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Sorptive Immobilization and Subsequent Release of Hen Egg Lysozyme

Valentina Krilova[1], Renate Steika[2], [1-2]*Riga Technical University*

***Abstract*: For lysozyme immobilization, synthesized carboxylic ion-exchanger has been used. Lysozyme immobilization was performed both from model solution and from diluted hen egg white. Enzymatic activity of lysozyme containing solutions was evaluated by *Micrococcus lysodeiktikus* cell lysis. Reversibly conjugated lysozyme release was ascertained to depend on the contacted medium composition. Lysozyme release from conjugates was stable for the duration of experiment (11 days). To evaluate the stability of conjugate over time, samples were stored at ambient temperature for 72 days. Lytic activity of desorbates was practically unchanged as compared to the initial activity.**

***Keywards*: lysozyme, immobilization, sorption, lytic activity.**

I. INTRODUCTION

Enzymes can be considered formations, which not only play an outstanding role in living organisms, but also are of significant importance in technology.

The use of different enzymes in food production and pharmaceutical technology, in chemical processing and analytics has become the usual practice nowadays.

To improve enzyme properties (usually – its stability) and to achieve the possibility of reusing for several technological cycles, enzyme immobilization is used. The technique of enzyme immobilization can be divided, according to an immobilization mechanism, into covalent immobilization and non-covalent one. Non-covalent immobilization offers the simplest approach, while the other usually involves complicated chemistry to produce covalent linkages between enzyme and support [1]. The immobilization technique, depending on support and enzyme nature, can be justified by ionic or polar interaction, hydrophobic interaction or the combination of the mechanisms. Immobilized enzymes may exhibit much better functional properties than the corresponding soluble enzymes [2].

Immobilized enzymes have a broad application in contemporary research, with the projection to expand and promote their use in future. The opportunity of use of immobilized proteins in bioreactors as biosensors, biochips, food pacing material and so on is intensively studied [2, 3, 4, 5, 6, 7, and 8].

For protein sorptive immobilization, different supports can be used, both native and synthetic. High mechanical resistance, large number of functional groups, good osmotic stability can be attributed to the advantages of synthetic support materials.

Macroporous carboxylic ion-exchange resins based on (meth)acrylic acid, cross-linked by ethyleneglycol dimethacrylate or methylene bisacrylamide were used for penicillin G acylase immobilization. The developed catalytic process allowed implementing up to 15 reaction cycles [9].

The understanding and control of the interaction between protein and support is of critical significance for protein immobilization. This approach has been successfully used for beta-galactosidases purification and immobilization on anion-exchangers – activated agarose [5]. Protein reactivity maintenance requires its native state preservation, which should not be altered by immobilization. This demand is problematic in covalent binding of proteins when reactive sites can be blocked by immobilization procedure, resulting in reduced activity of the protein [4]. The modification of polyurethane surface by a poly(ethylene glycol) layer and a chelator (quinolin-8-ol) ensured a relatively high target protein binding capacity. This approach ensured the bioactivity of immobilized proteins [4].

Immobilization of enzymes can furnish antimicrobial activity of conjugate formed. Lysozyme from hen egg white is the most appropriate enzyme for antimicrobial usage.

Lysozyme (muramidase, EC.3.2.1.17) performs its antimicrobial activity via lysis of the linkage between N-acetylmuramic acid and N-acetylglucosamine in a bacterial cell wall. Lysozyme isolated from hen egg white and other sources has a broad potential in food and clinical applications. Enzymatic lysis of microbial cell wall offers an advantage over mechanical disruption methods. Immobilized lysozyme is gaining importance in minimizing the microbial load during continuous operation of a bioreactor [10]. Lysozyme was immobilized on poly(hydroxyethyl methacrylate) film modified by covalently attached dye Procion Green. The resulting film shows bacteriolytic property and is a suitable candidate for hydrolysis of microbial cells in food industry [10].

It is expected that research and development of antimicrobial materials for food applications will grow in the next decade with the advent of new polymeric materials and antimicrobials [6].

