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Biochemistry

Purification and Characterization of Mannose-Specific Lectin Isolated from the Rhizomes of Georgian Endemic Plant *Polygonatum obtusifolium* Miscz.

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ABSTRACT. Mannose-Specific Lectin (SABA–1) was isolated from the rhizomes of Georgian endemic medicinal plant - mountainous Solomon's seal. Isolation and identification of lectin includes the following stages: 1. Obtaining of protein extract from the rhizome; 2. Chromatography on Toyopearl HW-55 column; 3. Fractionation using ammonium sulphate; 4. Thermal treatment at +60^{0}C for 30 minutes; 5. Treatment with acetone; 6. Chromatography on Toyopearl HW-55 column; 7. Affinity chromatography on rabbit erythrocyte column, fixed with glutar aldehyde. SABA-1 was purified 789 times, hemagglutination activity corresponds to 0.000095 mg/ml. As a result of fractionation of summary proteins on Toyopearl HW-55 column 9 protein fractions with different molecular masses were obtained. Hemagglutination activity was expressed only in the third peak, eluted from the column corresponding to 0.0080 mg/l. Using affinity chromatography on the column, stabilized with trypsin-treated rabbit erythrocytes, 2 protein fractions were obtained, of which only the fraction, eluted with 50 mM D-mannose, possessed hemagglutination activity, which corresponds to 0.000096 mg/ml. Using the marker proteins and analytical gel-filtration, molecular weight of the native SABA-1 protein was established, which corresponded to 30 000 Da molecular weight protein fraction. Using electrophoresis of marker proteins on polyacrylamide gradient gel (10-25%), in the presence of SDS, it was established that SABA-1, dissociated into subunits, migrates as a single strip and its molecular mass corresponds to 15 000 Da. Thus, a new mannose specific lectin was identified from the rhizome of Georgian endemic medicinal plant – mountainous Solomon's seal. It is shown, that its native molecular mass is 30 000 Da and it is composed of two equal molecular mass - 15 000 Da - polypeptide subunits. *© 2017 Bull. Georg. Natl. Acad. Sci.*

Key words: *Polygonatum obtusifolium* Miscz. rhizomes, mannose-specific lectin, purification, characterization

Physiologically active compounds of plant origin are widely used in biology, agriculture, medicine and other fields of science. Among them there are sugar binding proteins lectins, which are given special attention last time [1,2].

Proteins of various structures with the ability to

selectively and reversibly bind carbohydrates of glycoconjugates, causing agglutination of cells, are considered to be lectins. Lectin activity is based on the phenomenon of biological recognition, which is stipulated by specific, stereo chemical compatibility between the molecules. One of the compatible or complementary biomolecules is the ligand, carrying biological information, and another one is information-recognizing biomolecule – the receptor. As a result of interaction between the ligand and receptor, decoding of information takes place, providing responses of cells, tissues and organs. Of the components of biological recognition system carbohydrate-containing glycoconjugates and proteins, capable selectively and reversibly bind with them - lectins - are the carriers of the above mentioned functions [3].

Nowadays lectins are successfully used as research tools and affinity sorbents for identification of glycoconjugates, cells and organelles. As specific biological probes they are successfully applied for the sounding of terminal membrane carbohydrates. Because of this they are widely applied in foreign medicine as diagnostic and identification means.

Lectin activities, such as cell proliferation, hormonal, toxic, immune modulating, antitumor, antimicrobial, antiviral and others deserve special interest [4].

It is worth noting that nowadays isolation, identification and study of biological functions of plant lectins proceed intensively and remain one of urgent problems of modern biology, medicine, agriculture and biotechnology.

The present work was aimed at identification, purification and study of physicochemical properties of lectin, isolated from the Georgian endemic plant mountain Solomon's seal.

Material and Methods

Underground parts of Solomon's seal were chosen as objects of investigation. Young and old rhizomes of this plant are effectively used in folk medicine for the treatment of various diseases [5].

