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Research Article

Optimization of Affinity Chromatography Based on Sepharose 4B-chitin for Rapid Purification of *Urtica dioica* Agglutinin

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Background: Today, numerous antimicrobial and anticancer properties have been reported for plant lectins due to their ability to bind to carbohydrates. The *Urtica dioica* agglutinin (UDA lectin) is a monomeric, small, and low molecular weight glycoprotein. It has attracted the attention of many researchers for identification, treatment, and other clinical purposes.

Objectives: The aim of this study is the optimization of the chitin affinity chromatography based on Sepharose 4B (CNBr-activated Sepharose 4B) for the rapid purification of UDA lectin from *Urtica dioica* rhizome.

Materials and Methods: The chitin ligands were dissolved in 40% Trichloroacetic acid and attached to Sepharose 4B according to the Amersham-Biosciences instructions. The attachment of the ligand to the Sepharose 4B beads was investigated by Fourier transform infrared (FTIR) spectroscopy. An acidic crude extract of nettle rhizome passes from chromatographic columns in two sizes with dimensions: 24 x 0.51 cm and 8.44 x 0.86 cm. Quantity and quality of purified lectin were calculated by the Bradford microplate method: SDS-PAGE gel electrophoresis and human erythrocyte cell (RBC) hemagglutination, respectively.

Results: The analysis of FTIR spectrograms showed that major changes were observed in the fingerprint regions. Besides, due to the dissolution of Sepharose 4B and chitin in the aqueous phase, this difference was not significant in the Imine and Nitrile regions. On the other hand, the comparative results of purification chromatograms showed that increasing the column length causes a smaller half-width and increases the length of the purified peak. Also, it leads to high-quality purified UDA lectin, with a molecular weight of almost 12.5 kDa in gel electrophoresis. Hemagglutination activity on trypsinized red blood cells was displayed, and agglutination of purified UDA lectin started at least at 300 $\mu g.mL^{-1}$ concentration.

Conclusion: According to our findings, we suggested that dissolving chitin in the polar solvent of Trichloroacetic acid, using Sepharose 4B as the beads of a matrix, and increasing the column length might lead to a decrease in the half-width of the peak. These can increase the purity and concentration of purified UDA lectin, and speed up the purification process. These findings could be used by researchers to accelerate the purification of UDA lectin in other studies, dealing with drug delivery systems, ELISA techniques, and cell growth.

Keywords: Affinity chromatography, CNBr-activated Sepharose 4B, Hemagglutination, UDA lectin

1. Background

Plant lectins have recently drawn the interest of numerous researchers due to their plentiful biological origin, simplicity of access, and strong affinity to bind to specific carbohydrates (1). These plant glycoproteins are different in their intensity of the tendency to bind to carbohydrates as well as differences in the type of binding carbohydrates. Hence, they can be used directly as anti-fungal, anti-microbial and anti-pathogen as well as in the treatment of cancer (2). Also, these plant compounds can also be used indirectly in cell identification and isolation, drug delivery systems, ELISA techniques, and as mitogens to stimulate cells in cell culture (3). Before using lectins as glycoproteins in biological applications, they must first be purified from the plant and then used. The purity of purified lectins is especially important when these glycoproteins are used for therapeutic purposes. Therefore, the use of basic biochemical principles in the separation and the purification of lectin to optimize purification should be considered.

The *Urtica dioica* (Stinging nettle) agglutinin, also called UDA lectin, is a plant lectin found in plenty in the rhizomes and a lesser amount in the seeds. At a post-translational modification, UDA lectin pre-protein is proteolytically processed to yield a very small protein (8.5- 14 kDa), which is composed of ~89 amino acids with two high-affinity domains for N-acetylglucosamine (GlcNAc) oligomers or chitin (4, 5). This protein can alter the behavior of cancer cells by binding to the cell surface carbohydrates (6). It could be effective in inhibiting the proliferation of various cancer cells, such as those of the uterus (7), blood (8), and endothelial blood vessels (9).