The use of antimicrobials is subjected to the aim of the process developed. For the application of lysozyme in food industry, the use of partially purified lysozyme preparations obtained by cheaper and faster methods would be economically more feasible [11, 12].

The degree of sorptive immobilization strength is influenced mainly by a sorption mechanism, and the ionic interaction lead to the most resistant links. For the purpose of ion interaction, sorptive material must have ionizable functional groups, and carboxylic groups may be considered the most appreciable ones for sorption of basic protein lysozyme. Commercial ion-exchangers, such as Amberlite

IRC-50, Amberlite IRP-64 or similar ones, can be used for lysozyme immobilization.

To enhance accessibility of functional groups, poly(methacrylic acid) grafting to a natural support can be done. Poly(methacrylic acid) grafted cross-linked chitosan beads were found to link 65.7 mg/g of lysozyme [13].

The comparison of three types of 2-hydroxyethyl methacrylate gels: neutral, acidic (+ acrylic acid), and basic (+ dimethylaminoethyl methacrylate), showed that lysozyme uptake by the acidic gels was more rapid than that by the neutral gels. To sorb 90% of the protein, only 1 h was required for the acidic gels, but 15 days – for neutral gels. Lysozyme did hot adsorb into the basic gels [14].

Synthesized on the base of acrylic monomers carboxylic ion-exchanger having a porous structure and macroreticular network with enhanced content of cross-linking agent triethyleneglycol dimethacrylate was shown to exhibit high and reversible sorption of lysozyme [15]. The regularities of lysozyme sorption into a synthesized carboxylic ion-exchanger can be interpreted in the context of functional group condition. The data obtained allow elucidating the results of lysozyme sorption at different pH values and NaCl concentration connecting with the change of sorbent functional group ionization. The regularities testify the predominant role of ionic mechanism of lysozyme sorption onto a synthesized ion-exchanger.

The studied regularities are fruitful in polymer–lysozyme conjugate design or sorptive immobilization of lysozyme for different purposes.

The present paper studies the lytic activity of synthesized carboxylic ion-exchanger conjugates with model lysozyme and lysozyme isolated from hen egg white.

II. MATERIALS AND METHODS

Carboxylic ion-exchanger K-120 contained the units of methacrylic and acrylic acid and triethyleneglycol dimethacrylate (15% in monomer mixture). The diameter of porous spherical beads was 0.2-0.3 mm. Static exchange capacity in 0.1 N NaOH was 10.2 m-equiv/g.

Before sorption, K-120 beads were equilibrated with 0.04 M phosphate buffer solution having pH 7.3. Sorption of lysozyme was studied in a batch condition using axial rotation. 1 ml of equilibrated sorbent was mixed with 10 ml of model solution of lysozyme.

Lysozyme from hen egg white (Fluka), (M~14600, IP 10.4) was dissolved in 0.04 M phosphate buffer solution, pH 7.0.

Protein concentration was measured photometrically (spectrophotometer YENWEY 6300) using the Benedict method.

Hen egg white (HEW) was manually separated from yolk, diluted with 0.04 M phosphate buffer in mass proportion 1:3 and homogenized on the magnetic stirrer. Filtered mixture was used for sorption experiments. After sorption an ion-exchanger (0.5 ml samples) was filtered through a nylon filter, washed with 0.05 M NaCl, then with distilled water and transferred into a flask having 10 ml of 0.2 M phosphate solution containing 0.3 M NaCl and having pH 9. The mixture was mixed for 1 h.

Lytic activity (LA) of lysozyme in solutions was evaluated using *Micrococcus lysodeiktikus* lyophilized cells (SIGMA) dispersed in 0.1 M PBS, pH 7.0. The decrease in optical density was fixed for 3 min at 450 nm.

Lytic activity was calculated as follows:

LA = (ΔOD_{450} x F)/0.001 x C, where ΔOD_{450} – the decrease in optical density for 1 min; F – the dilution factor; C – the protein concentration; 0.001 – decrease in optical density, which corresponds to 1 unit of activity.