Hemagglutination activity was determined visually using 96-well immunological microtiter U-plates using a hemagglutination test on rabbits trypsin-treated erythrocytes with the method of Takatsy [6]. Lectin hemagglutinating activity (HA) was estimated according to the minimum protein concentration (mg/ml), which causes full agglutination of rabbit trypsin-treated erythrocytes. In order to estimate lectin activity we used also a specific activity (ml/mg), which is its inverse value and reflects the minimum concentration, which still causes agglutination: $SA=T^{-1}xC^{-1}$, where T^{-1} (titer) is the degree of minimum protein concentration in which agglutination is still noticeable, C is protein concentration, expressed in mg/ml. Lectins content was judged by the ratio of overall protein content to lectin activity (conventionally agglutination unit, hemagglutination unit – HU) or by the formula GAA=1/Tm (T - titer of lectin hemagglutinating activity; m - mass of the used fragment of *Polygonatum obtusifolium*. Kinetics of hemagglutination activity was measured in hemagglutination medium by the photocolorimetric method, at 670 nm light, a 200 µl 2% suspension of trypsin-treated erythrocytes was introduced into the control and test cuvettes. Lectin fraction was added to the testing cuvettes and PBS of the same volume to the control one. In the control cuvette, light transmission at 670 nm was considered as 100% and variation of this index was fixed with recorder, connected with a photo colorimeter (КФК-3) [7].

Purification of lectin (SABA 1). For the extraction of lectins, the rhizomes of Solomon's seal were ground up and homogenized in a homogenizer of a blender type. Soluble protein fraction was extracted using the extraction solution of the following composition: 0.9% NaCl, 40mM K^+-phosphate buffer, pH 7.4, 0.1% b-mercaptoethanol; extraction was made in conditions of (w/v = 1/5) ratio of raw material to the extraction solution (pH 7.4). The homogenate was placed on magnetic stirrer for 30 minutes at room temperature. Extract was filtered through the double gauze and the filtrate was centrifuged at 15 000 r/min for 15 minutes. For the partial purification and

fractionation of lectin the proteins were precipitated with ammonium sulphate in conditions of 0-60% saturation. Suspension of proteins was centrifuged at 8 000 r/min for 5 minutes. The supernatant was removed by decantation and the precipitated proteins dissolved in the minimum volume of PBS. Protein solution was dispersed in the glass homogenizer and centrifuged at 8000r/min for 10 min. The supernatant was collected by decantation and dialyzed chromatographically on G-10 column (1.6x40 cm) to remove the ammonnium sulphate salt. The dialyzed protein fraction was placed at 60°C temperature for 15 min, to remove the thermolabile proteins. The turbid protein solution was placed in icy bath for 30 min and centrifuged at 18 000 r/m for 10 min.

Treatment with acetone. The sediment was removed and the proteins precipitated from the supernatant with 5 volumes of cold acetone. The mixture was centrifuged at 3000 r/min per 15 minutes and the sediment dissolved in the minimum volume of PBS solution. The proteins labile to aceton were removed by centrifugation at 3000 r/min for 15 min and the lectin was further purified from the supernatant.

Gel-filtration on Toyopearl HW-55 column. Further purification of the protein fraction with lectin activity was carried out on Toyopearl HW-55 column (3.57x70cm), which was balanced with saline. Chromatograhy was preformed using the HPLC system (Knauer). For elution PBS solution was applied. Elution rate was 2 ml/min. Detection was done at 280 nm vawe length. Protein fractions, eluted from the column were tested on lectin activity.

Affinity chromatography on the column of trypsin-treated rabbit erythrocytes, stabilized with glutaraldehyde. Further purification of proteins, possessing lectin activity was performed using the method of affinity chromatography on the column with trypsin-treated rabbit erythrocytes, stabilized by glutaraldehyde (1.60x20 cm) using the HPLC (LKB). Elution rate was 0.5 ml/min. Detecton was carried out at 220 nm vawelength. Elution of the lectin, immobilezed on the sorbent of affine column, balanced with saline was perfomed using the acidic solution (40 mM Glycine-HCl, pH 3.5) (solution A), but in control experiments this was done using the saline solution of lectin-specific carbohydrate 50 mM D-mannose (PBS, pH 7.4) (Solution B). Protein fraction, eluted with the Solution-A was dialysed against PBS, while the protein fraction eluted with the Solution-B was dialysed with acidic solution for 24 hours at +4°C temperature. Lectins were precipitated with ammonium sulphate by the salting out method (0-60%). The precipitate of salted out proteins was dissolved in minimum volume of PBS and centrifuged at 8000r/min for 10 minutes. Dialysate, possessing lectin activity was dialyzed and used in further researches.

