INVESTIGATION OF KINETIC AND THERMODYNAMIC ASPECTS OF POLYMERIC SUBSTRATE HYDROLYSIS BY SOLUBLE AND IMMOBILIZED HYDROLASES

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ABSTRACT

In this study covalent immobilization of three hydrolysis enzymes by modified Glutaraldehyde method has been carriedout. Kinetic and thermodynamic aspects of the reactions substrate hydrolysis, catalysed by soluble and immobilized hydrolases have been studied. It has been shown that the kinetics of polymers hydrolysis does not correspond to Michaelis-Menten equation; sigma-like forms of the curves V(S) confirm that the quaternary structures of the hydrolytic ezymes stay intact after the immobilization. Besides Covalent immobilization of those enzymes is accompanied by an increase of K_m, E_{act}, ΔH and a decrease of V_{max} and ΔS.

دراسة الحركيات والثرموديناميك لبوليمرات المادة الأساس المتحللة مائياً باستخدام انزيمات التحلل المائي الذائبة وغير المتحركة

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المستخلص

استخدمت طريقة Glutaraldehyde المحورة لثلاثة انزيمات التحلل المائي باتباع طريقة عدم التحريك تساهمياً. تمت دراسة الحركيات والثرموديناميك للتحلل المائي للمادة الاساس باستخدام الانزيم الحال hydrolases الذائب وغير المتحرك. اوضحت النتائج ان حركيات البوليمرات المتحللة لا تخضع لمعادلة Menten- Michaelis ميكالس-منتن. يؤكد الشكل سكما للخط البياني V(s) بان التركيب الرباعي للانزيمات الحالة بقى غير متأثر بعدم الحركة. يصاحب عدم الحركة تساهميا للانزيمات الحالة زيادة في قيمة km ثابت ميكالس، Eact طاقة التنشيط ΔH الانثالبي وهبوط في السرعة القصوى V(max) و ΔS الانتروبي.

Introduction

One of the most important aspects of molecular biology is investigation of physicochemical properties of the enzymes. Enzymes significantly suprass chemical catalysts in accelerating the rate of a reaction, and in their specificity.

Commercial enzymes have been used in a number of processing areas such as in food processing and waste conversion to useful product ext. A development of medical enzymology promotes wide application of biological catalysts in carrying out of clinical analysis and in diagnosis of different diseases. Amylolytic and lipolytic enzymes are widely used as a medicines alternatives for the correction of alimentary canal hypofermentosis. Those hydrolases were mainly from microbical origin, because of their high activity and low cost. Application of the enzymes ensures significant acceleration of technological processes, improves the produce quality. In this way many periodical processes can be transferred to continuous conditions with formation of ecologically safe waste that can be applied as a raw material for different branches of industry.

However an application of enzyme preparations gives a rise to numerous problems, such as high prices of pure proteins, their instability under unsuitable storage condition and other influences. Enzymes can not be used many times because of the difficulties in their separation from the products of the reaction. In this regard a development of methods of obtaining synthetic insoluble derivatives, i.e.immobilized enzymes, solves these problems.

Immobilization refers to the localization of enzyme during the process which keep the enzyme separated physiclly from substrate. Immobilization of enzymes increases their stability toward denaturing factors and allows them for re-use. Several techniques are used to immobilize enzymes. Generally immobilization can be achieved by chemically or physicany associating the enzyme with support or by confining it by means of semi permeable membrane which can be used many times for the industrial and analytical purposes(2).

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Investigation of enzyme kinetic is a basis of up-to-date knowledge of molecular mechanisms of biological processes. A study of hydrolases kinetics is important from theoretical and practical points of view. It is necessary to know optimal conditions of enzymes active site functioning and the effect of different physicochemical factors on their activity.

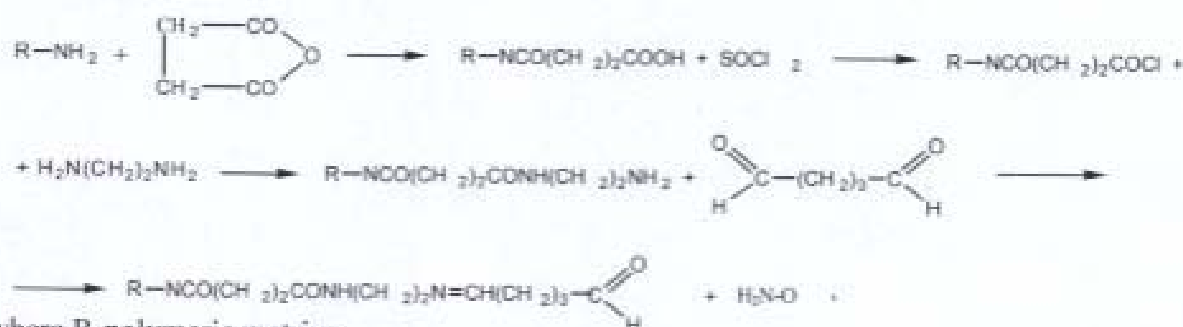
So the present study aimed to investigate the kinetic and thermodynamic characteristics of the reactions, catalysed by soluble and immobilized enzyme hydrolases.

