DETERMINATION OF SARS-COV-2 MAIN PROTEASE (M^{pro}) ACTIVITY BASED ON ELECTROOXIDATION OF THE TYROSINE RESIDUE OF A MODEL PEPTIDE

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Abstract. The proposed approach for determining the catalytic activity of SARS-CoV-2 main protease (M^{pro}) is based on the registration of the peak area of the electrochemical oxidation of the tyrosine residue of the model peptide substrate CGGGAVLQSGY immobilized on the surface of a graphite screen-printed electrode (SPE) modified with gold nanoparticles (AuNP). The AuNP were obtained by electrosynthesis. The steady state kinetic parameters of M^{pro} towards the model peptide were determined: catalytic constant (k_{cat}) was $(3.1 \pm 0.1) \cdot 10^{-3}$ s^{-1}; Michaelis constant (K_M) was $(358 \pm 32) \cdot 10^{-9}$ M; catalytic efficiency (k_{cat}/K_M) was 8659 s^{-1}/M. The limit of detection (LOD) determined for M^{pro} using the proposed electrochemical system was 44 nM. The proposed approach is a promising tool to search for new M^{pro} inhibitors as drugs for the treatment of coronavirus infections.

Keywords: *M^{pro} protease, tyrosine electrooxidation, screen-printed electrodes, gold nanoparticles, model peptide*

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INTRODUCTION

Coronavirus major proteases (M^{pro}), also known as 3C-like proteases ($3CL^{pro}$) or nonstructural proteins 5 (Nsp5), are highly conserved enzymes of the cysteine protease group that play an important role in the life cycle of β-coronaviruses, including severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) [1, 2]. The function of this group

of enzymes is post-translational processing of pp1a and pp1b proteins, which are essential for viral invasion, replication and transcription processes [1, 3, 4]. M^{pro} SARS-CoV-2 (EC 3.4.22.69) is a homodimeric enzyme that is 96% identical to $M^{(pro)\ of}$ severe acute respiratory syndrome coronavirus (SARS-CoV) [5]. This enzyme is a promising target for drug action to treat coronavirus infections [6]. M^{pro} has a number of unique properties that can be utilized to develop effective and selective drugs for the treatment of coronavirus infections, including Coronavirus Infectious Disease 2019 (COVID-19), without adverse effects on host proteases [2, 7-9]. For example, due to the lack of M^{pro} homologs among proteases in human cells with similar specificity to that of the coronavirus protease, peptidomimetics are promising compounds for the treatment of coronavirus infections [6]. It has been suggested that the use of M^{pro}, which has a highly conserved amino acid sequence and structure, as a target for therapy of coronavirus infections may allow reducing the risk of resistance to therapy of new coronavirus variants [10, 11]. Due to the need to quickly find an effective pharmacotherapy strategy for coronavirus infections in a pandemic, a number of M^{pro} inhibitor drugs have been approved by the Food and Drug Administration (FDA) for use in clinical practice. However, many of the approved drugs do not meet the optimal parameters of bioavailability, toxicity, and efficacy [12]. Moreover, the emergence of new virus strains, including those with mutant forms of M^{pro}, causes their resistance to approved drugs and makes it necessary to search for new antiviral drugs for the treatment of coronavirus infections [12, 13].

The values of kinetic parameters M^{pro} presented in the literature are in a wide range. The lack of consensus on the kinetic parameters of this enzyme complicates an objective and accurate assessment of the inhibitory properties of new compounds [7]. Currently, there are various approaches to study the properties of proteases based on the use of labeled peptides as substrates [14]. Previously, methods based on Förster resonance energy transfer (FRET) and liquid chromatography with mass spectrometry (LC-MS) have been used to study the kinetics of M^{pro}, the latter approach being characterized by high labor intensity [7]. FRET approaches also have a number of drawbacks, such as the distortion of the actual values of the enzyme kinetic parameters due to a decrease in the actual fluorescence intensity due to intermolecular interactions between the quencher molecule, 4-((4-(4-(dimethylamino)phenyl)phenyl)azo)benzoic acid (DABCYL), and the fluorophore, 5-((2-aminoethyl)amino)naphthalene-1-sulfonic acid (EDANS) [1, 7]. In addition, the use of substrates labeled with quencher-fluorophore pairs is associated with a labor-intensive process of their synthesis and low stability during long-term storage [1, 7, 15, 16]. In this regard, it seems urgent to develop new, highly efficient and convenient in

practical use systems for the determination of protease activity of M^{pro}, allowing to characterize the kinetic parameters of this enzyme and to analyze the inhibitory activity of new compounds promising for the treatment of COVID-19 [1, 16, 17].

