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Characterisation of Recombinant 3CL Protease from SARS-CoV-2 Produced in *E. coli* BL21 (DE3) for Screening Anti-Covid Drug Candidates using *Rhodamine* 110-Synthetic Peptide Conjugate as a Substrate

(Pencirian Protease 3CL Rekombinan daripada SARS-CoV-2 Dihasilkan dalam *E. coli* BL21 (DE3) untuk Menyaring Calon Dadah Anti-Covid menggunakan *Rhodamine* 110-Sintetik Peptida Konjugat sebagai Substrat)

I GEDE EKA PERDANA PUTRA[1], MARIA ULFAH[1], NAUFAL HAFIZH[2], ERWAHYUNI ENDANG PRABANDARI[3], FIRDAYANI[3] & IS HELIANTI[1,*]

[1]*Research Center for Applied Microbiology, National Research and Innovation Agency, Jalan Raya Bogor Km 46, Cibinong, Bogor, Indonesia*
[2]*Research Center for Agroindustry, National Research and Innovation Agency, Jalan Raya Bogor Km 46, Cibinong, Bogor, Indonesia*
[3]*Research Center for Vaccine and Drug, National Research and Innovation Agency, Jalan Raya Bogor Km 46, Cibinong, Bogor, Indonesia*

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ABSTRACT

The prediction that the pandemic is progressing towards becoming endemic does not change the fact that COVID-19 can still be fatal for individuals with weak immune systems. Therefore, anti-COVID drugs are still needed, even when the disease becomes endemic. With regards to SARS-CoV-2, the roles of 3CL protease are crucial in the formation of new virus particles. Therefore, inhibiting the function of these viral proteases will directly prevent viral replication in the human body. In this study, we report the production of a recombinant 3CL protease from SARS-CoV-2 in *E. coli* BL21 (DE3), which has not been extensively studied in Indonesia. The purified 3CL protease exhibited high solubility and functional activity. Additionally, the recombinant enzyme was characterised using the Rhodamine 110 fluorogenic peptide substrate. We showed that the recombinant 3CL protease was unstable in the presence of a DMSO concentration above 10%. Using the Rhodamine 110 fluorogenic peptide substrate, we found that the enzyme had a K_M of 47.0 μM, V_{max} of 0.41 RFU/s, and k_{cat}/K_M of 0.0088 RFU/μM^2.s while the IC$_{50}$ of the GC376 was 13.35 nM. We also tested three bioactive compounds (catechin, emodin, and 1,4-naphthoquinone) using this recombinant protease as a protein target, and 1,4-naphthoquinone was the most promising bioactive compound in inhibiting the SARS-CoV-2 virus.

Keywords: Characterisation; peptide substrate LGSAVLQ-Rh110; recombinant 3CL protease

ABSTRAK

Ramalan bahawa wabak itu sedang berkembang menjadi endemik tidak mengubah fakta bahawa COVID-19 boleh membawa maut bagi individu yang mempunyai sistem imun yang lemah. Oleh itu, dadah anti-COVID masih diperlukan, walaupun penyakit itu menjadi endemik. Berkenaan dengan SARS-CoV-2, peranan 3CL protease adalah penting dalam pembentukan zarah virus baharu. Oleh itu, merencat fungsi protease virus ini secara langsung akan menghalang replikasi virus dalam tubuh manusia. Dalam kajian ini, kami melaporkan penghasilan 3CL protease rekombinan daripada SARS-CoV-2 dalam *E. coli* BL21 (DE3), yang belum dikaji secara meluas di Indonesia. 3CL protease yang telah ditulenkan menunjukkan keterlarutan yang tinggi dan aktiviti berfungsi. Tambahan lagi, enzim rekombinan telah dicirikan menggunakan substrat peptida fluorogenik Rhodamine 110. Kami mendapati bahawa 3CL protease rekombinan ini tidak stabil dalam kepekatan DMSO melebihi 10%. Dengan menggunakan substrat peptida fluorogenik Rhodamine 110, kami mendapati bahawa enzim ini mempunyai nilai K_M 47.0 μM, V_{max} 0.41 RFU/s dan k_{cat}/K_M 0.0088 RFU/μM^2.s manakala nilai IC$_{50}$ GC376 ialah 13.35 nM. Kami juga menguji tiga sebatian bioaktif (katechin, emodin dan 1,4-naftoquinon) menggunakan protease ini sebagai sasaran protein dan 1,4-naftoquinon didapati adalah sebatian bioaktif yang paling berpotensi dalam menghalang virus SARS-CoV-2.

Kata kunci: Pencirian; rekombinan 3CL protease; substrat peptida LGSAVLQ-Rh110

Introduction

The SARS-CoV-2 virus, which causes COVID-19, first emerged in Wuhan, China in December 2019 and rapidly spread globally, leading to a widespread public health crisis that has tragically claimed over 6.8 million lives (https://www.worldometers.info/coronavirus/). While the pandemic may turn endemic, COVID-19 remains a threat to people with weak immune systems. Therefore, the search for new anti-COVID drugs, especially from Indonesia's rich herbal biodiversity, remains crucial.

