2016/2017

Univerza v Mariboru

Fakulteta za kemijo
in kemijsko tehnologijo

BIOSEPARATION PROCESSES AND BIOCATALYSIS

LABORATORY EXCERCISES

Prof. Dr. Maja Leitgeb

Assist. Prof. Dr. Mateja Primožič

Katja Vasić, univ. dipl. ing.

Maja Čolnik, univ. dipl. ing.

Proofreader: Dr. Victor Kennedy

Content:

Exercise 1: Release of enzymes from *Saccharomyces cerevisiae* using homogenization method........ 2
Exercise 2: Supercritical carbon dioxide chromatography........ 9
Exercise 3: Electrophoresis........ 15
Exercise 4: Gel chromatography........ 20
Exercise 5: Activity of different enzymes in detergents........ 29
References........ 34

Exercise 1: Release of enzymes from *Saccharomyces cerevisiae* using homogenization method

1) Theoretical background

1.1 Homogenization

Homogenizing or "breaking down the cells" is a process in which tissue loses its morphological and some biochemical properties. The bond between the cells of cell walls and membranes is broken, and the content of the cell can be released into a suitable medium. At the beginning, the tissue is cut into fine pieces and suspended in an isotonic sucrose solution or NaCl and, if necessary, other additives are added to suspensions to prevent the organelles and biomolecules from changing their properties.

1.2 Homogenization using a rotor-stator homogenizer

In the gap between the external stationary stator and the internal rotor turbulence arises as a result of large shear forces. This causes spraying of larger droplets into smaller ones. The aim of homogenization using a rotor-stator homogenizer (Figure 1) is to unify or to break down the internal phase droplets.

Figure1: Homogenizer.

1.3 Alcohol dehydrogenase

Alcohol dehydrogenase is a group of dehydrogenases that occur in many organisms and perform conversion between alcohols and aldehydes or ketones. In the human organism and in many animals, this enzyme plays a key role in ethanol detoxification. In the case of yeasts and various bacteria, the opposite reaction is catalyzed in the fermentation process (Figure 2).

Figure 2: Oxidation of ethanol by alcohol dehydrogenase.

2) Reagents preparation

A. 50 mM sodium pyrophosphate buffer, pH 8.0 at 25 °C
   Prepare 100 mL of buffer in deionized water.
B. 95% (v/v) ethanol
   Prepare 100 mL of ethanol in deionized water.
C. 15 mM β- nicotinamide adenine dinucleotide solution (β-NAD)
   Prepare 15 mL of β-NAD solution in deionized water.
D. 10 mM sodium phosphate
   Prepare 100 mL of sodium phosphate in deionized water.
E. 10 mM sodium phosphate buffer
   Prepare 100 mL of buffer in deionized water.
F. 10 mM sodium phosphate buffer with 0.1% (w/v) bovine serum albumin (BSA), pH 7.5 at 25°C
   Prepare 25 mL of BSA solution in reagent E.
G. sample (*S. cerevisiae*)

3) Calculations

a) Enzyme activity

$$\text{Unit/mL enzyme} = \frac{\Delta A340nm/\min TEST - \Delta A340nm/minBLANK\ (3)(df)}{(6.22)(0.1)}$$

3 = total volume
df = dilution factor
6.22 = milimolar extinction coefficient for β-NAD at 340 nm
0.1 = volume of the enzyme

b) Protein concentration using Bradford method

Equation (from calibration curve):
y = 0.7321 x

where it is:
x = concentration of proteins [mg/mL]
y = absorbance $_{average}$

4) Protocol

a) Homogenization

The *S. cerevisiae* yeast is inoculated into 10 mL of saline solution. The homogenizer is immersed in a physiological solution containing *S. cerevisiae* yeast and samples are taken from the solution after 10, 20, 30, 40, 50 and 60 min of homogenization. In the samples obtained, total protein concentration using the Bradford method and the enzyme activity using enzyme assay for ADH activity determination is studied.

b) Determination of enzyme concentration using the Bradford method

Preparation of Bradford's reagent:

Bradford's reagent was prepared by dissolving 100 mg of Coomassie Brilliant Blue in 50 ml of 95% ethanol and in100 ml of 85% (v/v) phosphoric acid (H_3PO_4) and diluting with Milli-Q water to 1 L.

Preparation of the calibration curve:

Albumin protein in the concentration range from 0 to 1 mg/mL was used to prepare the calibration curve (Figure 3).
Prepare albumin concentrations. Weigh 10 mg of albumin and add 1 mL Milli-Q water.

10 mg/mL → dilute according to the following principle:

only Milli-Q water (Blank) → 0.0 mg/mL

20 µL of albumin (10 mg/mL) + 980 µL of Milli-Q water → 0.2 mg/mL
40 µL of albumin (10 mg/ mL) + 960 µL of Milli-Q water→ 0.4 mg/mL
60 µL of albumin (10 mg/ mL) + 940 µL of Milli-Q water→ 0.6 mg/mL
80 µL of albumin (10 mg/ mL) + 920 µL of Milli-Q water→ 0.8 mg/mL
100 µL of albumin (10 mg/ mL) + 900 µL of Milli-Q water→ 1.0 mg/mL

