CHIKUNGUNYA VIRUS PROTEASE EXPRESSION IN ESCHERICHIA COLI ROSETTA STRAIN

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SUMMARY

Viral proteases play a crucial role during the replication cycle of various viruses, making them key targets for antiviral drug design. In this work, we describe the development and optimization of an in vitro assay to measure the enzymatic activity of the chikungunya virus protease. For this assay, the recombinant viral protease and a synthetic substrate that mimics one of the natural cleavage sites were required. Expression of both proteins was performed using the Rosetta strain of Escherichia coli. coli , which is optimized to improve the expression of proteins containing rare codons. Purification was carried out by affinity chromatography and allowed obtaining both proteins in pure form and with high yield.

Validation experiments showed that the enzyme activity assay is robust, quantitative and allows us to follow enzyme activity over time.

Our results highlight the potential of this method as a tool for the characterization of new antiviral compounds against the chikungunya virus, and may be useful in the development of assays to study other viral proteases.

KEYWORDS: *Chikungunya virus Viral protease Activity test Rosetta strain*

INTRODUCTION

Chikungunya virus (CHIKV) is a pathogen transmitted to humans by the bite of mosquito vectors of the genus *Aedes* , mainly *Aedes aegypti* and *Aedes albopictus* (1) (1). Infected individuals present various symptoms including muscle weakness, fever, myalgia, rash, and joint pain (2, 3). Although it rarely causes death, joint pain may last for months or years, or become chronic, causing disability for some people.

Although this virus was identified in the 1950s, it is considered re-emerging due to the presence of cases in regions where they had not been previously reported (4-7). During the first decades of the 21st century, CHIKV cases were reported on all continents, except Antarctica. According to the Pan American Health Organization, in the period 2023-2024, 810,684 cases were reported in America (8) .

The increasing prevalence of the disease, recurrent and unpredictable epidemic outbreaks, and the lack of pre-existing immunity make CHIKV a significant threat to global health. However, specific treatments for the disease are still lacking, so symptoms are mainly treated with analgesics and anti-inflammatory drugs (2) .

CHIKV has a compact RNA genome encoding essential proteins for the virus, including those that are part of the viral replication complex (nsP1, nsP2, nsP3 and nsP4) (9) .

These proteins are known to be synthesized as an immature polyprotein that is cleaved by the viral cysteine protease (nsP2), which sequentially releases the individual viral proteins (10) . Being an exclusively viral enzyme and essential for replication, viral proteases are the target of choice for the design of specific antiviral agents.

The aim of our work is to develop a simple method to measure the enzymatic activity of the CHIKV nsP2 protease. This requires expressing and purifying both the enzyme and its substrate.

The Rosetta strain is a modified variant of *E. coli* used for the expression of heterologous proteins.

It is designed to improve the expression of proteins containing rare codons, as it has genes encoding specific tRNAs .

This helps to optimize translation and obtain a higher amount of the desired protein compared to other *E. coli* strains .

In this work, we used the Rosetta strain to express the nsP2 protease and a designer substrate containing one of the natural cleavage sites.

Using affinity chromatography techniques, we were able to obtain both proteins in pure form and with high yield.

Using these proteins, we developed a simple and inexpensive assay to measure enzymatic activity, based on the resolution of reaction products in denaturing polyacrylamide gels.

In vitro assays of enzymatic activity are widely used to evaluate antiviral compounds, so our development could provide a valuable tool to identify new molecules with therapeutic potential, contributing to the treatment of viral infections.

MATERIALS AND METHODS

Cloning, expression and purification of the nsP2 protease and a designer substrate containing the cleavage site

To obtain the CHIKV nsP2 protease (Figure 1), we obtained the cDNA encoding the enzyme by PCR on the infectious clone of CHIKV, using the *primers* GGCAGCCATATGACCTTTGATACGTTCC (sense) and GTGGTGCTCGAGTTATCCTACAAAGGCTG (antisense). We cloned this cDNA into the expression vector pET28c (*Novagen*). We transformed competent bacteria of the *E. coli strain Rosetta with the vector and grew cultures in LB medium supplemented with 50 µg ml* $^{-1}$kanamycin and 35 µg ml^{-1}chloramphenicol . We induced protein expression by adding 0.4 mM isopropyl -β-D- thiogalactopyranoside (IPTG) , and after expression we harvested the bacteria by centrifugation.