Indonesia possesses a vast variety of plant species, exceeding 2,500 that can be employed for medicinal purposes, as stated in the Indonesian Herbal Dictionary (Elfahmi, Woerdenbag & Kayser 2014). Additionally, Zuhud and Siswoyo (2001) found approximately 1,845 species in Indonesian forests that could be utilised for medicinal purposes. These figures are potentially updated due to ongoing inventories and investigations of yet-to-be-identified species. The Indonesian Food and Drug Monitoring Agency (BPOM) has formally listed 283 plant species for medicinal use, with the majority of the remaining species being used in traditional medicine (Elfahmi, Woerdenbag & Kayser 2014).

To find antiviral drugs, it is usually necessary to target viral enzymes that are essential in viral replication. Regarding SARS-CoV-2, the functions of two proteases, 3CL protease and PL protease are vital for the creation of new viral particles. Therefore, inhibiting the function of these viral proteases will directly prevent viral replication in the human body (Chang et al. 2020). In this study, we focused on the 3CL protease. The enzyme is also known as the main protease (Mpro) and is a member of the chymotrypsin-like cysteine protease family. It is responsible for cleaving the polyprotein produced by the virus upon infection into functional proteins. Specifically, it performs 11 cleavages on the polyprotein, resulting in the generation of 16 non-structural proteins (NSPs) crucial for viral replication and transcription processes (Yan & Wu 2021). The active site of the 3CL protease is located in a cleft between the two subunits, and it contains a catalytic dyad composed of cysteine and histidine residues (Xiong et al. 2021). Because of its vital function in viral replication, the 3CL protease becomes an appealing target for the development of antiviral drugs. Currently, many publications related to the screening of active compounds as candidates for COVID-19 have been carried out (Arya et al. 2020; Kumar et al. 2020; Srivastava et al. 2022; Tahir ul Qamar et al. 2020; Utomo, Ikawati & Meiyanto 2020). These studies were carried out computationally by pairing target proteins such as the SARS-CoV-2 3CL protease with ligands derived in active compounds from herbs and looking at the inhibitory power of the ligands on their targets.

However, the mentioned research is only *in silico*. Although it is very helpful in the initial screening, *in vitro* bioassay proof still needs to be done. A computational approach also cannot be performed in cases of screening from natural product sources whose bioactive components are unknown. However, the availability of drug target proteins such as recombinant SARS-CoV-2 3CL protease to carry out these *in vitro* tests is very limited, especially in Indonesia. Therefore, the development of SARS-CoV-2 viral proteases intended for screening and confirmation of active compounds from herbs *in vitro* by Indonesian scientists is urgently needed. Our study focused on the characterisation of this recombinant 3CL protease from SARS-CoV-2 as a protein-target inhibitor for screening anti-COVID-19 compounds from herbal and other sources. For this application, 3CL protease must be produced and characterised. The completed characterisation for the purpose of the bioassay system related to the kinetics and stability of recombinant SARS-CoV-2 protease has not been performed. This characterisation is important to form a reliable bioassay system that can be used for valid *in vitro* testing and protease inhibitor screening.

In this study, we report the production of a recombinant 3CL protease from SARS-CoV-2 in *E. coli* BL21 (DE3). The protease was subsequently purified to ensure its high solubility and functional activity. Moreover, the recombinant enzyme was characterised using the Rhodamine 110 fluorogenic peptide substrate. We also tested three bioactive compounds (emodin, catechin, 1,4-naphthoquinone) using this protease as a protein target.

Materials and Methods

Construction of Plasmid Carrying DNA Sequence Encoding the 3CL Protease of SARS-CoV-2

The synthetic recombinant DNA was created using the SARS-CoV-2 3CL protease gene sequence and inserted into pET32b(+) vector with 6xHis tag sequence at the C-terminal as described previously (Haniyya et al. 2022). This recombinant DNA was introduced at the *Nde*I/*Xho*I site and confirmed as two proper fragments of 945 bp, and 5.4 kb. *E. coli* BL21 (DE3) was employed as an expression host.

PRODUCTION AND PURIFICATION OF RECOMBINANT 3CL PROTEASE

The recombinant 3CL protease was produced in an LB medium supplemented with ampicillin (100 μg/mL) as a selection marker. Shake-flask fermentation was conducted in 2 L Erlenmeyer containing a fifth of culture with 1% (v/v) of overnight seed culture. After reaching a cell density of λ_{600} 0.4 to 0.6, the culture was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). The following enzyme production took place at 37 °C for 5 h with an agitation rate of 200 rpm.

