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Preparation of the recombinant GST-GCK

Theoretical part:

Glucokinase (GCK), also known as hexokinase IV, is one of four isoforms of hexokinase that phosphorylate glucose at the hydroxyl group on the carbon 6 using ATP as a donor of phosphate. Glucokinase serves as a glycemic sensor in the pancreatic β-cells, provides glucose-6-phosphate for glycogen storage in the liver, is expressed in the brain and endocrine cells in the intestine. GCK has unique kinetic properties, which differentiates them from other hexokinases. These consist of $S_{0.5}$ for glucose about 8 mM, cooperativity in the case of glucose as a substrate – the Hill coefficient *h* about 1.7, the inflection point about 4 mM, and the fact that GCK is not inhibited by its product, glucose-6phosphate.

The English physiologist Archibald V. Hill formulated the Hill equation already in 1910, when he described the sigmoidal curve of hemoglobin binding oxygen. Archibald V. Hill was awarded the 1922 Nobel Prize in Medicine and Physiology for the elucidation of the production of heat and mechanical work in muscles.

Heterozygously present inactivating mutations in GCK cause either monogenic diabetes *GCK*-MODY (maturity-onset diabetes of the young) or, more rarely, persistent hyperinsulinemic hypoglycemia of infancy (PHHI). If both alleles carry an inactivating mutation (homozygosity), this leads to severe neonatal diabetes (permanent neonatal diabetes mellitus = PNDM). Inactivating mutations of GCK cause the increase of $S_{0.5}$ above 9 mM of glucose, the decrease in the activity of GCK or/concurrently any change of cooperativity (the changed value of Hill coefficient *h*).

On the contrary, activating mutations in GCK lead to severe hypoglycemia (PHHI*).* Activating mutations of GCK cause the decrease of $S_{0.5}$ values below 7 mM of glucose, the increase in the activity of GCK or/concurrently any change of cooperativity (the changed value of Hill coefficient *h*).

Throughout the GCK gene, hundreds of variations have been described, mainly identified as inactivating variations. Heterozygous variations usually manifest as mild hyperglycemia, therefore *GCK*-MODY is often diagnosed during the medical examination of other disorders or during preventative examinations (e.g., the oral glucose tolerance test carried out in some countries, including the Czech Republic, during pregnancy).

References:

Matschinsky, F.M. (2009) Assessing the potential of glucokinase activators in diabetes therapy. *Nat. Rev. Drug Discov.* **8**:399–416.

Šimčíková, D., *et al.* (2017) Evidence-based tailoring of bioinformatics approaches to optimize methods that predict the effects of nonsynonymous amino acid substitutions in glucokinase. *Sci. Rep.* **7**:9499.

Induction of protein expression by IPTG

Isopropyl β-D-1-thiogalactopyranoside (IPTG) is a substance mimicking allolactose. Allolactose is a disaccharide composed of D-galactose and D-glucose. It is a metabolite of lactose, which triggers transcription of *lac* operon, thereby inducing expression of genes, which are under the control of *lac* operator. IPTG, as well as allolactose, binds *lac* repressor and releases it from *lac* operator, thereby enabling transcription of genes in *lac* operon. Sulphur in the molecule of IPTG makes the bond nonhydrolysable for the cell, thus prevents the degradation of the inductor. The concentration of IPTG remains constant and protein expression is continuous in the course of an experiment.

Lac operon is the most commonly used system for expression of recombinant proteins in *E. coli*. The desired gene is inserted into a vector (pET, pGEX, etc.), which contains the gene encoding antibiotic resistance and the *lacI* gene derived from *lac* operon, which encodes *lac* repressor (LacI). The gene encoding an expressed protein is regulated by the T7 promoter, *lac* operator and the ribosome-binding site (the beginning of transcription).

Affinity chromatography

Affinity chromatography enables us to separate a group of similar proteins or even one specific protein from a complex mixture. The method is based on the use of an immobilized ligand interacting specifically with an enzyme (protein), which should be purified. Wash procedures ensure that only proteins that form strong bonds with a ligand remain bound to the affinity matrix, whereas those bound only weakly are washed out. The protein bound to the ligand is subsequently eluted from the ligand with high concentrations of salts or with solution, in which the soluble ligand is present. In a case of purification with Glutathione Sepharose, the desired protein is expressed with a Glutathione*S*-transferase tag, which mediates the binding of enzyme to the immobilized glutathione.

