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MIDDLE SIZED WATER SOLUBLE DEXTRIN AND ITS APPLICATION FOR FRACTIONATION OF CELL POPULATIONS

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Water soluble fraction of dextrin was isolated from the commercial specimen of corn dextrin using fractional precipitation with ethanol at 33–74 % of alcohol concentration. The obtained product (mol. mass 4–21 kDa with median point at 10 kDa) can be considered as dextrin with middle sized molecules. It develops specific red-brownish color with iodine which is expressed as an extended peak of light absorption with maximum at 395 nm. It is soluble in water, DMSO and pyridine. 40 % solution (w/v) in water or saline shows 1,15 ± 0,01 g/cm^3 density. Obtained dextrin was used for preparation of density gradient media to be used for fractionation of cell populations. Optimized procedure for separation of total white blood cells from the peripheral blood and of their mononuclear (MN) and polymorphonuclear (PMN) subpopulations is described. Separation of MN and PMN was achieved by centrifugation in a discontinuous 1,083 g/cm^3 density gradient which was prepared by mixing stock dextrin solution with blood plasma/serum. Thus the proposed water soluble middle sized dextrin can serve as a versatile natural compound for preparing density gradient media for cell separation in addition to commonly used media produced with the aid of xenobiotics (sodium diatrizoate or percoll).

Keywords: water soluble dextrin, density gradient, cell separation

Centrifugation in density gradient media defined as isopicnic sedimentation is commonly used for fractionation of heterogeneous cell populations. Several substances are used for preparation of media with higher density and physiological osmolarity, Hypaque–Ficoll solution is the most popular. It was introduced by Boyum A. for separation of human mononuclear (MN) and polymorphonuclear (PMN) white blood cells (WBC) [1,2]. Nowadays numerous modifications and improvements of that method were published [3–7]. Nevertheless in all versions sodium diatrizoate (Hypaque) is used for preparing dense solutions. The main disadvantage of diatrizoate is that it is light sensitive xenobiotic substance which may be decomposed during inappropriate storage to toxic products exerting harmful effect upon WBC.

Percoll (Pharmacia, Sweden) is another frequently used material which is a solution of colloidal silica coated with polyvinylpyrrolidone [8, 9]. It is also a xenobiotic, yet non-toxic, more expensive then Hypaque, and less accessible. For obtaining of density

gradients, especially continuous linear gradients, are used concentrated (30 %) solutions of bovine and human serum albumins, however, they are viscous enough thus complicating cell sedimentation [9]. Ready for use concentrated albumin solutions are proposed by «Sigma», USA, yet they are more expensive then two previously mentioned media.

We have addressed to oligosaccharides with medium sized molecules as possible substances for obtaining solutions with high density. These compounds are hydrophilic, possess almost no electric charge and reactive chemical groups, like proteins, and can produce concentrated solutions of low viscosity which do not retard cell sedimentation. The aim of this study was to obtain water soluble dextrin from commercial partially soluble dextrin specimen and to investigate a possibility of its application for cell separation by density gradient sedimentation.

Materials and methods.

Commercial specimen of corn dextrin (Sphera Sim, Lviv, Ukraine) was used in this study for isolation of water soluble dextrin fraction.

Isolation of water soluble dextrin. 50 g of dextrin powder was suspended in 250 ml of water and 125 ml of ethanol was added under the constant stirring. Mixture was leaved at room temperature for 24 hours and thereafter centrifuged at 3000 g during 20 minutes. Supernatant was saved and sediment was placed on paper filter for several days in order to obtain an additional portion of clear filtrate. Dextrin was precipitated from the clear solution with two volumes of ethanol and the turbid mixture was leaved for 24 hours in refrigerator. The sediment settled on the bottom of the flask and forms a yellow honey-like mass. Clear supernatant was carefully eliminated and sediment was treated with 2-3 volumes of acetone. By mincing with a glass rod this mass turns to a solid powder which is easily collected by centrifugation, washed with small volume of acetone and dried. Yield 23–25 g. Low molecular weight constituents were eliminated by dialysis. 20 g of powder were dissolved in 100 ml of water and dialyzed in visking tubes against 1 L of tap water during 6–7 hours at 4 °C. After dialysis solution (160 ml) was treated with 80 ml ethanol and leaved for 48 hours in refrigerator. Small precipitate was eliminated by centrifugation 15 min at 3000 g, clear supernatant was treated with two volumes of ethanol and leaved for 24–48 h in refrigerator. After formation of yellow viscous mass of sediment on the bottom of the flask a supernatant was carefully eliminated, the sediment was treated with several volumes of acetone, washed with acetone and dried. Yield 11–12 g.

