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Identification of an Antimicrobial Protease from *Acanthamoeba* via a Novel Zymogram

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Abstract: Proteases play a role in different processes for protozoans and for the free-living amoeba *Acanthamoeba*. Some of these processes are related to pathogenicity and to encystment. In this study we describe the discovery of a protease with antimicrobial activity produced by *Acanthamoeba*. To identify it, we developed a novel zymogram using bacteria as an in-gel substrate that can help identify proteins capable of bacterial degradation. We used chromatography to isolate the proteases and showed that it quickly degrades in the environment. Additionally, we identified overexpressed proteases during encystment. The study of proteases from *Acanthamoeba* can serve several purposes including new antimicrobial proteins that the amoeba can use for potentially predigesting prokaryotes. Secondly, it can help with the identification of potential new therapies against *Acanthamoeba* infection.

Keywords: *Acanthamoeba*; protease; encystment; zymogram; antimicrobial

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1. Introduction

Protozoans, such as the free-living amoeba *Acanthamoeba*, secrete various bioactive proteins that help modulate interactions between the amoeba and other microorganisms [1]. *Acanthamoeba* produces various antimicrobials, including hydrolytic enzymes such as proteases, which are involved in substrate degradation for nutrition [2,3]. Proteases are highly researched proteins in *Acanthamoeba* since they play a crucial role in pathogenicity. Proteases of varying molecular weights have been identified in this amoeba [4–11], and their activity profile has been suggested as a method to distinguish between potentially pathogenic and non-pathogenic strains of *Acanthamoeba* [6]. Proteases have been shown to increase cell permeability, degrade the human extracellular matrix, disrupt the cell monolayers, mediate tissue invasion, and even induce cytotoxic effects [3,12–15]. Additionally, proteases have been linked to the typically biphasic life cycle of *Acanthamoeba* as they play an important role during encystment [16–18].

In this study, we identified a novel *Acanthamoeba* protease of approximately 33 kDa, named AcPro33, that possesses antimicrobial properties. To facilitate the identification of proteins with antimicrobial properties against specific bacteria, we developed a zymogram that provides a simple and quick screening method. In addition, we discovered two cysteine proteases involved in the encystment process, which is critical to the persistence of *Acanthamoeba* in human infections. Understanding the molecular processes, by which *Acanthamoeba* feeds and the enzymes it secretes with this purpose, can have therapeutic implications in combatting infection.

2. Materials and Methods

2.1. Strains and Cultures

Acanthamoeba strain 53 genotype T4s were used for the experiments (de Obeso, 2018). Also, *Acanthamoeba castellanii* Neff strains (ATCC 30010), a T2 genotype strain labeled 61, a T4-labeled 64, and a T5-labeled BC were tested for proteolytic activity. All *Acanthamoeba* cultures were grown in AX2 media [19]. All *Acanthamoeba* cultures were grown at room temperature until confluency.

2.2. Electrophoresis SDS Page and Protease Zymography

An SDS-PAGE was performed in 10% acrylamide gel. Protease zymograms were performed using similar conditions to the SDS-PAGE gels, with the addition of fish gelatin at 0.1% to the resolving gel. After electrophoretic separation, the gels were incubated with a solution consisting of 2.5% Triton X-100; 50 mM of Tris-HCl pH 7.0 for 1 h, and then overnight in 50 mM of Tris-HCl pH 7.0, and finally in 2 mM of $CaCl_2$ at room temperature with continuous shaking. The gels were rinsed and stained with a Coomassie stain for one hour. The gels were deistained using a SDS-PAGE destaining solution.

2.3. Anitmicrobial Zymogram

An antimicrobial zymogram was developed and tested on *Escherichia coli* and *Arcobacter butzleri.* Bacterial cultures were grown overnight. The cultures were centrifuged, and the supernatant was discarded while the remaining bacteria were resuspended in 1 mL of H_2O. A gel similar to the SDS-PAGE was used by substituting 1 mL of water with the prepared bacterial solution which served as the substrate. Electrophoretic separation was carried out with 200 V at 4 °C. The gels were incubated with a solution consisting of 2.5% Triton X-100; 50 mM of Tris-HCl pH 7.0 for 1 h, and then overnight using a new solution consisting of 50 mM of Tris-HCl pH 7.0. The 2 mM $CaCl_2$Gels were rinsed and stained with a Coomassie stain and destained using a destaining solution. All incubations were performed at room temperature with continuous shaking. Acridine orange (20 mL at 0.02% of acridine orange, staining for 20 min and destained with methylene blue (20 mL with 0.02 g of MB + 0.002 g of NaOH+ 0.002 g of KCl, incubated for 1.5 h and destained with H_2O) and Alcian blue (18 mL of H_2O + 2 mL of 95% ethanol + 0.06 g of AB, incubated for 3 h and destained with H_2O) were also tested before identifying Coomassie as the better staining method.