For purification of nsP2, we lysed the bacteria by sonication pulses at 4 ºC in 50 mM Tris-HCl buffer pH 7.5, 500 mM NaCl , 5% (v/v) glycerol, 0.5 mM dithiothreitol (DTT) , 20 mM imidazole , 1% (v/v) Tween-20 and 50 µg m $^{-1}$ lysozime. We then centrifuged the cell lysates to separate the soluble and insoluble fractions.

Since the proteins expressed using the pET28c vector are produced bound to a histidine tract, we seeded the supernatant on a nickel ion affinity column (HisTrap , GE). After exhaustive washings, we eluted the enzyme with 200 mM imidazole. After dialyzing the enzyme to remove the imidazole, we stored it in liquid nitrogen.

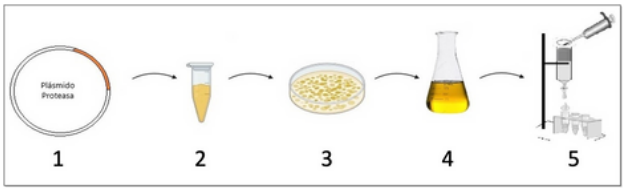

FIGURE 1. SCHEMATIC OF NSP2 CLONING, EXPRESSION AND PURIFICATION. WE CLONED THE HISTIDINE-LINKED PROTEASE CODING SEQUENCE INTO AN EXPRESSION PLASMID (1). WE INTRODUCED THE PLASMID INTO *E. COLI* BACTERIA OF THE ROSETTA STRAIN (2) AND CULTURED IT ON LB-AGAR TO ISOLATE COLONIES (3). WE GREW BACTERIA FROM ONE COLONY IN LIQUID CULTURE AND INDUCED PROTEASE EXPRESSION BY ADDING LACTOSE (4). WE LYSED THE BACTERIA AND PURIFIED NSP2 BY NICKEL ION AFFINITY CHROMATOGRAPHY (5).

In the same way as for nsP2, we transformed the construct containing the substrate sequence in the expression vector pET28c into competent bacteria of the Rosetta strain. E. coli . Then,we induced theexpression and purification of the protein. In this case, the elution of the substrate from the column was performed with 100 mM imidazole.

Cloning, expression and purification of the mutant nsP2

To generate an inactive mutant of nsP2 we replaced the active site cysteine with alanine (C478A).

To do this, we performed a restriction enzyme-free cloning that involved (i) a first PCR to amplify the sequence with the designed mutation using the *primers* 5'AACGTTGCTTGGGCTAAG3' (sense) and 5'GTGGTGCTCGAGTTATCCTACAAAGGCT3' (antisense), (ii) a second PCR using the product of the first PCR as *megaprimer* and the plasmid pET28c containing the sequence encoding nsP2 as template, and (iii) digestion with DpnI and transformation of the second PCR product.

The plasmids were sent for sequencing (*Macrogen* , Korea) and once the success of the cloning was confirmed, we proceeded to express the mutant nsP2 in E. coli Rosetta and purify it by chromatography, following the protocol previously described for wild-type nsP2.

Protease activity measurements

Protease activity assays consist of incubating the purified nsP2 enzyme with its substrate under optimal reaction conditions. In our assays, the composition of the reaction medium was 20 mM Hepes pH 7.5, 6 mM NaCl , and 1 mM DTT .

Recombinant nsP2 (0.5 µM) and its substrate (5 µM) were incubated at 25 ºC for different times, up to 30 minutes. In each case, we stopped the reaction by adding seeding

buffer, containing 50 mM Tris-HCl pH 6.8, 2% (w/v) sodium dodecyl sulfate (SDS), 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol , and 0.05% (w/v) bromophenol blue. Then, we resolved the reaction products by denaturing polyacrylamide gels (SDS-PAGE) and by densitometry (*Image J*), we estimated the concentration of the substrate and the products at each reaction time.

RESULTS

Substrate design

For the enzymatic activity assays, we used a substrate containing a 15 amino acid segment that displays the natural recognition site of the nsP2 protease located at the junction between nsP3 and nsP4 (DELRLDRAGG|YIFSS).

Since it is not possible to resolve the products of hydrolysis of a small peptide by SDS-PAGE, the substrate has two protein domains flanking the cleavage site. These domains are the green fluorescent protein (GFP) at the N-terminus and the thioredoxin (TRX) at the C-terminus (Figure 2A) (11 This substrate has an approximate molecular weight of 40 kDa and when cleaved by the protease, it generates products of 27 kDa (GFP) and 12 kDa (TRX) that can be resolved by SDS-PAGE.

