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MUCOADHESIVE GEL WITH IMMOBILIZED LYSOZYME: PREPARATION AND PROPERTIES

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The study of non-covalent immobilized lysozyme, as well as physico-chemical and biochemical properties of obtained mucoadhesive gel was the aim of the research. Lysozyme activity was determined by bacteriolytic method (*Micrococcus lysodeikticus* cells were a substrate). Lysozyme immobilization was conducted by the method of entrapment in gel. Enzyme/carrier interaction was studied by viscometric, spectrophotometric and spectrofluorimetric methods. Mucoadhesive gel with immobilized lysozyme, possessing antiinflammatory and antimicrobial activities, was prepared. Due to immobilization, protein-polymer complex with the original enzymatic activity was formed. The product is characterized by high mucoadhesive properties, quantitative retaining of protein and bacteriolytic activity, prolonged release of the enzyme, improved biochemical characteristics (extended pH-activity profile, stability in acidic medium and during storage for 2 years), and it is perspective for further studies. The proposed method for lysozyme immobilization in the carboxymethyl cellulose sodium salt gel allows to obtain a stable, highly efficient product, with high adhesive properties for attachment to the mucous membranes, that is promising for use in biomedicine.

Key words: lysozyme, immobilization, mucoadhesive gel.

The mucous membrane plays a special role in humans as it provides for them absorption the necessary materials and protection against foreign ones. However, continuous movement of mucous membrane damp surface prevents strong attaching and reliable retention of drugs. Adding of polymers prolongs the time of drugs staying on it, thus increasing their bioavailability. It has led to the creation of mucoadhesive medicinal forms, i.e. those that may be in close contact with the surfaces of the mucous membranes of the eyes, mouth, gastrointestinal (GI) tract, the respiratory system, women genitals [1].

Considering the limited number of studies in the field of lysozyme mucoadhesive forms development and widespread enzyme usage in antibacterial therapy, the elaboration of new lysozyme forms, promising for usage in dentistry and ophthalmology, is actual.

Bacteriolytic enzymes and complexes immobilization is viewed preferably concerning lysozyme. Recently its immobilized forms are obtained with following polymers usage: cellulose derivatives [2]; chitosan [3], high molecular weight polymers, activated by silane reagents [4], silicate nanoparticles [5], carboxymethyl cellulose modified, by calcium carbonate [6], polyamide [7], gelatin hydrogel [8], calcium alginate [9], cryogel of polyvinyl alcohol [10], etc. However mucoadhesive properties of polymers previously did not taken into account, besides there are no works on development of mucoadhesive gel with immobilized lysozyme for the usage in medical practice.

In this connection the development of immobilized lysozyme new forms, according to its purpose, and mucoadhesive polymeric carriers usage is perspective.

The aim of the work was the immobilization of lysozyme in carboxymethyl cellulose gel, and physico-chemical and biochemical properties of the developed mucoadhesive gel investigation.

Materials and Methods

Egg white lysozyme (EC 3.2.1.17) was used (M. m. 14.4 kDa, 68 000 units/mg;

Applichem, Belgium), cells of *Micrococcus lysodeikticus* 2665 (Sigma-Aldrich, Germany), carboxymethyl cellulose sodium salt (Na-CMC) (M. m. 125 kDa; FMCBiopolymer, Ireland).

Lysozyme activity was determined by bacteriolytic method in Na-phosphate buffer solution (NaH_2PO_4/Na_2HPO_4, pH 6.2, 0.1 mol/dm^3) using *M. lysodeikticus* 2665 cells as a substrate (1 mg/cm^3) [11]. The amount of enzyme that decreases the optical density of cell suspension by 0,001 per minute at 55 °C was taken as its activity unit — for the maximum activity of lysozyme determination, and at 37 °C — for the activity under physiological conditions of determination. Free lysozyme activity was taken as 100 % and it was compared with the activity of immobilized enzyme.

Protein content was determined by Lowry-Hartree [12].

Spectrophotometric study was performed at 25 °C using the Shimadzu UV-2401PC. Spectrofluorimetric — at a temperature of 25 °C using the Cary Eclipse Varian (Australia) with a 150 W xenon lamp. Distilled water was used to prepare Na-CMC solutions of 0.1 to 0.25% concentrations. The final concentration of lysozyme in solution was 0.02 %.

The viscosity of polymer aqueous solutions was measured using Ostwald viscometer with capillary diameter 0.73 mm at 25 °C according to [13]. The concentration of the sample solution was in the range of 0.05 to 0.25%. The measurements were performed 5 times.

