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Sorption of Lysozyme onto ter-Polymeric Cation-Exchanger and Lytic Activity of Desorbate

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Abstract **– Lysozyme (muramidase) is a well known antibacterial biopolymer which can be used in pharmaceutical and food technology. Synthesized carboxylic ion-exchanger on the base of acrylic monomers was used in experiments for lysozyme sorption study from model solutions. Ionization of ion-exchanger functional groups was studied by potentiometric titration in 0.1 M and 1.0 M NaCl solution. The results were correlated with the influence of solution ionic strength on sorption of lysozyme at different pH values. The data obtained suggested to choose desorption condition. Lytic activity of lysozyme in desorbates was evaluated using *Micrococcus lysodeiktikus* lyophilized cells. The results testified high recovery of lysozyme using macroreticular poly(methacrylic acid-co-acrylic acid) ter-polymeric ion-exchanger and preservation of enzymatic activity of lysozyme in desorbate.**

Keywords **- cation-exchanger, carboxylic group, ionization, sorption, lysozyme, lytic activity**

INTRODUCTION

Preparative procedure for isolation, separation and purification of proteins is connected with several technological manipulations, such as salting and precipitation, filtration and sedimentation, dissolution, fractionation and others. Usage of sorption processes allows to essentially diminish the number of stages and to enhance the quality of a product recovered. Moreover, sorption processes are not energy expensive. The basic problem is to develop a sorbent which is most applicable for the particular process.

Sorption of proteins is a complicated process where different mechanisms are realized but depending on the structure and composition of used sorbent some of the mechanisms might be predominant. It is mostly ion exchange, electrostatic interaction, weak and strong electron donor – acceptor, as well as hydrophobic interactions. Thus, ion-exchange chromatography, affinity chromatography and hydrophobic chromatography can be used for protein and enzyme recovery in preparative scale.

Most of sorbents used in these processes are based upon natural (polysaccharide) matrix. These sorption materials have good permeability for biomolecules at the same time possessing some essential disadvantages - mostly low sorption capacity, large swelling, poor osmo-mechanical stability, and susceptibility to microbial degradation.

Another way is to develop the synthesis of new sorbents upon synthetic or combined base [1-10]. Ion-exchange resins in existing market are usually few permeable for large biomolecules.

Lysozyme (muramidase, EC.3.2.1.17) is a well known antibacterial enzyme with activity against Gram-positive and Gram-negative bacteria [2]. Lysozyme is a commercially important enzyme and is currently used in food technology as antibacterial agent and in pharmacological technology as a drug for treating ulcers and infections [1, 3]. Enzymatic activity of lysozyme is expressed by hydrolysis of linkage between N-acetylmuramic acid and N-acetylglucosamine in mucopeptide wall structure of microbial cells.

Lysozyme has been found in a wide variety of biological sources. This enzyme is discovered in chicken and goose egg white, animal tissues, human saliva, tear fluid, as well as in plants, insects and reptiles [1, 2, 3, 4, 5, 6].

Dye-ligand chromatography is rather often used for separation and purification of proteins. Dye-ligand macroreticular poly(styrene-co-divinylbenzene) micro particles coated with poly(vinyl alcohol), and cross-linked with glutaraldehyde were synthesized and a dye was immobilized on these micro particles to obtain specific lysozyme adsorbent [7].

Poly(hydroxyethyl methacrylate)/chitosan composite membrane was prepared by photo-polymerization and dye ligands were immobilized onto the membrane. Separation and purification of lysozyme from aqueous solution and egg white were investigated [8, 3].

Poly(methacrylic acid) brush grafted crosslinked – chitosan beads were prepared and adsorption of lysozyme was investigated in batch system [1].

The crosslinked micro spheres of N-vinylpyrrolidone and 2-hydroxyethyl methacrylate copolymer were treated with methacryloyl chloride, and subsequently methacrylic acid was graft-polymerized on microspheres. Grafted microspheres possessed very strong adsorption for lysozyme by right of strong electrostatic interaction [9].

