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Determination and Purification of Ricin Protein from *Ricinus communis* L. Seeds using CLC (Column Liquid Chramotagoraphy) and FPLC (Fast Protein Liquid Chramatography)

Irma Erika Herawati[1,2], Ronny Lesmana[3,4], Jutti Levita[1] and Anas Subarnas[1]

[1]Department of Pharmacology and Clinical Pharmacy, Universitas Padjadjaran, Sumedang, Indonesia

[2]Department of Pharmacy, Universitas Al Ghifari, Bandung, Indonesia

[3]Physiology Molecular Laboratory, Universitas Padjadjaran, Sumedang, Indonesia

[4]Department of Medical Basic Sciences, Universitas Padjadjaran, Sumedang, Indonesia

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*Corresponding author: Irma.erika.h@gmail.com

Abstract

Ricin is the most toxic substance isolated from *Ricinus communis* L. seeds. It is a heterodimeric two-domain polypeptide protein that includes chain A (30 kDa) and chain B (35 kDa) linked by a disulfide bond. Ricin binds to cells by the B chain and is then internalized. Ricin has been reported as a potential chemical for cancer treatment. The measurement of protein concentration in an aqueous sample is an important assay in biochemistry research. However, so far, the quantification of ricin protein is not much reported. In this study, the quantification of ricin protein extracted from *R. communis* L. from Nganjuk, East Java, Indonesia, the techniques purification was used column liquid chromatography (CLC) and fast protein liquid chromatography (FPLC), followed by quantification protein content using the Bradford method. Results showed that all purification techniques positively confirm the presence of ricin protein. Ricin protein content was 0.171±0.021 mg/mL and 0.382±0.023 mg/mL using CLC and FPLC, respectively. This study might contribute to understanding the chemical properties of the ricin protein from *R. communis* L. The Bradford method was used to quantify purified ricin, and the fractions obtained from FPLC were higher than those obtained from CLC.

Keywords: Ricin, *Ricinus communis*, CLC, FPLC

Pengukuran dan Pemurnian Kandungan Protein Ricin dari Biji *Ricinus communis* Menggunakan CLC (*Column Liquid Chramotagoraphy*) dan FPLC (*Fast Protein Liquid Chramatography*)

Abstrak

Ricin merupakan zat paling beracun yang diisolasi dari biji *Ricinus communis* L. Senyawa tersebut merupakan protein polipeptida dengan dua domain heterodimer yang terdiri dari rantai A (30 kDa) dan rantai B (35 kDa) yang dihubungkan oleh ikatan disulfida. Ricin dapat berikatan dengan sel melalui rantai B. Ricin telah banyak diteliti dalam pengobatan kanker. Pengukuran konsentrasi protein dalam sampel merupakan pengujian penting dalam biokimia. Namun, sejauh ini, kuantifikasi protein ricin tidak banyak dilaporkan. Pada penelitian ini, dilakukan pengukuran protein ricin yang diekstraksi dari *R. communis* L. yang berasal dari Nganjuk, Jawa Timur, Indonesia, teknik yang digunakan dalam pemurnian protein menggunakan metode kromatografi cair kolom (CLC) dan kromatografi cair protein cepat (FPLC), dilanjutkan dengan pengukuran kadar protein menggunakan metode Bradford. Hasil menunjukkan bahwa semua teknik pemurnian secara positif mengkonfirmasi keberadaan protein ricin. Kandungan protein ricin berturut-turut adalah 0,171±0,021 mg/mL dan 0,382±0,023 mg/mL dengan menggunakan CLC dan FPL. Studi ini dapat berperan untuk memahami sifat kimia protein ricin dari *R. communis* L. Kandungan protein ricin menggunakan metode Bradford, yang didapat dari pemurnian dengan FPLC lebih tinggi dibandingkan dengan CLC.