Electrochemical systems based on the use of model peptides for the determination of the catalytic activity of proteases are of particular importance because of their high sensitivity and small volume of the analyte used [16, 18, 19]. A common principle of electrochemical systems for the determination of protease activity is the "Signal-off" approach, which allows to register the activity of proteases by signal reduction (current and/or peak area) of electrochemical oxidation or reduction of additional redox tags incorporated into peptides immobilized on the electrode surface. Despite the fact that such an approach is widely used in practice, the introduction of additional tags into the composition of peptides complicates the process of their synthesis, and can also affect the kinetic parameters of enzymes and reduce the stability of the analytical system [20-22]. Thus, actual task is to develop and increase the stability of electrochemical systems without the use of synthetic tags for the determination of protease activity.

A number of amino acids are known to be capable of irreversible electrochemical oxidation [23-27], which can be used to record the catalytic activity of proteases by electrochemical methods. Thus, tyrosine undergoes electrochemical oxidation according to Scheme 1.

Scheme 1. Putative mechanism of electrochemical oxidation of tyrosine

Previously, we developed an electrochemical system for the determination of trypsin activity and specificity based on the registration of the decrease in the peak area of electrochemical oxidation of the tyrosine residue of model peptides immobilized on the surface of a printed graphite electrode (PGE) after their proteolytic cleavage [28]. The advantage of this approach is that there is no need to introduce additional synthetic redox labeling during the peptide preparation process. We hypothesized that a similar approach could be applied to develop an electrochemical system for the determination of M^{pro} activity. Thus, the aim of the present work was to develop an electrochemical system for the determination of M^{pro} activity based on the registration of the peak area of the electrochemical oxidation of the tyrosine residue of a model peptide immobilized on the PGE surface.

MATERIALS AND METHODS

Reagents. The following reagents were used in this work: hydrochloric (III) gold-chloric acid trihydrate from "Alfa Aesar" (Germany); potassium dihydrophosphate (≥ 99%), potassium hydrophosphate trihydrate (≥ 99%), sodium chloride (99.5%) from "Acros Organics" (USA); glycerol (≥ 99%) from "PanReac AppliChem" (Spain); hydrochloric acid (30%) from Sigma-Aldrich (USA); N-9-fluorenylmethoxycarbonyl (Fmoc)-amino acids (≥ 99%), O-(1H-6-chlorobenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HCTU) (≥ 98%), 2,4,6-trimethylpyridine (TMP) (≥ 99%), 4-methyl-piperidine (Mpip) (≥ 98%) from Novabiochem (Germany); trifluoroacetic acid (99%), acetonitrile (≥ 99.9%), and anisole (99%) from Sigma-Aldrich (Germany); 3,6-dioxo-1,8-octandithiol (95%) from Sigma-Aldrich (USA); triisopropylsilane (98%) from Merck (Germany); N,N-dimethylformamide (DMF) (≥ 99.9%), methyl tert-butyl ether (≥ 98%) and petroleum ether 70-100 (≥ 95%) from "ECOS-1" (Russia); M^{pro} coronavirus SARS-CoV-2 (recombinant protein, lyophilized powder, purity by SDS-PAGE ≥ 90%) from "Sigma-Aldrich" (USA), Cat. # SAE0172. Lyophilized M^{pro} was diluted according to the manufacturer's recommendation in 100 μl of distilled water containing 10% glycerol. The resulting M^{pro} solution with a protein concentration of 59.2 μM was separated into aliquots and stored at -20 .°C

Synthesis of a model peptide. The peptide with the amino acid sequence CGGGAVLQSGY was prepared by solid-phase synthesis using Fmoc-protected amino acids in DMF, HCTU/TMP and Mpip on the Overture system (Protein Technologies, USA) as described previously [29]. Unprotection and peptide cleavage were performed by incubation with an acid cocktail (91.5% trifluoroacetic acid, 2.5% anisole, 2.5% water, 2.5% 3,6-dioxo-1,8-octandithiol, 1% triisopropylsilane) for 2 h at room temperature. Then an eightfold volume of a mixture of methyl tert-butyl ether and petroleum ether (1 : 2 by volume) was added to the peptide solution, and the resulting suspension was incubated for 30 min at− 20° C. Next, the peptide suspension was centrifuged at 4° C and 4000 *g* for 10 min, and then the supernatant was removed and the peptide was dissolved in a mixture of water-acetonitrile (1 : 1 by volume). The resulting peptide solution was analyzed by liquid chromatography and electrospray ionization mass spectrometry on an Agilent ChemStation 1200 Series chromatograph with an Agilent 1100 Series LC/MSD Trap XCT Ultra mass spectrometer. The resulting peptide was purified by high-performance liquid chromatography using an Agilent ChemStation 1200 Series chromatograph with an Agilent

