α-Amylase Inhibitor Peptides Derived from Goat Casein Tryptic Hydrolysate

Nurwardian Aulyawati[1], Chairil Anwar[1,2], and Tri Joko Raharjo[1*]

[1]*Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Gadjah Mada, Sekip Utara, Yogyakarta 55281, Indonesia*

[2]*Department of Medical Laboratory Technology, Universitas Aisyiah Yogyakarta, Jl. Siliwangi No. 63 Nogotirto, Gamping, Sleman, Yogyakarta 55292, Indonesia*

* ***Corresponding author:***

email: trijr_mipa@ugm.ac.id

Received: February 26, 2025
Accepted: September 11, 2025

DOI: 10.22146/ijc.105030

Abstract: *This study explores the potential of bioactive peptides derived from hydrolyzed goat milk casein as α-amylase inhibitors, with implications for diabetes management. Goat milk casein was hydrolyzed using trypsin at varying enzyme-to-protein ratios to achieve different degrees of hydrolysis (DH). The optimal enzyme-to-protein ratio of 1:40 yielded the highest DH (31.31%). The resulting hydrolyzates were fractionated using strong cationic exchange solid-phase extraction (SCX-SPE), and their α-amylase inhibitory activity was evaluated. The fraction collected at pH 5 exhibited the highest α-amylase inhibition, with an inhibition value of 76.88% and an IC_{50} of 221.69 μg/mL. This fraction also contained the highest total peptide mass (1,208.9 μg). The enzyme kinetic study demonstrates an uncompetitive inhibition by fraction 5. Molecular docking simulations revealed that peptides from the most active fractions, including EGIEELLTGTIR, SSPSKHQPPPIR, and LLPRKAK, interact with key residues in the active site of α-amylase. The docking results demonstrated favorable binding energies and stable interactions with critical binding pockets, indicating the peptides strong potential as α-amylase inhibitors. Furthermore, the docking analysis revealed that these peptides interact with both the active site and other regions outside the active site, indicating an uncompetitive inhibition mechanism.*

Keywords: *bioactive peptides; α-amylase inhibition; goat milk casein hydrolysate; uncompetitive inhibitors*

■ INTRODUCTION

Bioactive peptides are protein fragments with specific amino acid sequences that can benefit human health. The biological activity of these peptides is primarily determined by the composition and sequence of amino acids within the peptide structure, which in turn dictates their biological properties [1]. Milk is a primary source of bioactive peptides and has been recognized as a promising ingredient for developing functional foods to improve health [2]. Among various types of milk, goat milk is distinguished by its unique biological properties, including a higher buffering capacity, distinct alkalinity, and superior digestibility, due to its smaller fat globule size (approximately 3.49 μm). Additionally, the proteins in goat milk are generally more digestible, facilitating more efficient absorption of amino acids compared to cow's milk [3].

Bioactive peptides can be generated from milk proteins through enzymatic hydrolysis using digestive enzymes, such as pepsin, trypsin, and papain. Enzymatic hydrolysis is preferred due to its high precision, shorter reaction times, and versatility in application. For example, a previous study successfully identified bioactive peptides from the casein and whey fractions of goat milk proteins through hydrolysis with pepsin, trypsin, and papain enzymes [4-5]. Similarly, other research has employed enzymatic hydrolysis to investigate whey and casein protein hydrolysates from camel and cow milk, which were treated with protease and alcalase enzymes [6].

Selecting an appropriate protease is crucial in determining the nature and outcomes of milk protein hydrolysis. The trypsin enzyme has been widely used to identify a range of bioactive peptides. Trypsin is particularly favored for its specificity in cleaving peptide bonds involving lysine and arginine residues [1]. Milk protein-derived peptides have been recognized as promising to address various lifestyle-related health disorders, including diabetes [7]. For example, a previous study successfully identified specific bioactive peptides from goat milk casein hydrolysates, which improved insulin resistance in high-glucose-induced HepG2 cells [8].

Diabetes mellitus is a severe metabolic disorder characterized by elevated blood glucose levels that exceed normal limits [9]. Type 2 diabetes (T2DM) arises from insulin resistance and impaired secretion, increasing blood glucose levels [10]. Although various pharmacological treatments are available for T2DM, some, such as thiazolidinediones and metformin, may induce adverse effects, including weight gain, hypoglycemia, and gastrointestinal disturbances [8]. One approach to managing diabetes mellitus involves slowing glucose absorption by inhibiting the activity of the α-amylase enzyme [9]. This enzyme catalyzes the hydrolysis of complex carbohydrates, such as glycogen and starch, into simpler sugars, such as maltose and glucose, which are subsequently absorbed into the bloodstream. α-Amylase is predominantly expressed on the surface of intestinal cells, and excessive enzyme activity can contribute to elevated blood glucose levels. Therefore, inhibiting α-amylase activity can slow the conversion of carbohydrates into glucose, potentially aiding in reducing blood sugar levels [11].

■ EXPERIMENTAL SECTION

Materials

The materials used in this study were as follows: Etawa crossbred goat milk was obtained from Den Farm, while ammonium bicarbonate (NH_4HCO_3), trypsin, bicinchoninic acid (BCA), bovine serum albumin (BSA), hydrochloric acid (HCl), sodium hydroxide (NaOH), and methanol were purchased from commercial suppliers. The Amicon Ultra-15 centrifugal filter for protein concentration and the Supelco DSC-SCX Cartridge for solid-phase extraction were obtained from Sigma Aldrich. For the α-amylase inhibition assay, the following reagents were used: amylum, α-amylase, iodine, sodium phosphate dibasic dihydrate ($Na_2HPO_4{\cdot}2H_2O$), and monobasic sodium phosphate hydrate ($NaH_2PO_4{\cdot}H_2O$).

Instrumentation

The instruments used in this study were a Shimadzu UV-vis spectrophotometer for absorbance measurements and a liquid chromatography high resolution mass spectrometry (LC-HRMS) system with Proteome Discoverer ver. 2.5 software for peptide analysis, a Sorvall Biofuge Primo R centrifuge for sample centrifugation, and a vortex mixer for sample mixing were obtained from Thermo Scientific. Additional instruments were used, i.e., a pH meter for pH measurements, a vacuum freeze dryer (Labfreez FD-10-MR) for sample drying, and a Sakura incubator oven for temperature-controlled incubations.