2. Objectives

Due to the importance of UDA lectin as a plant-derived protein and its clinical applications in previous studies, the aim of this study is to optimize the chitin affinity chromatography based on CNBr-activated Sepharose 4B matrix for the rapid purification of UDA lectin from *Urtica dioica* rhizome.

3. Materials and Methods

3.1. Plant Crude Extraction

Stinging nettles were collected from Bahnamir (latitude: 371 ′ 43 ″ 361, longitude: 52 ′ 475 ″ 205, height: -22 meters), Mazandaran Province, North of Iran (**Fig. 1A**). Plant specimens were identified as *Urtica dioica,* and a sample was placed on a thin paper sheet in the herbarium of the University of Mazandaran (#HUMZW36).

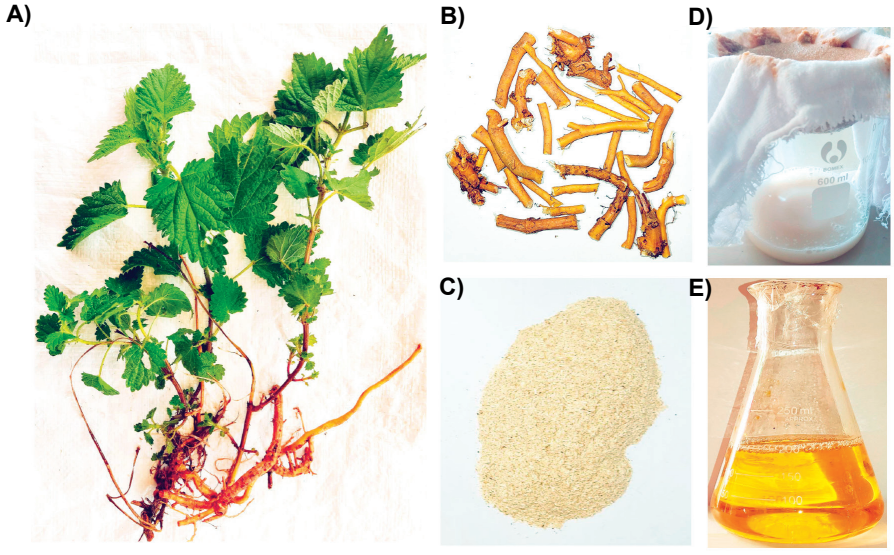

Figure 1. Steps of preparation of UDA crude extract: **A**) Collection of *Urtica dioica*; **B**) Isolation and cleaning of plant rhizomes; **C**) Preparation of plant powder of rhizome tissues; **D**) Preparation of plant rhizome suspension; **E**) Preparation of crude extract of plant rhizome.

The extraction of rhizome UDA lectin was performed by Peumans *et al*. (1984) method (4) with the following modifications. The rhizome of the *U. dioica* was separated, washed with running water and then placed in the air for a few hours to dry completely (**Fig. 1B**). Rhizomes were dried in a freeze dryer and ground by liquid nitrogen to achieve *U. dioica* rhizome tissue powder (**Fig. 1C**). This powder was kept at -20 °C until the aqueous extract was prepared. About 30 g of rhizome powder was mixed with 10 mL of cold solution (0.1 M, HCl; 1 mM, PMSF) in a mortar and sharply mortared for one to two hours to obtain a homogeneous suspension. Again, 170 mL of the above solution was added to the suspension and placed at 4 °C overnight (O/N) on a magnetic stirrer. The resulting suspension was centrifuged (12,000 rpm for 20 min) after passing through a double-layer mesh fabric (**Fig. 1D**). The aqueous extract was adjusted to pH 3.8 by 2M, NaOH and incubated at 4 °C for 2 hours. The rhizome extract was centrifuged at 12000 rpm for 20 min, and the supernatant was filtered with filter paper (Whatman3), resulting in a filterable solution known as UDA crude extract (**Fig. 1E**).

