ISOLATION OF TRYPSIN BY AFFINITY CHROMATOGRAPHY

To learn the molecular mechanism of an enzyme action, first the protein has to be purified. Because of its simplicity and reproducibility, affinity chromatography has become a adopted method throughout the scientific community. Affinity chromatography occupies a unique place in separation technology since it is the only technique which enables purification of almost any biomolecules on the basis of its biological function or individual chemical structure. The principle of the affinity chromatography is shown in Figure 1. and Figure 2.

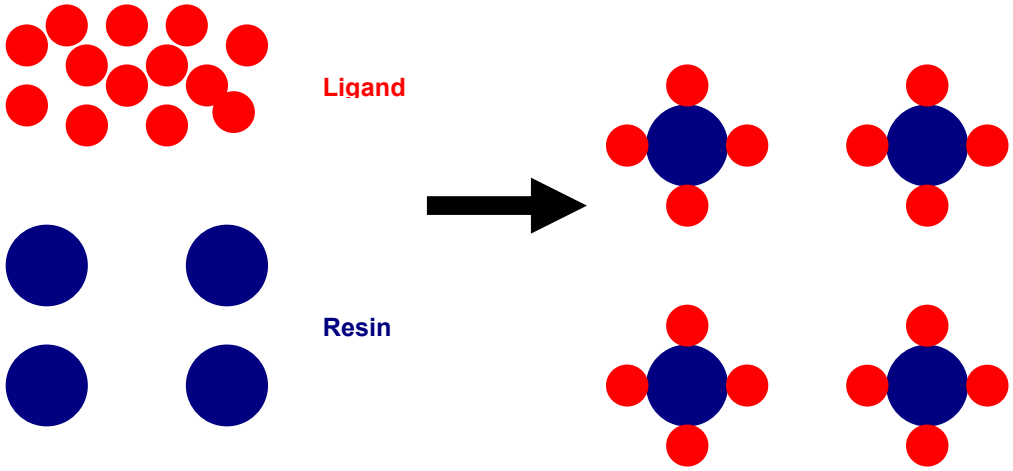


Figure 1. Immobilization of ligands

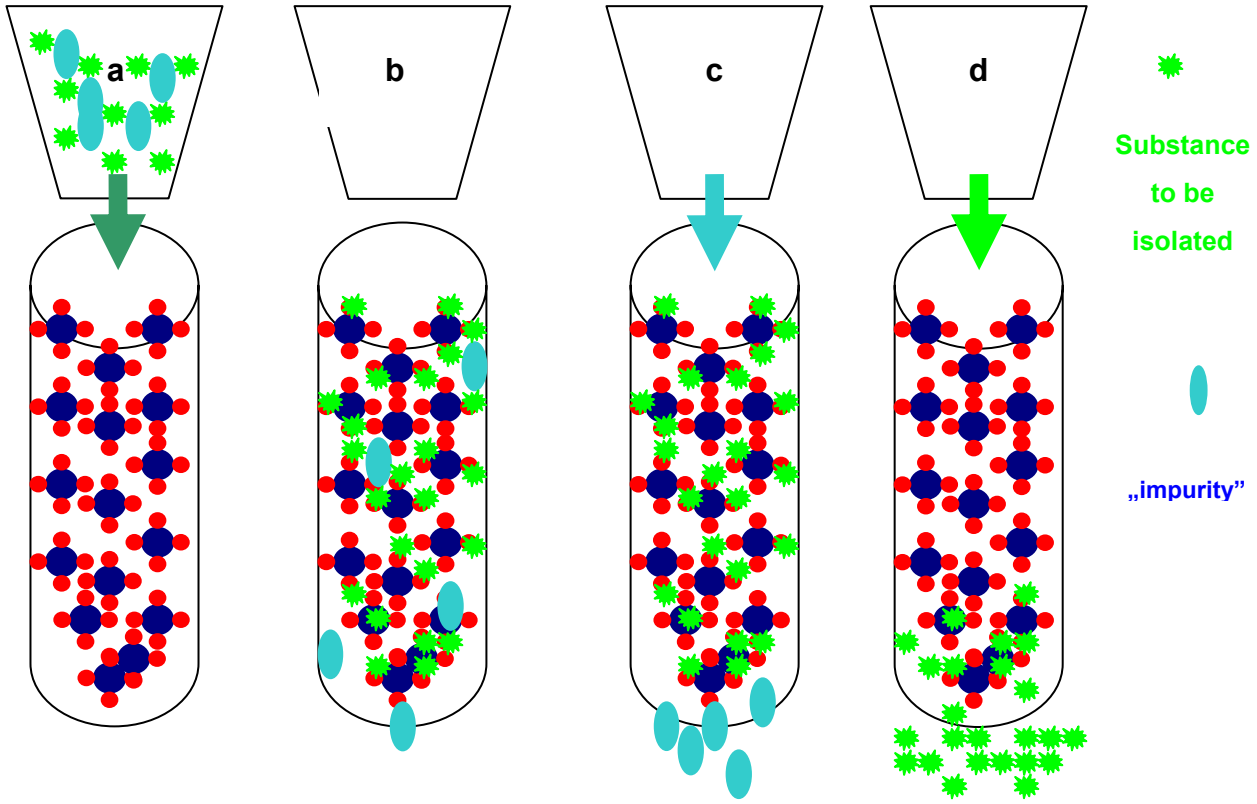


Fig 2: The principle of the affinity chromatography

a Sample (Substance to be isolated + “impurities”) application **b** Immobilized ligand specifically absorbs the substance to be isolated **c** Elution of the “impurities” **d** Desorbtion of the specific-bound substance

Table 1 shows the most important biological systems in which affinity chromatography is most frequently used.

SAMPLE SUBSTRATE	**LIGAND**
Enzymes	Substrate analogue, inhibitor, cofactor
Antibody	Antigen, cell, virus
Nucleic acid	Complementary base sequences, histone
Hormone	Receptor, carrier protein
Vitamin	Receptor, carrier protein

Table 1.: The most important biological systems used in the affinity chromatography

Bed-formed agarose gel (Sepharose) is the most fequently used matrix to which ligand molecules are immobilized. The selection of ligands is influenced by two factors: (i) the ligand should exhibit specific and reversible binding for the substance to