The cells were collected by centrifugation for 15 min at 8000 rpm, 4 °C (Hitachi, R9A rotor), and subsequently resuspended in 40 mL of lysis solution (20 mM sodium phosphate buffer, pH 8.0). The cell mixture was subjected to ultrasonication (Ultrasonicator Model UCD-2000, BIOBASE) on ice, at 30% amplitude for 50 cycles of 3 s each, followed by an 8 s break. After the centrifugation at 18,000 rpm and 4 °C for 30 min, the crude extract was separated and subsequently filtered through a 0.22-micrometer membrane filter. The crude extract was purified by using HisTrap HP affinity column (Cytiva), pre-equilibrated with buffer A (20 mM imidazole, 20 mM NaH_2PO_4, 300 mM NaCl, pH 7.4) of 10 column volumes (CVs). After loading 40 mL of sample onto the column, unbound proteins were washed out using 10 CVs of buffer A. Subsequently, a linear gradient elution of 20 CVs was performed using buffer B (500 mM imidazole, 20 mM NaH_2PO_4, 300 mM NaCl, pH 7.4). Fractions obtained from this linear gradient elution were pooled and applied onto HiLoad 26/600 Superdex 200 pg (Cytiva). The protein was then eluted from the column using gel filtration buffer (50 mM sodium phosphate buffer, 150 mM NaCl, pH 7.2). Following SDS-PAGE and western blot analysis of the fractions, the fractions containing 3CL protease were pooled and concentrated to a final concentration of approximately 0.5 to 1 mg/mL with 10% glycerol and stored at -20 °C. The protein concentrations were assessed through the Bradford assay, employing an albumin standard as described by Bradford (1976).

SDS-PAGE

SDS-PAGE examination of the samples was performed using the Bollag, Rozycki & Edelstein (1996) method. Samples were combined with 5X protein sample solution (0.5 M DTT, 50% glycerol, 10% SDS, 0.25 M Tris-HCl pH 6.8, 0.5% bromphenol blue) and heated at 100 °C for 5 min before being injected into the gel. The slab gel system included 5% stacking gel (334 μL of 30% polyacrylamide, 15 μL of 10% ammonium persulfate, 7 μL of TEMED, 25 μL of 10% SDS, 625 μL of 0.5 M Tris-HCl buffer pH 6.8, and 1.5 mL of ddH_2O) and 12.5% separating gel (3 mL of 30% polyacrylamide, 30 μL of 10% ammonium persulfate, 7 μL of TEMED, 60 μL of 10% SDS, 1.5 mL of 1.5 M Tris-HCl buffer pH 8.8, and 2.4 mL of ddH_2O). The protein marker was the SpectraTM Multicolor Board Range Protein Ladder from Thermo Fisher Scientific. Electrophoresis was carried out for 90 min, at 120 V with 1x running solution (0.25 M Tris-HCl, 1% (w/v) SDS, and 1.92 M glycine). The gel was then stained using Xpress BlueTM Protein Stain (Himedia) protocol. The gels were documented and the obtained images were analysed by densitometric analysis using ImageJ software version 1.53 to determine the purity of the recombinant 3CL protease.

WESTERN BLOT

Recombinant 3CL protease expression was confirmed by western blot. SDS-PAGE-separated samples were transferred in transfer solution by using the semi-dry tank western blot apparatus (Thermo Fisher Scientific). The PVDF membrane was submerged in methanol for 15 min before usage. The procedure of transfer was performed at 2.5 mA, 21 V for 30 min. Following the transfer, the membrane was blocked for an hour at 37 °C with a 5% skim milk blotting-grade blocker that was diluted in PBS with 0.05% Tween 20. The membrane was then shaken for an hour while being rinsed six times in PBST (PBS-Tween 20) buffer. The membrane was then incubated at 4 °C for 16 to 18 h with a diluted 1:3,000 mouse 6x His-Tag monoclonal antibody HIS H8 (Invitrogen) probe in 5% skim milk in PBST, followed by an additional hour at 37 °C with a gentle shaking. After 1 h of washing with fresh PBST buffer, every 10 min, goat anti-mouse IgG (H+L) horseradish peroxidase conjugate (1:100,000 dilution, Invitrogen) in 5% skim milk in PBST solution was applied as a secondary antibody. The membrane was incubated at 37 °C with gentle shaking for 1 h and washed every 10 min with fresh PBS-T solution for 1 h. To visualise the results, the PVDF membrane was treated for 5 min in a dark room with Super Signal™ West Femto chemiluminescent substrate (Thermo Fisher Scientific). PVDF membrane treated with Femto substrate was incubated for 1 to 15 min with high-performance chemiluminescence film (Amersham Hyperfilm™ ECL). Finally, the film was stained with a fixer and developer solution (Carestream Kodak autoradiography GBX fixer and developer) to show the protein target.