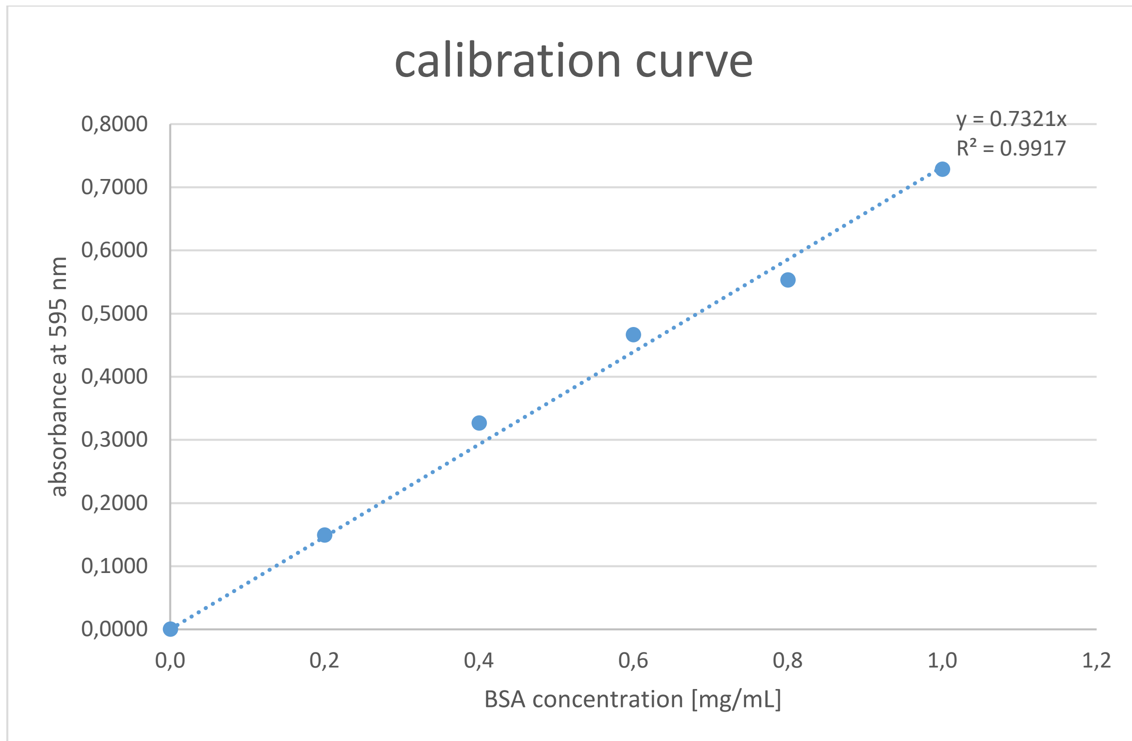

Figure 3: Calibration curve for Bradford method.

Sample preparation:

Pipette 1 mL of Bradford reagent into microcuvette and add 20 µL of the sample. The cuvette is put on the vortex and incubated for 15 minutes at room temperature. Then, the absorbance at 595 nm is measured. For a blank sample, instead of 20 µL of the sample, a blank test is taken (**or milliQ water or corresponding buffer**).

Example:

Blank sample:
1 mL of Bradford reagent
20 µL of milliQ water
put on vortex, 15 min of incubation at room T

Sample:
1 mL of Bradford reagent
20 µL of desired sample
put on vortex, 15 min of incubation at room T
measuring of absorbance at 595 nm

measuring of absorbance at 595 nm

c) Enzyme assay

Pipette the given reagents (in mL) into the tubes:

	Test	Blank
Reagent A (buffer)	1.30	1.30
Reagent B (ethanol)	0.10	0.10
Reagent C (β-NAD)	1.50	1.50

Intensely mix/shake at 25°C, then add:

	Test	Blank
Reagent G (sample)	0.10	/
Reagent F (medium)	/	0.10

Immediately mix/shake intensely and measure kinetics at λ = 340 nm for 6 minutes. Calculate the results in units per mL of enzyme (Unit/mL).

Present the homogenization time influence on total protein concentration and ADH activity on a diagram.

Observations:

5) Calculations

6) Results and discussion

Exercise 2: Supercritical carbon dioxide chromatography

1) Theoretical background

Supercritical fluid chromatography (SFC) (Figure 1) is one of the separation processes that has been intensively investigated and developed over recent years. Supercritical carbon dioxide (SC CO_2) is a fluid state of carbon dioxide where it is held at or above its critical temperature and critical pressure.

Carbon dioxide behaves as a gas in air at standard temperature and pressure (STP), or as a solid called dry ice when frozen. If the temperature and pressure are both increased from STP to be at or above the critical point for carbon dioxide, it can adopt properties midway between a gas and a liquid. More specifically, it behaves as a supercritical fluid above its critical temperature (31.1°C) and critical pressure (73.8 bar), expanding to fill its container like a gas but with a density like that of a liquid.

Supercritical CO_2 is becoming an important commercial and industrial solvent because of its role in chemical extraction in addition to its low toxicity and environmental impact. The relatively low temperature of the process and the stability of CO_2 also allows most compounds to be extracted with little damage or denaturing. In addition, the solubility of many extracted compounds in CO_2 varies with pressure, permitting selective extractions. In Figure 2, chromatography parameters are presented.

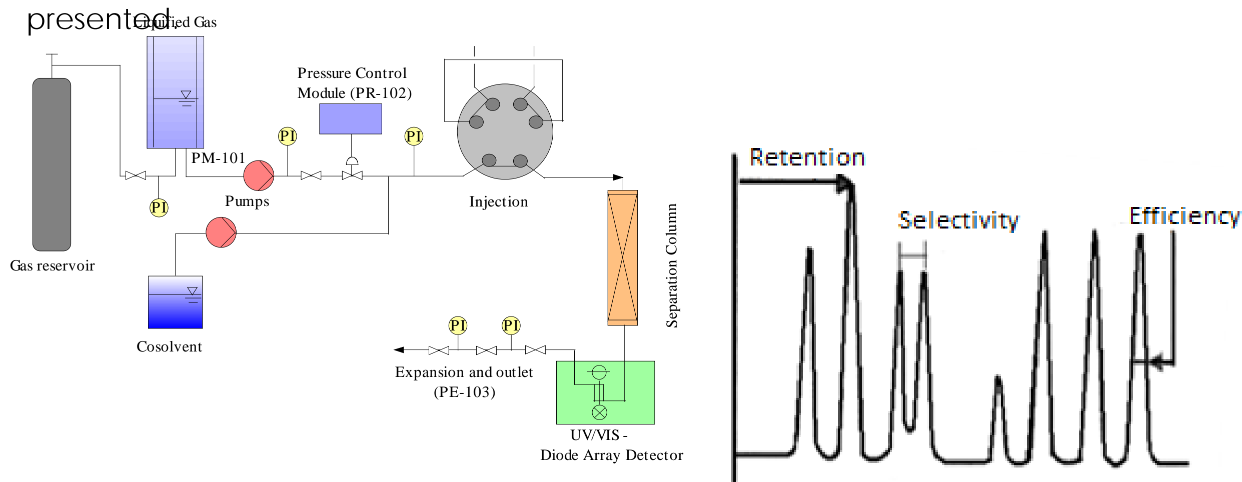

Figure 1: Scheme of SFC.

