LABORATORY OF BIOPHYSICS

FOR ADVANCED

Experimental exercises for III year of the First cycle studies

Field: "Applications of physics in biology and medicine"

Specialization: "Molecular Biophysics"

Determination of oligomeric state of a protein using gel filtration chromatography

(PBdZ 29)

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PBdZ 29

Determination of oligomeric state of a protein using gel filtration chromatography

The goal of the experiment

The aim of the exercise is to determine the oligomeric state of the enzyme - purine nucleoside phosphorylase (PNP) - by gel filtration chromatography, known also as molecular sieve chromatography. Molecular weight of the protein will be determined using markers - globular proteins of known mass. As the molecular sieve (gel) Superdex 200 column will be used. Based on the molecular weight obtained in the experiment and the known molecular weight of one subunit of the enzyme, the oligomeric state of the native PNP molecule will be determined. It will also be checked whether the change in the pH of the environment or the presence of ligands may affect the oligomeric state.

Description of the problem to be solved and the methods used

Introduction

Chromatography is an analytical and a preparative method, which separates a mixture of molecules using the differences in the behavior of the individual components of the mixture under certain conditions. In a chromatography two phases: mobile and stationary are present. Mobile phase comprising a mixture of the tested molecules moves with a certain speed in a certain direction through a stationary phase. The mobile phase is usually a gas or liquid, and the stationary phase - usually solid.

In this exercise, one of many possible chromatographic techniques will be poresented – the so-called. gel filtration chromatography, also known as a molecular sieve chromatography, that distinguishes molecules according to their mass. This method is used to:

• purification of macromolecules, mainly proteins, and oligonucleotides

• determination of their molecular weight

• desalt solutions of macromolecules or change buffer

In gel filtration chromatography, the solution containing a mixture of components, which we want to separate, passes through the column filled with a porous bed, so-called molecular sieves, consisting of porous granules with a well-defined diameter, typically ranging from a

few to several hundred μm (Figure 1). In contrast to several other chromatographic techniques, in gel filtration chromatography molecules do not bind to the chromatographic media, but pass through the column with different speeds.

Depending on the size of the components of the mixture, they more or less easily penetrate into the pores of the gel during the flow through the column, and hence their path before they appear in the effluent from the column, is not the same - the longer the smaller is the. Large molecules with a diameter larger than the pores of the gel cannot penetrate into the pores. Such molecules flow only between grains of a gel, therefore have the shortest route, and will appear first at the outlet of the column. The smallest molecules present in the mixture, which are those that can more effectively penetrate the pores of the grains of the gel and therefore will be reach the outlet of the column later.

To obtain separation, depending only on the size of the molecules is necessary to reduce non-specific interactions of molecules examined with the silica grains, particularly electrostatic interactions. Therefore, gel filtration chromatography use solutions having very low ionic strength (not lower than 20 mM). In order to further reduce electrostatic inteactions interactions buffer is supplemented with salts, e.g. 100 or 150 mM KCl or NaCl. In order not to clog the column, it is necessary also to limit the viscosity of the passing solution.

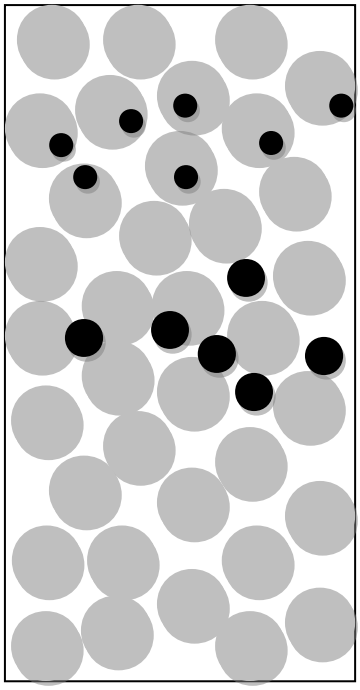
Separation will be the more efficient the longer is the column and the slower the mixture will flow through. The efficiency of separation increases as the square root of the length of the column. A very important parameter is the volume of the mixture applied. Small volume provides almost "common start" of all the molecules on their way through the column. If this volume is too large, greater than approx. 3-5% of the column volume, separation is not effective.