Protein purification device (FPLC = fast protein liquid chromatography) Äkta pure 25 L

FPLC enables us to optimize and standardize a process of protein purification, which is essential for applications requiring large amounts of proteins (in the order of milligrams up to grams) as well as their high purity (protein engineering, structural analysis of macromolecules, the production of antibodies, etc.). A typical FPLC consist of piston pumps, which ensure that the buffers are flowing through the purification system. Another component are injection loops, which are filled with the sample before it is injected onto the separation column. The injection loops are connected to the system by injection valves, which typically have positions for loading the sample loop, for injecting the sample from the loop onto the column, and for allowing the flow-through of the buffers directly to the column. . The various columns, which differ in volume, matrices, pressure limitations, etc., can be connected with

the FPLC system. They are stored in distilled water or 20% ethanol, and must be equilibrated with binding buffer prior to purification. Following the column, there are usually one or more flow cells, which allow measuring various parameters of column eluates; there, you may measure, for example, the UV absorbance, to check for the presence of proteins. The FPLC is also equipped with the fraction collector, which is filled with test tubes and allows collecting fixed volumes of eluted samples.

Practical part:

A/*E. coli* transformation

In molecular biology, transformation means a genetic change of a cell, which results from taking up an exogenous genetic material into the cell. The recipient bacterial cell must be in a state of competency, thus the cell must have an ability of taking up an exogenous DNA (cells for these purposes are prepared in the way to be competent, e.g., cells are treated by the solution of RbCl or $CaCl_2$). One of the procedures how to transform competent cells is "heat shock", when cells are exposed to higher temperature than their optimal temperature range. By the sudden increase of temperature, the competent cells form pores in the plasmatic membrane and take up an exogenous DNA.

1/ Let thaw the competent cells *Escherichia coli* strain **BL21 pLysS(DE3)** for several minutes on ice. The cells are stored in the solution containing glycerol, thus the suspension becomes liquid several degrees below the freezing point.

2/ Add carefully **1 µl of the vector pGEX-5X-2-GCK** into the cell suspension, mix gently and incubate for **20 min on ice**. pGEX-5X-2-GCK is the expression vector carrying fusion gene encoding GCK and glutathione-*S*-transferase. The presence of the glutathione-*S*-transferase tag enables one-step purification – affinity chromatography. This expression vector also carries the gene for ampicillinresistance, which serves as the selectable marker during the bacteria growth.

3/ Insert the tube with cells and the vector from ice directly in the **pre-heated** heat block for **2 min, 42°C** (generally the of "heat shock" ranges from 30 s to 2 min), **do not mix!**

4/ Insert the tube back into ice and incubate for **5 min**.

5/ Add **1 ml of LB medium** and incubate the tube for **1 hour at 37°C** and mix at **300 rpm**.

6/Transfer **50 µl** of the suspension of transformed cells on the plate with LB agar and ampicillin (100 µg/ml) and spread it throughout the provided plate. Incubate the plate overnight at **37°C**.

B/ Expression and purification of GST-GCK

We moved by three days to the future – in the course of these days, the colonies of cells grew on the plate with ampicillin at 37°C, we picked one of them and inoculated 5 ml of LB medium with ampicillin with it and incubated the suspension at 37°C overnight. The following day, we inoculated 75 ml of LB medium with ampicillin in each flask by 750 µl of this overnight culture (4 flasks in total) and incubated the culture at 37°C up to OD 0.7 (the optical density measured at 600 nm – its value corresponds to the number of cells). Afterwards, we added IPTG to the final concentration of 0.2 mM. We incubated the flasks for 16 hours at 22°C, 200 rpm.

1/ Centrifuge the bacterial culture at **4000 rpm, 10 min**.