An important issue to consider in the design of the substrate is the accessibility of the cleavage site, since the GFP and TRX domains could cause it to be hidden.

To study the accessibility of the cleavage site, we performed a prediction of its three-dimensional structure using the *Alphafold algorithm.*(12) .

As seen in Figure 2B, GFP and TRX fold into independent domains (in green and blue, respectively), while the sequence containing the cleavage site is located in a flexible and fully exposed *loop* (in pink).

This result suggests that GFP and TRX do not affect the recognition of the cleavage site of the substrate by nsP2, so that its conformation would be suitable for use in the enzymatic activity assay.

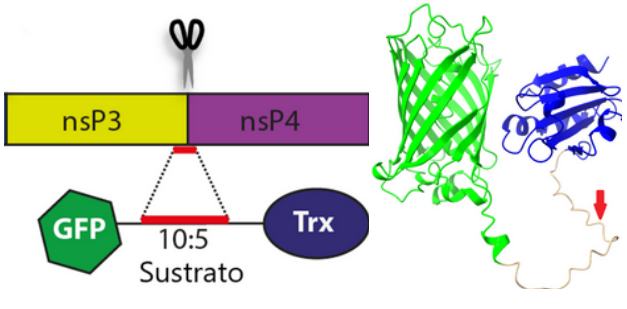

FIGURE 2. PROTEASE SUBSTRATE. (A) SCHEMATIC OF THE SUBSTRATE, COMPOSED OF THE GFP AND TRX PROTEIN DOMAINS LINKED BY A SPACER CONTAINING THE NATURAL NSP3/4 RECOGNITION SEQUENCE OF THE PROTEASE (10 RESIDUES UPSTREAM AND 5 DOWNSTREAM OF THE CLEAVAGE SITE). (B) PREDICTION OF THE SUBSTRATE STRUCTURE BY ALPHAFOLD . GFP IS SHOWN IN GREEN, TRX IN BLUE, AND THE SEGMENT CONTAINING THE CLEAVAGE SITE IN PINK. THE RED ARROW INDICATES THE NSP2 CLEAVAGE SITE.

Purification and yield of wild-type nsP2, mutant nsP2 and recombinant substrate

Once nsP2 and its substrate were designed and cloned, we proceeded to express and purify them. At the end of this procedure, we examined the purity of the preparations by SDS-PAGE, which was higher than 98% (Figure 3A). Then, we quantified the proteins spectroscopically based on absorbance at 280 nm, using a molar extinction coefficient of 51255 and 43695 M^{-1} cm^{-1} for nsP2 and the substrate, respectively (Figure 3B).

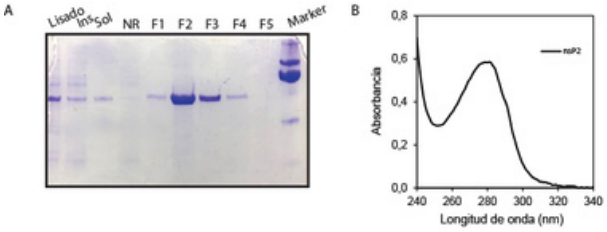

FIGURE 3. PROTEIN PURIFICATION. (A) REPRESENTATIVE SDS-PAGE OF THE INTERMEDIATE STEPS IN THE PURIFICATION OF NSP2 VARIANTS AND THEIR SUBSTRATE. LANES CORRESPOND TO THE TOTAL BACTERIAL LYSATE (LANE 1), THE INSOLUBLE FRACTION AND THE SOLUBLE FRACTION (LANES 2 AND 3), THE FRACTION NOT RETAINED ON THE AFFINITY COLUMN (LANE 4), THE FRACTIONS RECOVERED AFTER ELUTION WITH IMIDAZOLE (LANES 5 TO 9), AND THE MOLECULAR WEIGHT MARKER (LANE 10). (B) ABSORPTION SPECTRUM OF PURIFIED NSP2.

In the case of the substrate, since it contains GFP at its N-terminal end, it is possible to demonstrate its expression and identify the fractions where it is enriched by detecting fluorescence under ultraviolet light (Figure 4).

