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Kallikrein immobilized on magnetic beads for activity-based assays using mass spectrometry

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A crucial step in drug discovery involves identifying active molecules, which depends on fast and efficient screening assay methods. Kallikreins a family of serine protease enzymes, play a pivotal role in biological fluids and tissues. Deregulated activity and expression of human KLKs have been implicated in various pathologies, so these enzymes constitute attractive biological targets for discovering molecules that can modulate their activity. The novelty of the present study is the IMER-pKLK-MB bioreactor resulting from immobilization of porcine pancreas kallikrein (pKLK) on magnetic beads which proved highly active and stable. For example, over 60% of IMER-pKLK-MB activity was maintained after it was incubated in 70% methanol. In addition, even after being stored for 11 months, IMER-pKLK-MB allowed for at least 10 consecutive cycles of activity, which attested to its excellent stability. Parameters such as K_{Mapp} and IC_{50} for leupeptin confirmed that the immobilized pKLK retained its ability to recognize both the substrate and reference inhibitor. We optimized an off-flow assay based on high-performance liquid chromatography coupled to mass spectrometry (HPLC-MS) and IMER-pKLK-MB to evaluate the inhibitory activity of some molecules toward pKLK. We also evaluated the kinetic parameter (K_{Mapp} = 81.2 ± 18 μmol.L^{-1}) and qualified the method by using leupeptin as standard inhibitor (IC_{50} = 2.15 ± 0.4 μmol.L^{-1}). The developed and qualified method proved an important and reliable approach for screening ligands and can be used to screen KLK inhibitors.

KEYWORDS

kallikrein, activity assay, immobilized enzyme, HPLC-MS, screening assay

Introduction

Identifying potentially active compounds is a critical step in drug discovery research and demands efficient screening assays. In this sense, an interesting approach is to develop analytical tools and assay strategies that allow enzyme inhibitors to be identified. Immobilized enzyme assays as an alternative to classic free enzyme assays offer advantages such as enhanced activity and stability in the assay conditions, allowing enzymes to be better handled and even reused in many screening cycles (de Moraes et al., 2019). Chromatographic supports such as magnetic particles (Trindade Ximenes et al., 2021; Vanzolini et al., 2015), Sepharose (de Carvalho et al., 2021), and silica gel microspheres (Li et al., 2019; Shi et al., 2015), have been used to prepare bioreactors for ligand screening assays. Such bioreactors can be employed to immobilize biomolecules by means of off-line or on-line approaches, resulting in different formats and applications (De Simone et al., 2019; Moraes et al., 2016; Girelli and Mattei, 2005; de Moraes et al., 2014). In general, the support must be inert and not retain biomolecules by adsorption (Hanefeld

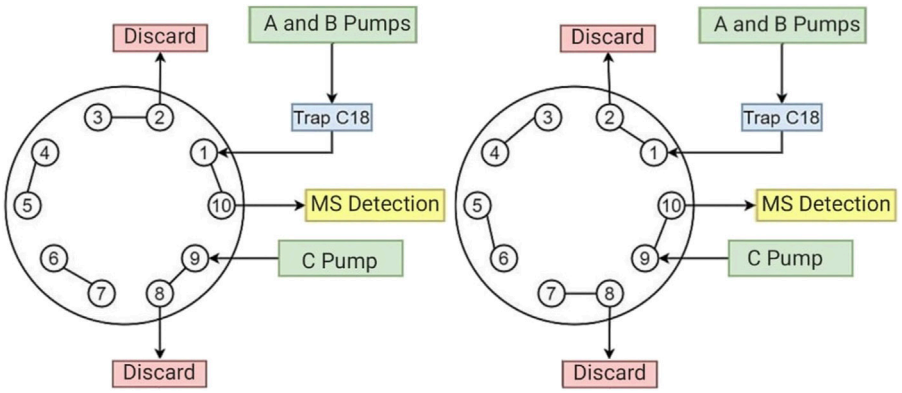


FIGURE 1
Schematic representation of the LC-MS system for monitoring the IMER-pKLK-MB activity. Source: Designed by the authors.

TABLE 1 LC method configuration.

Time (min)	Mobile phase (A:B v/v)	Flow rate A+ B (mL.min^{-1})	Event	Valve position
0.0–2.0	50:50	0.2	Enzymatic reaction analyses	1
2.01–5.0	0:100	1	Pre-column Cleanup	2
5.01–6.0	50:50	1	Return to initial condition	2
6.01–7.0	50:50	0.2		1
7.01	*Stop*			

Obs.: Pump C constant flow rate 0.2 mL/min.

et al., 2013). Several enzyme immobilization methods have been described in the literature, and covalent reactions involving amino, carboxyl, or epoxide functional groups have been the most widely reported (Cao, 2006). Bioaffinity chromatography, employed at various stages of drug development, is based on an enzyme immobilized on a solid support. The support stabilizes the enzyme in the presence of organic solvents and temperature variations, allowing small amounts of the enzyme to be used and reused. In addition, immobilized enzymes can be applied in numerous assay formats, including continuous process reactors on-flow assays (de Moraes et al., 2019; De Simone et al., 2019; Girelli and Mattei, 2005; De Oliveira et al., 2022; Guo et al., 2019; Hou et al., 2020) or off-flow systems (Liu et al., 2024; Carvalho et al., 2022; Miranda de Souza Duarte-Filho et al., 2023; Wubshet et al., 2019) to screen new ligands fast. Moreover, bioaffinity chromatography allows the kinetics, thermodynamics, and affinity of a ligand to be rapidly characterized and is considered a high-throughput screening (HTS) technique.