Gel filtration method has been used since the 40's of the twentieth century. In the first work, which appeared in the 50's, corn starch was employed, but it never came into widespread use due to the poor mechanical properties (Lindqvist and Storgards, 1955). Only later, due to introduction of cross-linked dextran yielded completely satisfactory results.

Cross-linked dextran, so-called Sephadex, is still one of the most popular materials used in gel permeation chromatography. Others, also popular, are Sepharose - cross-linked or not cross-linked agarose, Superose - densely cross-linked agarose, and Superdex – a mixture of agarose and dextran, as well as other matrices, such as Sephacryl it means dextran-bisacrylamide.

The pore size of the bed must be selected based on the size of molecules that we want to separate. The smaller are the pores the better smaller particles can be separated. The diameter of bed particles determines the flow rate, because larger grains yield smaller resistance when the solution flows through the column. Although smaller grains usually limit flow rate but at the same time slow flow rate increases the efficiency of the separation.

In general, both the size and the shape of molecules determine their flow rate through the gel filtration column. If we assume that the studied molecules are spherically symmetric and have similar density, the molecular weight may be determined. However, the column must be standardized by a set of globular molecules of known molecular weight. It is one of the most important applications of analytical gel filtration chromatography. In this exercise, we apply this method to determine the molecular weight of the enzyme - purine nucleoside phosphorylase - which will allow us, with known molecular weight of one subunit, to determine the oligomeric state of the protein.



Rysunek 1
Schemat kolumny filtracji żelowej. Porowate ziarna żelu (szare kulki) wypełniają kolumnę – jest to faza stacjonarna. Przez tę fazę przepływa roztwór mieszaniny molekuł, które chcemy rozdzielić. Bardzo duże molekuły (większe czarne kulki) przemieszczają się najszybciej, bo są zbyt duże, żeby wędrować wewnątrz porów żelu. Mniejsze cząsteczki wnikają, mniej lub bardziej, w pory żelu, zatem ich droga wędrówki jest tym dłuższa im są mniejsze, i ostatecznie te najmniejsze pojawia się na wylocie kolumny później niż większe molekuły.

Studied objects

Protein for which oligomeric sate will be determined is purine nucleoside phosphorylase (PNP). PNP is present in every living cell and is essential for metabolism of nucleic acids. It catalyzes the phosphorolytic cleavage of the glycosidic bond of purine nucleoside with the participation of inorganic phosphate, resulting in the release of purine base and pentose 1-phosphate:

purine nucleoside + orthophosphate ↔ purine base + α–D-pentozo-1-phopsphate

In this exercise, we will examine the recombinant calf purine nucleoside phosphorylase. Crystallographic studies (Koellner et al., 1997) show that the enzyme, at least a crystalline form, is a trimer (Figure 2). However, in the literature there is a report (Ropp and Traut, 1991), claiming that at low enzyme concentration and in certain environmental conditions (pH, temperature, the presence of ligand) phosphorylase dissociates into monomers (moreover, monomers exhibit much higher activity than the trimer). The aim of this exercise is to check by gel filtration chromatography whether that is really the case.

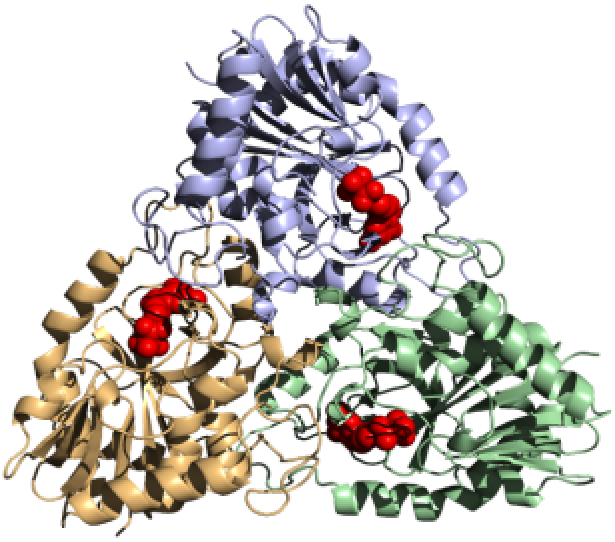


Figure 2
Three dimensional structure of calf purine nucleoside phosphorylase obtained from X-ray diffraction on protein crystals. Ligand molecules in the active site are shown in red. Figure from PhD thesis of Dr. Katarzyna Breer (studies done in Division of Biophysics, faculty of Physic, University of Warsaw).

