



Optimization of Soy Protein Hydrolysis with Papain Enzyme and Its Cytotoxic Activity Against MCF-7 Cancer Cells

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ABSTRACT: Soybean is a source of vegetable protein that is rich in nutrients and a source of producing anticancer bioactive peptides. This study aims to determine the optimum conditions for hydrolysis of soybean protein with papain enzyme and their cytotoxicity against the MCF-7 cells. Soybean protein was isolated using an acid precipitation technique. Then, the protein isolate was hydrolyzed using papain enzyme with variations of papain concentration 0,5; 1; and 5% (v/v) and variations of incubation time 0, 1, 2, 3, and 4 hours at 50 °C. The hydrolysates were tested for their degree of hydrolysis (%DH), molecular weight profile using SDS-PAGE, and cytotoxicity against the MCF-7 cells through an in-vitro assay. The most active hydrolysate was fractionated using Sephadex G-15 and characterized by the molecular weight by LCMS/MS. The result showed that the optimum condition for hydrolysis was 1% (v/v) of enzyme concentration and 3 hours of incubation time with a %DH value of 3.01%. Based on the SDS-PAGE result, the hydrolysate had protein bands in a lower range (<25 kDa). That hydrolysate has cytotoxicity with an IC_{50} value of 1.87 mg/mL, and the molecular weight of its bioactive peptide is 7.70 kDa.

Keywords: bioactive peptide; cytotoxicity; MCF-7 cells; papain; soybean.

Introduction

Cancer is a frequent and severe health problem all over the world. Based on data from the World Health Organization (WHO), in 2020, around 2.3 million women were diagnosed with breast cancer and 685000 deaths globally [1]. Currently, there are various ways to treat breast cancer: surgery, chemotherapy, or radiotherapy. However, these treatments have adverse side effects due to drug resistance in the cancer cells. These include the inactivation of the drug, cell death inhibition (apoptosis suppression), epigenetic changes, changes in the drug targets, and target gene amplification [2]. Meanwhile, alternative methods for treating cancer have increased in recent years.

Soybean (*Glycine max* (L.)) is a complex matrix containing an inexpensive source of protein with high nutritional value that can be provided for millions of people. Bioactive peptides isolated from soybeans have been shown to benefit the body's biological functions, including as antioxidants, ACE inhibitors, antiobesity and anticancer. Soybean contains a complex mixture of different protein types. Glycinin and β -conglycinin, the precursor of most isolated peptides, represent approximately 65-80% of total

seed protein in soybeans [3]. Moreover, bioactive peptides isolated from soybeans, such as lunasin and the Bowman-Birk protease inhibitor (BBI), are now being intensively studied as cancer chemopreventive agents [4,5].

Bioactive peptides can be isolated through enzymatic hydrolysis with protease enzymes such as papain, trypsin, pepsin, and alkalase [6-9]. Soybean peptides from hydrolysis using alkalase and pepsin enzymes showed cytotoxic activity in breast cancer cells [8,9]. Therefore, this study will explore the potential of peptides from hydrolyzed soybeans using a different enzyme cleavage pattern by papain enzyme.

Hydrolysis was performed using variations of papain concentration 0.5; 1; and 5% (v/v) and variations of incubation time 0, 1, 2, 3, and 4 hours at 50 °C [6]. The optimum condition was determined based on the degree of hydrolysis value and cytotoxic activity against the human breast cancer MCF-7 cell line. The protein profile of hydrolysates was determined through SDS-PAGE, and the anticancer peptide's molecular weight was determined

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using LCMS/MS. Consequently, the potential products with bioactive peptides from this enzymatic hydrolysis are expected to be applied as an alternative chemopreventive agent.

