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ISOLATION OF A *GALEGA OFFICINALIS* L. FRACTION AND A TENTATIVE EVALUATION OF THE CHEMICAL STRUCTURE OF BIOLOGICALLY ACTIVE SUBSTANCES, INHIBITING PLATELET AGGREGATION

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Summary

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A biologically active fraction (BAF) IV that inhibited platelet aggregation was isolated via elution from an aqueous extract of *Galega officinalis* L. and its chemical composition was tentatively determined. The aqueous extract was purified from impurities by column chromatography on Sephadex G-25 (BAF I), Sepharose 4B (BAF II), DEAE-Cellulose (BAF III) and Sephadex G-100 (БАФ IV). The maximum purified BAF IV fraction contained substanced with molecular mass of 100–140 kDa. The amino acid analysis and NIR spectrometry showed that the fraction contained 19-23% protein and about 74% polysaccharides. The consecutively isolated fractions inhibited platelet aggregation, induced by 25 μM adenosine diphosphate at concentrations, decreasing in conformity with the sequence of isolation. The IC_{50} of the aqueous extract was 1100 ± 30.6 μg/mL and that of BAF IV – 9.3 ± 0.45 μg/mL. When both IC_{50} were compared, a 118.3-fold purification of BAF IV was determined. The high biological activity of BAF IV was supposed to be due to the protein component of the fraction.

Key words: *Galega officinalis* L., glycoprotein fraction, medicinal plant, platelet aggregation

INTRODUCTION

Galega officinalis L. (goat's rue) is a medicinal plant that, according to literature data, is widely distributed in Eastern Europe, Italy and Bulgaria. Goat's rue is used in medicine for the treatment of diabetes mellitus and for increase in the lactic secretion (Benigni *et al*., 1972). Over 15 biologically active substances: alkaloids, flavonoids, glycosides, saponins etc. have been isolated from *Galega officinalis*. The biologically active alkaloid galegine, exhibiting an *in vivo* hypoglycaemic activity has also been isolated from the plant (Hoppe, 1975). More recent experimental data (Atanasov, 1994) evidenced that its aqueous extract inhibited *in vitro* platelet aggregation, induced by adenosine diphosphate, thrombin and collagen.

The present report presents the purification procedure of the fractions from the aqueous extract as well as the isolation and the biological characterization of the activities of four consecutive biologically active fractions (BAF) (BAF I, BAF II, BAF III and BAF IV), obtained by chromatographic procedures. An attempt for chemical characterization of the most purified fraction (BAF IV) has been made.

MATERIALS AND METHODS

Materials

The aqueous extract was prepared from commercial dried *Galega officinalis* L. blades using the dextranes Sephadex G-25, Sephadex G-100, Sephadex G-150, Sepharose 4B and the ion-exchange medium DEAE-Cellulose (SIGMA Chemical Co., USA). As protein standards β-amylase, alcohol dehydrogenase, albumin, ovalbumin and carbonic anhydrase (ICN Pharmaceuticals, Inc) were applied.

The aggregation of platelet-rich human plasma was induced by adenosine 5'-diphosphate (Reanal, Hungary).

Preparation of the aqueous extract and elution of BAF I, BAF II, BAF III and BAF IV

The aqueous extract was obtained by maceration of 200 g dried *Galega officinalis* blades in 2000 mL distilled water for 20–24 h at 18–20 °C. Distilled water was alkalized to pH 8 with sodium hydrogen carbonate. The raw extract was filtered and concentrated at a temperature not higher than 35°C to a concentration of 7.5 mg/mL. Thereafter it was purified from low molecular substances with molecular weight below 20 kDa using column chromatography on Sephadex G-25. The elution curve was determined in accordance with UV absorption at 280 nm. The biological activity of each 10 mL eluent was determined. Four biologically active fractions – BAF I, BAF II, BAF III and BAF IV were isolated from the concentrated extract. The eluate of chromatographic columns was scanned with an UV monitor and the eluent curve was registered. The eluent was distributed into tubes by means of a fraction collector FRAC-100 (Pharmacia).

BAF I was isolated by desalting the aqueous extract through a Sephadex G-25 column. Ten mL concentrated aqueous extract, containing 75 mg dry residue were desalted on a Sephadex G-25 column (700 x 65 mm), equilibrated with aqueous ammonia with pH 7.3. BAF I was eluted with the same equilibrating solution at 16 °C at a rate of 720 mL/h. The fraction was concentrated and lyophilized.

BAF II was obtained by purifying BAF I lyophilisate through a Sepharose 4B column (550 x 24 mm). The gel filtration was used for eliminating the substances with molecular weight over 200 kDa from BAF I, containing colorants. Two hundred mg of the lyophilized BAF I were applied to the second column, equilibrated with 0.01M Tris-HCI buffer; pH 7.3. The BAF II was eluted with the same buffer at 16°C at a rate of 10 mL/h. The biological activity of each 10 mL eluent was determined. The fraction was desalted, concentrated and lyophilized.