Important facts regarding calf purine nucleoside phosphorylase necessary to make the experiment:

- Amino acid sequence contains 289 residues, including 3 tryptophan molecules and several tyrosine and phenylalanine residues
- Extinction coefficient at 280 nm is 30.809 cm^{-1} M^{-1} (at pH 7.0)
- $E_{1\%,280}$ =9.6 (at pH 7.0)
- Molecular weight of one subunit is 32 093 kDa

Execution of the experiment

1. Prepare 2 ml solution of PNP in 50 mM citric buffer pH 6.5 with addition of 250 mM KCl. Concentration of the enzyme should be about 2 mg/ml. Presence of salt, due to higher ionic strength of the solution, reduces the electrostatic interactions and prevents nonspecific binding of a protein to the column.

Determine PNP concentration by measuring the UV spectrum in the quartz cuvette with 1 cm optical path length. Check whether stock solution should not be diluted to obtain good UV spectrum

Determine PNP specific activity using the spectrophotometric method, which was used in experiment PPB7w1 or PPB7w2 („Enzymes – kinetics and inhibition of enzymatic reaction: purine nucleoside phosphorylase (PNP)") - using 7-methylguanosine as a substrate, in 50 mM phosphate buffer pH 7.0,

2. The following markers will be used to calibrate the column: LMM (*low molecular mass*) and/or HMM *(high molecular mass*) from GE-Healthcare consisting of globular protein with molecular weight in the range 6.5-75 kDa and 43-669 kDa,, respectively.

Table 1. Characteristics of Gel Filtration Calibration Kit LMW

Protein (weight per vial)	Molecular weight (M_r)	Source
Aprotinin (10 mg)	6500	Bovine lung
Ribonuclease A (50 mg)	13 700	Bovine pancreas
Carbonic anhydrase (15 mg)	29 000	Bovine erythrocytes
Ovalbumin (50 mg)	43 000	Hen egg
Conalbumin (50 mg)	75 000	Chicken egg white
Blue dextran 2000 (50 mg)	2 000 000	

Table 2. Characteristics of Gel Filtration Calibration Kit HMW

Protein (weight per vial)	Molecular weight (M_r)	Source
Ovalbumin (50 mg)	43 000	Hen egg
Conalbumin (50 mg)	75 000	Chicken egg white
Aldolase[1] (50 mg)	158 000	Rabbit muscle
Ferritin[1] (15 mg)	440 000	Horse spleen
Thyroglobulin (50 mg)	669 000	Bovine thyroid
Blue dextran 2000 (50 mg)	2 000 000	

[1] These proteins are supplied mixed with sucrose or mannitol to maintain stability and aid their solubility.

3. Separation will be carried out on a Superdex 200 pg preparative column (pg - preparative grade) with dimensions 16 mm/600 mm or on a Superdex 200 HR high resolution column (HR - high resolution) with dimensions 10 mm/300 mm, both from GE-Healthcare. The experiment is conducted at the temperature 4-6 ºC, in 50 mM citrate buffer, pH 6.5 containing 250 mM KCl, flow rate should be 1 ml/min. Markers

LMM and/or HMM should be applied in a volume of 0.5 ml. Markers contain also blue dextran, with molecular weight so large that it does not allow to penetrate pores of grains. Thus appearance of blue dextran in the column effluent determines the free volume of the column, hence elution volume zero.

We will be monitoring the column effluent by measuring absorbance at 280 nm. We expect to obtain a plot similar to that shown in Figure 2 below. When absorbance shows that no more proteins are eluted from the column, we will apply a sample of a studied protein - in our case PNP.