Molecular mass of the obtained dextrin was determined by column gel filtration on Sephadex G-50 [10]. Bovine serum albumin (67 kDa), soybean trypsin inhibitor (20 kDa), cytochrome c (12,3 kDa) and raffinose (504 Da) were used as reference substances. Detection of sugar in eluate was performed by anthrone method [11] and of protein – by Lowry method [12].

Samples of human blood were obtained from one of authors (M. L.) and from donors of blood after their consent at Lviv Regional Station of Blood Transfusion (Ukraine). Blood samples of mouse, rat and rabbit were obtained at vivarium of Lviv National Medical University (Ukraine) with preservation of all bioethical rules. Blood samples (5 ml) were taken by venipuncture (human) or cardiac puncture (rats under the ether narcosis) and stabilized with 1/20 volume of 1.3 % EDTA in the syringe or by defibrination. In the last case, blood was transferred immediately from a syringe into the plastic tube and defibrinated by gentle swirling with a glass hook during 15–20 min and fibrin clot was discarded. Mouse blood was taken after animal decapitation and collected

drop wise in equal volume of solution A (10 mM tris, 0.13 M NaCl, 5 mM KCl, 2 mM $MgCl_2$, 0.6 mg/ml EDTA, 0.1 mg/ml heparin, pH 7,4) by mixing on a magnetic stirrer. Rabbit blood was taken similarly by the puncture of ear marginal vein.

For isolation of WBC from the peripheral blood, the following solutions were used:

10 mM tris, 0.13 M NaCl, 5 mM KCl, 2 mM $MgCl_2$, 0.6 mg/ml EDTA, 0.1 mg/ml heparin, pH 7,4;

5 % solution of Dextrane T-500 (Fluka) in PBS. Solution we sterilized by filtration and it can be stored for several years in sealed ampoules at 4 °C.

Hemolytic buffer: 0.83 % solution of NH_4Cl containing 0.5 mg/ml EDTA, adjusted to pH 7.4 with 10 % ammonia;

Preparation of total pool of WBC. Stabilized with EDTA or defibrinated blood was diluted two fold by addition of an equal volume of solution A (blood of mouse and rabbit was not diluted as it was already two fold diluted during its withdrawal). Blood was mixed with 1/8–1/10 volume of 5 % dextran (solution B), transferred to appropriate syringe and installed in the inclined position at 45° (syringe tip upward) for 15 min. Thereafter syringe was placed strictly vertical for 30-35 min. In the presence of dextran sedimentation of red blood cells is greatly accelerated and blood in syringe is separated into upper layer of blood plasma with leucocytes, platelets, residual red blood cells, and lower layer of packed red blood cells and the rest of leucocytes. The needle was replaced with a thin polyethylene canula and upper layer was carefully transferred to plastic 2 ml Eppendorf tubes and cells were sedimented by centrifugation at 1,000 rpm during 5 min. Supernatant material of blood plasma/serum was maximally removed, the cell sediment was treated with hemolytic buffer for elimination of red blood cells. For this purpose, cells were suspended in a residual plasma by shaking and 0.4 ml of the hemolytic buffer was added. The tube was incubated in water bath at 30 °C during 3–4 min, then 0.5 ml of the autologous plasma/serum was added, and suspension was centrifuged for 2 min at 1,000 rpm. Supernatant was carefully removed, white sediment of cells was resuspended in 0,4 ml of blood plasma and cells were collected by centrifugation for 2 min at 1,000 rpm. Cells were suspended in appropriate volume of the autologous plasma or serum, or processed further according to a subsequent protocol of investigation.