Kallikreins (KLKs), a family of serine proteases involved in many crucial biological functions (Yousef and Diamandis, 2003; Prassas et al., 2015) comprise 15 types and are found in human tissues and organs (KLK-1 to KLK-15), human plasma (KLK1B), and venom of certain snakes (Prassas et al., 2015; Felicori et al., 2003). The KLK family is known for the role played by KLK1 in the kallikrein-kinin system and the use of KLK3 as a biomarker of prostate cancer (Prassas et al., 2015; Tan et al., 2015). However, over the past decade, great advances have been made toward understanding the localization, regulation, and physiological functions of most tissue KLKs (Prassas et al., 2015; Tan et al., 2015; Kryza et al., 2016; Stefanini et al., 2015). Among other functions, KLKs are directly involved in the inflammatory process cascade. Patients with COVID-19 may present pulmonary edema early in the disease. In recent studies, this has been suggested to occur due to a local vascular problem associated with activation of the bradykinin 1 (B1R) and 2 (B2R) receptors in endothelial cells in the lungs. SARS-C oV-2 enters the cell via angiotensin-converting enzyme 2 (ACE2) which, among other functions, is necessary to inactivate des-Arg9 bradykinin, a potent B1R ligand. Without ACE2 acting as gatekeeper to inactivate B1R ligands, the pulmonary environment is subject to local vascular leak, which culminates in angioedema. Therefore, it has been proposed that blocking B2R and inhibiting KLK activity may have an ameliorative effect on early COVID-19 and may even prevent acute respiratory distress syndrome (ARDS) (van de Veerdonk et al., 2020). These associations have prompted research efforts to develop specific KLK inhibitors as therapeutic agents (Sotiropoulou and Pampalakis, 2012; Swedberg et al., 2010; Teixeira et al., 2011; de Souza et al., 2019). Researchers have been actively studying KLKs given that alterations in this enzyme family may lead to medical conditions such as cancer over time (Prassas et al., 2015). Due to structural similarities within the KLK family, in this study we have employed porcine pancreas KLK (pKLK) as a model enzyme to develop an active, stable, and applicable tool to screen KLK ligands. Studies have indicated that both human and

porcine KLK can cleave peptides derived from human and bovine kininogen (Del Nery et al., 1999). Bearing in mind the advantages of enzyme immobilization and the growing search for KLK inhibitors, here we present an off-line assay based on pKLK immobilized on magnetic beads (MB) and MS detection to screen inhibitors of this enzyme. We will demonstrate that the IMER-pKLK-MB bioreactor is a valuable, stable, and reusable tool for off-line ligand screening, and that it can be integrated with an HPLC-MS method.

Materials and methods

Chemicals and reagents

Porcine pancreas kallikrein (pKLK, 250 units) and amine-terminated magnetic particles (50 mg mL^{-1}) were purchased from Sigma-Aldrich (St. Louis, MO, United States). Buffer components and other chemicals were acquired from Sigma-Aldrich, Merck (Darmstadt, Germany), Synth (São Paulo, Brazil), or Acros (Geel, Belgium). The water used in all the preparations was obtained from a MILLI-Q® system (Millipore®, São Paulo, Brazil). All the chemicals and solvents used here were analytical or HPLC grade and were employed without any further purification.

Instrumentation and system configuration for analyses

The magnetic separator (model DynaMagTM-Spin Magnet) was purchased from Thermo Fisher Scientific (São Paulo, SP, Brazil). Orbital Microplate shaker (IKA, model MS3 D, Staufen, Germany). HPLC-MS analyses were carried out on a high-performance liquid chromatography system (model NexeraXR) purchased from Shimadzu (Kyoto, Japan) and equipped with three LC 20ADXR pumps, an SIL-20A automatic injector, a DGU-20A degasser, a 10-port two-position high-pressure switching valve (Valco Instruments Co. Inc. Houston, United States), and a CBM-20A system controller. The HPLC system was coupled to an ion trap mass spectrometer (model AmaZon speed) acquired from Bruker Daltonics (Bremen) and equipped with an electrospray ionization source (ESI) interface. The system was controlled with the Bruker Compass Hystar software (version 4.5), and data were acquired and analyzed by using the Compass DataAnalysis software (version 4.3).