Figure 2: Chromatography parameters.

Chromatography parameters:

- ***Retention time (t_r)***; the amount of time a compound spends on the column after it has been injected.
- ***Retention factor (k')***; a measure of the time the sample component resides in the stationary phase relative to the time it resides in the mobile phase; it expresses how much longer a sample component is retarded by the stationary phase than it would take to travel through the column with the velocity of the mobile phase. Mathematically, it is the ratio of the adjusted retention volume (time) and the hold-up volume (time).
- ***Selectivity (a)***; the ability of the chromatographic system to chemically distinguish between sample components. It is usually measured as a ratio of the retention (capacity) factors (k) of the two peaks in question and can be visualized as the distance between the apices of the two peaks.
- ***Column Efficiency (N)***; the number of theoretical plates.
- ***Resolution (R_s)***; the parameter describing the separation power of the complete chromatographic system relative to the particular components of the mixture, expressed as the ratio of the distance between two peak maxima to the mean value of the peak width at the base line.

2) Materials

Chemicals: methanol, ethanol, benzene, caffeine, theophylline

Colons: silica 2-ethylpyridine and silica SI60, dimensions: l = 250 mm, d = 4.6 mm,

$d_{particles}$ = 5 µm

3) Protocol

a) Sample preparation

Weigh 5 - 7 mg of theophylline in a 10 mL volumetric flask, and 5 - 7 mg of caffeine into another one. Dilute with methanol. Put volumetric flasks in an ultrasound bath for complete dissolving of components. Put 1 mL of each component into a third volumetric flask and dilute with methanol. Again put the flask in the ultrasound bath. The prepared samples will be used throughout the whole experiment.

Preparation of sample t_0: put one drop of benzene into a 10 mL volumetric flask and dilute with methanol.

b) Pressure influence on SCF

Pressure influence will be observed at a certain co-solvent concentration, which has to be the same as the solvent in the solution. Measurements will be performed on a silica 2-ethylpyridine column at three different pressures (150 bar, 180 bar, 230 bar) with constant temperature of 45°C and 0.45 mL/min methanol. When conditions are set, the baseline is set to 0. Firstly, solvent is injected with a syringe and secondly the solution of benzene is injected. Then, each component in the solvent is injected. Clean the syringe every time with the solvent 4 to 6 times. In between sample injections, solvent must be injected. Write data into Table 1.

c) Temperature influence

Temperature influence at a certain pressure and certain co-solvent concentration will be investigated. Measurements will be performed on silica 2-ethylpyridine column at 180 bar at different temperatures (35°C, 45°C and 55°C) at 0.45 ml/min methanol flow. Injection protocol is the same as in section b). Write data into Table 1

d) Influence of amount and co-solvent type

Influence of the type and amount of co-solvent will be investigated at 180 bar and 45°C. Ethanol and methanol as co-solvents will be used. Measurements will be performed on silica 2-ethylpyridine column at 180 bar and 45°C. Flow rate of ethanol will be 0.2 ml/min, 0.45 ml/min and 0.7 ml/min. Injection protocol is the same as in section b). Write data into Table 1.

4) Calculations

flow CO_2 = ________ e x 0.0492 = _______ g/min, $\rho_{ethanol\ room}$ =______ g/ml,

$\rho_{methanol\ room}$ = _________g/ml

$$Rs = \frac{1.77 \cdot (t_{r2} - t_{r2})}{w_{0.5(1)} - w_{0.5(2)}} \qquad N = 5.54 \left(\frac{t_r}{w_{0.5}}\right)^2$$

$$k' = \frac{t_r - t_0}{t_0} \qquad \alpha = \frac{k'_2}{k'_1}$$

Relations: k'=f(pv.), k' =f(T) in k' =f(mass fraction of solvent) for both components shall be presented on a single diagram.

5) Table 1

p_v (bar)	p_i (bar)	T (°C)	$q_{co\text{-}solvent}$ (ml/min)	Mass fraction of solvent (%)	t_0 (min)	Substance	t_r (min)	k'	h (mAU)	$w_{0,5}$ (min)	α	N	R_s

6) Results and discussion

Exercise 3: Electrophoresis

1) Theoretical background

Electrophoresis could be described as the traveling of charged particles in an electric field using a carrier medium. Positively charged particles travel towards the cathode and negatively charged particles travel towards the anode. Particle separation usually takes place in a gel, which, because of its cross-linking, impedes the travel of molecules from one to the other electrode; the larger the molecule is, the more slowly it will travel. Through the extent of crosslinking of the gel, the speed of travel of the molecules can be controlled. With increase in the tension, the travel speed of the molecules through the gel increases, since they are influenced by the stronger force. The condition for the separation of molecules by electrophoresis is their charge. Many organic molecules are amphoteric and their charge in the solution depends on the pH. In biochemistry, electrophoresis is used primarily for the separation of nucleic acids (DNA and RNA molecules) and proteins. Because of the presence of a phosphate group, DNA not only has a negative charge, it also always equals the relative charge on a particular nucleotide. This is why the separation of DNA with electrophoresis is very simple, because no additional reagents that would charge DNA molecules are needed. Separation usually occurs in agarose gels that are less cross-linked than those used for protein separation.