BAF III was obtained after purification of the BAF II lyophilisate through a DEAE-Cellulose column. One hundred mg of the lyophilized BAF II fraction was applied to the third in the line ion-exchange column on DEAE-Cellulose (80 x 10 mm), equilibrated with 0.02M potassium phosphate buffer with pH 7.3. The BAF III fraction which inhibits the platelet aggregation, was eluted with an equili-

brating buffer and was desalted, concentrated and lyophilized.

BAF IV was obtained after purification of the BAF III lyophilisate through a Sephadex G-100 column. Twenty-four mg of the lyophilized BAF III was applied to the fourth column in the line Sephadex G-100 (52 x 16 mm), equilibrated with 0.01M Tris-HCl buffer with pH 7.3. The BAF IV fraction was eluted with the same buffer at 16°C at a rate of 16 mL/h. The biological activity of each 5 mL eluent was determined.

Determination of the molecular mass of BAF IV

To determine the molecular mass of BAF IV, a Sephadex G-150 (100 x 1.5 cm) column equilibrated with 0.01M Tris-HCl buffer with pH 7.3 was used. As protein standards, we used β-amylase (MW 200 kDa), alcohol dehydrogenase (MW 150 kDa), albumin (MW 66 kDa), ovalbumin (MW 43 kDa) and carbonic anhydrase (MW 29 kDa).

Amino acid analysis of BAF IV

The amino acid analysis of the final purified BAF IV fraction was performed according to the method described in the Bulgarian National Standard 11374-86. on a amino acid analyser T 339 M column (Microtechna, Praha).

Determination of the chemical composition of BAF IV by means of NIR spectroscopy

The chemical composition of the final purified fraction BAF IV was determined by near infrared analysis (NIR spectroscopy) in aqueous solution with pH 7.3. The experiments were performed in the Laboratory of NIR Spectroscopy to the Agricultural Faculty, Kobe University (Japan) with a NIR System 6500 (NIR System Inc., Silver Spring, MD, USA).

Determination of the biological activity of the aqueous extract and the fractions

The inibiting effect of the aqueous extract and the fractions on platelet aggregation was determined via the photometric method of Born and Zucker (Zucker, 1989). The aggregation of human platelet-rich plasma was induced with 25 μM adenosine 5'-diphosphate (ADP). During the purification of the fractions from the aqueous extract, we used the method of Born and Zucker for detection of the fractions, containing platelet inhibitors. The biological activity (IC_{50}) was determined with lyophilisates of the aqueous extracts and the purified fractions. The IC_{50} was expressed in μg/mL platelet-rich human plasma. The aggregation of platelet-rich plasma induced by 25 μM ADP without addition of extract or any fraction was accepted as 100% during the IC_{50} determination. To determine IC_{50}, a control sample with adenosine 5'-diphosphate was performed. Additional four determinations were made after prior incubation of plasma for 10 min with different concentrations of the aqueous extract and the fractions with different degree of purification. The concentrations were chosen in such a way that platelet aggregation inhibition was within the range of 16-84%. The average IC_{50} of the aqueous extract and each fraction was calculated from five measurements.

RESULTS

Elution of BAF I–IV

The elution curve of BAF I is presented in Fig. 1A. It was located between volumes of 400 and 2000 mL. The eluent between

