Short Communication:

Identification of α-Amylase Inhibitory Peptides from Tryptic Hydrolysate of *Caulerpa racemosa* Green Algae Protein

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Abstract: *Peptides have been reported as a potential alternative for antidiabetic therapy by inhibiting α-amylase, one of the key enzymes involved in carbohydrate metabolism. This study aimed to identify α-amylase inhibitory peptides derived from trypsin hydrolysate of* Caulerpa racemosa *protein. The protein was extracted using trichloroacetic acid (TCA)/acetone. The resulting peptides were fractionated using a strong cationic exchanger-solid phase extraction (SCX-SPE) column. The peptide fractions were tested for α-amylase inhibitory activity, and the peptides in the most active fraction were identified using liquid chromatography-high resolution mass spectrometry (LC-HRMS). Seven fractions were obtained from SCX-SPE elution at pH 3 to 9. The fraction eluted at pH 5 exhibited the highest α-amylase inhibitory activity, with an IC_{50} value of 43.70 μg/mL. Four peptides with the sequences VQKEKR, MNFYCISSK, DLCDYIHNK, and ISICYEK were identified from this fraction. Molecular docking studies revealed that the peptides with sequences MNFYCISSK and VQKEKR had the strongest interactions with α-amylase. The peptide binding affinity energies of MNFYCISSK and VQKEKR were −10.3 and −9.4 kcal/mol, respectively, with RMSD values of 0.3 ± 0.0 and 0.3 ± 0.2 Å. It can be concluded that the peptides with sequences MNFYCISSK and VQKEKR could be proposed as potential antidiabetic peptides with an α-amylase inhibition mechanism.*

Keywords: *peptide; α-amylase; inhibitor;* Culerpa racemosa*; antidiabetic*

■ INTRODUCTION

Diabetes mellitus is a chronic metabolic disorder characterized by hyperglycemia resulting from impaired insulin production and/or action. Type 2 diabetes mellitus is a disease associated with numerous complications and contributes to the highest mortality rate worldwide. According to the International Diabetes Federation, the number of people with diabetes aged 18–79 reached 570 million in 2021 and is projected to increase to 783 million by 2045 [1]. In 2021, the prevalence of diabetes in Indonesia was estimated to be 7.11% of the Indonesian population [2]. Diabetic patients are at high risk of developing complications from various serious diseases, such as retinal damage, cardiovascular disease, kidney and nerve damage, and triggering cancer cell growth [3]. Additionally, people with diabetes are more susceptible to developing various types of infections, including viral and bacterial infections [4]. Despite the availability of various diabetes medications, long-term use of synthetic medicines can have adverse health effects.

Natural foods contain bioactive peptides that are safer than synthetic medicines have attracted global research interest. Peptides have been reported to be a potential alternative therapy for diabetes by inhibiting α-amylase [5-6]. α-Amylase is an enzyme in human saliva and the pancreas that hydrolyzes polysaccharides into oligosaccharides, such as dextrin and glucose or maltose. Inhibiting α-amylase can slow down carbohydrate

digestion, resulting in decreased glucose absorption and preventing postprandial glucose level increases [7].

Macroalgae are a rich source of protein with high diversity. One such macroalgae is *Caulerpa racemosa*, which has high density and is widely cultivated in Indonesia [8]. *C. racemosa* is a consumable macroalgae known to contain various essential compounds, including proteins and bioactive compounds with numerous health benefits [9]. Therefore, the current study aimed to discover new α-amylase inhibitory peptides using ion exchange chromatography, purification with solid phase extraction with PEP cartridge, and identification with liquid chromatography-high resolution mass spectrometry (LC-HRMS).

■ EXPERIMENTAL SECTION

Materials

The primary materials used in peptide preparation were *C. racemosa*, acetone (Merck, Germany), water (MS grade, Merck, Germany), trypsin (Bioworld, USA), citric acid monohydrate ($C_6H_8O_7{\cdot}H_2O$, Merck), trisodium citrate dihydrate ($C_6H_5Na_3O_7{\cdot}2H_2O$, Merck), sodium phosphate dihydrate ($NaH_2PO_4{\cdot}2H_2O$, Merck), sodium phosphate monobasic (NaH_2PO_4, Merck), methanol (CH_3OH, Merck), and ammonium bicarbonate (NH_4HCO_3, Sigma). The antidiabetic test utilized α-amylase (HIMEDIA GRM638-100G), 0.2% iodine solution (Merck, Germany), starch (Merck, Germany), and acarbose (OGBdexa).