Lysozyme immobilization was performed by its entrapment into the gel according to the developed technique [14]: lysozyme solution was introduced into the prepared Na-CMC gel (final concentration of the enzyme in the gel was 0.5%), thoroughly stirred. As a supplement to the gel promising for usage in dentistry, peppermint alcoholic extract (10%), glycerol (2%), chlorhexidine bigluconate (2%) were added. An alcoholic extract of peppermint was not added into the gel intended for the usage in the treatment of eye burns. The mixture was adjusted by calculated volume of water to a final concentration of Na-CMC 3% and was emulsified for uniform distribution of components in the gel, and then tightly packed.

Determination of obtained gel adhesion was performed according to [15], using TA.XT2 Texture analyzer (UK).

As a mucous membrane model the surface of the porcine small intestine was used. Before the experiment, the tissue was washed with saline, and morphologically homogeneous mucosal samples of 5 cm^2 area were excised. During the experiment mucosa was additionally moistened.

Mucoadhesive gel with a layer thickness of 0.3 mm was fixed on a moving rod and connected with mucosa. Load on the rod and retention time (60 s) were fixed. Then abruption of the rod with a fixed gel on was performed at 90° angle. Adhesion was calculated as the abruption force of rod, coated with the gel, from the mucosa.

To assess the dynamics of lysozyme release from immobilized sample physiological solution was added to the gel (volume ratio 1: 5). Solution samples of 0.1 cm^3 were taken out at regular intervals for 3 hours and bacteriolytic enzyme activity was determined.

PH-optimum of enzyme activity was established by adding substrate suspension (1 mg/cm^3) in a buffer solution of 0.1 mol/dm^3 concentration and pH in the range of 3.0 to 10.0 to the equal by activity samples of free and immobilized preparation.

To determine the pH-stability, an equal by the activity samples of free and immobilized lysozyme were incubated in Na-phosphate buffer (0.1 mol/dm^3, pH 5.5; 37 and 25 °C) for 3 h, activity was controlled at intervals of 10–30 minutes.

Thermo-optimum of specimens' enzyme activity was found by the activity of lysozyme in the temperature range of 20–80 °C.

The final immobilized preparation was stored at 4 °C. Bacteriolytic activity of immobilized lysozyme was measured at intervals of 1 month for two years.

Experimental data were subjected to statistical analysis according to [16]. The degree of difference significance of results between series of experiments at $n = 5$ number of parallels and relative to initial activity of free lysozyme (68 000 units/mg of enzyme) was estimated.

Results and Discussion

Sodium carboxymethyl cellulose is widely used in the manufacture of drugs — for oral and external use, especially to increase their viscosity; at a concentration of 4–6% it is part of ointments, pastes as hydrogel base, as well as of drugs for parenteral usage. It is applied also as binding and fluffing material in the manufacture of tablets (concentration 2.3%), of liquid medicines like anion-active emulsifier and emulsions stabilizer.

In addition, Na-CMC is one of the main ingredients of adhesive-absorbing systems in the treatment of problem wounds, for removal

of wound content, exudates etc. Through mucous binding property, Na-CMC is used to increase the stability of systems active ingredients in contact with mucous membranes, and to modify their release kinetics [17]. Considering the above, Na-CMC was selected as a carrier for lysozyme immobilization.

The completeness of lysozyme inclusion in Na-CMC was investigated at different mass ratio enzyme: polymer (Table 1).

Table 1. **Visual assessment of gel with immobilized lysozyme**

Lysozyme: Na-CMC	Characteristics of the sample
1:0,4	Lysozyme is not included, gel is muddy
1:2	
1:4	Lysozyme is fully included, transparent gel
1:6	
1:8	
1:10	

From these results it follows, that lysozyme immobilization occurs at mass ratios of 1:4–1:10, while the use of Na-CMC in lower concentrations does not lead to full enzyme entrapment-that is visible to the naked eye.

Working with water-soluble polymeric matrices you can explore the conformational changes of protein macromolecule at immobilization by spectrophotometric methods. Fig. 1 shows the absorption spectra of free and included in the Na-CMC solutions lysozyme. It should be noted that at polymer presence, absorption spectrum increases (hyperchromic effect), indicating the presence of interactions between the matrix and the enzyme and protein-polymer complex formation.