Procedure

Casein protein isolation from goat milk

Casein was isolated using a modified method based on Mohsin et al. [12]. Initially, milk was heated to 45 °C and centrifuged at 1,650 g for 30 min to separate the fat layer, which was subsequently removed. The skimmed milk was then adjusted to room temperature, and the pH was adjusted to 4.2 using 1 M HCl to induce casein precipitation. The mixture was stirred for 30 min and centrifuged at 1,650 g for 15 min to recover the casein precipitate. The precipitate was then washed by suspending it in purified water (10 times the initial volume of milk) and stirring for 1 h before re-centrifugation. All centrifugation steps were performed at room temperature. Finally, the isolated casein was lyophilized and stored at −20 °C until further analysis.

Hydrolysis of goat milk casein

A total of 60 mg of casein was dissolved in 5 mL of 100 mM NH_4HCO_3 solution. The mixture was then vortexed to ensure thorough mixing, followed by

sonication for 60 min to facilitate complete dissolution, according to the method described by Ningsih et al. [13].

$$DH = \frac{\text{Abs of hydrolyzed proteins} - \text{Abs of hydrolyzed proteins}}{\text{Abs of hydrolyzed proteins}} \times 100\% \quad (1)$$

The samples with the highest degree of hydrolysis (DH), which was calculated using Eq. (1), were subsequently filtered using Amicon Ultra-15 Centrifugal Filter Devices to separate the peptides from larger molecular weight components. This was achieved through centrifugation at 4000 g for 40 min. The resulting filtrate was then collected and used for further fractionation and analysis.

Fractionation of peptides from protein hydrolysate

The hydrolysate was fractionated using a DSC-SCX SPE cation exchange column, as described by Raharjo et al. [14]. The column was conditioned with 5 mL of 2 M HCl and citrate buffer (pH 3) for 15 min each. Subsequently, 5 mL of the sample (pH 3) was loaded onto the column and incubated for 30 min at room temperature. Stepwise elution was performed using citrate buffer (0.2 M, pH 3–6) and phosphate buffer (0.2 M, pH 7–9), with 5 mL of each buffer being used. The eluted fractions were collected in separate containers, and the column was washed with 100% methanol and distilled water between buffer changes. Further purification of the fractions was performed using a 5 mL HyperSep Retain PEP column, as described by Ningsih et al. [12]. The column was conditioned with 1 mL of 100% methanol and 2 × 1 mL of distilled water. The sample (5 mL) was incubated for 20 min, followed by stepwise elution with 5 mL of 100% methanol, repeated four times. The column was washed with 2 × 1 mL of 5% methanol. The peptide concentration in each fraction was determined by measuring the absorbance at 562 nm. The purified hydrolysate was then air-dried for 3 days.

Assessment of α-amylase inhibition

The α-amylase inhibitory activity was determined using a modified procedure based on the methods described by Mudgil et al. [7] and Rafique et al. [15]. A 0.5 g/L amylum solution was prepared by dissolving 25 mg of amylum in 50 mL of phosphate buffer (pH 6.9), followed by heating at 70 °C to facilitate dissolution. An α-amylase enzyme solution (50 µL/mL) was prepared by dissolving 1 mg of α-amylase in 40 mL phosphate buffer (pH 6.9). The reaction mixture, comprising buffer, enzyme, sample, and substrate, was incubated at 37 °C for 10 min to allow for enzyme-substrate interaction. The reaction was then terminated by heating at 85 °C for 15 min. Subsequently, 3 µL of 0.2% iodine solution was added to the reaction mixture, and the absorbance was recorded at 570 nm.

Determination of α-amylase IC_{50} inhibition of the peptide fractions

The IC_{50} value was determined for the fraction exhibiting the highest inhibition percentage by evaluating concentrations ranging from 31.25 to 1,000 ppm. The assay was performed according to the standard α-amylase inhibition protocol, where absorbance was measured at 562 nm using a UV-vis spectrophotometer. Inhibition percentages were calculated from the absorbance data using Eq. (2);

$$\%\text{inhibition} = \frac{\left(A_{control} - A_{test}\right)}{A_{control}} \times 100\% \quad (2)$$

where $A_{control}$ = absorbance of the blank control and A_{test} = absorbance of the test sample. The IC_{50} value was the concentration required to inhibit 50% of the α-amylase activity.

Study of peptide fraction inhibition type

The Michaelis-Menten constant (K_m) and the maximum reaction rate (V_{max}) were determined using a modified method based on the work of Sun et al. [16]. A range of amylum substrate concentrations (0.125, 0.250, 0.375, 0.500, 0.750, and 1.000 mg/mL) was used to assess enzyme kinetics. For each substrate concentration, a 100 µL aliquot was mixed with 20 µL of the sample (inhibitor) and 20 µL of the α-amylase enzyme solution. The mixture was then incubated at 37 °C for varying intervals (0, 2, 4, 6, 8, and 10 min). The reaction was terminated by heating at 85 °C for 10 min, followed by the addition of 5 µL of iodine solution. The absorbance was measured at 570 nm using a UV-vis spectrophotometer. The K_m and V_{max} values were calculated using the Lineweaver-Burk equation in Eq. (3);

$$\frac{1}{V_0} = \frac{K_m}{V_{mas}[S]} + \frac{1}{V_{max}} \quad (3)$$

where V_0 = the initial reaction rate, [S] = the substrate concentration, K_m = the Michaelis-Menten constant, and V_{max} = the maximum rate of the reaction. The type of inhibition on α-amylase was identified by analyzing the changes in K_m and V_{max} values using the Lineweaver-Burk method. The procedure for determining K_m and V_{max} was identical to that previously described, except that 20 µL of phosphate buffer was used as a control in place of the inhibitor. The inhibition type was determined by comparing the K_m and V_{max} values obtained from systems with and without the inhibitor. This allowed for the distinction between competitive, non-competitive, and uncompetitive inhibition mechanisms.

Identification of active peptides using LC-HRMS

The peptide fraction exhibiting the highest α-amylase inhibitory activity was subjected to LC-HRMS analysis to identify the peptides. A 5 µL sample was injected into the LC-HRMS system, which was equipped with an Acclaim PepMap 100 C18 column (1.0 × 150 mm, 3 µm, 100 Å). Elution was performed using a gradient of mobile phase A (water with 0.1% formic acid) and mobile phase B (acetonitrile with 0.1% formic acid) at a 0.1 mL/min flow rate. Ionization was achieved using an ESI source set at 3800 V, with a transfer tube temperature of 320 °C. The analysis was conducted in full-scan mode (*m/z* 150–2000) for peptide ion detection, followed by data-dependent MS2 (dd-MS2) mode for fragmentation and sequence identification. The resulting data were analyzed using Thermo Xcalibur and Proteome Discoverer 2.5 software, concerning the *Capra hircus* protein database obtained from UniProt (https://www.uniprot.org/).