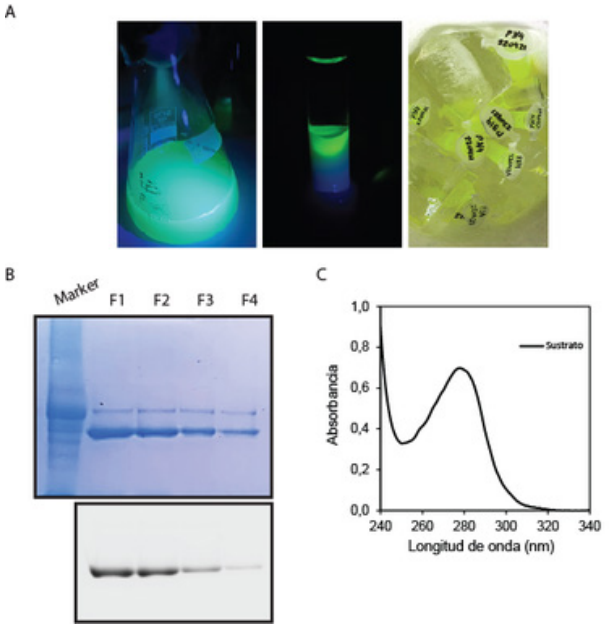

FIGURE 4. EXPRESSION AND PURIFICATION OF THE SUBSTRATE. (A) UV PHOTOGRAPHS OF A CULTURE OF *E. COLI BACTERIA* EXPRESSING THE SUBSTRATE (LEFT), AFFINITY CHROMATOGRAPHIC COLUMN ONTO WHICH THE SOLUBLE FRACTION OF THE BACTERIAL LYSATE WAS SEEDED (CENTER), AND VISIBLE LIGHT PHOTOGRAPH OF THE PURIFIED ENZYME, ONCE ELUTED FROM THE AFFINITY COLUMN (RIGHT). (B) REPRESENTATIVE SDS-PAGE OF SUBSTRATE PURIFICATION, REVEALED BY COOMASSIE BLUE STAINING (TOP) AND GFP FLUORESCENCE (BOTTOM). MOLECULAR WEIGHT MARKER (LANE 1) AND FRACTIONS RECOVERED AFTER ELUTION WITH IMIDAZOLE (LANES 2 TO 5). (C) ABSORPTION SPECTRUM OF THE PURIFIED SUBSTRATE.

Enzymatic activity of wild-type nsP2 and nsP2 with an active site mutation

To study the enzymatic activity, we incubated the wild-type nsP2 with the purified substrate for different times at 25 ºC . We evidenced the hydrolysis product of the nsP2 activity from the disappearance of the band corresponding to the complete substrate, and the appearance of the band corresponding to GFP, visualized by staining with Coomassie blue or using a fluorescence reader (Figure 5A).

Using densitometry, we quantified the intensity of each band and calculated the concentration of the product obtained at each time based on the spectrophotometric estimate of the substrate concentration.

Finally, we represented the concentration of the product as a function of the reaction time (Figure 5B).

Our results indicate that the appearance of product is directly proportional to the reaction time during the first two minutes (dotted red line in Figure 5B).

Therefore, it is possible to determine the initial rate of proteolysis as the slope of this line. The estimated initial rate was 50 nmoles of product per hour.

Evidence in the literature shows that the catalytic dyad residues C478 and H548 are absolutely essential for proteolysis and viral replication (13) .

Therefore, to validate the measurement method, we decided to clone, express and purify a CHIKV protease containing a mutation in the active site of nsP2 (C478A).

Then, we performed the activity assay with this mutant enzyme. As expected, the substrate is stable during the incubation time and nsP2 C478A is unable to hydrolyze it.

Overall, the results indicate that our method is robust and specific, and allows for rapid and easy quantitative measurements of nsP2 activity.

Since viral proteases are the targets of choice for the design of antivirals, assays such as the one proposed can also be extrapolated to the study of drugs targeting the proteases of other viruses.