Separation of MN and PMN populations of WBC was performed by centrifugation on a density gradient medium with a specific gravity 1.083 g/ml, which was prepared as follows. Stock 40 % (weight/volume) solution of dextrin was prepared by dissolving substance in Tris-buffered saline (TBS) in a proportion of 400 mg of dextrin, 0,55 ml TBS and 0,15 ml of water. For preparing of density gradient medium the following data were taken into account: 40 % (w/v) solution of dextrin in saline shows a density of 1.15 ± 0.01 g/ml. Mixture of equal volumes of this solution and blood plasma/serum has density of 1.086 g/ml, and in proportions of 0.45 dextrin solution – 0.55 plasma/serum density is 1.079 g/ml.

The medium with 1.083 g/ml density was prepared *ex tempore* by mixing stock solution of dextrin with autologous blood plasma/ serum in 1:1 (sometimes 1:1.1) proportion. Thereafter, 1 % solution of heparin in saline was added to a final concentration 0.1 mg/ml. After 20–30 min a turbidity appeared due to aggregation of lipoproteins, and solution was clarified by centrifugation 15 min at 5,000 rpm. Clear liquid was carefully collected not disturbing the floated layer of the lipoproteins on the surface of liquid. Density of the obtained solution was controlled by weighting in a calibrated pipette (picnometry). The solution could be stored for no more than 3 days at 4 °C.

The initial steps of blood processing were the same as at obtaining total pool of WBC, up to the point after the first centrifugation of cells. After centrifugation, upper layer of blood plasma was eliminated leaving about 0,5 ml of residual volume in which the sedimented cells were resuspended. Cell suspension (0.5–0.7 ml) in 2 ml Eppendorf tube was carefully underlayered with 0,5 ml of medium with an appropriate density, i.e. 1.083 g/ml. Tube was centrifuged for 20 min at 2,000 rpm (580 g) without brake («Janetzky» centrifuge, bucket rotor, r = 13 cm). Mononuclear cells were retained at the border of sample/medium interface and aspirated by pipette with fine bent tip. The obtained suspension was diluted with an equal volume of solution A, cells were collected by centrifugation for 3 min at 1,000 rpm, and suspended in a small volume of the autologous plasma.

PMN and red blood cells passed the medium and were collected at the bottom of tube. Cells of the sediment were suspended in 0.4–0.5 ml of hemolysing buffer and incubated in water bath for 3–4 min at 30 °C. Hemolysis was stopped by addition of an equal volume of plasma, and centrifugation 2 min at 1,000 rpm. Cells were washed with blood plasma/serum and suspended in 0.3–0.4 ml of blood plasma/serum.

WBC count was performed according to standard procedure using hemocytometric chamber. Cell suspension was diluted 11-fold with Turck solution (0,1 mg/ml gentian violet in 3 % acetic acid) [13]. Proportion of PMN and MN cells was estimated simultaneously in hemocytometric chamber according to the shape of cell nuclei stained with gentian violet, which could be distinctly differentiated at x 600 magnification. Cell morphology and their integrity were investigated on cytological smears after staining by Romanovsky-Giemsa method [14].

All quantitative data were statistically evaluated by M±m parameters using Microsoft Excel 2003 program.

Results and discussion.

Properties of water soluble dextrin specimen. The obtained dextrin specimen is a powder of light beige color, readily soluble in water. It is also soluble in DMSO and pyridine. Molecular mass was determined by gel filtration on Sephadex G-50 column (Fig. 1). Substance was eluted as a broad peak corresponding to molecular mass of 2–24 kDa. 80 % of material was eluted between 4–21 kDa, the median point of the molecular mass is 10 kDa, which corresponds to approximately 62 glucose residues. This permits considering the obtained dextrin as a substance with middle sized molecular mass. It develops specific red-brownish color with iodine expressed as an extended peak of light absorption with maximum at 395 nm. The product demonstrates negative Trommer reaction which suggests that it does not contain reducing sugars, like glucose or its di- and trisaccharides. A commercial corn dextrin is available in great quantities and water soluble fraction can be prepared in a sufficient amount for reserve. Dry product is stable and can be stored in closed vessel at room temperature for more then a year without changing its properties.