Instrumentation

The instrumentation used in this research include a sonicator, incubator 37 °C (Sakura), oven (Memmert), aerator, hot plate (Thermo Scientific Cimarec), Amicon® Ultra-15 centrifugal filter (Sigma), vortex mixer (Thermo Scientific), vacuum freeze dryer (Christ), SPE Supelco DSC-SCX Cartridge column (Sigma), Sorvall Biofuge Primo R centrifuge (Thermo Scientific), multimode microplate reader (SPARK TECAN), Ultimate 3000 Rapid Separation Ultra High Performance Liquid Chromatography connected to Q-Exactive Orbitrap Mass Spectrometer and Proteome Discoverer Ver software. 2.5 (Thermo Scientific).

Procedure

Protein extraction

C. racemosa freeze-dried powder was extracted using cold trichloroacetic acid (TCA)/acetone. A 4 g of *C. racemosa* powder was added to 30 mL of distilled water and sonicated for 45 min. The resulting supernatant was then mixed with cold TCA/acetone (1:1) and incubated at 4 °C for 12 h. The mixture was centrifuged at 1500× g for 15 min at room temperature, and the resulting pellet was washed twice with acetone. Finally, the protein content was measured using a multimode microplate reader at a wavelength of 562 nm.

Protein hydrolysis

The extracted protein was hydrolyzed with trypsin using enzyme-to-protein ratios of 1:20, 1:25, 1:30, and 1:35 (w/w). The mixture was incubated for 20 h at 37 °C and further incubated for 15 min at 90 °C. The protein hydrolysate was collected from the supernatant after centrifugation at room temperature at 1500× g for 20 min. The supernatant was then applied to an Amicon Ultra-3KDa column and centrifuged for 60 min at 4000× g. The hydrolysate that passed through the Amicon membrane was collected as the protein hydrolysate. The degree of hydrolysis was determined using the BCA protein assay reagent. Absorbance was measured using a multimode microplate reader at 562 nm. The degree of hydrolysis (DH, %) was expressed as the ratio of the percentage of absorbance after and before enzymatic hydrolysis, as shown in Eq. (1).

$$DH = \frac{\text{Total proteins (g)} - \text{Concentrate (g)}}{\text{Totat proteins (g)}} \times 100\% \quad (1)$$

Peptide fractionation

The cation exchange SPE column was pretreated with 5 mL of methanol and 2 × 5 mL of distilled water. After washing with distilled water, the column was conditioned using a 0.1 M citrate buffer at pH 3 and incubated for 15 min at room temperature. The buffer was then aspirated using a manifold pump. The protein hydrolysate was added to the column and incubated for 30 min at room temperature. The peptide fractions were collected by eluting the hydrolysate from the column using a 0.1 M citrate buffer at pH 3, 4, and 5, followed by

0.1 M phosphate buffer at pH 6, 7, 8, and 9, respectively. All elution processes used a 5 mL volume of the buffers. Each eluate was then neutralized and desalted using a HyperSep PEP SPE column. The absorbance of the eluate obtained in each fraction was measured at 562 nm using a multimode microplate reader.

α-Amylase inhibitory test

The α-amylase inhibitory activity test was carried out using a previously established procedure [10]. Briefly, 100 µL of 1% starch solution was added to 20 µL of peptide sample. The mixture was then added to 20 µL of α-amylase (1 U/mL) dissolved in 0.1 M phosphate buffer (pH 7). The resulting mixture was incubated for 15 min at 37 °C, followed by the addition of 2 µL of 0.2% iodine solution. The absorbance of the mixture was then measured using a microplate reader at 562 nm. The inhibitory activity of the peptide fraction against α-amylase was determined using the formula in Eq. (2);

$$\%\text{Inhibition} = \frac{\text{Abs(blank} - \text{control)} - \text{Abs(AS0} - \text{AS1)}}{\text{abs(blank} - \text{control)}} \times 100\% \quad (2)$$

where control = the absorbance from the system that only consisted of the enzyme, blank = the absorbance from the system that did not consist of either the enzyme and samples, AS1 = the absorbance from system that consisted of enzyme and samples, and AS0 = the absorbance from system that only included samples without enzyme. The peptide fraction with the highest inhibitory activity was further analyzed to determine its IC_{50} value. Various concentrations of the peptide fraction were tested, and their inhibition percentages were calculated. The IC_{50} value was then calculated using probit analysis.