Lytic activity of lysozyme calculated with the aim to evaluate its release into the contacting medium was as follows:

LA = (ΔOD_{450} x F)/0.001.

Lysozyme release was controlled for 3 h. 0.5 ml of conjugate was axially mixed with 20 ml of a solution, and 0.2 ml portions were analysed every 30 min.

To evaluate cell lysis caused by a direct contact of conjugate, a decrease in the number of *Micrococcus lysodeikticus* cells was controlled for 5 min contact of lysozyme conjugate with 10 ml portions of cell dispersion.

Micrococcus lysodeikticus cell lysis by immobilized (onto 1ml of sorbent) and non-immobilized lysozyme was defined in a constant system having 50 ml of phosphate buffer and 50 ml of cell dispersion.

III. RESULTS

Bioactive protein immobilization onto different supports is undoubtedly perspective for future technology. Enzyme properties have to be usually improved before their implementation at industrial scale where many cycles of high yield process are desired [2]. The main problem is to find an appropriate support and immobilization protocol. Sorptive immobilization of enzymes can be considered less traumatic for enzyme and easier in comparison with covalent immobilization. Adsorption of proteins is governed by different mechanisms, the implementation of which depends on characteristics of the protein, as well as nature, condition and number of sorbent functional groups and the medium composition and temperature. Depending on a sorption mechanism, the stability of complex formed may be different and, accordingly, desorption may take place at different conditions.

The use of immobilized enzyme in technology provides for its controllable release in an exploitation condition or functioning in a conjugated form. This signifies that sorptive immobilization must be rather strong, and multipoint coupling to an ion-exchanger having a high content of functional groups may be the most advantageous.

High sorption capacity (330–350 mg/g) and selectivity of synthesized carboxylic ion-exchanger K-120 towards lysozyme have been ascertained.

The release of lysozyme from an ion-exchanger depended on the surrounding medium. As experiments showed, more lysozyme was released into 0.04 M phosphate buffer solution containing 0.1 M NaCl and less into 0.1 M NaCl, while the content of enzyme in 0.1 M phosphate buffer was mediocre.

All solutions had a pH value of 7. Lysozyme release was evaluated as the lytic activity of contacted solutions for 3 h, the equilibrium was achieved after 1–2 h (Fig. 1).

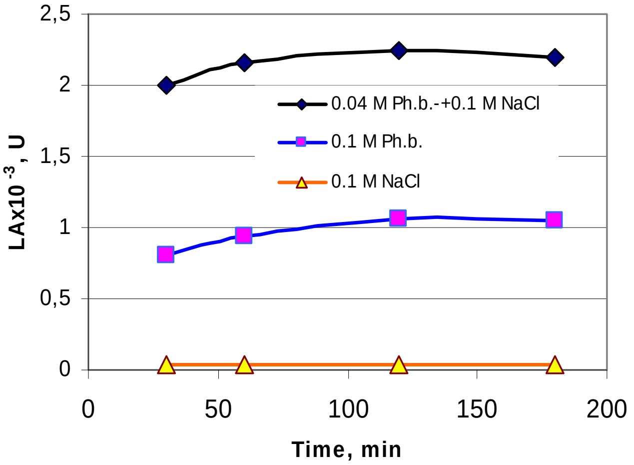

Fig. 1. Lysozyme release from the newly obtained conjugate in different solutions

The value of lytic activity of the solutions after 24 h contact testified its stability, whilst after the substitution with fresh solutions lytic activity change had similar character, but equilibrium was achieved later (Fig. 2).

Analogous experiments with conjugates achieved by lysozyme sorption from HEW showed similar regularities, but equilibrium was achieved later (Fig. 3), and the activity preservation was noticed even on the eleventh day (Fig. 4).

The equal result was obtained using conjugates loaded with 28 mg/ml and 8 mg/ml lysozyme. Moreover, the conjugates were stored for 336 h at room temperature before use. The result testified little amount of enzyme needed for lytic activity and high storage resistance of sorbent-lysozyme conjugates.

The data obtained allowed predicting much more prolonged activity of sorbent–lysozyme conjugates.

