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Some physicochemical characteristics of anti-platelet fraction isolated from *Galega officinalis* L.

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Some physicochemical characteristics of a 100-140 kDa fraction isolated from *Galega officinalis* L. were studied. The water soluble fraction inhibits platelet aggregation initiated by ADP, thrombin and collagen.The enzyme-treated fraction changes negligible inhibiting effect on platelet aggregation. The isolated fraction appears to have a polysaccharide nature, including 23 % protein. No loss in the activity of the fraction after storage for several months at 4 ºC in N_3H-H_2O solution with neutral pH, and after freesing of liophilized fraction at -10 ºC. The fraction shows maximum activity in the temperature diapason of 10°C-42°C and pH diapason of pH 5.5-9.8. The micro-calorimetric analyses show two protein subunits in the fraction. Anti-platelet fraction may find application in medicine, similarly to dextrans.

Key words: *Galega officnalis* L.**,** isolation, anti-platelet fraction, characteristics

INTRODUCTION

Galega officinalis L.. is a medicinal plant wide spread in West Europe, Italy and Bulgaria. The plant has been used in a traditional medicine system in treatment of *diabetes mellitus* [1, 2]. Over 15 biologically-active substances are isolated from *Galega officinalis*: galegine, hydroxygalegine, peganine, vasicinone, lutein (alkaloids), penthahydroxyflavone 5-glucoside, luteolin, galuteoline, luteolin 5-glucoside (glucosides); flavonoids, glucosidessaponins and γ-γ dimethylallylamidin [3, 4]. The previous experimental investigations of Atanasov et.al indicate that the 100-140 kDa fraction isolated from *G. officinalis* has wide spectrum of effects on platelet and blood-plasma functions [5, 6] as :

- Inhibition of platelet release reaction [3]
- Inhibition of spontaneous platelet aggregation [3]
- Inhibition of platelet aggregation initiated by ADP, thrombin and collagen [5, 6]
- Inhibition of platelet aggregation initiated by free-radical compounds [3]
- Inhibition of spontaneous blood-plasma coagulation [3]

In vivo inhibition of platelet aggregation after intravenous injection in animals [7]

In manuscript are presented some physicochemical characteristics of fractions from *Galega officinalis*, which are important for biomedical approach on living organisms.

EXPERIMENTAL

Platelet aggregation measurement and fraction's activity

Blood was taken from volunteers (3 males and 3 females aged 20-23 years) wich was not treated with medicine for 15 days prior to blood collection. Blood was collected in disposable syringes and diluted at a ratio of 1 part 3,8 % trisodium citrate and 9 parts venous blood. Platelet-rich plasma (PRP) was prepared by centrifugation (180 x g for 10 min) and diluted to 300 x 10^6 platelets per ml with autologous platelet-poor plasma (1800 x g for 15 min). The platelet aggregation was studied by a photometric method according Born and Zucker [8]. The extinction change that takes place during the aggregation of 400 μl platelet-rich plasma compared with platelet-poor plasma (whose extinction was taken as zero) after adding aggregating agent at final concentration 25 μM ADP, 100 μg/ml collagen or 0.8 U/ml thrombin at 37°C was the basis of measurement of the aggregating effect. Aggregation (A) is calculated by the formula:

$$A, \% = (Eo - Esample)/(Eo - Eplasma).100\%,$$

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where E_0 is the initial extinction of the platelet-rich plasma, E_{plasma} is the extinction of the poor plasma and E_{sample} is he extinction after platelet aggregation. The fraction activity was evaluated by degree of platelet aggregation (A, %).

Purification procedures

The purification of the fraction was made by 4 steps: 1) gel-filtration of crude extract on Sephadex G-25; 2) gel-filtration on Sepharose 4B ; 3) ion-exchange chromatography on DEAE-cellulose and 4) gel-filtration on Sephadex G-100, accordingly to procedures given in [3, 9].