3.2. Chitin-Sepharose 4B Column

About 50 mg of chitin (CAS number: 1398-61-4; Sigma-Aldrich) was dissolved in 5 mL of 40% Trichloroacetic acid (TCA) and then placed on a magnetic stirrer for 12 hours to prepare the active ligand. To wash chitin from contaminants and TCA, chitin solution was dialyzed against coupling buffer (0.1M, $NaHCO_3$, pH 8.3; 0.5M, NaCl) using falcon dialysis (3 kDa, Amicon® Ultra-15, USA). Activation of 2.5 g of CNBr-activated Sepharose 4B column beads (with code number: 17-0430-01), and attachment of chitin to column beads-activated were performed by Amersham-Biosciences (10) protocol. Matrix beads (Chitin- Sepharose 4B) dissolved in twice time volume of the column equilibrium buffer (0.05M, NaOAc, pH 3.8; 0.1M, NaCl). This solution was poured into handmade glass tubes and allowed to settle matrix beads and get a regular uniform shape. In this study, we prepared two handmade columns, including 24 x 0.51 cm, as a long-length column (LLC), and 8.44 x 0.86 cm, as named a short-length column (SLC). All of the columns were poured with 4.9 cm^3 bead.

3.3. Ligand Binding Analysis

Fourier transform infrared spectroscopy (FTIR) by the Cary 630 FTIR spectrometer (Agilent Technologies, Inc; USA), in the range of 600- 4000 cm^{-1} was used to confirm the binding of chitin to Sepharose 4B. For this aim, 100 µL of activated- Sepharose 4B (dissolved in 1mM, HCl), chitin (dissolved in coupling buffer), and chitin coupling Sepharose 4B (dissolved in equilibrium buffer) were prepared and used for analysis at room temperature. Also, a solid powder of Sepharose 4B was used for the analysis before being dissolved in HCl.

3.4. UDA Lectin Purification

UDA lectin was purified by Peumans *et al*. 1984 method (4). Before loading the crude extract, the column was equilibrated with 2.5 times the volume of the column using an equilibrate buffer (0.05M, NaOAc, pH 3.8; 0.1M, NaCl) by a peristaltic pump, Heidolph 5001, Heidolph Co, Germany, (**Fig. 2A**). The crude extract was then loaded at a flow rate of 85 drops per 16 minutes (**Fig. 2B**). The solution was collected by the fraction collector (Bio-Rad 2110, Bio-Rad Co, USA) and the absorbance of fractions was read at OD_{280nm} from the column in an approximate volume of 3 mL (85 drops) in the tubes. Impurity components were washed using washing buffer (0.05M, NaOAc, pH 3.8; 1M, NaCl) until OD_{280nm} to achieve below 0.01 (**Fig. 2C**). The 0.5M acetic acid was used to elute UDA lectin from the column (**Fig. 2D**). Fractions containing UDA lectin protein were concentrated against PBS buffer (0.137M, NaCl; 0.0027M, KCl; 0.01M, Na_2HPO_4; 0.0018M, KH_2PO_4) using the above Falcon dialysis.

3.5. Lectin Concentration and Quality

The Bradford microplate method was used to determine the concentration of purified UDA lectin (11). Based on the Bradford reagent (0.01% W/V, Coomassie brilliant blue G-250; 5% V/V, EtOH; and 10% V/V of 85% H_3PO_4), a standard plot was drawn for different concentrations of bovine serum albumin (BSA) in OD_{595nm}.

Purification of UDA lectin was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), which was discussed by Laemmli, 1970 (12) in mini-gel (10 x18 cm) with 4% stacking gel (pH 6.8.) and 15% resolving gel (pH 8.8) in a Tris-Glycine