IMER-KLK-MB preparation

Porcine pancreas kallikrein (pKLK) was immobilized on the surface of amine-terminated magnetic beads (MBs). Briefly, 20 mg of MBs was washed three times with 1 mL of 100 mM KH_2PO_4 buffer, pH 7.0. After each wash, the MBs were magnetically separated by using a magnetic separator, and the supernatant was removed. Then, 1 mL of glutaraldehyde (5% in 100 mM KH_2PO_4 buffer, pH 7.0) was added to the MBs and incubated under smooth rotation at room temperature for 3 h. After that, the supernatant was removed, and the MBs were washed again three times with 1 mL of 25 mM KH_2PO_4 buffer, pH 8.0. Next, 400 µL of pKLK solution was added to the MBs and incubated under smooth rotation at 4°C for 16 h. After immobilization, the supernatant was removed, and IMER-pKLK-MB was washed three times with 100 mM Tris-HCl buffer, pH 8.0, and stored in the same buffer (20 mg mL^{-1}) at 4°C until it was used.

Immobilization efficiency

After enzyme immobilization as described above, 100 µL of the supernatant was added to 100 µL of substrate Z-Phe-Arg-AMC (200 µM) and incubated for 15 min under agitation (800 rpm) at room temperature. Afterward, 5 µL of this solution was directly injected into the MS. Additionally, 100 µL of enzyme solution before the immobilization was incubated with 100 µL of substrate under the same conditions described above. Then, 5 µL of this solution was directly injected into MS. The ESI ionization parameters were as follows: positive ionization mode, SIM mode, capillary voltage = 4500 V, end plate voltage = 550 V, drying gas flow rate = 9 $L{\cdot}min^{-1}$, drying temperature = 275°C, and nebulizer pressure = 40 psi. The enzymatic reaction product, Z-Phe-Arg-OH, was monitored at m/z 456 $[M + H]^+$. We evaluated the immobilization efficiency on the basis of immobilization yield (IY) by applying the following equation:

$$IY\ (\%) = \left(\frac{A_0 - A_f}{A_0}\right) \times 100$$

where A_0 corresponds to the initial activity of the enzyme solution before immobilization, and A_f corresponds to the activity of the supernatant solution after the immobilization procedure.

LC-MS method for off-flow analysis of IMER-pKLK-MB activity

The LC-MS method was developed on the basis of a previous method described by Carvalho et al. (2022). LC analysis was carried out by using a Phenomenex Security Guard TM (model AJ04287) C18 micron guard cartridge (4.0 × 3.0 mm, Phenomenex, Torrance, California, EUA) connected to a 10-port/two-position valve in the LC-MS system to redirect the flow during pre-column cleaning to a waste line (Figure 1). The mobile phase consisted of water (solvent A and C) and methanol (solvent B); the injection volume was 5 µL. With the valve in position 1, the enzymatic reaction products were eluted from the trap column to the mass spectrometer with an initial mobile phase composed of 50% solvent B at a flow rate of 0.2 mL min^{-1}. After 2.0 min, the valve was switched to position 2, and solvent B in the mobile phase was increased to 100% at a flow rate of 1.0 mL min^{-1}, which was maintained until 5 min, to wash the column and to remove excess substrate. At 5.1 min, solvent B in the mobile phase was returned to 50% at a flow rate of 1.0 mL min^{-1} until 6.0 min. At 6.1 min, the valve was switched to position one again, under the initial condition. The total run time was 7 min. Pump C (solvent water) at a flow rate of 0.2 mL min^{-1} was used to deliver the mobile phase to the MS during the column washing step. All the analyses were performed at 21°C (controlled room temperature). The ESI ionization parameters were as follows: positive ionization mode, SIM mode, capillary voltage = 4,500 V,

end plate voltage = 550 V, drying gas flow rate = 9 L min^{-1}, drying temperature = 275°C, and nebulizer pressure = 40 psi. The enzymatic reaction product, Z-Phe-Arg-OH, was monitored at m/z 456 $[M + H]^+$.

The mobile phase and method configuration are shown in Table 1.

Method optimization

The IMER-pKLK-MB off-line assay conditions were optimized. More specifically, the optimal $AcNH_4$ concentration, pH, incubation temperature, incubation time, and IMER-pKLK-MB mass were determined.

$AcNH_4$ was tested at 5 or 10 mmol.L^{-1}, at pH 7.0, 8.0, 8.5, or 9.0, in triplicate. For this purpose, 0.5 mg of IMER-pKLK-MB (25 µL of the stock suspension) was added to a 0.5-mL conical microtube. IMER-pKLK-MB was washed three times with 200 µL of 5 mmol.L^{-1} $AcNH_4$, pH 9.0, to remove the storage buffer. After each wash, IMER-pKLK-MB was separated by using a magnetic separator for 1 min, and the supernatant was removed. Following the washing procedure, 75 µL of 5 or 10 mmol.L^{-1} $AcNH_4$ at pH 7.0, 8.0, 8.5, or 9.0, and 25 µL of 200 µmol.L^{-1} substrate solution (in $AcNH_4$ and at the tested pH) were added to IMER-pKLK-MB. The suspension was homogenized by vortexing and incubated under agitation at room temperature for 5 min. After incubation, the supernatant of the enzymatic reaction was removed by using a magnetic separator and analyzed by the LC-MS method described above. For the LC-MS analysis, 25 µL of the enzymatic reaction supernatant and 25 µL of 5 mmol.L^{-1} AcNH4, pH 9.0, were added to a vial; the injected volume was 5 µL. To optimize the incubation temperature (10, 26, or 37°C), incubation time (5, 15, or 30 min), and IMER-pKLK-MB mass (0.1 or 0.5 mg, corresponding to 5 or 25 µL of the stock suspension, respectively), 5 mmol.L^{-1} AcNH4, pH 9.0, was used, and the procedure described above was followed.