Amino acid analyses

Aminoacid analysis of the fractions was performed by method given in BNS (Bulgarian National Standard) 11374-86. The amino acid content was determined by hydrolysis of the active component with 6N HCI. For determination of S-content amino acids methionine and cysteine were used [3, 9]. The amino acid quantity was determined over the ionit " OSTION LG ANB" on "AMINO ACID ANALYSER T 339 M" column (Microtechna-Praha), D(0.37 x 36 cm) using sodium citrate buffers with pH 3.5, 4.25 and 9.45. Ninhidrin was used as reagent for visualization. The absorbance was measured by photometry at 525 nm using interferent filter. "Computing integrator-CI 100" (Laboratorni Pristoje-Praha) was used for computing of the signal from the aminoanalyser.

Solubility of the fraction

The solubility of the final biologically active fraction (BAF) was studied after dissolution in various hydrophilic and hydrophobic solvents. 1mg IVBAF was dissolved in H_2O:EtOH solutions at 18°C for 24h. The activity of the IVBAF was determined by ability of 50μg BAF to inhibits aggregation of 1ml PRP, initiated by 25μM ADP. The activity of the fraction was presents like level of platelet aggregation (A, %).

Ultraviolet, visible and infrared spectrum of BAF

Spectrophotometric study of a fraction in ultraviolet (UV) and visible (VIS) area was meseared by spectrophotometer "LKB - ULTROSPEC" – Sweden. The infrared (IR) spectrum of BAF was meseared on spectrophotometer Bruker IFS 113v into tablets of potassium bromid (KBr).

pH dependence of a fraction activity

4% water solution of the fraction was treated with different amount of HCI and NaOH in pH range of 3.0-11.0. The activity of fraction was measured and present by mean ± SD.

Temperature dependence of a fraction activity

Water solution of 3mg final sample in 6 ml H_2O-NH_3 (pH 7.4) was heated for 20min in temperature range of 20-100°C. The activity of the fraction was measured and present by mean ± SD. A non-treated fraction was taken as control.

Microcalorimetric study of a fraction

The differential scanning microcalorimetric measurement was made on DASM-4 microcalorimeter.The sample with final concentration 200 μM (pH 7.3) was heated with in range of 1.5 °C/min. All heating curves were corrected using an instrument baseline obtained by heating the buffer.

Fraction activity after treatment with chemical substances and enzymes

Denaturing effect of chemical agents (containing divalent metal ions Ca^{2+}, Zn^{2+}, Mg^{2+}, Mn^{2+}, Co^{2+}) and effect of proteolytic enzymes (Chymotrypsin,Chymopapain, Trypsin, α-amilase, β-amilase and alkaline protease were purched from Sigma Co.,USA) on fraction activity was studies. 1mg BAF was incubated for 24h at 30°C in 0.01 M Tris-HCI buffer, pH 7.0 with chemical agents and enzymes. On 1ml platelet-rich plasma was added 15-20μg BAF (control or treated). Aggregation was initiated by 25μM ADP. The level of platelet aggregation (A, %) was present as mean ± S.D.

RESULTS AND DISCUSSION

Purification steps and fraction's activity

The purification steps from the crude extract to the final farctions are given on Table 1.

For crude extract: IC_{50} was 1.1mg; after Sephadex G-25: IC_{50} was 0.030mg; after Sepharose 4B: IC_{50} was 0.012mg; after DEAE-cellulose: IC_{50} was 0.011mg; after Sephadex G-100: IC_{50} was 0.0093mg.

The activity of the fractions was detrmined by photometric method according Born and Zucker and the protein content was determined method of Loury accordingly to procedures given in 'Aminoacid analyses'.

Table 1. The purification steps from crude extract to fractions

Purification step	Total (mg)	Specific activity (U/mg)*	Total activity (U)	Protein content (%)
Crude extract	1000×103	0.9	900×103	0.17 %
Sephadex G-25	24×103	32	770×103	6.3 %
Sepharose 4B	6×103	83	500×103	15.1 %
DEAE-Cellulose	2.5×103	90	225×103	16.9 %
Sephadex G-100	2.0×103	108	216×103	23 %

*One unit for specific activity for extract or fraction was taken to be IC_{50} - this quantity extract or fraction that inhibits 50% platelet aggregation of 1ml platelet-rich plasma initiated by 25μM ADP.