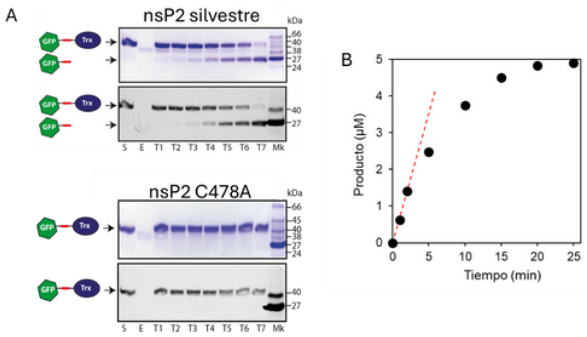

FIGURE 5. NSP2 ACTIVITY ASSAY. (A) WE INCUBATED WILD-TYPE NSP2 ENZYME (TOP) OR MUTANT C378A (BOTTOM) WITH SUBSTRATE FOR 0, 2, 5, 10, 16, 20, OR 30 MIN (T1 TO T7) AND RESOLVED THE REACTION PRODUCTS BY SDS-PAGE, WHICH WAS REVEALED BY COOMASSIE BLUE STAINING (TOP) AND GFP FLUORESCENCE (BOTTOM). SUBSTRATE DEGRADATION AS A FUNCTION OF REACTION TIME IS SHOWN. SUBSTRATE IN THE ABSENCE OF ENZYME (S) AND ENZYME WITHOUT SUBSTRATE (E) AND A MOLECULAR WEIGHT MARKER (MK) WERE ALSO INCLUDED. (B) AMOUNT OF PRODUCT AS A FUNCTION OF REACTION TIME, OBTAINED FROM QUANTIFICATION BY DENSITOMETRY OF THE SDS-PAGE BANDS FOR WILD-TYPE NSP2 ENZYME.

DISCUSSION

In this work, we describe the expression and purification of the chikungunya virus protease and a synthetic substrate containing one of the enzyme's natural cleavage sites.

Using the purified recombinant proteins, we were able to develop a simple, inexpensive, robust, and quantitative assay of enzymatic activity.

The design of specific antivirals is a challenge in the treatment of viral diseases, since viruses use cellular machinery for most of their metabolic processes. However, eukaryotic cells do not have certain enzymes necessary for

viral replication, so viral genomes contain the information that encodes them.

These enzymes include the polymerase, helicase and viral protease. Since these enzymes are exclusively viral, they have been the targets of choice for the design of specific antivirals for decades.

The first protease inhibitors were targeted at the HIV-1 (human immunodeficiency virus type 1) protease.

In 1990, Roberts and coworkers (14) designed a series of peptide derivatives based on the transition state mimetic concept that inhibit HIV-1 and HIV-2 protease in the nanomolar range .

These compounds showed little effect at 10 µM against structurally related human aspartic proteases, indicating a high therapeutic index. In 1995, saquinavir (*Invirase , Roche*), the first HIV protease inhibitor, was approved, marking a significant advance in the treatment of HIV/AIDS.

Subsequently, due to the low oral availability of saquinavir and the rapid selection of viruses with mutations that confer resistance, the development of new inhibitors (ritonavir, indavir , lopinavir, among others) was necessary (15) .

New generation inhibitors present pharmacokinetic improvements and provide alternatives in the case of patients who do not respond to treatment.

The background with HIV inhibitors was the foundation stone for the design of inhibitors against other viral proteases, such as Hepatitis C and SARS-Cov2 (16) .

In light of this evidence, viral protease inhibitors must be frequently designed and redesigned.

his is due to the high viral capacity to mutate to generate resistant variants and the emergence of new viruses against which there are no specific treatments available. In the case of CHIKV, there are currently no inhibitors approved for use in humans. However, a variety of peptide inhibitors are being studied (17, 18) , inhibitors based on the repositioning of drugs already approved for other diseases (19, 20) , and inhibitors identified by computational techniques (*docking*). molecular) (21, 22) .

in vitro measurement methods of protease activity. The most widely used assays are based on the Förster resonance energy transfer (FRET) technique (23) , where the substrate is a small peptide (10-15 amino acids) containing the cleavage site, a fluorophore at one end and a *quencher* at the other.

When these labeled peptides are cleaved by nsP2, the dissociation of the FRET pair occurs with the consequent increase in the fluorescent signal. These assays have the advantage that they allow proteolysis to be determined in real time. However, due to their small size, it is possible that the cleavage conditions are far from physiological conditions, and the peptides fit into the enzyme structure without too many restrictions.

On the other hand, *quenching could occur* intermolecular at high peptide concentrations, which could make the interpretation of the results difficult. Finally, the use of FRET peptides is expensive and not very versatile, since these molecules are commercial and cannot be modified by site-directed mutagenesis.

Based on the above, we consider that the measurement method proposed in this work is an interesting alternative for the study of potentially protease-inhibiting molecules, with advantages in its cost and experimental design.

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