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ORIGINAL ARTICLE

Development of a rapid purification method for Ricin A chain from *Ricinus communis* seeds

Nasr N[1], Razazan N[1*], Athayi Sh[2]
1- Department of Biology,Payame Noor University (PNU) , IRAN
2- Bonab Office of Education and Training, IRAN
*corresponding: nrazazan@gmail.com and aybalar@yahoo.com

ABSTRACT
Ricin is a highly toxic, naturally occurring lectin (a carbohydrate binding protein) produced in the seeds of the castor oil plant Ricinus communis, and composed of two chains(A and B)(8). Ricin A chain inactivates 60S ribosomal subunit by disrupting the binding site for elongation factor (EF-2), and thus prevents the formation of the initiation complex (7). This toxin specifically influences on eukaryotic cells and affects protein synthesis.Ricin A chain (RTA), has been investigated as a potential candidate for cancer chemotherapy in the form of immunotoxins. Immunotoxins made with RTA have been used in clinical trials for the treatment of patients with bone-marrow transplantation, lymphoma, lupus nephritis, metastatic tumors, and leukemia (11). Ricin B chain (RTB) is a lectin that is able to bind terminal galactose residues on cell surfaces .This chain has no catalytic activity, but can mediate transport of the A-B protein complex from the cell surface to the lumen of the endoplasmic reticulum (ER). In this research we tried to design a simple and rapid method for RTA purification from castor bean. The crude ricin was loaded on column containing acid treated sepharose-4B gel.The column was washed with PBS for several hours (till the optical density reached below 0.05) to remove the unbound proteins. Due to RTB binding to the galactose moiety on sepharose-4B, whole toxin was attached to the sepharose. Then 2ME [Mercapto ethanol] containing buffer (0.2 molar 2ME in PBS) was added to the column for disulfide bound disruption. Fractions were collected manually, and absorbance was recorded at 280 nm. Protein containing fractions were pooled and concentrated. The purity of RTA was evaluated by the sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Purified RTA gives a single band in SDS-PAGE, under reduced condition. The molecular weight of purified RTA was estimated to be 30.1 KDa. The western-blot analysis with anti-RTA monoclonal antibody revealed one strong bound in 30 KDa regions.
Keywords: *Ricin A chain, castor bean, rapid purification, chromatography.*

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INTRODUCTION

Ricin is a highly toxic, naturally occurring lectin (a carbohydrate binding protein) produced in the seeds of the castor oil plant *Ricinus communis*, and composed of two chains(A and B)(2). Ricin A chain inactivates 60S ribosomal subunit by disrupting the binding site for elongation factor (EF-2) [5], and thus prevents the formation of the initiation complex [10]. This toxin specifically influences on eukaryotic cells and affects protein synthesis [9, 2].Ricin A chain (RTA), has been investigated as a potential candidate for cancer chemotherapy in the form of immunotoxins. Immunotoxins made with RTA have been used in clinical trials for the treatment of patients with bone-marrow transplantation, lymphoma, lupus nephritis, metastatic tumors, and leukemia

Ricin B chain (RTB) is a lectin that is able to bind terminal galactose residues on cell surfaces [12] .This chain has no catalytic activity, but can mediate transport of the A-B protein complex from the cell surface to the lumen of the endoplasmic reticulum (ER) [1].

Since there are a lot of researches currently performing to treat cancer and different types of methods for designing treatment procedures [13], along with the world up to dated knowledge, is going on, a group of researchers are developing methods of treatment based on immunotoxins which a part of that is the use

of ricin toxin and guiding it to cancer cells [3]. Therefore development of a rapid method for isolating chain A toxin for using in the manufacture of immunotoxins can be an effective step in introducing new methods in cancer treatment. Introduced methods for purification of this material was actually based on classical methods of protein purification and its result is separation of toxin containing A and B chains. This study is an attempt to introduce a rapid method using biological properties of chain B, for easily purification of chain A in order to meet the need of medical research sector to this material. Since B chain of the complete toxin has lectin property and binds easily to sugar such as galactose [4], it can be helpful because of this property for purification of chain A using affinity chromatography.

MATERIALS AND METHODS

Preparing homogeneous solution of ricin granules, the first step is defatting which is performed by dissolving in ether. 100 grams of castor seeds were dried away from sunlight. To ensure about drying the seeds, they were placed at 45 °C temperature of fore for 2 days. All seeds were peeled and completely crushed, in an electric mill and 100 ml ether was added to the crushed seeds. The resulting solution was centrifuged for 10 min at 3000g around. The surfactant was discarded and the precipitate was re-dissolved in 100 ml ether and the stage of centrifugation was repeated. This step was done 3 consecutive times. The final precipitate was collected and dried at room temperature.