Weakly acid cation-exchange derivatives of glycol-methacrylate gel Spheron 300 were prepared by carboxymethylation, succination and oxidation. The samples were tested by chromatographic separation experiments using a natural mixture of egg proteins and a synthetic mixture of albumin, chymotrypsinogen and lysozyme [10].

Carboxylic cation-exchangers for sorption of proteins was developed on the base of poly(methacrylic-co-acrylic acid) having macroreticular and macroporous structure [11]. These sorbents synthesized as spherical beads were permeable for bovine serum albumin, the sorption of which was differently influenced by neutral salt concentration at different pH value [12]. Also the change of physico-chemical parameters with the increase in ionization degree has a complicated character [13].

In the present study the regularities of lysozyme sorption onto synthesized carboxylic cation-exchanger in context of functional group condition have been considered. Lytic

activity of initial solution and desorbates has been evaluated using *Micrococcus lysodeiktikus* lyophilized cells.

MATERIALS AND METHODS

Carboxylic cation-exchanger (in the present study named as K-120) was synthesized by water-suspension polymerization. Briefly, functional monomers were methacrylic and acrylic acids (molar ratio 3:1 accordingly), crosslinking agent was triethyleneglycol dimethacrylate (15% in monomer mixture). Benzoyl peroxide was used as polymerization initiator. Polymerization took place in the presence of a diluent.

Unsaturated acids were purified by vacuum distillation, triethyleneglycol dimethacrylate was treated with 1 N NaOH to extract stabilizer and then washed with water until the neutral reaction. All other reagents, GR, were used as received.

Suspension polymerization was carried out in glass reactor, stirring of polymerization mixture being regulated to obtain polymeric beads diameter predominantly 0.3-0.6 mm in air-dried state.

Potentiometric titration of cation-exchanger was conducted in 0.1 N NaCl or 1.0 N NaCl solution containing different amount of 0.1 N NaOH. 100 mg of air-dried sorbent with the known mixture was in a contact with 10 ml of the titrant. A separate sampling method has been used; equilibrium was achieved after 15 days.

Sorption of lysozyme was studied in batch experiments. Before sorption the samples of cation-exchanger were equilibrated with buffer solution at the desired pH value. Samples were mixed using Orbital Shaker OS-10 (Biosan). Lysozyme from hen egg white (muramidase) – M ~ 14600 (Fluka) (IP 10.4) was dissolved in 0.05 M buffer solution, and NaCl was added to achieve the desired concentration.

Sorption experiments were done in 0.05 M acetate (pH 5.0, 5.5) or phosphate (pH 6.0-8.0) buffer solution with definite NaCl concentration. Lysozyme concentration was 1 mg/ml. 50 mg (or 500mg in experiments at optimal conditions) of air-dried sorbent with the known moisture was located into flasks containing 10 ml of the solution and shaken at 110 rpm during 1 h. Desorption solution contained 0.2 M of phosphate salt and 0.3 M of NaCl, pH being 9.0. Desorption of lysozyme was done after washing the isolated beads with fitting buffer solution. Filtered samples were located in flasks with 10 ml of desorption solution and shaken during 0.5 h. Protein concentration in the solutions was determined by biureth reaction using Benedict solution.

Sorption capacity was calculated as:
$SC = (CxV) / mx[(100-W)x10^{-2}]$, mg/g; where C – a) difference of protein concentration in initial solution and in solution after sorption, mg/ml; b) protein concentration in desorbate, mg/ml; V – a) volume of sorption solution, ml; b) volume of desorbate, ml; m – air-dried sorbent mass, g; W – moisture content in the sorbent, %.

Lytic activity of solutions was evaluated using *Micrococcus lysodeikticus* lyophilized cells (SIGMA) dispersed in 0.1 M phosphate buffer, pH 7.0. Optical density decrease was fixed during 3 min at 450 nm using Spectrophotometer YENWEY 6300.