Identification of α-amylase inhibitory peptides

Peptides in the fraction were separated using a liquid chromatography system equipped with an Acclaim® PepMap RSLC column (C_{18}, 1 mm × 150 cm, with a particle size of 3 µm). The peptides were eluted from the column using a gradient mobile phase consisting of 0.05% trifluoroacetic acid (TFA) in water (mobile phase A) and water/acetonitrile (20:80, v/v) with 0.1% TFA (mobile phase B). The mobile phase composition was as follows: 96% A and 4% B at a flow rate of 0.3 µL/min from 0 to 2.99 min, followed by a linear gradient to 90% A and 10% B at 3 min, and then to 80% A and 20% B at 30 min. The composition was then changed to 5% A and 95% B at 70 min and maintained until 90 min. Mass spectrometry (MS) and tandem mass spectrometry (MS/MS) analyses were performed using electrospray ionization. The positive ion mode was operated in high-resolution and accurate mass mode for MS detection. MS/MS peptide analysis was performed in the *m/z* range of 350–1800 using full-MS/dd-MS2 mode. The resolving power was set at 140,000 full width at half maximum (FWHM) for the full-MS scan, while the resolution was set at 17,500 FWHM for the dd-MS2 scan.

For peptide identification, the raw MS data were processed using Proteome Discoverer Software version 2.5. Peptide identification was performed by processing the data using the Fusion Basic Sequest HT workflow and a basic consensus workflow. The Sequest HT algorithm was employed for peptide analysis. The genomic database of the green algae (*C. racemosa*) was used and downloaded from the UniProt Consortium. Peptide identification was completed by comparing the experimental data with the database used.

■ RESULTS AND DISCUSSION

Protein Extraction of *C. racemosa*

The dried *C. racemosa* was ground into a homogeneous powder using a blender and sieved to obtain a uniform particle size. The total protein content of *C. racemosa* was analyzed prior to protein precipitation. The results showed that *C. racemosa* contained 11.15% protein, which is relatively high for a macroalga typically rich in carbohydrates. Protein precipitation from *C. racemosa* was carried out using the TCA/acetone method with a 1:1 ratio of *Caulerpa* supernatant to TCA/acetone. This method was chosen because it can effectively remove secondary metabolites, lipids, phenolic compounds, and pigments [11]. The resulting *C. racemosa* protein was a white powder that had been purified of green pigments and secondary metabolites. However, a limitation of this method is that it yields a relatively small amount of protein, approximately 1% of the initial sample weight. Specifically, 16 g of *C. racemosa* yielded 160 mg of protein that showed lower yield than previous study (0.67%) [9].

Protein Hydrolysate of *C. racemosa*

Protein hydrolysis was performed to enhance protein bioactivity. Enzymatic hydrolysis was carried out on *Caulerpa* protein to obtain bioactive peptides with α-amylase inhibitory activity. The enzymatic hydrolysis method was employed because it is easy to control and can preserve the amino acid structure [12]. The bioactive peptides obtained are primarily determined by the enzyme specificity, making the selection of enzymes crucial in the hydrolysis process [13]. Protein hydrolysis was performed using a single type of protease enzyme, namely trypsin. Trypsin was chosen for the hydrolysis process because it can specifically cleave lysine (K) or arginine (R), which are cationic amino acids.

The protein-to-enzyme ratio used in this study was 1:25 (w/w). This optimal ratio was determined through the optimization of the degree of hydrolysis, as shown in Fig. 1. The optimization process involved comparing enzyme-to-protein ratios of 1:20, 1:25, 1:30, and 1:35. The results revealed that the highest percentage of hydrolysis (89.93%) was achieved with an enzyme-to-protein ratio of 1:25.

Fractionation of *C. racemosa* Protein Hydrolysate

Peptide fractions were separated using SCX-SPE. The SCX-SPE cartridge possesses a benzene sulfonic acid functional group polymerically bonded to H^+ ions, acting as strong cation exchangers. Under acidic conditions, peptides with a positive charge (cations) bind to the sorbent [13]. pH variations ranging from pH 3 to 9 were employed to separate peptides using SCX, and citrate and phosphate buffers were used to prepare the pH gradients. The fraction collected using SCX SPE was further purified using HyperSep Retain PEP SPE. The concentration of the fractionated and purified peptides was determined using the BCA protein assay. The peptides from *C. racemosa* were predominantly found in the pH 5 fraction, with a total mass of 940 μg. The distribution of the peptide fractions is shown in Fig. 2. The varying amounts of peptides in each fraction can be attributed to the protein hydrolysis process with trypsin, which results in diverse peptide compositions with varying numbers of amino acids.

