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The changing of enzymatic activity of hydrolases immobilized on natural polysaccharide matrix for purulent and burn wounds treatment during storing and exploitation

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ABSTRACT

This research is devoted to creating and studying of special properties of modified cellulose medical materials based on immobilized enzymes for wound healing. We studied different parameters of materials production (glycerin addition during immobilization process, technological features of materials production), the changing of their properties during storing and exploitation (effect of temperature conditions). Also we proposed a structural scheme of obtained materials and mechanisms of interaction of all of the components altogether. The understanding of this scheme will lead to scientifically based designing of the new wound care materials with requires properties in future.

Keywords: *chitosan, chitosan-coated dialdehyde cellulose, immobilized enzymes, proteinases, wound healing, drug design.*

1. INTRODUCTION

The problem of creating of the medical materials for wound healing is still relevant these days. There is a lot of new researches devoted to the designing of cellulose derivatives containing enzymes and other therapeutic agents, the development of scientific base of the production of such materials, special technological features of this process, the study of the properties and new fields of these materials application [1-3].

Modern wound dressing materials are quite different from the traditional ones by their properties and design. Nowadays wound dressings appear to be not only textile materials but also various powders, films, sponges, gels and combinations of those things [2,3]. The application of biodegradable polymers is one of the most relevant trends in the designing of new wound care products [1,3-6]. It should be noticed that wound dressings are disposable materials with a small exploitation term (about 72 hours) so the biological activity should be completely realized by this time [1].

The objects of many researches today are the materials based on natural polysaccharide – cellulose and its derivatives. Great potential of the cellulose fibers is related to the molecular structure that offers magical possibilities as a carrier for the creating of the bioactive, biocompatible and so-called intellectual materials [1,5,7]. The features of exploitation of the wound dressings should determine the selection of polymer and the way of immobilization.

The drugs based on the enzymes for wound treatment have been used for a long time [1]. Proteolytic enzymes could clean the wound from the exudate, provide a normal blood circulation in the wound and also decrease the number of pathogenic microorganisms on the wound surface, which could cause a secondary infection [8,9]. In nowadays in modern medicine there is a trend of using mostly poly-enzymatic preparations rather than pure medical enzymes. Poly-enzymatic drugs contain a complex of different enzymes what helps to hydrolyze a wound exudate better by affecting different chemical bonds in wound exudate and destroying them. Also the cost of poly-enzymatic preparations is far lower than the cost of pure enzymes because of the lack of the enzyme high purification stage in the process of the wound healing drugs production [1, 9-10].

One more substance that is also used quite often in modern medicine for wound care for various purposes is chitosan. There are a lot of studies where chitosan is used as a carrier for different therapeutic agents [9,11] or as an active compound with antibacterial properties against *Escherichia coli* and *Staphylococcus aureus* - microorganisms that make up the largest percentage of the pathogenic microflora on the wound surface [11-13]. Chitosan is a biodegradable and biocompatible polymer.

In this article we designed different compositions of immobilized materials for wound healing, studied their properties and general characteristics and proposed a possible structural scheme of obtained materials.

2. EXPERIMENTAL SECTION

2.1. Materials and methods.

2.1.1. Reagents.

In this work we used: *collitin* (Col) – proteolytic complex of enzymes from pancreas of pig (VFS42-2483-95); *PC* – proteolytic complex from hepatopancreas of crab (“Bioprogress”, Schelkovo, Moscow region, Russia; *trypsin (Tr)* – from pancreas of cattle (“Spofa”, Czech Republic); *chitosan (Ct)* (“Bioprogress”, Schelkovo, Moscow region, Russia, humidity 10%, 9289-067-00472124-03, deacetylation degree 80.0%, kinematic viscosity 383.7 cSt, MW 478 kDa). All chemicals were used as received as analytical grade.

2.1.2. Apparatus and Instruments.

UV-Vis spectra were measured on the recording spectrophotometer Shimadzu UV-2600 (Japan).

Zeta potential and the average particles diameter were measured on a Zetasizer Nano ZS, Malvern Instruments, UK.

