

ANTIBACTERIAL PEPTIDE FROM SOLID PHASE EXTRACTION (SPE) FRACTIONATION ON TRYPSIN HYDROLYSIS OF JATROPHA (*Ricinus communis*) SEED PROTEIN ACID EXTRACT

D. R. Atmawati, Z. Andriana, R. T. Swasono and T. J. Raharjo✉

Department of Chemistry, Faculty Mathematics and Natural Sciences, Universitas Gadjah Mada, Bulaksumur, Yogyakarta, Indonesia, 55281
 ✉Corresponding Author: trijr_mipa@ugm.ac.id

ABSTRACT

The antimicrobial peptide is a new potential antibiotic type that can be isolated not only from free peptides but also peptides from hydrolyzed protein with proteolytic enzymes. The protein of jatropha seed is toxic due to RIP, ribosome-inactivating protein, and a potential source of AMP. Trifluoroacetic acid was used to isolate jatropha seed protein. The protein was hydrolyzed using trypsin and the hydrolysate separated with a cation exchange solid phase extraction (SPE) column. Each fraction was tested for antimicrobial activity and resulted in the pH 3 and pH 4 fractions being able to inhibit bacterial growth against *Staphylococcus aureus* (Gram-positive bacteria) and *Escherichia coli* (Gram-negative bacteria). The pH 3 fraction has IC₅₀ value 17.95 µg/mL to *E. coli* and 17.03 µg/mL to *S. aureus*, while the pH 4 fraction has IC₅₀ value 37.30 µg/mL and 95.31 µg/mL respectively. There are three peptides with amino acid sequences of GAGLVPKR, MGACCSKEPSFAEGR and VALASLLSQPLQISDK identified from those fractions. These peptides potentially inhibit bacterial growth through membrane destabilization mechanism, due to their amphipathic and ionic properties.

Keywords: TFA Extraction, Anti-Bacterial Peptide, *Ricinus Communis*, Cationic Exchange, SPE

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INTRODUCTION

Antibiotic resistance was declared by World Health Organization as a global health threat two decades ago. Genetic mutation of the pathogen is one of the factors that caused this health concern due to antibiotic over-prescribing.^{1,2} It is predicted there will be over 10 million deaths predicted by 2050 due to antimicrobial resistance.³ Unfortunately, the development of new antibiotics was not grown as fast as other medicine. Antimicrobial peptides (AMPs) are potentially developed as a new type of antibiotics. AMP is a small compound consisting of amino acids that have biological activity against bacteria. AMP is an amphipathic compound because it has both hydrophilic and hydrophobic sites.⁴ Compared to traditional antibiotics, AMPs' ability to work faster with some mechanisms is an excellent trait to limit antibiotic resistance.⁵ Cell membrane destabilization is the most common mechanism of the AMPs to attack bacteria. Cationic AMPs can generate pores by binding on the negative charge of the bacteria cell membrane and causing cell lysis. Nisin is an antimicrobial peptide from *Lactococcus lactis* that follows this mechanism against gram-positive bacteria.⁶ Different from cationic AMPs, anionic AMPs work with the presence of divalent metal ions, to facilitate peptides with the bacterial cell membrane. Kapacins are anionic AMPs from the κ-casein hydrolysate enzyme where is in the presence of Zn²⁺, it was able to reduce > 90% viable cell numbers in the biofilm of *S. mutans*. Bacteria need to redesign their cell membrane structure as a defense mechanism against AMPs, but it will require a long period over multiple mutations.³ AMPs can be isolated not only from free peptides but also peptides from hydrolyzed protein with proteolytic enzymes such as trypsin.⁷ Moscoso-Mujica, *et al.* (2021) purified AMP from hydrolysates of kanihua (*Chenopodium pallidicaule* Aellen) seed protein fractions that showed antibacterial activity against both Gram-positive and Gram-negative bacteria.⁸ AMPs can be hydrolyzed from a toxic protein, like ricin. Jatropha plant (*Ricinus communis* L.) is famous for its seed that contains ricin. Ricin is a ribosome-inactivating protein (RIP) for its ability to inhibit protein synthesis.⁹ Ricin consists of two RIP chains, ricin toxin A (RTA) connected by