Methodology

Materials and Chemicals

Yellow variety soybean obtained from the market in South Tangerang-Indonesia, aquadest, n-hexane, hydrochloric acid, sodium hydroxide, Bradford's solution, BSA (Bovine Serum Albumin) (Sigma-Aldrich), papain enzyme obtained from Nanning Pangbo Biological Engineering Co., Ltd. China (100 u/mg), 0.1 M phosphate buffer pH 7.5, buffer Tris-Cl pH 8 0.5 M, TCA 10%, cisplatin, dimethyl sulfoxide (DMSO), phosphate buffered saline (PBS), PrestoBlue™ cell viability reagent, Roswell Park Memorial institute Medium (RPMI), fetal bovine serum (FBS), trypsin-EDTA, trypan blue, MCF-7 cancer cell line (ATCC HTB-22), gel acrylamide solution (bio-rad), bis-acrylamide, stacking buffer (Tris-HCl 0.5M pH 6.8 bio-rad), resolving buffer (Tris -HCl 1.5M pH 8.8 bio-rad), sodium dodecyl sulfate (SDS) 10% and 20%, ammonium peroxide disulfate (APS) 10%, N,N,N'-Tetramethylethylenediamine (TEMED) (Sigma-Aldrich), staining solution coomassie blue (bio-rad), protein marker (thermofisher #26643) and Sephadex G-15 gel (Sigma-Aldrich).

Soy Protein Isolation

70 g of yellow soybeans were mashed using a blender and then sieved through a 60 mesh. The flour was defatted by repeatedly soaking in n-hexane (technical) (ratio 1:5 w/v) for 3x1 hour at room temperature. Fat-free flour was mixed with aquadest (ratio 1:10 w/v), then 1 N NaOH was added until the pH of the suspension reached 8.5, then stirred with a magnetic stirrer for 1 hour at room temperature centrifuged at 3000 RPM for 20 minutes at 4 °C. The pellet obtained was discarded while the supernatant was added with HCl until it reached pH 4.5 and then centrifuged at 3000 rpm for 20 minutes at 4 °C. The precipitated protein was rinsed with distilled water and neutralized to pH 7.

Soy Protein Hydrolysis

Soy protein hydrolysis is done by adding a papain enzyme. A total of 0.1 g of protein isolate was dissolved in 50 mL of 0.1 M phosphate buffer pH 7.5. The solution was added with papain enzyme according to variations in concentration (0.5, 1, and 5% (v/v)) and then incubated

for 4 hours. During the incubation process, 5 mL of the mixture was taken at intervals of 0, 1, 2, 3, and 4 hours and the degree of hydrolysis was measured. Each hydrolysate mixture was inactivated by adding Tris-HCl pH 8 0.5 M and heated at 80-90 °C for 10 minutes. Each hydrolysate obtained was measured by the degree of hydrolysis (%DH). The hydrolysate with the highest degree of hydrolysis will then be preserved using the freeze-drying method.

5 mL of protein hydrolysate and 5 mL of 10% TCA (w/v) were added. The mixture was allowed to settle for 30 minutes and then centrifuged (speed 4000 rpm, for 20 minutes). The supernatant was analyzed for protein content using the Bradford method [10]. The degree of hydrolysis of each hydrolysate can be calculated using the following equation:

$$\%DH = (\text{TCA-Soluble Protein} / \text{Total Protein in the Sample}) \times 100\%$$

Protein Profile Analysis with SDS-PAGE

The protein profile for soybean hydrolysate was determined by the Laemmli method which electrophoresis was carried out using a 4% stacking gel and a 15% separating gel with the SDS-PAGE device [11]. Protein hydrolysate from enzymatic hydrolysis was denatured using sample buffer (containing Tris-Cl, SDS, glycerol, mercaptoethanol, and coomassie brilliant blue) and then heated at 100 °C for 5 minutes. The sample was injected into a well, and in one of the wells, a protein marker Spectra Multicolor Broad Range Protein Ladder (Thermofisher #26643) was placed with a molecular weight of 10-260 kDa. The electrophoresis process was carried out for 60 minutes at a voltage of 150 V. Protein staining was conducted using coomassie brilliant blue. The stained results are washed in a destaining solution.

Cytotoxicity Test

Samples with various concentrations were placed on 96-well plates containing RPMI media and MCF-7 cells and then subjected to an incubation period for 24 hours with 5% CO₂ gas at 37 °C. The media in each well was discarded, and 9 mL of media was prepared in a tube to which 1 mL of PrestoBlue™ Cell Viability Reagent was added (10 L of reagent for 90 L of media). A volume of 100 L of the solution mixture was transferred into each well of the microplate and subjected to another incubation period for 1-2 hours until a color change was seen. The absorbance was then measured at a wavelength of 570 nm and normalized to 600 nm values (reference wavelength) using a multimode reader.