2.2. Determination of enzymatic activity.

Enzymatic activity was measured by the casein or azocoll hydrolysis [1, 14]. For the proteolytic activity determination we used 2% solution of Hammarsten casein in 1/15 M K-Na phosphate buffer (PB) pH 8,0. Samples of immobilized material were put in 2,0 ml of PB, specified amount of gel or solution of enzyme (2,0 ml in sum).

Then tubes have been incubated in thermostat at 7,0 ± 0,5 °C using a shaking device (VXR-Vibrax-IKA 400 min^{-1}, Janke & Kunkel, Germany). In 10 minutes 2,0 ml of 2% casein were added. After that we returned tubes in thermostat for another 60 minutes (±5 seconds). The reaction was stopped by addition of 4,0 ml 10% solution of trichloroacetic acid. In 10-15 minutes (after precipitation forming) obtained solutions were filtered using filter paper (blue ribbon filter). As a control we used samples where the sequence of reagents addition was changed: after incubation we added 4,0 ml 10% trichloroacetic acid and then 2,0 ml of casein solution in those tubes. Absorbance was measured at 280 nm. Zero was set up using solution of 1:1 phosphate buffer and 10% trichloroacetic acid.

Proteolytic activity was calculated:

$$PA = \frac{(A - A_{control}) \times D}{g \times K \times t}, \text{ (PU/g)} \quad (1)$$

where: A – absorbance of studied sample;
$A_{control}$ – absorbance of control tube;
D – dilution (8);
K – tyrosine coefficient (1,2 ml/mcM Tyr);
t – incubation time (minutes);
g – weight of sample (considering humidity, g.)

Enzymatic activity using azocoll was measured with azocoll suspension 3,5 mg/ml in 1/15M phosphate buffer pH 8,0 [1, 14]. Sampless of immobilized material were put in 3,0 ml of PB (pH 8,0). Then tubes have been incubated in thermostat at 7,0 ± 0,5 °C using a shaking device (VXR-Vibrax-IKA 400 min^{-1}, Janke & Kunkel, Germany). In 10 minutes 7,5 mg of azocoll were added. After that we returned tubes in thermostat for another 60 minutes (±5 seconds).

The reaction was stopped by addition of 3,0 ml of cold distilled water. After that obtained solutions were filtered using filter paper. Then 20 µl 5n NaOH were added in tubes. Absorbance was measured at 440 nm.

For the determination of enzymatic activity using azocoll as a control we used a cellulose carrier with the same mass and oxidation degree as in studied samples.

Carriers in control tubes do not contain an immobilized enzyme because of the fact that during the immobilization process cellulose carrier undergoes a hydrolytic destruction [1, 15] with a reduction of the amount of aldehyde groups on the carrier so the adsorption of azodye on the carriers would be different. Enzymatic activity was calculated:

$$EA = \frac{(A - A_{control}) \times \sum V}{g \times t}, \quad (2)$$

where: A – absorbance of studied sample;
$A_{control}$ – absorbance of control tube;
t – incubation time (hour);
g – weight of sample (considering humidity, g.)

2.3. The obtaining of immobilized materials.

We activated our cellulose materials by the periodate oxidation as it was shown in [1,15]. The amount of aldehyde groups on the carrier we determined using a 3,5 - dinitro salicylic acid (DNS), a calibration curve was constructed using glucose [16].

The materials without chitosan we obtained following these steps: enzyme solution was immobilized on the cellulose carrier and was air-dried. To obtain chitosan-containing materials we immobilized chitosan gel on the cellulose and after air-drying process we immobilized our enzymes from the water solutions or we just immobilized an enzymes-containing chitosan gel right on the cellulose matrix [1,17].

Dried materials with a residual humidity no more than 6% were stored in polyethylene bags at room temperature in a dark place. In our studies we controlled a biological activity of obtained immobilized samples.

For the determination of the effect of drying on the preservation of biological activity of the individual drugs or some mixtures with chitosan we dissolved a studied system in a solution of specified composition, 50 or 100 µl (depending on its activity) of obtained solution were applied on a clean dry polyethylene film and then were air-dried. In the process of drying and after a complete drying we cut off a little piece of a film with a studied drug and determined a biological activity of the samples (100% of activity is an activity of the studied composition before drying). In the special experiments it was stated that polyethylene film does not affect a biological activity of studied systems [17,18].