6100 Series Quadrupole LC/MS mass spectrometer.

Electrodes and electrochemical equipment. In this work, PGE with graphite working (geometric area 0.0314 $cm^{2)}$ and auxiliary electrodes, as well as silver chloride (Ag/AgCl) reference electrode obtained from "ColorElectronics" (Moscow, Russia) were used.

Electrochemical measurements were performed using a μStat 400 potentiostat/galvanostat ("Metrohm Autolab BV", The Netherlands) with DropView 8400 software.

All potentials in this work are given relative to the Ag/AgCl reference electrode.

Modification of PGEs with gold nanoparticles (AtsNPs) and immobilization of model peptide. AtsNPs for the modification of working PGEs were obtained by electrosynthesis according to the method described previously [30]. Briefly: 60 μL of 5 mM $HAuCl_4$ in 0.1 M HCl was applied to the surface of PGE in a horizontal mode. Electrosynthesis was performed at a working electrode potential of− 0.5 V for 180 s. After electrosynthesis, the solution was washed off the electrode surface with distilled water. Immobilization of model peptide with amino acid sequence CGGGAVLQSGY on the surface of modified AtsNCH working electrodes was carried out due to the formation of chemical bonds between mercapto groups of *N-terminal* cysteine residues of model peptide and AtsNCH molecules. For this purpose, 50 μl of aqueous solution of the peptide with a concentration of 0.5-6 mM was applied to the surface of the working electrodes modified with АцНЧ. The electrodes with the applied peptide solution were incubated for 2 h at 4℃ . After incubation, the electrodes were washed from unbound model peptide with distilled water.

Determination of protease activity of M^{pro}. The protease activity of M^{pro} was determined by recording the decrease in the peak area of tyrosine residue electrooxidation in the model peptide CGGGAVLQSGY immobilized on the surface of PGE modified with acNCh (PGE/acNCh) due to proteolytic cleavage of the peptide bond formed by the carbonyl group of glutamine residue and the amino group of serine residue. For this purpose, 25 μl of 0.1 M potassium phosphate buffer (pH 7.4) containing 20% glycerol (by volume) and various concentrations of M^{pro} (0-1500 $nM^{)}$were applied to the surface of PGE/ACNCH with immobilized peptide. PGE/ACNH were incubated at 37℃ between 300-1200 s. After incubation, the solution was washed off the surface of the PGEs/AcNTs with distilled water. Next, 60 μl of 0.1 M potassium-phosphate buffer (pH 7.4) containing 50 mM NaCl was applied to the PGE/ACNF with immobilized peptide, followed by recording the area of the oxidation peak of tyrosine residues in the 0.55 V region by cyclic

voltammetry. Cyclic voltammetry voltammetries were recorded in the potential range from 0.35 to 0.7 V and scan rate of 50 mV/s. The areas of the peaks corresponding to the oxidation of the tyrosine residue of the immobilized peptide were calculated after bringing the voltammetric curve to the baseline using DropView 8400 software to a potentiostat/galvanostat. All electrochemical measurements were performed at room temperature (22± 3° C).

The surface concentration of the electroactive model peptide was calculated using Equation 1 [31]:

$$\Gamma_0 = \frac{Q}{nF\mathrm{A}}, (1)$$

where G_0 is the surface concentration of the electroactive model peptide on the electrode surface (mol/cm^2); Q is the charge calculated by integrating the oxidative peak of the tyrosine residue of the model peptide (Cl); n is number of electrons involved in the electrochemical process (equal to 2 for the tyrosine residue); F *is* the Faraday constant (96,485 Cl/mol); A is the surface area of the electrode (cm$^{2)}$.

G_0 was calculated at the initial time point and after time-dependent incubation of the immobilized model peptide on PGE/AcNCH with the enzyme ($G_{0,\,t}$).

The fraction of unreleased model peptide immobilized on the PGE/AcNCH surface was expressed as the ratio of $G_{0,\,t}$ to the G_0 value multiplied by 100%, according to Equation 2:

$$\text{Нерасщеплённый пептид}, \% = \frac{\Gamma_{0,\,t}}{\Gamma_0} \times 100\%, \qquad (2)$$

where $G_{0,\,t}$ is the surface concentration of the electroactive peptide on the PGE/AcNCH surface after time-dependent incubation with M^{pro} (mol/cm^2).