2.4. Protease and Antimicrobial Zymogram Testing Conditions

Different conditions were tested to observe and characterize the enzymatic activity. The different incubation temperatures, EDTA, and PMSF were used to test for inhibition and characterization of the protease. PMSF is an inhibitor of serine proteases, while EDTA, while not a proper inhibitor, is a chelator that affects enzyme activity. Both the supernatant and cell phases were tested for different conditions.

2.5. UPLC System and Chromatography

Chromatography was performed using an Acquity® UPLC class HXBridge Peptide BEH C18 1.7 m column (150 mm × 2.1 mm). Chromatography was carried out at a flow rate of 0.4 mL/min. The samples were loaded directly on the analytical column. The mobile phase was filtered via a degassed 0.22 µm nylon membrane. The injection volume consisted of 10 µL with an analytical wavelength at 280 nm. The wavelength was selected using a scan mode in a photodiode array detector ranging from 200 nm to 400 nm. The data were acquired for 18 min and processed by using an Empower Pro data handling system.

The mobile phase was prepared via 0.1% phosphoric acid in acetonitrile and methanol (85:15). Here, phosphoric acid was used to increase the sharpness of the peaks and for good resolution; the 85:15 ratio of the mobile phase was used to minimize the time of elution of the two peaks with a good resolution.

The column temperature was set at 25 °C. The sample temperature was maintained at 4 °C to compensate for protease instability at room temperature.

2.6. Protein Purification

All purification steps were performed at 4 °C on an AKTA Purifier 10 system (GE Healthcare Biosciences, Uppsala, Sweden). The supernatant from the cultures grown for 48 h was used as a crude source of enzyme. The crude enzyme solution of *Acanthamoeba* was separated via anion exchange chromatography eluted with a stepwise NaCl gradient. The *Acanthamoeba* culture was diluted using 50 mL of a 50 mM sodium phosphate buffer, pH 7.0, and filtered with a 0.45-µm pore size. The enzyme solution was loaded onto a HiLoad 26/10 Q Sepharose High-Performance column (GE Healthcare Biosciences) and then equilibrated with 50 mM of a sodium phosphate buffer, pH 7.0. The fractions were eluted at a flow rate of 1 mL/min using an equilibration buffer with a NaCl stepwise gradient of 0–0.17 M by using a volume of 20 mL and 0.30–1.0 M for 20 mL. The fractions were detected via UV absorbance at 280 nm for collection. *Acanthamoeba* protease activity in the collected fractions was observed with the zymograms.

2.7. RNA Sequencing

Finally, RNAseq data [20–22] previously obtained to study encystment was used to analyze protease expression throughout the amoeba life cycle to look for differentially expressed proteases. Automated TruSeq-stranded mRNA-seq from total RNA were used to prepare the libraries. Sequencing experiments were performed using HiSeq-4000 75PE. Sequencing experiments were carried out by Edinburgh Genomics. The reference genome (FASTA and GTF files) from *A. castellanii* was obtained from ENSEMBL Protists [23]. STAR software (version 2.6.0) was used to index the genome and align the reads [24]. The differential expression analysis was performed using R studio and edgeR [25]. FeatureCounts software (version 1.6.0) generated counts per gene using reverse stranded reads [26]. A logFC (logarithmic fold change) above 1 or below −1 was the threshold for differential expression.

3. Results

3.1. Antimicrobial Zymogram

A zymogram to test for antimicrobial activity was developed. Different staining methods were tested. Acridine orange (incubation for 20 min with 20 mL of H_2O, 0.02% of acridine orange and destaining with H_2O). did not show any visible bands. Alcian blue (incubation for 3 h in 18 mL of H_2O, 2 mL of 95% ethanol, and 0.06 g Alcian blue while destaining with H_2O) and methylene blue (90 min with 20 mL of H_2O with 0.02 g of methylene blue, 0.002 g NaOH, and 0.002 g KCl while destaining with H_2O) did not destain properly. Coomassie blue showed proper destaining and contrast to observe the band of enzymatic activity [20]. The zymogram showed similar results using *E. coli* and *A. butzleri*, which are both Gram-negative bacteria (Figure 1A).