a sulfide bridge to ricin toxin B (RTB). RTB is a lectin that attached glycolipids and glycoproteins of the bacteria membrane cell to initiate translocation of RTA by endocytosis. RTA is an RNA N-glycoside that catalyzes ribosome inactivation.¹⁰ Peptides with RIP activity will be active as an antimicrobial agent.¹¹ Peptides jatropha seed protein hydrolyzed are interesting objects to study because of their potential AMPs activity.¹² Surfactant is commonly used to isolate protein by increasing protein solubility in water. This method requires additional treatment to remove surfactant using membrane dialysis, but this additional process also increases the loss of protein.¹³ Jatropha seed protein extraction using sodium dodecyl sulfate (SDS) successfully extracted 41,35% protein.¹⁴ To increase the yield of protein extracted, an isolation procedure has to be performed with a different method. AMPs of jatropha seed protein hydrolysate can be obtained by hydrolyzing its protein with an enzyme. Mostly, AMPs have a positive charge due to the presence of lysine (K), arginine (R), and histidine (H). According to this theory, protein hydrolysis can be done with the trypsin enzyme. Trypsin can cleave lysine (K) and arginine (R) on C-terminus, and it will generate positive charge peptides.¹⁵ Peptides from protein hydrolysate need to be fractionated. A cation exchange column is used to fractionate cationic peptides based on their isoelectric points. The solid-phase extraction (SPE) column has some advantages, such as an easy and fast process and requiring a small amount of buffer.^{13,16,17} Raharjo *et al.* (2021) fractionated jatropha seed hydrolysate using strong cation exchange (SCX).¹⁴ Peptides analysis can be performed using high-resolution mass spectrometry (HRMS) to determine the amino acid sequences. This process includes matching fragmented MS data of peptides with the jatropha seed (*Ricinus communis* L.) database using Proteome Discoverer 2.5 software.^{14,18} This study mainly aimed to identify amino acid sequences of the peptides present in jatropha seed protein hydrolysate that potential to be antimicrobial (AMP).

EXPERIMENTAL

Material

The jatropha seed was provided by EcoWell Indonesia. The protein extraction and enzyme digestion used trifluoroacetic acid (TFA) (Sigma), trypsin USP grade (Bioworld) iodoacetamide (IAM) (Sigma), dithiothreitol (DTT) (Promega), tris base (Calbiochem). Peptide fractionation employed cation exchange column SPE Supelco DSC-SCX Cartridge (Sigma). Bacterial activity test used *Escherichia coli* (ATCC) and *Staphylococcus aureus* (ATCC, USA), chloramphenicol (Kimia Farma).

Protein Extraction and Trypsin Hydrolysis

Dried defatted jatropha seed powder was stirred in 1:10 (m/v) trifluoroacetic acid for 10 minutes at room temperature. Tris base 1:10 (v/v) was added to neutralize the solution and centrifuged at 6,000 rpm for 30 minutes. The extracted protein absorbance was measured with a spectrophotometer UV – Vis at 280 nm.¹⁹ The DTT was added to the extract solution to a final concentration of 5 mM, then incubated at 56 °C for 25 minutes. The solution was cooled at room temperature, then add with IAM (14 mM) and incubated for 30 minutes at room temperature in the darkroom. The solution was reduced again with DTT (5 mM) and incubated for 15 minutes in the dark at room temperature. The protein was added with 1:40 (w/w) trypsin solution (50 mM in ammonium bicarbonate). Digestion was carried out for 24 hours at 37 °C. Then, further incubation was done at 80 °C for 15 minutes to start trypsin denaturation. Protein hydrolysate cooled at room temperature before being filtered with Amicon® Ultra – 15 Centrifugal Filter Device for 60 minutes at 6,000 G. Both filtrate and concentrate absorbance were measured at 280 nm with UV – Vis spectrophotometer.