be purified, and (ii) the ligand should have chemically modifiable groups which allow the ligand to be coupled to the matrix without losing its binding capacity. The ligand should ideally have an affinity for the substance to be purified in the range of 10^{-4}M-10^{-8}M in free solution.

$$S + L \leftrightarrow SL \; K_d = \frac{[S] * [L]}{SL}$$

If the K_d is higher than 10^{-4} M the interaction between the ligand and sample substance is likely to be too weak to perform an efficient affinity chromatography. On the other hand, if K_d value is less than 10^{-8} M, elution of the bound substance without inactivation is likely to be difficult. The substance to be purified (S) is incubated with the affinity matrix under conditions favorable for the S/ligand interaction. The unbound substances and impurities are washed out of the matrix and finally S is eluted using conditions favorable for the dissociation of the S-L complex. This can happen either by changing the temperature, pH, and ionic strength of the buffer used, or by applying an unbound ligand or ligand analogue in the elution buffer to compete with the matrix- bound ligand.

The purification of trypsin by affinity chromatography

The soybean trypsin inhibitor (Kunitz Soybean Inhibitor, $SBTIA_2$, Mw. about 22 kD) inhibits the enzyme by a competitive type of inhibition. A stoichiometric complex is formed. The higher the pH value (range of 4.5-8.0), the faster the formation of the trypsin-$SBTIA_2$ complex. This complex can be destroyed by lowering the pH of the medium (dissociation of the complex is very fast at pH=2.0).

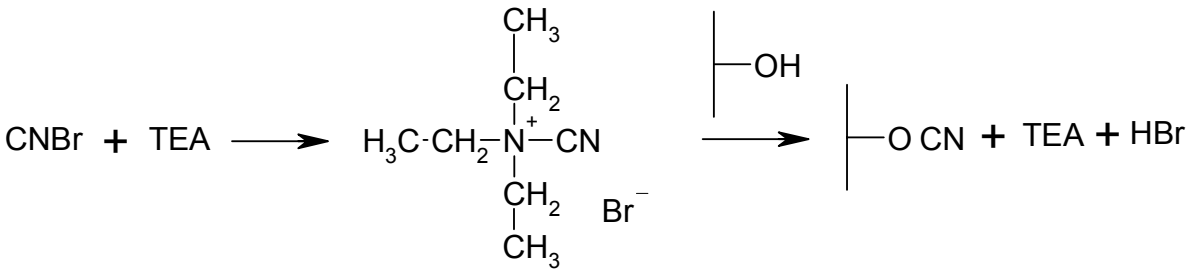
Reagents used:

1. Affinity column packed with soybean-trypsin-inhibitor-Seph-arose 4B resin
2. 0.1 M TRIS-HCl buffer pH=8.5
3. 0.1 M Na-formate buffer pH=4.5
4. 0.1 M Na-formate buffer pH=2.6
5. BAPNA solution (O.1 mM). BAPNA = (N-α_-benzoyl-DL-arginin-p-nitroanilid) was dissolved in solution of 0.1 M TRIS-HCl 1 % dimethylsulfoxyd pH=8.5 (Prepare a solution of 100 mM BAPNA in dimethylsulfoxyd and finally dilute it 100 fold in the buffer of 0.1 M TRIS-HCl).
6. mixture of trypsin (1 mg/ml) and bovine serum albumin (BSA) 10 mg/ml.

Synthesis of soybean-trypsin-inhibitor-Sepharose 4B resin

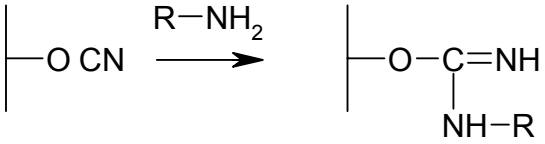
Soybean-trypsin-inhibitor-Sepharose 4B resin was prepared according to the following procedures and equations: (i) activation of Sepharose 4B matrix with BrCN under the catalytic influence of triethylamine (TEA), and (ii) coupling of ligand to the activated resin.

Activation of Sepharose 4B matrix



Temporary triethylammoniumnitril complex

The immobilization of ligand



R = soybean trypsin-inhibitor

Itinerary of the experiment

1. A column of $SBTIA_2$-Shephadex 4B is poured (1 ml bed volume) and the matrix is equilibrated with 6 ml of 0.1 M TRIS-HCl pH=8.5 buffer.
2. Load 200 µl of crude trypsin solution on the column. The column is washed to remove unbound proteins ("impurities") with the following buffer solution.

   Begin to collect 1 ml of fractions.

   (i) 3 ml of 0.1 M TRIS-HCl buffer pH=8.5

   (ii) 3 ml of 0.1 M Na-formate buffer pH=4.5
3. The bound trypsin is eluted with 5 ml of 0.1 M Na-formate buffer pH=2.6 with continued fraction collection.

Re-equilibrate the matrix by washing with 10ml of 0.1 M TRIS-HCl buffer pH=8.5

Determination of the tryptic activity and the protein content of the collected chromatographic fractions

Detection of protein content of fractions

Measure the absorption of the fractions at 280 nm. Distilled water serves as a blank. Determine the absorbance of buffers applied in the washing process and elution. Subtract these values from the corresponding absorption values. Plot the OD_{280nm} values as a function of fraction number.

Determination of the tryptic activity of fraction with BAPNA solution

Tryptic activity is determined by the amidolytic effect of trypsin on small peptide substrate BAPNA. Trypsin hydrolises the substrate, leading to the formation of p-nitroanilid with a new absortion value at 405 nm. The rate of hydrolysis depends on the tryptic activity of the collected fractions. Calculate the tryptic activity of samples in U/ml. 1 U of activity = conversion of 1 µmol substrate/min, the extinction coefficient of p-nitroanilid:

$\varepsilon = 8270\ M^{-1} * cm^{-1}$

Calibrate the photometer at 405 nm against 1 ml of BAPNA solution. Pipette 100 µl aliquotes from all fraction to the BAPNA solution and read the optical density at 405 nm after 5 minutes. Calculate the enzyme activities in U/ml of the trypsin containing fractions according to the following equation:

$$Acticity = \frac{\Delta E \bullet 10^6 \bullet V}{\varepsilon \bullet l \bullet v_o}$$ Where

V stands for the reacting volume: [ml]

$\boldsymbol{\varepsilon}$ is 8270 $M^{-1} * cm^{-1}$ (molar extinction coefficient)

l is 1 cm

$\boldsymbol{v_o}$ denotes the volume of enzyme used in the experiment

Summary of the result:

Fraction#	puffer pH	OD_{280nm}	Tryptic activity
1.	8.5		
2.	8.5		
3.	8.5		
4.	4.5		
5.	4.5		
6.	4.5		
7.	2.6		
8.	2.6		
9.	2.6		