2.4. Statistical analysis.

All experiments in this study were performed in triplicate if not specifically noted. Analysis of variance (ANOVA) was performed with the Matlab program version 8.4 (USA). The results are expressed as mean ± standard deviation (SD) and the least significant differences for comparison of means were computed at p>0,95.

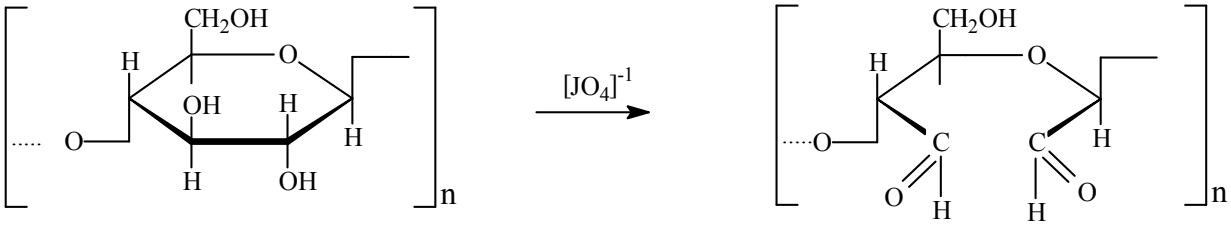
3. RESULTS SECTION

3.1. The scheme of obtaining of studied materials.

The scheme of obtaining of our materials consists of a few stages:
1) Activation of a cellulose carrier [1,15] (Figure 1).
2) Immobilization of the biologically active compounds (BAC) (chitosan and enzymes)
a) Immobilization of chitosan (Ct) or enzymes on the obtained dialdehyde cellulose (Figure 2)

b) Immobilization of the enzyme in chitosan gel (Figure 3). This scheme shows 3 possible ways of mechanisms of interaction between immobilized substance and chitosan molecule [19].

3) Scheme of sequential immobilization (chemisorption immobilization) (Figure 4).



Cellulose (C) Dialdehyde cellulose (DAC)

Figure 1. Scheme of obtaining of dialdehyde cellulose (DAC).

DAC Ct or enzyme DAC-Ct (enzyme)

Figure 2. Scheme of immobilization of the BAC on dialdehyde cellulose.