Preparing the trypsin-treated erythrocyte chromatoraphic column fixed with glutaraldehyde. With the aim of fixing erythrocytes with glutaraldehyde the erythrocytes were treated with trypsin using the above described method. Trypsin-treated erythrocytes 2% suspension was prepared in 2.5 or 5% solution of glutaraldehyde, agglutination buffer (pH 7.4) and placed on the stirrer for 17 hours at 4°C. The percipitate was washed in quadriple volume of agglutination buffer, centrifuged (700 g, 10 min), hematocrite determined and 2% suspension of erythrocytes was prepared in 1M glycine solution (pH 7.4), prepared on agglutination buffer. The suspension was allowed to stay on a stirrer for 17 hours at +4°C. The precipitate was again washed off in a quadriple volume of agglutination buffer, centrifuged (700 g x10 min); after determination of hematocrite the sediment was mixed with biogel P-150 at 1:10 ratio and the affine column (75x20 mm). The column, fixed with glutaraldehyde was prepared. The column was well washed, first, by agglutination buffer and, then, with 0.2M glycine/HCl buffer (pH 3.0). Before the use the column was balanced with agglutination buffer (PBS).

Determination of SABA-1 molecular weight by the method of gel-filtration. For determination of the protein, purified by affine chromatography, the HPLC

Table. Main stages of purification of SABA-1

Main stages of purification of SABA-1	Hemagglutination activity (mg/ml)	Purification degree
Extract	0.075	0
Chromatography	0.0080	9
Fractioning with ammonium sulphate	0.0045	17
Thermal treatment	0.0030	25
Treatment with acetone	0.0010	75
Chromatography	0.0008	94
Affine chromatography	0.000095	789

system on the Toyopearl HW-55 column (1.0x60 cm), equilibrated with PBS was used. Chromatography was carried out in the following conditions: elution rate - 1.0ml/min, detecton was performed at 280 nm wave length. For the standard and experimental protein fractions the distribution coefficients were determined by the following formula: $K_{av}=(V_e-V_o)/(V_n-V_o)$. The following standard proteins were used: Albumin (66,000 Da), Carbonic Anhydrase (29,000 Da) and Cytochrom C (12,400 Da). (Sigma Gel Filtration Molecular Weight Markers Kit for Molecular Weights 12,400–200,000 Da).

Analytical electrophoresis of SABA-1 in dissociated conditions. Electrophoresis of proteins was carried out in dissociated state, using the system of Laemmlu on 2mm thick 10-25% polyacrylamide gradient gel in the presence of 0.1% SDS [8]. Elecrophoresis lasted 3.5 hr (on 1 ml gel at 2mA amperage (current). The following low molecular weight marker proteins were used for establishing the molecular weight (SIGMA - Molecular Weight Marker KIT for Molecular Weight Range 14,000–70,000 Da): Albumin, Bovine- 66, 000; Ovalbumin, Chicken-45,000; Pepsin, Porcine Stomach Mucosa-34,700; Trypsinogen, Bovine, PMSF treated-24,000; I-Lactoglobulin, Bovine-20,100; Lysozyme, Egg White-14,200.

Protein concentration was measured by the method of Lowry, et al. [9]. All experiments were performed in triplicate. Statistical analysis was performed using Student's t-test and p-values < 0.05 considered to be significant.