The fraction of cleaved model peptide after incubation with M^{pro} (θ) was calculated using Equation 3 [32]:

$$\theta = 1 - \frac{\Gamma_{0,\,t}}{\Gamma_0}, (3)$$

where θ is the fraction of cleaved model peptide.

The dependences of θ on incubation time (t) of the model peptide immobilized on PGE/ACNCH with different concentrations of M^{pro} were approximated by an exponential function according to Equation 4 [33]:

$$\theta = a[1 - e^{-tk_{\text{eff}}}], (4)$$

where a is the limiting value of the fraction of cleaved peptide at the individual concentration M^{pro}, which is the horizontal asymptote to which the graph of the dependence of θ on t at incomplete cleavage of the model peptide tends; e is the exponent (2.718); t is the incubation time (s); k_{eff} is the effective rate constant (c^{-1}).

The tangents of the initial linear sections of the dependences of θ on t at each concentration M^{pro} corresponded to the values of k_{eff}. Further, from the dependence of k_{eff} on the concentration of M^{pro} approximated by a hyperbolic function, the values of the catalytic constant (k_{cat}) and Michaelis constant (K_M) were determined in accordance with Equation 5 [32, 33]:

$$k_{eff} = \frac{k_{cat}}{1 + \frac{K_M}{[E]}}, (5)$$

where k_{cat} is the catalytic constant (from^{-1}), K_M is the Michaelis constant (M), [E] is the enzyme concentration M).

Mathematical and statistical processing of data. Curves were constructed and analyzed using OriginPro software (version 8.1). Statistical processing of the data was performed using Microsoft Office Excel 2019 software. All measurements were performed at least three times. Mean values ± standard deviations are presented.

RESEARCH RESULTS

Design of an electrochemical system for the determination of M^{pro} activity. PGEs modified with AtsNF (PGE/AtsNF) were chosen for the design of the electrochemical system. PGEs allow enzymatic activity assays that do not require labor-intensive preconditioning and allow the use of small volumes of analyte [34]. The possibility to modify PGEs with nanomaterials allows to create a surface suitable for immobilization of biological molecules, including enzymes, to miniaturize the analytical system, as well as to increase its sensitivity and selectivity [34]. AtsNCHs possess an optimal set of physicochemical properties such as electrical conductivity, biocompatibility, as well as the convenience of synthesis and functionalization by forming stable and strong bonds with thiol groups of biological molecules [35-38]. In this work, AtsNCHs were obtained by electrosynthesis at the working electrode by reducing $AuCl_4^-$ to $Au^0 + 4Cl^-$ from 60 μL of 5 mM $HAuCl_4$ in 0.1 M HCl at a− potential of 0.5 V for 180 s. The size of the obtained AtsNFs on the PGE surface was 100-700 nm, which we have previously shown using scanning electron microscopy (SEM) [28].

We modeled the sequence of the peptide as a substrate of M^{pro} for subsequent immobilization on the surface of PGE/AcNCH. The substrate specificity of M^{pro} was investigated in a number of studies and it was shown that the enzyme cleaves the peptide bond formed by the carbonyl group of glutamine, whereby the amino group must belong to an amino acid residue having a small radical (serine, alanine, or glycine) and, in addition, a hydrophobic amino acid residue (leucine, phenylalanine, or valine) must be located before the glutamine residue [39-42] (Scheme 2). The glutamine and amino acid residues at the P_1' and P_2 positions are most important for the protease activity of M^{pro}. The amino acid residues surrounding the described consensus sequence are less conserved and are essential for substrate recognition and stable substrate binding. It has been shown that the AVLQS sequence is most efficiently cleaved by M^{pro} under *in vitro* conditions [3, 43, 44].

Scheme 2. Fragment of a peptide cleaved by M^{pro}, where P_1 is a glutamine residue; P_1' is a small radical amino acid residue (serine, alanine, or glycine); P_2 is a hydrophobic amino acid residue (leucine, phenylalanine, or valine). The arrow shows the cleavage site under the action of M^{pro}

To immobilize the peptide on the PGE/ACNCH surface, *the N-terminal* cysteine residue was incorporated into the amino acid sequence of the modeled peptide, so that the mercapto group of cysteine formed a chemical bond with ACNCH [45, 46]. The amino acid sequence acting as a natural spacer gives the peptide greater mobility and provides a higher density of the peptide immobilized on the electrode surface [18, 47, 48]; therefore, three glycine residues were included in the model peptide. A tyrosine residue was chosen as a natural redox tag, which has the ability to undergo irreversible electrochemical oxidation, which can be recorded by electrochemical methods [27, 49, 50]. Thus , a peptide with the amino acid sequence CGGGAVLQSGY capable of cleavage under the action of M^{pro} to form octapeptide CGGGAVLQ and tripeptide SGY was modeled and obtained by solid-phase synthesis.

