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Obtaining Stabilized Forms Of Hydrolytic Enzymes For Technical And Pharmaceutical Purposes

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ABSTRACT

The unique specificity of enzymes makes biocatalytic processes more technologically advanced compared to chemical ones. Hydrolytic enzymes are the most widely used. Currently, enzyme preparations (EP) based on hydrolytic enzymes are increasingly used in pharmaceutical production, medicine, food and chemical industries. The practical use of proteolytic enzymes is varied, the most significant is their use in surgery for the treatment of wound surfaces of various etiologies, which is due to the locality of action: the rapid rejection of non-viable tissue, without affecting healthy areas. The goal of the work is to develop stabilized forms of hydrolytic enzymes that can be used for technical and pharmaceutical purposes, as well as to evaluate their effectiveness based on the analysis of their kinetic and thermodynamic parameters.

Keywords: hydrolytic enzymes, technology, method, treatment, chemical industry, effect.

INTRODUCTION

Engineering enzymology is a scientific and technical field that deals with the development of new ways of practical use of enzyme preparations[1].

Enzymes are unique biocatalysts of various biochemical processes that have valuable properties that determine their practical use. These properties include, first of all, high selectivity, catalytic activity and stability. Enzymes are able to retain their properties outside of cells, which makes it possible to use them in various fields of industry. In terms of production volume, enzyme preparations occupy key positions among biologically active substances after antibiotics and amino acids. Of the huge list of enzyme preparations, hydrolytic enzymes are the most widely used in industry[2].

Currently, an increasingly important direction is the production of biofuels based on the use of waste from the processing of biological raw materials, as well as waste oils from food production. A larger number of

[1] Berezin I.V. Engineering enzymology. Biotechnology series. M.: Higher School, 2017. 143 p.
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researchers have been studying the structure of lipids and the impact on them of various factors of technological processes in the production of fat and feed products, as well as the directions of their use[3].
The versatility of lipase-catalyzed reactions has made this enzyme a unique heterogeneous catalyst in the transesterification reaction. Research has shown that biodiesel can be produced enzymatically with the help of lipase catalyzing the transesterification reaction, which allows easy reduction of glycerol, making the biodiesel purification process easier. For cost-effectiveness and greater lipase stability, it is preferable to use immobilization-modified lipase in biodiesel production. This facilitates easier separation of the catalyst from the reaction product, simplifies process control, allows the reuse of the enzyme preparation and the organization of continuous operation of the production as a whole[4].

MATERIALS AND METHODS

There are several types of enzyme stability:
• operational stability (the ability to maintain enzyme activity in an expanded range of operating values - temperature, pH, etc.);
• functional stability (the ability to maintain enzymatic activity under conditions of protein denaturation);
• storage stability (the ability to maintain enzymatic activity for a long time).
According to the literature, several approaches can be used to improve enzyme stability.
The first approach to solving the problem of increasing the stability of enzymes is to strengthen the protein molecule due to a change in its primary structure - replacing amino acid residues using genetic engineering methods[5]. Using targeted mutagenesis, it is possible not only to change the catalytic activity of enzymes, but also to increase their stability. Covalent modification and cross-linking in the internal regions of the protein globule help to increase the stability of the enzyme with respect to inactivating agents. In [6], collections of thermophilic and hyperthermophilic microorganisms were screened in order to detect enzymes with the required specificity and stability. In the process, the analysis of hyperthermophiles poses significant challenges due to the difficulty of isolating and culturing them. Genetic engineering approaches require a detailed study of the enzyme structure; this method is technically difficult to implement.
The second way to improve enzyme stability is to create an optimal macroenvironment for the enzyme. It is based on changing the composition of the solvent by adding various soluble substances, for example, salts, organic solvents, etc. As a result, the hydration shell of the enzyme changes and, as a result, a change in its structure and stability is possible. This method is simple and universal and does not require precise knowledge of the structure of enzymes. However, the literature provides conflicting data that does not allow us to accurately state the effect of low-molecular-weight agents on enzyme stability and the effectiveness of this method in general.
The third approach does not have the disadvantages of the first two methods for increasing enzyme stability. It is based on protecting the tertiary structure of the enzyme from the influence of unfavorable environmental factors. This method is implemented by immobilizing the enzyme (covalent or non-covalent modification of the protein)[7].