To compare the lytic activity of non-immobilized and immobilized lysozyme, cell lysis was noticed in constant volume containing dissolved lysozyme or immobilized one (Fig. 5). The result in 100 ml volume was similar. Some increase of the effect in the system comprising immobilized lysozyme might be attributed to sorption of cell debris by the sorbent. The control experiment of cell sorption showed that this fact decreased cell content in the solution by 2–7 %.

To evaluate the conjugate stability for a long time, samples were stored at ambient temperature for 1728 h. Lytic activity of desorbates was practically not changed compared to the initial activity (24095 U/mg and 23976 U/mg, respectively). Decrease in the number of *Micrococcus lysodeikticus* cells in close contact with the conjugate has the same character as in the case of newly developed conjugate.

Immobilized lysozyme can be desorbed using solution that has the enhanced salt concentration or increased pH value. As experiments showed, the most suitable composition of desorbing solution was 0.2 M potassium phosphate that contained 0.3 M NaCl and had pH 9. Under experimental conditions, desorbates had pH value of about 7.3 and preserved lytic activity. The resulting pH decrease till a neutral level was due to the high buffer capacity of ion-exchanger which, in its turn, was the result of large content of carboxylic groups in it.

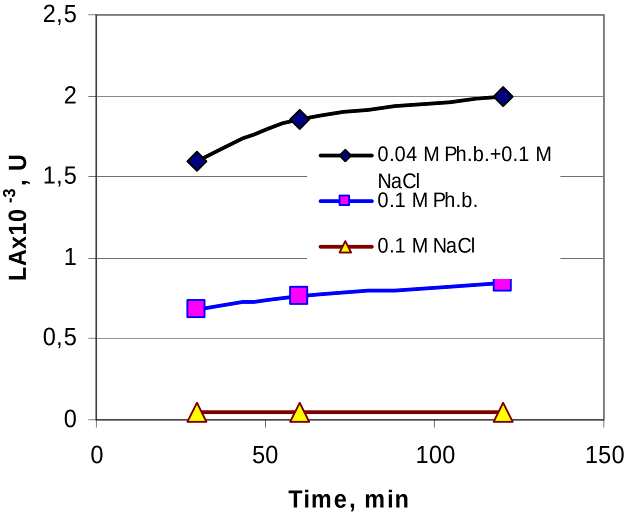

Fig. 2. Lysozyme release from conjugate into fresh solutions after 24 h storage

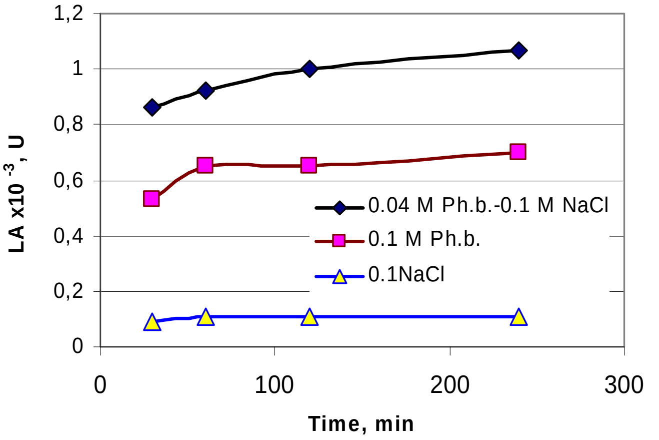

Fig. 3. Lysozyme release from conjugates obtained by sorption from HEW

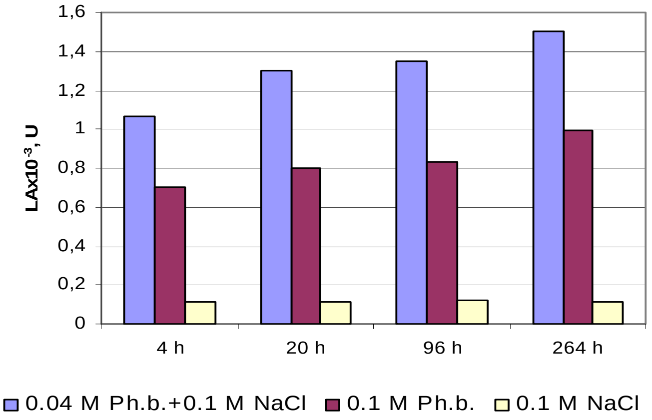

Fig. 4. Immobilized lysozyme release from conjugates stored at different points of time; lysozyme sorption was conducted from HEW

