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IMMOBILIZATION OF PROTEOLYTIC ENZYMES IN CARBOXYMETHYLCHITIN FILMS AND SPONGES (REVIEW)

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The possibility of immobilization of the proteolytic enzymes collagenase and terrilytin in chitin carboxymethyl ester films and sponges was demonstrated and some characteristics of this process were investigated. It was found that the optimum pH for immobilization of collagenase and terrilytin lies in the range of 6.5-7.5, which approximately corresponds to the optimum pH of the effect of native enzymes. According to the data from in vitro *experiments, the activity of the immobilized enzymes at the optimum pH of immobilization is 75-50% for collagenase and 80-90% for terrilytin. An increase in the molecular weight of carboxymethylchitin in the range of 60-600 kilodaltons significantly strengthens the films and simultaneously decreases the activity of the immobilized enzymes, probably due to the stronger binding of the molecules of the enzyme in the matrix of higher molecular weight. In immobilization of enzymes in sponges, the molecular weight of the polymer matrix has no effect on the activity of the immobilized enzymes. Changing the degree of substitution of carboxymethylchitin in the 0.7-1.3 range has almost no effect on the activity of the enzymes immobilized in the films and sponges.*

Of the scientific directions successfully developed over many years by Z. A. Rogovin and his scientific collective, the study of chemical transformations of cellulose and other polysaccharides has rightfully occupied a leading position. The research in this area was generalized in [1], translated into German and published in the FRG in 1983. The reactivity of the functional groups of polysaccharides, possibilities of synthesis, and the products of derivatives which contain different functional groups and are analogs of natural polymers, aquatic polysaccharides in particular (alginic acid, chitosan) were examined with respect to the effect of the structure on the chemical, physicochemical, and other properties of these materials.

The studies related to methods of creating active materials, primarily for medical applications, based on synthesized derivatives occupy a special position in this research; these materials are based on formation of chemical bonds of different types between the polymer support and the biologically active substance. Drugs of different structure were used as the biologically active substances immobilized on the polymer supports in the first stage. The evolution of these studies led to the development of methods of fabricating biologically active fibre and film materials containing immobilized enzymes, and their use in medicine and biotechnology will solve important social and technological problems [2].

Incorporating enzymes in the structure of films or sponges of water-soluble polymers is an effective method of modifying the properties of proteolytic enzymes used for treatment of burns and purulent wounds in particular which is comparatively easily implemented on the industrial scale. Immobilization of enzymes in a polymer matrix increases the stability and efficiency of use of the enzymes and in many cases, eliminates an undesirable effect, allergy, for example. The polysaccharide chitin and the products of its chemical modification which have film- and fibre-forming capabilities, low toxicity, and biodegradability, are of great interest as supports for drugs, including proteolytic enzymes. The possibility of immobilizing enzymes on chitin and chitosan was communicated in [3, 4]. The results obtained in studying some characteristics of immobilization of the proteolytic enzymes terrilytin (TER) and crab pancreatic collagenase (CPC) in chitin carboxymethyl ester (CTCM) films and sponges are reported below. The presence of a wide set of functional groups (hydroxyl, acetyl, amino, and carboxyl) in its macromolecules, the solubility of the polymer in water in almost the entire pH range, and hydrolyzability with lysozyme, which makes it possible to decompose it during metabolism in the body, are advantages of CTCM as a polymer support for drugs [5].

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TABLE 1. Characteristics of the Structure of Samples of CTCM

CTCM sample No.	Concentration, %			DS by groups		[η], dl/g*, for ionic strength of 0.2 mole
	COONa	N_{ac}	N_{tot}/C	CH_2COONa	$NHCOCH_3$	
1	27,1	4,4	4,51	1,17	0,85	1,52
2	26,1	3,8	4,51	1,08	0,75	3,95
3	28,0	4,0	4,41	1,22	0,85	6,78
4	27,4	4,0	4,48	1,15	0,80	7,64
5	30,3	4,2	4,32	1,33	0,85	8,10
6	18,4	3,1	5,50	0,63	0,52	1,80
7	19,1	3,9	5,06	0,70	0,70	3,00
8	18,9	2,4	–	0,65	0,40	3,24

*A change in [η] in the 1.52-8.10 dl/g range corresponds to an increase in the MW from approximately 60 to 600 kilodaltons. A preliminary calculation of the MW of CTCM was performed with the equation $[\eta] = 4.3 \cdot 10^{-4} MW^{0.74}$ proposed for CMC in [10].