RESULTS AND DISCUSSION

Enzyme immobilization methods
During the last two decades, many papers and literature reviews have been published on enzyme immobilization, with considerable attention paid to immobilization methods.
There are four ways to prepare (activate) the enzyme and carrier for immobilization[8].

[3] Belov A. A. Development of industrial technologies for obtaining medical materials based on modified fiber-forming polymers containing biologically active protein substances: dis. ...doc. tech. Sci. M., 2019. 393 p.
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1. Activation of the enzyme before the immobilization process, which is performed before binding it to the carrier. This method is used quite rarely due to the high probability of loss of enzyme activity due to chemical modification of not only specific reactive groups, but also the catalytic center.
2. Activation of the carrier before immobilization of the enzyme. This is the best known and widely used method for covalently linking a native enzyme to a carrier.
3. The use of reactive bi- or multifunctional coupling agents that serve as intermediaries between the enzyme and the carrier.
4. Modification of the enzyme using recombinant DNA methods in order to introduce functional groups into its molecule that can be adsorbed on certain carriers.

Immobilization methods are extremely diverse, which makes it possible to select the most optimal method of incorporating biomolecules, taking into account their subsequent use.
It is customary to distinguish two methods of immobilization of biologically active substances: physical and chemical[9].

Physical methods of immobilization
• Adsorption. During adsorption immobilization, the bioactive substance is fixed on the surface of the carrier due to hydrophobic, electrostatic, dispersion and hydrogen bonds. The effectiveness of this method is determined by the specific surface area and porosity of the carrier. The disadvantages of adsorption immobilization include the low strength of binding of the protein to the carrier, which limits the possibilities of using the immobilized drug obtained by this method due to its desorption from the carrier[10].
Examples of studies of adsorption immobilization are described in a significant number of scientific papers. So ChenZun, ZhouHui, LiWei, etc.
[11]immobilized glucoamylase on starch gel. The research results showed that the immobilized enzyme had an 8-fold higher specific activity, a 10-fold higher temperature optimum of action, and was more stable than the free enzyme.

Cellulose
Cellulose is a polysaccharide containing up to 10,000 glucose residues linked together by β-glycosidic bonds and without side branches. The molecules of this polymer are connected by hydrogen bonds, which give it mechanical strength[12].
Cellulose, like starch, is hydrolyzed stepwise in an acidic environment, has virtually no reducing properties and does not participate in other reactions with the carbonyl group characteristic of monosaccharides[13].
When cellulose is exposed to oxidizing agents (hydrogen peroxide, ozone, hypochlorous acid salts, potassium permanganate, dilute nitric acid), complex oxidation products are obtained, which are called hydroxycelluloses. The chemical properties of cellulose are determined by the presence of hydroxyl groups. The main reactions of cellulose as a polyhydric alcohol are the formation of alcoholates and cellulose ethers. Cellulose, when treated with concentrated solutions of caustic alkalis, forms strong compounds, which most researchers classify as alcoholates.
As noted [14], cotton cellulose (in the form of, for example, medical or surgical gauze) contains both carbonyl and carboxyl groups, which may also be present in native cellulose, but are formed to a greater extent during the processing of cellulose .
Cotton cellulose consists of 65-75% crystalline and 25-35% amorphous parts[15]. It has been proven that the process of cellulose oxidation with periodic acid occurs on the surface and affects the structure of the