Proteins are built from 20 different amino acids, each separately delivering a specific charge, and the sum of all partial charges is determined by the total protein charge at a certain pH value. This is different for each protein. The pH value at which a protein does not carry a net electrical charge is called the isoelectric point (pI). At a pH value lower than pI, the protein is charged positively, and at a higher pH than pI it is negative. Even in extreme pH, where almost all proteins would be charged positively or negatively, they would still not have the same relative charge per individual amino acid. Additionally, extreme pH values would denature the proteins, which would precipitate in the detergent-free buffer. Therefore, protein electrophoresis can be carried out in several ways:

• in the presence of SDS (sodium dodecylsulfate) (SDS-PAGE),

• isoelectric focusing,

• under native conditions.

2) SDS-PAGE (polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate)

SDS-PAGE is a type of electrophoresis in which proteins with anionic detergent (SDS, sodium dodecyl sulphate or sodium lauryl sulphate) are dissociated into their basic units (polypeptides). Proteins are denatured by heating at 100°C; the addition of SDS further accelerates denaturation. Under such conditions, most proteins bind SDS in a constant ratio (1.4 g of SDS/g polypeptide). SDS polypeptide complexes have identical charge ratios (m/z) and they travel in a polyacrylamide gel in an electric field based solely on different molecular weights. SDS results in complete denaturation of proteins (Figure 1), which means loss of tertiary structure, and in most cases the potential quartar structure is destroyed. The sample is usually treated with a reducing agent (-mercaptoethanol or dithiothreitol) prior to application to the gel, which reduces disulphide bonds (S-S bridges) in the protein. The polypeptide chains associated with disulphide bridges are decomposed into individual polypeptide chains. The electrophoresis gel is usually a polyacrylamide, of which the degree of crosslinking could be changed. To separate small proteins, the gel is more cross-linked - and less to separate large proteins.

First, a separating gel is poured, the function of which is to separate proteins. Once it is dried, a concentrated gel is added into the top, into which a comb is inserted to create the wells. When the gel is dried the comb is removed. The gel retains the wells for applying the sample. When the sample is applied, the negative pole is connected to the side of the samples (at the top, usually the vertical version), and the proteins travel into the gel. The task of the concentration gel is to concentrate all the proteins in the applied sample into a single line, which then begins to separate in the separation gel. In principle, therefore, the height of sample in the column which is added to the wells in the concentration gel doesn't matter. Once the electrophoresis is complete, the gel proteins are coloured with dyes (e.g. Coomassie blue or silver) that show the position of individual proteins in the gel. The molecular weight of the sample is determined by comparing standard polypeptides of known molecular weights. Figure 2 shows separation of proteins by electrophoresis

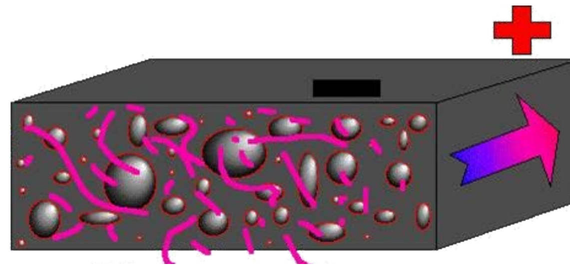

Figure 1: Scheme of denatured proteins in the gel and the travel direction of molecules in electrophoresis.

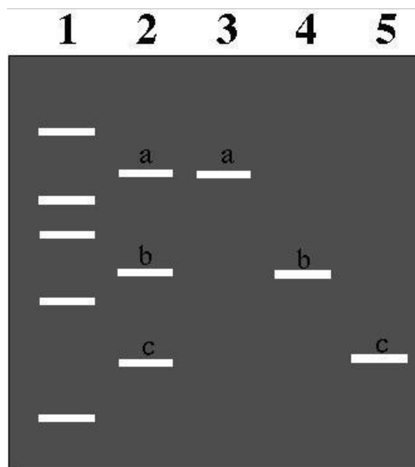

Figure 2: Separation of proteins by electrophoresis (A schematic presentation of SDS gel containing standard and samples).

Line1 - standard (markers)- proteins with a known molecular weight.

Line 2 - a mixture of three proteins (a, b, c)

Lines 3, 4, 5 – individual proteins (a - with a maximum and c - with a minimum molecular weight).

3) Materials and methods

Prepared apparatus for SDS-PAGE electrophoresis, gel preparation reagents, protein marker, dye solution and solution for gel discoloration, samples - enzymes.

4) Protocol

a) 12% separation gel

Pipette into the tube (**the order is important!**):

3.35 mL	miliQ
2.5 mL	1.5 M TRIS HCl, < pH 8.8
100 μL	10% (w/v) SDS
4 mL	acrylamide/bis solution 30%
50 μL	10% (100 mg/mL) ammonium persulfate
5 μL	TEMED

b) Concentration gel

Pipette into the tube (**the order is important!**):

3.05 mL	miliQ
1.25 mL	0.5 M TRIS HCl, pH 6.8
50 µL	10% (w/v) SDS
665 µL	acrylamide/bis solution 30%
25 µL	10% (100 mg/mL) ammonium persulfate
5 µL	TEMED

First, add 1 mL of agarose gel to the prepared gel cast model. Then pour the separation gel and wait for one hour to solidify. Initially, the concentration gel is made and poured into the top of the gel casting model after 1 hour. Insert the comb and make sure there is no air.

c) Sample preparation

Prepare solutions of selected enzymes with a concentration of 10 mg/mL dissolved in milliQ water. The samples for electrophoresis are prepared in microtubes:

- 20 µL of enzyme
- 33 µL DTT + LB (loading buffer)
- Denaturation at 95°C for 3-5 min
- Centrifugation of samples at 11,000 rpm for 2 min

Insert the prepared gel into electrophoresis and carefully remove the comb; 10 µL protein marker should be carefully applied to the first gap on the gel. Then apply 15 µL of the prepared sample to the gel. It is very important to indicate from which side of the gel samples were applied. When all samples are applied, put the buffer into electrophoresis and turn it on. Electrophoresis expires at U = 200 V and t = 45 min.

d) Dyeing and decolouring the gel

After electrophoresis is finished, rinse the gel with distilled water and start dyeing. Into the crystallizer pour approx. 12 mL of Coomasie Blue dye, put a gel on it and shake gently on the shaker for 10 min. After finishing the dyeing, the colouring matter is

cleared. Pour the discolouring solution into the crystallizer and gently shake for 5 min. Pour out the solution, pour in a new amount and shake again for 5 min. For the third time, pour out and add a new discolouring solution, shake for 30 minutes and read the results.