Molecular docking of the peptides to α-amylase

Molecular docking simulations were performed to investigate the interaction mechanism between peptide molecules and α-amylase, and to assess their potential for inhibiting enzyme activity. Peptide fragments (5–15 amino acids) derived from LC-HRMS analysis were modeled in three dimensions using PEP-FOLD4 and saved in .pdb format. The crystal structure of α-amylase (PDB ID: 1AMY) with a resolution of 2.80 Å was retrieved from the RCSB Protein Data Bank (https://www.rcsb.org/structure/1AMY) and optimized using UCSF Chimera 1.14 and Biovia Discovery Studio 2020 [17-18]. Two docking approaches were employed: CABS-dock (https://biocomp.chem.uw.edu.pl/CABSdock), which generated the top 10 clusters, and HADDOCK2.4 (https://rascar.science.uu.nl/haddock2.4/), which generated the top 4 clusters based on peptide sequences and protein structure [19-20]. The affinity energy of the top clusters was analyzed using PRODIGY [21]. Binding analysis was conducted using Biovia Discovery Studio 2020, with interaction precision assessed based on RMSD values.

■ RESULTS AND DISCUSSION

Fractionation of Casein Hydrolysate

The hydrolysis of casein in this study highlights the significant influence of the enzyme-to-protein ratio on the efficiency of casein breakdown into smaller peptides. The experimental results demonstrate that a trypsin-to-protein ratio of 1:40 yielded the highest DH compared to ratios of 1:10 and 1:20. This observation suggests that increasing the enzyme concentration enhances the hydrolysis process, resulting in a greater number of peptide bonds being cleaved and the formation of smaller peptides with lower molecular weights. In contrast, lower DH values indicate that the quantity of trypsin is insufficient to optimally catalyze peptide bond cleavage within the substrate [22]. A summary of the hydrolysis results is presented in Table 1, which provides a clear comparison of DH values obtained for each trypsin-to-protein ratio tested.

The results presented in Table 1 reveal that a trypsin-to-protein ratio of 1:40 yielded the highest DH at 31.31%, which is significantly higher than the DH values obtained for the 1:10 and 1:20 ratios. This finding suggests that a higher trypsin concentration promotes hydrolysis, leading to the cleavage of more peptide bonds and the production of peptides with lower molecular weights. The effectiveness of trypsin in hydrolyzing casein at this ratio is consistent with its known catalytic

Table 1. Degree of hydrolysis of casein hydrolyzed by trypsin at various trypsin-casein ratios

Trypsin-casein ratio	1:10	1:20	1:40
DH(%)	25.64	16.85	31.31

properties, which involve the cleavage of peptide bonds adjacent to specific amino acids [23]. These results are in agreement with previous studies that have investigated the relationship between enzyme concentration and hydrolysis efficiency. For instance, Ningsih et al. [13] reported that a 1:40 enzyme-to-protein ratio resulted in the highest DH in their experiments, providing further support for the findings of this study. Similarly, Liu et al. [24] demonstrated that increasing the enzyme-to-protein ratio positively influenced the degree of hydrolysis, with higher ratios leading to more extensive peptide bond cleavage.

However, it is essential to note that despite the higher DH achieved at the 1:40 ratio, the hydrolysis process does not reach completion (100% DH). Additionally, excessive enzyme concentrations may lead to autohydrolysis, a phenomenon in which trypsin hydrolyzes itself. This self-digestion reduces the availability of active enzyme molecules for catalyzing the breakdown of casein, ultimately diminishing the efficiency of the hydrolysis process [25]. The hydrolysate was subjected to preliminary separation using an Amicon column before sample fractionation. This step ensured that only peptides were present in the hydrolysate, as the molecular weight cut-off (MWCO 3,000 Da) of the Amicon column excluded larger molecules of unhydrolyzed casein. The fractionation was then performed using a strong cation exchange solid-phase extraction (SCX-SPE) column. This technique effectively separates peptides based on differences in their electrostatic charge, which varies with pH. The fractionation process yielded peptide fractions corresponding to pH values ranging from 3 to 9, and the peptide content of each fraction was quantified. The results are presented in Table 2.

The data in Table 2 indicate that the total amount of collected peptides is 6,594 μg. Based on the DH of 31.31%, the theoretical yield of peptides would be approximately 18 mg. Despite the effective fractionation process, some peptides were lost during the procedure. Furthermore, some peptides may have remained bound to the cation exchange resin at higher pH values, making them challenging to elute even at pH 9. The fraction collected at pH 5 contained the highest total peptide mass (1,208 μg), suggesting that the majority of peptides in the hydrolysate possess an optimal positive charge for interaction with the SCX resin at this pH. As the pH approaches 5, the electrostatic interactions between the peptides and the resin weaken, causing the peptides to elute from the column. The varying peptide contents in other fractions indicate differences in the electrostatic properties of the peptides. Notably, the lowest peptide masses were observed in fractions corresponding to pH 8 (767 μg) and pH 9 (768 μg).

Table 2. Peptide yield of each fraction following SPE-SCX

Fraction based on pH for elution	Amount of the peptide in μg (percentage of the total)
3	888 (13.56%)
4	1,082 (16.52%)
5	1,208 (18.45%)
6	777 (11.87%)
7	1,059 (16.17%)
8	767 (11.70%)
9	768 (11.72%)
Total	6,594 (100.00%)

The effectiveness of the SPE-SCX method in separating peptides based on their charge characteristics underscores its utility in peptide fractionation. This finding aligns with previous studies, such as those by Raharjo et al. [14] and Habibie et al. [26], which successfully employed SPE-SCX to isolate peptides from hydrolyzed Jatropha seed proteins. Similarly, Dephoure and Gygi [27] demonstrated the capability of SCX to achieve high-quality peptide separation in their study involving yeast cell extracts, further validating the efficacy of this method.