2/ Prepare **40 ml of fresh lysis buffer** – mix **40 ml of PBS, pH 7.4** (phosphate buffered saline) with **40 µl of 1 M $MgCl_2$**, **266 µl of 150 mM PMSF** (phenylmethylsulfonyl fluoride – an inhibitor of serine proteases), **0.031 g of dithiothreitol** (DTT – a reducing reagent, which prevents free thiol groups of cysteines from oxidation) and **enzymes** (DNase I, lysozyme) according to the instructions of the assistant.

3/ Pour out the medium, resuspend each pellet (the sedimented cells) by repeated slow pipetting the content in-and-out in **5 ml of lysis buffer**.

4/ Lyse the cells for **30 min at room temperature**.

5/ Sonicate the cells **10× 30 s, with 30 s–pauses on ice** between the 30 s-lasting sonication steps.

6/ Centrifuge the lysate at **4000 rpm, 10 min, 4°C**.

7/ Pipette the supernatant (the liquid above the pellet, containing GST-GCK) into the new tube.

8/ **Sample preparation for SDS-PAGE:** mix 80 µl of the supernatant (step 7) with 20 µl of 5× sample loading buffer, label it as **S1** and boil it in the heating block for 5 min. Resuspend the pellet in 5 ml of distilled water and mix 80 µl of this suspension with 20 µl of 5× sample loading buffer, label it as **S2** and boil it in the heating block for 10 min.

9/ Connect the **GSTrap FF** (5 ml) column to the FPLC Äkta pure protein purification device according to the instructions of the assistant. Set up the limit pressure on **0.5 MPa** (at higher pressure, the device stops the purification process, thereby preventing the destruction of matrix and the column).

10/ Wash the column with **30 ml of binding buffer** (PBS, pH 7.4, 5 mM DTT) at the flow rate of **1 ml/min**.

11/ Apply **the sample on the column** (the supernatant prepared in the step 7) at the flow rate of **1 ml/min**. Collect the solution of proteins going through the column without being captured into the beaker.

12/ Wash the column with **25 ml of binding buffer** or adjust the amount of binding buffer based on the decline of UV signal to the baseline (the concentration of proteins is detected at the wavelength of 280 nm) at the flow rate of **5 ml/min**. Collect again the solution of proteins, which were washed out from the column.

13/ **Sample preparation for SDS-PAGE:** mix 80 µl of the solutions from the steps 11 and 12 with 20 µl of 5× sample loading buffer. Label them as **W1** a **W2** and boil in the heating block for 5 min.

14/ For the release of GST-GCK from the column, wash it with **25 ml of elution buffer** (50 mM Tris, 200 mM KCl, pH 8.0, 5 mM DTT, 10 mM glutathione) at the flow rate of **5 ml/min**. Concurrently begin to collect fractions of the **3 ml** volume.

15/ **Sample preparation for SDS-PAGE:** mix 80 µl of each fraction with 20 µl of 5× sample loading buffer. Label them as **F1, F2, F3, etc.**, and boil in the heating block for 5 min.

C/ SDS-PAGE

1/ Use two **10% SDS-PAGE gels** for analysis of expression and purification. Assemble the SDS-PAGE apparatus and pour the sufficient amount of 1× running buffer between the two gels and to the bottom of the apparatus.

2/ Pipette **5 µl of LMW-SDS marker** (rabbit muscle phosphorylase b – 97 kDa, bovine serum albumin – 66 kDa, ovalbumin – 45 kDa, bovine carbonic anhydrase – 30 kDa, soybean trypsin inhibitor – 20.1 kDa, bovine α-lactalbumin – 14.4 kDa) into the first well of each gel. 3/ Pipette **10 µl of samples S1 and S2** into the wells of one gel.

4/ Pipette **10 µl of samples W1 a W2 and F1, F2, F3**, etc., into the wells of the second gel.

5/ Set up the following values on the power supply device: **90 V, 50 mA, 7 W**.

6/ When samples passed through the upper stacking gel into the separating gel, increase the value of voltage on **130 V**.

7/ After finishing the separation, move the gels carefully to the **staining solution** (0.25% Coomassie Brilliant Blue R-250 dissolved in 45% methanol and 10% acetic acid) and stain them for 15 min.

