

Fractionation of Biologically Active Poly[3-(3,4-Dihydroxyphenyl)Glyceric Acid] Preparation from *Symphytum asperum* Using HPSEC-MALLS-RID and Membrane Ultrafiltration Methods

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Fractionation of biologically active poly[3-(3,4-dihydroxyphenyl)glyceric acid] (PDPGA)-preparation from *Symphytum asperum*, distribution of PDPGA and polysaccharides impurity using high performance size exclusion chromatography (HPSEC) coupled with multi angle laser light scattering (MALLS), refractive index detector (RID) and membrane ultrafiltration methods were carried out. Three absorption maxima $\lambda_{\max}$ at 212, 236, 286 nm in the UV spectra of PDPGA-preparation in H₂O, 10 mM NaOH and 100 mM NaOH were observed. Supernatants after centrifugation as well as precipitates dissolved in 100 mM NaOH were tested on the presence of carbohydrates admixture by phenol-sulfuric acid method. Polysaccharides fraction was water soluble. The solubility of PDPGA-preparation increased with increasing alkaline concentration. Two fractions of the PDPGA-preparation obtained by HPSEC analysis were fractionated by using membrane ultrafiltration method with the cut off value of 10 kDa and got the permeate and retentive fractions. Retentive fraction has high UV absorbance and contained mainly PDPGA. Sugars mainly are in permeate fraction. © 2021 Bull. Georg. Natl. Acad. Sci.

Poly[oxy-1-carboxy-2-(3,4-dihydroxyphenyl)ethylene], poly[3-(3,4-dihydroxyphenyl)glyceric acid], *Symphytum asperum*, high performance size exclusion chromatography, multi angle laser light scattering, refractive index detector

In our previous study the crude polysaccharides preparation from medicinal plant *Symphytum asperum* (Boraginaceae) was ultrafiltered on membrane filter and additionally fractionated by gel chromatography on a Sepharose 2B column. These fractionation procedures allowed us to remove most

biologically inactive ballast polysaccharides and to obtain water-soluble high-molecular (>1000 kDa) biologically active preparation [1]. Its main chemical constituent according to data of different techniques of NMR spectroscopy was found to be caffeic acid-derived biopolymer, namely poly[oxy-

1-carboxy-2-(3,4-dihydroxyphenyl)ethylene] or poly[3-(3,4-dihydroxyphenyl)glyceric acid] (PDPGA) (Fig. 1) [2].

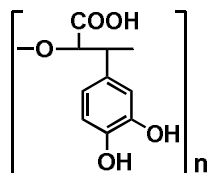


Fig. 1. Poly[3-(3,4-dihydroxyphenyl)glyceric acid] (PDPGA).

However, this PDPGA-preparation contained some amounts of polysaccharides admixture. Later we attempted to fractionate PDPGA-preparation by HPLC on two types of gel-filtration chromatography columns – silica-based Biosep 4000 and polymer-based Polysep 6000 with the use of both UV and RI detectors. However, with these methods the polysaccharides admixture could not be completely separated from PDPGA [3]. Recently molecular weights and contents of different fractions for PDPGA-preparation were simultaneously determined using high performance size exclusion chromatography (HPSEC) on column of TSK-Gel G6000PWXL coupled with multi angle laser light scattering (MALLS) and refractive index detector (RID) [HPSEC-MALLS-RID] with the refractive index increment (dn/dc) [4].

We continue our attempt to fractionate PDPGA-preparation by modern methods and to get as much information as possible. Consequently, subject matter of present work was fractionation of biologically active PDPGA-preparation from *S. asperum*, distribution of PDPGA and polysaccharides admixture using HPSEC-MALLS-RID and membrane ultrafiltration methods.

Materials and Methods

PDPGA-preparation of *S. asperum* was obtained as described in earlier work [1]. Fractionation of PDPGA-preparation was performed using

HPSEC-MALLS-RID method according to previous reports [4,5].