Inhibition of α-Amylase Activity by Peptide Fractions

The α-amylase inhibition assay was performed to evaluate the potential of peptide fractions derived from casein hydrolysate to inhibit α-amylase enzyme activity. The results of the inhibition test, which utilized iodine-starch staining as a visual indicator of enzyme activity, are presented in Fig. 1. The assay was conducted with an

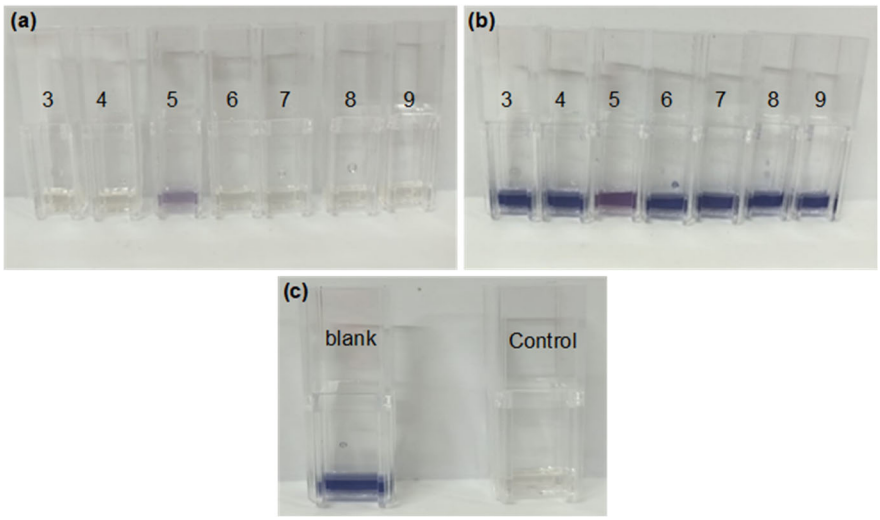

Fig 1. Qualitative analysis of α-amylase inhibition with iodine indicator. The systems consisted of: (a) α-amylase, amylum, and peptide fraction pH 3–9; (b) amylum and peptide fraction pH 3–9; and (c) (left) a blank (amylum) and (right) a positive (α-amylase and amylum)

initial concentration of 500 ppm for the peptide fractions. The results revealed substantial variation in inhibition activity among the fractions across a pH range of 3 to 9, indicating pH-dependent differences in α-amylase inhibition. The α-amylase inhibition results are summarized in Table 3.

The results reveal that the peptide fraction at pH 5 exhibited the most potent inhibition of α-amylase activity, with an inhibition value of 76.88%. This suggests that the peptides present in this fraction possess a strong inhibitory effect on the enzyme. Notably, the purple color of the iodine-amylum complex remained unchanged in this fraction throughout the assay, indicating that amylum was not hydrolyzed by the α-amylase enzyme [28]. This observation can be attributed to the unique structural or biochemical properties of the peptides in the pH 5 fraction, which likely exhibit a high affinity for α-amylase, thereby effectively inhibiting its activity.

In contrast, the pH 3, 4, 6, 7, 8, and 9 fractions exhibited significantly lower inhibition values, ranging from 3.12 to 17.50%. These results suggest that the amylum in these fractions was predominantly hydrolyzed, as evidenced by the color change of the iodine-amylum complex to clear or transparent. To further quantify the inhibitory potential of the pH 5 fraction, the IC_{50} value was determined. The IC_{50} value represents the concentration required to inhibit 50% of α-amylase enzyme activity. The analysis revealed that the IC_{50} value for the pH 5 fraction was 221.69 μg/mL, indicating that a concentration of 221.69 μg/mL is required to achieve 50% inhibition of α-amylase activity. According to the classification of inhibitory potential, this IC_{50} value suggests that the pH 5 fraction exhibits potential inhibitory activity against α-amylase considering the non toxic property of peptides.

This IC_{50} value is relatively high compared to previous studies, indicating lower inhibitory effectiveness. For example, Mudgil et al. [7] reported an IC_{50} value of 12.90 μg/mL for peptides derived from goat milk hydrolysis via microbial fermentation. In another work, Mudgil et al. [29] found that cow's milk protein hydrolysate (BC) and camel's milk hydrolysate (CC) had IC_{50} values of 0.58 and 0.59 mg/mL, respectively, against α-amylase. The observed differences in IC_{50} values are

Table 3. α-Amylase inhibition activity of peptide fractions at a concentration of 500 ppm

Fraction	pH 3	pH 4	pH 5	pH 6	pH 7	pH 8	pH 9
%Inhibition	17.50	7.50	76.88	5.62	6.89	8.12	3.12

likely due to the composition of the pH 5 fraction, which remains a complex mixture of peptides with diverse bioactive properties. As a result, not all peptides in this fraction act as α-amylase inhibitors.

The relatively low inhibitory activity of the pH 5 fraction may also be attributed to its suboptimal purity, which could compromise its overall bioactive effectiveness. Further purification steps could be employed to enhance the inhibitory potential, such as isolating specific bioactive peptides that act as α-amylase inhibitors. Alternatively, synthesizing individual peptides based on identified sequences could also be explored. Moreover, optimizing reaction conditions, including pH and temperature settings, may facilitate improved interactions between the peptides and α-amylase, potentially leading to enhanced inhibitory activity.

Kinetics Study for α-Amylase Inhibition by the Most Active Peptide Fraction

The kinetics of α-amylase inhibition by the pH 5 peptide fraction were investigated using the Lineweaver-Burk method, which enabled the determination of key enzyme kinetic parameters, including K_m and V_{max}. Additionally, this analysis enabled the elucidation of the inhibition mechanism type, as presented in Fig. 2. Using the Lineweaver-Burk plot, the K_m and V_{max} values were calculated for both the uninhibited enzyme and the enzyme inhibited by the peptide fraction at pH 5. The estimated kinetic parameters are summarized in Table 4, which presents the K_m and V_{max} values obtained under both conditions.

Under uninhibited conditions, the K_m value was 0.2526 g/L, and the V_{max} was 0.0353 g/L/min, reflecting the enzyme's intrinsic affinity for the substrate and its catalytic efficiency. After the pH 5 peptide fraction was added as an inhibitor, the K_m value decreased to 0.2296 g/L, while the V_{max} decreased to 0.0321 g/L/min. The reduction in K_m suggests that the inhibitor enhances the enzyme's affinity for the substrate by stabilizing the enzyme-substrate complex. Conversely, the decrease in V_{max} indicates that the inhibitor interferes with the conversion of the enzyme-substrate complex to the product [30]. These observations are consistent with the pH 5 peptide fraction functioning as an uncompetitive inhibitor. Uncompetitive inhibitors bind specifically to the enzyme-substrate complex, forming an enzyme-substrate-inhibitor (ESI) complex. This interaction enhances the enzyme's affinity for the substrate, as