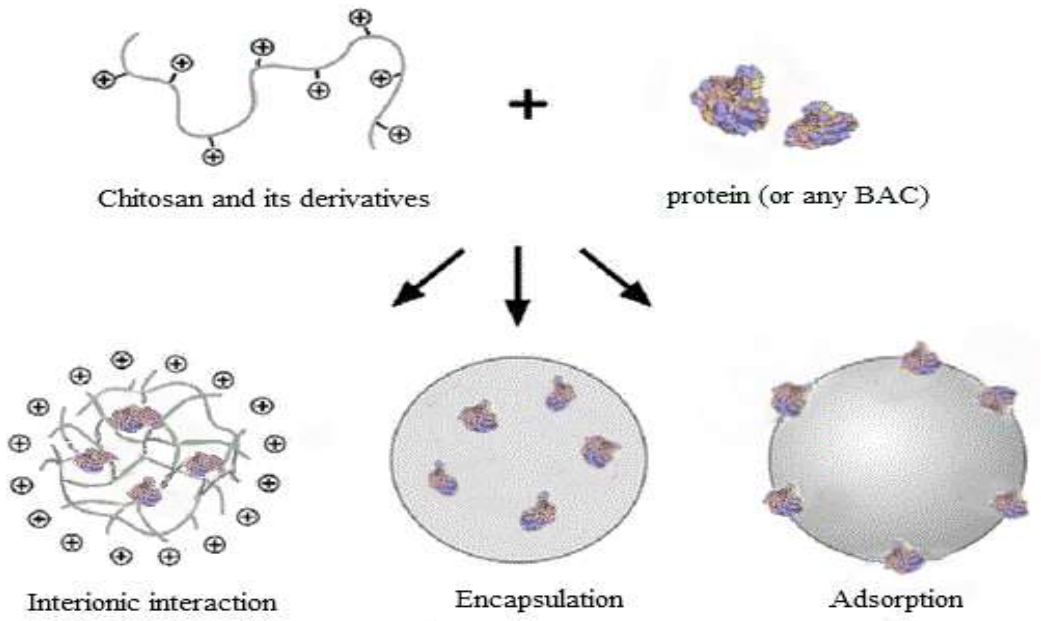


Figure .3. Possible ways of mechanisms of interaction between immobilized substance and chitosan molecule.

Table 1 summarizes our data on changing of hydrodynamic diameter (average size, nm) and ζ-potential (mV) of the used systems (PC and chitosan) and of the obtained compositions depending on the quantitative ratio PC:Chitosan.

These results show that interionic interaction can be formed when the concentration of chitosan is rather small (Fig.3), and increasing of chitosan concentration leads to formation of our system using another mechanism (Fig.3) – absorption. This data corresponds to our results of changing of proteolytic activity of compositions PC:Chitosan [18].

Table 1. Average size (nm) and ζ-potential (mV) of studied systems.

Sample	Characteristics			
	d, nm			ζ, mV
PC in 0,1M NaCl	789,0± 79,2 (84,1±8,5%)	33,1±3,3(10,5±0,9%)	5,2±0,5 (5,4±0,5%)	-12,0±2,3
Chitosan (5 mg/ml)	5237±628 (2,7±0,3%)	1449±174 (97,3±11,7%)	-	+48,9±7,3
PC-Chitosan (1:5)	1635±360 (100%)	-	-	+60,2±1,2
PC-Chitosan (50:1)	138,8±22,2 (57,4±9,2%)	330±56 (42,6±7,2%)	-	-24,0±0,5
PC-Chitosan (1:50)	4386±526 (100%)	-	-	+63,5±3,8

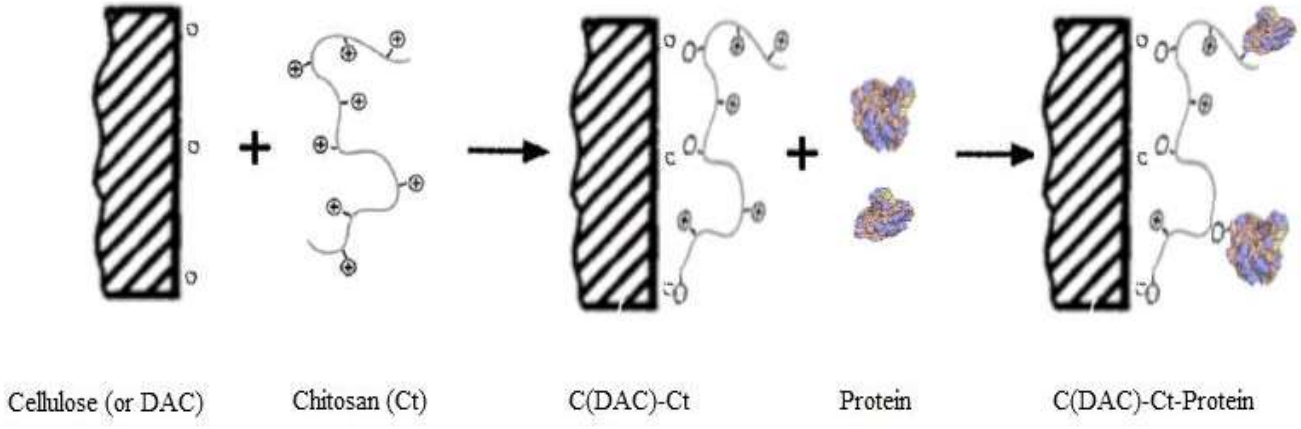


Figure. 4. Scheme of sequential immobilization (chemisorption immobilization).

For the creation of an optimum technology of production of chitosan-containing materials we studied an interaction between chitosan solutions and different cellulose carriers used in this research (cellulose, dialdehyde cellulose).