The design of AtsNF electrode modification and peptide immobilization is shown in Fig. 1.

Fig. 1. Modification of PGEs by ACNCH obtained by electrosynthesis and immobilization of the model peptide CGGGAVLQSGY due to the formation of a chemical bond between the mercaptogroup of cysteine and ACNCH. The arrow shows the site of peptide cleavage

under the action of M^{pro}

After incubation of PGE/ACNF with different concentrations of peptide (0-6 mM) and washing of non-immobilized molecules, a peak in the region of 0.55 V was recorded by cyclic voltammetry, corresponding to electrochemical oxidation of tyrosine residues of model peptide molecules (Fig. 2).

Fig. 2. Cyclic voltammetry voltammetry of PGE/AcNH after incubation with different concentrations of the model peptide CGGGAVLQSGY (0-6 mM) for 2 h at 4°C . Measurements were performed in 60 μL of 0.1 M potassium phosphate buffer (pH 7.4) containing 50 mM NaCl. The scanning speed was 50 mV/s. The arrow indicates the scanning direction

It is known that high peptide density on the electrode surface can lead to steric limitations of protease cleavage of the peptide, as well as reduce the signal-to-noise ratio in electrochemical measurements [51]. In the work of Anne et al. when immobilizing model peptides on the surface of Ultra-Flat TS-Gold Rotating Disc Electrodes for the study of trypsin and thrombin activity, it was shown that at G_0< 10 pmol/$cm^{(2),}$ a peptide density on the electrode surface was achieved that promoted efficient proteolysis by reducing the probability of intermolecular peptide interactions [32]. In this connection, we analyzed the dependence of G_0 on the concentration of peptide applied to the PGE/AcNF surface for immobilization (Fig. 3).

Fig. 3. Dependence of the surface concentration ($G_{0)}$ of the electroactive peptide CGGGGGAVLQSGY on its concentration in solution applied to PGE/ACNCH followed by incubation for 2 h at 4°C . Mean values from at least three repetitions of experiments ± standard deviations are presented

Based on the relationship presented in Fig. 3, we assumed that the 1.5 mM concentration of peptide applied to PGE/ACNCH, at which the G_0 value of 5.4± 0.1 pmol/cm^2 is reached, is optimal, because at higher values of G_0 the density of peptide on the electrode surface may prevent efficient protease cleavage, and a lower concentration

may be insufficient to record the kinetics of the enzymatic reaction due to faster depletion of the peptide. Thus, to further investigate the proteolytic activity of M^{pro}, the concentration of peptide applied on PGE/ACNCH for its immobilization was chosen to be 1.5 mM.

To evaluate the possibility of electrochemically recording the proteolytic activity of M^{pro}, we incubated PGE/AcNCH with immobilized peptide in the presence of different concentrations of M^{pro} (0-1500 nM) over a time range of 300-1200 s at 37°C . As can be seen in Fig. 4, a decrease in the current and peak area of electrochemical oxidation of tyrosine residue in the region of 0.55 V is recorded with increasing enzyme concentration after incubation for 1200 s, indicating proteolytic cleavage of the peptide with the release of tyrosine-containing fragment (SGY).

Fig. 4. Cyclic voltammetry voltammetry patterns of PGE/AcNH with immobilized model peptide CGGGAVLQSGY after incubation with different concentrations of M^{pro} (0-1500 nM) for 1200 s at 37°C . Measurements were performed in 60 µl of 0.1 M potassium phosphate buffer (pH 7.4) containing 50 mM NaCl. The scanning speed was 50 mV/s. The arrow indicates the scanning direction

According to Equation 2, we determined the value of the fraction of unsplit peptide (%) when it was incubated with different concentrations of M^{pro} (0-1500 nM) between 300-1200 s at 37°C (Fig. 5).