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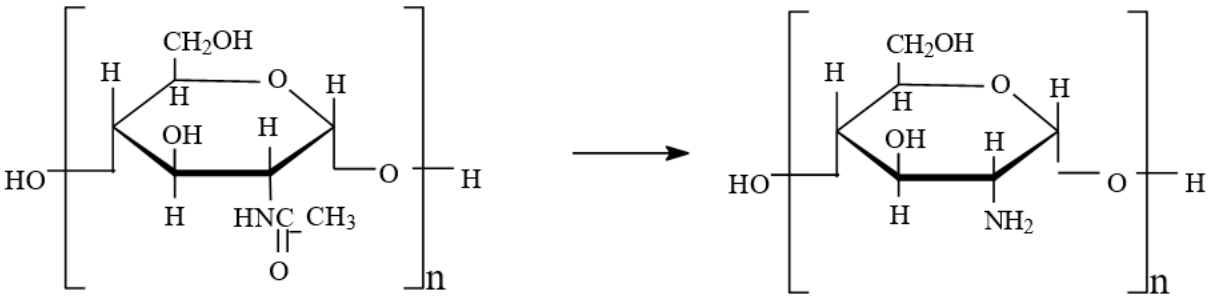
crystalline part of cellulose[16]. With an increase in the degree of oxidation of cellulose with a solution of sodium periodate, the degree of crystallinity decreases[17]. Cellulose, as a hydrophilic polar compound, is capable of swelling in water and polar solvents. This occurs due to the fact that solvent molecules, penetrating into the amorphous fractions of cellulose, break intermolecular hydrogen bonds and form hydration and solvate shells around the hydroxyl groups of the polymer. The degree of cellulose swelling depends on the nature of the fiber, temperature, pH and solvent composition. Fiber micropores can increase several times. The average pore size of cotton fiber in the dry state is 3.5 Å, and in the swollen state it is 30-50 Å; the cross section increases with swelling by approximately 50%[18].

Chitosan: physicochemical properties, characteristics as a carrier for immobilization and areas of application

Chitosan is the simplest derivative of chitin, aminopolysaccharide 2-amino-2-deoxy-β-D-glucan (Figure 1.2).



Chitin Chitosan

Figure 1.2 - Structural formula of a chitosan unit

Chitosan is obtained using the reaction of elimination of the acetyl group from the chitin molecule (N-acetyl-D-glucosamine) (deacetylation reaction) under the influence of concentrated alkali (more than 30%) at a temperature of 120°C for several hours[19].

Using IR spectroscopy, it was revealed that during the formation of chitosan, the intensity of the carbonyl absorption bands (1625 cm-1) of the amide group (3260 and 3110 cm-1) decreases and the intensity of the bands of the NH2 group (3365-3445 cm-1) increases.

X-ray studies of chitosan have shown that it has the same amorphous-crystalline structure as chitin, but less ordering of macromolecules. The main parameters characterizing the solubility, mechanical and other properties of chitosan are the degree of crystallinity, the degree of deacetylation and molecular structure.

The question of enzymatic reaction rates is of paramount importance when studying the characteristics of any group of enzymes. To estimate the rate of an enzymatic reaction, two parameters of the Michaelis-Menten equation (the maximum rate Vmax and the Michaelis constant Km) are usually determined. The value of the Michaelis constant is used to determine the substrate concentration required to achieve maximum reaction rate and to compare the catalytic activity of enzymes of the same class. Also, the Michaelis constant serves as a measure of the affinity of the enzyme for the substrate: the higher the rate of formation of the enzyme-substrate complex, the lower Km.

To determine the kinetic parameters of enzymatic reactions catalyzed by the above enzymes, experiments were carried out on the influence of temperature and pH of the environment on the rate of enzymatic reactions.

To graphically find the values of the Michaelis constant and the maximum reaction rate, dependences were plotted in double inverse Lineweaver-Burk coordinates at various temperatures (Fig. 3).