This assay helped identify the protease that is thought to be the Peptidase_S8 domain-containing protein (ACA1_222700) as it is the correct size, type of protease, and it has been linked to pathogenicity previously [7]. We show in Figure 1A that the protease is present in the cells and the supernatant. Additionally, we verified that the protease was the same protein with antimicrobial activity performing an antimicrobial zymogram and using the gel as the sample to run a protease zymogram (Figure 1B). We then tested different strains to check if the same proteolytic pattern showed in the gel (Figure 1C). Finally, samples were incubated with protease inhibitors and EDTA as a chelator. We identified the protease as a serine protease, as it could be completely inhibited with PMSF in the cells and the supernatant fractions from zymography. EDTA, that is regularly used as a metalloprotease inhibitor, showed a slight inhibition. The results for the protease inhibitors are shown in Figure 1D.

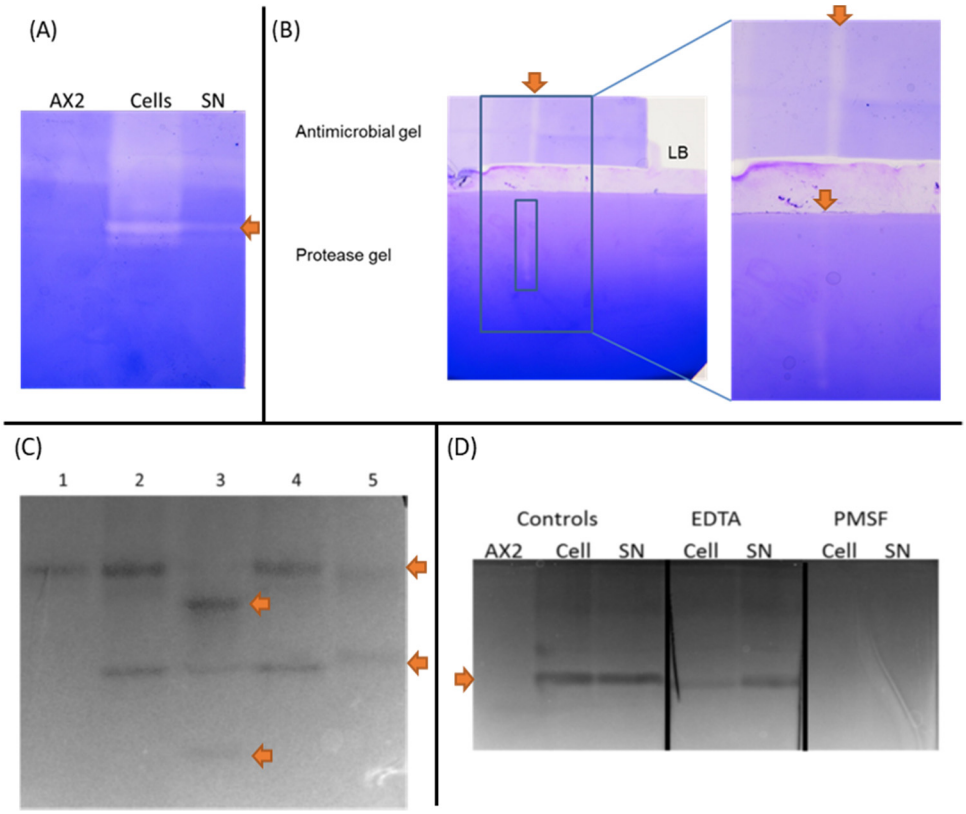

Figure 1. Antimicrobial and protease zymograms from *Acanthamoeba* cultures using a Coomassie blue stain. Small orange arrows are used to point at the bands shown. (**A**) Antimicrobial zymogram using *A. butzleri* as the substrate. AX2 media was used as a negative control. Two samples, one including cells and another one with the supernatant (SN) for the culture were loaded into the gel. (**B**) Protease zymogram using an antimicrobial zymogram as the loaded sample. With this method, we proved that the antimicrobial protein also has proteolytic capabilities. On the right lane, we used LB media as a control. The antimicrobial gel was used using *E. coli* as the substrate. (**C**) Protease zymogram from five different strains: 1. Neff strain, 2. Strain 65 genotype T4, 3. Strain 61 genotype T2, 4. Strain 64 genotype T4, and 5. Strain BC genotype T5, where different proteolytic patterns are shown. (**D**) Protease inhibition zymograms using EDTA and PMSF. Slight inhibition was observed in the cell sample of EDTA, but complete inhibition was observed when using PMSF. AX2 media was used as the control. As in (**A**), the cells and the supernatant were loaded into the wells. (**C**,**D**) The images have been processed to greyscale, with the colors inverted and the contrast altered to improve the resolution.