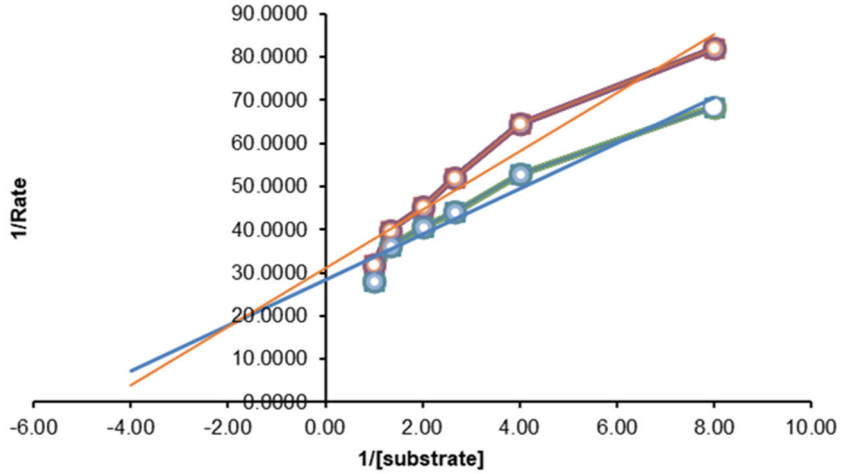

Fig 2. Lineweaver-Burk plot for the enzymatic reaction kinetics of amylum hydrolysis by α-amylase with inhibitor (pH 5 peptide fraction, red) and without inhibitor (blue)

Table 4. Enzyme kinetic parameters: K_m and V_{max} values for α-amylase-catalyzed amylum hydrolysis

Reaction conditions	K_m (g/L)	V_{max} (g/L/min)	Inhibitor type
Without inhibitor	0.2526	0.0353	-
Peptide fraction pH 5 as an inhibitor	0.2296	0.0321	uncompetitive

evidenced by a decrease in K_m. Simultaneously, it impedes the conversion of the enzyme-substrate complex to the product, reducing V_{max}. Although the enzyme-substrate affinity is increased, the presence of the inhibitor at a specific site on the enzyme prevents efficient product release, thereby reducing the overall reaction rate.

Identification of Bioactive Peptides Using LC-HRMS

The pH 5 fraction exhibiting potent inhibitory activity against α-amylase was further analyzed using LC-HRMS to elucidate its peptide sequences. Three peptides were identified by comparing the detected peptide masses to their theoretical counterparts, corresponding to possible *Capra hircus* protein peptide fragments. The peptide identification was further confirmed by analyzing the fragment masses generated from MS^2 experiments. The amino acid sequences of the identified peptides are summarized in Table 5. Three peptides were identified from the pH 5 fraction with high confidence and a low cross-correlation score. The fraction contained peptides with 7 to 15 residues, with molecular weights below 3,000 Da, corresponding to the MWCO of the Amicon column used. This indicates that the separation of peptides from non-hydrolyzed proteins was successful. Notably, peptides with fewer than 5 residues are likely to possess limited bioactive potential, whereas those exceeding 15 residues may be prone to reduced stability or impaired cellular uptake.

Peptides' Interaction with α-Amylase Studied Using Molecular Docking

HADDOCK2.4 was employed to analyze peptide interactions involved in the inhibition of α-amylase computationally [20]. This approach enables more accurate modelling of biomolecular complexes. A key advantage of HADDOCK2.4 is its ability to accommodate small conformational changes in the receptor structure, thereby enhancing its representation of the ligand binding process. Using the HADDOCK2.4 docking method, the interactions between three peptides and the α-amylase enzyme were assessed, with a focus on hydrogen bond formation and interactions with the enzyme's catalytic triad, comprising Asp179, Glu204, and Asp289, as illustrated in Fig. 3.

The best top cluster for all complex models was selected by their HADDOCK score. The best cluster of the EGIEELLTGTIR-α-amylase model exhibited −70.0 ± 3.8 kcal/mol HADDOCK score with 0.9 Å RMSD. The second peptide, SSPSKHQPPPIR, exhibited a binding energy of −89.3 ± 3.1 kcal/mol and an RMSD of 0.5 Å. The HADOOCK2.4 score and RMSD for the best cluster of LLPRKAK −84.1 ± 4.3 kcal/mol and 0.2 Å, respectively. All selected clusters were used to show the interaction of peptide-α-amylase complexes.

The EGIEELLTGTIR peptide forms two hydrogen bonds, one of which is with Asp289, thereby contributing to stabilizing the reaction transition state. This interaction suggests that the peptide may interfere with enzyme activity by affecting substrate binding. In contrast, the SSPSKHQPPPIR peptide exhibits stronger interactions, forming six hydrogen bonds, including those with Glu204 and Asp289, which are critical to the catalytic process of α-amylase. Specifically, Asp289 helps stabilize the reaction intermediate, while Glu204 functions as an acid/base catalyst, facilitating the breakdown of glycosidic bonds. These interactions suggest that the SSPSKHQPPPIR peptide could act as a potent inhibitor. The LLPRKAK peptide demonstrates a powerful interaction, forming 6 hydrogen bonds, 3 directly involving

Table 5. Identified peptides from the pH 5 fraction via LC-HRMS analysis

The amino acid sequence	Molecular weight ($[MH]^+$, Da)	XCorr Sequest*	Confidence**
EGIEELLTGTIR	1,330.72127	0.24	High
SSPSKHQPPPIR	1,330.72261	0.33	High
LLPRKAK	825.56688	0.24	High

(*) Scores the number of fragment ions common to two different peptides with the same precursor mass and calculates the cross-correlation score for all candidate peptides queried from the database. A low value of XCorr means the cross-correlation is low.

(**) The level of confidence of the identified peptide.