Luminescence spectra of free and included in the Na-CMC solutions lysozyme given in Fig. 2 display thet the enzyme luminescence intensity decreasing is observed along with polymer concentration increase, which also indicates protein-polymer complex formation. However, in both cases while investigating absorption (280 nm) and luminescence (350 nm) λ_{max} was unchanged, i.e. conformational changes are minor and does not affect lysozyme native conformation.

As a result of lysozyme immobilization by entrapment in Na-CMC gel, the product is obtained with quantitative protein and bacteriolytic activity preservation. Into the composition of the gel promising for usage in dentistry, peppermint alcoholic extract was

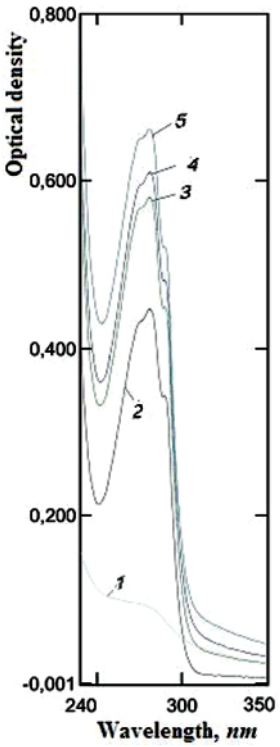

Fig. 1. **Electronic absorption spectra of free and included in the Na-CMC lysozyme aqueous solutions:**
1 — absorption spectrum of Na-CMC 0.25% solution;
2 — 0.02% lysozyme;
3 — lysozyme with 0.1% Na-CMC addition;
4 — lysozyme with 0.2% Na-CMC addition;
5 — lysozyme with 0.25% Na-CMC addition

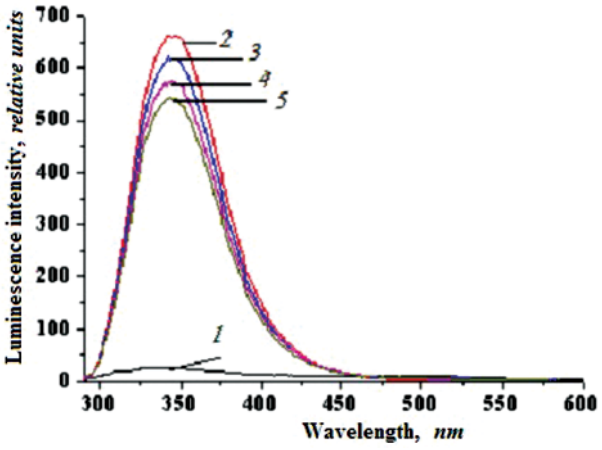

Fig. 2. **Luminescence spectra of aqueous solutions of free and included in the Na-CMC lysozyme:**
1 — luminescence spectrum of Na-CMC 0.25% solution;
2 — 0.02% lysozyme;
3 — lysozyme with 0.1% Na-CMC addition;
4 — lysozyme with 0.2% Na-CMC addition;
5 — lysozyme with 0.25% Na-CMC addition

added to improve the organoleptic properties, glycerol — to improve elasticity, chlorhexidine bigluconate — as a preservative to increase shelf life. The main characteristics of the product are given in Table 2. It should be noted that the chosen immobilization method saves bacteriolytic enzyme activity on the initial level.

In studying of lysozyme release dependence on incubation time in the solution the prolonged quantitative enzyme release from mucoadhesive gel was observed (Fig. 3). Maximum activity of immobilized preparation is achieved after 180 min of incubation under conditions close to physiological ones (pH 6.2, t = 37 °C).

One of the most important characteristics of mucoadhesive gel is its adhesion to the mucous membranes. Adhesion strength of the developed gel based on Na-CMC is 6000 Pa regarding the mucosa, while for the most used mucoadhesive polymers — in the range of 2 000–9 000 Pa [15], that indicates a high mucoadhesive properties of the selected product. It should be noted that the gel is distributed on the mucosa surface in thin layer, and glycerol in its composition prevents drying in case of contact with air.

The study of viscosity showed that lysozyme addition into the solution of Na-CMC leads to a pronounced increase in kinematic viscosity (22.4%). Thus, for the Na-CMC solution the viscosity is 2.63 ± 0.08 mm^2/s, whereas for (Na-CMC + lysozyme) — 3.39 ± 0.07 mm^2/s. These results may be caused by the interaction of the enzyme and the polymer molecules due to electrostatic, hydrogen bonds.