Method qualification

The method was qualified on the basis of criteria outlined in the literature (Cassiano et al., 2009; Food and Drug Administration, 2001) The linearity of the method was assessed by constructing a calibration curve. For this purpose, 0.1 mg of IMER-pKLK-MB was incubated with the following final substrate concentrations (n = 3): 6, 10, 15, 30, or 50 µmol.L^{-1} in 5 mmol.L^{-1} $AcNH_4$, pH 9.0, according to the procedure described above. Analyses were performed by using the LC-MS method described above. The curve was constructed by applying linear regression in Excel software, and the area of the product ion at *m/z* 456 $[M + H]^+$ was plotted against substrate concentration. The selectivity of the method was evaluated by incubating IMER-pKLK-MB with buffer only, i.e., without substrate, under the same conditions described above. The lower limit of quantification (LLQ) and the limit of detection (LD) were assessed by incubating 0.1 mg of IMER-pKLK-MB with the following final substrate concentrations: 1, 6, 10, or 15 µmol.L^{-1}. LLQ was defined as the lowest concentration that can produce precision expressed as relative standard deviation (RSD) of less than 20%, while LD was defined as the lowest concentration generating a signal greater than twice the baseline noise.

IMER-KLK-MB kinetic constant (KMapp)

To determine K_{Mapp}, 0.1 mg of IMER-pKLK-MB (equivalent to 5 µL of the stock suspension) was added to a 0.5-mL conical microtube. IMER-pKLK-MB was washed three times with 200 µL of 5 mmol.L^{-1} $AcNH_4$, pH 9, to remove the storage buffer. After each wash, IMER-pKLK-MB was magnetically separated by using a magnetic separator for 1 min, and the supernatant was discarded. Subsequently, IMER-pKLK-MB was incubated with 75 µL of 5 mmol.L^{-1} AcNH4, pH 9.0, and 25 µL of substrate at 1, 6, 10, 15, 30, 40, 50, 70, 200, or 300 µmol.L^{-1} in 5 mmol.L-1 $AcNH_4$, pH 9.0. Each substrate concentration was tested in triplicate. The suspension was homogenized by vortexing and incubated under agitation at room temperature for 5 min. After incubation, the enzymatic reaction supernatant was removed by using a magnetic separator and analyzed by the LC-MS method described above. For the LC-MS analysis, 25 µL of the enzymatic reaction supernatant and 25 µL of 5 mmol.L^{-1} $AcNH_4$, pH 9.0, were added to a vial; the injected volume was 5 µL. A curve was constructed by plotting the peak area of the product ion at m/z 456 $[M + H]^+$ as a function of substrate concentration. Data were fitted by using nonlinear regression into a Michaelis-Menten plot, and K_{Mapp} was obtained with the GraphPad Prism 8.0 software.

Inhibition studies

Inhibition studies were carried out by using leupeptin as standard inhibitor. To determine the half maximum inhibitory concentration (IC_{50}), 0.1 mg of IMER-pKLK-MB (equivalent to 5 µL of the stock suspension) was added to a 0.5-mL conical microtube. IMER-pKLK-MB was then washed three times with 200 µL of 5 mmol.L^{-1} $AcNH_4$, pH 9.0, to remove the storage buffer. After each wash, IMER-pKLK-MB was magnetically separated using a magnetic separator for 1 min, and the supernatant was discarded. Next, IMER-pKLK-MB was incubated with 65 µL of 5 mM $AcNH_4$, pH 9.0, 25 µL of 200 µmol.L^{-1} substrate (in 5 mmol.L^{-1} $AcNH_4$, pH 9.0), and 10 µL of leupeptin at different concentrations. The final leupeptin concentration, tested in triplicate, was: 0.5, 1, 2, 5, 10, 20, 40, 70, 100, 200, or 400 µmol.L^{-1}. As control, the same assay was performed without adding leupeptin. The suspension was then homogenized by vortexing and incubated under agitation at room temperature for 5 min. After incubation, the enzymatic reaction supernatant was removed using a magnetic separator and analyzed by the LC-MS method described above. The enzymatic activity was monitored by quantifying the area of the product ion at *m/z* 456 $[M + H]^+$. The enzymatic activities in the presence (Ai) and absence (A0) of leupeptin were compared, and the percentage of inhibition was calculated by employing the equation: %I = 100 - [(Ai/A0) × 100]. The inhibition curve was constructed by plotting the percentage inhibition versus the corresponding leupeptin

concentration, and IC50 was determined from the curve built by plotting [leupeptin] versus % inhibition with the GraphPad Prism 5.0 software.