TABLE 2. Physicomechanical Characteristics of CTCM Films

No. of CTCM sample in Table 1	Concentration of CTCM in solution, %	σ, mPa	ε, %
1	10	23/20	8/13
2	5	–/40	–/14
3	4	50/–	10/–
6	4	42/–	11/–
7	4	54/43	8/26
8	4	46/–	9/–

Note. The indexes of a film prepared without glycerin are given in the numerator and those of a film prepared with 10% glycerin are given in the denominator.

Samples of CTCM which differed in the concentration of functional groups and the intrinsic viscosity (Table 1) were synthesized and analyzed according to the recommendations in [6, 7]. Laboratory samples of a highly active, but little studied, protease of animal origin, CPC with specific activity (*A*) of 5.0 ± 0.6 units/mg and an isoelectric point (*pI*) equal to 2.5 [8], and the microbial proteolytic enzyme TER manufactured by Biokom, with $A = 3.3 \pm 0.5$ units/mg and $pI = 4.6$, widely used in medical practice, were used as the enzymes.

CTCM films were formed by the dry method from 3-10% aqueous solutions with a defined pH. In preparation of spinning of spinning compositions with enzymes, an aqueous solution of the enzyme was poured into an aqueous solution of CTCM while stirring. After deaeration of the spinning solution for 0.5 h, the films were poured onto a glass or polyethylene support with a slit spinneret. The slit gap was adjusted as a function of the concentration of the polymer so that the thickness of the finished film was 20-60 μm. The duration of drying at 20-25°C was 20 h. Sponges were prepared by lyophilic drying of frozen spinning compositions.

The breaking stress (σ) and elongation at break (ε) were calculated with the stress–strain curves obtained in studying the films on an Instron machine.

The activity (caseinolytic) of the immobilized enzymes was determined with the method in [9], expressed in units/mg of enzyme and in % of the activity of the native enzyme.

TABLE 3. Effect of DS of CTCM on the Activity of TER Immobilized in CTCM Film*

CTCM	DS	A, units/mg (%)
CTCM-2	1,08	3,6 (92)
CTCM-7	0,70	3,5 (89)
CTCM-5	1,33	3,6 (92)
CTCM-8	0,65	3,4 (87)

*CTCM:TER = 1:0.05; immobilization pH = 7.0.

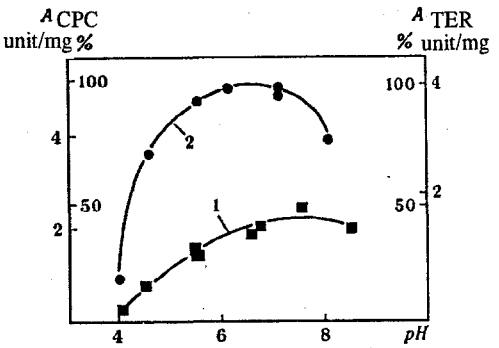

Fig. 1. Effect of the pH of CTCM-1 forming solution on the activity of immobilized CPC (1) and TER (2) for a weight ratio of CTCM:CPC = 1:0.02 and CTCM:TER = 1:0.05.

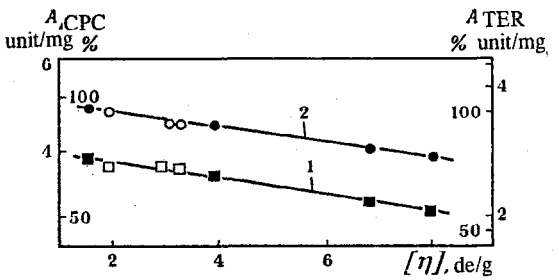

Fig. 2. Effect of MW and DS of CTCM on the activity of CPC (1) and TER (2) immobilized in films for DS = 1.1-1.3 (•, ■) and 0.6-0.7 (o , □), CTCM:enzyme ratio by weight = 1:0.02.