Peptides Fractionation from the Hydrolysate

SPE column was pre-conditioned using methanol, water, and citrate buffer pH 3 (0.1 M). The protein hydrolysate was set at pH 3 by adding citrate buffer at 50 mM pH 3 and then applied through the column. The peptides were then eluted by various pH starting with pH 3 (0.1 M), citrate buffer M pH 4 (0.1 M), citrate buffer M pH 5 (0.1 M) and phosphate buffer pH 6 (0.1 M), phosphate buffer pH 7 (0.1 M), phosphate buffer pH 8 (0.1 M), phosphate buffer pH 9 (0.1 M) were eluted to the column. All the eluted fractions were collected.²⁰

Antibacterial Activity Assay

Both *E. coli* and *S. aureus* bacterium was grown in LB medium (NaCl, tryptone, and yeast extract) at 37 °C for 24 hours as a bacterial stock solution. The disc diffusion used the bacteria stock diluted in melted LB

agar and then put on a sterilized petri dish. The tested disc was prepared by dipping the paper disc into peptides of fraction solution, chloramphenicol as a positive control, and buffers as negative control followed by placing the disc on the surface of the bacterial culture in the petri dish. The incubation processes were carried out at 37 °C for 24 hours. Diameters of inhibition were measured in every paper disc. The IC₅₀ was performed to the most active fraction based on the inhibition zone of the disc diffusion method. The 96well plate was filled with 10 µL bacteria stocks and 80 µL LB medium. The microdilution assay employed various concentrations of peptide fractions, and positive and negative control were applied to the respected well 96-well. The plates were also incubated at 37 °C for 24 hours. The bacteria turbidity was measured with a microplate reader at 600 nm. The percent of inhibition of each concentration was determined to calculate the IC₅₀.

Identification of Antibacterial Peptides

The peptides identification was carried out with a ThermoExact high-resolution mass spectrometer (Thermo Scientific) equipped with Acclaim® PepMap RSLC column (C18, 1 × 150 mm, particle size, 3 µm). The mobile phase used a gradient of 0.05% TFA in water (A) and 0.1% TFA in water/acetonitrile 20/80 and (B). The mobile phase was flow at 50 µL/min of 4% B for 3 minutes, then changed 10% B to 29th min, changed linearly to 20% B for 30th to 70th min, and finally, 95% B until 90th min. The MS/MS was conducted using positive ion mode using the range of m/z (250–1,800) with full scanning MS1 and data-dependent MS2 mode. The resolving power for the MS1 was set at 140,000 FWHM while the resolution for the dd-MS2 was set at 17,500 FWHM. The MS raw data were analyzed with Proteome Discoverer Software ver. 2.5 using Sequest HT algorithm. Peptide identification was performed by comparing the raw data with the UniProt database of the *Ricinus communis* protein database.

RESULTS AND DISCUSSION

Jatropha Seed Protein Extract and the Hydrolysate

Extraction of protein from plant material is challenging because of the complexity of the plant cell structure. Protein extraction in principle can be carried out by increasing protein solubility in water. With the addition of strong acid, amine groups on the protein will be protonated into a positive charge and the carboxylic group will be protonated, leading to the protein having a positive total charge and increasing protein solubility in water.¹⁷ Extraction of jatropha seed protein using trifluoroacetic acid yields up to 60.4% protein. This result is much higher than the previous extraction method using SDS 0.01% only able to extract 20.94%.¹⁴ In protein extraction, SDS works by disrupting cell membranes and interfering with the protein structure. Although the content of the protein in the seed is also influenced by the different botanical environments where the sample was planted the yield of TFA extraction is similar to base extraction reported previously with the yield of up to 69.2%. These results confirm that in jatropha seed protein extraction, protonation of the protein is more effective to increase the protein solubility than the disruption of the tertiary and quaternary structure of the proteins. In general, the yield of the protein extraction using TFA has been reported relatively high whatever the sample type. Doellinger *et al.* (2019) successfully extract protein from mouse lung tissue that has a difficult-to-lyse cell with trifluoroacetic acid and yielded about 68% protein extract.¹⁹ To obtain a sufficient amount of peptide, hydrolysis protein should be performed effectively. The folding structure of the protein not only affects the function of the protein but possibly also inhibits protease enzymes working in protein hydrolysis. One of the interactions that play a role in protein folding is the disulfide bond. A disulfide bond is formed by two cysteines which have a thiol group as a side chain. Disulfide bond cleavage needs to be carried out by disulfide reduction and alkylation to increase the efficiency of protease enzyme work.^{21,22} Dithiothreitol as a reducing agent and iodoacetamide as alkylating agent is the most common combination for disulfide bond cleavage in protein analysis by mass spectrometry.^{23,24} Employing protein hydrolysis with trypsin can hydrolyze up to 65.9% protein. Hydrolysis degree depends on incubation time, amount, and quality of enzymes used. The higher the hydrolysis degree, the more peptides will be produced.^{14,25}