Lytic activity was calculated as:

$LA = (\Delta OD_{450} \text{ x } F)/0.001C$, U/mg; where ΔOD_{450} – optical density decrease in 1 min; F – dilution factor; C – protein mass, mg; 0.001 – optical density decrease which corresponds to 1 activity unit.

RESULTS

Sorbent beads permeability for large molecules is the structural factor that is of great importance for the sorption of proteins. Synthesized beads of carboxylic cation-exchanger K-120 – crosslinked poly(methacrylic-co-acrylic acid) - possesses microglobular structure with channels inside (Fig.1), the size of which allows to propose that they have to be permeable for large molecules.

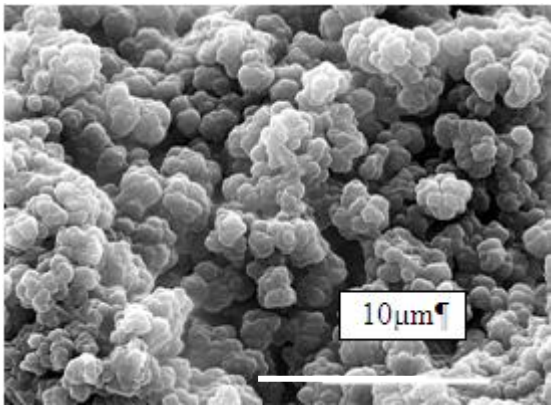

Fig.1. SEM image of cation-exchanger K-120 bead inside

The main physico-chemical parameters of the synthesized cation-exchanger are:

- total exchange capacity in 0.1 N NaOH - 10.2 meq/g;
- specific density - 0.43 g/ml;
- coefficient of swelling in water - 1.4;
- specific swelling in water - 3.2 ml/g;
- specific swelling in 0.1 N NaOH - 6.1 ml/g.

The most important characteristic of an ion-exchanger is its ionization. Ionization process can be described by several methods, potentiometric titration being the most popular. Potentiometric titration curves are usually obtained by the usage of separate samples method, the equilibrium being achieved after 10-15 days. Ionization parameters of polyelectrolyte can be found from titration curves using Henderson-Hasselbach equation for weak electrolytes which contains Gregor constant n (nonideality constant) [14]:

$$pH = pK_{app.} - n \lg [(1-\alpha)/\alpha];$$

where α – ionization degree;
$pK_{app.}$ – apparent ionization constant;
n – nonideality coefficient.

Representing titration results in pH – $\lg[(1-\alpha)/\alpha]$ coordinates, it is possible to find ionization parameters of ion-exchanger, that is half – ionization constant (pK_{app} at $\alpha = 0.5$) and coefficient n, which characterises mutual influence of functional groups during ionization, or, in other words, the intensity of ionization constant changes depending on ionization degree. As experiments show, the most intensive

change of ionization constant takes place at the beginning and at the end of titration ($0.2 \geq \alpha \geq 0.8$). Therefore ionization parameters of cation-exchanger K-120 have been estimated by potentiometric titration in the interval $0.3 \leq \alpha \leq 0.7$ (Fig.2). To estimate the influence of neutral salt concentration on ionization of cation-exchanger titration has been done in solutions containing 0.1 M and 1.0 M NaCl.