Kata Kunci: Ricin, *Ricinus communis*, CLC, FPLC

1. Introduction

Proteins are the most abundant class of biomolecules since they represent over 50% of the dry weight of cells. The measurement of protein concentration in an aqueous sample is an essential assay in biochemistry research. Various platforms and methods are available to quantitate proteins; one of them is dye-binding assays.[1] In comparison to other methods, dye-binding assays can be run at high throughput, using inexpensive reagents with equipment found in most biochemical laboratories.[2] The Bradford assay was first described by Bradford and become the preferred method for quantifying protein in many laboratories.[2]

Protein can be separated based on the size and shape, total charge, hydrophobic groups on the surface and binding capacity with the stationary phase. Column chromatography is one of the most common methods of protein purification.[3] In column chromatography technique, the stationary phase is attached to a matrix, packed in a glass, plastic, or metal column. The mobile phase flows through the column, either using hydrostatic pressure or with the aid of a pump system or applying gas pressure. Mainly, column liquid chromatography is one of the most important techniques used for analytical or preparative protein purification, which can be achieved at low pressure as well as by fast protein liquid chromatography.[4] Fast Protein Liquid Chromatography (FPLC) is a type of high performance chromatography that has the advantage of small-diameter stationary phases to achieve high resolution. The method can be used on a variety of biological molecules, such as oligonucleotides and plasmids.[5]

Ricin isolated from the seeds of *Ricinus communis* L., is a heterodimeric two-domain polypeptide protein that comprises an enzymatically active domain (namely ricin-A or rRNA N-glycosidase, and a lectin-like domain (chain B). The single-chain A is capable of inactivating over 1500 ribosomes/minute. Ricin could induce apoptosis and autophagy. It has been reported that a single molecule of ricin in the cytosol has been widely explored for its anticancer activity, e.g., in HeLa cells (IC50 for cell viability = 1 µg/mL). Ricin can induce activation of the caspase in immortalized epithelial cell lines (MAC-T).[6] Ricin is the deadliest poison with LD50 in humans of 5-10 µg/kg through inhalation or 350-700 µg for a 70 kg person.[7] This study was conducted to quantify ricin protein in *Ricinus communis* L. seed that is purified by CLC and FPLC using the Bradford method.

2. Material and Methods

2.1. Plant Materials

Ricinus communis L. seeds were purchased from a local farmer in Nganjuk, East Java, Indonesia (Figure-1). They were taxonomy-identified by a certified biologist at the Plant Taxonomy Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Padjadjaran, Indonesia, with several certificates 30/

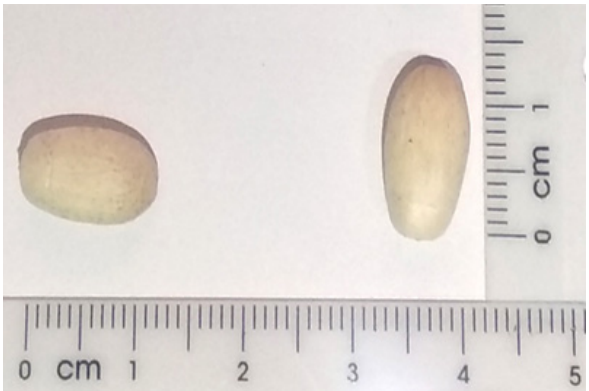

Figure 1. The castor seeds (*Ricinus communis* L.) obtained in Nganjuk, East Java, Indonesia

HB/09/2019. The seeds were confirmed as *Ricinus communis* L.

2.2. Chemicals

The chemicals used were Sephadex G-100 (Sigma-Aldrich), Bovine Serum Albumin (Sigma-Aldrich), Coomassie Brilliant Blue (CBB), ethanol 95%, phosphoric acid 85%, and other chemicals were purchased from Biochem.

2.3. Methods

2.3.1. Extraction

Ricinus communis L. seeds were extracted using Kumar et al. (2004) method. The fat in the seeds was removed by grinding in ether and centrifuged at 3000 g for 10 minutes. The clear liquid was discarded, and the residue was resuspended in ether. This procedure was repeated four times. The final residue was collected, air-dried, and the dry fat-free castor seeds residue was soaked in five-volume distilled water at pH 4.0 by adding diluted CH_3COOH. The resulted suspension was homogenized and left for 24h. The homogenate was further centrifuged at 8000 g for 10 minutes, and the supernatant was collected, adjusted to 60% $(NH_4)_2SO_4$ saturation, left for 24h at 4°C, and centrifuged for 30 minutes. Pellet was collected and dissolved in a mixture of 0.005 M of Na_3PO_4 0.2 M NaCl buffer at pH 7.2 Phosphate Buffer Saline (PBS). The dissolved pellet was concentrated by lyophilization and denoted as crude ricin.