EFFECT OF DMSO ON THE RECOMBINANT 3CL PROTEASE ACTIVITY

To determine the effect of dimethyl sulfoxide (DMSO) on 3CL protease activity, varying concentrations of DMSO ranging from 0 to 50% (v/v) were added to a 40 μL reaction mixture comprising 1 μM 3CL protease and 20 μM LGSAVLQ-Rh110 fluorogenic peptide substrate (R&D Systems). This reaction was carried out in a reaction buffer (20 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, 0.01% bovine serum albumin, 1 mM 1,4-dithiothreitol, pH 7.3) (Zhu et al. 2020). The reactions were conducted in triplicate at 37 °C for 30 min in a black 384-microwell plate (Thermo Fisher Scientific). A Varioskan LUX Multimode Microplate Reader (Thermo Fisher Scientific) was employed to measure the relative fluorescence unit (RFU) values, with excitation and emission wavelengths of 485 nm and 535 nm, respectively.

KINETIC PARAMETERS BASED ON MICHAELIS-MENTEN

The kinetic parameters of the purified SARS-CoV-2 3CL protease were determined through triplicate assays employing LGSAVLQ-Rh110 (R&D Systems) as the substrate. The substrate solutions were prepared at concentrations ranging from 2.5 to 50 μM and then mixed with 1 μM of 3CL protease in reaction buffer (20 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, 0.01% bovine serum albumin, 1 mM 1,4-dithiothreitol, pH 7.3) with a total reaction volume of 40 μL in a black 384-well plate (Thermo Fisher Scientific). The RFU value was measured using a Varioskan LUX Multimode Microplate Reader (Thermo Fisher Scientific) at 37 °C for 1 h, with excitation and emission wavelengths of 485 nm and 535 nm, respectively. The first 10 min of the kinetic progress curves were used to determine the initial reaction rate using linear regression. The initial reaction rates were fitted to the Lineweaver-Burk equation (Equation 1),

$$v_0 = \frac{V_{\max}[S]}{K_M + [S]} \frac{1}{v_0} = \frac{K_M}{V_{\max}} \cdot \frac{1}{[S]} + \frac{1}{V_{\max}} \qquad (1)$$

where $v_0\, v_0$ is the initial reaction rate at the substrate concentration S, V_{max} is the maximum reaction rate, and K_M is the Michaelis-Menten constant. Kinetic parameters were calculated using Microsoft Excel (Microsoft Inc., USA).

DETERMINATION OF THE HALF MINIMUM INHIBITORY CONCENTRATION (IC_{50}) VALUE OF GC376

Inhibition assays were conducted to determine the IC_{50} value of GC376, a known inhibitor of the SARS-CoV-2 3CL protease. In all, 4 μL GC376 (Selleck Chemicals) diluted in DMSO at concentrations ranging from 0.01 nM to 1000 nM were added to 36 μL of 1 μM 3CL protease enzyme and 20 μM LGSAVLQ-Rh110 substrate in reaction buffer (20 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, 0.01% bovine serum albumin, 1 mM 1,4-dithiothreitol, pH 7.3) in a black 384-well plate. The RFU values were measured as described earlier. The calculation of percent inhibition was performed in comparison to control reactions where DMSO was used instead of the GC376 solution. Subsequently, the obtained values were plotted against different concentrations of GC376 to create a dose-response curve. The half-maximal inhibitory concentration (IC_{50}) value was calculated by performing a nonlinear regression analysis on the dose-response curve using Microsoft Excel.

CATHECIN, EMODIN, AND 1,4-NAPHTHOQUINONE INHIBITION ASSAY

Catechin and emodin were purchased from Shaanxi Pioneer Biotech (China) and 1,4-naphthoquinone was purchased from Sigma-Aldrich. The three compounds were prepared for primary screening by diluting them in DMSO to a final concentration of 100 μM. To initiate the inhibition assay, 3CL protease with a final concentration of 1 μM was pre-incubated with the compounds with a final concentration of 100 μM for 15 min before the addition of substrate with a final concentration of 20 μM. The resulting mixture was then brought to a final reaction volume of 40 μL and subjected to RFU measurements at 37 °C for a period of 1 h. The excitation wavelength was adjusted to 485 nm, while the emission wavelength was set to 535 nm. Pure DMSO was employed as the negative control in place of the compounds. The percentage of inhibition was determined by comparing the reactions to the control. The compound showing the strongest inhibitory effect against 3CL protease was selected for the IC_{50} value determination. A range of at least five inhibitor concentrations was employed to establish a dose-response curve. The IC_{50} value was obtained through nonlinear regression analysis of the inhibitor concentration plotted against percent inhibition.