In the process of producing such chitosan-containing cellulose dressing materials we should consider that obtained materials are getting extra stiff after immobilization of chitosan on cellulose carriers and drying (unlike medical gauze) and according to our results an amount of immobilized chitosan should not exceed 40mg of chitosan/ g. of cellulose carrier [1,20].

We showed that interaction between cellulose matrix and chitosan takes about 30-40 minutes, and modification degree of cellulose carriers does not affect the amount of associated chitosan [17].

In the process of studying of the association of proteins with chitosan-containing materials it was stated that chitosan could slightly reduce a proteolytic activity of the obtained materials [18].

3.2. The study of biological activity's changing of the obtained systems.

One of the main technological problems in the process of producing of the materials with immobilized enzymes is a fall of activity of biologically active compounds during immobilization of a drug (especially of proteins) on the carriers, during the process of drying, sterilization and storing. Also activity loss takes place during an exploitation of drugs by the patients because of the human body temperature, pH of the wound, effect of different inhibitors etc. [1,5].

So nowadays the developers of wound dressings and technologists are facing a problem of the creation of materials not only with a minimal activity loss of immobilized enzymes in the process of immobilization and storing but also with the preservation of all of the necessary features and characteristics.

3.2.1. The study of enzymatic activity's changing of obtained materials in the processes of immobilization, drying and storing.

For our researches we chose a monoenzymatic drug – trypsin – and polyenzymatic compositions: PC and collitin. Trypsin is a proteolytic enzyme that is successfully used in wound healing as a cleaning and anti-inflammatory agent [1,2,20-22].

However today we can see a tendency of using mostly of polyenzymatic drugs in wound treatment [1, 2]. As it was noted before polyenzymatic composition contain a complex of different enzymes that can affect different bounds in wound exudate and destroy them helping to clean the wound and to hit the pathogenic microflora on the wound surface.

Table 2 shows the presence of trypsin', chymotrypsin' and elastase' activities in the studied samples of polyenzymatic compositions.

Table 2. Determined values of enzymatic activities of studied compounds.

Enzyme	Substrate, conditions	Activity (units)	Method of enzymatic activity determination
Trypsin	Azocoll (pH 8,0; 37°C)	3200±80 U/mg	[14,23]
	BApNa (pH 8,0; 25°C)	800±38 nM/mg*min	[1,24]
	Casein (pH 8,0; 37°C)	3,0±0,2 PU/mg	[1,14,25]
PC	Azocoll (pH 8,0; 37°C)	28,0±2,5 U/mg	[14,23]
	BApNa (pH 8,0; 25°C)	43±4 nM/mg*min	[1,24]
	Casein (pH 8,0; 37°C)	0,9±0,1 PU/mg	[1,14,25]
	BocAlaONp (pH 6,2; 25°C)	250±26 nM/mg*min	[27]
	BzArgOEt (pH 8,0; 25°C)	3,3±0,3 mcM/mg*min	[26]
	BzTyrOEt (pH 8,0; 25°C)	0,54±0,10 mcM/mg*min	[28]
Collitin	Azocoll (pH 8,0; 37°C)	5380±482 U/mg	[14,23]
	BApNa (pH 8,0; 25°C)	60±6 nM/mg*min	[1,24]

Enzyme	Substrate, conditions	Activity (units)	Method of enzymatic activity determination
	Casein (pH 8,0; 37°C)	1,23±0,30 PU/mg	[1,14,25]
	BocAlaONp (pH 6,2; 25°C)	280±31 nM/mg*min	[27]
	BzArgOEt (pH 8,0; 25°C)	3,6±0,3 mcM/mg*min	[26]
	BzTyrOEt (pH 8,0; 25°C)	7,7±0,8 mcM/mg*min	[28]

The kinetics of enzymes inactivation in the processes of immobilization, drying and storing can be presented as a complex exponential function typical for hydrolases [1].
For the immobilization process:

$$A/A_0 = e^{-k(imm)*t} \quad (3);$$

for the drying process:

$$A/A_0 = e^{-k(d)*t} \quad (4);$$

for the process of storing:

$$A/A_0 = x_1 e^{-k(1)*t} + x_2 e^{-k(2)*t} \quad (5);$$

where k_{imm} – is an effective constant of inactivation of immobilization; k_d – an effective constant of inactivation rate in the process of drying (for 20 hours); k_1 – constant that characterizes a labile part of enzyme; k_2 characterized a stable one [1]. To compare out systems we calculated effective constants of inactivation rate for obtained materials, considering that the smallest constant corresponds to the minimal loss of enzymatic activity.