Results and Discussion

The main stages of purification of lectin isolated form from rhizomes of Solomon's seal, hemagglutination activity of protein fractions obtained at each stage of purification and quality of purification of SABA-1 lectin are presented in Table 1. The data given in Table 1 evidence that the process of purification of lectin, isolated from Solomon's seal rhizome consists of 7 stages: 1. Isolation of protein extract from the rhizome; 2. Chromatography on the Toyopearl HW-55 column; 3. Fractionation of SABA-1 containing proteins with ammonium sulphate; 4. Thermal treatment at +60°C for 30 min; 5. Treatment with acetone; 6.Chromatography on the Toyopearl HW-55 column; 7. Affinity chromatography on the column, stabilized with glutaraldehyde.

Table 1 shows that at each next step of purification hemagglutination activity of protein fraction containing SABA-1 increases. Correspondingly increases purification quality of SABA-1. In particular, in the protein fraction of crude extract, obtained as a result of chromatography, showing hemagglutination peak, hemagglutination activity was 0.0080 mg/ml. This indicates that the degree of purification of SABA-1 makes 9. Applying the next stages of purification, including consecutive fractioning with ammonium sulphate, thermal and acetone treatment, chromatography via gel-filtration and affine chromatography at the final stage, showed that after each stage of purification hemagglutination activity increased correspondingly 17, 25, 75, 95-times and af-

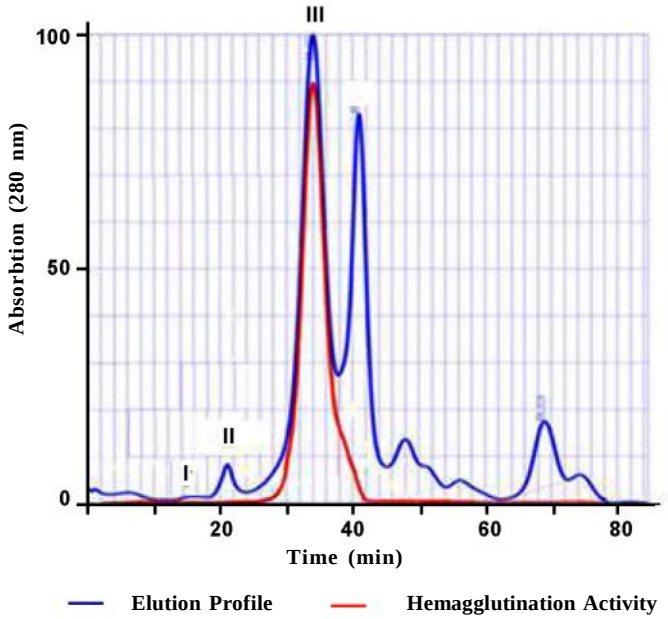

Fig. 1. The profile of elution of raw extract, obtained from rhizomes of Solomon's seal on the Toyopearl HW-55 column.

ter the final step of activity of SABA-1 corresponding to 0.000095 mg/ml, and purification degree attained 789. Data presented in the Table indicate that using the method developed by us SABA-1, isolated from the raw extract of Solomon's seal rhizome as individual molecules, was purified 789-times.

The profile of fractioning of summary proteins, extracted from the rhizome on the Toyopearl HW-55 column is presented below, in Fig. 1. As seen from Fig. 1, 9 protein fractions of different molecular weight are eluted from the column. In parallel to this in each eluted fraction hemagglutination activity was determined with the aim of revealing the lectin. As shown in Fig. 1, hemagglutination activity was detected only in the most enriched protein fraction, which corresponds to the 3rd peak, eluted from the column. Hemagglutination activity of the mentioned fraction corresponds to 0.0080 mg/ml.

For the further purification of SABA-1 lectin, protein fraction, possessing hemagglutination activity (peak 3) was fractionated with ammonium sulphate (0-60%), then treated thermally (+60°C), and finally treated with cold acetone. Further purification of SABA-1 form the protein fraction, obtained using the described biochemical methods, was perfomed by means of repeated chromatography on Toyopearl HW-55 column.