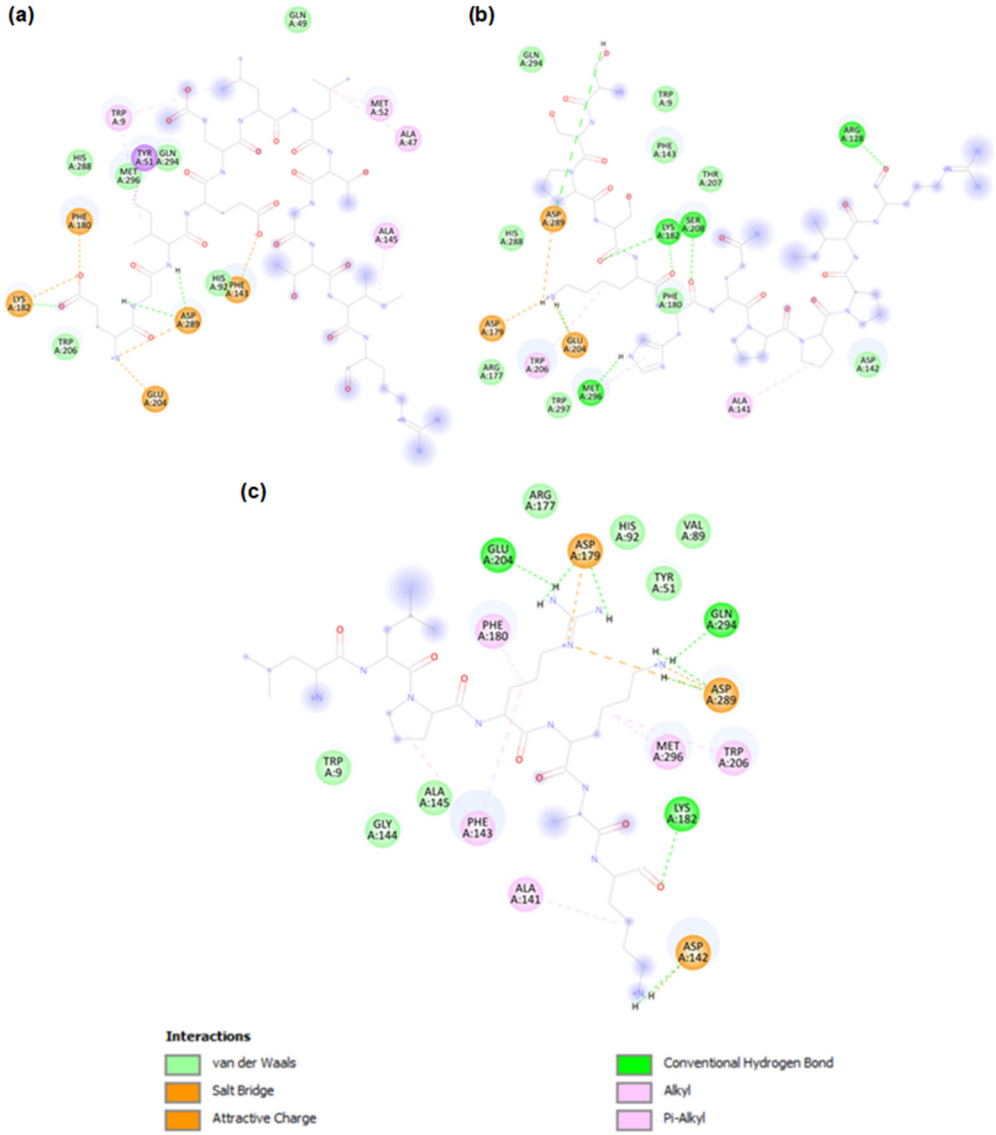

Fig 3. Interaction of α-amylase with peptides (a) EGIEELLTGTIR, (b) SSPSKHQPPPIR, and (c) LLPRKAK using HADDOCK

Asp179, Glu204, and Asp289. This combination of interactions suggests that this peptide has high binding stability and significant potential to inhibit α-amylase activity by disrupting the function of the catalytic triad.

Notably, all peptides appear to interact effectively with the catalytic triad. However, based on the enzyme kinetic testing discussed earlier, it was found that the inhibition type is competitive. Upon re-analysis of the docking results, it was observed that several of the tested peptides form hydrogen bonds and pi-sigma interactions with residues outside the catalytic triad of α-amylase, such as Gln294 and Trp9. For instance, the EGIEELLTGTIR peptide forms hydrogen bonds with Trp9, Glu204, and Gln294, and creates a pi-sigma bond with Tyr51. Similarly, the SSPSKHQPPPIR peptide forms hydrogen bonds with Ser208 and Gln294, while the LLPRKAK peptide forms hydrogen bonds with Asp142, Lys182, and Gln294. These interactions suggest that the peptides influence the stability of the enzyme-substrate complex by binding to residues outside the catalytic triad, potentially inhibiting the conversion of the substrate to the product. The enzymatic kinetic data, which indicate

an uncompetitive inhibition mechanism, reveal a decrease in K_m, suggesting that the inhibitor enhances the enzyme's affinity for the substrate. In contrast, the decrease in V_{max} reflects a reduction in the number of available enzyme-substrate complexes for the reaction. This supports the notion that these peptides act as uncompetitive inhibitors, which do not prevent substrate binding but impede further enzymatic reactions by stabilizing an inactive enzyme-substrate complex. Furthermore, HADDOCK is a targeted docking tool that docks peptides at the binding and active site; therefore, molecular docking analysis with HADDOCK2.4 was invalid for analyzing interactions between peptides and proteins with an uncompetitive inhibition mechanism [30].

To investigate the peptide-protein interactions with an uncompetitive inhibition mechanism, the second approach for the docking study was performed by CABS-dock [20]. CABS-dock is a web-based molecular docking tool designed to model interactions between proteins and peptides without requiring prior knowledge of the binding site locations. CABS-dock can generate protein-peptide interaction models with high or moderate accuracy in over 80% of cases, for both bound and unbound interactions. The results of the analysis, detailing interactions between individual peptides and key residues such as those within the catalytic triad (Asp179, Asp180, Glu204, Glu205, Asp289, and Asp291), which are crucial for the catalytic mechanism of α-amylase, are presented in Table 6.

Docking results obtained using CABS-dock revealed that the tested peptides interacted not only with the active site of α-amylase but also with non-active site residues, including those within the catalytic triad (Asp179, Glu204, and Asp289) and other residues involved in non-catalytic triad interactions. Notably, the peptide EGIEELLTGTIR exhibited several interactions with residues outside the catalytic triad while maintaining direct interactions with the triad. Fig. 4 showed that almost all identified peptides interacted with α-amylase outside of the binding pocket. Although several interactions in the binding pocket are still observed (Table 6), findings suggest that these peptides may function as uncompetitive inhibitors, which inhibit enzyme activity by binding to the enzyme-substrate complex rather than directly to the enzyme's active site. In the uncompetitive inhibition mechanism, inhibitor binding occurs subsequent to substrate binding, inducing conformational changes that reduce reaction efficiency without affecting the enzyme's affinity for the substrate. The results also suggested that SSPSKHQPPPIR potentially exhibited the best inhibition of α-amylase, with 60 total interactions, followed by EGIEELLTGTIR with 48 total interactions and LLPRKAK with 33 interactions.

The enzymatic kinetic data, which reveal a decrease in K_m and V_{max}, strongly support this hypothesis. The reduction in K_m indicates that the inhibitor enhances the enzyme's affinity for the substrate. In contrast, the decrease in V_{max} reflects a reduction in the amount of enzyme-substrate complex available for the reaction, characteristics typical of an uncompetitive inhibitor. Furthermore, the CABS-dock results, which demonstrate peptide interactions with non-catalytic triad sites, reinforce the notion that the peptides inhibit α-amylase activity by indirectly modulating the enzyme's conformation and stability. This mechanism enables enzyme inhibition without compromising the enzyme's ability to bind to the substrate, a hallmark of uncompetitive inhibition.