An employment of density gradient centrifugation for fractionation of cell populations is mostly used for separation of the peripheral blood leukocytes. Since introduction of A.Boyum's method in 1968 [2], methods of purification and isolation of WBC and their subpopulations are constantly modified and improved as it can be seen from publications [3–7]. Taking this into account it was reasonable to prove obtained soluble dextrin in separation of blood leukocytes.

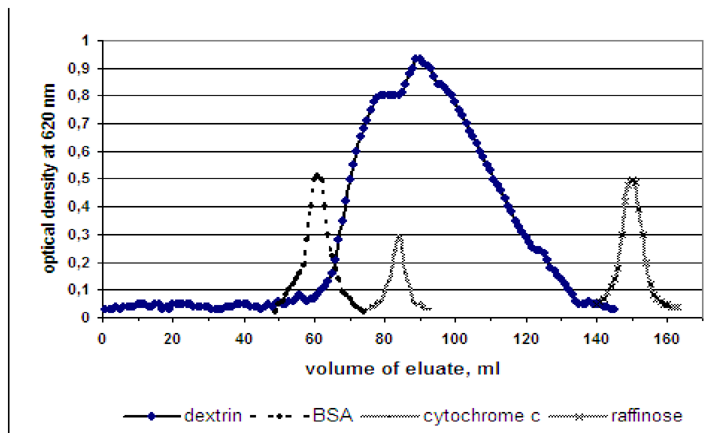

Fig. 1. Gel filtration profile of water soluble dextrin and reference protein mol. mass markers (bovine serum albumin – 67 kDa, cytochrome c – 12,4 kDa, raffinose – 504 kDa) on Sepadex G-50 column. Column 98×1.8 cm, eluent – tris-buffered saline, pH 7.4. Sugars were determined by the anthrone reagent, and proteins were measured by Lowry method. In both cases optical density was measured at 620 nm.

The isolation process consists of three steps: 1) separation of total pool of WBC using the method of accelerated sedimentation of red blood cells (RBC) in presence of dextrane T500; 2) separation of the thepopulations of MN and PMN cells by centrifugation in density gradient medium; 3) purification the PMN cells by lysis of the RBC with ammonium chloride buffer.

During our preliminary experiments it was noticed that separation of WBC, and especially MN cells, proceeds more efficiently with the defibrinated blood due to elimination of blood platelets which induce aggregation and damage of the PMN cells, as well as occasional formation of fibrin clots, thus complicating separation of cells. We compared the effect of blood stabilization with EDTA used as anticoagulant recommended in protocols of Lymphoprep procedure [15] and defibrination of blood on characteristics of obtained total population of WBC (Table 1). It was found that defibrination significantly reduced WBC count up to 65 % of that in EDTA treated blood. The proportion of PMN to MN did not change significantly. The overall yield and the rate PMN/MN in final WBC pool did not differ significantly in both types of blood stabilization. It was drawn a conclusion that anticogulants and defibrination can be used equally well for purification of total WBC pool. For purification of MN population blood defibrination is preferred since elimination of blood platelets improves a yield of the MN cells due to a blockage of fibrin network formation. The only inconveniency of defibrination is that it takes some time (15–20 min) just after obtaining blood by the venipuncture which is undesirable in clinical conditions but is acceptable in research.

Separation of MN and PMN cells was achieved by centrifugation on density gradient formed by soluble dextrin. Fraction of MN cells was obtained in a sufficiently pure state without additional treatment with hemolytic buffer (Table 2). 97.5 % of cells in this fraction were represented by MN cells, the rest were mainly damaged PMN cells and single RBC. It should be noted that lower value of density gradient, e.g. 1.077 g/ml,

provides higher uniformity of MN cells up to 99,5 % due to a retention of small resting lymphocytes and a loss of enlarged MN cells (activated lymphocytes and monocytes) passing to the PMN cells fraction. The cells of this fraction moved up to the bottom of the tube and were significantly enriched with RBC that we eliminated by treatment with the ammonium chloride buffer at strictly controlled conditions. After proving several recommended receipts [15] we have found that treatment of cell suspension with lysis buffer at 30 °C for 3–4 min with a subsequent addition of 2 volumes of autologous plasma/serum, provides minimal damage of PMN cells. Hosts of RBC are eliminated with two washings of cells with plasma/serum. Counting and cytological investigation of PMN cell fraction showed that it contained about 15 % of large MN cells (Table 2). Generally, a yield of PMNs was higher than that of the MNs.