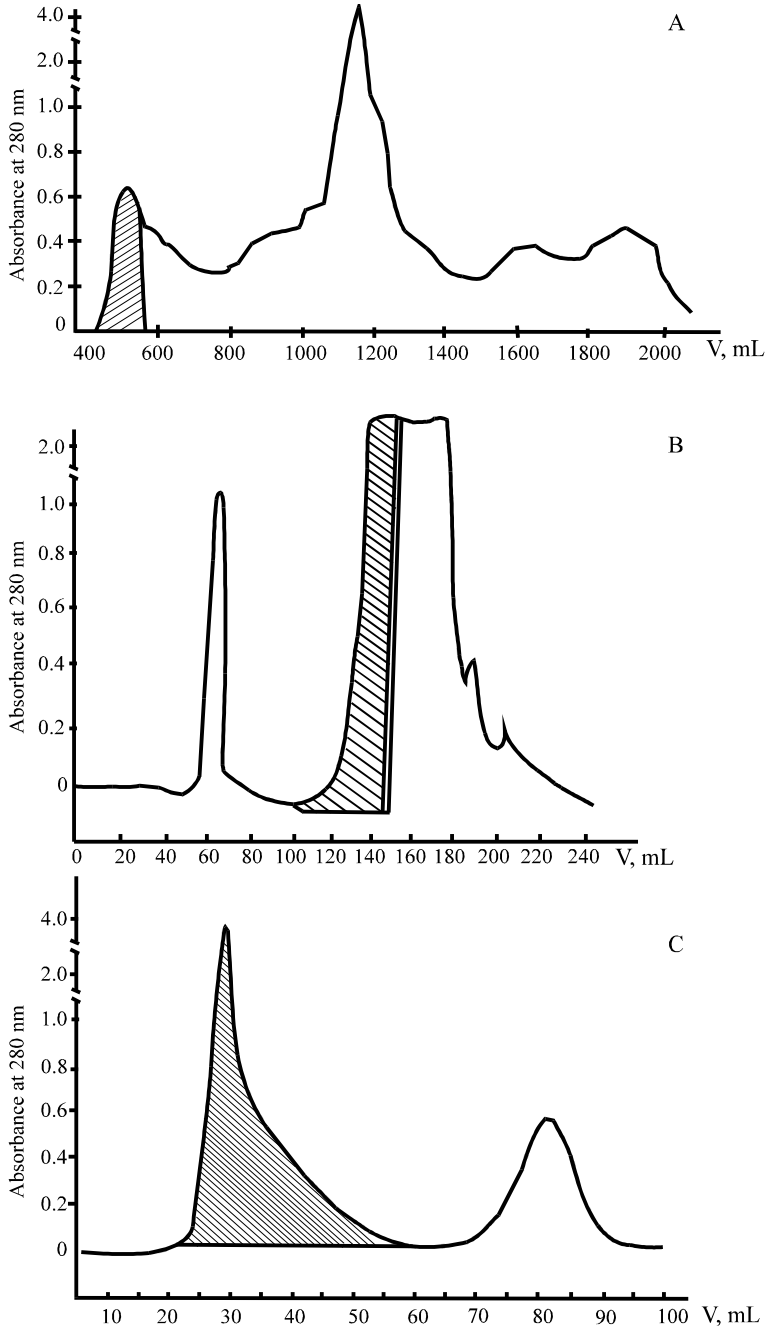

Fig. 1. Elution curves of BAF I (A), BAF II (B) and BAF IV (C) showing an absorption of the eluent at 280 nm. The biologically active fractions are hatched. The X axis presents the volume (V) of the eluent (mL); the Y axis – the absorption of the eluent at 280 nm.

400 and 550 mL contained the substances, inhibiting platelet aggregation (BAF I).

The elution curve of BAF II after Sepharose 4B column (Fig. 1B) determined by UV absorption at 280 nm was between 60 and 240 mL. The eluent between 120 and 160 mL contained platelet aggregation inhibitors (BAF II).

BAF III was highly decoloured by removing the colour substances from BAF II that were inactive and were absorbed on the ion-exchange medium DEAE-Cellulose.

The eluent curve of BAF IV, determined by the UV absorption at 280 nm, is shown in Fig. 1C. It was between volumes of 20 and 100 mL. Platelet aggregation inhibitors (BAF IV) were in the eluent between 20 and 60. BAF IV was the final, most purified fraction, that allowed its molecular mass, amino acid content and NIR spectrum to be characterized.

Molecular mass of BAF IV

The molecular mass of BAF IV was determined on Sephadex G-150 using protein markers. Thus, it was determined as being between 100 and 140 kDa.

Amino acid and NIR spectrometric analysis of BAF IV

The amino acid analysis showed that the total protein of BAF IV was 23% and contained the following amino acids: aspartic acid (3.037 %), glutamic acid (3.886 %), leucine (1.884 %), serine (1.300 %), threonine (0.995 %), alanine (2.181 %), glycine (1.436 %), valine (1.508 %), proline (1.217 %), lysine (1.198 %), isoleucine (1.064 %), phenylalanine (0.921 %), tyrosine (0.680 %), arginine (0.573 %), histidine (0.397 %), methionine (0.390 %), cysteine (0.294 %).

The principal chemical components determined by the NIR spectrum of BAF IV were proteins and polysaccharides with absorption peaks at: 2048 nm (proteins); 1440, 1448 nm (polysaccharides); 1496 nm (polysaccharides); 1948 nm (polysaccharides and water). The total protein content determined by NIR spectrometry was 19 ± 2.8 % and the polysaccharide content was 74 ± 3.9 %.

Biological activity

The correlation “concentration–effect" for the aqueous extract and the fractions after each step of purification was characterized by decreasing IC_{50} values (concentrations of the extract and the fractions, that inhibited platelet aggregation induced by 25 μM ADP by 50%). The IC_{50} values of the aqueous extract was 1100 ± 30.6 μg/mL, and those of BAFs: 31 ± 2.1 μg/mL, 12 ± 0.64 μg/mL, 11.2 ± 0.6 μg/mL and 9.3 ±0.45 μg/mL for BAF I, II, III and IV respectively .

DISCUSSION

When the IC_{50} values of the aqueous extract and BAF IV are compared, it could be seen that IC_{50} of BAF IV was 118.3 times lower than that of the extract, so the four consecutive procedures resulted in a 118.3-fold purification of the initial aqueous extract.

The high biological activity of BAF IV was possibly due to the protein component of the fraction, similarly to snake venom peptides, isolated by Huang *et al.* (1992). It is known that synthetic tri-, tetra- and hexapeptides with peptide sequences equal to that of snake venoms, inhibit platelet aggregation in low concentrations (Nicholson *et al*., 1991; Vezenkov *et al*., 1999).

Another experimental fact, suggesting that the protein component of the fraction

is responsible for its biological activity, was the dependence of the extract activity on the ambient temperature and medium pH (Atanasov, 1994). The aqueous extract lost its ability to inhibit platelet aggregation at temperatures higher than 60 °C and pH below 5.5.

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