The primary protein purification: 10 g precipitate obtained from the previous step was dissolved in 50 ml distilled water. The solution pH was titrated to 4 by 0.2 M acetic acid. The resulting solution was stirred overnight at 4 °C temperature. After centrifuge at 8000 g for 10 min, the supernatant was collected. 30 ml of saturated ammonium sulfate was added to 50 ml collected supernatant. (Until 60% saturated solution was obtained.), and stirred overnight at 4 °C temperature. Then the resulting solution was centrifuged at 10000 g for 30 min at 4 °C temperature. The obtained precipitate was solved in phosphate-buffered saline (PBS) and pH = 7.2. To remove ammonium sulfate salt, the obtained samples was dialyzed overnight at 4 °C in phosphate-buffered saline in pH = 7.2. The dialyzed solution was divided into 5 ml vials and was lyophilized.

Affinity chromatography: in order to preparing 100 ml gel, sepharos-4B gel (Pharmacia, 17-0120-01) in a hydrochloric acid solution 0.1M was stirred for 3 hours at 50 °C temperature. The prepared gel was added to the chromatography column and after the gel was packed, it was washed with 1 ml PBS buffer in pH= 7.2. (It is necessary to be explained that the pH of output solution must be 2/7.)

10 ml ricin extract was added to the column. Washing the column with PBS buffer continued with rate of 2 ml per min until the effluent solution absorption reached to zero at 280 nm. Afterwards 2ME (2 - Mercaptoethanol) ((MERCK, 805740) 0.1M was added to the PBS buffered column and the 2 ml fractions were collected. Velocity of fluid was 1 ml per min. Absorption of samples was read at 280 nm.

Regeneration column: in order to regeneration and reuse of the column in next stages, galactose solution was used and 200 ml solution containing galactose 0.1M and sodium chloride 0.5 M was passed through the column. Then the column was washed with one liter of PBS buffer. Checking the antigen purity: in order to checking the purity of protein, the SDS-PAGE electrophoresis method in 10% gel was used. Performing Western blotting: the bands existing in the gel using transfer tank buffer Tris - glycine containing methanol and SDS(2 g of Tris (121/1 = MW) and 11.3 g glycine (75.07 = MW) was dissolved in800 ml distilled water and 200 ml methanol was added to it and with a 50 V voltage was applied for 16 hours. After passing appropriate time the current was cut and the nitrocellulose membranewere dried in the open air. A piece of nitrocellulose membrane was stained with a solution of ponso S 1% and other segments were stained for enzymatic detection. For this purpose first the nitro-cellulose membrane in a solution of 1% BSA(A-3912 SIGMA) was placed on Shaker for 45 min. Then the BSA solution was discharged and the solution anti-chain A of ricin antibody (HyTest Cat no: 2R1, clon: CP75) was added and was placed on shaker for an hour and a half. After passing this time the antibody solution was removed and it was washed twice, each timefor 3 min with BSA 1% in order to remove additional antibodies. Then the antibody solution of anti-mouse antibody linked to HRP (Anti-mouse-HRP) was added and was placed for an hour on shaker. Finally nitrocellulose was washed three times each time for 5 min and after washing the substrate solution, the dye was added and it was placed for 20 min in the dark. At this point the color bands gradually appeared. Then the substrate solution was discharged and the coloring reaction was stopped by placing the nitrocellulose paper in distilled water.

Molecular weight calculation (14): to calculate the molecular weight of protein after electrophoresis, the calibration curve of log molecular weight of standard proteins is drawn versus the RF of the same samples. The calibration curve is resulted through connecting the obtained points. By drawing the regression line which encompasses all obtained points, Rf of unknown sample can be determined on the curve and the molecular weight of protein can obtained.