To determine the operational stability of IMER-pKLK-MB, 0.5 mg of IMER-pKLK-MB (equivalent to 25 µL of the stock suspension) in a 0.5-mL conical microtube was subjected to 10 consecutive analysis cycles. IMER-pKLK-MB was then washed three times with 200 µL of 5 mmol.L^{-1} $AcNH_4$, pH 9.0, to remove the storage buffer. After each wash, IMER-pKLK-MB was separated using a magnetic separator for 1 min, and the supernatant was discarded. Following the washing procedure, 75 µL of 5 mmol.L^{-1} $AcNH_4$, pH 9.0, and 25 µL of 200 µmol.L-1 substrate solution (in 5 mmol.L^{-1} $AcNH_4$, pH 9.0) were added to IMER-pKLK-MB. The suspension was homogenized by vortexing and incubated under agitation at room temperature for 5 min. After incubation, the enzymatic reaction supernatant was removed by using the magnetic separator and analyzed by the HPLC-MS method described above, immediately after the reaction had ended. To analyze the enzymatic activity, 25 µL of the enzymatic reaction supernatant and 25 µL of 5 mmol.L^{-1} $AcNH_4$, pH 9.0, were added to a vial. The injected volume was 5 µL. To initiate a new reaction cycle, IMER-pKLK-MB was washed three times with 5 mmol.L-1 $AcNH_4$, pH 9, and the reaction procedure was restarted.

The storage stability of IMER-pKLK-MB was assessed by measuring its activity over time. The procedure and HPLC-MS method described above were followed. The evaluation periods included 1, 2, 3, 4, 5, 6, and 11 months after immobilization. A specific aliquot was set aside and used exclusively for this study.

Results and discussion

Compared to assays using enzymes in solution, assays with enzymes immobilized on magnetic particles are particularly useful for conducting ligand screening studies in an optimized manner. In this context, assays based on immobilized enzymes coupled with HPLC-MS systems have emerged as a promising alternative to colorimetric screening assays in microplates. In this study, we developed an off-line assay that couples HPLC-MS with pKLK-immobilized magnetic particles as an alternative method for ligand screening. The immobilization of pKLK was successful, resulting in a highly active bioreactor (IMER-pKLK-MB) with an immobilization efficiency of 97%.

Figure 2 shows the chromatograms of the reaction products recorded for the enzyme solution containing either the free enzyme or the supernatant after the enzyme immobilization. The product at *m/z* 456 $[M + H]^+$ was monitored. The solution remaining after enzyme immobilization did not produce any reaction product.

HPLC-MS analysis method development

We developed the HPLC-MS method to analyze reaction products while preventing ionic suppression or contamination of the ionization source by the substrate. To achieve this, we employed a C18 pre-column as a trap column, which effectively separated the products from the substrate and allowed only the products to enter the mass spectrometer, while the substrate was retained on the stationary phase. A 10-port, two-position switching valve was used for this purpose. In position 1, the reaction products are eluted from the trap column and transferred to the mass spectrometer using a methanol mobile phase (1:1, v/v), as detailed in Table 1. Changing the valve to position two allows excess substrate to be removed by washing the pre-column with mobile phase of increasing strength, containing up to 100% solvent B. We evaluated the enzymatic activity by integrating the area of the extracted ion chromatograms (EIC) for the product Z-Phe-Arg-OH, at *m/z* 456 $[M + H]^+$, which showed a more intense signal compared to the other product (AMC, at *m/z* 176 with $[M + H]^+$). Scheme 1 illustrates the catalytic reaction involving pKLK.

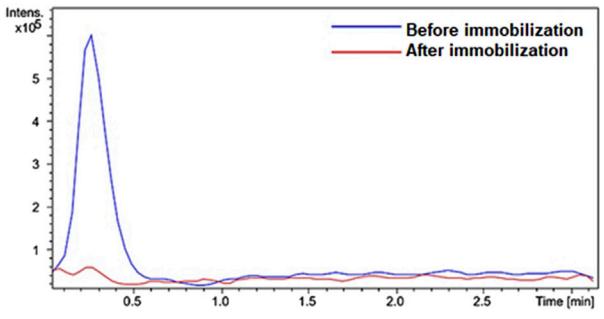


FIGURE 2
Overlap of peaks in the extracted ion chromatograms recorded for IMER-pKLK-MB activity. The HPLC-MS conditions are described in the experimental section. Source: Designed by the authors.

Figure 3 displays the chromatogram obtained when we analyzed the IMER-pKLK-MB activity by using the developed LC-MS method. By applying the method, we successfully separated the reaction products from the substrate and effectively removed most of the substrate from the system, thereby minimizing potential issues related to ionic suppression resulting in a fast analysis that lasted only 6 min.