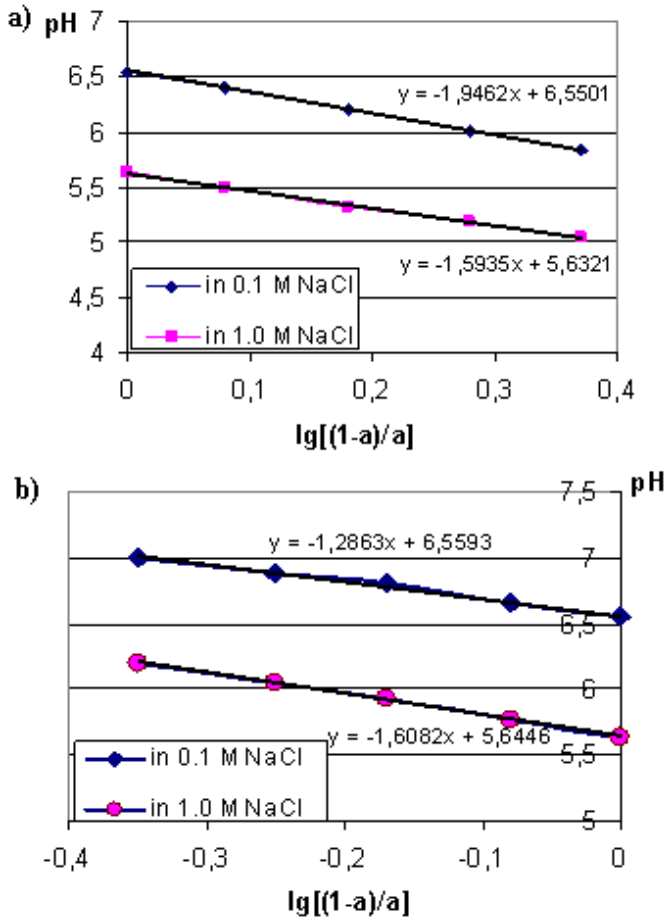

Fig.2. Potentiometric titration curves of cation-exchanger K-120: a) $\alpha \leq 0.5$; b) $\alpha \geq 0.5$

Ionization parameters of carboxylic cation-exchanger depend essentially on ionic strength of a solution. Tenfold increase of neutral electrolyte concentration caused also tenfold increase of K-120 carboxylic groups' ionization at $\alpha = 0.5$, that is cation-exchanger became more acidic.

The value of parameter n also differed depending on neutral electrolyte concentration. Moreover, titration in 0.1 M NaCl solution showed that n value at $\alpha \geq 0.5$ was smaller than at $\alpha \leq 0.5$ (Table 1). The same phenomenon was noticed when cation-exchangers of similar structure were titrated [11]. Small difference in methacrylic and acrylic acid pK value did not express in potentiometric titration.

TABLE 1

IONIZATION PARAMETERS OF K-120

NaCl concentration, M	$pK_{app.}$ at $\alpha = 0.5$	n before $\alpha = 0.5$	n after $\alpha = 0.5$
0.1	6.6	2.0	1.3
1.0	5.6	1.6	1.6

Sharp sensibility of the condition of functional groups to the composition of a solution defines criteria of sorption – desorption process.

Sorption of lysozyme from the model solution depends on pH value and neutral salt concentration (Fig.3).

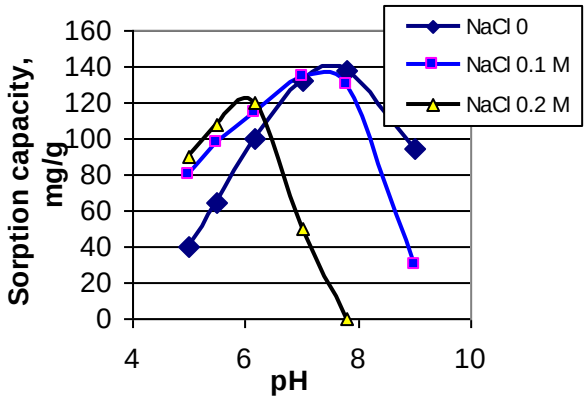

Fig.3. Sorption of lysozyme at different pH values and NaCl concentration

Neutral salt concentration increase from 0 till 0.2 M caused the decrease of maximal sorption value and the deviation of acidic area.

More detailed dependence is shown in Fig.4. NaCl concentration increase from 0 till 0.2 M aroused the increase of lysozyme sorption at pH 5.0 and pH 5.5. At the pH interval 7.0 – about 8.0 NaCl concentration increase to 0.1 M influenced little lysozyme sorption capacity, but further increase to 0.2 M caused sharp decrease of sorption value.