2.3.2. Protein Purification

The purification of crude ricin was carried out by employing two methods, Column Liquid Chromatography (CLC) according to Kumar et al. (2004) and FPLC-ÄKTA start column according to Trung et al. (2016) with modification.

2.3.3. FPLC-ÄKTA Start Column

Ricin was purified by gel filtration using gel Sephadex G-100. Firstly, the gel was activated in distilled water at room temperature (1g/10mL H_2O) for 6 hrs. Then, gel was loaded on a glass column (1.6 cm x 13 cm) and soaked in phosphate buffer 0.002 M at pH 7.2 with ratio of protein solution/gel = 1:6 (v/v). Protein fractions were eluted with phosphate buffer 0.002 M at a rate of 0.2 mL/minute. Protein fractions were collected with 2 mL for each fraction.

2.3.4. Column Liquid Chromatography

The crude ricin (17.5 mL) was loaded on an affinity column of Sephadex G-100 in 0.5 M NaCl solution. The column was washed with PBS for six to remove the unbound proteins. Each 2 mL of the fractions was collected manually, and its absorbance was measured at 280 nm (the maximum wavelength of ricin). Protein-containing fractions were pooled, concentrated, and quantified by using the Bradford method.

2.3.5 Quantification using Bradford Method

The quantification preparation was assessed using the Bradford method (1976). First, Bradford's solution was made using the method of reacting 25 mg coomassie brilliant blue G-250 with 12.5 mL of 95% ethanol. Next, the result of the reaction was diluted using 500 mL of distilled water. Then, 30 µL of the sample was reacted with a 1 mL dye reagent of Bradford's solution in a test tube. The solution was incubated for ten minutes, and the absorbance was measured with a spectrophotometer at 595 nm.

3. Result

The FPLC and CLC chromatogram of crude ricin is presented in Figure-2 and Figure-3. Bovine Serum Albumin (BSA) was used as a positive control in quantifying purified ricin protein using the Bradford method because it is inexpensive, readily available, and exhibits the best linearity.[9] The protein content of purified ricin obtained from FPLC and CLC showed in Table 1.

4. Discussion

The chromatograms FPLC and CLC indicated a doublet peak consisting of one central peak with a smaller tailed peak. This doublet peak was predicted due to the similar polarity of two compounds, in this case,

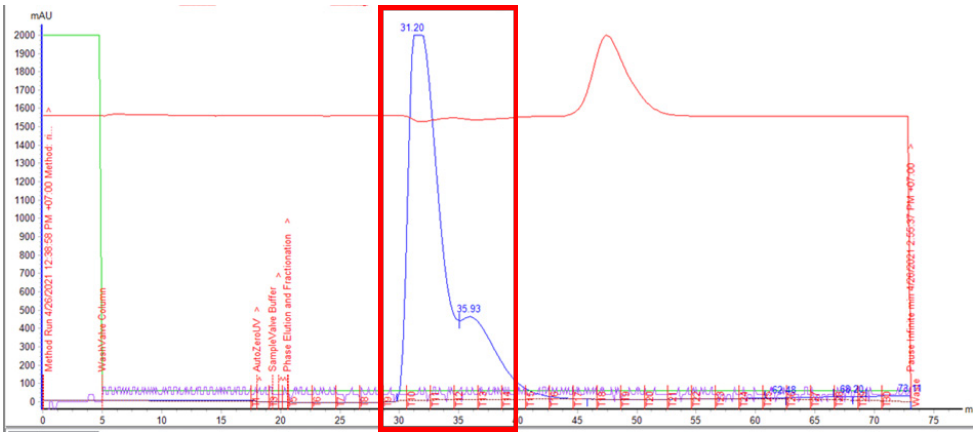

Figure 2. FPLC chromatogram of the fractionated crude ricin using ÄKTA st[illegible]olumn. The red rectangle indicates the doublet peak of the fractions of ricin (fraction 8-14)

proteins. These peaks are predicted to belong to chain A and the lectin-like B chain. Both FPLC and CLC chromatograms (Figure-2 and Figure-3) also indicated a doublet peak of ricin. These doublet-peak chromatograms are in agreement with that of Worbs et al. (2015). Thus, ricin protein can be separated by using both methods.