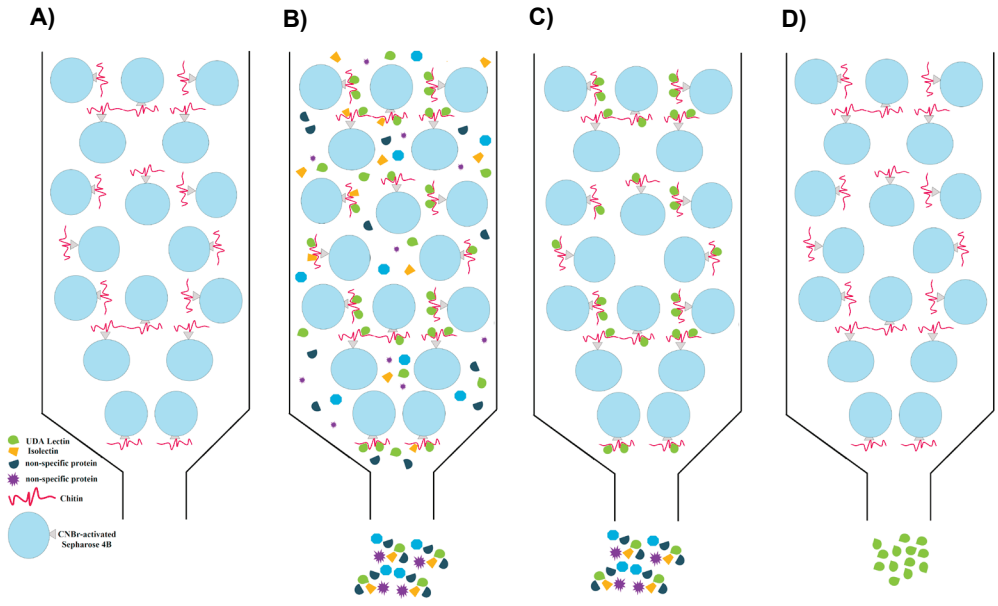

Figure 2. Schematic of the UDA lectin purification process. **A**) Equilibrated column containing chitin-Sepharose 4B as matrix; **B**) Column containing loaded UDA lectin crude extract; **C**) Column containing UDA lectin obtained by using wash buffer; **D**) Elution of the column and UDA lectin collection.

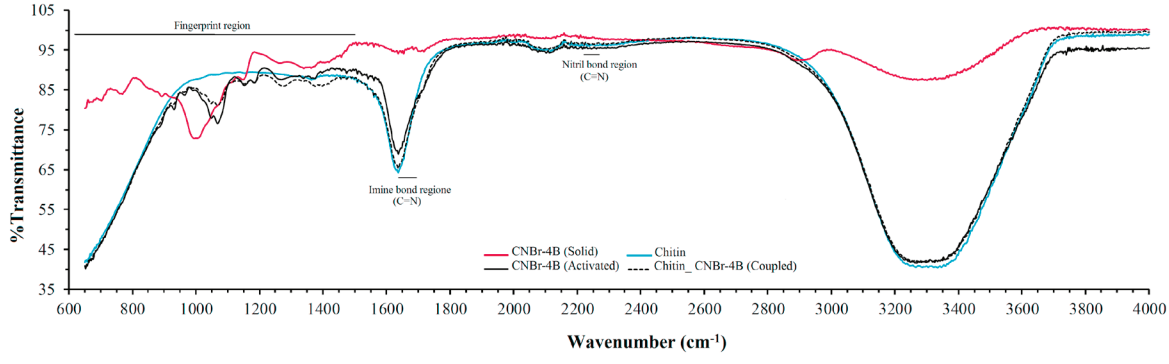

Figure 3. FTIR spectrogram. Region of 1500-600 for fingerprints; 2260 to 2222 for nitrile (C≡N) and 1690 to 1640 for imine (C = N).

SDS buffer tank (pH 8.3) for 4 hours at 90 volts. After electrophoresis, the mini-gel was stained with 0.1%, Coomassie Brilliant Blue R-250 dye by general methods (13).

Determining the molecular weight (MW) of UDA lectin was done using an equation of the logarithm MW of a protein marker versus the relative migration distance (R_f) of all of its bands, as discussed by Hames in 1998 (14).

3.6. Hemagglutination Assay

The activity of purified UDA lectin was evaluated by hemagglutination assay, which was discussed by Liener 1955 (15) with little change. An amount of 200 µL of human red blood cells was washed with five times the volume of PBS and centrifuged at 3000 rpm for 3 min. The erythrocyte pellet was mixed with 200 µL of 1% trypsin to obtain red blood cells (RBCs) that were 100% Trypsinized. Then, a 2% RBC working

suspension was prepared from 100% Trypsinized RBC in PBS to use for the hemagglutination test.