4. Stock solution of PNP, 0.5 ml, should be applied on the column. The flow rate should be 1 ml/min. As in the case of markers, monitor the effluent by measuring the absorbance at 280 nm. Collect the effluent, divided into 2 ml fractions in order to examine the eluted PNP, in particular its specific activity. According to Ropp and Tratut (1991) specific activity should increase when the enzyme dissociates into monomers.
5. Molecular weight of PNP should be determined by comparison of the PNP elution volume with the standard curve obtained using markers. What is the oligomeric form of PNP, monomeric or trimeric? Standard curve is obtained by plotting elution volumes of markers vs logarithm of its molecular weight.
6. The column should be washed with buffer that will be used in the next experiment (see 7, below).
7. The experiment, points 4 and 5, should be repeated using 100 mM phosphate buffer pH 6.5, and (only when time allows and students are interested in doing this additional part) points 3, 4 i 5 using 50 mM citrate buffer, pH 6.5 containing 250 mM KCl and phosphate buffer pH 6.5, and the experiment conducted at room temperature. What is the oligomeric state of the enzyme in these conditions?

Figure 2
Separation on Superdex 200 pg (*preparative grade)* gel filtration column with dimensions 16 mm/600 mm (GE-Healthcare). Figure is taken from GE-Healthcare web page.
Plot of absorbance of the eluted solution (in mAU units, it means 0.001 A) vs volume of the effluent. Volume of the blue dextran elution is taken as volume zero (see above).
Abbrevaitions: Apr – aprotinin 6.5 kDa, R – ribonuclease 13.7 kDa, CA – carbonic anhydrase 29 kDa, O – ovalbumin 43 kDa, CA – conalbumin 75 kDa, Ald – aldolase 158 kda, F – ferrytin 440 kDa, thyroglobulin 669 kDa.
Insert shows elution volume of the particular protein vs logarithm of its molecular weight.

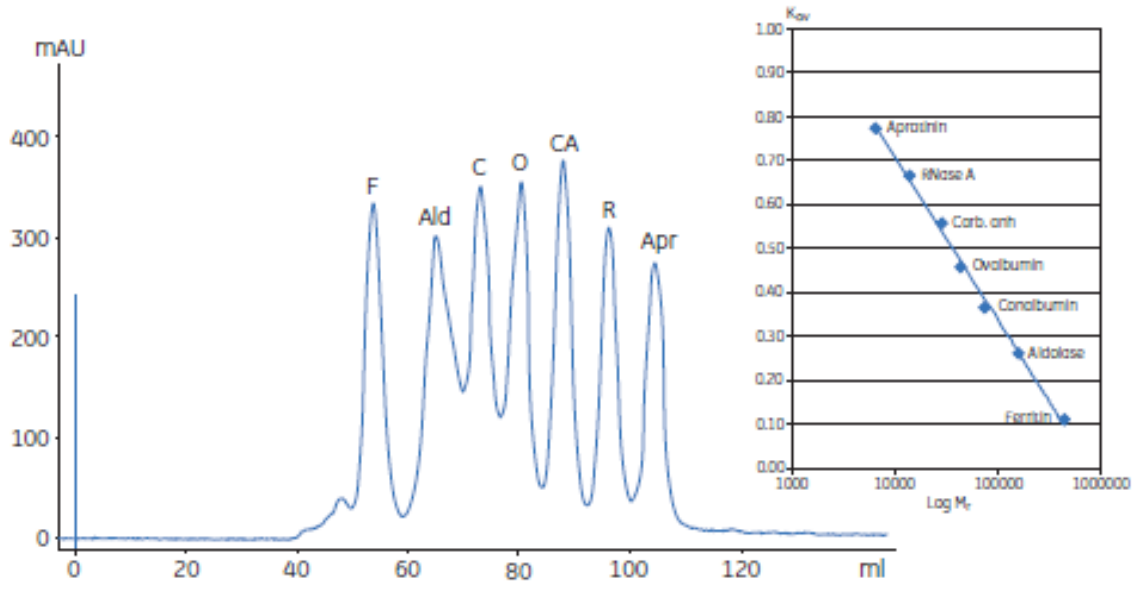


Fig 7. Chromatographic separation and calibration curve for the standard

The requirements for the pre-test

The condition to start the experimental part of this exercise is to pass the pre-test.

The choice of how to carry out a pre-test, i.e. its form (written or verbal questions and answers, open or closed questions etc., depends on the person supervising students during the experiments.