This phenomenon might be explained by assumption of dominant role of ion interaction in the sorption process taking place in the system carboxylic sorbent – basic protein. As potentiometric titration showed, neutral salt concentration increase caused the increase of cation-exchanger acidity, that is, ionization of carboxyl groups. Obviously, this factor had negative effect on lysozyme sorption at higher pH when the carboxylic groups of ion-exchanger were substantially ionized. It also has to be mentioned the response of charged groups in lysozyme to solution ionic strength increase. The molecule of lysozyme has 10 ionizable carboxylic groups; some of them have highly abnormal pK values: 2.0 and 6.5, while the others vary between 3.5 and 5.5 [15].

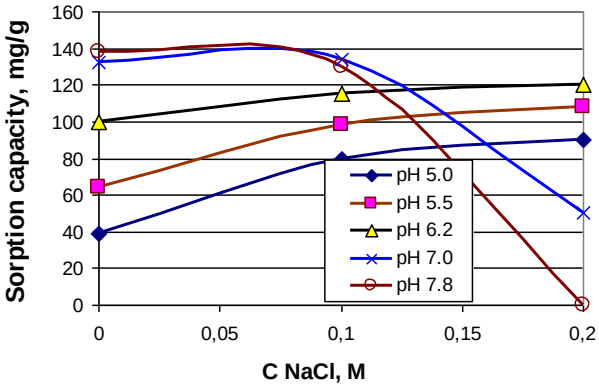

Fig.4. Influence of NaCl concentration on sorption of lysozyme at pH from 5.0 till 7.8

The obtained data allowed choosing optimal conditions for lysozyme sorption and desorption processes. pH value interval 7.0-7.3 and NaCl content 0.1 M had been chosen for further sorption experiments.

Desorption of lysozyme seemed to be more effective in basic solution rather than in acidic. Moreover, neutral salt concentration increase had to facilitate the desorption process. Really, the desorption solution containing 0.2 M phosphate and 0.3 M NaCl was more effective than phosphoric acid solution which had to be at least of 1 M concentration for quantitative desorption (Fig.5).

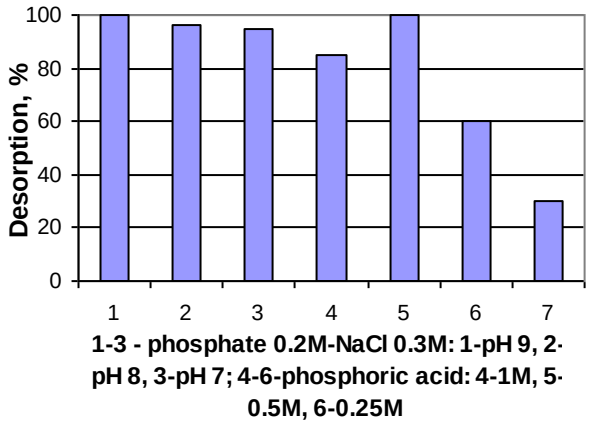

Fig.5. Desorption of lysozyme from cation-exchanger K-120

To evaluate enzymatic activity of lysozyme, lysis of *Micrococcus lysodeikticus* cells has been watched as OD diminishing during first 3 minutes from the beginning of cells dispersion contact with the solution tested. Cell concentration was chosen to be sufficient to ensure OD of about 1.500. Optimal concentration of lysozyme was found experimentally in order to provide constant OD decrease during 3 min. This concentration was 0.1 mg/ml (Fig.6).

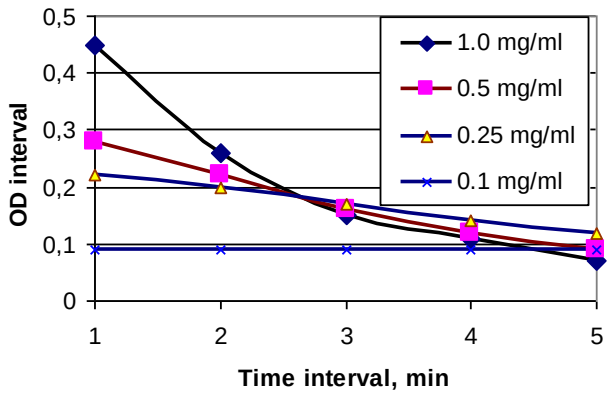

Fig.6. Lysis of *Micrococcus lysodeikticus* cells by different concentration of lysozyme