For the calculations we took a biological activity of wet material (right after immobilization and before an air-drying) as 100% - maximum of enzymatic activity of the immobilized material. Further changes of activity of our systems are calculated relatively this value of 100% of activity.

One of the problems in cellulose fiber materials application is theirs traumaticity - adhesion of a dressing to the wound surface after the evaporation of wetting solution (dry drugs with immobilized enzymes are not practically active). That's why it is so important to create an optimum environment for wound treatment: both excess and lack of the moisture can harm the healing process. Thus a wetting solution should be a key for a few environment for the wound healing process. As a wetting solution we chose a water solution of glycerine that is approved for medical use [22], possesses high hygroscopicity and is known to be a stabilizer for biologically active compounds when storing.problems: it makes a dressing less traumatic, provides an optimum environment for the enzymatic reaction and creates an optimum

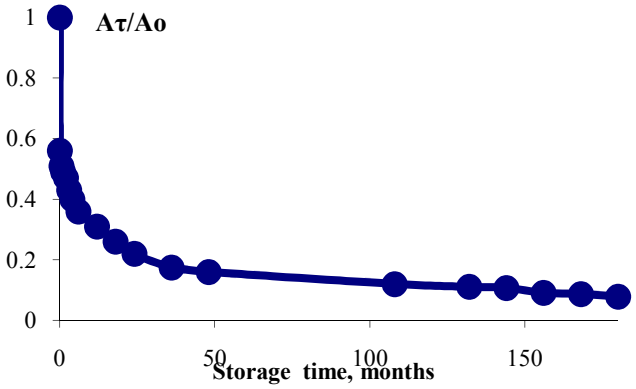


Figure 5. Changing of enzymatic activity (casein as a substrate) of trypsin immobilized on DAC in the processes of producing, drying and storing.

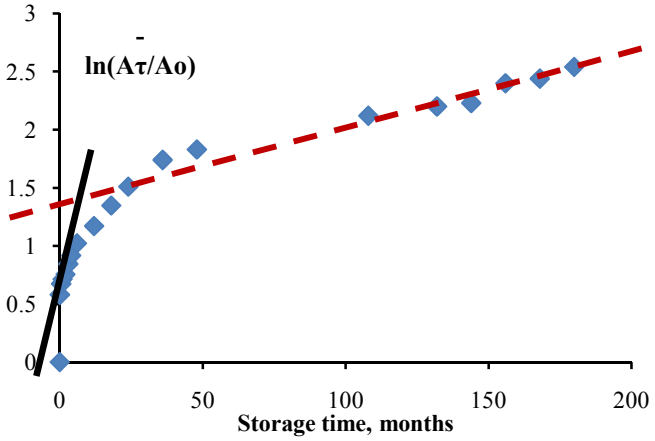


Figure 6. Calculation of kinetic constants of trypsin immobilized on DAC in the processes of producing, drying and storing.

Table 3. Effective constants of inactivation rate (month^{-1}) of modified and non-modified materials in the processes of immobilization (k_{imm}), drying (k_d, about 20 hours) and storing at 25°C.