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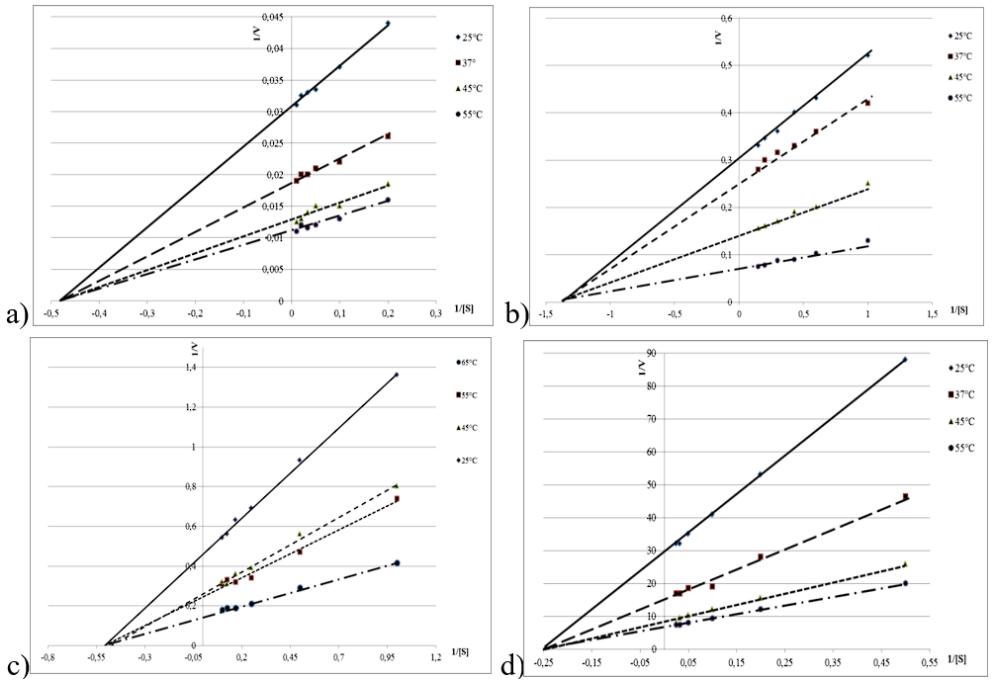

Figure 3 - Dependencies in Lineweaver-Burk coordinates for native hydrolases: a) trypsin; b) proteolytic complex; c) amylase; d) lipases

According to the literature, the low efficiency of an enzymatic reaction is most often due to the low specific activity of enzyme preparations, inactivation under the influence of various factors, insufficient stability at non-optimal pH and temperature values, as well as a significant decrease in enzymatic activity during storage of the enzyme preparation.

It is known that conformational changes in the enzyme molecule, leading to a decrease or increase in its activity, are mainly determined by two parameters: temperature and pH of the environment.

Table 1 – Values of the parameters of the Michaelis-Menten equation of native forms of hydrolases at different temperatures

Enzyme preparation	Values of V_{max} ($\cdot 10^3$) (mol/dm^3·s) at temperatures,ºC			
	25	37	45	55
Trypsin	0,54±0,03	0,94±0,05	1,33±0,07	1,48±0,07
	K_m = 2,12±0,11 g/dm^3			
Proteolytic complex	0,06±0,01	0,07±0,01	0,12±0,01	0,24±0,01
	K_m = 0,76±0,04 g/dm^3			
Lipase	0,03±0,01	0,07±0,01	0,11±0,01	0,13±0,01
	K_m = 4,00±0,20 g/dm^3			
Enzyme	Values of V_{max} ($\cdot 10^3$) (mol/dm^3·s) at temperatures,ºC			
	25	45	55	65
Amylase	34,08±1,71	57,42±2,87	65,33±3,27	77,50±3,88
	K_m = 1,78±0,09 g/dm^3			

For each enzyme, certain temperature and pH values are established at which their activity is maximum. When the temperature and pH of the enzymatic process deviate from the optimal value, significant changes can occur in some parts of the structure of the protein molecule, which in most cases lead to a decrease or complete loss of functional activity, i.e. denaturation of the enzyme[20]. During denaturation, the structure of the enzyme usually changes significantly: the protein globule unfolds[21]. The ability of an enzyme to maintain activity over a wide range of temperatures and pH values is called operational stability.

[20] Polyenzyme drugs in purulent surgery: methodological recommendations / Ed. corresponding member RAMS N.A. Efimenko. M., 2015. 73 p.