Antibacterial Activity of the Peptide Fractions

Jatropha peptides were fractionated using a strong cation exchange solid phase extraction column. Peptides from trypsin hydrolyzed will have a positive total charge, due to lysine (K) and arginine (R) on the C-

terminus, amine group on the N-terminus, and the presence of lysine (K), arginine (R), and histidine (H) amino acid on peptide chain. These positive peptides will have electronic interaction with the anionic sorbent surface. Peptide's selectivity separation was based on the difference in the isoelectric point (pI) of each peptide. Peptides at pH below their pI will have a positive charge and then restrain in the SPE column. By increasing pH for elution, peptides that reach their pI will be neutralized and flowed with buffer as a mobile phase. Raharjo *et al.* (2021) successfully fractionated jatropha peptides trypsin hydrolysate using SPE. Peptides in the pH 4 fraction have the highest peptides content about 19.85% and the lowest is peptides in the pH 9 fraction with 2.60% peptides.¹⁴

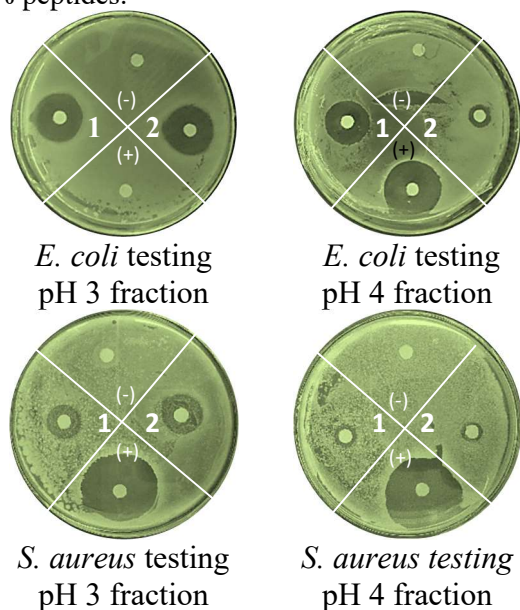


Fig.-1: Growth Inhibition of *E. coli* and *S. aureus* Resulted in pH 3 and pH 4 Fractions of Jatropha Seed Protein Hydrolysate. Chloramphenicol and Citrate Buffer was used as a Positive Control (+) and Negative Control (-), Respectively, While a Replicated of Fractions was Indicated with 1 and 2.

Table-1: Inhibition Zone Diameter, MIC, and IC50 pH Fractions of Jatropha Seed for *S. aureus* and *E. coli*

Bacteria	Sample	Diameter of inhibition (mm)	MIC (μg/mL)	IC50 (μg/mL)
<i>E. coli</i>	pH 3 fraction	22	6.6	17.9
	pH 4 fraction	20	18.3	37.3
	Chloramphenicol	21	13.9	20.8
<i>S. aureus</i>	pH 3 fraction	16	6.6	17.0
	pH 4 fraction	10	36.7	95.3
	Chloramphenicol	28	13.9	23.9

From Fig.-1 and Table-1, the antibacterial activity of the fraction in *S. aureus* was smaller than in *E. coli*. *S. aureus* is a Gram-positive bacterium with a cell wall structure made of a 20-80 nm thick layer of peptidoglycan polymer with a combination of teichoic acid, while *E. coli* bacteria have a cell wall structure made of a 2-3 nm peptidoglycan layer covered by a lipid bilayer.²⁶ The presence of a lipid bilayer on the cell wall of *E. coli* bacteria not only increases the hydrophobic interactions between the peptides and the cell membrane surface but also increases the destabilization process by peptides⁴. More membrane destabilization process occurs in *E. coli*, bigger chances for peptides to inhibit *E. coli* bacteria growth. It will make inhibition zone diameter larger and concentrations of both MIC and IC50 in *E. coli* bacteria smaller than in *S. aureus* bacteria.