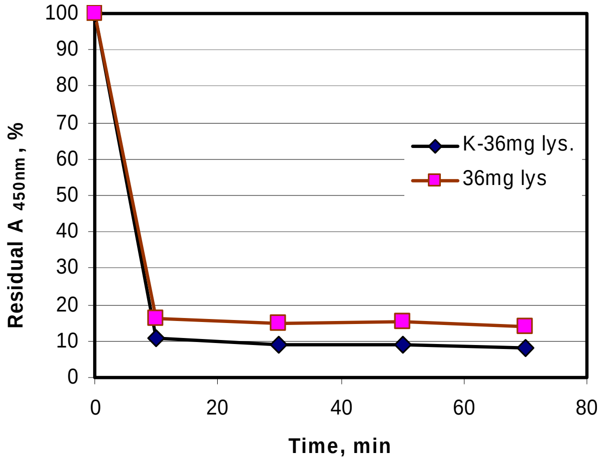

Fig.5. *Micrococcus lysodeikticus* cell lysis caused by immobilized and non-immobilized lysozyme

In accordance with sorbent K-120 structure and composition characteristics, which were discussed in previous publications, immobilized lysozyme protection might be predicted due to the buffering possibility of sorbent network possessing a high ion-exchange capacity.

IV. CONCLUSION

Synthesized porous carboxylic ion-exchanger containing methacrylic and acrylic acid units and cross-linked by long-chain triethyleneglycol dimethacrylate might be considered the appropriate support for basic enzyme lysozyme reversible immobilization. Lysozyme release from conjugates depends on the medium composition. From three media studied having an equal pH value the greatest lytic activity was noticed in the solution containing 0.04 M phosphate buffer and 0.1 M NaCl, but the lowest – in 0.1 M NaCl. Obviously, this fact can be explained by thermodynamic affinity of lysozyme to a definite level of ionic strength and concentration of K^+ and Na^+ ions, as well as by a change in cationite ionization.

The conjugate obtained by one-step sorption from diluted hen egg white demonstrates lysozyme release, which defines stable and long-term lytic activity.

When conjugate was in close contact with *Micrococcus lysodeikticus* cell dispersion, the cell lysis happened quickly and this ability was preserved for all trial time.

Immobilized lysozyme was shown to have same lytic activity as dissolved lysozyme.

Carboxylic ion-exchange K-120 used as support for lysozyme immobilization has a high buffer capacity and, therefore, may preserve enzyme from medium pH change.

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Valentina Krilova, Dr. chem., Leading Researcher at the Institute of General Chemical Engineering of Riga Technical University. Main field of study – synthesis of ion-exchange resins, the research of structure, properties and sorption processes.
Address: 14/24 Azenes Str., Riga, LV-1048, Latvia.
E-mail: v.krilova@inbox.lv

Renate Steika is a student of the Institute of General Chemical Engineering at Riga Technical University.
Address: 14/24 Azenes Str., Riga, LV-1048, Latvia.
E-mail: renate.steika@gmail.com

Valentīna Krilova, Renāte Steika. Vistu olu lizocīma sorbtīva imobilizācija un sekojoša izdalīšana