To evaluate lytic activity of lysozyme in desorbates, sorption experiments were done in optimal conditions: pH 7.1 and NaCl concentration 0.1 M. Desorption of sorbed lysozyme in batch system with definite amount of solution containing 0.2 M phosphate and 0.3 M NaCl and having pH 9.0 was accompanied by the recharging of cation-exchanger. The optimal amount of desorption solution ensured pH value of desorbate equal to 7.0. In batch system with mixing, lysozyme sorption from the solution having initial concentration 1 mg/ml onto cation-exchanger K-120 beads was almost quantitative. Sorbent: solution ratio was 0.5g : 10 ml. Residual concentration of lysozyme was only 2-5 % of initial concentration.

Lytic activity of the initial solution of lysozyme was near the value specified by manufacturer. Lysozyme activity in the solution after sorption was substantially smaller, but lytic activity of desorbates was a little larger than the initial activity (Table 2). This might testify the purification of lysozyme to some extend during sorption – desorption processes without denaturation.

TABLE 2

LYTIC ACTIVITY OF LYSOZYME CONTAINING SOLUTIONS IN SORPTION – DESORPTION PROCESS

Initial solution, U/mg	Solution after sorption, U/mg	Desorbate, U/mg
90900-96000	34500-37000	96500-97400

CONCLUSIONS

Sorption of lysozyme onto synthesized carboxylic ion-exchanger K-120 – poly[(methacrylic acid -co- acrylic acid) – triethyleneglycol dimethacrylate] depends essentially from the medium composition.

The data of potentiometric titration of ion-exchanger testify sharp decrease of $pK_{app.}$ value at ionization degree equal to 0.5 caused by the increase of neutral salt concentration: the increase of NaCl concentration from 0.1 M to 1.0 M aroused tenfold increase of functional groups ionization. The character of mutual influence of functional groups during ionization also changes, that is expressed in the change of parameter n value in Henderson-Hasselbach equation.

The data obtained allow elucidation of the results of lysozyme sorption at different pH values and NaCl concentration connecting with the change of sorbent functional groups ionization. The regularities testify the predominant role of ionic mechanism of lysozyme sorption onto ion-exchanger K-120.

The results obtained indicate high level of lysozyme recovery from model solution at optimal sorption conditions.

Desorption of lysozyme is more effective in basic solution rather than in acidic. The combination of pH and NaCl concentration increase allows to achieve quantitative desorption with preservation of lysozyme lytic activity.

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Valentīna Krilova. Līzocīma sorbcija uz ter-polimērā katjonīta un desorbāta lītiska aktivitāte