RESULTS AND DISCUSSION

Ricin is a protein toxin found in seeds of *Ricinus communis* as a plant specious. This toxin is a small di-peptide molecule with a molecular weight of about 65 kDa which contains two chains A and B. A chain weighs approximately 32 kD and B chain weighs about 34-32 kDa. These two chains are connected by a disulfide bond to each other. A chain of ricin inactivates subunit 60 S of ribosome and by prohibiting Elongation Factor-2 function [9], it prevents performance of ribosomes and the degradation of the complex starts and it stops protein synthesis[6]. Differences in glycosylation of this protein in different species of castor causes that the differences in the molecular weight of this protein is seen. Stilmark [14] for the first time succeeded in purification and isolation of this toxic protein. In the past years, different methods for purification of the toxin were raised which mainly were based on molecular weight of the toxin using chromatography gels [13].The result of all of the above methods is whole toxin with two chains. However, regarding to the aim of the present study which is preparing only A chain, the steps of the procedure were designed so that the purification of the A chain is performed in the shortest time. Conventional methods so far mentioned in the literature for the preparation of chain A was completely separation of the toxin and then breaking it into two chains, A and B, and finally using the column chromatography again for the separation of the two chains from each other. In our study, based on this fact that the B chain has lectin properties, first the complete toxin was connected to the sepharose gel made from a galactose polymer and then by breaking the disulfide bond of the complete toxin, the A chain was collected. This method has the advantage that during one step procedure, a chain is separated as quickly as possible.

Crudepreparation: The first step in procedure is crude preparation from the castor plant seeds. Thus the seed was dried so that they can easily be separated from the skin because the seed skin does not contain the toxin and in this way apart of proteins and excessive material were removed from the system easily. On the other hand this toxin is soluble in aqueous solutions. Thus, eliminating fats in castor seeds, which their amount is relatively high, a part of unwanted material can be removed. Therefore ether was used for fat removal. On the next stage while it was attempted to isolate the proteins, the concentration was also done. The amount of salt for protein precipitation is enough to precipitate all the proteins with molecular weight 65 kDa and greater than it.

Purification: The purification stage included a complete toxin binding to the gel and then breaking the chains A and B and ultimately collecting a chain. The toxin B chain has lectin properties and binds to galactose. Therefore during the passage of the obtained extracts from castor seeds, the toxin remains in the gel, because of the B chain binding to the sepharose gel.

As a result, washing the gel helps the unattachedmaterials be eliminated and only whole toxins remain on the gel. In fact, this method can be called a kind of affinity chromatography because the binding of toxin to the sepharose gel is performed based on tendency of B chain to galactose. As it can be seen in Figure 1, the washing stages continued until the protein content in the effluent solution reached to zero. After ensuring about discharge of the column from unwanted proteins, the A chain was separated using 2ME solution. Adding 2ME soluble caused to breaking the disulfide bonds between chains A and B so that the A chain and B chain were separated and while the B chain was still attached to the column, the A chain exit condition was provided. During this stage of the procedure, enough time was needed for desirable function of 2ME solution. For this purpose, after adding 2ME solution to the column and entering the solution to column containing gel, fraction collection was stopped for half an hour until the attached toxin to the column exposed to 2ME solution. Then the process continued, effluent fractions were collected and collecting the effluent proteins continued as it can be also seen in the diagram that the effluent solution contains protein. At the end of collecting the fractions, galactose solution was used to regenerate the column so that theconnected chains are separated. To continue the process and to evaluate the effluent proteins, in the second stage it was necessary to examine the collected samples in respect of the presence of the target protein.

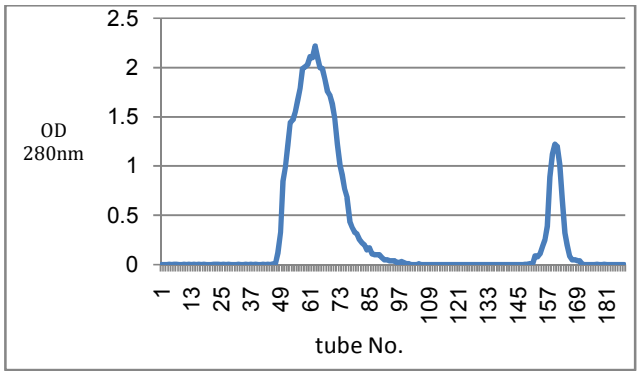

Figure 1: Optical absorption diagram of collected fractions from the column

Assessment of protein purity: The method of SDS-PAGE gel electrophoresis in gel concentration12.5% was used for evaluation the collected protein purity. As the figure shows in the collected samples of second stage, effluent of the column contained a single-chain protein with a molecular weight of about 30 kDa . Up to this stage of the process, it was determined that the steps of purification led to a pure protein isolation and its comparison with the protein extracts obtained from castor seeds showed that the above band exists at the lower doses in the extract.