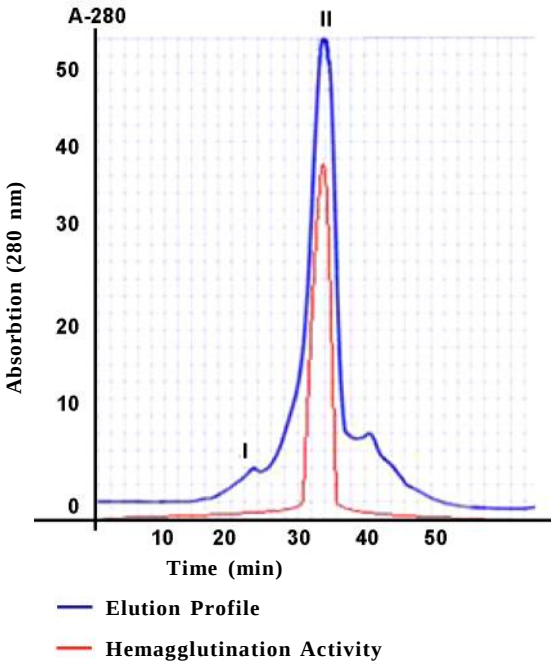

Fig. 2. Profile of purification with ammonium sulphate, thermo- and acetone treatment of the protein fraction, isolated from the Solomon's Seal rhizome on Toyopearl HW-55.

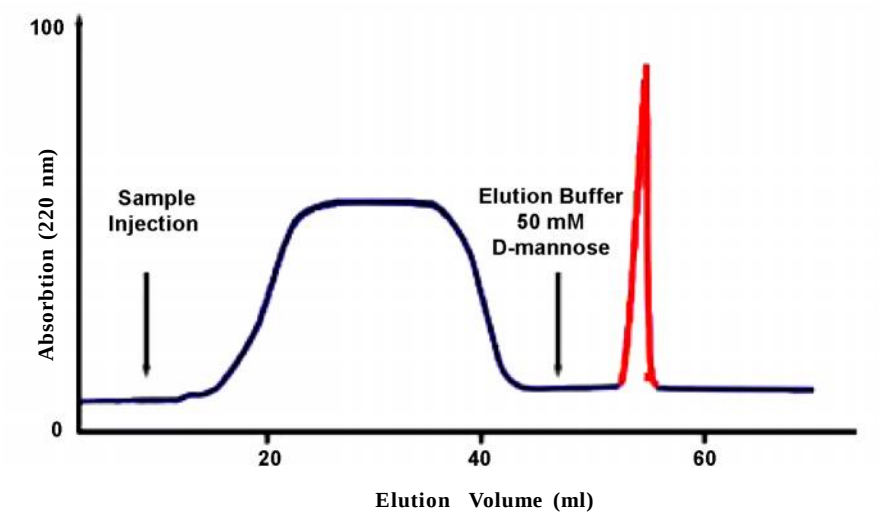

Fig. 3. Profile of affine chromatography of SABA-1 on the column of trypsin-treated erythrocytes, stabilized with glutaraldehyde.

As shown in Fig. 2. two small and one big protein fractions were eluted from the column. Hemagglutination activity was detected in the big protein fraction (peak 2). Hemagglutination activity was 0.0008 mg/ml (Table 1).

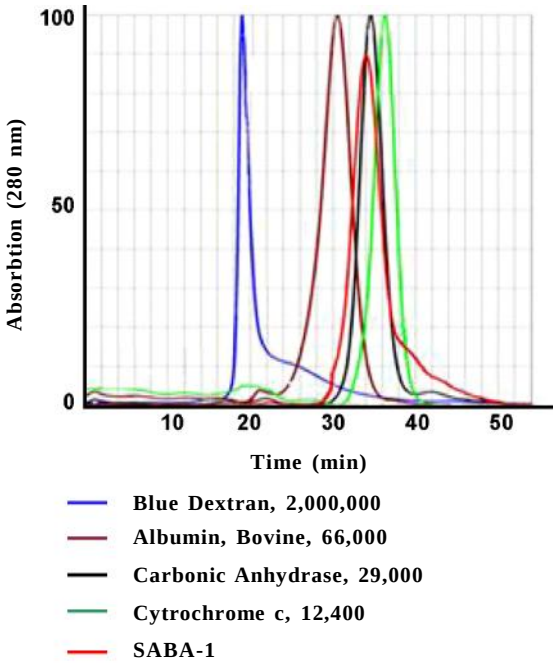

Fig. 4. Elution profiles of Calibration Kit proteins and affinity purified SABA-1 on Toyopearl HW-55 column. Elution volumes (V_0) and (Ve) are found at maximum peak height of each respective protein.