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According to literature data, immobilization provides the greatest stability of the enzyme and increases its stability. This process makes it possible to stabilize the enzyme molecule both through chemical modification (chemical interaction with various natural polymers - i.e. covalent immobilization) and physical limitation of the mobility of the enzyme molecule due to its mechanical capture in the polymer shell (physical immobilization method)[22].

When choosing carriers for the immobilization of hydrolases, we assessed the effectiveness of using a common group of natural polymers of a polysaccharide nature for this purpose: cellulose, chitosan and sodium alginate.

The above polymers are hydrophilic and are multifunctional compounds that can interact with proteins. Cellulose has mechanical strength, and chitosan and sodium alginate form fairly dense gels. In addition, chitosan and sodium alginate have a number of valuable biological properties, such as biocompatibility, biodegradability, hemostatic effect, antitumor and anti-inflammatory activity, which makes it possible to use both these polymers directly and enzymes immobilized on them in medicine and pharmaceuticals.

Also, modified cellulose, dialdehyde cellulose (DAC), which was obtained by oxidation of cellulose with sodium periodate, was used as a carrier for immobilization. The choice of this carrier was due to the fact that, according to literature sources, cellulose carriers based on DAC have the ability to maintain the sanitary and hygienic characteristics of unmodified cellulose and interact with the amino groups of enzymes under optimal conditions. However, DAC, compared to cellulose, is less resistant to high temperatures and acidic environments, which can lead to the destruction of DAC-based preparations. In this work, DAC was obtained with an oxidation degree of 2% (i.e., per 100 cellulose units, 2 units were oxidized to form two aldehyde groups). It is known that with an increase in the degree of oxidation of DAC, the mechanical strength and durability of this carrier decrease; in addition, the reactivity of DAC with low degrees of oxidation (2-5%) is higher than for a carrier with an oxidation degree of 20-25%, therefore, In this work, we obtained DAC with an oxidation degree of no more than 2%.

Table 2 - Values of effective rate constants for inactivation of native hydrolases, hydrolases in polysaccharides and forms of hydrolases immobilized on a solid support during storage time

Enzyme preparation	Inactivation constant k_{in}, ($\cdot 10^{-3}$), c^{-1}	
	Native (or polysaccharide-stabilized) form	Drug mobilized on a solid carrier
Trypsin	0,78±0,04 0,11±0,01	0,76±0,04 0,11±0,01
Chitosan-trypsin	0,70±0,04 0,10±0,01	0,68±0,04 0,09±0,01
Sodium alginate-trypsin	0,83±0,04 0,14±0,01	0,82±0,04 0,12±0,01
Proteolytic complex	1,18±0,06 0,23±0,02	1,10±0,06 0,16±0,01
Chitosan-proteolytic complex	1,08±0,06 0,15±0,01	0,78±0,04 0,14±0,01
Sodium alginate-proteolytic complex	4,41±0,22 0,31±0,01	4,32±0,22 0,28±0,01
Amylase	0,11±0,01	0,10±0,01
Chitosan amylase	0,08±0,01	0,06±0,01
Sodium alginate amylase	0,11±0,01	0,10±0,01
Lipase	0,19±0,01	0,18±0,01
Chitosan lipase	0,16±0,01	0,08±0,01
Sodium alginate lipase	0,17±0,01	0,15±0,01

From the results obtained it follows that enzymes immobilized on a polyethylene matrix, and in the presence of polysaccharides, are more stable during storage than unstabilized enzymes. The values of the inactivation constants of stabilized immobilized enzymes are close to the corresponding values obtained for solutions of hydrolases stabilized by polysaccharides. This indicates that immobilization of enzymes on a solid support

[22] Polyenzyme drugs in purulent surgery: methodological recommendations / Ed. corresponding member RAMS N.A. Efimenko. M., 2015. 49 p.

slightly reduces their activity. It was found that dried films with immobilized enzymes retained enzymatic activity at a level of 40 - 70% of the original for more than 5 months.