Imobilizācija uz nesēja dod iespēju paaugstināt fermenta stabilitāti un pagarināt pielietošanu tehnoloģiskajos procesos. Sorbtīva imobilizācija paredz aktīva nesēja spēju saistīt fermentu bez tā struktūras būtiskas izmaiņas un, attiecīgi, bez enzimātiskās aktivitātes samazināšanas. Lizocīma imobilizācijai izmantoja sintezētu poraino karboksilkatjonītu, saturošu metakrilskābes un akrilskābes posmus un krosaģentu trietilēnglikoldimetakrilātu; sorbenta struktūra optimizēta proteīnu sorbcijai. Karboksilkatjonītam piemīt augsta lizocīma sorbcijas kapacitāte (330-350 mg/g) un selektivitāte. Lizocīma imobilizāciju veica gan no modeļa šķīdumiem, gan arī no atšķaidīta vistu olu baltuma. Imobilizēta fermenta izmantošana tehnoloģiskajos procesos prasa to kontrolētu izdalīšanu vai spēju funkcionēt saistītā veidā. Atrasts, ka atgriezeniski imobilizēta lizocīma izdalīšana kontaktējošā vidē atkarīga no vides sastāva: vislielākā bija 0.1 M NaCl saturošā fosfāta buferšķīdumā, bet vismazākā – 0.1 M NaCl šķīdumā. Lizocīma izdalīšana tika novērota 3 stundu laikā; tā stabilitāte saglabājās pēc 24 stundām. Tas tika konstatēts, lietojot konjugātus, iegūtus, sorbējot lizocīmu no modeļa šķīduma un no atšķaidīta vistu olu baltuma. Šķīdumu lītisku aktivitāti novērtēja pēc *Micrococcus lysodeikticus* šūnu līzisa. Lizocīma izdalīšana no konjugātiem, kas iegūti, lietojot vistu olu baltumu, bija praktiski nemainīga pēc 96 un 264 stundām. Konjugātu ilgtermiņa stabilitātes novērtēšanai paraugi tika uzglabāti 1728 stundas istabas temperatūrā. Desorbātu lītiskā aktivitāte maz atšķīrās no lizocīma sākotnējas aktivitātes. Konjugātiem kontaktējot ar šūnu dispersiju eksperimenta apstākļos, izraisīts līzis notika ātri un vienādi vismaz 336 stundu laikā. Rezultāti liecina par iegūto konjugātu augstu uzglabāšanas stabilitāti. Karboksilkatjonīta bufera kapacitāte zināmā mērā pasargā konjugātu no vides pH izmaiņām. Katjonīta daudzkārtīgas izmantošanas iespēja, bufera kapacitāte, lizocīma regulējama vienstadijas sorbcija no olu baltuma ļauj prognozēt efektīvu konjugātu izmantošanu tehnoloģiskajos procesos.

Валентина Крылова. Ренате Стейка. Сорбционная иммобилизация и последующее выделение лизоцима куриных яиц

Иммобилизация на носителе позволяет улучшить стабильность фермента и повысить длительность его использования в технологических процессах. Сорбционная иммобилизация предполагает наличие активного носителя, способного связать фермент без существенного изменения его нативной структуры и, соответственно, его энзиматической активности. Для иммобилизации лизоцима использован синтезированный пористый карбоксильный катионит, содержащий звенья метакриловой и акриловой кислот и длинноцепочный кроссагент триэтиленгликольдиметакрилат; структура сорбента оптимизирована для сорбции белков.

Обнаружено, что выделение сорбированного из модельного раствора лизоцима в контактирующую среду зависит от её состава при одинаковой величине pH. Аналогичным образом проявляется литическая активность растворов, полученных после контакта с катионитом, содержащим извлечённый из разбавленного белка куриных яиц лизоцим. Литическую активность оценивали по лизису клеток *Micrococcus lysodeikticus.* Литическая активность конъюгата сохранялась в течение всего времени эксперимента.

При непосредственном контакте конъюгата с дисперсией клеток в условиях эксперимента лизис происходил быстро и одинаково в течение по меньшей мере 336 ч. При этом сходный результат наблюдался при большом и малом содержании белка в конъюгате, что свидетельствовало о достаточности малых количеств лизоцима для проявления антимикробных свойств конъюгата. Возможность многократного использования катионита, его высокая буферная ёмкость, регулируемый уровень связывания лизоцима при одностадийной сорбции из яичного белка позволяют предположить перспективность применения образованных конъюгатов в технологических процессах.