Separation of Anticancer Peptides from Hydrolysate

The most active hydrolysate was separated using the filtration gel chromatography method. The stationary phase used was Sephadex G-15, PBS pH 7.4, as the mobile phase at a flow rate of 0.3 mL/minute. The separation results obtained as many as 50 fractions with a volume of 0.5 mL each. 50 L of each fraction was added by 50 L of Bradford's reagent at a 96-well plate. The identified fractions containing peptides (indicated by the appearance of blue color) were then analyzed for their molecular weights using LCMS/MS.

Peptide Characterization by Molecular Weight

The identified fractions containing bioactive peptides were analyzed using the LCMS/MS instrument with electrospray ionization (ESI) ionization mode. The fraction was injected into the LCMS/MS instrument through a C-18 column (1.8 m 2.1 x 100 mm) using acetonitrile:aqueous mixture as a mobile phase at a flow rate of 0.2 mL/min. The molecular weight of the samples was analyzed based on the mass spectrum ($M+nH^+$) and deconvolution calculations using ESIprot online (www.bioprocess.org/esiprot/).

Result and Discussion

Soy protein hydrolysate was obtained using the papain enzyme. Hydrolysis was carried out at a temperature of 50 °C with a phosphate buffer of pH 7.5, as these conditions were the optimum conditions for the papain enzyme to work on previous research [6]. Enzyme concentration and hydrolysis time were varied. Variations in papain

enzyme concentration were 0.5, 1.0, and 5.0% (v/v), and variations in hydrolysis time of 1, 2, 3, and 4 hours. The hydrolysis process is controlled by determining the value of the degree of hydrolysis (%DH). The high value of the degree of hydrolysis indicates the best conditions because the number of peptide bonds broken during the hydrolysis process is higher. The results of measuring the %DH of soybean hydrolysate can be seen in Figure 1.

The highest %DH value was obtained at 1.0% enzyme concentration with a hydrolysis time of 3 hours (Figure 1). The %DH value increased between 1-3 hours, with the maximum %DH value at 3 hours and then decreased at 4 hours. An increase in DH% had positive effects on the solubility property and oil holding ability, while a reduced emulsifying ability was observed up to four hours of hydrolysis. The highest degree of hydrolysis value indicates the best hydrolysis time because the amount of protein hydrolyzed continues to increase over time until the enzyme cannot carry out peptide-cutting activity again and indirectly causes the hydrolysis rate to reach a stationary state [12]. Moreover, the increase in %DH was experienced at the papain enzyme concentration of 0.5% to 1% and decreased at 5%. This is influenced by the rate of reaction between the substrate and the enzyme. The higher value of the degree of hydrolysis is due to the willingness of more enzymes to hydrolyze a fixed amount of substrate. At lower concentrations, the value of the degree of hydrolysis is also lower due to the smaller number of enzymes so the peptide-cutting speed on the same amount of substrate will be lower. However, the decrease in the value of the degree of hydrolysis at higher enzyme concentrations was due to the speed of

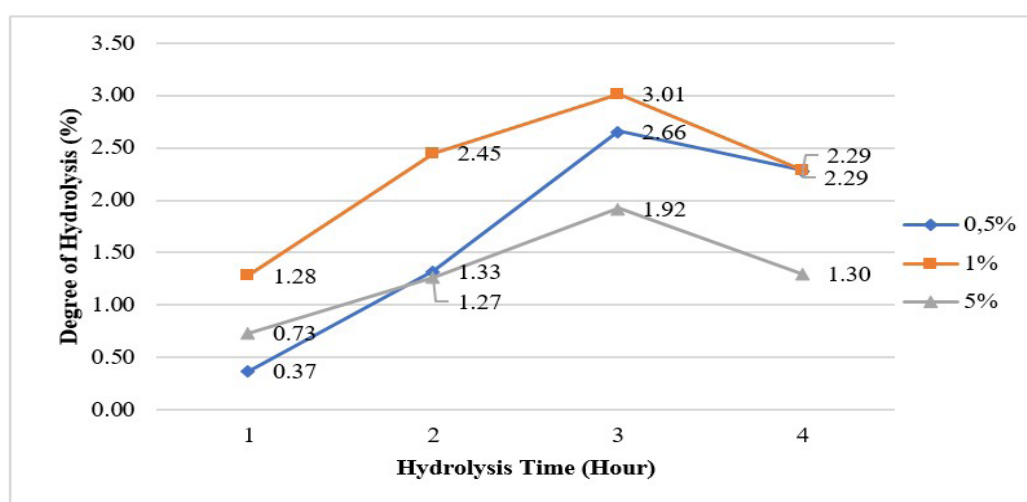


Figure 1. Soy protein isolate hydrolysis degree value at 0,5, 1,0, and 5,0% enzyme concentration