Extraction of ricin protein from cas[illegible] beans had resulted in an extract yield [illegible] 5.21%. The ricin protein extract ob[illegible]d was in the form of a white powder.

The study on protei[illegible] qu[illegible]ification used the Bradford me[illegible]d beca[illegible]e this method has a practical me[illegible]surement and staining method fo[illegible]esting prote[illegible]s and has a high sensitivity [illegible]lue, s[illegible]bility, and selection. The Bradford meth[illegible] has a [illegible]gher sensitivity value than the L[illegible]wry an[illegible] Biu[illegible]est methods. Method Brad[illegible]fo[illegible] i[illegible] also faster and more accurate, r[illegible]quires n[illegible] wa[illegible]-up, and responds more st[illegible]ble [illegible]lorimetry than other methods. Brad[illegible]rd metho[illegible] also can measure protein s[illegible]mples in the range of numbers 0.2-1.4 mg/m[illegible] for stan[illegible]ard assays.[12]

The process of salting-out (protein [illegible]urification) in this study used ammonium [illegible]sulfate. The use of ammonium sulfate is due to its high solubility, inexpensive, easily soluble at low temperatures, and able to stabilize protein deposits. Protein deposition is referred to as salting out because the salt concentration increases to the ideal point, leading to protein instability and deposition. The salting-out process can stabilize the protein so that it is not denatured and

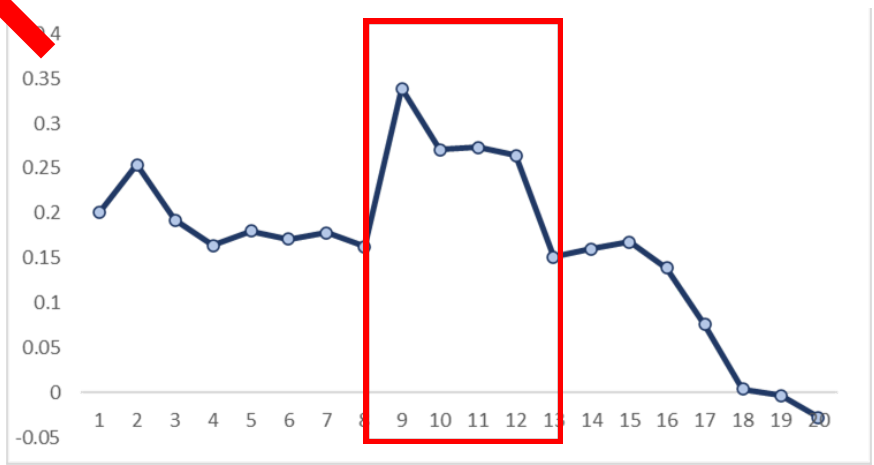

Figure 3. CLC chromatogram of fractionated crude ricin. The red rectangle indicates the doublet peak of the fractions of ricin (fraction 8-13)

Table 1. Protein Content from Purified Ricin

Sample	Absorbance (595nm)	Content (mg/mL)
Fraction FPLC-AKTA	0.399	0.382±0.023
Fraction CLC	0.188	0.171±0.021

contaminated with bacteria. The salting-out method is also a method that is suitable when combined with the Bradford method because the Bradford method is stable to the presence of impurities such as ammonium sulfate.[13]

The primary mechanism of the Bradford method is the binding of coomassie brilliant blue dye at acidic pH to essential amino acid residues such as arginine, histidine, phenylalanine, tryptophan, tyrosine residues, and hydrophobic interactions in proteins which results in a color change to blue.[5] Thus, the quantity of protein can be estimated by determining the amount of dye in the blue ionic form. This is usually achieved by measuring the absorbance of the solution at 595 nm. The advantages of the Bradford assay include the ease of use, relative sensitivity, low cost of the reagents, and low interference by other substances.[12]