The experiment was performed in 12 wells of 96 U-bottom microplates (SPL Life Sciences Co. Korea). For this purpose, the first, 25 µL of PBS was poured into each of the 12 wells. Then 25 µL of UDA lectin was added to PBS in the first well. For serial dilution, 25 µL was taken from the first well and was added to the next well, and this process was repeated until the next ten wells, and finally, 25 µL was taken from the 11th well and was discarded. Thus, no lectin was added to the 12th well, which was selected as a control. An amount of 75 µL of 2% trypsinized RBCs was added to each of the wells and mixed. The agglutination of RBCs was examined with the naked eye at room temperature after 2 hours.

4. Results

4.1. Analysis of Chitin Bonded to Sepharose 4B

Chitin binding to CNBr-4B beads was investigated using FTIR spectrogram analysis. Comparisons of the Chitin (ligand), CNBr-4B (bead), and Chitin-CNBr-4B (bead-ligand) spectrograms showed that the major changes in the fingerprint region were in the range of 600 to 1500 nm (**Fig. 3**). However, these changes are not significant in the areas of nitrile and imine uptake, which is probably due to the aqueous solvent base in the three components. Also, results showed aqueous CNBr-4B has a stronger peak in the imine region compared to solid CNBr-4B.

4.2. Lectin Purification

Purification of UDA lectin was performed by loading crude extract into two short and long-length columns (SLC and LLC), with a flow rate of 3 mL per 16 minutes. Thus, the two columns contained a matrix of equal volume with 4.9 cm^3. Evaluating the chromatogram (by adsorption at OD_{280nm} of each fraction) showed that four fractions (84-88) and three fractions (84-86) in the elution step (elution of UDA lectin from chitin Sepharose 4B matrix) had an absorption peak in the column with short and long length, respectively (**Fig. 4A**). The comparison of the peaks showed that the eluted peak in the SLC had a greater half-width than the eluted peak in the LLC. Also, the absorption peak in the smaller column (with 0.11) was less than compared to the 0.6 in the larger column (**Fig. 4A**).

4.3. Analysis of Quality and Activity of Purified Lectin

Four fractions 84-88 of SLC and three fractions 84-86 of LLC were pooled and concentrated by falcon dialysis against PBS buffer. The concentrations of UDA lectin showed 38.4 and 8.92 mg. mL^{-1} from SLC and LLC, respectively. On the other hand, the results of purified UDA lectin protein electrophoresis in SDS-PAGE showed that purified UDA lectin from LLC has a clear band compared to SLC (**Fig. 5A**). These results confirm that purified UDA lectin from LLC has a higher purity than SLC. Also, the molecular weight of purified UDA lectin was calculated, by the plot of log MW vs. R_f for approximately 12.5 kDa in LLC (**Fig. 5B**).

Evaluating RBCs agglutination test showed that purified UDA lectin from LLC is needed for hemagglutination and is less than SLC (**Fig. 6**). Started-concentration was 300 (**Fig. $6A_8$**) and 1115 mg. mL^{-1} (**Fig. $6B_4$**) for LLC and SLC, respectively. Hence, these results could confirm that the purity and activity of UDA lectin from LLC are higher than SLC.

5. Discussion

Plant lectins are glycoproteins that can be used directly and indirectly in clinical treatment and diagnosis (2). The UDA lectin is one of the Stinging nettles (*Urtica dioica*) rhizome lectins with an approximate weight of 8.5-14 kDa that can have different biological effects, especially anti-cancer by binding to N-acetylglucosamine on the cell surface (16). This molecular weight difference may be a reasonable difference in the expression of lectin isoforms (17), which consist of 11 isolectins (18), and its glycosylation rate. Ganzera *et al*. (2003) isolated some of these isolectins by reversed-phase high-performance liquid chromatography. However, they have not characterized all types of UDA isolectin properties (18). Our study was performed to optimize the UDA lectin purification with less cost and a higher yield than the previous studies. For this purpose, in the first step (or for the first time), we purified UDA lectin from the rhizome by chitin-based CNBr-Sepharose 4B chromatography at minimum column lengths. Therefore, we showed CNBr-4B, as a column matrix, that can react with two functional groups, Nitrile (C≡N) and Imine (C=N), to form urea and isocarbonate derivatives, respectively (10). Therefore, due to the peaks of activated beads bound of the spectrogram should be expected that the