Table 6. Interaction of peptides with the catalytic triad via CABS-dock

Peptide	Total interaction	Number of interactions with catalytic triads			Number of interactions with catalytic non-triads
		Asp179	Asp289	Glu204	
EGIEELLTGTIR	48	1	2	-	45
SSPSKHQPPPIR	60	-	2	-	58
LLPRKAK	33	2	3	1	27

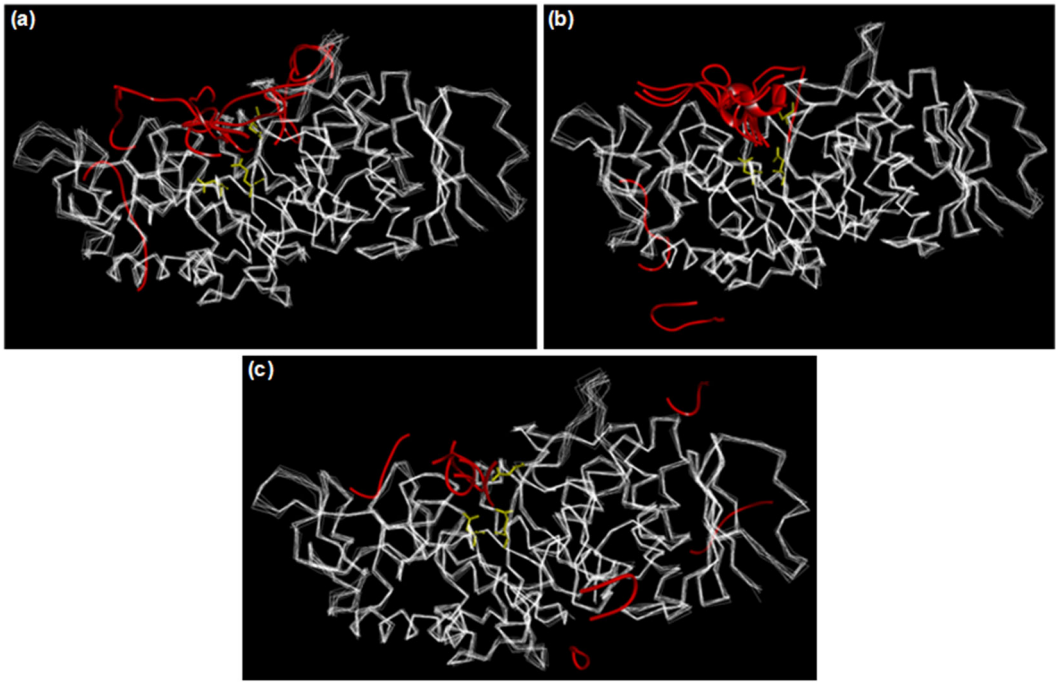

Fig 4. The 10 lowest energy complex α-amylase and peptides (a) EGIEELLTGTIR, (b) SSPSKHQPPPIR, and (c) LLPRKAK using CABS-dock. The binding pocket and peptides were colored yellow and red, respectively

■ CONCLUSION

This study successfully identified bioactive peptides derived from hydrolyzed goat milk casein, demonstrating their potential as inhibitors of α-amylase and offering promising implications for diabetes management. Optimization of the hydrolysis process, achieved at an enzyme-to-protein ratio of 1:40, yielded peptides with varying inhibitory effects, with the pH 5 fraction exhibiting the most potent α-amylase inhibition. Molecular docking analysis revealed that the peptides, including EGIEELLTGTIR, SSPSKHQPPPIR, and LLPRKAK, interact with both the enzyme's active site and residues outside, suggesting a uncompetitive inhibition mechanism.

■ ACKNOWLEDGMENTS

This research was supported by Universitas Gadjah Mada through the Regular Fundamental Research Scheme 2023, contract number 3150/UN1/DITLIT/Dit-Lit/PT.01.03/2023. The authors would like to acknowledge Tri Wahyudi of the Universitas Gadjah Mada Integrated Laboratory for his assistance with LC-HRMS analysis.

■ CONFLICT OF INTEREST

The authors do not have a conflict of interest.

■ AUTHOR CONTRIBUTIONS

Tri Joko Raharjo was the principal architect of the idea, led the team, designed the experiment, analyzed the results, and contributed to manuscript preparation. Chairil Anwar provided supervision for both the experiment and manuscript preparation. Nurwardian Aulyawati played a key role in conducting the experiment, analyzing the results, drafting the manuscript, and revising and verifying the manuscript and results. All authors have read, approved, and made significant contributions to this research, drawing on their collective expertise and knowledge.

■ REFERENCES

[1] Kashung, P., and Karuthapandian, D., 2025, Milk-derived bioactive peptides, *Food Prod., Process. Nutr.*, 7 (1), 6.

[2] Mohanty, D.P., Mohapatra, S., Misra, S., and Sahu, P.S., 2016, Milk derived bioactive peptides and their

impact on human health – A review, *Saudi J. Biol. Sci.*, 23 (5), 577–583.

[3] Ahmed, A.S., El-Bassiony, T., Elmalt, L.M., and Ibrahim, H.R., 2015, Identification of potent antioxidant bioactive peptides from goat milk proteins, *Food Res. Int.*, 74, 80–88.

[4] Cakir, B., and Tunali-Akbay, T., 2021, Potential anticarcinogenic effect of goat milk-derived bioactive peptides on HCT-116 human colorectal carcinoma cell line, *Anal. Biochem.*, 622, 114166.

[5] Sumlang, G.H.P., Handoyo, R.L., Laksitorini, M.D., Astuti, E., and Raharjo, T.J., 2025, Goat milk casein peptides as potential α-amylase inhibitors: A computational and experimental approach, *Indones. J. Chem.*, 25 (3), 770–786.

[6] Mudgil, P., AlMazroui, M., Redha, A.A., Kilari, B.P., Srikumar, S., and Maqsood, S., 2022, Cow and camel milk-derived whey and casein protein hydrolysates demonstrated effective antifungal properties against selected *Candida* species, *J. Dairy Sci.*, 105 (3), 1878–1888.

[7] Mudgil, P., Kamal, H., Yuen, G.C., and Maqsood, S., 2018, Characterization and identification of novel antidiabetic and anti-obesity peptides from camel milk protein hydrolysates, *Food Chem.*, 259, 46–54.

[8] Gong, H., Gao, J., Wang, Y., Luo, Q.W., Guo, K.R., Ren, F.Z., and Mao, X.Y., 2020, Identification of novel peptides from goat milk casein that ameliorate high-glucose-induced insulin resistance in HepG2 cells, *J. Dairy Sci.*, 103 (6), 4907–4918.