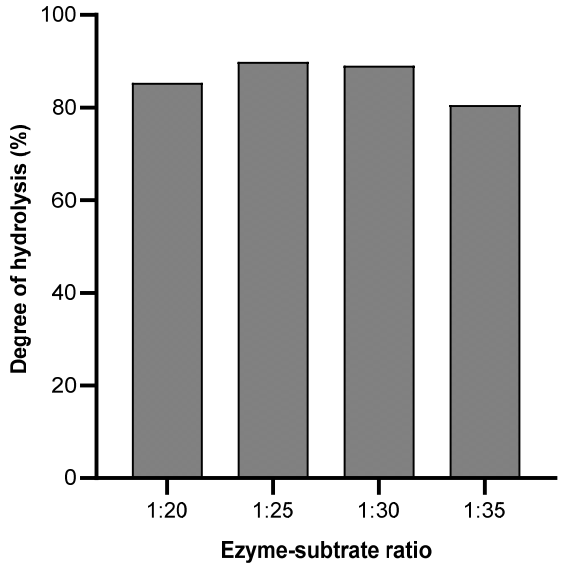

Fig 1. The degree of trypsin hydrolysis on *C. racemosa* protein at various trypsin/protein ratios

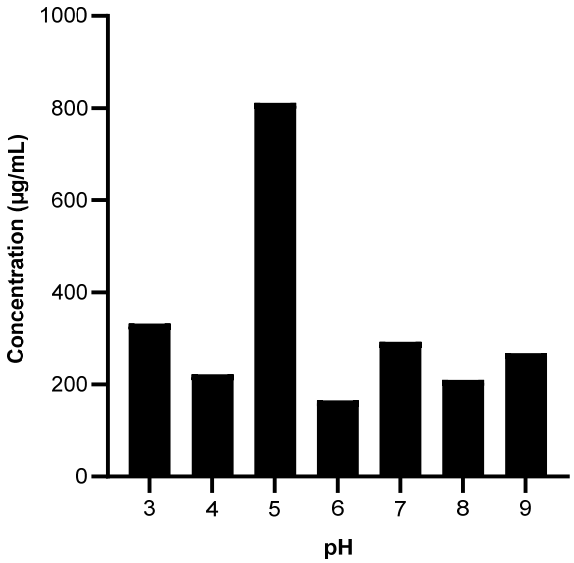

Fig 2. The concentration of peptides presents in the fractions resulted from SCX fractionation of *C. racemosa* protein hydrolysate

α-Amylase Inhibitory Peptide Activity of the Peptide Fractions

The isolated peptide fraction was subsequently tested for its inhibitory activity against α-amylase. Iodine was used as an indicator in this assay. The starch substrate reacts with iodine to form a starch-iodine complex, which produces a purple color [14]. α-Amylase catalyzes the hydrolysis of starch to form maltose,

maltotriose, and oligosaccharides (6-8 glucose chains) by breaking glycosidic bonds. The higher the α-amylase activity, the less starch remains in the solution, resulting in reduced purple color intensity. This is evident from the decrease in absorbance value of the system containing only the substrate compared to the system comprising both the enzyme and substrate. This study employed acarbose, a commonly used antidiabetic drug, as a positive control. Acarbose has been proven to inhibit α-amylase, but its use is associated with side effects [15].

The inhibitory activity of the peptide fraction can be visually determined by the color of the resulting solution. If the peptide fraction exhibits inhibitory activity against α-amylase, the substrate and enzyme solution will retain a purple color. This indicates the inhibition of starch hydrolysis into maltose, maltotriose, and α-(1,6) and α-(1,4)-oligoglucans. The peptide fraction with α-amylase inhibitory activity, specifically the pH 5 peptide fraction, is evident from the purple color that appears during the inhibition test (Fig. 3). In contrast, the other pH peptide fractions did not exhibit inhibitory activity, as indicated by the clear color produced after the addition of iodine. The clear coloration suggests that α-amylase has completely hydrolyzed the starch substrate into glucose.

Based on Fig. 4, it is evident that the pH 5 peptide fraction at a concentration of 1000 ppm exhibits a higher percent inhibition (97.77%) compared to acarbose (92.82%). At concentrations ranging from 200 to 800 ppm, the pH 5 peptide fraction demonstrated good activity, similar to the positive control acarbose. At the lowest concentration (200 ppm), acarbose showed an inhibition value of approximately 82.75%, while the pH 5 peptide fraction had a lower inhibition value of around 71.18%. The pH 5 peptide fraction had an IC_{50} value of 43.70 μg/mL, whereas acarbose had an IC_{50} value of 35.49 μg/mL. The results indicate that the inhibition activity percentage and IC_{50} values of acarbose and the pH 5 peptide fraction are comparable, suggesting that the pH 5 peptide fraction has promising potential for development as an antidiabetic agent.