At the final stage of purification of SABA-1 lectin the method of affine chromatography was used. Column of trypsin-treated erythrocytes, stabilized with glutaraldehyde was used as absorbent.

As seen from Fig. 3, after running the lectin-containing protein fraction through the column of trypsin-treated erythrocytes, the fraction of ballast proteins, unbound with the column was eluted with PBS, which did not have hemagglutination activity or it did not contain lectins. Elution of the protein fraction with hemagglutination activity from the column was performed using the SABA-1 specific carbohydrate, 50 mM D-mannose-containing PBS solution and its hemaggutination activity corresponds to 0.000095 mg/ml (Table 1).

In further series of research, using the gel-filtration method and calibration standard proteins (markers), individual native molecular mass of affinity purified SABA-1 was determined (Fig. 4).

As seen from the chromatography profile, presented in Fig. 4, the fraction of affinity-purified SABA-1 (red curve), is eluted from the column between the albumin (66,000 Da) and carbonic anhydrase (29,000 Da) and is approximated to the elution profile of the latter.

Fig. 5 presents the calibration curve, built according to the elution profile of chromatography of Cali-

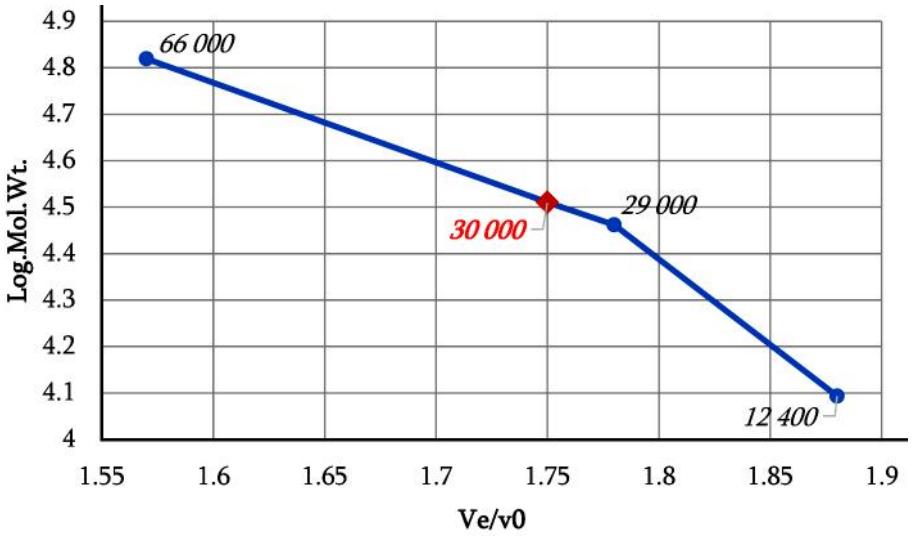

Fig. 5. Calibration curve for Calibration Kit proteins and affinity purified SABA-1 on Toyopearl HW-55 column.

bration kit proteins, which was used for the determination of molecular mass of the native molecule of SABA-1. According to calculations, done with the use of the calibration curve, it was established, that molecular weight of SABA-1 is 30,000 Da.

With the aim of establishing the quaternary structure of SABA-1, on the next steps of experiment native SABA-1 molecules were denaturated in the presence of sodium dodecyl sulphate (SDS) and molecular masses and quantity of its constituent subunits was determined by the method of electrophoresis in polyacrylamide gel (10-25%), using standard calibration proteins (markers).