Table 1

Characteristics and yields of WBC population depending on a mode of blood stabilization

Mode of blood treatment	Initial blood sample		Elimination of RBC by dextran induced sedimentation			Lysis of residual RBC with NH_4Cl solution		
	Total WBC count, $\times 10^6$/ml	Proportion PMN % / MN %	Total WBC count, $\times 10^6$/ml	WBC yield, %	Proportion PMN % / MN %	Total WBC count, $\times 10^6$/ml	WBC yield, %	Proportion PMN % / MN %
Stabilization with EDTA	26.0	61 / 39	15.3	59.0	58 / 42	11.0	42.3	51 / 49
Defibrinated blood	16.9	56 / 44	8.7	51.5	53 / 47	7.0	41.4	45 / 55

Note on abbreviations: WBC – white blood cells, RBC – red blood cells, PMN – polymorphonuclear cells, MN – mononuclear cells.

Table 2

Characterization of MN and PMN cell populations separated by density gradient centrifugation

Cell population	Purity of cell population (%)	Yield (%)
MN cells	95.7±2.0	39.8±2.4
PMN cells	85.8±1.9	56.5±3.3

Note: the yield of MN cells after separation was calculated relatively to the count of MN cells in a volume of plasma collected after sedimentation of RBC in presence of dextran. The same with PMN cells.

Conclusion. The proposed specimen of water soluble middle sized dextrin can serve as a versatile natural compound for preparing density gradient medium for cell separation in addition to commonly used media prepared with the aid of xenobiotics such as sodium diatrizoate or percoll. Its efficiency was demonstrated at separation of WBC populations from human peripheral blood.

Application of the developed water soluble dextrin for preparing a continuous linear density gradient for accurate fractionation of heterogeneous cell populations is expected.

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РЕЗЮМЕ

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ВОДОРОЗЧИННИЙ ДЕКСТРИН СЕРЕДНЬОЇ МОЛЕКУЛЯРНОЇ МАСИ І ЙОГО ЗАСТОСУВАННЯ ДЛЯ ФРАКЦІОНУВАННЯ КЛІТИННИХ ПОПУЛЯЦІЙ

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Із промислового зразка кукурудзяного декстрину отримано фракцію водорозчинного декстрину шляхом фракційного осадження етанолом в межах концентрації алкоголю 33 % – 74 %. Мол. маса отриманого продукту становить 4–21 кДа із середнім значенням 10 кДа, що дозволяє віднести його до речовин середньої мол. маси. Речовина дає червоно-коричневате забарвлення із йодом, якому відповідає широка смуга світлопоглинання із максимумом при 395 нм. Препарат розчинний у воді, ДМСО і піридині. Густина 40 % розчину (вага/об'єм) у воді або фізіологічному сольовому середовищі становить 1,15 ± 0,01 г/мл. Отриманий декстрин застосували для приготування середовища із градієнтом густини для фракціонування клітинних популяцій. Описано оптимізований процес виділення сумарного пулу лейкоцитів із крові і популяцій мононуклеарних (МН) і поліморфонуклеарних (ПМН) клітин. Розділення МН і ПМН клітин проводили центрифугуванням у ступеневому градієнті густини 1,083 г/мл, який створювали змішуванням запасного розчину декстрину із плазмою/сироваткою крові. Отриманий препарат водорозчинного декстрину середньої молекулярної маси може застосовуватись як речовина природного походження для приготування середовищ із градієнтом густини для фракціонування клітинних популяцій на додаток до звичайно вживаних градієнтних середовищ, створюваних з допомогою ксенобіотиків (діатризоату натрію або перколлу).

Ключові слова: водорозчинний декстрин, градієнт густини, фракціонування клітин.

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