Results and Discussion

PURIFICATION RESULTS

The highly soluble recombinant 3CL protease was successfully expressed in *E. coli* BL21 (DE3). The molecular weight of the enzyme was estimated to be approximately 33.8 kDa, and SDS-PAGE analysis verified that it was expressed at the anticipated size (Figure 1(a), lane 1). Pure 3CL protease was achieved using affinity chromatography and gel filtration. Linear gradient elution was employed to collect recombinant 3CL protease from the HisTrap affinity column. Most of the non-specific binding proteins were removed by washing with 100% buffer A (20 mM imidazole), as shown in Figure 1(a), lane 2. The target enzyme was eluted at 40% buffer B (212 mM imidazole), leading to a noticeable peak on the chromatogram, as demonstrated in Figure 1(b). During the initial purification runs, a minor peak was observed while eluting with low buffer B concentration. Nonetheless, analysis of the corresponding peak through SDS-PAGE showed that the target enzyme was not being washed out of the column together with impurities, as seen in Figure 1(a,) lane 3. The purity of recombinant 3CL protease after the HisTrap column was 89%, as determined by densitometric analysis of an SDS-PAGE image (Figure 1(a), lane 4).

Gel filtration with HiLoad Superdex column was performed to enhance the purity of recombinant 3CL protease. Isocratic elution with 100% gel filtration buffer resulted in a sharp and prominent peak as shown in Figure 1(c). SDS-PAGE analysis of the peak showed a single band of the purified recombinant 3CL protease (Figure 1(d)) with an estimated purity of 99%.

The purity of the recombinant 3CL protease was further confirmed by western blot analysis, using an anti-6xHis antibody, which specifically detects the C-terminal 6xHis-Tag fusion (Figure 1(e)). The typical yield from the two purification steps was approximately 5 mg of protein from 1 L of culture. The yield was comparable to those reported in a previous study (De Marco Verissimo et al. 2022).

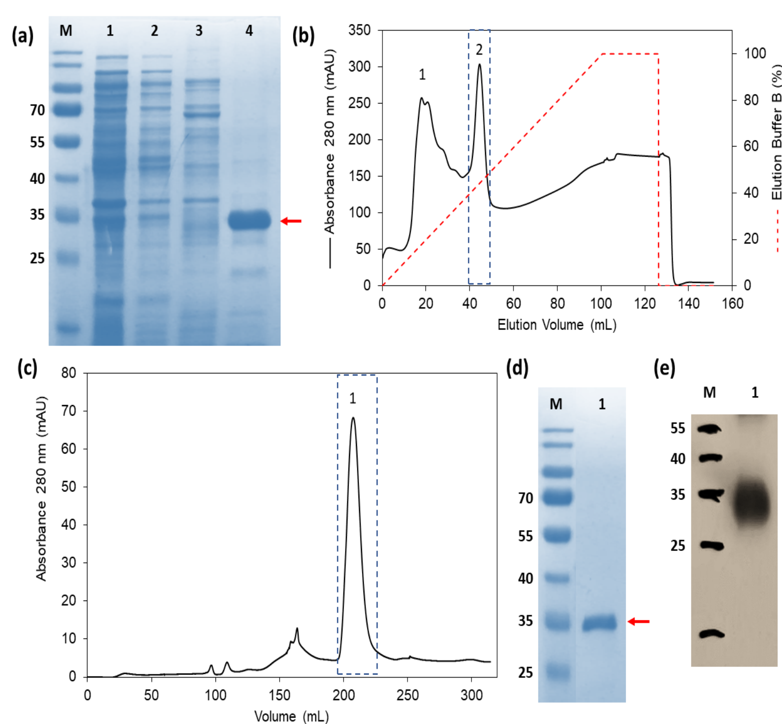

FIGURE 1. (a) SDS-PAGE analysis (M: marker, lane 1: crude extract, lane 2: flow-through fractions, lane 3: peak 1, lane 4: peak 2) and (b) chromatogram of 3CL protease purification using affinity HisTrap column. (c) Chromatographic profile, (d) SDS-PAGE, and (e) western blot analysis of the 3CL protease purification in gel filtration column

EFFECT OF DMSO ON THE RECOMBINANT 3CL PROTEASE ACTIVITY

Dimethyl sulfoxide (DMSO) is commonly used in drug discovery studies due to its ability to dissolve a variety of organic compounds. To maintain intrinsic activity, DMSO is usually used at low concentrations in assays. However, at higher concentrations, DMSO can perturb the conformation of enzymes, leading to a reversible and gradual decrease in their catalytic activities (Tomohara et al. 2019). The effect of DMSO on the activity of 3CL protease has been investigated. Figure 2 shows that DMSO significantly reduced the activity of 3CL protease. At DMSO concentrations above 10%, the activity of 3CL protease decreased drastically, leaving only 22% activity compared to 5% DMSO. 3CL protease completely lost its activity at a DMSO concentration of 40%. These results were slightly different from those reported in another study (Nguyen et al. 2021), where 3CL protease remains stable in the presence of up to 10% DMSO. The difference can be attributed to the variation in enzyme concentrations used. Nguyen et al. (2021) employed a higher enzyme concentration in their assay. Additionally, differences in the fluorogenic peptide substrate employed in the assay can also lead to discrepancies in the activity of 3CL protease in DMSO solutions.