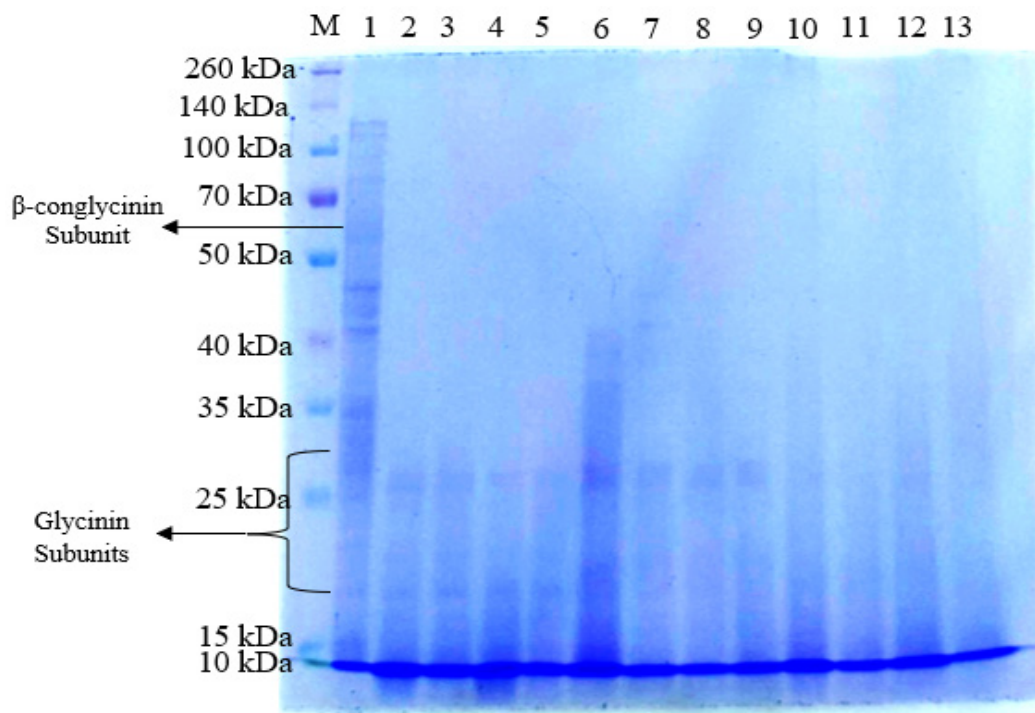


Figure 2. Soy protein electrophoregram (M= Marker, 0 = Soy protein isolate, 2-5 = Hydrolysate 0,5% 1-4 hour, 6-9 = Hydrolysate 1,0% 1-4 hour, 10-13 = Hydrolysate 5,0% 1-4 hour)

the enzymatic reaction which was directly proportional to the enzyme concentration to a certain extent so that the reaction was in equilibrium. At equilibrium, the addition of enzyme concentration has no effect anymore [13].

The value of the degree of hydrolysis obtained in this study is known to be lower than in previous research which obtained the highest degree of hydrolysis at 35.39% [6]. This can be caused by differences in hydrolysis conditions which include enzyme activity (units/mg) and enzyme concentration used. The enzyme activity used in this study was 100 u/mg. If the hydrolysis use enzyme with higher activity, it can give more product results so that the degree of hydrolysis obtained is higher [14]. The difference in the

value of the degree of hydrolysis can also be caused by the concentration of the enzyme used, where our previous research used an enzyme concentration of 0.2% (w/v) which gave a hydrolysis degree of 35.39%. In this study, the highest degree of hydrolysis was 3.01% at 1% (v/v) enzyme concentration.