According to the data obtained (Table 2), all films formed from CTCM have sufficient strength for practical use, and the strength of the films increases when the molecular weight (MW) increases and the degree of substitution (DS) of the polymer with respect to carboxymethyl groups decreases. The dependence of the strength on DS is probably due to the level of preservation or degradation of the supermolecular structure of the chitin. It should be noted, however, that the elasticity of the films, characterized by their elongation at break, is inadequate. The films were plasticized using glycerin as the plasticizer, added to the solution in the amount of 10-20% of the weight of CTCM, in order to improve this index. As Table 2 shows, plasticization of the films increased the elongation at break and improved the elasticity with a slight decrease in the strength.

TABLE 4. Effect of γ-Sterilization and Storage of CTCM Films on the Activity of the Enzymes Immobilized in Them*

Composition of film	Relative activity, %, after			
	forming	γ-sterilization	heating**	storage**
CTCM-1/TER	88	77	72	69
CTCM-1/CPC	70	57	52	59

*CTCM:enzyme ratio by weight = 1:0.02.
**At 40°C for 4 h.
***At 20°C for 40 h.

TABLE 5. Effect of Glycerin on the Activity of TER Immobilized in CTCM-1 Films*

Expt. No.	Concentration of glycerin, %	pH of forming solution	*A*, units/mg (%)
1	0	6	2,90 (82)
2	10	6	2,93 (82)
3	0	7	2,89 (81)
4	10	7	2,84 (79)

*Concentration of CTCM in forming solution of 10%.

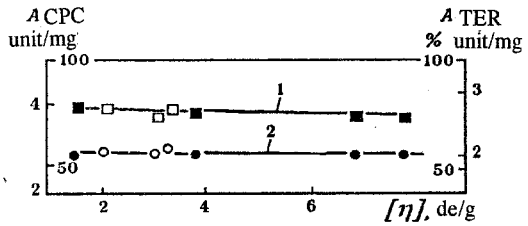

Fig. 3. Effect of MW and DS of CTCM on the activity of CPC immobilized in sponges for a weight ratio of CTCM:CPC = 1:0.02 (1); 1:0.05 (2) and DS = 1.1-1.3 (•, ■) and 0.6-0.7 (○, □).

Preliminary experiments showed that no less than 95 and 90% of the activity of the native enzymes is retained in the solutions when they are held for 1 and 24 h, respectively.

The water solubility of CTCM in a wide range of pH allows varying the pH of the forming solution from 4.0 to 8.5. In studying the effect of the pH of the forming solution on the activity of the immobilized enzymes (Fig. 1), it was shown that the optimum activity of collagenase incorporated in the structure of CTCM is within the 7.0-7.5 pH range, close to the optimum pH of the effect of native collagenase (~7.8). The proteolytic activity of collagenase is 48-50% at the immobilization optimum. The optimum activity of immobilized TER is at pH 6-7, and the optimum effect of the native enzyme is equal to 7.6. The relative activity of immobilized TER in the region of the extreme attains 80-90%, which is significantly higher than the activity of collagenase incorporated in the structure of CTCM.

In further studies of the effect of the structure of CTCM on the activity of the immobilized enzymes, the pH of the forming solutions was brought to the pH_{opt} found for immobilization. The data in Fig. 2 show that the activity of TER and

TABLE 6. Effect of the CTCM:TER Ratio and Drying Conditions on the Activity of the Immobilized Enzyme*

No. of CTCM sample in Table 1	Concentration of CTCM, %	Polymer: enzyme ratio	Drying conditions	A, units/mg (%)
1	4,1	1:0,02	22±2°C	3,3 (95)
1	4,0	1:0,05	22±2°C	3,0 (87)
1	4,6	1:0,05	Sublimation	3,1 (91)
1	4,0	1:0,02	"	3,1 (91)
3	2,6	1:0,05	"	3,0 (87)
1	2,6	1:0,05	"	3,0 (87)

*pH of forming solution of 6.55.