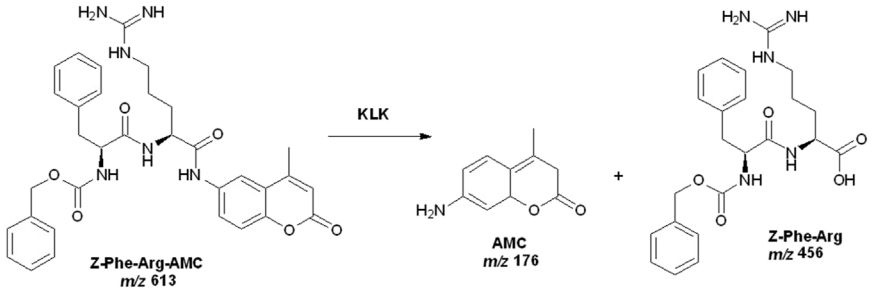
Method optimization

Buffer

Buffer conditions influence the enzymatic activity. As illustrated in Figure 4A, unfavorable buffer concentration or pH causes the enzymatic activity to be reduced. Additionally, during LC-MS analyses, using low buffer concentration enhances sensitivity and reduces ionic suppression by the matrix (García-Moreno et al., 1991). Here, we achieved optimal IMER-pKLK-MB activity by using 5 mmol.L^{-1} $AcNH_4$ and pH 9.0.

Temperature

The IMER-pKLK-MB activity increased slightly, but not significantly, upon rising temperatures, as shown in Figure 4B. Therefore, we selected the room temperature to conduct further assays given that the temperature in the laboratory was approximately 26°C. This temperature was sufficient to maintain the enzymatic activity and dismissed additional temperature control, thereby simplifying the experimental procedure.



SCHEME 1
Catalytic reaction involving pKLK and the substrate Z-Phe-Arg-AMC. Source: Designed by the authors.

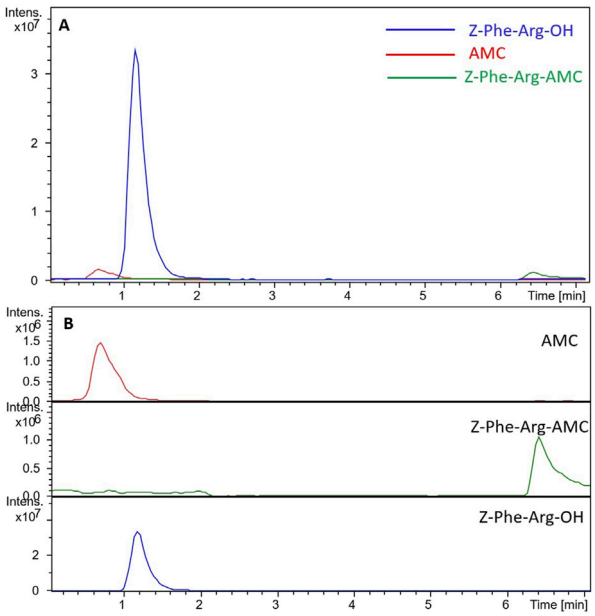


FIGURE 3
Chromatograms obtained during analysis of IMER-pKLK-MB activity **(A)** Superposition of the substrate and reaction product chromatograms **(B)** Separate chromatograms of the substrate and reaction products. Source: Designed by the authors.

Incubation time

Longer incubation yielded a greater amount of reaction product, with significant difference between incubation times of 5 and 15 min, as shown in Figure 4C. However, even at 5 min, the amount of product was more than sufficient for detection in the mass spectrometer. To avoid exceeding the MS detection limits, we diluted the reaction solution 5 mmol.L^{-1} $AcNH_4$, pH 9.0, at a 50: 50 v/v ratio, before analysis.

Bioreactor mass

We found that 0.1 mg of IMER-pKLK-MB was sufficient to obtain a measurable product area within 5 min of reaction, as illustrated in Figure 4D. Therefore, we employed 0.1 mg of IMER-pKLK-MB hereafter, which ensured that the method was sensitive, and that the available resources were effectively used.

Off-flow activity assay protocol

We established the assay protocol on the basis of the previously optimized conditions. In a 0.5-mL conical microtube, we added 0.1 mg of IMER-pKLK-MB (equivalent to 5 µL of the stock suspension). We washed IMER-pKLK-MB three times with 200 µL of 5 mmol.L^{-1} $AcNH_4$, pH 9, to remove the storage buffer. After each wash, we magnetically separated IMER-pKLK-MB by using a magnetic separator for 1 min and discarded the supernatant. Subsequently, we added 75 µL of 5 mmol.L^{-1} AcNH4, pH 9, and 25 µL of 200 µmol.L^{-1} substrate solution (in 5 mmol.L^{-1} $AcNH_4$, pH 9.0) to IMER-pKLK-MB. We homogenized the suspension by vortexing and incubated it under agitation at room temperature for 5 min. After incubation, we removed the enzymatic reaction supernatant by using a magnetic separator and analyzed it by the LC-MS method (Table 1). To analyze the enzymatic activity, we added 25 µL of the enzymatic reaction solution and 25 µL of 5 mmol.L^{-1} $AcNH_4$, pH 9.0 to a vial. The injection volume was 5 µL.

Determination of K_{Mapp}

The IMER-pKLK-MB activity as a function of substrate concentration revealed a Michaelian-type curve. This behavior indicates that the IMER-pKLK-MB activity exhibits a hyperbolic dependence on substrate concentration, thus following Michaelis-Menten kinetics. In previous studies by our group, we found that free pKLK in solution has K_{Mapp} of 23.5 ± 3.3 µmol.L^{-1} (de Carvalho et al., 2021). We found that IMER-pKLK-MB has K_{Mapp} of 81.2 ± 18 µmol. L^{-1} (Figure 5), so immobilized pKLK can still recognize its substrate.