5) Results and discussion

Exercise 4: Gel chromatography

1) Theoretical background

Chromatography comprises various separation techniques of molecules in the mixture. In chromatography, the sample in the mobile phase descends through a column in which the stationary phase is bound. While traveling, the molecules are distributed between the stationary and the mobile phase with respect to affinity with both phases. The molecular distribution between the mobile and the stationary phase is influenced by the chemical and physical properties of the stationary and the mobile phase and the solubilities in the mixture.

Depending on the aggregate state of the mobile phase, gas chromatography (the mobile phase is gas) and liquid chromatography (the mobile phase is a liquid) are distinguished. In biochemistry, chromatography is often used to separate and purify proteins from complex mixtures. The basis for separation are the differences in the chemical and physical properties of proteins used by the following chromatographic techniques:

- *Protein charge:* Ion exchange chromatography
- *Hydrophobic areas in the protein:* hydrophobic chromatography
- *Size or Hydrodynamic protein radii:* gel filtration
- *Affinity of proteins to different ligands:* affinity chromatography

2) Ion exchange chromatography

Each protein is composed of amino acids that, because of their side-groups, bring the protein its unique properties in terms of the total electrical charge. The pH value at which the protein is not charged at all is called the isoelectric point of the protein (pI). At a pH value lower than pI, the protein is positively charged, and at a pH higher than pI, it is negatively charged. Knowing the pI of unknown proteins is essential for determining the optimal conditions in ion exchange chromatography. There are many services on the Internet where you can calculate the point pI from a known primary sequence of proteins.

There are two kinds of chromatography, cationic and anionic exchange chromatography. As the name suggests, in cationic chromatography, cations from

the mobile phase are exchanged with bound cations from the stationary phase. Therefore, the stationary phase must be negatively charged, and cations compete for negatively charged binding sites at the stationary phase. Carboxymethyl (CM)-O-CH_2-COO- (weak) or sulphopropyl (SP)-O-CH_2-CHOH-CH_2-O-CH_2-CH_2-CH_2SO_3- (a strong exchanger) is used as the cation exchanger. If it is desired to establish a bond between the target protein and the stationary phase in the column, it is essential that the protein mixture be applied to the column at a pH that is lower than the pI of the target protein (at that time the protein will be positively charged). All negatively charged molecules will be rinsed out from the column with the mobile phase.

If we want to rinse the protein from the column, its binding to the stationary phase has to be broken, which can be done in two ways:

- Increase the pH value in the mobile phase buffer above the point pI. In this case, the bound protein will receive a negative charge and terminate the binding to the negative charge of the ion exchanger.
- Increase the neutral salt content of the mobile phase (e.g. increase the concentration of NaCl). Consequently, the concentration of positive ions in the mobile phase (Na^+) is increased. Therefore, they will only compete with the positively charged proteins for binding to the exchanger. If the salt concentration is increased sufficiently, additional cations will supplant the positively charged protein, which will then be washed out from the column.

In anion exchange chromatography, quaternary ammonium salts are most commonly used as an exchanger, e.g. quaternary aminoethyl (QAE) -O-CH_2-CH_2-N+(C_2H_5)$_2$-CH_2-CHOH-CH_3. The principles of binding and rinsing with respect to the pH value are exactly the same as for cation exchange chromatography, and elution of the protein from the column with an elevated neutral salt concentration also works in this case.

3) Hydrophobic chromatography

Most proteins have a smaller or larger hydrophobic region on the surface. The aqueous medium is an ideal environment for the formation of hydrophobic bonds between hydrophobic regions; consequently, water is excluded from contact with hydrophobic regions, which is thermodynamically favourable. When a neutral salt is added to the

protein sample (e.g. NaCl), the strength of hydrophobic interactions is increased. Therefore, if strongly hydrophobic groups (phenyl, octyl) are bonded to the stationary phase, the proteins will be bound to these hydrophobic groups at the stationary stage through their hydrophobic regions; because of the differences in hydrophobicity of proteins, a selection at the binding will appear. Reduction of salt concentration in the mobile phase reduces hydrophobic interactions, which allows elution of the protein. Alternatively, the addition of a small amount of an organic non-polar solvent (which, however, must be water-miscible), accelerates the desorption and elution of proteins.

4) Affinity chromatography

This is a separation method based on very specific biological interactions. Separation takes place on the basis of reversible interactions between proteins and ligands bound to the inert carrier. A ligand is a molecule that reversibly binds a specific molecule or group. When choosing an affinity chromatography ligand, two things must be considered: the ligand must have a specific and reversible affinity to the target molecule, and it must have groups through which it can covalently bind to the carrier. After binding to the carrier, the ligand´s binding affinity to the target molecule should not be changed.

Examples of interactions between the ligand and the target molecule:

- Enzyme – substrate, inhibitor, cofactor
- Receptor – vitamin, hormone
- antibody – antigen
- Nucleic acid - complementary nucleic acid, histone, a protein that binds to nucleic acid
- Lectin - glycoprotein

Finally, the affinity bound protein is washed from the column by a change in pH (changes in interaction with ligands), or by the addition of a soluble ligand competing for a protein with a bound ligand.

5) Gel filtration

Gel filtration is used to separate proteins based on their different molecular sizes or hydrodynamic radii (which usually coincide with the molecular weight of the proteins). The separation of proteins takes place as a result of the different velocity of vertically traveling molecules in the mobile phase (solvent) through the carrier (matrix gel), which is placed in a glass or plastic tube. For gel filtration purposes, synthetic, polymeric organic substances that act as so-called molecular sieves (Figure 1- for gel chromatography) are used.