Fig. 5. Dependences of the fraction of unrepaired CGGGAVLQSGY peptide (%) on its incubation time (t) with different concentrations of M^{pro} (0-1500 nM). Mean values from at least three repetitions of experiments ± standard deviations are presented

The presented dependence of the fraction of unsplit peptide on t shows that the fraction of split peptide (θ)increases with increasing M^{pro} concentration. At the same time, after incubation of PGE/AcNCH with immobilized peptide with a solution without M^{pro}, there is practically no decrease in the peak area of electrochemical oxidation of the tyrosine residue of the model peptide in the given time range. The selected range of M^{pro} concentrations at which time-dependent cleavage of the peptide is registered was used for further determination of the kinetic parameters of M^{pro}.

Thus, the possibility of recording M^{pro} activity using the developed electrochemical system was shown.

We determined the analytical characteristics of the developed electrochemical system. The dependence of the fraction of unsplit peptide (%) on the logarithm of M^{pro} concentrations was linear and described by an equation of the form: $y = -(17.752 \pm 1.827)x + (101.19 \pm 4.69)$ with the value of $R^2 = 0.9303$ (Fig. 6). From this equation, the limit of detection (LOD)for M^{pro} was calculated considering three times the standard deviation of the mean value of the tyrosine oxidation peak area (3σ) as 44 nM. The coefficient of variation (CV) was calculated as 7% ($n = 3$) for an M^{pro} concentration of 1500 nM. For comparison, in Sondag et al. the LOD for fluorescence assay with a peptide sample containing the specific cleavage site of M^{pro} was in the range of 4-80 nM [52], and in Xu et al. the LOD value was 35 nM for titration with a self-cleavage fluorescent substrate to detect M^{pro} [53].

Fig. 6. Dependence of the fraction of unspliced peptide (%) on the logarithm of M^{pro} concentrations. Mean values from at least three repetitions of experiments are presented± standard deviations

Kinetic analysis of the protease activity of M^{pro}. To determine the parameters of the steady-state kinetics of M^{pro} toward the model peptide CGGGAVLQSGY using the developed electrochemical system, a mathematical model for quasi-saturated enzyme systems in which the concentration of M^{pro} exceeded the concentration value of the immobilized peptide was applied [54]. The dependence of θ on t in such heterogeneous enzyme systems is exponential and is described by Equation 6 [32]:

$$\theta = 1 - e^{-tk_{\text{eff}}} \quad (6).$$

However, in order for the experimentally obtained data to be consistent with this equation, the value of θ should reach 1, which would correspond to complete cleavage of the immobilized peptide. Since in such systems the complete cleavage of immobilized peptide most often does not occur, Equation 4 was used to approximate the dependence of θ on t, containing an additional variable a, corresponding to the maximum possible value of the fraction of cleaved peptide at a given concentration M^{pro}. For the immobilized model peptide at different concentrations of M^{pro}, the dependences of θ on t were obtained, as

shown in Fig. 7.

Fig. 7. Dependences of the fraction of cleaved model peptide CGGGAVLQSGY under the action of M^{pro} (θ) on the incubation time (t) with different enzyme concentrations (50-1500 nM). Mean values from at least three repetitions of experiments ± standard deviations are presented

The value of a after incubation of the model peptide immobilized on the PGE/ACNCH surface for 1200 s at a maximum concentration of M^{pro} equal to 1500 nM was calculated as 0.582, which is consistent with the assumption of incomplete cleavage of the peptide. Incomplete cleavage of immobilized peptide under the action of the enzyme can be explained by steric constraints arising from the uneven coverage of the peptide on the structurally heterogeneous surface of the working electrode, as well as the presence of electrochemically inert components in the composition of the working electrode that can affect the kinetics of heterogeneous electrochemical processes [55]. In addition, the dimeric form of M^{pro} is known to have protease activity, whereas the monomeric form has lower catalytic activity or is inactive [56-58]. Thus, the ratio of the dimeric and monomeric forms of M^{pro} in solution may also influence the kinetics of protease cleavage of the peptide in an electrochemical system.

From the obtained dependences of θ on t, the values of k_{eff} were calculated. The dependence of k_{eff} on the concentration M^{pro} had a hyperbolic character (R^2= 0.9948) and obeyed equation 5, which is characteristic of heterogeneous systems (Fig. 8).