As seen from the electrophoregram presented in Fig. 6, on the first track the migration profile of markers of the known molecular weight is shown. On the third track the profile of migration of protein fractions obtained as a result of the third stage of purification is presented, which evidences that the mentioned fraction represents partially purified SABA-1, whose share in the mentioned fraction of proteins is insignificant. The track 2 shows that SABA-1, purified as a result of affinity chromatography, in the dissociated state migrates as one compact strip, which proves that it corresponds to the individual polypeptides of SABA-1, obtained as a result of purification.

Fig. 7 shows the calibration curve, built according to the electrophoregram for the determination of molecular weights of the SABA-1 constituent subunits. Using the calibration curve it was determined that molecular weight of subunits of SABA-1 is equal and it comprises 15.000 Da.

Thus, the method of isolation and purification of D-mannose specific lectin from the rhizome of

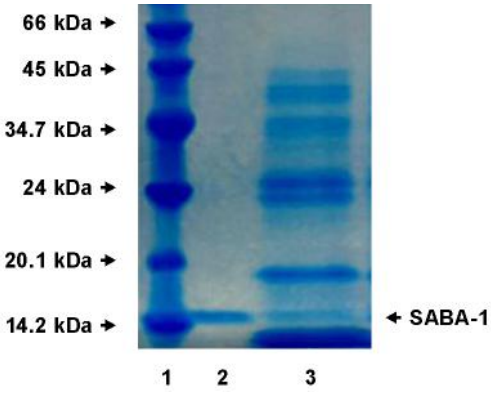

Fig. 6. SABA–1 electrophoresis in polyacrylamide gradient (10-25%) gel in the presence of the anionic detergent, sodium dodecyl sulfate (SDS).
1. SDS Molecular Weight Markers, Molecular Weight Range 14,000–70,000 Da, gel loading volumes are 1-10 µl. (Albumin, Bovine-66, 000; Ovalbumin, Chicken-45,000; Pepsin, Porcine Stomach Mucosa-34,700; Trypsinogen, Bovine, PMSF treated-24,000; I-Lactoglobulin, Bovine-20,100; Lysozyme, Egg White-14,200);
2. SABA–1 purified by affinity chromatography;
3. SABA-1containing protein fraction obtained after the gel-filtration on Toyopearl HW-55 column.

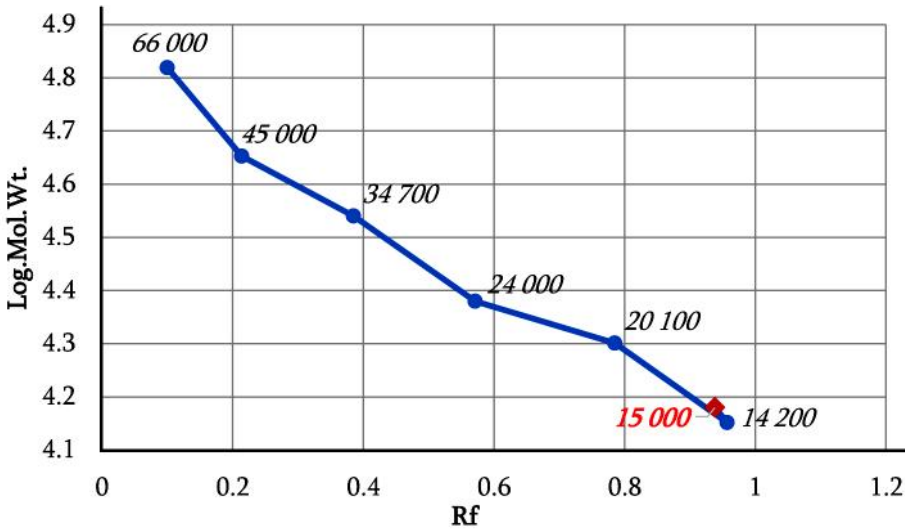

Fig. 7. Calibration curve obtained with proteins from SIGMA - LMW Range kit 14,000–70,000 Da, run on polyacrylamide gradient (10-25%) gel in the presence of sodium dodecyl sulfate (SDS).

