gene in the pTPBf-EGFP plasmid. Bands corresponding to the appropriate size of pTPBf-EGFP plasmid (5680bp) plus Ddpcp insert (1220bp) are shown in (Figure 4A). The resulting cutting pTPBf-EGFP plasmid and pcp gene with *Xho*I containing PCR insert are shown in (Figure4B).

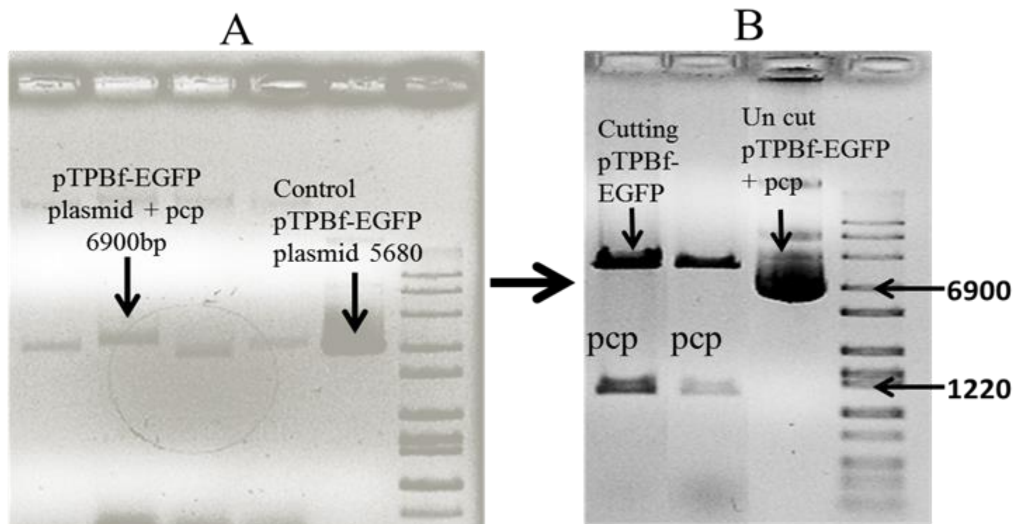


**Figure (4): Agarose gel electrophoresis photograph. A. Plasmid pTPBf-EGFP with *Xho*I pcp gene containing insert (6900bp). B) pTPBf-EGFP plasmid linerized using *Xho*I restriction digestions to confirm the presence of pcp gene in the plasmid and the control which represent un cut pTPBf-EGFP with *Xho*I pcp gene containing insert (6900bp).**

**DNA sequencing**

DNA sequencing was performed for the final confirmation of successful ligation of pcp gene with *Xho*I containing PCR insert in pTPBf-EGFP plasmid using an automatic sequencer. DNA sequencing was analyzed and similarity searches were approved out with the Basic Local Alighment Search Tool (BLAST) in National Center of Biotechnology Information (NCBI) website. The nucleotide sequence of the paracaspase in the present study has been deposited in the gene bank sequence data base in the flowing accession number (NCBI Gene Bank: AF316600). Additional sequence analysis of the paracaspase was documented in present study through alignment system using ClustalX 1.83 program and no deletion and insertion were seen in this protein (Figure 5).

**Figure (5): DNA sequencing photograph of pTPBf-EGFP plasmid with pcp gene with *Xho*I containing PCR insert using ClustalX 1.83 showing a perfect match.**

Then, Ddpcp sequence analysis was done again for reading the open frame using Open Reading Frame Finder (ORF Finder) http://www.ncbi.nlm.nih.gov/gorf/gorf.html) to make sure that there is not any stop codon inside the expressed protein (Figure 6).

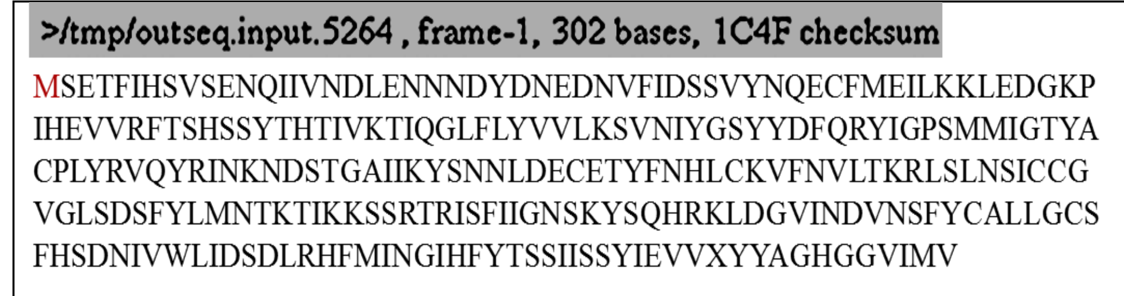


**Figure (6): DNA sequencing photograph of pcp protein using in frame program showing that there is no stop codon within this protein. Sequence of paracaspase in the destination vector using fram-1 program.**

**Discussion**

*A. castellanii* is grown in a low cost media (18, 19). Several systems are suitable for the creation of recombinant proteins including *D. deiscoideum, E. coli*, *Bacillus* sp., *Saccharomyces cerevisiae*, and mammalian tissue culture cells (20-27). Regarding *Leishmania* parasite, it was reported that, when *L. major* metacaspase (LmjMCA) was over-expressed in *S. cerevisiae* yca1 null mutants. The results proposed that the LmjMCA protein is involved in the stress response (28). In other study, *P. falciparum* metacaspase (PfMCA1) over-expressing in yeast induced cellular growth inhibition [29]. *A. castellanii* and the slime mold *D. discoideum* are closely related amoebozoa (30). Both microorganisms have been practical as model organisms in different biological studies. The two model organisms *Aanthamoeba* and *Dictyostelium*, each having a non-traditional caspase, are being used to gather insight into their novel roles, especially important would be results indicating to proteins or processes that could be targeted for treatment of parasitic infections. A recent study in *Dictyostelium* explores the localization and the molecular role of the *A. castellanii* metacaspase (Acmcp). The study determined the intracellular location of metacaspase protein via the creation a cell lines of *Dictyostelium* that express the full length of Acmcp using GFP-tagged protein. The original observations concerning Acmcp and Ddpcp proposed that they were associated and function with the contractile vacuolar system. The indication suggests a role in the plasma membrane fusion of CVs, which block water release. Moreover, a primary result has revealed that both proteins (Acmcp and Ddpcp) cooperate with other proteins that are associated with the contractile vacuole in *Dictyostelium* [14, 15]. Recently, plasmids for the stable transfection of *A. castellanii* have been described (31). In order to determine the function of Ddpcp in *A. castellanii*, we cloned *D. discoideum* paracaspase gene in the *Acanthamoeba* expression plasmids (pTPBf-EGFP plasmid). This cloned plasmid is suitable to be expressed in *Acanthamoeba* which then allow us to investigate the possibility that pcp protein have the same function in *Acanthamoeba*. In addition, finding that Acmcp and Ddpcp share the same location and function within the cell gives insight into the possibility that the caspase-like proteins in protozoan amoeba might share similar functions and could be potential targets for treatment therapy against the harmful amoebas.

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