Soybean hydrolysate was then electrophoresed to determine the success of the hydrolysis process based on the comparison of the isolate protein profile (before hydrolysis) and the hydrolysate (after hydrolysis). The results of the SDS-PAGE analysis in Figure 2 show as many as 12 protein bands in soybean protein isolates with molecular weights between 10 - 260 kDa. There is a thick

Table 1. The IC₅₀ value of soy protein isolate, hydrolysates, and cisplatin against MCF-7 cell line

Sample	IC ₅₀ (mg/mL)
Soy Protein Isolate	3.70
Hydrolysate 0.5% 3 Hour	8.69
Hydrolysate 1.0% 3 Hour	1.87
Hydrolysate 5.0% 3 Hour	40.51
Cisplatin	0.05

IC50 = 50% inhibiton concentration

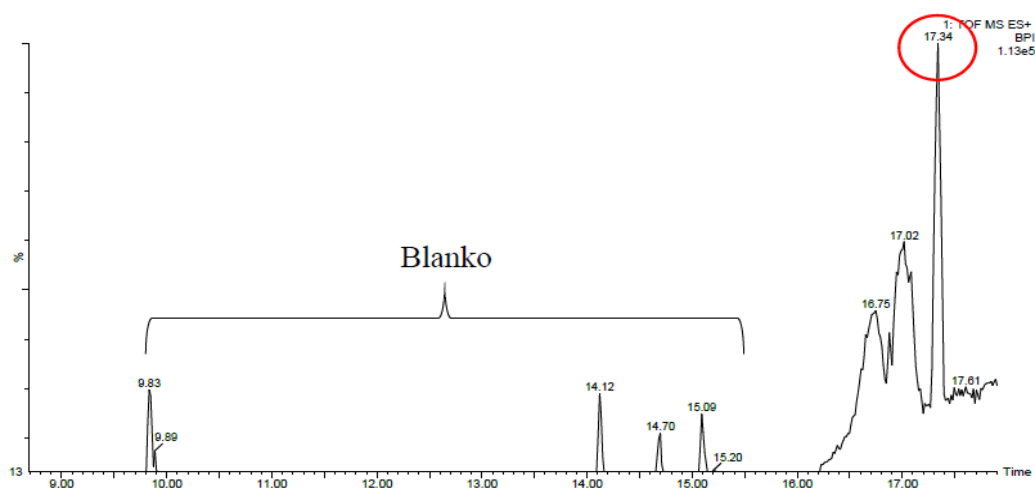


Figure 3. Chromatogram of the fraction 11

band at the molecular weight between 15-35 kDa which indicates the presence of two glycinin subunits, namely acidic polypeptide and base polypeptide, with a molecular weight of 24.20 and 34.35 kDa. In addition to glycinin, protein isolate bands indicate the presence of β -conglycinin subunits at molecular weights between 50 - 70 kDa which are referred to α β -conglycinin with a molecular weight of 72.73 kDa [15]. Thin bands are also present at molecular weights >100 kDa which may indicate the presence of lipoxygenase which has a molecular weight of about 102 kDa or hemagglutinin which has a molecular weight of about 110 kDa [16].

The hydrolysate bands show bands at lower molecular weights. After 1 hour of each enzyme concentration

variant, the subunits of β -conglycinin were completely degraded, as were as the protein components at a molecular weight of >100 kDa. Gel bands indicating the presence of glycinin subunit (15 – 25 kDa) were still visible in several hydrolysate variants. Based on this, it is known that glycinin shows a higher resistance to the hydrolysis process. This could be due to the presence of disulfide bonds in glycinin. Each constituent of the glycinin subunit is connected by a chain of disulfide bonds, whereas the β -conglycinin subunit does not contain disulfide bonds [15]. The electrostatic and hydrophobic interactions accompanied by the presence of disulfide bonds cause the quaternary structure of glycinin to be more stable against denaturation [16,17].