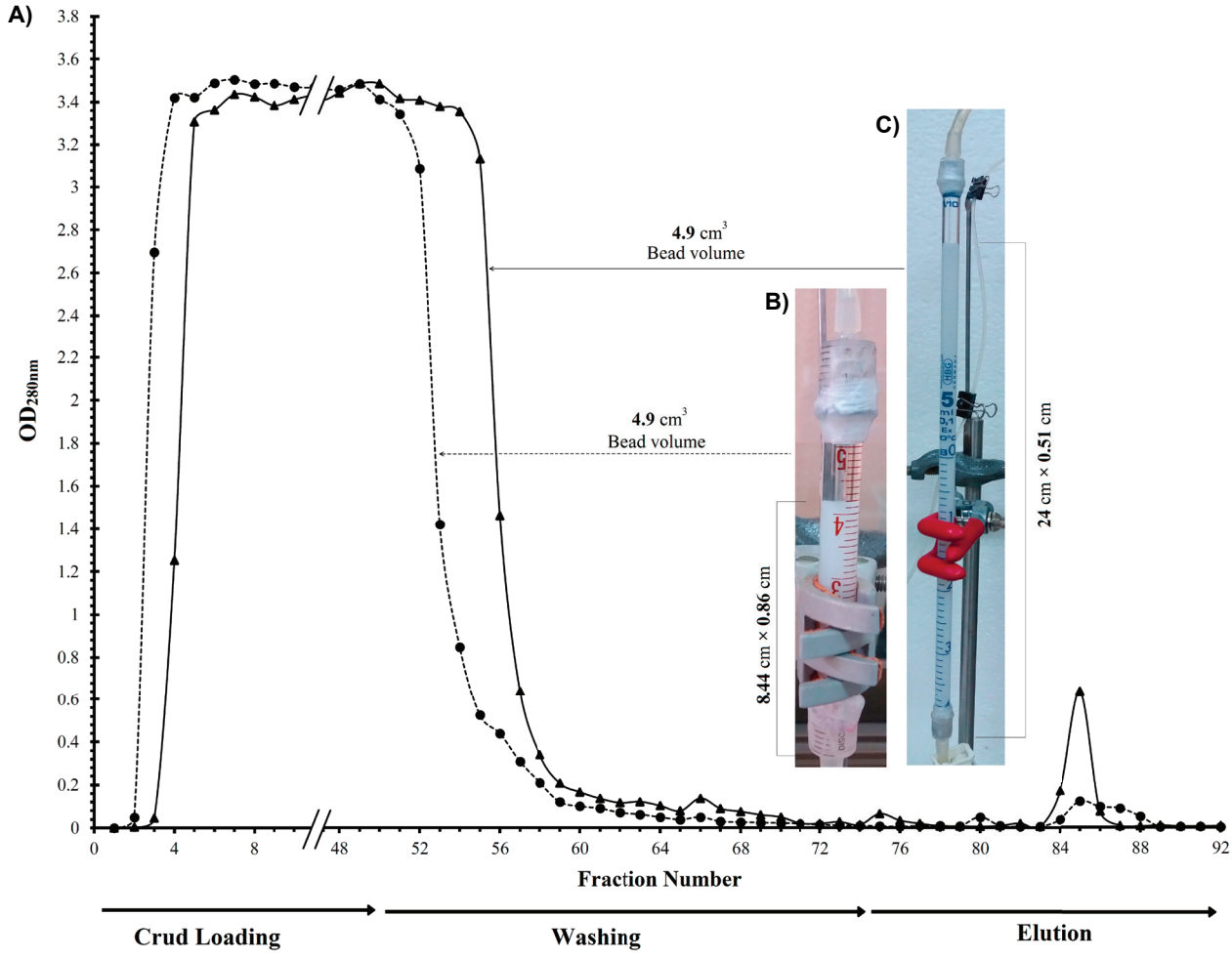

Figure. 4. Chromatograms and purified columns of chromatography. **A**) Chromatogram obtained by purification of two columns with different dimensions; **B** and **C**) Two handmade columns, which used for purification of UDA lectin including SLC with 8.44 x 0.86 (B), and LLC; with 24 x 0.51 cm.