The homogeneity of the IV BAF was tested by SDS electrophoresis with and without mercaptoethanol. In both cases it was found one band only on, colored as polysaccharide. No loss in the activity of the fraction after storage for several months at 4 ºC in N_3H-H_2O solution at neutral pH, and after freesing of liophilized fraction at -10 ºC.

The results of gel-filtration procedures can be used as a base for semi-industrial production of the BAF by two steps. By filtration through a capillary dialyzer to remove low molecular weight fraction (less than 10-15 kDa) at the first step. By second step realizing ultrafiltration trough separation membranes to remove fractions with molecular weight less than 100 kDa fig.2.

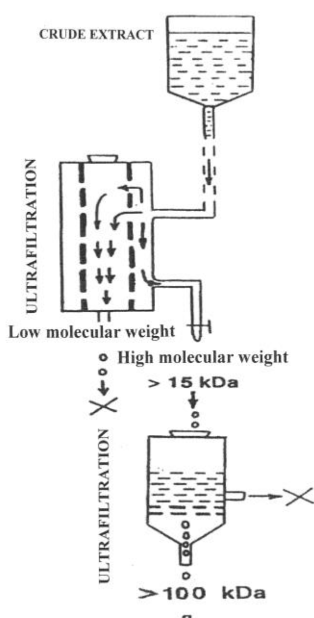

Fig. 1. Scheme of a semi-industrial yeald of IV BAF from *Galega officinalis* L.

Protein content in crude extract and fractions

The protein content of crude extract is about 0.1% [3]. Seven amino-acids (aspartic acid, arginine, alanine, glycine, lysine, serine and valine) consist 46.23% from the total amino-acid content of the final IV fraction [9]. In the final fraction the hydrophilic amino-acids consist 70% while the hydrophobic amino-acids consist 30%. These data are in good agreement with high solubility of the fraction in water solution and low slubility in hydrophobic solvents. The specific activity of the fraction increases with each step of purification (Table 1). This shows a strong relationship between fraction's specific activity and protein content - Fig.2.

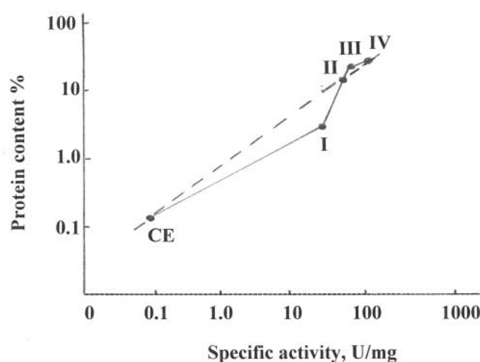

Fig.2. Relationship between protein content and specific fraction activity in log-log plot.

Solubility of the final IV fraction

The solubility of the IV fraction was studied in hydrophilic and hydrophobic solvents-Table 3. The fraction was 100% soluble and active in water solutions. In binary H_2O: EtOH solutions the fraction was well soluble and active up to ratio 4/1 between H_2O and EtOH. For H_2O: EtOH solutions in ratio 3/1 the fraction was maximum 50% active.

For H_2O: EtOH solutons in ratio 1/1 the fraction was about 8% active. In hydrophobic solvents the fraction was not soluble and has no activity.

Table 2. Solubility and residual activity of IV fraction (mean ± SD, n=3)

Solvents	Solubility	Activity of BAF (A%)
H_2O (pH 6-9.5)	soluble	100% active
H_2O : EtOH	90 : 10	96% ± 3.4 %
	80 : 20	84% ± 3.8 %
	70 : 30	52% ± 4.1 %
	60 : 40	24% ± 4.0 %
	50 : 50	8% ± 4.3 %
Chlorophorm, eter, aceton	non-solubility	non-active
Dimethilformamide dimethilsulfoxide	poorsoluble	non-active

Ultraviolet (UV), visble (VIS) and infrared (IR) spectra of BAF

The UV and VIS spectrum of IV BAF in water (pH 7.0) is given on Fig.3. The UV and VIS spectrum of the BAF was similar to heparin spectrum in diapason of 200-420 nm. In the two spectra was observed maximum of absorbance (extinction) at 280 nm. This result could be explained with the fact that BAF and heparin appear high-molecular polysaccharides.