Results and Discussion

The PDPGA-preparation could be partially dissolved in water and almost completely dissolved in 0.1 M NaOH solution. It could not be dissolved in organic solvents. Then we tried to study how the solubility of PDPGA-preparation increased. A: 3.0 mg/mL PDPGA-preparation in H₂O; 50°C, 4 h; B: 3.0 mg/mL PDPGA-preparation in 10 mM NaOH; 50°C, 4 h; C: 3.0 mg/mL PDPGA-preparation in 100 mM NaOH; 50°C, 4 h. Solutions A, B and C were centrifuged at 6000 g for 10 min. Soluble mass of the PDPGA-preparation increased with increasing alkaline concentration.

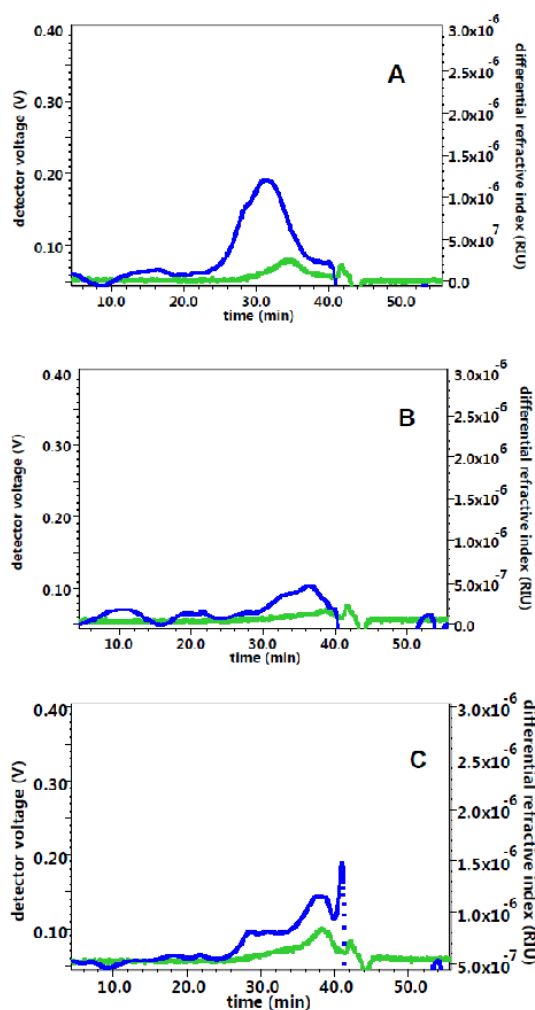


Fig. 2. HPSEC-MALLS-RID analysis of A, B and C. Green peak indicates detection of compounds by UV

detector; blue peak indicates detection of compounds by RI detector.

3.0 mg/ml **PDPGA**-preparation in H₂O; 50°C, 4 h; centrifuged at 6000 g for 10 min. A:Supernatant; B:Precipitate dissolved in 100 mM NaOH; C:High concentration of B. Then A, B and C were analyzed using HPSEC-MALLS-RID method (Fig. 2).

UV detector indicates the presence of only **PDPGA** as compound of phenolic nature, whereas RI detector indicates the presence of both **PDPGA** and polysaccharides impurity.

A: 3.0 mg/mL **PDPGA**-preparation, in H₂O; B: 3.0 mg/mL **PDPGA**-preparation, in 10 mM NaOH; C: 3.0 mg/mL **PDPGA**-preparation, in 100 mM NaOH. Each supernatant after centrifugation at 6000 g for 10 min diluted 128 times with H₂O and scanned at 200-350 nm. Three absorption maxima λ_{max} at 212, 236, 286 nm in the UV spectra were observed.