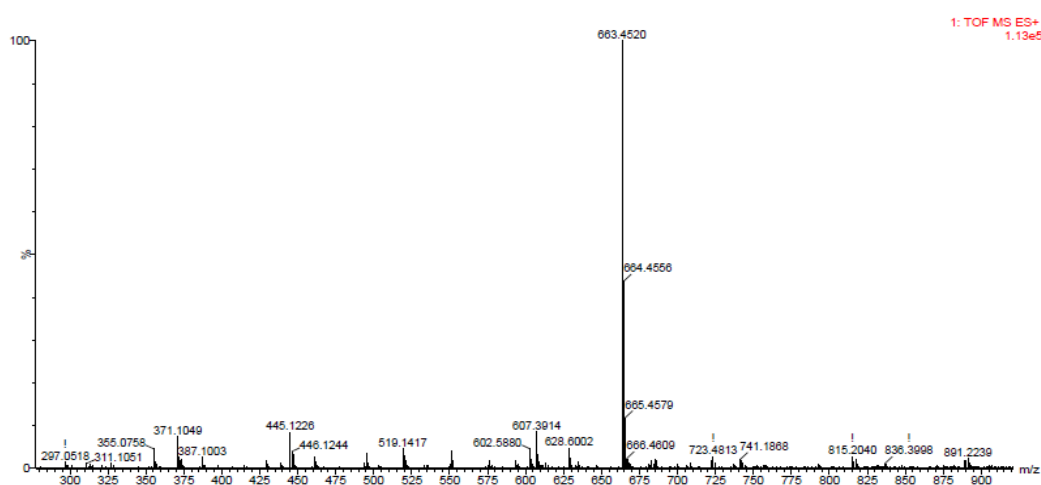


Figure 4. Chromatogram of the fraction 11

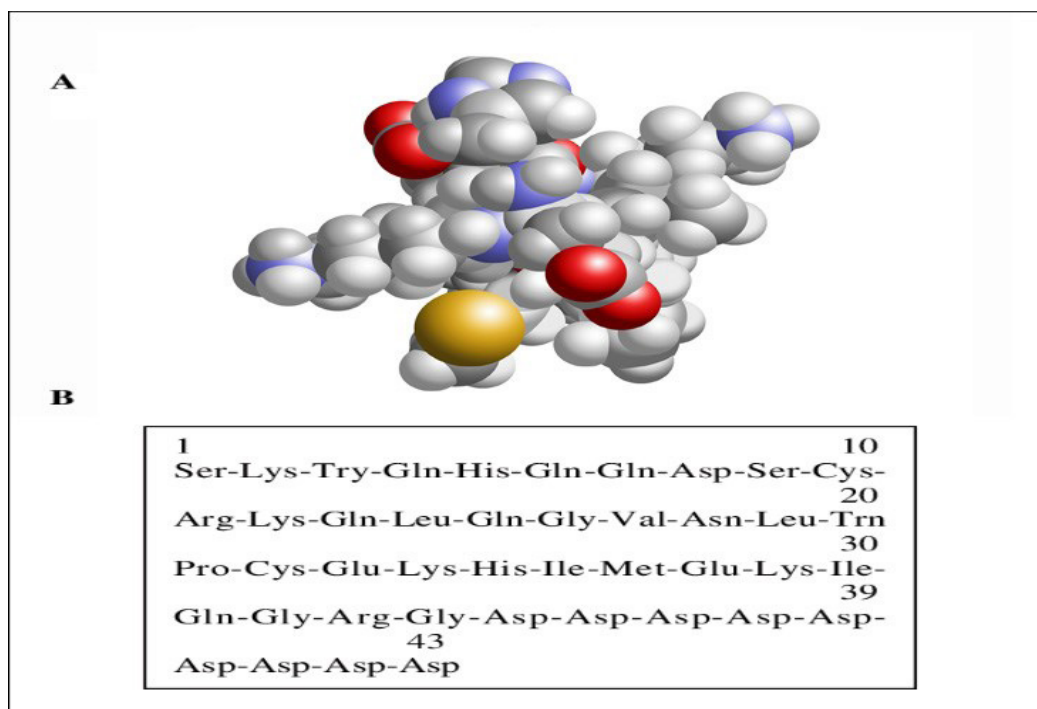


Figure 5. Chromatogram of the fraction 11

The hydrolysate fraction shows a thick band at a molecular weight below 10 kDa, while the molecular weight of the marker has a range between 10 - 260 kDa. Therefore, it is difficult to predict accurately the size of the peptide with a molecular weight of <10 kDa due to the ability of the SDS-Page gel which is only able to separate proteins up to 10 kDa in size.

Soybean protein isolate and three variants of soy protein hydrolysates with the best degree of hydrolysis were tested for cytotoxicity against MCF-7 breast cancer cells in vitro using PrestoBlue (PB) reagent. Analysis of the cytotoxicity against MCF-7 resulted in IC_{50} values, it was expressed through a linear regression equation that relates the absorbance value and concentration. Cisplatin, an intercalating agent, was used as the positive control. IC_{50} values of isolate, hydrolysates, and the positive control are synopsised in [Table 1](#).