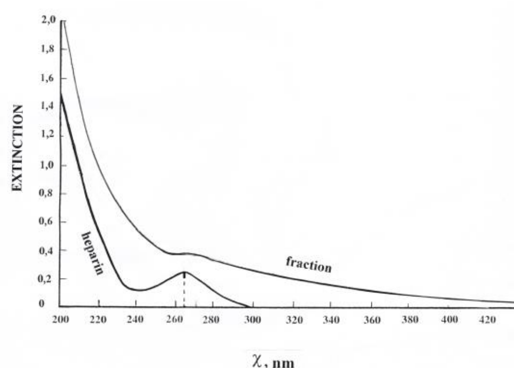

Fig.3. UV and VIS spectrum of IV BAF and heparin (0.1mg BAF and heparin in 5ml dH_2O, pH 7)

The IR spectrum of BAF into tablets of potassium bromid (KBr) gives information for amount of polysaccharides in the fraction-Fig.4. The first part of IR spectrum of BAF from 400 to 2200 cm^1 (signed as 1) was similar to infrared spectrum of poor alginic acid (Fig.5), which is polysaccharide with high molecular weght. Infrared spectrum of IV fraction consist some bands with absorbance at 1400, 1650, 1050, 3400 and 2925 cm^{-1}. The well expressed band with maximum at 3400 cm^{-1} corresponds to hydrohillic O - H groups, while the weekly expressed band at 2925 cm^{-1} corresponds to aromatic hydrohillic O - H groups. A well expressed band at 1650 cm^{-1} is spcific for C = O group, and at 1050 cm^{-1} is specific for C - O group. At 1400 cm^{-1} is observed a week band specific for C - H group [10]. Alginic acid contained same bands of absorbance as IV BAF : at 3400 cm^{-1} , 2925 cm^{-1} , 1050 cm^{-1} and 1400 cm^{-1}. The only exception is the absorbance at 1610 cm^{-1} in alginic acid, unlike absorption at 1650 cm^{-1} of BAF. Accordingly to previous NIR spectrometry analyses of BAF about 74% of total fraction content was polysaccharides [9]. Giving in the mind 23% protein in a fraction, about 97% of the fraction's content was determined.