3.2. Chromatography Acanthamoeba Protease

Chromatographic analysis of AcPro33 was initiated under isocratic conditions to obtain an adequate response, a sharp peak shape, and a short run time. The results of the chromatographic profile are shown in Figure 2. The chromatogram shows two peaks at about minute one, of which these peaks are related with the AcPro33. Follow-up injections showed the second peak decreasing or disappearing over time via comparisons of different subsequent injections (Figure 2).

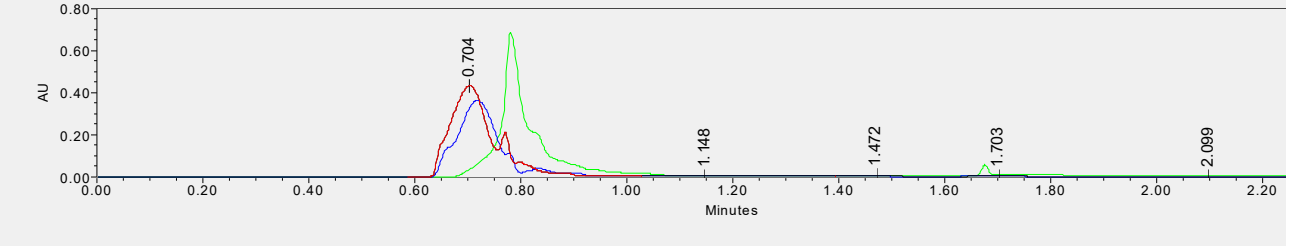

Figure 2. Chromatogram of AcPro33 in the XBridge Peptide BEH C18 column. Three different injections and runs of the chromatogram with the same sample, one after the other. The samples show changes in the patterns with one of the peaks being lost. The red line represents the first sample that shows two clear peaks, while the blue line is the second sample with only one peak. The green one represents the third injection showing a displacement of the samples, showing possible degradation.

3.3. Purification of Acanthamoeba Protease

The crude extract separated presented similar activity to the protease characterized in the present work. The elution happened at 0.17–0.20 M of the NaCl-contained active protein. The specific activity of the pooled active fractions represented a 90.6% yield of the original initial mix. The proteases purified from *Acanthamoeba* showed high specific activity against *E. coli*. Enzyme activity was confirmed via the zymogram.

3.4. RNA-Seq Data

The gene identified to code for AcPro33 (ACA1_222700) was analyzed using RNAseq data during the trophozoite and encysting stages. The data showed the third highest LogCPM (Logarithmic counts per million) during the 0 (trophozoite stage) versus 24 h of encystment comparison of the 13,271 genes tested. The LogCPM was 12.185 while the average for all other genes was 4.651. However, despite the high expression rates of the gene in both the trophozoites and encysting cells, no differential expression of the protease was observed.

In relation to protease activity, the RNAseq data was screened for related genes during encystment. Two new cysteine proteases (with geneIDs of ACA1_115390 and ACA1_138380) were observed to be overexpressed at 24 h after induction of the encystment alongside the encystation mediating proteinase (ACA1_321400) used as a control. The differential expression analysis showed a LogFC for ACA1_11530 of 3.915 and for ACA1_138380 of 6.259.

4. Discussion

Prokaryotic proteases, such as AcPro33, have been researched for their antimicrobial capabilities, with lysostaphin from *Staphylococcus simulans* being a commonly studied example [27]. However, the use of prokaryotic proteases for combating other prokaryotes is limited due to the possibility of horizontal gene transfer leading to community-wide antimicrobial resistance [28]. Therefore, bacteriolytic eukaryotic proteases offer a more promising option. Such eukaryotic proteases are not widespread, but there are some examples such as an aspartic protease produced by potatoes, a serine protease from horseshoe crabs, another serine protease from jackfruit, and proteinase 3 from neutrophils [29–32]. Therefore, proteases from *Acanthamoeba* and other free-living amoebae could be great options to combat infections and antimicrobial resistance.