Usually, cross-linked polymers, which swell in aqueous solutions to form a gel with pores, are used. The pore size is determined by the degree of binding of individual polymer chains. Molecules larger than the pores cannot penetrate the gel, so they are most rapidly rinsed with the mobile phase (solvent) - they have access only to the external volume of the mobile phase (V_o); that is, the volume which is outside of the gel polymeric material. V_o is also called the void volume.

The smaller the molecules are, the more easily and deeply into the pores of the gel they can penetrate; they are eliminated more slowly because they have access to the volume of the mobile phase (solvents) within the gel carriers (V_i) and the void volume of the column (V_o).

(Figure 2: A schematic view of the traveling of large and small molecules in gel chromatography (gel filtration)).

Figure 1: Gel chromatography.

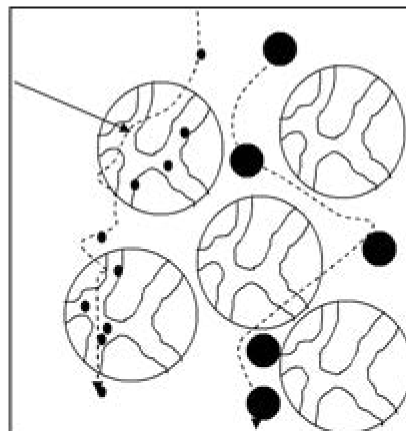

Figure 2: A schematic view of the traveling of large and small molecules in gel chromatography (gel filtration).

For the description of gel column parameters, the following symbols are used (Figure 3):

V_t = the total volume of the prepared gel column. It can be calculated as the volume of the entire column (base surface × height).

V_o = void cell volume outside the gel carrier

V_i = the volume of the mobile phase present inside the gel carriers can be calculated according to the formula: $V_i = a \times W_r$ (a - mass of dry gel, W_r - volume of bound water to dry mass). W_r is determined for each gel separately, e.g. for sephadexG - 100 is 7.5 mL/g. This part of the gel carrier is accessible to small molecules that penetrate the pores.

V_g = volume inside the gel carrier that is not accessible for the mobile phase (solid matrix).

V_e = eluting volume represents the volume of the mobile phase needed to remove a substance from the column.

Since V_g is usually negligible, we can write: $V_t = V_o + V_i$

$K_d = (V_e - V_o) / (V_t - V_o)$

where K_d represents the distribution coefficient ranging between 0 and 1. Large molecules that do not pass into the pores of the gel have a K_d = 0 value, while the smallest molecules have a value of K_d = 1.

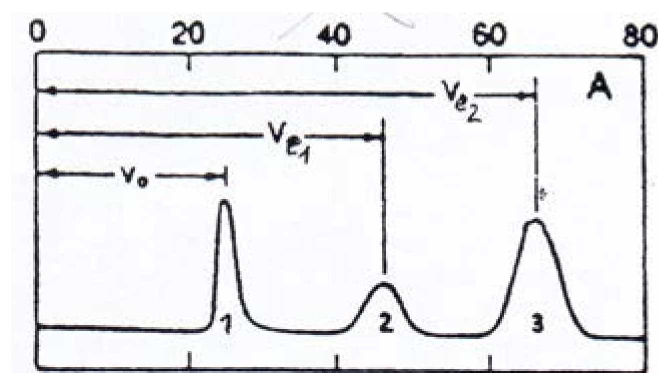

Figure 3: An elution profile of three different substances.

Legend to Figure 3:

V_0 = volume of substance 1 (with the highest molecular weight)

V_{e1} = volume of substance 2

V_{e2} = volume of substance 3

Elution volume (mL)

Gel filtration is used for:

- separation and purification: through appropriate gels, viruses, proteins, enzymes, hormones, antibodies, nucleic acids and polysaccharides can be separated and purified,
- determination of relative molecular masses.

On Figure 4, calibration curve for determining the molecular mass of the unknown compound in the sample by gel filtration is presented.

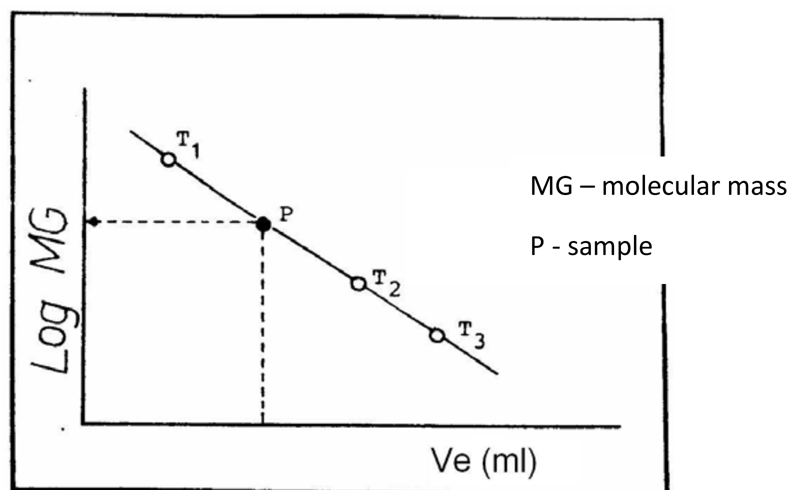

Figure 4: Calibration curve for determining the molecular mass of an unknown compound in a sample by gel filtration. The molecular mass of the washed proteins is calculated by extrapolation, in comparison with markers of known molecular weights. (A standard curve should be constructed).

Legend to Figure 4:

T_1,T_2,T_3 : Compounds with a known molecular weight (for construction of standard curve)

P: A sample of which the molecular mass is determined

6) Materials and methods

Trypsin (30 mg/mL in 0.1 M TRIS pH = 7.2); liver extract (source of catalase); 5% solution of dextran blue (or concentrated) with a known molecular weight (M ≈ 2,000,000 g/mol); 0.25% methylene blue solution (M = 355.89 g/mol); Chromatographic buffer of 0.1 M PBS, pH = 7.2; 3% hydrogen peroxide; BATCH (50 M in 0.1 M TRIS, pH = 7.2).