D-mannose specific SABA-1 lectin was developed. Hemagglutination activity of SABA-1, identified by using the mentioned method is equal to 0.000095 mg/ml and its purification degree is 789. Using the methods of gel-filtration and electrophoresis it was showed that the mass of its native molecule is 30,000 Da and it consists of two equal polypeptide subunits of 15,000 Da molecular mass.

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ბიოქიმია

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** საქართველოს საპატრიარქოს წმიდა ანდრია პირველწოდებულის სახელობის ქართული უნივერსიტეტი; მედიკო-ბიოლოგიური სამეცნიერო-კვლევითი ცენტრი შპს „ალექსისი“, თბილისი, საქართველო*
***აკადემიის წევრი, საქართველოს საპატრიარქოს წმიდა ანდრია პირველწოდებულის სახელობის ქართული უნივერსიტეტი, თბილისი, საქართველო*

საქართველოს ფლორის ენდემური სამკურნალო მცენარის, მთის სვინტრის ფესურადან გამოყოფილი და გასუფთავებულია მანოზა-სპეციფიკური ლექტინი SABA–1. ლექტინის გამოყოფისა და გასუფთავების ძირითადი ეტაპებია: 1. ფესურადან ცილოვანი ექსტრაქტის მიღება; 2. ქრომატოგრაფია Toyopearl HW-55-ის სვეტზე; 3. ფრაქციონირება ამონიუმის სულფატით; 4. თერმული დამუშავება +60°C-ზე 30 წთ-ის განმავლობაში; 5. აცეტონით დამუშავება; 6. ქრომატოგრაფია Toyopearl HW-55-ის სვეტზე; 7. აფინური ქრომატოგრაფია გლუტარალდეჰიდით ფიქსირებული ბოცვრის ერითროციტების სვეტზე. SABA–1 გასუფთავებულ იქნა 789-ჯერ, ჰემაგლუტინაციურმა აქტივობამ შეადგინა 0,000095 მგ/მლ. Toyopearl HW-55-ის სვეტზე სუმარული ცილების ფრაქციონირების შედეგად მიღებულ იქნა 9 განსხვავებული მოლეკულური მასების მქონე ცილოვანი ფრაქცია. ჰემაგლუტინაციური აქტივობა ფიქსირდებოდა მხოლოდ სვეტიდან ელუირებულ მე-3 პიკში და იგი შეადგენდა 0,0080 მგ/მლ. აფინური ქრომატოგრაფიის გამოყენებით გლუტარალდეჰიდით ფიქსირებული ტრიფსინიზით დამუშავებული ერითროციტების სვეტზე მიღებულ იქნა 2 ცილოვანი ფრაქცია, რომელთაგან მხოლოდ 50 mM D-მანოზას შემცველი ფიზიოლოგიური ხსნარით ელუირებულ ფრაქციას გააჩნდა ჰემაგლუტინაციური აქტივობა, რომელმაც შეადგინა 0,000096 მგ/მლ. მარკერული ცილების და ანალიზური გელ-ფილტრაციის გამოყენებით დადგენილ იქნა, რომ ნატიური SABA-1-ის მოლეკულური მასა შეესაბამება 30,000 Da-ს. მარკერული ცილების ელექტროფორეზით პოლიაკრილამიდის გრადიენტულ გელში (10-25%), SDS-ის თანაობისას დადგენილ იქნა, რომ სუბერთეულებად დისოცირებული SABA–1 მიგრირებს ერთი ვიწრო ზოლით და მისი მოლეკულური მასა შეესაბამება 15,000 Da.

ამრიგად, საქართველოს ენდემური მცენარის მთის სვინტრის ფესურადან გამოყოფილი და იდენტიფიცირებულია მანოზა-სპეციფიკური ახალი ლექტინი. ნაჩვენებია, რომ მისი ნატიური მოლეკულური მასა 30,000 Da-ია და შედგება ორი თანაბარი, 15,000 Da მოლეკულური მასის მქონე, პოლიპეპტიდური-სუბერთეულისაგან.

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