Determination of inhibitory potential

To validate the assay as a tool to screening inhibitors, we selected the reference inhibitor leupeptin. Previous studies identified that leupeptin has IC_{50} of 1.62 ± 0.18 µM for the free enzyme in solution and 0.13 ± 0.01 µM for IMER-KLK-Sepharose-NHS, determined through fluorescence detection assays (de Carvalho et al., 2021). Here, we obtained IC_{50} of 2.15 ± 0.4 µmol.L^{-1} concerning IMER-pKLK-MB inhibition by leupeptin. The IC_{50} values vary when the test conditions are modified, so it is a relative comparison parameter (Holdgate et al., 2018). Thus, pKLK immobilization did not affect its ability to recognize the inhibitor Figure 6.

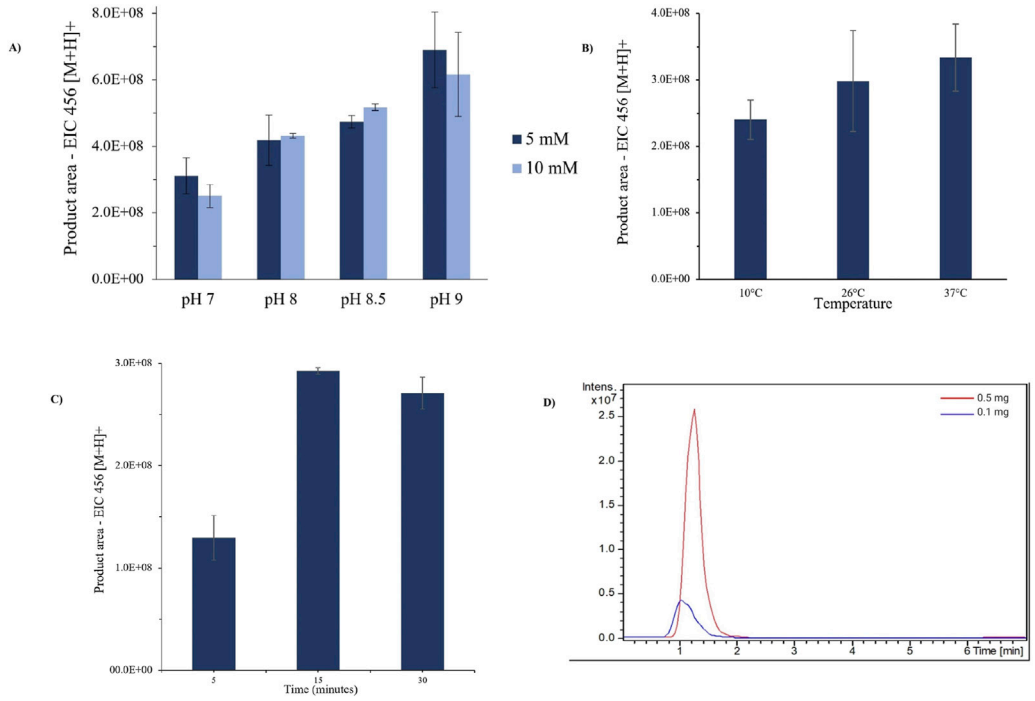


FIGURE 4
(A) Effect of $AcNH_4$ buffer conditions on IMER-pKLK-MB activity. **(B)** Effect of temperature on IMER-pKLK-MB activity. **(C)** Effect of incubation time on IMER-pKLK-MB activity **(D)** Effect of IMER-pKLK-MB mass on IMER activity. Source: Designed by the authors.

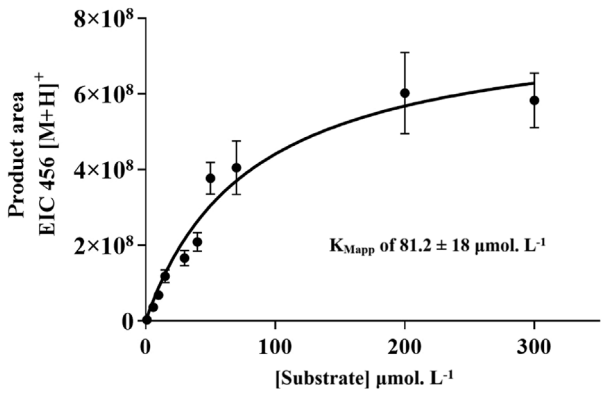


FIGURE 5
IMER-pKLK-MB activity upon varying substrate concentration: Michaelian curve for determining K_{Mapp}. Source: Designed by the authors.

Bioreactor stability

Stability between consecutive cycles of activity

IMER-pKLK-MB kept its high activity even after 10 consecutive analysis cycles, as illustrated in Figure 7A. This finding indicates that the analytical method was robust, that pKLK was not significantly lost during the washing steps, that immobilization was successful, and that pKLK was securely anchored to the support.