Table 2. **Main characteristics of mucoadhesive gel with immobilized lysozyme**

Indices	Results of the determination
Bacteriolytic activity, units/mg	68 200 ± 3 410 (*$P < 0,005$, **$P > 0,05$)
The lysozyme content of, mg/g of the gel	2,0 ± 0,1 (* $P < 0,001$)
Water content, %	95 ± 5,6 (* $P < 0,005$)
pH of the gel	7,0
Organoleptic properties	Uniform, homogeneous, keeps its shape, elastic, plastic; the smell of mint, yellow-green (dental gel); odorless, transparent (ophthalmic gel)

Note: * — the difference significance between the series of experiments for $n = 5$;

** — relative to free lysozyme initial activity (68 000 units/mg of the enzyme).

The effectiveness of lysozyme immobilization was evaluated by comparing the main biochemical characteristics of free and immobilized enzyme.

Studying the effect of temperature on immobilized lysozyme activity, the enzyme activity reduction at 60–80 °C (Fig. 4) was noted due to the partial destruction of Na-CMC under the high temperatures that could destabilize the protein molecule.

By contrast, pH-profile of immobilized enzyme applies to the area of acid values: at pH 5.5 for the immobilized enzyme activity 42.4% higher than for free enzyme (Fig. 5).

One of the most important parameters is pH stability that should be controlled during the development of antibacterial and anti-inflammatory drug, because through the damage of various etiology, usually through existing microbial contamination, acidosis is observed [18]. Lysozyme is stable under physiological conditions at pH 6.0–7.4, but the shift to acid value leads to a partial loss of activity. This disadvantage can be avoided by enzyme immobilization.

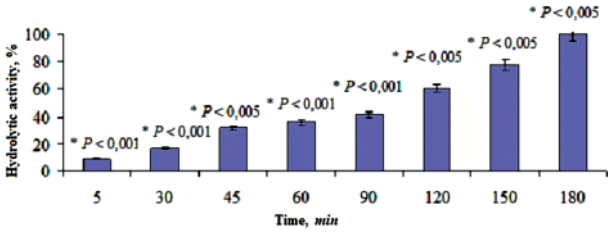

Fig. 3. **Immobilized lysozyme hydrolytic activity dependence on its incubation time**
(pH 6.2; t = 37 °C; here and in Fig. 6: as 100% maximum enzyme activity was taken)

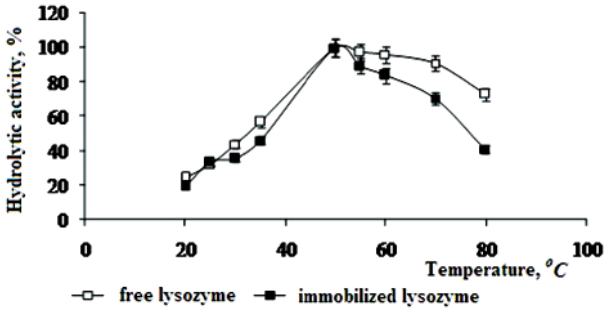

Fig. 4. **Thermo profile of free and immobilized lysozyme lytic activity**
(here and in Fig. 5: 100% — initial activity, that is 68 000 units/mg for free and 68 200 units/mg for immobilized lysozyme under optimal conditions)

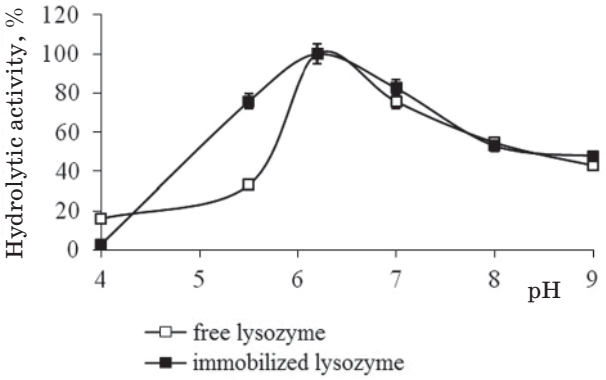

Fig. 5. **Bacteriolytic activity of free and immobilized lysozyme dependence on the pH of incubation medium (t = 55 °C)**

The simulated wound environment conditions is pH 5.5 because acidosis is observed at various injuries through existing microbial contamination. Therefore at drugs developing, special attention is paid to the investigation of the stability and activity at pH 5.5. Investigation of lysozyme bacteriolytic activity pH-stability showed that immobilization in Na-CMC promotes activity in slightly acidic environment (pH 5.5) and its preservation of high level (95–100%) for 3 h at 25 °C is well as at 37 °C (Fig. 6). Result of immobilization is positive because free enzyme at pH 5.5 is active at the level of 25–30% (Fig. 5).