Procedure:

- As a chromatographic buffer, 0.1 M PBS will be used, pH = 7.2.
- Mix the sample for the column: 50 µL of dextran blue; 50 µL of methylene blue; 100 µL of liver fractions (catalase enzyme) and 100 of µL trypsin (30 mg/mL).
- The top of the column must always be covered with buffer; always add the buffer to the side slowly with the pipettor. Be careful not to pull the buffer in a pipettor.
- Leave the buffer through the column until it covers the surface of the gel. Shut the exit from the column on the bottom to stop the flow. Apply your sample evenly over the entire surface with your automatic pipette.
- Place the first tube under the column and start collecting the fractions.
- Unscrew the column and wait until the sample has sunk into the gel. Shut the column again and carefully add 0.5 mL buffer to the top of the column and again lower it down to the surface of the gel. Repeat this procedure for as long as the added buffer loses the blue colour upon addition to the surface of the gel.
- At the same time, change the tubes into which you collect 3 mL of fractions.
- Collect fractions until all the blue colour from the column (40 fractions) is eliminated. In sequence, you mark them. At the same time, be careful that there is always enough buffer on the top to ensure that the column never dries out.
- When the entire blue colour is washed, add the buffer at the top of the column and shut it at the bottom so that the buffer is no longer flowing out.

7) Analysis of chromatographic fractions

Note the dimensions of the column (the height up to the top of the gel) and the column diameter that you will need to calculate V_t. Also note in which fraction the dextran blue and in which methylene blue were eliminated, as the elution volume of these two substances represents the void volume (dextran blue) and the total volume of the column (methylene blue). The fractions will be analyzed for the presence of the liver enzyme of catalase. Catalase in the liver catalyses the following reaction:

$$2H_2O_2 \longrightarrow 2H_2O + O_2$$

Trypsin as protease catalyses the degradation of the BAPNA (Na-Benzoyl-D,L-arginine 4-nitroanilide hydrochloride) substrate.

Procedure:

Determination of the presence of catalase

- Pipette 200 µL of each fraction into the cavity wells of a microtiter plate. Do not forget the blank test, where you have to pipet 200 µL of chromatographic buffer (0.1 M PBS, pH = 7.2).
- Then add 100 µL of 3% H_2O_2 to each well and observe the intensity of bubble formation.

Determination of the presence of trypsin

- Pipette 400 µL of each fraction into microtubes. Do not forget the blank sample, where you have to pipet 400 µL of chromatographic buffer (0.1 M PBS, pH = 7.2).
- Add 100 µL of BAPNA substrate (50 M in 0.1 M TRIS pH = 7.2) to each microtube vortex, to mix the reagents.
- Measure the absorbance at 410 nm with UV-vis spectrophotometer.

Prepare a table in which you will enter the obtained results on the basis of which you will draw a graph.

Determination of Bradford protein concentration

- Pipette 120 μL of each fraction into microtubes.
- For the construction of a calibration curve, pipette 120 μL of each standard solution (BSA) into microtubes (two repetitions): [10, 20, 30, 50, 100, 200, 400, 1000] μg/mL. Do not forget the blank sample, that is, 120 μL of chromatographic buffer (PBS, pH = 7.2).
- Add 480 μL of Bradford reagent to all microtubes with samples and standard solutions.
- Vortex, to mix the reagents.
- Measure absorbance at 595 nm with UV-vis spectrophotometer.

8) Results and discussion

Exercise 5: Activity of different enzymes in detergents

1) Theoretical background

Modern laundry powders (washing powders) contain a number of components besides detergents, including optical and chemical bleaches, water softeners, fragrances and enzymes. Enzymes are essential for effective dirt removal even at low temperatures, and they represent a very low load for the environment.

Washing powders contain:

- proteases, that help in the removal of protein stains (stains of blood, grass, ...);
- lipases for removing fatty stains (oil, butter, sauces, ...);
- amylases for removing stains based on starch (potatoes, pasta, ...);
- cellulases, that are not primarily designed to remove stains, but remove loose cellulose fibres in cotton fabrics, thereby making the fabric softer, indirectly allowing other washing components easier access to trapped dirt and preventing the appearance of "gray fabrics."

2) Materials and methods

Materials:

Dyes, reagents, microtubes, washing powder, heater, glasses, crystallization dish glass, stopwatch, thermometer, pipettes.

Methods:

The activity of enzymes, α-amylase, lipase, cellulase and protease, in washing powders is to be determined at different temperatures (room temperature, 30°C, 40°C, 60°C and 95°C) and at incubation time of 1 hour. The enzyme activity is calculated from the measurements obtained.

a) Cellulase

Pipette 4 mL of Reagent B (Sigmacell solution) into the tubes. After addition of the incubated solution of the washing powder (1 mL), shake the tubes on the shaker for 2 hours at a temperature of 37°C. For a blank sample, use distilled water. After two hours, transfer the tubes to an ice bath. Centrifuge the samples for 2 min at 11,000 rpm. Transfer the supernatant into microtubes and Pipette 25 μL of the supernatant into 0.75 mL of D-glucose reagent. Measure the absorbance of the prepared sample at 340 nm.

Calculate the cellulase activity using the equation:

$$\text{Unit/mL of enzyme in detergent solution} = \frac{(\Delta A_{340\,nm}) \times (V_1) \times (V_2)}{(k) \times (f) \times (V_3) \times (V_4)}$$

V_1 = volume of washing powder solution and D-glucose reagent (0.775 mL)

V_2 = volume of the washing powder sample solution and the reagent B (5 mL)

k = milimolar extraction coefficient β – NADH at 340 nm (6.22)

f = a conversion factor for converting two hours to one hour, as specified in the definition of unit (2)

V_3 = volume of washing powder solution added to 4 mL of reagent B (1 mL)

V_4 = volume of centrifuged washing powder solution of solution, reagent B and D-glucose reagent (0.025 mL)

$\Delta A_{340\,nm}\, sample$ = absorbance value of the washing powder sample solution at a wavelength of 340 nm

$\Delta A_{340\,nm}\, blank\ sample$ = absorbance value of the blank sample solution at a wavelength of 340 nm

b) α-Amylase

Determination of α-amylase activity is carried out by weighting 1.08 g of Starch azure colorant into tube and adding 13.5 mL of reagent A. Reagent A is potassium phosphate buffer with sodium chloride.