Ferments līzocīms ir labi zināms kā antibakteriālais biopolimērs, kurš ir atrodams dažādos bioloģiskajos objektos: olu baltumā, dzīvnieku audos, augos un citos. Dotajā darbā parādīta iespēja izmantot uz akrila monomēru bāzes sintezētu karboksilkatjonītu līzocīma izdalīšanai no modeļa šķīdumiem. Sintezētais karboksilkatjonīts K-120 ir makroretikulārais metakrilskābes un akrilskābes ter-polimērs, sfēriskām granulām piemīt poraina struktūra. Tā kā proteīnu sorbcijas uz karboksilkatjonītiem dominējošais mehānisms ir jonu mijiedarbība, dotajā procesā īpaša nozīme ir sorbenta un sorbāta funkcionālo grupu jonizācijai. Katjonīta K-120 jonizācija izpētīta lietojot potenciometriskās titrēšanas metodi. Parādīta būtiska katjonīta funkcionālo grupu skabacitātes palielināšana, ko izraisa šķīduma jonu spēka paaugstināšana. NaCl koncentrācijas palielināšana no 0.1 M līdz 1.0 M izraisa desmitkārtīgu funkcionālo grupu jonizācijas palielināšanu pie jonizācijas pakāpes 0.5. Izteikta katjonīta funkcionālo grupu jūtība pret šķīduma sastāvu ļauj smalki regulēt sorbcijas-desorbcijas procesu. Parādīts kā pH lieluma un NaCl koncentrācijas izmaiņa ietekmē līzocīma sorbciju. Sāls koncentrācijas palielināšana līdz 0.2 M izraisa sorbcijas maksimālā lieluma samazināšanu un optimālas pH nobīdi skābā pusē. Sāls koncentrācijas palielināšana līdz 0.1M, kad $pH<pH_{opt}$, izraisasorbcijas nelielu paaugstināšanu. Koncentrācijas paaugstināšana līdz 0.2 M, kad $pH>pH_{opt,}$ izraisa asu sorbcijas lieluma samazināšanu. Līzocīma desorbcija notiek efektīvāk palielinot pH, nekā samazinot. Līzocīma kvantitatīva desorbcija notiek, lietojot 0.2 M fosfāta un 0.3 M NaCl saturošu šķīdumu (pH 9.0), pie kā desorbāta pH var pazemināt līdz 7.0 sorbenta karboksilgrupu pārlādēšanas rezultātā.
Līzocīma šķīdumu enzimātiskās aktivitātes novērtēšanai tika izmantots *Micrococcus lysodeiktikus* liofilizēto šūnu līzis. Eksperimentāli atrasta optimāla šūnu un līzocīma koncentrācija. Līzocīma sorbcija optimālos apstākļos un sekojoša desorbcija raksturojas ar līzocīma augstu izdalīšanas pakāpi un enzimātiskās aktivitātes pilnu saglabāšanu.

Валентина Крылова. Сорбция лизоцима на тер-полимерном катионите и литическая активность десорбата

Фермент лизоцим является известным антибактериальным биополимером, обнаруженным в различных биологических объектах: в яичном белке, тканях животных, некоторых растениях и т.д. В данной работе приводятся результаты по использованию карбоксильного катионита, синтезированного на основе акриловых мономеров для сорбционного выделения лизоцима из модельных растворов. Синтезированный карбоксильный катионит К-120 является макросетчатым тер-полимером метакриловой и акриловой кислот, сферические гранулы которого обладают пористой структурой. Поскольку доминирующим механизмом сорбции белков на карбоксильных катионитах является ионное взаимодействие, в данном процессе особое значение имеет ионизация функциональных групп как сорбента, так и сорбата. Ионизация катионита К-120 изучена методом потенциометрического титрования. Показано значительное увеличение кислотности функциональных групп катионита с повышением ионной силы раствора. Увеличение концентрации соли с 0.1 М до 1.0 М вызывает десятикратное увеличение ионизации функциональных групп при степени ионизации 0.5.
Выраженная чувствительность состояния функциональных групп катионита к составу раствора позволяет тонко регулировать процесс сорбции-десорбции. Показано, как влияет изменение величины pH и концентрации хлорида натрия на сорбцию лизоцима. Увеличение концентрации соли до 0.2 М вызывает уменьшение максимальной величины сорбции и сдвига оптимума pH в кислую область. При значениях $pH<pH_{опт.}$ увеличение концентрации соли до 0.1 М вызывает небольшое увеличение сорбции. При $pH>pH_{опт.}$ повышение концентрации до 0.2 М вызывает резкое уменьшение величины сорбции. Десорбция лизоцима более эффективна при повышении величины pH, чем при понижении. Использование раствора, содержащего 0.2 М фосфата и 0.3 М хлорида натрия, имеющего pH 9.0, позволяет количественно десорбировать лизоцим; при этом pH десорбата можно понизить до 7.0 за счёт перезарядки карбоксильных групп сорбента.
Для оценки энзиматической активности растворов лизоцима использовался лизис лиофилизированных клеток *Micrococcus lysodeiktikus*. Экспериментально найдена оптимальная концентрация клеток и лизоцима. Проведённая при оптимальных условиях сорбция лизоцима и последующая десорбция характеризовались высокой степенью извлечения лизоцима с полным сохранением его энзиматической активности.