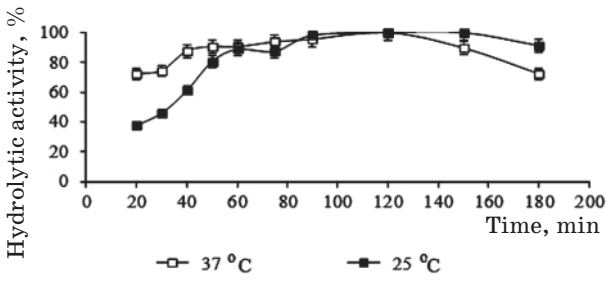

Fig. 6. **Bacteriolytic activity of immobilized lysozyme dependence on incubation time at pH 5.5 and temperature 25 °C and 37 °C**

One of the key indicators of immobilization efficiency is protein and immobilized enzyme bacteriolytic activity storage time. The product remained stable for 2 years, did not significantly alter the activity, which was determined at the level of 95–100% of the original.

Thus by the proposed method of immobilization through complex formation in sodium carboxymethyl cellulose gel highly stable product of prolonged action is obtained — mucoadhesive gel with immobilized lysozyme having high adhesive properties in respect of the mucous membranes, improved biochemical properties compared with the free enzyme (activity 70% higher at acidic environment, the shelf life of the final product is 2 years) and is promising for further investigation.

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МУКОАДГЕЗИВНИЙ ГЕЛЬ З ІММОБІЛІЗОВАНИМ ЛІЗОЦИМОМ: ОДЕРЖАННЯ ТА ВЛАСТИВОСТІ

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Метою роботи було дослідження нековалентно іммобілізованого лізоциму, а також фізико-хімічних і біохімічних властивостей одержаного мукоадгезивного гелю. Активність лізоциму визначали бактеріолітичним методом (субстрат — клітини *Micrococcus lysodeikticus*). Іммобілізацію лізоциму проводили методом включення в гель. Взаємодію ензиму з носієм досліджували методами віскозиметрії, спектрофотометрії та спектрофлуориметрії. Отримано мукоадгезивний гель з іммобілізованим лізоцимом із протизапальною й антимікробною активністю. Унаслідок іммобілізації утворився протеїн-полімерний комплекс з вихідною ензиматичною активністю. Продукт характеризується високими мукоадгезивними властивостями, кількісним збереженням протеїну і бактеріолітичної активності, пролонгованим вивільненням ензиму, поліпшеними біохімічними характеристиками (розширеним pH-профілем активності, стабільністю в кислому середовищі й під час зберігання впродовж 2 років) і є перспективним для подальших досліджень. Запропонований метод іммобілізації лізоциму в гель натрієвої солі карбоксиметилцелюлози дає змогу одержувати стабільний високоефективний продукт з високими адгезивними властивостями для закріплення на слизових оболонках, що є перспективним для використання у біомедицині.

Ключові слова: лізоцим, іммобілізація, мукоадгезивний гель.

МУКОАДГЕЗИВНЫЙ ГЕЛЬ С ИММОБИЛИЗОВАННЫМ ЛИЗОЦИМОМ: ПОЛУЧЕНИЕ И СВОЙСТВА

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Целью работы было исследование нековалентно иммобилизованного лизоцима, а также физико-химических и биохимических свойств полученного мукоадгезивного геля. Активность лизоцима определяли бактериолитическим методом (субстрат — клетки *Micrococcus lysodeikticus*). Иммобилизацию лизоцима проводили методом включения в гель. Взаимодействие энзима с носителем исследовали методами вискозиметрии, спектрофотометрии и спектрофлуориметрии. Получен мукоадгезивный гель с иммобилизованным лизоцимом с противовоспалительной и антимикробной активностью. Вследствие иммобилизации образовался протеин-полимерный комплекс с исходной энзиматической активностью. Продукт характеризуется высокими мукоадгезивными свойствами, количественным сохранением протеина и бактериолитической активности, пролонгированным высвобождением энзима, улучшенными биохимическими характеристиками (расширенным pH-профилем активности, стабильностью в кислой среде и при хранении в течение 2 лет) и перспективен для дальнейших исследований. Предложенный метод иммобилизации лизоцима в гель натриевой соли карбоксиметилцеллюлозы позволяет получить стабильный высокоэффективный продукт с высокими адгезивными свойствами для закрепления на слизистых оболочках, перспективный для использования в биомедицине.

Ключевые слова: лизоцим, иммобилизация, мукоадгезивный гель.