1. Chromatographic methods:
   -describe overall properties of chromatographic techniques
   -name several types of chromatographic methods, describe their physical principles and their applications

2. Gel filtration chromatography:
   -describe their physical principles of this method
   -describe gel filtration media and differences between them
   -describe applications of this technique

-what assumptions are made and discuss how good they are met

3. Determination of molecular weight by gel filtration chromatography:
   -physical basis
   -assumptions
   -give and discuss the equation that describes how elutin volume and molcuar weight are related

4. Structure of proteins:
   -primary
   -secondary
   -tertiary

5. Specific activity of enzymes:
   -definition
   -units
   -methods of determination

6. How enzymatic reaction depends on:
   -enzyme concentration
   -substrate concentration
   -temperature

7. Basic definitions related to electromagnetic radiation, spectroscopic measurements and absorption of proteins in UV:
   -wavelength, frequency, wave number
   -describe absorption of proteins in UV
   -molar extinction coefficient, definition, units
   -Lambert-Beer law

Execution of the experiment – overall remarks

Students obtain the solutions and buffers necessary to conduct the experiment.

1. PNP in 50 mM citrate buffer pH 6.5 with 250 mM KCl, concentration about 2 mg/ml (2 ml for each two-students group)

2. LMM i HMM protein markers for column calibration:

3. 50 mM citrate buffer pH 6.5 with 250 mM KCl (300 ml for each two-students group)

4. 50 mM citrate buffer pH 6.5 with 250 mM KCl and 100 mM phosphate buffer pH 6.5 (600 ml for each two-students group)

5. 7-metyloguanosine about 200 μM for determination of PNP activity
   ε = 8 500 $M^{-1}cm^{-1}$ at pH 7.0

(5 ml for ml each two-students group)

6. 200 mM bufor fosforanowy pH 7.0 do wyznaczenia aktywności PNP (ok. 10 ml każda grupa dwuosobowa)

The best wavelength to monitor proteins in an effluent is 280 nm – close to maximum of absorption of tryptophan, tyrosine and phenylalanine.

The experiment will be done with the help of the chromatograph for the low- and medium-pressure, so-called FPLC. It allows to program the flow-rate and measurement of some parameters of the effluent such as for example absorbance in the wavelength of choice. The chromatograph is in the refrigerator, to make studied protein more stable during the relatively long period f time necessary to make the experiment. Proteins are typically more stable at 4-6^{o}C, than at room temperature. In the case of analytical experiment it is not so crucial, but when gel filtration is done to purify a protein is important to keep is stable during the purification process.

For one part of this experiment (not obligatory) the column will be placed in the room temperature to carry out separation in the temperature in which, according to Ropp i Tratut, 1991, PNP dissociates into monomers.

How to make the report

The report should start from the Introduction, in which general issues relevant to the task performed are discussed, followed by Materials and methods, in which equipment used, compounds studied and measurement conditions should be described. Then, in the Results and Discussion one should show, evaluate, describe and interpret the results obtained it means determine the molecular weight of PNPO at various conditions and check if there are conditions in which PNP is in a monomeric state.

For data analysis Origin or GrapPad Prism programs may be used.

Literature

GE Healthcare “Gel filtration. Principles and methods”
B. Lindqvist, T. Storgards Molecular-sieving Properties of Starch, Nature (London) 175, 511-512, 1955
P. Ropp, T. Traut, Purine nucleoside phosphorylase. Alloseric regulation of a dissociating enzyme, J. Biol. Chem. 266, 7682-7687, 1991.
A. Bzowska, Calf spleen purine nucleoside phosphorylase: complex kinetic mechanism, hydrolysis of 7-methylguanosine, and oligomeric state in solution. Biochim. Biophys. Acta 1596, 293-317 (2002)
G. Koellner, M. Luić, D. Shugar, W. Saenger, A. Bzowska, Crystal structure of calf spleen purine nucleoside phosphorylase in a complex with hypoxanthine at 2.15 Å resolution. J. Mol. Biol. *265*, 202-216 (1997).
A. Fersht, „Structure and mechanism in protein science” W.H. Freeman and Company, New York, 1998
R.A. Copeland, „Enzymes” Wiley-VCH
B.D. Hames, N.M. Harper „Biochemia. Krótkie wykłady”, PWN 2006
J. Kłączkowski, „Podstawy biochemii” WNT 2005, rozdział 5.
J.M. Berg, J.L. Tymoczko, L. Stryer, „Biochemia” PWN 2007

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