Fig. 8. Dependence of the effective constant (k_{eff}) on the concentration of M^{pro} applied to the surface of PGE/AcNCH with immobilized CGGGAVLQSGY peptide. Mean values from at least three repetitions of experiments ± standard deviations are presented

From the resulting relationship shown in Fig. 8, we calculated k_{cat}, K_M, and the catalysis efficiency expressed as k_{cat}/K_M, which corresponded to values of $(3.1 \pm 0.1) \cdot 10^{-3}$ c^{-1}, $(358 \pm 32) \cdot 10^{-9}$ M, and 8659 c^{-1}/M, respectively. We compared the kinetic parameters of M^{pro} obtained using the developed electrochemical system with those obtained using alternative systems for the determination of M^{pro} activity based on fluorescence methods and LC-MS. For example, in Sacco et al. the kinetic parameters of M^{pro}, $M^{(pro)}$with a

histidine tag and M$^{(pro)}$containing additional *N-terminal* histidine and methionine residues were obtained using the FRET method with respect to the peptide substrate DABCYL-KTSAVLQSGFRKME(EDANS) [59]. The k_{cat} values for these enzymes ranged from 0.01 s^{-1} to 0.21 s^{-1}, $K_{()}$values ranged from 27.8 · 10^{-6} M to 53.1 · 10^{-6} M, and the k_{cat}/K_M values ranged from 214 $s^{-(1)}$/M to 6689 s^{-1}/M. In Rut et al. similar parameters were obtained for M^{pro} with respect to various fluorescently labeled peptides, including those containing non-proteinogenic amino acids (Ac-Abu-Tle-LQ-ACC, Ac-Thz-Tle-LQ-ACC, Ac-VKLQ-ACC) [40]. The k_{cat} values ranged from 0.050 from^{-1} to 0.178 from^{-1}, the $K_{(M)}$values ranged from 189.5 · 10^{-6} M to 228.4 · 10^{-6} M, and k_{cat}/K_M values ranged from 219 from$^{-(1)}$/M to 859 from^{-1}/M. Since M^{pro} had low activity toward tetrapeptides in this work, the authors synthesized the longer peptide substrates ACC-G-Abu-Tle-LQSGFRK$_{(}$DNP)K-NH_2, ACC-G-Thz-Tle-LQSGFRK$_{(}$DNP)-K-$NH_{(2)}$, and ACC-GVKLQSGFRK$_{(}$DNP)-K-NH_2 containing quencher-fluorophore pairs. For the corresponding substrates, k_{cat}/K_M values ranging from 6755 with^{-1}/M to 19424 with^{-1}/M were calculated. In Li et al, FRET and LC-MS methods were applied to determine the kinetic parameters of M^{pro} and labeled M^{pro}. The values of k_{cat}, K_M and $k_{(cat)}/K_M$ obtained using FRET with respect to the FRET substrate DABCYL-KTSAVLQSFRKME(EDANS) were calculated as 0.23± 0.01 with^{-1}, (34.2 ± 4,8) · 10^{-6} M and 6800 ± 976 s^{-1}/M for the unlabeled enzyme, respectively, and 0.9 ± 0.1 · 10^{-2} $s^{-(1)}$, (139 ± 22.2) · 10^{-6} M and 67.5± 11.8 s^{-1}/M for the labeled enzyme, respectively [7]. The values of similar parameters obtained in this work for unlabeled M^{pro} relative to TSAVLQSGFR peptide using LC-MS method were calculated as 2.2± 0.07 s^{-1}, (903.5 ± 86.9) · 0^{-6} M and 2444± 248 s^{-1}/M, respectively. In Abian et al. the values of k_{cat}, K_M and k_{cat}/K_M M^{pro} with histidine tagged against FRET substrate DABCYL-KTSAVLQSGFRKME(EDANS) were calculated as 0.040 s^{-1}, 11 · 10^{-6} M and 3640 s^{-1}/M, respectively [60]. Table 1 summarizes the steady-state kinetics parameters obtained using alternative analytical methods and the electrochemical system developed by us.

Table 1. Stationary kinetics parameters M^{pro} obtained using different analytical approaches

Enzyme	k_{cat}, from^{-1}	K_M, M	k_{cat}/K_M, from^{-1}/M	Substrate	Reference
Fluorescence methods					
M^{pro}	0,16	(27.8± 5.2)· 10^{-6}	5748	DABCYL-KTSAVLQSGFRKME(EDANS)	[59]
	0.178± 0.016	(207.3± 12)· $10^{-(6)}$	859± 57	Ac-Abu-Tle-LQ-ACC.	[40]