Storage stability

Evaluation of the storage stability of IMER-pKLK-MB revealed it was highly stable in 100 mM Tris-HCl buffer at pH 8.0 even after 11 months elapsed since it was immobilized. These results indicate that immobilization effectively preserved the IMER-pKLK-MB activity over an extended period. Additionally, we observed that the IMER-pKLK-MB activity increased during the first months following immobilization probably because pKLK was adapting to its new environment and adjusting its active conformation in the immobilized state. However, after this initial period, the IMER-pKLK-MB activity remained at a high level, which means that the immobilized enzyme sustained (Figure 7B). These results highlight one of the main advantages of immobilized enzymes: their reuse in multiple reaction cycles, which provides both economic and environmental benefits.

Method qualification

We assessed the linearity of the method by constructing a calibration curve in which we plotted the product area as a function of substrate concentration. The calibration curve demonstrated a linear response for substrate concentrations ranging from 1 to 50 $\mu mol.L^{-1}$. The regression equation was y = 3606771.127x + 5817619.093; the coefficient of determination (R^2) was 0.999 (n = 3). The RSD values for the curve construction were less than 19% for all triplicates. We confirmed that the method was selective by noting the absence of a detectable signal for the product at *m/z* at 456 $[M + H]^+$ with when we incubated only the buffer with IMER-pKLK-MB under the same assay conditions. The limit of

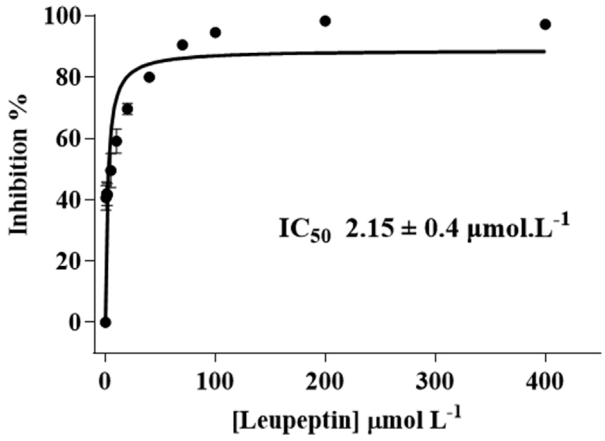


FIGURE 6
Dose-response curve plot of inhibition percentage for IMER-pKLK-MB in the presence of leupeptin. Source: Designed by the authors.

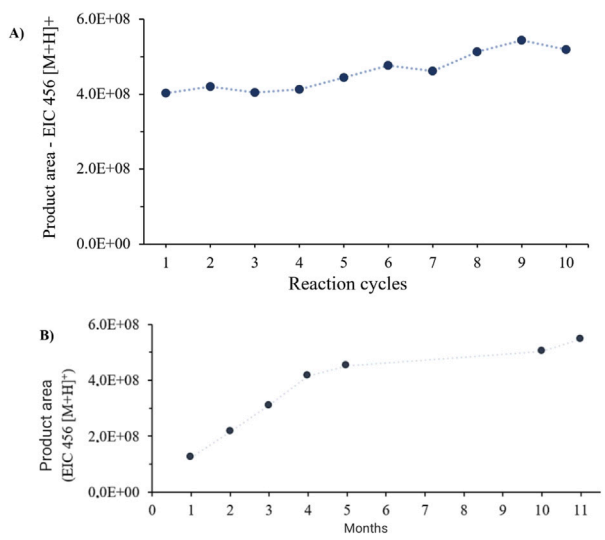


FIGURE 7
(A) IMER-pKLK-MB activity over 10 consecutive analysis cycles. **(B)** IMER-pKLK-MB activity along time. Source: Designed by the authors.

detection (LD) and lower limit of quantification (LLQ) were 1 and 6 µmol.L-1, respectively. On the basis of literature parameters, the developed method proved adequate for monitoring the IMER-pKLK-MB activity.

Compared to traditional kallikrein activity assays that use the enzyme in solution with fluorimetric detection, the proposed method offers several advantages. It allows for enzyme reuse, reducing costs and increasing storage stability, and is effective in the presence of organic solvents. This is particularly beneficial for ligand screening applications, as it enables testing of a broader range of samples, including those that are insoluble in aqueous solutions. Additionally, samples that present fluorescence can interfere with fluorimetric assays, leading to false positives (Simeonov, 2018). This issue is avoided by using mass spectrometry, which offers high selectivity, sensitivity, and efficiency.

Conclusion

IMER-pKLK-MB displays high activity and stability even after being stored for 11 months. It also exhibits excellent operational stability, which allowed it to be reused for at least 10 consecutive cycles of activity. IMER-pKLK-MB proved remarkably stable in the presence of organic solvents—60% and 10% activity was recovered after it was incubated in 70% and 100% methanol for 15 min, respectively. Additionally, the K_{Mapp} and IC_{50} parameters regarding leupeptin confirmed that IMER-pKLK-MB can still recognize both the substrate and the reference inhibitor. The developed LC-MS method proved suitable and reliable for monitoring the IMER-pKLK-MB activity, effectively overcoming ion suppression and contamination of the ESI source caused by the substrate.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

CR: Formal Analysis, Investigation, Methodology, Validation, Writing–original draft. CC: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Visualization, Writing–review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Generative AI statement

The author(s) declare that no Generative AI was used in the creation of this manuscript.

Publisher's note

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