In [Table 1](#), it is known that the hydrolysate has the highest activity with a 1% papain enzyme concentration variation and a hydrolysis time of 3 hours with an IC_{50} value of 1.87 mg/mL. The cytotoxic activity of the hydrolysate was higher than the soy protein isolate which had an IC_{50} value of 3.70 mg/mL. Meanwhile, the IC_{50} of hydrolysate 0.5% for 3 hours and hydrolysate 5% for 3 hours had a lower value than the soy protein isolate with values of 8.69 and 40.51 mg/mL, respectively.

The result showed that the hydrolyzed protein at

optimum conditions with 1% papain enzyme concentration and 3 hours of hydrolysis time could increase the cytotoxic activity against MCF-7 cells compared to the protein in the isolate. The increase in the anticancer activity of the 1% 3-hour hydrolysate is thought to be due to the role of peptides that have been successfully solved from the initial form of larger polypeptides through the hydrolysis process. Our results were in accordance with the previous findings that short peptides from protein hydrolysate usually exert high anticancer activity than their parent native protein or large polypeptides. Rayaprolu et al. [8] stated that the peptide fraction 5-10 kDa from soybean R95-1705 had a higher antiproliferative effect on MCF-7 cells than the fraction 10-50 kDa. Chen et al. [18] revealed that black soybean protein hydrolysate with peptide fraction <4 kDa had the highest anticancer activity compared to fractions 4-6 kDa and >6 kDa against MCF-7 cells.

The bioactive peptides with the highest cytotoxicity were then separated using filtration gel chromatography. There are 50 fractions of separation which were tested qualitatively with Bradford's reagent. The selected fractions (fraction 11) were then characterized using LCMS/MS to determine their molecular weights. LCMS/MS data analysis was performed using MassLynx software. The chromatogram obtained can be seen in [Figure 3](#).

In the chromatogram ([Figure 3](#)) it is shown that the fraction 11 produces a major peak at a retention time

of 17.34 minutes. [Figure 4](#) demonstrated mass spectra of molecule with m/z data, while the molecular weight was analyzed using ESIprot online (www.bioprocess.org/esiprot/). The calculation of the protein molecular weight is based on the results of the deconvolution of the peaks of the mass spectrum generated in each fragment. Based on the deconvolution results using ESIprot online (www.bioprocess.org/esiprot/) it is known that the peptide fragment in this fraction has a molecular weight of 7.70 kDa with a standard deviation of 561.78 Da. Protein hydrolysate which has anticancer properties with an IC_{50} value of 1.87 mg/mL is predicted to contain bioactive peptides at a molecular weight of 7.70 kDa.

The size of the peptide and the distribution of the molecular weight of the hydrolysate are known to have a close relationship with their biological activity. In several cases, peptides with molecular weights <10 kDa can increase the bioactivity [9,19,20]. Galvez & De Lumen [19] stated that lunasin, a peptide isolated from soybean with a molecular weight of 5.5 kDa whose sequence and structure are shown in [Figure 5](#), has anticancer activity due to the sequence RGD, a cell adhesion motif, that is responsible for the attachment of lunasin to the extracellular matrix [20]. The role of RGD peptides inducing apoptosis in different cell lines via the caspase-dependent mechanism. The difference in the enzymes used also causes the hydrolysate to have different qualities and bioactivity due to differences in the amino acid sequence and the size of the peptide produced. Papain preferentially cleaves peptide bonds involving basic amino acids, particularly lysine, arginine, and residues following phenylalanine [21]. Differences in the specificity of peptide cleavage could affect the amino acid product, the molecular weight of the peptide, and the cytotoxic activity [22,23]. However, the continuation of this research to determine the sequence of amino acids and testing the pure peptides are required to ascertain this assertion. For the next step, in order to predict the molecular structure of bioactive peptides, it is necessary to isolate the bioactive peptide and perform amino acid sequencing in vitro or through an in silico approach by simulating papain hydrolysis of soy protein.

Conclusion

Enzymatic hydrolysis using papain enzyme has been proven to be a promising approach to obtaining anticancer peptides from soy protein. The results of this study indicate the potential for hydrolysis with papain enzymes at optimum conditions to increase the cytotoxic activity of soy protein against MCF-7 breast cancer cells. Hydrolysis

of soybean protein with papain enzyme (1% papain enzyme concentration and hydrolysis time of 3 hours) degraded protein with a smaller molecular weight of 7.70 kDa with the activity of inhibiting MCF-7 breast cancer cells at 1.87 mg/mL. Therefore, further in-vivo testing and studies on peptide fractions that subjected have anticancer activity are expected to influence future drug development.

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