Identification of the Antidiabetic Peptides

Peptide identification was performed using LC-HRMS interfaced with Proteome Discoverer software version 2.5, utilizing the *C. racemosa* database obtained from UniProt. The pH 5 fraction peptide, which exhibited antidiabetic activity, was subjected to amino acid sequence identification using LC-HRMS, resulting in the identification of four peptide sequences (Table 1). The identified peptide sequences, consisting of 6–9 amino acids, varied in length. Notably, the four successfully identified peptide sequences contained lysine (K) or arginine (R) residues, indicating successful

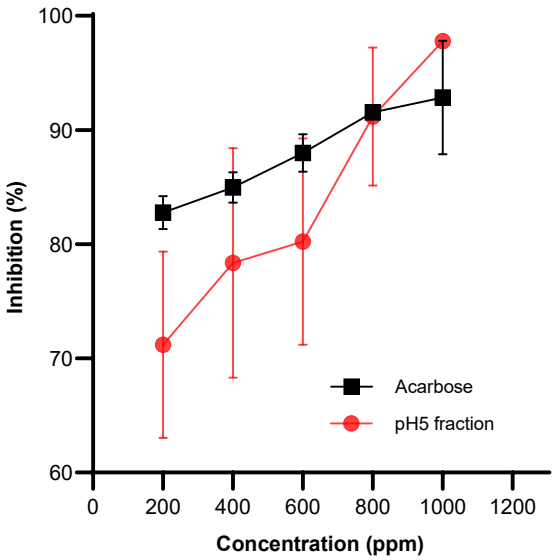

Fig 4. The percentage of inhibition of the peptide fraction at various concentrations compared to acarbose

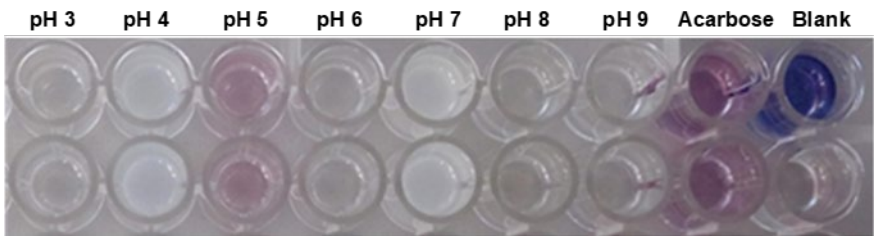

Fig 3. The α-amylase inhibitory activity test using a 96-well plate. The pH 5 fraction shows a purple color as a result of α-amylase inhibition

Table 1. Identified peptides at pH 5 fraction using LC-HRMS

Peptide sequences	Protein of peptide origin	[MH⁺]Da	pI
VQKEKR	DNA polymerase I	787.48075	9.99
MNFYCISSK	Plastid-encoded RNA polymerase subunit alpha	1191.52201	7.95
DLCDYIHNK	Site-specific DNA-methyltransferase (adenine-specific)	1177.53321	5.21
ISICYEK	DNA-directed RNA polymerase	912.45142	5.99

trypsin-mediated cleavage. The four peptide sequences of the pH 5 fraction were VQKEKR, MNFYCISSK, DLCDYIHNK, and ISICYEK.

The peptides were characterized by their retention times, mass-to-charge ratios (*m/z*), and precursor ion masses. Specifically, the peptides exhibited the following characteristics: VQKEKR: eluted at 15.7394 min, *m/z* value of 263.16510 Da, ion charge of +3, and precursor ion mass of 787.48075 Da. The second peptide, MNFYCISSK: eluted at 10.3677 min, *m/z* value of 397.84552 Da, ion charge of +3, and precursor ion mass of 1191.52201 Da. The third peptide, DLCDYIHNK: eluted at 10.3833 min, *m/z* value of 393.18259 Da, ion charge of +3, and precursor ion mass of 1177.53321 Da. The fourth peptide, ISICYEK: eluted at 17.5140 min, *m/z* value of 304.82199 Da, ion charge of +3, and precursor ion mass of 912.45142 Da. As a representation, the MS1 and MS2 spectra of the MNFYCISSK peptide are presented in Fig. 5 and 6, respectively.

The bioactivity of peptides is significantly influenced by their constituent amino acids. Several amino acids are crucial for inhibiting α-amylase, including leucine, tryptophan, arginine, tyrosine, methionine, phenylalanine, and amino acids with acidic