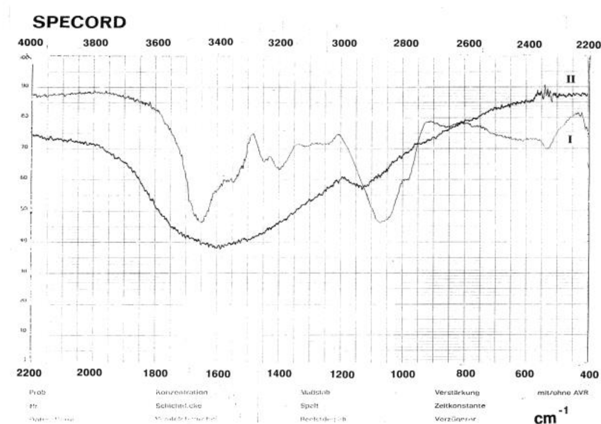

Fig.4. Infrared spectrum of IV BAF into tablets of KBr

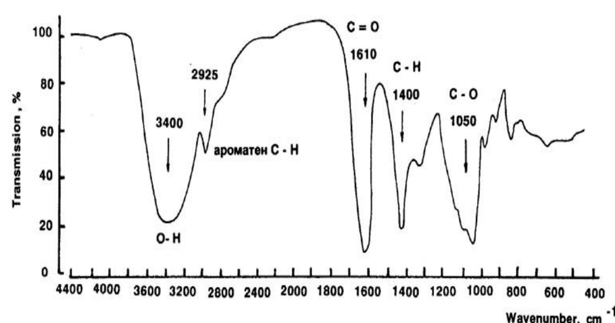

Fig.5. Infrared spectrum of alginic acid into tablets of KBr

pH dependence of the fraction activity

The pH dependence of a fraction activity was studied in pH range from 3.0 to 11.5 - Fig.6. The optimum of the fraction's activity was between pH 5.5 and 9.8.The fraction show losses activity below pH 3.5 and over 11.0. In the range of pH 4.0 – 5.5

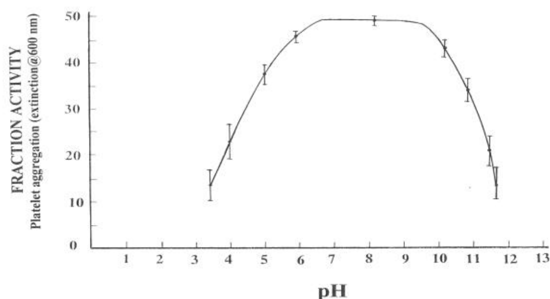

Fig. 6. Effect of pH on fraction's activity

and pH 10.0 -10.5 the fraction inhibits platelet aggregation between 10% and 60%. We did not observed decrease in inhibitory activity of the fraction stored for three monts at pH 7.3 and temperature 4°C-18°C.

Temperature dependence of the fraction activity

The temperature dependence of a fraction activity was study in temperature interval 10-100°C, cf. Fig.7.

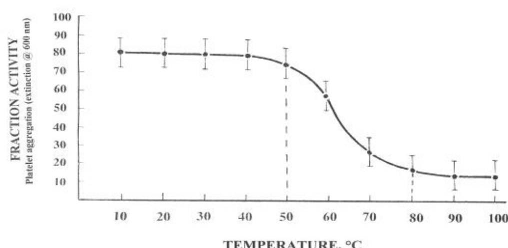

Fig.7. Effect of temperature on fraction's activity

The fraction keeps high inhibitory activity in range of 10- 42°C. In temperature rangre of 42 - 80°C the activity slow decreases and over 80°C the activity losses. The activation energy of the fraction inhibitory deactivation was evaluated to be 70 ± 4.5 kJ/mol. This activation energy is typical for polysaccharides. This result shows that it is possible the polysaccharides participate in inhibiting mechanism of the fraction.

Microcalorimetric study of the BAF

Upon uniformly heating of BAF in differential scanning microcalorimeter it was registered endothermic process within range of 65°-92°C with two maximums-Fig.7: Ist - corresponding to denaturation process at 73° C and IInd – corresponding to denaturation process at about 85ºC. This is indication that the fraction contains two proteins and/or protein subunits. The beginning of denaturation at 65°C coincides with the most significant loss of fraction's activity. This fact confirms the participation of fraction's protein in inhibitory mechanisms of the fraction.

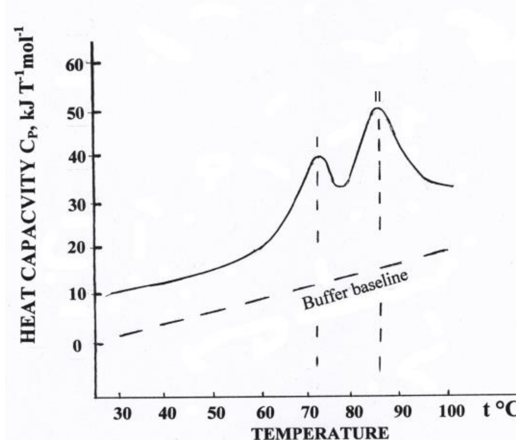

Fig. 8. Microcalorimetric thermogramm of BAF.Concentration of sample and a heating rate are 2, 4%, and 1.5°C/min, respectivelly

Fraction actvity after treatment with chemical substances and enzymes

The residual fraction activity after treatment with chemical agents and enzymes presents interest before *in vivo* study on laboratory animals. The influence of chemical substances and enzymes on the fraction's activity is given on Table 4 and Table 5.