Test of the Carbohydrates Fraction Using Phenol-Sulfuric Acid Method [6]

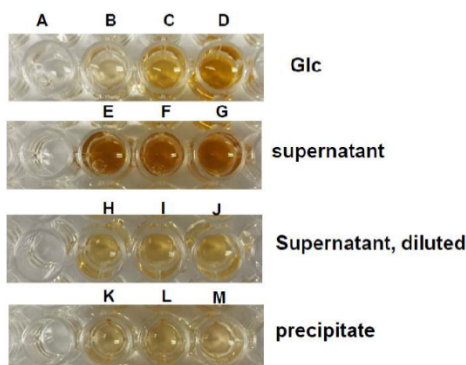


Fig. 3. Presence of polysaccharides admixture of **PDPGA**-preparation in H₂O, 10 mM NaOH, 100 mM NaOH solutions using phenol-sulfuric acid reaction.

Table. Optical density values of phenol-sulfuric acid reaction with polysaccharides impurity

A	B	C	D	E	F	G
0	0.344	1.484	1.796	1.723	1.792	1.800
	H	I	J	K	L	M
	0.958	1.011	1.068	0.503	0.460	0.421

A: H₂O as blank control (Fig. 3, A; Table, A); B, C and D: 0.01, 0.1 and 0.4 mg/mL glucose (Glc) (Fig. 3, B, C, D; Table, B, C, D); E, F and G: Supernatants of **PDPGA**-preparation in H₂O, 10 mM NaOH and 100 mM NaOH, respectively, after centrifugation at 6000 g for 10 min (Fig. 3, E, F, G; Table, E, F, G); H, I and J: Four times dilution of E, F and G, respectively, with H₂O (Fig. 3, H, I, J; Table, H, I, J); K, L and M: Precipitate of **PDPGA**-preparation in H₂O, 10 mM NaOH and 100 mM NaOH, respectively, after centrifugation at 6000 g for 10 min (Fig. 3, K, L, M; Table, K, L, M). Polysaccharides fraction was water soluble (Fig. 3, E, H; Table, E, H).

Primary Fraction of the **PDPGA**-Preparation

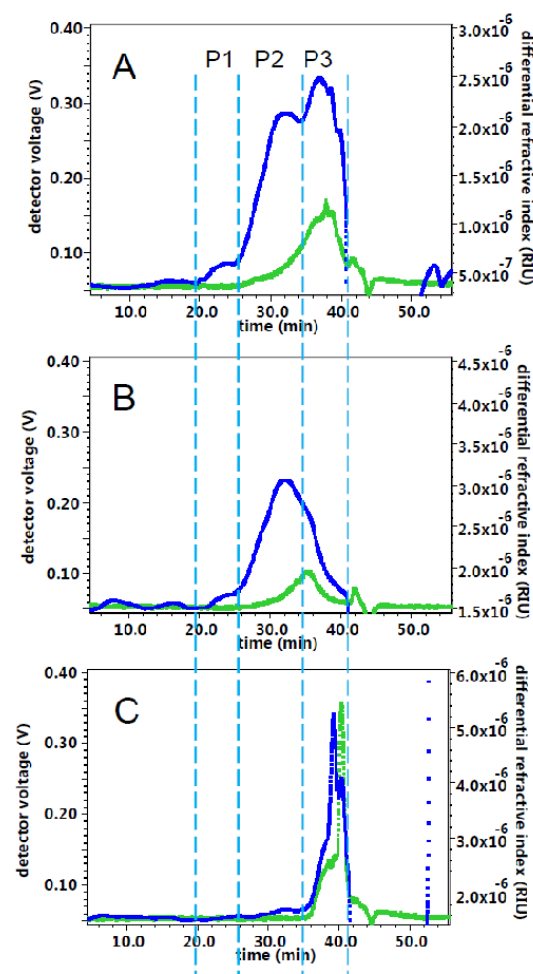


Fig. 4. HPSEC-MALLS-RID analysis of A, B and C. Green peak indicates detection of compounds by UV detector; blue peak indicates detection of compounds by RI detector.

UV detector indicates the presence of only **PDPGA** as compound of phenolic nature, whereas RI detector indicates the presence of both **PDPGA** and polysaccharides admixture.