	0.144± 0.006	(189.5± 2.7)· 10^{-6}	760± 50	Ac-Thz-Tle-LQ-ACC.	
	0.050± 0.002	(228.4± 9.9)· 10^{-6}	219± 3	Ac-VKLQ-ACC	
	unlisted	unlisted	14748± 684	ACC-G-Abu-Tle-LQSGFRK(DNP)K-NH_2	
	unlisted	unlisted	19424± 1176	ACC-G-Thz-Tle-LQSGFRK(DNP)K-NH_2	
	unlisted	unlisted	6755± 208	ACC-GVKLQSGFRK(DNP)K-NH_2	
	0.23± 0.01	(34.2± 4.8)· 10^{-6}	6800± 976	DABCYL-KTSAVLQSFRKME(EDANS)	[7]
Sworn $M^{(pro).}$	(0.9± 0.1)· 10^{-2}	(139± 22,2)· 10^{-6}	67.5± 11.8	DABCYL-KTSAVLQSFRKME(EDANS)	[7]
	0,21	(30.9± 3.8)· 10^{-6}	6689	DABCYL-KTSAVLQSGFRKME(EDANS)	[59]
	0,01	(53.1± 8.1)· 10^{-6}	214	DABCYL-KTSAVLQSGFRKME(EDANS)	
	0,040	11· 10^{-6}	3640	DABCYL-KTSAVLQSGFRKME(EDANS)	[60]
LC-MS					
M^{pro}	2.2± 0.07	(903.5± 86.9)· 10^{-6}	2444± 248	TSAVLQSGFR	[7]
Electrochemical systems					
M^{pro}	(3,1± 0,1)· 10^{-3}	(358± 32)· 10^{-9}	8659	CGGGAVLQSGY	this work

Note. Abu, amino butyric acid; ACC7-amino-4-carbamoylmethylcoumarin; DNP, 2,4-dinitrophenyl; Thz, thiazolidine-4-carboxylic acid; Tle, 2-amino-3,3-dimethyl butyric acid.

DISCUSSION OF RESULTS

Coronavirus proteases, in particular, the major protease of coronaviruses M^{pro}, are potential targets for therapy of viral diseases, which determines the importance of studying the properties and searching for new highly effective inhibitors of these enzymes. Current methods for the determination of M^{pro} activity have a number of drawbacks, including the need to use labeled peptide substrates and the high labor intensity of the analytical process. In this regard, the development of new analytical approaches for the determination of M^{pro}

activity is an urgent task. We have developed an electrochemical system for the determination of M^{pro} activity based on the registration by cyclic voltammetry of the peak area of electrochemical oxidation of a tyrosine residue of a model peptide immobilized on the surface of PGE/ACNCH and used as a substrate. The model peptide contained an *N-terminal* cysteine residue for its covalent immobilization on the PGE/ACNCH surface, three glycine residues as a spacer, an AVLQS sequence (most efficiently cleaved by M^{pro} at the peptide bond formed by the carbonyl group of a glutamine residue and the amino group of a serine residue), and a *C-terminal* dipeptide consisting of glycine and tyrosine residues. The decrease in the peak area of the electrochemical oxidation of the tyrosine residue served as an analytical signal of the catalytic activity of M^{pro}. The LOD in the developed system was comparable to that of other systems for the determination of M^{pro} activity. Using the developed approach, we determined the steady-state kinetics parameters of M^{pro} toward the model peptide CGGGAVLQSGY. The k_{cat} and K_M values we obtained for M^{pro} relative to the model peptide CGGGAVLQSGY differed from similar parameters obtained using other analytical approaches. This seems to be due to the different amino acid sequence of the peptide substrates used in the present and the aforementioned works. In addition, in the electrochemical system, the peptide substrate is in an immobilized state on the electrode surface. However, the value of k_{cat}/K_M for M^{pro}, which was determined using the electrochemical system we developed, is within the range of values of this parameter obtained using the above alternative systems for the determination of M^{pro} activity (67.5-19424 with^{-1}/M).

The advantage of the developed approach is that there is no need to include an additional chemical label in the peptide substrate. We believe that the developed electrochemical system can be used to search for new M^{pro} inhibitors promising for the treatment of coronavirus infections.

AUTHORS' CONTRIBUTIONS

V.V. Shumyantseva, S.A. Moshkovsky, A.V. Kuzikov - concept and guidance of the study; T.A. Filippova, R.A. Masamreh, T.E. Farafonova, Y.Y. Khudoklinova - conducting experiments; T.A. Filippova, R.A. Masamreh, A.V. Kuzikov - writing the text of the article; T.A. Filippova, R.A. Masamreh, T.E. Farafonova, Yu. Shumyantseva, S.A. Moshkovsky, A.V. Kuzikov - editing the text of the article.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

ETHICS DECLARATION

This article does not describe any research involving humans or animals as subjects.

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