8/ Pour out the staining solution and pour the **destaining solution** on the gels (45% ethanol, 10% acetic acid).

9/ Store the gels in **1% acetic acid**.

D/ Enzyme kinetics of GST-GCK

The catalytic activity of glucokinase is measured spectrophotometrically by a coupled reaction with glucose-6-phosphate dehydrogenase. In the second reaction, NADP (does not absorb at 340 nm) is reduced to NADPH (absorbs at 340 nm), thus absorbance at 340 nm is increased.

You are going to measure the dependency of the reaction rate on the concentration of glucose of the wild-type glucokinase, which you prepared, and a mutant form of GCK, which you have received. According to your measurements, you should identify whether your patient suffers from GCK-MODY or PHHI.

1/ Prepare the **fresh reaction mixture** for every series of measurements – the composition for 10 reactions:

500 µl of 2× concentrated buffer (200 mM Tris, 300 mM KCl, 12 mM $MgCl_2$, pH 7.8),

2 µl of mercaptoethanol (a reducing reagent),

0.2 µl of glucose-6-phosphate dehydrogenase,

200 µl of 2.6 mM NADP, **50**

µl of 100 mM ATP.

2/ Measure the enzyme kinetics at the following concentrations of glucose and pipette the respective solutions into wells of a microtitration plate as follows:

Glucose concentration (mM)	**0**	**1**	**2**	**4**	**8**	**15**	**30**	**60**	**100**
Reaction mixture (µl)	75	75	75	75	75	75	75	75	75
Distilled water (µl)	15	14	13	11	7	0	12	9	5
0.1 M glucose (µl)	0	1	2	4	8	15	0	0	0
1 M glucose (µl)	0	0	0	0	0	0	3	6	10
GST-GCK (µl)	10	10	10	10	10	10	10	10	10

Start the reaction by the addition of GST-GCK and measure the reaction proceeding at 340 nm, in the course of time you can observe the increase of absorbance.

3/ Before starting measurements of both GCK variants, you must determine **an appropriate dilution of your GST-GCK** aliquot in order to avoid too fast or slow reaction, both of which would be difficult to measure. Dilute GST-GCK 2×, 4×, 10× and 20× in the elution buffer (the total volume is 200 µl). Then, measure the activity of the diluted enzyme at 100 mM glucose in the course of 5 min. If the reaction still proceeds too fast, dilute the enzyme more (40×, …).

4/ Measure both GCK variants according to the step 2. Note values of absorbance at 5 min.

5/ Evaluate your measurements by the statistical software **SigmaPlot 12.0** by non-linear regression according to instructions of the assistant.

E/ Protein concentration according to Bradford

This method for determination of protein concentration is rapid; therefore, it has become widespread. It has been published by an American biochemist Marion M. Bradford in 1976 and the publication is the third most cited publication of all the time, with almost 200,000 citations. Nevertheless, this method is rather tentative, since it depends on the composition of amino acids in a protein. Thus, every protein should serve as its own standard, but it is unfeasible. Therefore, bovine serum albumin is mostly used as a standard for many proteins.

The colorimetric assay is based on the shift in absorbance of the Coomassie Brilliant Blue G-250 dye. If the solution is without proteins, it remains brown-reddish. When Coomassie Brilliant Blue G-250 interacts with proteins, the blue color is stabilized because strong non-covalent bonds between carboxyl group and amino group of proteins are formed. The concentration of the complex proteindye is measured at the wavelength of 595 nm.

1/ For the measurement of protein concentration, use your GST-GCK sample and determine its concentration as follows:

Dilute the sample 2×, 5×, 10× (the total volume is 100 µl).

2/ Pipette **50 µl of non-diluted and diluted sample** into the wells of the microtitration plate and add **200 µl of Bradford reagent**. Pipette **50 µl of distilled water (as a blank)** into the well and **200 µl of Bradford reagent**.

3/ Incubate for **5 min** at room temperature.

4/ Measure the plate at **595 nm**.

5/ Calculate the protein concentration of the sample from the calibration curve, which you have received.