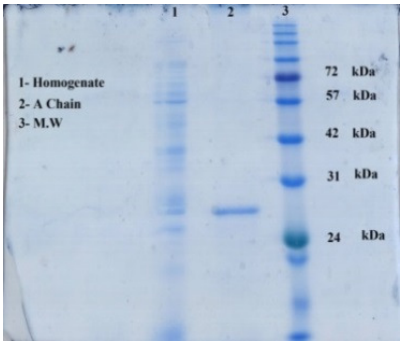

Figure 1: the SDS-PAGE electrophoresis stained gel with Coomassie Blue

Molecular weight calculation (4): for molecular weight calculation of a purified protein, molecular weight standard were used. By visual observation of Figure 1 it can be guessed that the weight of purified protein is approximately 24-31 kDa .However precise calculation needs to draw standard diagram. Using existing protocols for individual bands, molecular weight standard and relative factor (RF) were calculated and through drawing diagram of RF versus the log of the molecular weight, the standard diagram (Figure 2), was obtained. Then for the target protein also, the rate of RF was calculated equal to 0.6 and using the standard diagram, the molecular weight log was obtained equal to 1.48. Based on carried out calculations, the purified proteins weight was obtained approximately equal to 30.1 kDa, using SDS-PAGE method.

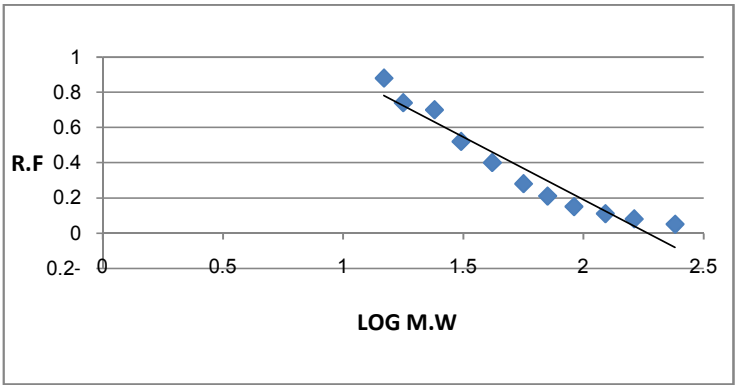

Figure 2: Standard curve of molecular weight log versus RF

Western blotting tests(16,15): The most important and sensitive part of the work was proving the nature of the isolated protein.The Western blotting method with anti A-chain monoclonal antibody was used to prove the existence of the target protein in purified band.First of all, it was necessary to transfer protein bands on a bed. In this method nitrocellulose membrane was used as a bed. Transfer tankers were used for transferring proteins on nitrocellulose paper. Then it was required to saturate the empty position of the paper by a protein to prevent the nonspecific antibody reaction. Afterwards monoclonal antibody was added to identify the target proteins. As shown in figure 2, the obtained result of this test was the reaction of specific antibody with the purified protein which showed that the purified protein was ricin a chain.However as it can be seen in the extract band, this band is also identified in the area of approximately 30 kDa in extracts and also a pale band can be seen in the area of 60 kDa. It seems that the formed band in the area of 60 kDa has been created because of the existence of complete toxin in the extract obtained from the seeds of castor. However, since in the sample preparation for SDS-PAGE electrophoresis there was 2ME in sample buffer, it was anticipated that the existing toxins were broken into two chains. However, presence of small quantities of complete toxin in obtained bands shows that the amount of 2ME solution in the sample buffer was not enough and it required more amount and longer exposure time to break the chains completely.

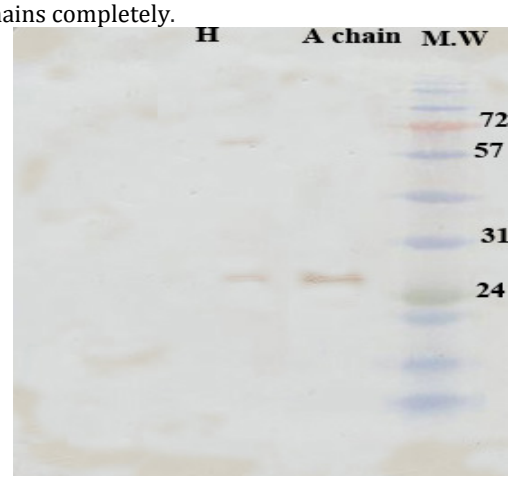

Figure 2: Western blotting test

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