A larger understanding of amoebic proteases will not only help combat bacteria but can help with amoebic infections as proteases play a role in pathogenicity and are involved in the encystment process. Protease inhibitors have been studied for their potential in controlling microorganisms, including *Acanthamoeba* [33]. For example, maslinic acid, a protease inhibitor that can trigger programmed cell death, can successfully inhibit *Acanthamoeba* destruction of the corneal epithelial cells' death [34]. Also, human protease inhibitors are involved in extracellular matrix synthesis, inflammation, and tissue repair, reaffirming the possibility of using such compounds to combat microorganisms as they

naturally play a role in human physiology [35]. Some protease inhibitors have been tested against other amoebae such as *Entamoeba hystolitica* and *E. invadens* [36,37].

Newly identified proteases involved in the encystment process of *Acanthamoeba* could offer new alternatives for treatment and decrease infections [38–40]. Cysteine proteases have been reported to play an important role during encystment and have been linked to mitochondrial degradation [41–43]. In this paper, we have also identified two proteases that are overexpressed during the encystment process (ACA1_115390 and ACA1_138380), increasing our understanding of the process.

Finally, the novel antimicrobial zymogram that has been described has helped identify the *Acanthamoeba* protease capable of degrading at least two distinct Gram-negative bacteria, offering the opportunity to establish treatments for the disease caused by either the organisms producing the enzyme, or the organisms affected by it. Gram-negative bacteria were selected for different reasons including the ability of *Acanthamoeba* to feed from them [44]. *E. coli* was selected as a target due to the importance as an opportunistic pathogen, its availability in most laboratory settings, and its prevalence as an excellent food source for *Acanthamoeba* [45]. *A. butzleri* was selected as an endosymbiont capable of surviving after being phagocytized by the amoeba [46,47]. Accessible and easy assays to identify new antimicrobials could help the identification of potential molecules we have to combat infections. This is especially important if we can use common techniques such as electrophoresis and easy to grow bacterial organisms such as *E. coli* so that the essay can be replicated in almost every setting. It would be important to test different *E. coli* strains (we used DH5α) since some can survive inside *Acanthamoeba* as endosymbionts, such as the K11 and 0157 strains [44,48].

Future studies should confirm the identity of AcPro33 as the protease reported in the literature and focus on understanding the pathogenic factors to develop new treatment alternatives [7,33,49]. Bacterial growth assays could be added to the characterization of the protein. Unfortunately, the fast degradation of the protease made these studies challenging and did not offer reliable results. It has been reported that a 33 kDa serine protease plays an important role in the pathogenicity and invasion of corneal tissue. This protease, or a similar one, was identified in *A. castellanii*, *A. healyi*, and *A. ludgunensis* [7,8]. AcPro33 can help combat different bacteria, at least some Gram-positive organisms. However, if AcPro33 were to be used as an antimicrobial agent, protein engineering would be recommended to increase stability [50,51].

Since AcPro33 is secreted in axenic cultures and its expression does not appear to change through the life cycle of *Acanthamoeba* (as observed from RNAseq data), we hypothesize that the secretion of AcPro33 is a method used by *Acanthamoeba* to preemptively combat bacteria and to pre-digest some of its food sources with or without the presence of other microorganisms. Additionally, secreted proteins can be a first line of defense against bacterial colonization since some bacteria are able to survive inside *Acanthamoeba* [52]. For example, *Legionella pneumophilla* can survive inside *Acanthamoeba* at around 20 °C, but when the temperature increases to 37 °C, as happens in the human body, it lyses the amoeba and is capable of human infection [53]. Several other bacteria are capable of becoming intracellular hosts of *Acanthamoeba*, such as *Bacillus anthracis*, *Chlamydia pneumoniae*, *Helicobacter pylori*, *Vibrio cholerae*, and *Salmonella typhimurium* [54–58]. Secreted proteins, such as AcPro33, that can have bacteriolytic effects provide *Acanthamoeba* with an evolutionary mechanism to try to avoid bacterial colonization. Understanding this process at a deeper level could help elucidate some of the unknown aspects of *Acanthamoeba*'s feeding habits, the relation with intracellular bacteria, and help prevent human infections.

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Data Availability Statement: The data presented in this study are available throughout the article and more in depth can be found in de Obeso Fernández del Valle [20].

Conflicts of Interest: The authors declare no conflict of interest.

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