CPC immobilized in CTCM films decreases slightly with an increase in the intrinsic viscosity [η] (molecular weight). The relative activity of TER immobilized in low-molecular-weight CTCM-1 ([η] = 1.5 dl/g; MW ~ 60 kilodaltons) and in the highest molecular weight CTCM-8 ([η] = 8.1 dl/g; MW ~ 6000 kilodaltons) is 95 and 80% of the activity of the native enzyme, and the relative activity of the immobilized CPC is equal to 80 and 50%, respectively. The decrease in the activity of the enzymes with an increase in the molecular weight of the polymer matrix is probably due to its stronger binding with the matrix and the lower accessibility of the molecules of the enzyme immobilized on the more extensive and bulky macromolecules of CTCM.

The effect of DS of carboxymethylchitin on the activity of immobilized TER was investigated in two pairs of samples of CTCM: 2, 6 and 7, 8 (Table 1), synthesized from chitin with a lower and higher MW. In this case, in our opinion, the effect of possible differences in the molecular weight of the samples of CTCM was eliminated *a fortiori*. As Table 3 shows, a change in DS for carboxyl groups in the 0.7-1.3 range has almost no effect on the activity of the immobilized enzyme.

The data in Table 4 suggest that immobilization of TER and CPC in CTCM films significantly increases the stability of the enzymes in γ-sterilization and subsequent holding at high and low temperatures: the decrease in the activity of the immobilized enzymes is 10-20%, versus 50% in the native enzymes. The effect of increasing the stability in immobilization was marked for CPC.

It was previously shown that the elasticity of CTCM film can be increased by plasticizing it with glycerin. In studying the effect of the plasticizer on the activity of immobilized terrilytin (Table 5), it was found that incorporation of glycerin in the amount of 10% of the weight of CTCM in the forming solution does not decrease the activity of the terrilytin in the films at pH 6-7. Concentration of the forming solutions takes place during hardening of the film, which can cause rearrangement of polycomplexes formed by macromolecules of CTCM and the enzyme, stronger binding of the enzyme with the polymer matrix, and consequently a change in its activity.

In addition to the films obtained by evaporation of solvent from the liquid phase of the forming solutions, porous sponges fabricated, for example, by lyophilic drying of frozen forming solutions, could be a potential type of dressing. Since elimination of the solvent is not accompanied by concentration and a change in the structure of the starting solution, the structure which arises is of interest for studying the character of the bonds formed between the macromolecules of the enzyme and the polymer matrix in the solution.

The effect of the conditions of elimination of the solvent (evaporation at room temperature and sublimation at −5°C) from forming solutions containing CTCM and the enzyme in weight ratios of 1:0.02 and 1:0.05 was investigated in low-molecular-weight CTCM-1 and the enzyme terrilytin. The results reported in Table 6 (expts. 1-4) showed that the activity of terrilytin immobilized in films and sponges is at a high level (90% of the activity of the native enzyme) and no significant differences were observed in the activity in going from films to sponges.

It is also interesting that the molecular weight of CTCM has no effect on the activity of immobilized terrilytin (Table 6, expts. 4-6) and collagenase (Fig. 3), like the DS for carboxyl groups and the polymer:enzyme ratio (see Fig. 3; Table 6, expts. 3, 4) when the enzymes are immobilized in sponges.

The lack of an effect of the structure of the polymer matrix and polymer:enzyme ratio in immobilization of the enzymes in carboxymethylchitin sponges could indicate the lack of significant interaction between them in the starting form

solutions and the appearance of such an interaction only in concentration of the system during drying by evaporation. However, this hypothesis requires additional confirmation in a more detailed study of the mechanism of the interaction between the polymer support and the enzyme.

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