A: Original **PDPGA**-preparation solution; B, C: Two fractions from A; Sample A (1 mL) was neutralized by HCl; Peaks 2 and 3 (Fig. 4) were fractionated by using membrane ultrafiltration method with cut off value of 10 kDa (Millipore), 15 mL. The permeate (B) and retentive (C) fractions were adjusted to 1 mL.

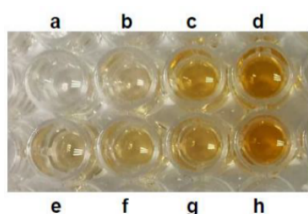


Fig. 5. Presence of polysaccharides admixture in four times diluted fractions C (e), B (f) and fractions C (g), B (h) without dilution using phenol-sulfuric acid reaction.

a: H₂O; b: 0.01mg/mL Glc; c: 0.1mg/mL Glc; d: 0.4 mg/mL Glc (Fig. 5, a, b, c, d); Four times diluted fractions C (e) and B (f) (Fig. 5, e, f). Fractions C (g) and B (h) without dilution (Fig. 5, g, h). Fraction C

has high UV absorbance due to the presence of mainly **PDPGA** (Fig. 4, C; Fig. 5, e, g); Sugars are mainly in fraction B (Fig. 4, B; Fig. 5, f, h).

Conclusion

Thus, biologically active **PDPGA**-preparation from *S. asperum* partially dissolved in water and completely dissolved in 0.1 M NaOH solution. The solubility of **PDPGA**-preparation increased with increasing alkaline concentration. Polysaccharides admixture of **PDPGA**-preparation was water soluble. The fractionation of **PDPGA**-preparation using HPSEC-MALLS-RID and additional fractionation by membrane ultrafiltration method resulted in obtaining of permeate and retentive fractions. Retentive fraction has high UV absorbance. Sugars do not have chromophores and consequently do not absorb light in the UV range. It is therefore highly likely that the UV absorption could arise due to presence of compound of phenolic nature, namely caffeic acid-derived biopolymer **PDPGA**. Sugars are mainly in permeate fraction. Thus, aforementioned fractionation procedures of **PDPGA**-preparation allowed us to determine predominant distribution of **PDPGA** and polysaccharides impurity in different fractions.

ფარმაკოქიმია

Symphytum asperum-ის ბიოლოგიურად აქტიური პოლი[3-(3,4-დიჰიდროქსიფენილ)გლიცერინის მჟავას] პრეპარატის ფრაქციონირება HPSEC-MALLS-RID-ის და მემბრანული ულტრაფილტრაციის მეთოდების გამოყენებით

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**ჩინური მედიცინის ხარისხის კვლევის სახელმწიფო საკვანძო ლაბორატორია, ჩინეთის მედიცინის მეცნიერებათა ინსტიტუტი, მაკაუს უნივერსიტეტი, მაკაო, ჩინეთი

§აკადემიის წევრი, ივანე ჯავახიშვილის სახ. თბილისის სახელმწიფო უნივერსიტეტი, ფიზიკური და ანალიზური ქიმიის ინსტიტუტი, ზუსტი და საბუნებისმეტყველო მეცნიერებების სკოლა