KINETICS OF THE RECOMBINANT 3CL PROTEASE

The SARS-CoV-2 virus has caused a global health crisis known as COVID-19. To address this urgent situation, there is a need to develop effective treatments. One promising drug target for SARS-CoV-2 is the 3CL protease, which plays a crucial role in the viral polyprotein proteolytic process and viral replication and transcription (Jin et al. 2021, 2020). It is crucial to characterise the 3CL protease to create a dependable bioassay system that can be utilised for accurate *in vitro* testing and screening of protease inhibitors. The kinetic characterisation of 3CL protease from SARS-CoV-2 using various types of fluorogenic substrates has been carried out (Al Adem et al. 2023; Li et al. 2020; Liu et al. 2020; Zhu et al. 2020). In this study, recombinant 3CL protease was characterised using LGSAVLQ-Rh110 as a substrate. This fluorogenic peptide substrate features a Rhodamine 110 dye conjugated to a naturally occurring cleavage site for the 3CL protease. Specifically, the 3CL protease acts on the C-terminal side of the glutamine amino acid, resulting in the release of the dye and subsequent fluorescence (De Marco Verissimo et al. 2022). This distinctive property allows for real-time monitoring of 3CL protease activity through the fluorescence signal generated upon cleavage.

The proteolytic activity of purified recombinant 3CL protease was characterised by measuring the K_M, V_{max}, and k_{cat}/K_M values. A Lineweaver-Burk plot (Figure 3) was plotted to study the Michaelis-Menten kinetic parameters of 3CL protease by measuring the initial reaction rate (v_0 v_0) when 1 µM 3CL protease was mixed with different concentrations of substrate (2.5 to 50 µM). The kinetic parameters K_M of 47.0 µM and V_{max} of 0.41 RFU/s were obtained by fitting the Lineweaver-Burk equation. The K_M value of the recombinant 3CL protease was comparable to the values obtained in previous studies that used different substrates (Ma et al. 2020; Pattaro-Júnior et al. 2023; Vuong et al. 2020; Zhu et al. 2020). The catalytic efficiency (k_{cat}/K_M) of the 3CL protease is 0.0088 RFU/µM2.s, which is 160 times lower than that obtained by Pattaro-Júnior et al. (2023). This difference may be caused by variations in the equipment used in the assay.