Bovine serum albumin (BSA) is a globular protein often used in lab experiments as a protein concentration standard. BSA's advantage is that it is small and can be easily stored. BSA is the preferred standard in protein assays and its ability to increase signal in assays. It also has been prevalent in the use of protein assays since the Bradford Protein Assay was first introduced in 1976.[9]

Using linear regression, a mathematical equation for the protein standard solution obtained from the standard absorbance value will be obtained, which will be used to measure the protein content (Figure 4).

The results of quantification using the Bradford method can be seen in Table 1. The results of quantification of protein content in this study are relatively low, and this is due to the possibility of the working principle of the Bradford method based on direct enhancement of Coomassie Brilliant Blue (CBB) dye by proteins containing amino acid residues with aromatic side chains (tyrosine, tryptophan, phenylalanine) or alkaline (arginine, histidine, and leucine). Thus proteins outside the aromatic and essential side chains cannot be measured.[12]

Ricin, one of the most toxic substance isolated from *Ricinus communis* L. seeds, is a heterodimeric two-domain polypeptide protein that includes chain A (30 kDa) and chain B (35 kDa) linked by a disulfide bond. Ricin binds to cells by the B chain and is then internalized. Ricin has been reported as a

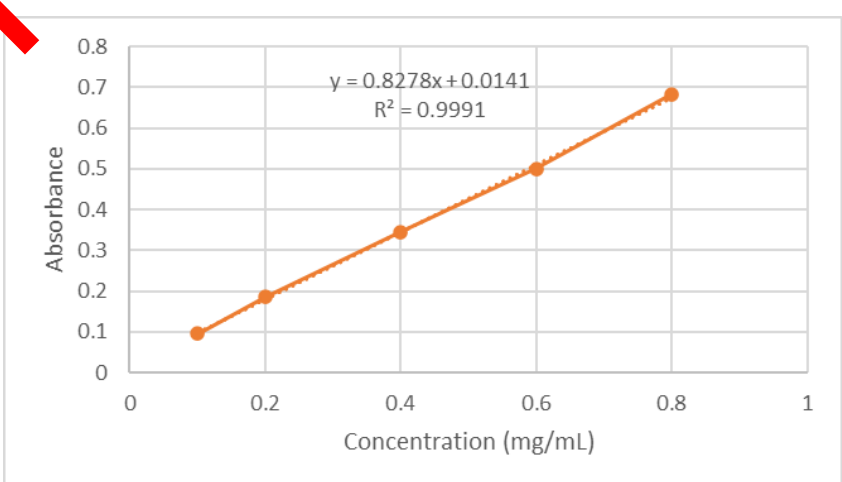

Figure 4. Standard Curve of Bovine Albumin Serum (BSA) as Positive Control in Bradford Method

potential chemical for cancer treatment.[15]

Several studies have shown that protein ricin contains the amino acid residues aspartate, threonine, serine, cysteine, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, tyrosine, tryptophan, alanine, valine, and arginine.[16]

The amino acid data above shows that many proteins may not be measured, such as threonine, serine, glutamic acid, proline, glycine, etc. This is why the protein content of the Bradford method is relatively low.

The ricin protein content obtained by purification using FPLC is higher than CLC. This proved that FPLC is a better method for purifying ricin protein from crude ricin. This may be because protein solubility refers to the total amount of protein entering the solution under certain conditions. It depends on the protein structure, pH, extraction time, temperature, and other intrinsic factors. The increase in protein solubility was indicated by the increasing amount of dissolved protein contained in the supernatant.[17]

5. Conclusion

The purified ricin extracted from *Ricinus communis* L. seeds originated from Nganjuk, East Java, Indonesia, employing two techniques, Column Liquid Chromatography (CLC) and Fast Protein Liquid Chromatography (FPLC). A doublet peak in all chromatograms indicates the presence of ricin protein. The quantification method of purified ricin using the Bradford method showed that fractions obtained from FPLC were higher than CLC fractions.

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