Nitrile peak (from the range of 2222-2260) will shift to the Imine peak (in the range of 1640-1690) in the FTIR spectrogram (19). However, the analysis of the FTIR spectrogram results showed that this change may not have occurred or is difficult to detect in the range of 2222-2260. Also, it seems that the binding between chitin and CNBr-4B was not done through Nitrile shift to Amine because chitin lacks a free amine group (20). On the other hand, FTIR spectrum analysis showed that most of the changes occurred in the fingerprint region (range 600 to 1500), which is unique and specific to each compound. Hence, considering the major changes in the fingerprint range, it can be concluded that the binding between Sepharose 4B and chitin ligand probably was done in regions other than Nitrile. Since the peak output of UDA lectin at the elution step showed that Sepharose 4B beads alone (not coupled to ligand) were not able to separate UDA lectin. The reason is that previous studies showed that Sepharose 4B beads have a large pore, which is the resolution of proteins isolated by them ranges from 60-20,000 kDa (21). However, the results of this study showed that purified UDA lectin has a molecular weight of about 12.5 kDa (**Fig. 5A**) which is a reason for chitin binding to Sepharose 4B.

Some reports showed that the UDA lectin band in the SDS-PAGE was located below 14 kDa (4, 9, 17, 22). The reason might be the differences in the expression

of the lectin isoforms and their glycosylation rate but Samadian *et al*. (2022) have reported other reasons for the aberrant migration in the SDS-PAGE. First, it is the lower isoelectric point (pI) of UDA lectin (about 3.8) and the applied ionic strength ranges of Tris-Glycine buffer (from 3 to 9) for running this protein in the SDS-PAGE (9).

Second, our study showed that increasing the length of the column can be a good way to get a better peak when purifying UDA lectin. Increasing the column length from about 8.44 cm to 24 cm causes a significant increase in the read OD_{280nm} (approximately 5 times) in the collected fractions containing lectin. It is due to the increased collision of UDA lectin with chitin ligands coupled with Sepharose 4B. Thus, the increased purification efficiency caused by the small half-width and high peak height of an LLC caused the purified UDA lectin not to need to be repurified. Also, UDA lectin isolated from the SLC with a wider half-width containing other impurities was observed as a band in