Antibacterial Peptides of Jatropha Seed Protein

Analysis of amino acid sequence in peptide fractions pH 3 and pH 4 were done using Proteome Discoverer 2.5 software with a database of *R. communis* plants. Several peptides do not have lysine (K) or arginine (R) ends, which are called missed cleavage sites. The non-specific cleavage of the enzyme can be caused by an increase in the enzyme: protein ratio.¹⁴ In several studies, an enzyme: protein ratio of 1:50 to 1:100 was

used.^{19,27} Excessive amounts of enzymes lead to non-specific cuts. Due to the unavailability of targeted amino acid for enzymes to cleavage, it forms peptides with non-specific ends and occurs of autolysis. Perutka and Sebela (2018) stated that missed cleavage happens due to the activity of the chymotrypsin enzyme (chymotrypsin-like activity) because of chymotrypsin and pseudotrypsin (Ψ -trypsin) contamination as the result of the autolysis process.²⁸ Amino acid modifications also occur in peptides. The modification often occurs in the formation of a carbamidomethyl group on the amino acid cysteine (C) during the alkylation process of thiol groups to prevent the formation of disulfide bonds.²⁹ Modifications also occur in the amino acid methionine (M) by an oxidation process. However, oxidation of the amino acid methionine happens naturally in cells or during the sample preparation process. Makes methionine oxidation itself is used as one of the parameters in peptide analysis.³⁰

Table-2: Characteristic of the Peptides in the pH 3 Fractions of *R. communis* Seed Protein Tryptic Hydrolysate

No.	Peptide Amino Acid Sequences	Predicted pI	[M-H] ⁺	The Protein Identity	Position of amino acid in the protein
1.	GAGLVPKR	11.00	797.49870	Cyclic nucleotide-gated ion channel, putative	55 - 62
2.	MGACCSKEPSFAEGR	5.89	1686.69233	Protein phosphatase-2c, putative	1 - 15

Table-2 shows data of two peptides identify from the pH 3 peptide fraction with their mass confirmed with MS¹ of HRMS. The GAGLVPKR peptide with a retention time of 65.5200 minutes was identified in MS² fragmentation with an m/z value of 399.25299 Da. This peptide has [M-H]⁺ = 797.49870 and z = +2. The mass spectra of these peptides are presented in Fig.-2. The confirmed MS² spectra in the theoretical fragment table are b²⁺ ions. The ion is formed as a result of the termination of the C - N bond between alanine (A) and glycine (G). The ion formed from the alanine (A) and glycine (G) has a theoretical mass of 128.05857 then it gets one proton, appearing at m/z of 129.05441.

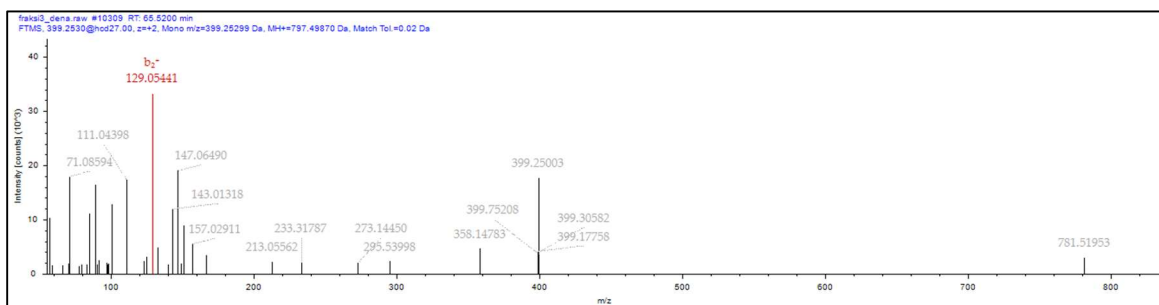


Fig.-2: MS/MS Spectra GAGLVPKR Peptide of the pH 3 Fraction

The MGACCSKEPSFAEGR peptide with a retention time of 87.9416 minutes was identified in MS² fragmentation with an m/z value of 338.14429 Da. This peptide has [M-H]⁺ = 1686.69233 and z = +5. The mass spectra of these peptides are presented in Fig.-3. The confirmed MS² spectra in the theoretical fragment table are y⁵⁺ ions. The ion is formed as a result of the termination of the C - N bond between phenylalanine (F) at the N - terminus and serine (S). The theoretical mass of the phenylalanine (F), alanine (A), glutamic acid (E), glycine (G), and arginine (R) is 578.28130. With the addition of one proton, the m/z value appears in the spectra of 579.28796.