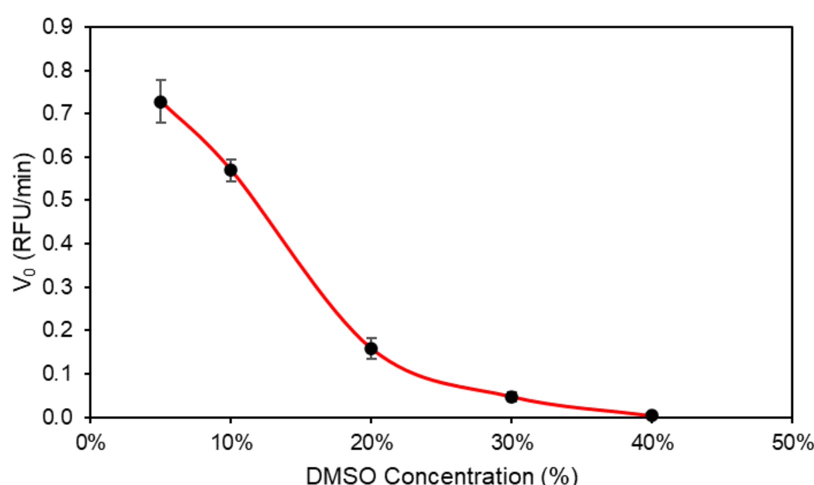

FIGURE 2. Effect of DMSO on the recombinant SARS-CoV-2 3CL protease activity

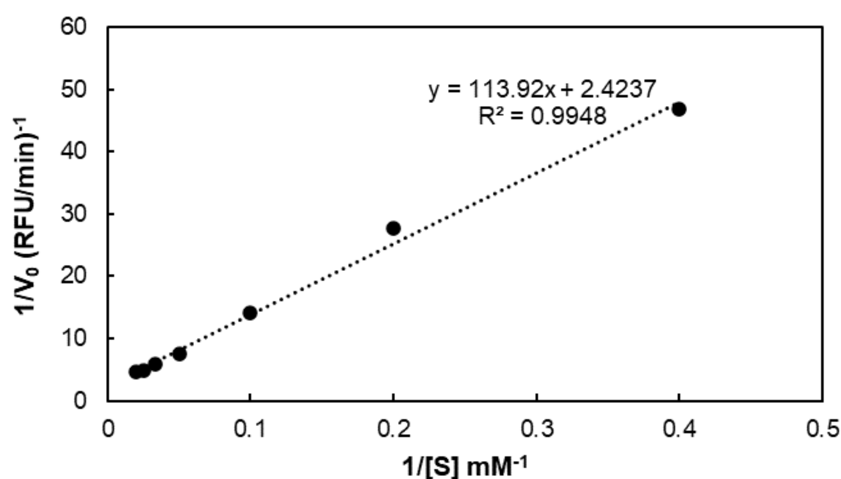

FIGURE 3. Lineweaver-Burk plot of recombinant 3CL protease of SARS-CoV-2

DETERMINATION OF IC_{50} VALUE OF GC376

Several previous studies used C376, a commonly known 3CL protease inhibitor, to validate screening assays for potential 3CL protease inhibitors (Gurard-Levin et al. 2020; Ma et al. 2020; Narayanan et al. 2022; Yan et al. 2021; Zhu et al. 2020). In this study, GC376 was used at concentrations ranging from 0.01 to 1000 nM to test the ability of the developed assay to detect inhibitory activity. As anticipated, GC376 exhibited inhibitory activity at low concentrations and displayed strong inhibition of 3CL protease with an IC_{50} value of 13.35 nM (Figure 4), which was in line with previously reported values (Gurard-Levin et al. 2020; Ma et al. 2020). These results demonstrated that the developed screening assay was a feasible method for rapidly screening 3CL protease inhibitors.

INHIBITORY ACTIVITY ASSAY OF CATECHIN, EMODIN, AND 1,4-NAPHTHOQUINONE

To test the 3CL protease produced as a protein target for screening anti-COVID compounds, three bioactive compounds (catechin, emodin, and 1,4-naphthoquinone) with potential as anti-COVID agents based on *in silico* studies (Nawrot-Hadzik et al. 2021; Wang et al. 2021) were used for inhibition assay. Firstly, we evaluated the inhibitory activities of the three bioactive compounds in a primary screening using a single-point concentration assay. The compound with the highest inhibitory activity then had its IC_{50} value determined. The three compounds were tested at a final concentration of 100 µM with DMSO as a negative control. Figure 5 shows that at 100 µM, 1,4-naphthoquinone had the strongest inhibitory activity (98.9%), followed by emodin (35.6%) and catechin (6.6%).

Catechin is a compound from the flavan-3-ol group. A published study (Nguyen et al. 2021) showed catechin inhibition activity of only 9% at a concentration of 200 µM using the substrate Dabcyl-KTSAVLQ↓SGFRKME-Edans in their experiment. Furthermore, Nguyen et al. (2021) evaluated the inhibitory activity of seven flavan-3-ol compounds, including catechin derivatives. The results showed that catechin and its isomer epicatechin had the lowest activity compared to its derivatives. They attributed this to the absence of hydroxyl groups at C3’, C4’, and C5’ in the B-ring of catechin and epicatechin.

In another study (Nawrot-Hadzik et al. 2021), the inhibitory activity of emodin was evaluated using a fluorescent peptide substrate (QS1, Ac-Abu-Tle-Leu-Gln-ACC) at a concentration of 100 µM, resulting in 51.5% inhibition. Furthermore, their molecular docking study showed that the carbonyl group at C10’ interacts with residues Cys145 and His41 on the 3CL protease, suggesting emodin as a potential inhibitor of 3CL protease.

Further experiments on the inhibitory activity of 1,4-naphthoquinone showed an IC_{50} value of 25.6 µM (Figure 6). This IC_{50} value was higher than the one reported previously (Wang et al. 2021). The difference in results could be due to the use of different substrates. In the study, the substrate used was Dabcyl-KNSTLQSGLRKE-Edans. A molecular docking study of

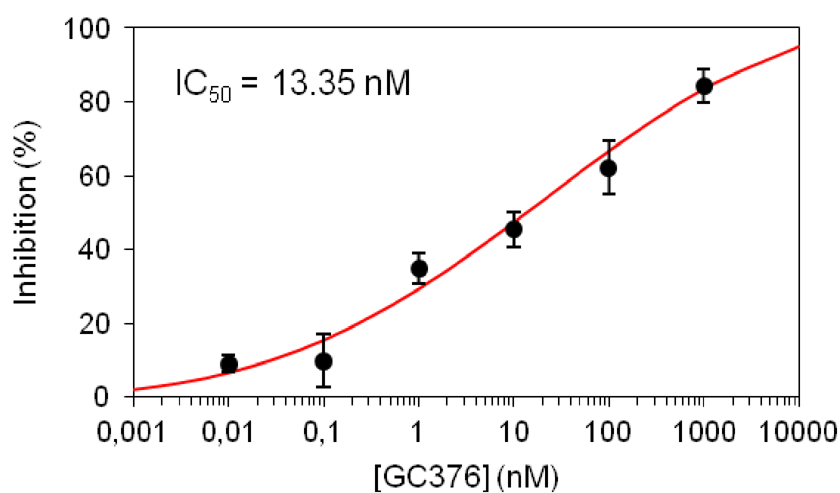

FIGURE 4. Dose-response curve of GC376, known 3CL protease inhibitor. At an enzyme concentration of 1 μM and substrate concentration of 20 μM, the IC_{50} value was 13.35 nM