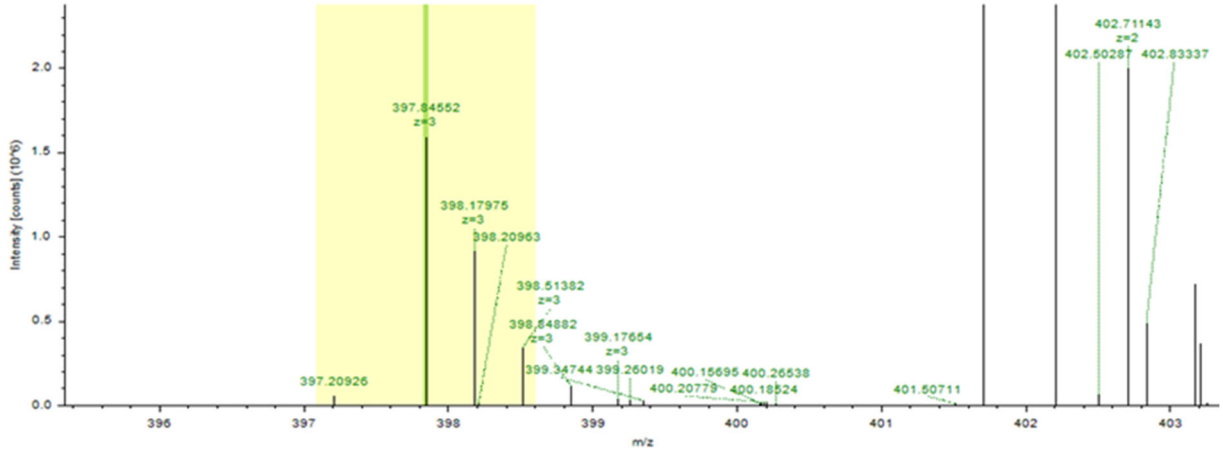

Fig 5. The MS 1 spectrum of the MNFYCISSK peptide

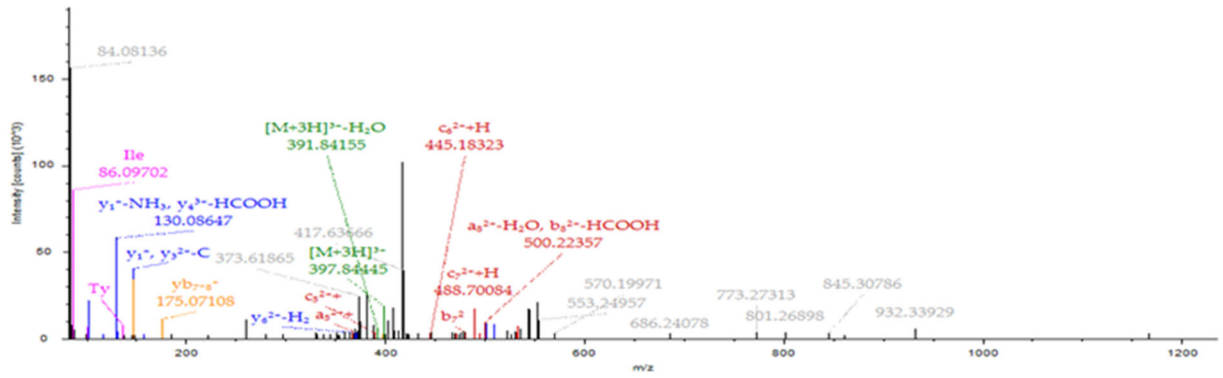

Fig 6. The MS 2 spectrum of the MNFYCISSK peptide

or positively charged properties. Research has shown that peptides containing the tryptophan-arginine-tyrosine (WRY) motif, such as those found in tendamistat-type α-amylase inhibitors, function by binding to amino acids in the active site of α-amylase [16]. Similarly, the LPLLR peptide from *Juglans mandshurica* Maxim has been identified as an α-amylase inhibitor [17]. Additionally, peptides from quinoa, such as the MMFPH peptide, have been shown to bind to residues in the active and allosteric sites of α-amylase at low concentrations [18]. The peptides obtained from *Caulerpa racemosa*, namely VQKEKR, MNFYCISSK, DLCDYIHNK, and ISICYEK, contain several key amino acids that are known to inhibit α-amylase.

In Silico Analysis

To investigate the interaction between the peptide and α-amylase, a docking study was performed using Haddock. Redocking between α-amylase and acarbose was conducted prior to the docking process of the receptor protein (α-amylase PDB ID: 1XD0) with the proposed compound (peptides). The docking method was validated by examining the binding affinity and root mean square deviation (RMSD) values. Binding affinity is a measure of the ability of a ligand to bind to the receptor, where a high binding affinity value indicates low affinity between the receptor and ligand, and a low binding affinity value indicates high affinity [19]. RMSD is a parameter used to evaluate the similarity between two structures based on the difference in distances of similar atoms [20]. An RMSD value ≤ 2 Å is considered good, indicating that the ligand pose obtained from redocking is similar to the initial shape, suggesting a successful docking process. The 3D structures of α-amylase and acarbose are shown in Fig. 7, while the 3D structures of *C. racemosa* peptides generated using PEPFOLD4 are presented in Fig. 8.