In the sample of the pH 4 fraction, several peptides were present in the sample of the pH 3 fraction. This may occur during the fractionation process, where too many samples make sorbent saturated. Saturated sorbent causes not all of the peptides to bind electrostatically with the sorbent, making the peptide eluted before reaching the pI value in a buffer with higher pH.

Table-3 shows data of two peptides identify from the pH 4 peptide fraction with their mass confirmed with MS¹ of HRMS. The VALASLLSQPLPQISDK peptide with a retention time of 31.6397 minutes was identified in MS² fragmentation with an m/z value of 594.01788 Da. This peptide has [M-H]⁺ = 1780.03910

and $z = +3$. The mass spectra of these peptides are presented in Fig.-4. The confirmed MS² spectra in the theoretical fragment table are y_1^+ and b_6^{2+} ions. The y_1^+ ion is formed due to the cleavage of the C - N bond of lysine (K) at the N - terminus with aspartic acid (D). Lysine (K) has a theoretical monoisotopic mass of 146.1553 which then gets one proton, appearing at m/z 147.09695. The b_6^{2+} ion is formed due to the cleavage of the C - N leucine (L) at the C - terminus with leucine (L). Because it has a charge of +2, the spectra that appear are half of the theoretical value of m/z b_6^+ of 555.35012.

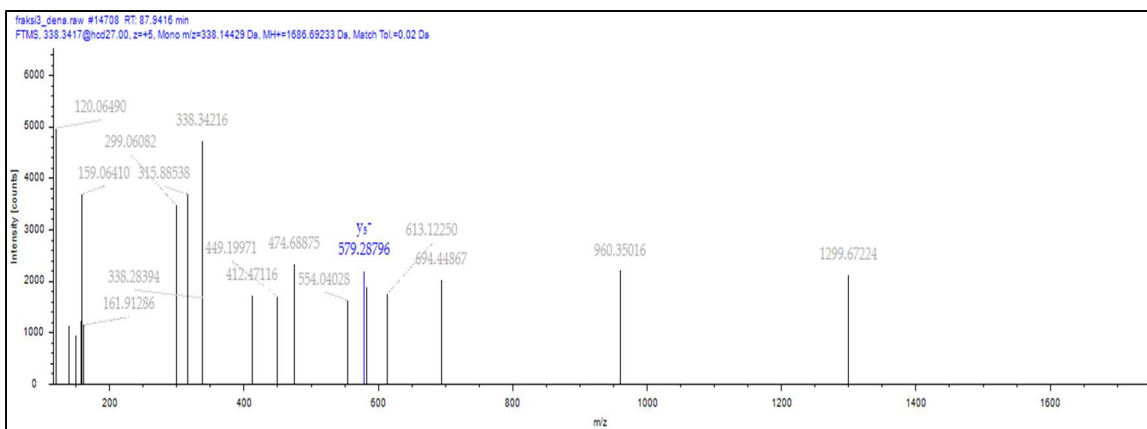


Fig.-3: MS/MS Spectra MGACCSKEPSFAEGR Peptide of the pH 3 Fraction

Table-2: Characteristic of the Peptides in the pH 4 Fractions of *R. communis* Seed Protein Tryptic Hydrolysate

No.	Peptide Amino Acid Sequences	Predicted pI	$[M-H]^+$	The protein identity	Position of amino acid in the protein
1.	VALASLLSQPLPQISDK	5.81	1780.03910	Uncharacterized protein	51 - 68
2.	GAGLVPKR	11.00	797.49693	Cyclic nucleotide-gated ion channel, putative	55 - 62