Sample	k_{imm}	k_d		k_1	k_2
Trypsin (drying and storing on the polyethylene film) *	-	33,14		-	-
DAC(0,30)-Trypsin**	0	17,48		0,080	0,008
DAC(0,38)-Collitin*	0	37,81		0,367	0,030
PC (drying and storing on the polyethylene film) *	-	3,90		0,550	0,033
PC (drying and storing on the polyethylene film) **	-	0		0	-
PC-Chitosan (PC as a film)*	-	1,00		0,140	0,012
PC-Chitosan (PC was mixed with a chitosan solution)*	-	1,30		0,230	0,011
PC-Chitosan (drying and storing on the polyethylene film)**	-	0		0	-
PC-Chitosan+5% glycerine*	-	0		0	0,033
Cellulose-PC**	0	28,47		0,054	-
Cellulose-Chitosan-PC**	0	11,73		0,133	0,059

DAC(0,32)-Chitosan-PC (25°C) **	0	0		0,170	0,042
DAC(0,32)-Chitosan-Glycerine(5%)-PC (25°C) **	0	0		0,140	0,007
DAC(0,32)-Chitosan-PC (5°C) **	0	0		0	0,048
DAC(0,32)-Chitosan-Glycerine(5%)-PC (5°C) **	0	0		0	0,011
DAC(0,32)-Chitosan-PC (37°C) **	0	0		0,490	0,090
DAC(0,32)-Chitosan-Glycerine(5%)-PC (37°C) **	0	0		0,390	0,004
DAC(0,28)-Chitosan-PC (solution of enzyme) **	0	0		0,540	0,100
DAC(0,28)-Chitosan-PC (enzyme as a powder) **	0	0		0,440	0,056

*-azocoll as a substrate, **-casein as a substrate.

Proteolytic activity of wet immobilized materials (k_{imm}=0 in table 2) does not change under the optimum conditions of immobilization for at least 70 hours at 5÷7ºC. The loss of proteolytic activity can start during air-drying and the first months of storing at room temperature [1].

The process of drying of immobilized material is a "fast" stage of the loss of proteolytic activity of immobilized materials that can be explained by the loss of water in proteins during drying (change of the micro environment of enzyme). This stage can be characterized by the significant loss of proteolytic activity. Decrease of proteolytic activity can be explained by the enzyme conversion from the state of 100% moisture to 4 ÷ 6%. Dehydration of protein in lyophilization process can lead to its partial denaturation. Modification of the accessible amino groups in enzyme molecule leads to cleavage of different sensitive (non-covalent) bonds and changing of the properties of immobilized material. Dehydration of enzymes in a process of drying of the immobilized materials can lead to the changes in protein structure. Protein inactivation can also take place even when native proteins are stored in a dry state because of the different undesirable processes such as an aggregation [29].

The second stage of inactivation is characterized by the relatively fast loss of the proteolytic activity of immobilized enzymes. For our samples this stage takes about 2-6 months of storing under conditions noted before. This stage characterizes a labile fraction of our samples.

The third, slow inactivation stage, occurs in a subsequent observation period of time. Stable fraction of enzymes in immobilized materials remains active when storing for 6 months and more.

Obtained results can be explained not only by the presence of "labile" and "stable" fractions of immobilized enzyme that are known for the enzymes immobilized on the derivatives of cellulose [1,30-32]. Inactivation of the immobilized materials can be also caused apparently by the same reasons as in solutions of enzymes: high hygroscopicity of the carriers (humidity of the studied cellulose materials ~ 5%) and non-stability of the azomethine bond except the fact that in solutions these processes occur with smaller velocity.

In different researches [33-35] it was shown that enzymatic reactions with proteases participation proceed in a solid phase without water as a dissolvent but require a necessary hydration degree of protein. We stated [1] that samples of polyvalent inhibitors of proteinases immobilized on different fiber carriers do not lose biological activity during the process of storing at least for 5 years.

It is known that immobilization of enzyme leads to the changing of its conformational mobility when its decrease can significantly decrease an activity. On the other hand the decreasing of conformational mobility prevents such processes as denaturation and autolysis and lets an immobilized enzyme to stay active under extreme conditions (for the native protein) [1].

That's why there should be an optimum value of the binding stiffness that is regulated by different parameters such as way of the binding of the enzyme and carrier, nature, length and stiffness of the linker that connects enzyme with a polymer carrier, and nature of the carrier itself. As it was shown before [1], swollen polymer matrix can cause a protein denaturation by the multipoint binding what is noticed for some immobilized proteinases while storing under standard conditions.