The binding affinity of acarbose to α-amylase was found to be −11.1 kcal/mol, with an RMSD of 0.1 ± 0.1 Å. The negative binding affinity value indicates

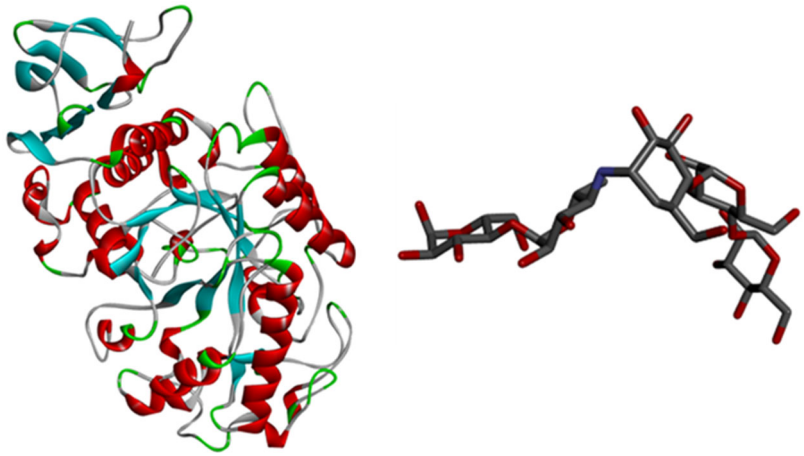

Fig 7. The 3D structure of the protein (α-amylase PDB ID: 1XD0) and acarbose

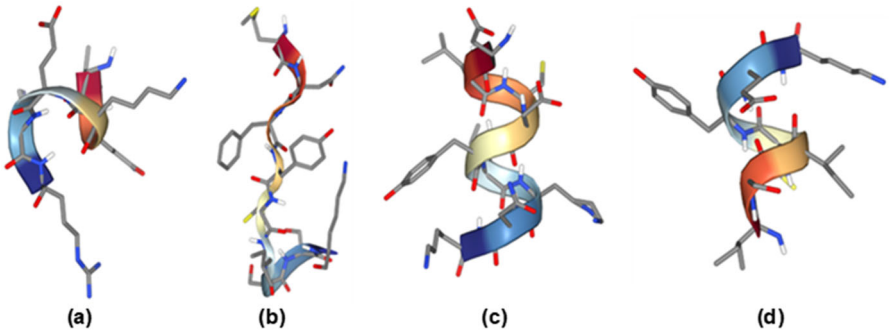

Fig 8. The 3D structure of *C. racemosa* peptides (a) VQKEKR, (b) MNFYCISSK, (c) DLCDYIHNK, and (d) ISICYEK

a stable interaction between acarbose and α-amylase. The docking results of α-amylase with four peptide compounds revealed that the MNFYCISSK peptide exhibits the best binding affinity energy, with a value of −10.3 kcal/mol and an RMSD of 0.3 ± 0.0 Å. Notably, the affinity and RMSD values of the MNFYCISSK peptide are comparable to those of the positive control, acarbose. This suggests that MNFYCISSK peptide forms a stable interaction with the binding site of α-amylase, demonstrating its potential as an antidiabetic agent.

The docking analysis results reveal that the MNFYCISSK peptide exhibits the most potent inhibitory activity among the proposed peptides, with a binding affinity energy of −10.3 kcal/mol and an RMSD of 0.3 ± 0.0 Å (Table 2). The MNFYCISSK peptide forms hydrogen bonds with amino acid residues Gly306, Tyr151, Gly308, and Glu240 in α-amylase (Fig. 9). Hydrogen bonding interactions are crucial for the stable binding of ligands to protein binding sites. Water-mediated hydrogen bonds provide additional stability to the protein-ligand system. Furthermore, the MNFYCISSK peptide engages in phi-cation, phi-sigma, phi-sulfur, and phi-alkyl interactions, which contribute to inhibitor binding. Notably, pi bonds are insensitive to solvation/desolvation effects, resulting in minimal enthalpy/entropy penalties on the free binding energy [21]. Specifically, the MNFYCISSK peptide forms a pi-sigma bond with Ile235, a pi-sulfur bond with His101, a pi-alkyl bond with Lys200, Ala198, Ala307, and Leu237, and a pi-cation interaction with His305. Pi-cation interactions can enhance binding affinity, specificity, selectivity, lipophilicity, bioavailability, and metabolic stability, desirable drug physicochemical features [22]. Additionally, the MNFYCISSK peptide interacts with key amino acids Asp300 and Asp197, which are crucial for the α-amylase inhibition mechanism.