Distribute the prepared solution containing reagent A and Starch azure dye to the tubes for further test performance. Incubate the tubes at 37°C for 3 min, then add 125 μL of the corresponding sample. After addition of the sample, stir the mixture at 37°C

for 10 min. Further, add 0.5 mL of reagent C (HOAc - acetic acid solution). Mix tubes intensively using vortex and then filtrate the suspensions. Use the filtrate to measure the absorbance on the UV-Vis spectrophotometer at 595 nm.

Calculate the activity of α-amylase using the equation:

$$\Delta A_{595\ nm}/min/mL = \frac{\Delta A_{595\ nm}}{(t)\times(V\ of\ washing\ powder\ solution\ in\ mL)}$$

$V_{washing\ powder\ solution}$ = volume of washing powder solution (0.125 mL)

t = time of measuring absorbance in the washing powder solution (5 min)

$\Delta A_{595\ nm}$ = absorbance value of the washing powder sample solution at a wavelength of 595 nm

c) Protease

First, prepare a casein solution for the protease test. Measure 0.2 g of casein into the beaker. Add 20 mL of pre-prepared phosphate buffer and slowly heat to boiling. Prepare the phosphate buffer by weighing KH_2PO_4 and $Na_2HPO_4 \cdot 2H_2O$.
In each tube, for the blank sample and for the washing powder solution samples, pipette 1 mL of casein and incubate for 2-3 min at 35°C. Then, add 500 µL of phosphate buffer and 500 µL of sample. Incubate the prepared tubes for 20 min at 35°C. After incubation, add 3 mL of TCA to each tube and store for 30 min at room temperature. After 30 min, centrifuge the samples for 20 min at 11,000 rpm. Carefully transfer the supernatant into a new plastic tube and measure the absorbance at 280 nm. Calibrate the apparatus with a blank sample.

To calculate the proteolytic activity of the washing powder solution, use the following equation:

$$1\ Tucas\ (\mu L) = \frac{V\ (sample)}{\Delta A_{280\ nm}}$$

V = volume of the added washing powder solution sample (500 µL)

$\Delta A_{280\ nm}$ = absorbance value of the washing powder sample solution at a wavelength of 280 nm

d) Lipase

Prepare a solution of 4-nitrophenylbutyrate (NPB) by dissolving 0.0157 g of NPB in 0.5 mL of acetonitrile. First, prepare a blank sample; pipette 1.8 mL of PBS buffer into the tube and add 13.4 µL of NPB solution. Then, add 1.8 ml of PBS buffer, 13.4 µL of NPB solution and 13.4 µL of washing solution to the microtubes, and vortex for 2 min. Then, measure the absorbance at a wavelength λ = 346 nm for all samples.

The enzyme activity of lipase is calculated using the equation:

$$\text{Unit/mL of washing powder solution} = \frac{(A_{346\ nm}) \times (V_k)}{(0.0148) \times (V_e)}$$

$U/mL_{\text{washing powder solution}}$ = lipase specific activity, (mL^{-1})

V_k = final volume (mL)

0.0148 = micromolar extinction coefficient (*p*-nitrophenol at 400 nm)

V_e = volume of washing powder solution (mL)

Plot the diagrams; determine enzyme activities vs. temperatures and compare the temperatures where the maximal activity for defined enzymes were detected.

3) Results and discussion

REFERENCES

Bergmeyer, H.U. (1983). Methods of Enzymatic Analysis, (3rd ed.), Verlag Chemie, Weinheim.

Bradford, M.M. (1976). Rapid and sensitive method for quantitation of microgram quantities of protein utilising principle of protein dye binding. Analytical Biochemistry. 72, 248-254.

Flickinger, M.C. (2013). Downstream Industrial Biotechnology: Recovery and Purification. New Jersey, Hoboken: John Wiley & Sons, Inc.

Gallagher, S.R. (2012). SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE). Current Protocols Essential Laboratory Techniques. 6:7.3:7.3.1–7.3.28.

Gibbins, J.M. (2004). Techniques for Analysis of Proteins by SDS-Polyacrylamide Gel Electrophoresis and Western Blotting. In: Gibbins J.M., Mahaut-Smith M.P. (eds). Platelets and Megakaryocytes. Methods in Molecular Biology™, vol 273. Humana Press.

Goldberg, S. (2008). Mechanical/Physical Methods of Cell Disruption and Tissue Homogenization. In: Posch A. (eds). 2D PAGE: Sample Preparation and Fractionation. Methods in Molecular Biology™, vol 424. Humana Press.

Ito S., Kobayashi T., Hatada Y., Horikoshi K. (2005). Enzymes in Modern Detergents. In: Barredo J.L. (eds) Microbial Enzymes and Biotransformations. Methods in Biotechnology, vol 17. Humana Press.

Janson, J.-C. (2011). Protein Purification: Principles, High Resolution Methods, and Applications, (3rd ed.). New Jersey, Hoboken: John Wiley & Sons, Inc.

Maurer, K.-H. (2010). Enzymes, Detergent. Encyclopedia of Industrial Biotechnology. 1–16, New Jersey, Hoboken: John Wiley & Sons, Inc.

Reymond J.-L. (2006). Enzyme Assays. Wiley-VCH Verlang GmbH&Co. KGaA.

Webster, G.K. (2014). Supercritical Fluid Chromatography: Advances and Applications in Pharmaceutical Analysis. New York: CRC Press, Taylor&Francis Group.