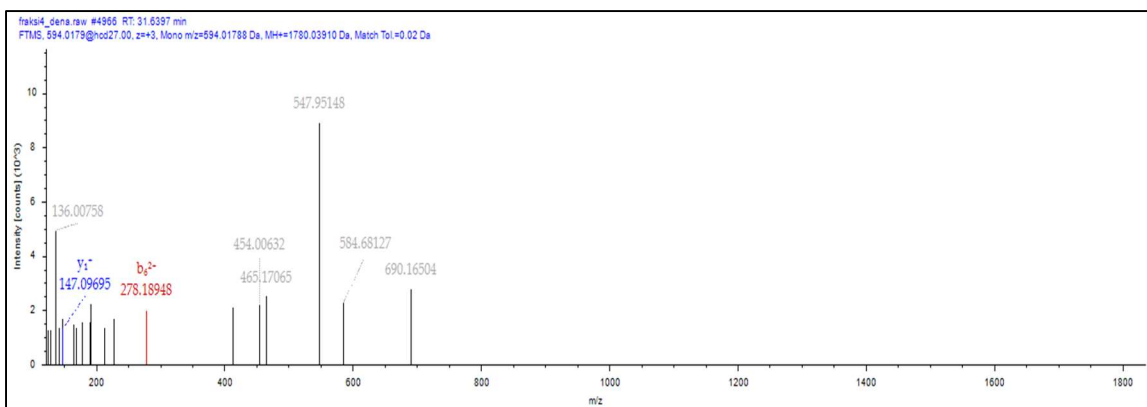


Fig.-4: MS/MS Spectra VALASLLSQPLPQISDK Peptide of the pH 4 Fraction

The GAGLVPKR peptide with a retention time of 65.7912 minutes was identified in MS² fragmentation with an m/z value of 399.25211 Da. This peptide has $[M-H]^+ = 797.49693$ and $z = +2$. The mass spectra of these peptides are presented in Fig.-5. The confirmed MS² spectra in the theoretical fragment table are b_2^+ ions. The ion is formed due to the termination of the C - N bond on the alanine (A) at the C - terminus with glycine (G). The ion formed from the amino acids glycine (G) and alanine (A) at the N - terminus of the peptide, has a theoretical mass of 128.05857. However, due to the addition of one proton, the m/z value of 129.05446 appears in the spectra.

The result of missed cleavage can increase the potential for the mismatched peaks with m/z spectra.¹⁴ In general, AMP is unstable and easily denatured, so it is necessary to synthesize AMP to determine the antibacterial mechanism of action of each peptide and test its activity against bacteria, microbes, and cells.³¹

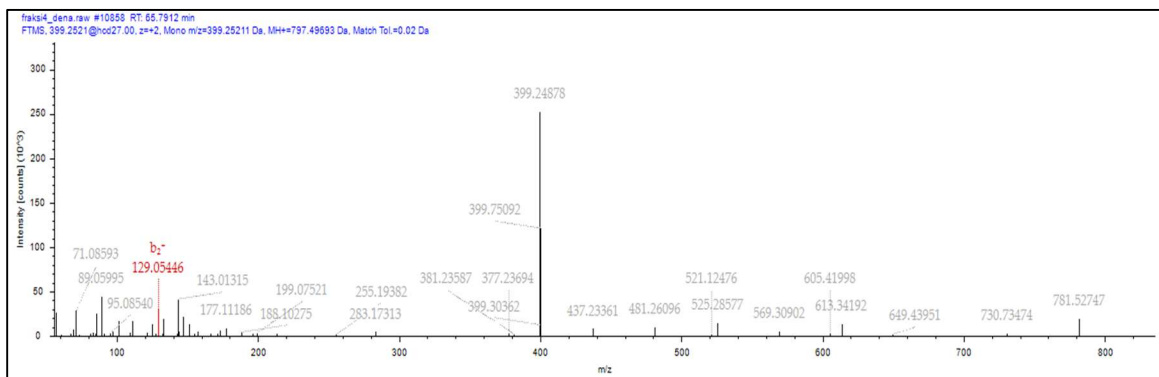


Fig-5: MS/MS Spectra GAGLVPKR Peptide of the pH 4 Fraction

CONCLUSION

Jatropha seed (*Ricinus communis*) protein extraction with trifluoroacetic acid has a higher result compared to sodium dodecyl sulfate. Protein digestion with trypsin followed with fractionation using strong cation exchange solid phase extraction column showed that pH 3 and pH 4 fractions have antibacterial activity against both gram-positive (*S. aureus*) and Gram-negative (*E. coli*) bacteria. The peptide with sequences GAGLVPKR was found on both pH 3 and pH 4 fractions, while a peptide with sequence MGACCSKEPSFAEGR was found on pH 3 fraction and another peptide with sequence VALASLLSQPLQISDK was found on pH 4 fraction. Those peptides are potentially classified as antimicrobial peptides, due to their ionic and amphipathic characteristics, which support the disruption of membrane cells of bacteria.

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