[9] Karimi, A., Azizi, M.H., and Ahmadi Gavlighi, H., 2020, Fractionation of hydrolysate from corn germ protein by ultrafiltration: *In vitro* antidiabetic and antioxidant activity, *Food Sci. Nutr.*, 8 (5), 2395–2405.

[10] Chaudhury, A., Duvoor, C., Reddy Dendi, V.S., Kraleti, S., Chada, A., Ravilla, R., Marco, A., Shekhawat, N.S., Montales, M.T., Kuriakose, K., Sasapu, A., Beebe, A., Patil, N., Musham, C.K., Lohani, G.P., and Mirza, W., 2017, Clinical review of antidiabetic drugs: Implications for type 2 diabetes mellitus management, *Front. Endocrinol.*, 8, 6.

[11] Samtiya, M., Samtiya, S., Badgujar, P.C., Puniya, A.K., Dhewa, T., and Aluko, R.E., 2022, Health-promoting and therapeutic attributes of milk-derived bioactive peptides, *Nutrients*, 14 (15), 3001.

[12] Mohsin, A.Z., Sukor, R., Selamat, J., Meor Hussin, A.S., Ismail, I.H., Jambari, N.N., and Jonet, A., 2020, A highly selective two-way purification method using liquid chromatography for isolating αS2-casein from goat milk of five different breeds, *J. Chromatogr. B*, 1160, 122380.

[13] Ningsih, D.R., Raharjo, T.J., Haryadi, W., and Wikandari, R., 2023, Antifungal activity and identification of bioactive peptide from Etawa crossbreed goat (*Capra hircus*) milk protein hydrolyzed using trypsin enzyme, *Arabian J. Chem.*, 16 (11), 105249.

[14] Raharjo, T.J., Utami, W.M., Fajr, A., Haryadi, W., and Swasono, R.T., 2021, Antibacterial peptides from tryptic hydrolysate of *Ricinus communis* seed protein fractionated using cation exchange chromatography, *Indones. J. Pharm.*, 32 (1), 74–85.

[15] Rafique, R., Khan, K.M., Arshia, A., Kanwal, K., Chigurupati, S., Wadood, A., Rehman, A.U., Karunanidhi, A., Hameed, S., Taha, M., and al-Rashida, M., 2020, Synthesis of new indazole based dual inhibitors of α-glucosidase and α-amylase enzymes, their *in vitro*, *in silico* and kinetics studies, *Bioorg. Chem.*, 94, 103195.

[16] Sun, L., Warren, F.J., Netzel, G., and Gidley, M.J., 2016, 3 or 3′-Galloyl substitution plays an important role in association of catechins and theaflavins with porcine pancreatic α-amylase: The kinetics of inhibition of α-amylase by tea polyphenols, *J. Funct. Foods*, 26, 144–156.

[17] Pettersen, E.F., Goddard, T.D., Huang, C.C., Couch, G.S., Greenblatt, D.M., Meng, E.C., and Ferrin, T.E., 2004, UCSF Chimera—A visualization system for exploratory research and analysis, *J. Comput. Chem.*, 25 (13), 1605–1612.

[18] BIOVIA, Dassault Systèmes, 2020, *Discovery Studio Visualizer v.21.1.0.20298*, Dassault Systèmes, San Diego, US.

[19] Kurcinski, M., Jamroz, M., Blaszczyk, M., Kolinski, A., and Kmiecik, S., 2015, CABS-dock web server for the flexible docking of peptides to proteins without prior knowledge of the binding site, *Nucleic Acids Res.*, 43 (W1), W419–W424.

[20] Honorato, R.V., Trellet, M.E., Jiménez-García, B., Schaarschmidt, J.J., Giulini, M., Reys, V., Koukos, P.I., Rodrigues, J.P.G.L.M., Karaca, E., van Zundert, G.C.P., Roel-Touris, J., van Noort, C.W., Jandová, Z., Melquiond, A.S.J., and Bonvin, A.M.J.J., 2024, The HADDOCK2.4 web server: A leap forward in integrative modelling of biomolecular complexes, *Nat. Protoc.*, 19 (11), 3219–3241.

[21] Jiménez-García, B., Elez, K., Koukos, P.I., Bonvin, A.M., and Vangone, A., 2019, PRODIGY-crystal: A web-tool for classification of biological interfaces in protein complexes, *Bioinformatics*, 35 (22), 4821–4823.

[22] Charoenphun, N., Cheirsilp, B., Sirinupong, N., and Youravong, W., 2013, Calcium-binding peptides derived from tilapia (*Oreochromis niloticus*) protein hydrolysate, *Eur. Food Res. Technol.*, 236 (1), 57–63.

[23] Deng, Y., Gruppen, H., and Wierenga, P.A., 2018, Comparison of protein hydrolysis catalyzed by bovine, porcine, and human trypsins, *J. Agric. Food. Chem.*, 66 (16), 4219–4232.

[24] Liu, X., Luo, Y., and Li, Z., 2012, Effects of pH, temperature, enzyme-to-substrate ratio and reaction time on the antigenicity of casein hydrolysates prepared by papain, *Food Agric. Immunol.*, 23 (1), 69–82.

[25] Vorob'ev, M.M., Açıkgöz, B.D., Güler, G., Golovanov, A.V., and Sinitsyna, O.V., 2023, Proteolysis of micellar β-casein by trypsin: Secondary structure characterization and kinetic modeling at different enzyme concentrations, *Int. J. Mol. Sci.*, 24 (4), 3874.

[26] Habibie, A., Raharjo, T.J., Swasono, R.T., and Retnaningrum, E., 2023, Antibacterial activity of active peptide from marine macroalgae *Chondrus crispus* protein hydrolysate against *Staphylococcus aureus*, *Pharmacia*, 70 (4), 983–992.

[27] Dephoure, N., and Gygi, S.P., 2011, A solid phase extraction-based platform for rapid phosphoproteomic analysis, *Methods*, 54 (4), 379–386.

[28] Xiao, Z., Storms, R., and Tsang, A., 2006, A quantitative starch–iodine method for measuring alpha-amylase and glucoamylase activities, *Anal. Biochem.*, 351 (1), 146–148.

[29] Mudgil, P., Kamal, H., Priya Kilari, B., Mohd Salim, M.A.S., Gan, C.Y., and Maqsood, S., 2021, Simulated gastrointestinal digestion of camel and bovine casein hydrolysates: Identification and characterization of novel anti-diabetic bioactive peptides, *Food Chem.*, 353, 129374.

[30] Silverstein, T.P., 2019, When both Km and Vmax are altered, Is the enzyme inhibited or activated?, *Biochem. Mol. Biol. Educ.*, 47 (4), 446–449.