Table 2. The interaction of the identified peptides with the α-amylase (PDB ID: 1XD0) from the Haddock docking study

Peptide	Binding affinity (kcal/mol)	RMSD (Å)	Amino acid interactions					
			Hydrogen bond	Attractive charge	Pi-sulfur	Pi-alkyl	Pi-sigma	Pi-cation
Acarbose	−11.1	0.1 ± 0.1	Glu240, Arg195, Asp300, Trp59, Thr163, His305, Tyr62, Leu162	Asp300, Asp197, Glu233	-	-	-	-
VQKEKR	−9.4	0.3 ± 0.2	Asp353, Asp356, Arg303, Gly306, Thr163, Ile148, Asp300, Tyr62, Tyr151	Asp356, Asp300	-	Tyr151	-	-
MNFYCISSK	−10.3	0.3 ± 0.0	Gly306, Tyr151, Gly308, Glu240	Asp300	His101	Lys200, Ala198, Ala307, Leu237	Ile235	His305
DLCDYIHNK	−8.2	0.4 ± 0.2	Lys261, Gly308, Gly306, Gly304, Tyr151, Thr163, Gly309	-	-	Leu23, Tyr151, Leu16, Ala307, Ile235	-	-
ISICYEK	−7.5	1.0 ± 0.1	His305, Asp300, Trp59, Asp353, Asn352	-	-	Tyr151, His305	-	-

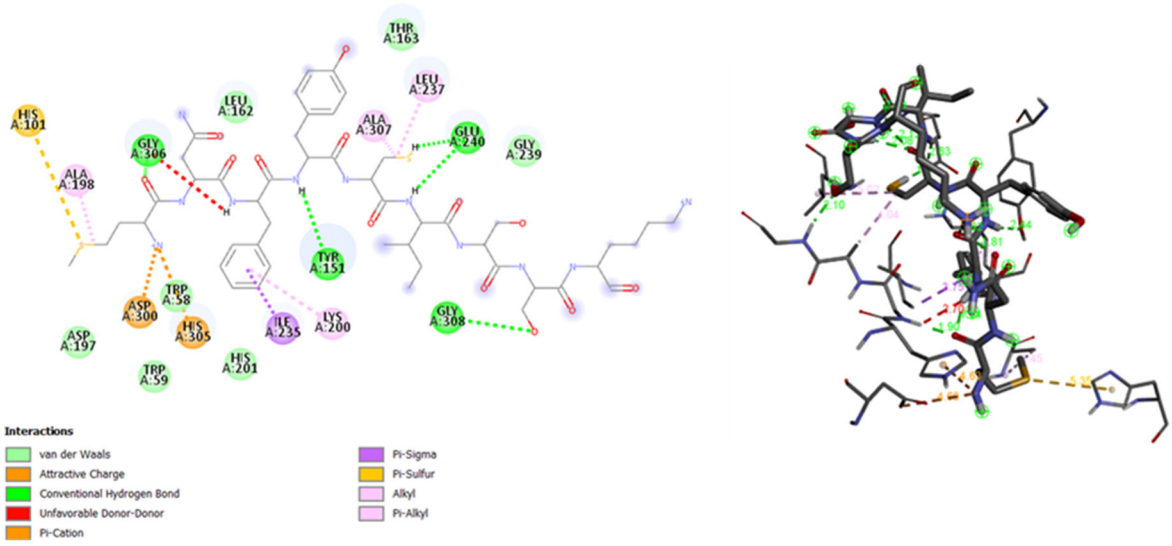

Fig 9. Interaction of peptide MNFYCISSK with the α-amylase (PDB ID: 1XD0)

■ CONCLUSION

The protein from *C. racemosa* can serve as a rich source of α-amylase inhibitor peptides. Tryptic digestion of the protein, followed by cation exchange fractionation, revealed that the pH 5 fraction of the hydrolysate exhibits α-amylase inhibitory activity with an IC_{50} value of 0.0437 mg/mL. Four peptides, namely VQKEKR, MNFYCISSK, DLCDYIHNK, and ISICYEK, identified in the pH 5 hydrolysate fraction, are likely responsible for this activity. The antidiabetic properties of these peptides, attributed to their ionic properties and structure, support their inhibitory activity on the active site of α-amylase. Molecular docking studies demonstrated that peptides MNFYCISSK and VQKEKR exhibit the strongest interactions with α-amylase, with binding affinity energies of −10.3 and −9.4 kcal/mol, respectively, and RMSD values of 0.3 ± 0.0 and 0.3 ± 0.2 Å.

■ ACKNOWLEDGMENTS

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■ CONFLICT OF INTEREST

Authors do not have a conflict of interest.

■ AUTHOR CONTRIBUTIONS

Tri Joko Raharjo conceived the idea, designed the experiment, and led the team. Endang Astuti analyzed the results and contributed to the manuscript preparation. Ahmad Habibie supervised the experiment, while Izatul Husna conducted the experiment, analyzed the results, drafted the manuscript, and revised and verified the manuscript and results. All authors have read, approved, and contributed significantly to this research, leveraging their expertise and knowledge.

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