Since the studies carried out showed a significant increase in the storage stability of hydrolases immobilized on a polyethylene matrix, the next stage of the work was devoted to studying the process of immobilization of hydrolytic enzymes on prepared textile carriers, which were cellulose and dialdehydecellulose. During the experiment, several types of samples were prepared: in the first case, the enzyme was immobilized on unoxidized cellulose and DAC, and in the second, the enzyme immobilized on cellulose was stabilized with alginate and chitosan. In the first case, a sample of the cellulose matrix (cellulose or DAC) was placed in a solution of the native enzyme of a given concentration and the required pH value at a hydromodulus of 10 (the weight of the impregnating solution was related to the weight of the carrier as 10:1). After a certain time of immobilization, the carrier was squeezed out of the excess impregnating solution, passing it through rubber rollers, then the material was placed in a dark place at a temperature of +5 ÷ +7ºC for 18-20 hours, after which it was dried in air. Next, the protein content in the resulting samples was determined by the Lowry method and residual enzymatic activity during storage. It should be noted that the residual activity was determined in wet samples (A0) after removing excess moisture and immediately after drying.

To obtain an enzyme preparation containing chitosan or sodium alginate, the cellulose matrix was first treated with a 0.5% polysaccharide solution at a hydromodulus of 10 for two hours. The material was then dried in air at room temperature, after which the enzyme was immobilized in accordance with the method described above.

The analysis of literary and experimental data proves the advantage of using natural polysaccharide carriers for the immobilization of hydrolases in comparison with synthetic ones.

Another promising method of immobilization is microencapsulation, i.e., the inclusion of high-molecular biologically active substances in semi-permeable membranes. Currently, this method is one of the most common immobilization methods. This method is classified as a physical (mechanical) immobilization method, in which enzyme molecules are retained in a polysaccharide capsule due to weak interactions without the formation of covalent bonds. Enzymes incorporated into polymer microparticles are used for analytical, medical and industrial purposes.

CONCLUSION

1. The kinetic and thermodynamic properties of native hydrolytic enzymes (bovine trypsin, PC from crab hepatopancreas, amylase from the fungus Aspergillus oryzae, bovine lipase) were characterized;
2. The most effective conditions for the immobilization of hydrolytic enzymes were selected: for proteases (bovine trypsin and PC) - on a cellulose-chitosan carrier; for fungal amylase - on a cellulose-chitosan carrier; for bovine lipase: microencapsulation in chitosan-alginate microparticles. It has been shown that immobilization on natural polysaccharides is more effective than on synthetic ones: the degree of preservation of enzymatic activity in the case of using natural carriers is on average at least 80%, while in the case of synthetic ones it is 50-80%.
3. The effect of immobilization on conformational, operational, functional and storage stability was studied. It has been proven that the immobilization of proteases and amylase on a cellulose-chitosan carrier and lipases into chitosan-alginate microparticles leads to increased stability relative to native forms of hydrolases;
4. The dynamics of the release of proteolytic enzymes (trypsin and PC) into the wound environment was studied, which made it possible to establish a prolonged action period of the drug of 72 hours, which meets the requirements for medical drugs. Concentrations of the softening agent (glycerol) and vitamins (C and ascorutin) were selected that do not cause a decrease in proteolytic activity. Based on the data obtained, a new type of atraumatic dressing material has been developed with the following composition: cellulose - chitosan - proteolytic enzyme (trypsin or PC) - 5% glycerol - 0.2% ascorutin (or vitamin C), which has a prolonged effect for 72 hours for the trypsin preparation and 24 hours for the PC drug;
5. The most effective conditions for hydrolysis of barley malt have been proposed: immobilized amylase preparation on a cellulose-chitosan carrier, 55°C, pH 5.0, 4 loads of EP, 8 hours, degree of hydrolysis 92%;
6. The most effective conditions for hydrolysis of fat-containing waste using lipase microparticles were proposed: pH 8.0, 37°C, 2 loads of FP, 70 hours, degree of hydrolysis 57.5%.

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