Kinetics of inactivation of immobilized enzymes in the process of storing can be described by a complex exponential dependence typical for hydrolases ($A\tau/Ao=a_1*e^{-k_1\tau} + a_2*e^{-k_2\tau}$) [1]. In semi logarithmic scale this dependence is presented as a line that is described by a first order equation. Figure 6 shows our kinetic data of the proteolytic activity changing of PC immobilized on modified textile carriers. Two stages of the inactivation of immobilized proteinases is a well-known fact [1,30-32,35]. These kinetic laws can be explained by the general inactivation mechanism that implies the existence of two forms of enzymes differ in activity and stability to denaturation. Thus two-exponential character of inactivation shows that inactivation mechanism of immobilized enzyme implies two types of enzyme differ in activity and stability regardless of the special mechanism features of and constants ratio.

Selection of the conditions of the production of materials with immobilized proteinases for medical use is not only about achieving a maximum of activity in the end of storing term (minimum value of effective constant of inactivation rate), but also is about observance of the necessary sanitary-hygienic characteristics of the end-product (pH of a water extract, mechanical strength etc.). In our research [1] we showed sanitary-chemical and toxicological data of the used carriers and some of the immobilized materials.

These results confirm a safety of our materials use for medical purposes.

4. Structural scheme of obtained immobilized materials.

We believe that it is essential not only to know how to product our materials but also have a complete understanding of their structure to have a scientifically based possibility to improve these materials in future and design some new ones. So the study and analysis of all of the interactions between single compounds and complexes lead us to understanding of the possible structural

scheme of obtained materials immobilized on modified cellulose (Figure 7).

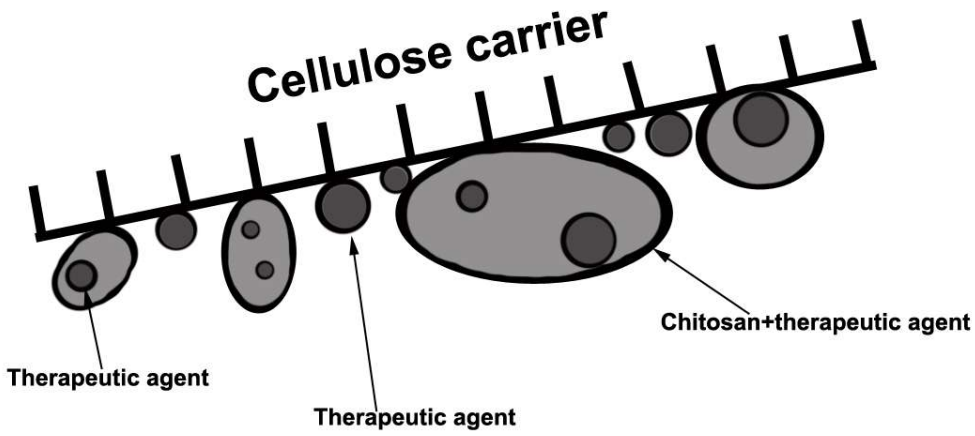


Figure.7. Structural scheme of obtained immobilized materials.

This scheme has a lot of advantages such as: controlled release of the therapeutic agent because of the irregular destruction of a complex carrier (cellulose and chitosan gel); possibility of the co-immobilization of the compounds that are incompatible because of the "co-annihilation" (proteins and enzymes); prolonged action of therapeutic agent because of the constant drug flow in wound. All of these factors are necessary parameters of an ideal wound care product.

Thus this structural scheme shows how all of the components of a wound dressing (cellulose carrier, chitosan and enzymes) can interact with each other and what we could change in a technology of producing of our materials to obtain a new composition with other properties.

4. CONCLUSIONS

We stated that increasing of storing temperature (from 5°C to 37°C) leads to increasing of values of effective constants of inactivation rate of enzymatic activity of our modified materials. Addition of glycerine to the polyenzymatic composition stabilizes modified forms of enzymes when storing at increased temperature (37°C). Polyenzymatic compositions are much more stable than monoenzymatic ones. Proposed structural scheme of multifunctional enzyme-containing material allows us to design new drugs with special properties in future.

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