Symphytum asperum-ის ბიოლოგიურად აქტიური პოლი[3-(3,4-დიჰიდროქსი-ფენილ)გლიცერინის მჟავას] (პდფგმ)-პრეპარატის ფრაქციონირება და ნარჩენი პოლისაქარიდების ფრაქციების განაწილება და განსაზღვრა განხორციელდა მაღალეფექტური ზომა ექსკლუზიური ქრომატოგრაფიის (მეზექ) გამოყენებით, რომელიც შეწყვილებული იყო მრავალკუთხოვანი ლაზერული სინათლის გაფანტვის (მკლსგ) და რეფრაქციის ინდექსის დეტექტორთან (რიდ). პდფგმ-პრეპარატის ხსნარები წყალში, 10 მილიმოლ NaOH-სა და 100 მილიმოლ NaOH-ში ამჟღავნებდა შთანთქმის მაქსიმუმებს ულტრაიისფერ სპექტრში λ_{max} 212, 236, 286 ნმ-ზე. აღნიშნული ხსნარების ცენტრიფუგირების შედეგად მიღებული სუპერნატანტები, ისევე როგორც ნალექები გახსნილი 100 მილიმოლ NaOH-ში შემოწმებულ იქნა ნარჩენი ნახშირწყლების არსებობაზე ფენოლ-გოგირდმჟავასთან რეაქციის მეთოდით. პოლისაქარიდების ფრაქცია უპირატესად წყალში ხსნადია. განხორციელდა პდფგმ-პრეპარატის მეზექ-ის ანალიზის შედეგად მიღებული ორი ფრაქციის ფრაქციონირება მემბრანული ულტრაფილტრაციის მეთოდით მემბრანის ფორების ზომით 10 kDa და მიღებულ იქნა მემბრანის ფორებში გამავალი და შეკავებული ფრაქციები. აღნიშნული მემბრანით შეკავებულ ფრაქციას გააჩნია ინტენსიური შთანთქმა ულტრაიისფერ სპექტრში. შაქრები ძირითადად არის მემბრანაში გამავალ ფრაქციაში.

REFERENCES

1. Barbakadze V., Kemertelidze E., Usov A.I., Kroes B.H., Quarles van Ufford H.C., van den Worm E., Beukelman C.J., van den Berg A.J.J., Labadie R.P. (1999) Evaluation of immunomodulatory activity of some plant polysaccharides. *Proc. Georgian Acad. Sci., Biol. Ser.*, 25, 4-6: 207-216. https://www.researchgate.net/publication/285495080_Evaluation_of_immunomodulatory_activity_of_some_plant_polysaccharides
2. Barbakadze V., Kemertelidze E., Targamadze I., Mulkijanyan K., Shashkov A.S., Usov A. I. (2005) Poly[3-(3,4-dihydroxyphenyl)glyceric acid], a new biologically active polymer from *Symphytum asperum* Lepech. and *S. caucasicum* Bieb. (Boraginaceae). *Molecules*, **10**, 9: 1135-1144. <https://doi.org/10.3390/10091135>
3. Barbakadze V., Gogilashvili L., Amiranashvili L., Merlani M., Mulkijanyan K., Wang Y., Hoang J., Rustamov I. (2013) HPLC analysis of poly[3-(3,4-dihydroxyphenyl)glyceric acid] preparations from *Symphytum asperum* and *Anchusa italica* (Boraginaceae) using different gel-filtration columns. *Bull. Georg. Natl. Acad. Sci.*, **7**, 1: 83-88. <http://science.org.ge/bnas/vol-7-1.html>
4. Barbakadze V., Gogilashvili L., Amiranashvili L., Merlani M., Li Sh-P., Chankvetadze B. (2021) Fractionation of biologically active poly[3-(3,4-dihydroxyphenyl)glyceric acid] preparation from *Symphytum asperum*, simultaneous determination of molecular weights and contents of its fractions using HPSEC-MALLS-RID. *Bull. Georg. Natl. Acad. Sci.*, **15**, 1: 115-121. <http://science.org.ge/bnas/vol-15-1.html>
5. Wu D.T., Lam S.C., Cheong K.L., Wei F., Lin P.C., Long Z.R., Lv X.J., Zhao J., Ma S.C., Li S.P. (2016) Simultaneous determination of molecular weights and contents of water-soluble polysaccharides and their fractions from *Lycium barbarum* collected in China. *J. Pharm. Biomed. Anal.*, **129**: 210-218. <http://dx.doi.org/10.1016/j.jpba.2016.07.005>
6. Dubois M., Gilles K.A., Hamilton J.K., Rebers P.A., Smith F. (1956) Colorimetric method for determination of sugars and related substances. *Anal. Chem.*, **28**, 3: 350-356. <https://doi.org/10.1021/ac60111a017>

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