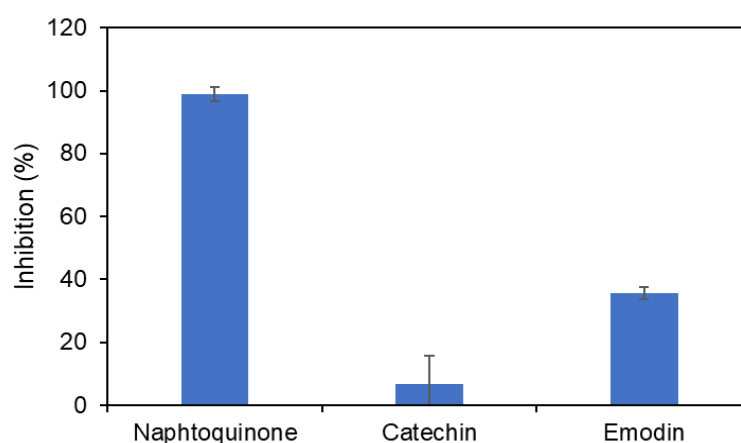

FIGURE 5. Inhibitory activity of three bioactive compounds against SARS-CoV-2 3CL protease. An enzyme concentration of 1 μM and a substrate concentration of 20 μM was used in the experiment

juglone, a derivative compound of 1,4-naphthoquinone with the crystal structure of 3CL protease, predicted that the carbonyl group at C1' forms a hydrogen bond with the Gly143 residue (Cui & Jia 2021). Another *in silico* study on a 1,4-naphthoquinone derivative compound, Vitamin K3 with 3CL protease, showed that inhibitory activity could be caused by the covalent bonding that occurs with the Cys145 residue (Wang et al. 2021). The results of this study confirmed 1,4-naphthoquinone as a potential inhibitor of the 3CL protease of SARS-CoV-2.

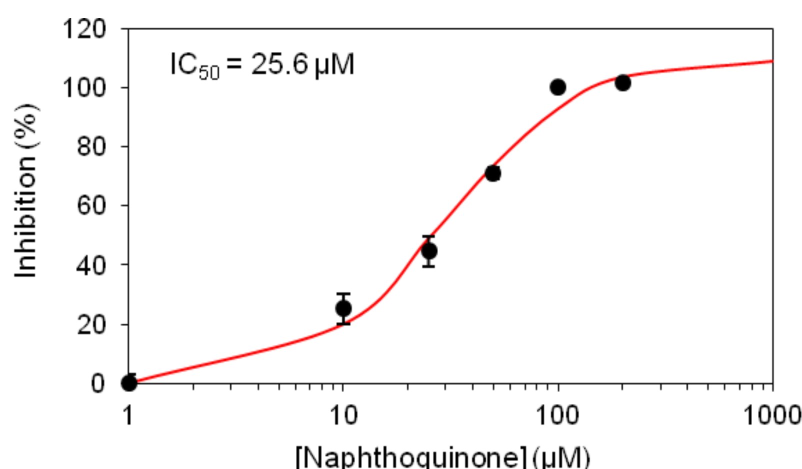

FIGURE 6. Dose-response curve of 1,4-naphthoquinone. At an enzyme concentration of 1 μM and substrate concentration of 20 μM, the IC_{50} value was 25.6 nM

CONCLUSIONS

In conclusion, in screening therapeutic drugs for the treatment of COVID-19, the 3CL protease was investigated as an outstanding target because it cleaves the peptide bonds that are not found in human proteins. The enzyme plays a significant role during the replication of the virus by cleaving sites within the polyprotein of the virus, forming non-structural proteins, and then producing a new SARS-CoV-2 virus inside human cells. The activity is attributed to the ability of the enzyme to recognise and cleave at the cleavage site in the motif Leu/Phe/Met-Gln ↓ Gly/Ser/Ala (↓ denotes the cleavage site). This study successfully demonstrated the characterisation of the recombinant 3CL protease produced in *E. coli* BL21 (DE3) and purified using His-Tag and gel filtration column. This was accomplished by utilising the Rhodamine 110 fluorogenic peptides substrate. When we tested the inhibitory effect of 3 potential compounds namely catechin, emodin, and 1,4-naphthoquinone, it was shown that 1,4-naphthoquinone was very potent in inhibiting 3CL protease activity. Overall, this assay can provide valuable information about the potency of 1,4-naphtoquinone as an inhibitor of the SARS-CoV-2 3CL protease, which can be useful for designing and optimising potential therapeutics for COVID-19 treatment. However, it is worth noting that the potential of 1,4-naphthoquinone as a therapeutic agent against COVID-19 is still being investigated, and further studies are needed to evaluate its efficacy and safety.

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*Corresponding author; email: is.helianti@brin.go.id