Table 3. Inhibition effect of BAF after treatment with chemical agents (mean± SD, n=5)

Chemical agent	mg agent/mg BAF	Activity of BAF (A, %)
Control		95.0 ± 4.9 %
Control + BAF		10.0± 3.2 %
ЕДТА, Urea	1/4	9.5 ± 2.3 %
Guanidin hydrochloride	3/4	12.3 ± 2.7 %
Bacitracin	1U/4mg	11.9 ± 1.8 %
$CuSO_4.5H_2O$, $MgCl_2.6H_2O$	0.5/4	10.2 ±1.5 %
$Zn(CH_3COO)_2Pb$, $(CH_3COO)_2Pb$	0.5/4	6.6 ±0.94 %

The ion salts (Cu^+, Mg^+ , Zn^+ and Pb^+) had no influence on fraction activity. Exceptions are the Fe^+ ions, which inactivate BAF. EDTA as agen forming chelate complexes with metal ions does not alter the inhibitory effect of BAF. The agents that denatured protein structures (urea, guanidine hydrochloride and bacitracin) do not affect the inhibitory effect of BAF on platelet aggregation.

The treatment of the fraction with α-amilase, β-amylase, papain, tripsin and hymotripsin did not influence the activity of the fraction-Fig.5. The treatment with alkaline protease only leads to decreases of the fraction activity. The results show the protein part of the fraction to play major role in platelet-inhibiting effect

Table 4. Effects of enzymes on fraction activity (mean ± SD, n = 5)

Enzyme	Enzyme/BAF ratio	Activity of BAF (A %)
Control extract		100 %
Control + BAF		50 ± 4.0 %
Chymotrypsin	1/10	46± 5.6 %
Chymopapain	1/10	10± 1.8 %
Trypsin	1/10	45± 3.0 %
α-amylase	1/5	38± 3.3 %
β-amylase	1/10	51± 3.6 %
Albumin	1/10	49± 4.0 %
Protease (alkaline)	1/10	70± 6.0 %
Blood plasma	0.4 mg BAF/0.1ml plasma	50 ± 3.3 %

The incubation of BAF in albumin and blood plasma did not change their activity. An additional, we found that the fraction do not lysate and do not agglutinate erythrocytes or platelets and do not precipitate the serum and plasma proteins.

CONCLUSSION

Sustainability of BAF to action of denaturing chemical agents and proteases (α-amylase, β-amylase, tripsin, hymotripsin and hymopapain) and lack of destructive influence of fraction on blood cells gives us reason to think that BAF can find practical app like dextran, as a component of blood plasma.

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НЯКОИ ФИЗИКОХИМИЧНИ ХАРАКТЕРИСТИКИ НА АНТИТРОМБОЦИТНА ФРАКЦИЯ ИЗОЛИРАНА ОТ *Galega officinalis* L.

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(Резюме)

Проучени са някои физикохимични характеристики на 100-140 kDa фракция, изолирана от лечебното растение *Galega officinalis* L. Получената фракция е водноразтворима и потиска тромбоцитната агрегация инициирана с аденозиндифосфат, тромбин и колаген. Третирането на фракцията с протеолитични ензими не влияе върху способността и да инхибира тромбоцитната агрегация. Изолираната фракция представлява високомолекулен полизахарид, съдържащ 23% протеин. Активността на фракцията се запазва след съхраняване и за няколко месеца при -4ºC в N_3H-H_2O разтвори при неутрално pH, както и след лиофилизация. Фракцията проявява максимална активност в температурния диапазон 10°C-42°C и в разтвори с pH 5.5- 9.8. Микрокалориметричният анализ на фракцията показа, че тя съдържа две белтъчни субединици. Получената антиромбоцитната фракция може да намери приложение подобно на високомолекулните декстрани.