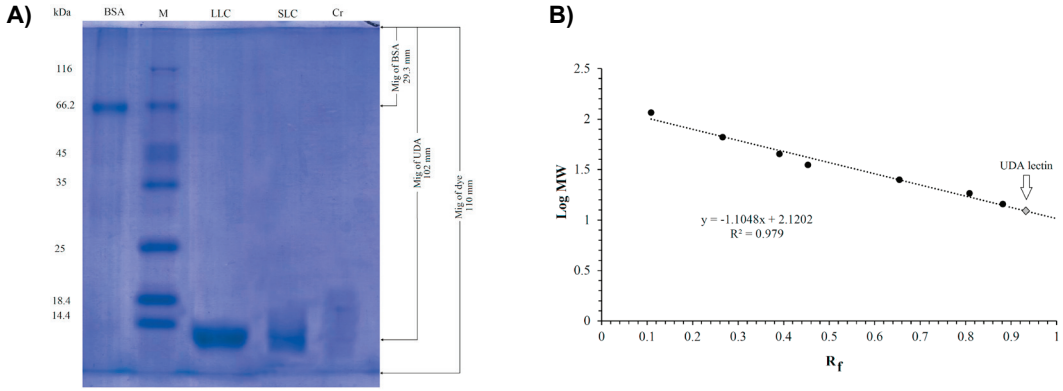

Figure 5. SDS-PAGE protein profile and MW calculation. A) Comparison of protein profiles of purified UDA in the gel electrophoresis, showing the intensity of UDA bands from long length column (lane: LLC), higher than that in short length column (lane: SLC), **B)** The plot of logarithmic plot of MW marker bands (lane: M) versus the relative migration distance (R_f) of bands. The Rf of the UDA was calculated by (migration of UDA lectin=102 mm) ÷ (migration of dye = 110 mm) and the MW of UDA, was determined by the plot's equation: y= -1.1048x-2.1202. BSA was used as a migration control (lane: BSA). R_f is defined by the ratio of the migration distance of the protein band to the migration distance of the dye.

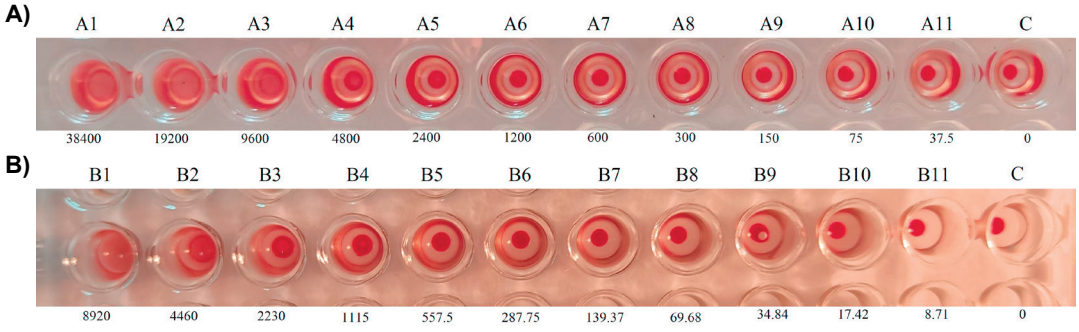

Figure 6. Hemagglutination test of purified UDA lectin. A) A1-A11 showed different concentrations of purified UDA from LLC, in which minimum concentration was 300 µg.mL^{-1} (in A8 well), **B)** Different concentrations of purified UDA from SLC for hemagglutination in B1-B11 wells, with a minimum concentration of 1115 µg.mL^{-1} (in B4 well).

SDS-PAGE gel (**Fig. 5A**). While previous studies by Peumans *et al*., 1984 (4) and Samadian *et al*., 2022 (9) showed the isolated UDA lectin needed to be repurified whereas purified UDA lectin in this study did not require further purification.

Perhaps one of the reasons for re-purification in previous studies (4, 9) was the nature of the chitin and its reaction in different solvents. Chitin polymer contains N-acetylglucosamine with α1-4 is highly hydrophobic. Therefore, the acetyl groups in chitin polymers cause increased insolubility in aqueous solvents (20). Further, in previous studies to produce active ligands, chitin was first dissolved in NaOH and HCl (4, 9) which led to chitin being converted to the deacetylation form (chitosan) in NaOH and then dissolved in HCl (23). Therefore, in the deacetylation reaction, chitin polymer converting to glucosamine polymer may reduce the affinity of UDA lectin to chitin and ultimately reduce the purity of purified UDA lectin. Hence, in this study, 40% polar TCA solvent was used to prepare chitin as a ligand. Thus, TCA is a strong corrosive polar solvent that breaks down chitin polymers (20).

6. Conclusion

The findings of this study indicated that some factors can make UDA lectin purification from *Urtica dioica* rhizomes purer and faster. They include dissolving chitin in a polar solvent like TCA, the use of the Sepharose 4B matrix, and making the chromatographic column longer. Since UDA lectin is one of the plant glycoproteins, it has a high affinity for cell surface carbohydrates, and can bind to glycocalyx at the cell surface of many pathogens, including fungi and bacteria, as well as cancer cells. Therefore, this optimized method for UDA lectin purification can be used by the next researchers to accelerate the UDA lectin purification with greater purity for further studies dealing with drug delivery systems, ELISA techniques, and cell growth.

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