Materials and Methods

The main objects of this study were hydrolytic enzymes: glucoamylase (α-1, 4:1, 6-glucan-4, 6-glucanohydrolase, EC 3.1.2.3) and inulase (2,1-β-D-fructanfructanohydrolase, EC 3.2.1.7) from *Aspergillus awamori*; lipase (triacylglycerolacilhydrolase, EC 3.1.1.3) from *Rhizopus japonicus*. A covalent immobilization of hydrolases on ion-exchangers Duolit A 368 PR and AV-17-2P was carried out by modified glutaraldehyde method. Purification of hydrolases was carried out by ion-exchange and gel-chromatography. Homogeneity of these preparations of hydrolases was confirmed by electrophoresis.

Catalytic activity of inulase was determined by spectrophotometric method with resorcine, of glucoamylase – by glucosooxydative method, of lipase – by spectrophotometric method with rhodamine 6G (1). As a substrate for inulase we used inulin, for glucoamylase – soluble starch, for lipase-tributirin. Determination of protein content was carried out by Lowry method (4). Kinetic and thermodynamic parameters of the reaction of polymers hydrolysis was studied by graphical and quantitative methods.

The hydrolytic enzymes were covalently immobilized on ion-exchangers Duolit A 368 PR and AV-17-2P by modified glutaraldehyde method, its essence is the following. Anionite matrix has been treated consecutively by succinic anhydride, thionile chloride, ethylenediamine and glutaric aldehyde. After this a covalent bonding of enzyme has been carried out. The scheme of modified immobilization of hydrolases has a following view:



where R-polymeric matrix;

Φ-the protein molecule residue.

Results and Discussion

A research of kinetic aspects of enzymatic reactions under the conditions of rate regulation by different chemical substances allows discovering of the behaviour peculiarities of hydrolases under normal conditions of cell functioning, and a study of catalysis by immobilized enzymes is necessary for clarifying of the functioning of membrane-connected and olygomeric enzymes and also of polyenzymatic systems.

The main points of the theory of heterogeneous catalysis are correct for immobilized enzymes. As a rule, immobilization of the enzymes leads to the change of kinetic and thermodynamic parameters of catalytic reaction, that is why they are marked as "seemed". In this way the choice of the support plays an essential role in catalysis by immobilized enzymes (6).

An increase of a distance between the support matrix and enzyme molecule leads to an increase of bonded protein quantity and, after that, to the growth of catalytic activity of the immobilized enzyme (3). The received "arm" gives to enzyme a possibility to form a solid bond with the support and promotes an increase of mobility of tertiary protein structure, responsible for catalytic transformation of substrate molecules.

The results of present study has proven that the covalent immobilization of hydrolytic enzymes does not disturb

olygomeric structure and complex character of hydrolysis rate on substrate concentrations (Fig. 1, 2, 3). It has been shown that the kinetics of the reaction of starch hydrolysis by immobilized glucoamylase does not correspond to Michaelis-Menten equation.

Analysis of experimental data indicated that a covalent bonding of glucoamylase with Duolit A 368 PR stabilized quaternary structure of the enzyme and decreases value of constant of conformational transition from the state R to the state T (for soluble glucoamylase L_o = 6,69; for immobilized one –2,10).

Some authors have proven that the enzyme glucoamylase has a complex structure, consisting of two subunits. It is an example of protein, including two domains: catalytic (residues 1-470) and C-terminal (509-616), connected with mannose and containing 70% of carbohydrates (8,9). Williamson, *et. al.* (7) stated that the structure of glucoamylase I grows longer due to catalytic domain, which interacts starch-bonding domain. Catalytic and starch-bonding domains are independent globular proteins, separated by the distance ~10 nm. Interaction between domains occurs due to O-glycosylated sites that causes significant interior mobility (7).

In connection with references and experimental data it is possible to suppose that glucoamylase immobilization on the anionite Duolit 368 PR by modified glutaraldehyde method leads to multiple bonding with catalytic and starch-bonding domains. That is why immobilization causes a decrease of enzyme catalytic activity, an increase of quaternary structure stability and it is accompanied by the shift of equilibrium, taking place during the transition from state T to state R. Also insignificant changes in conformation of separated globules occur, which make interaction of catalytic and starch-bonding domains difficult.

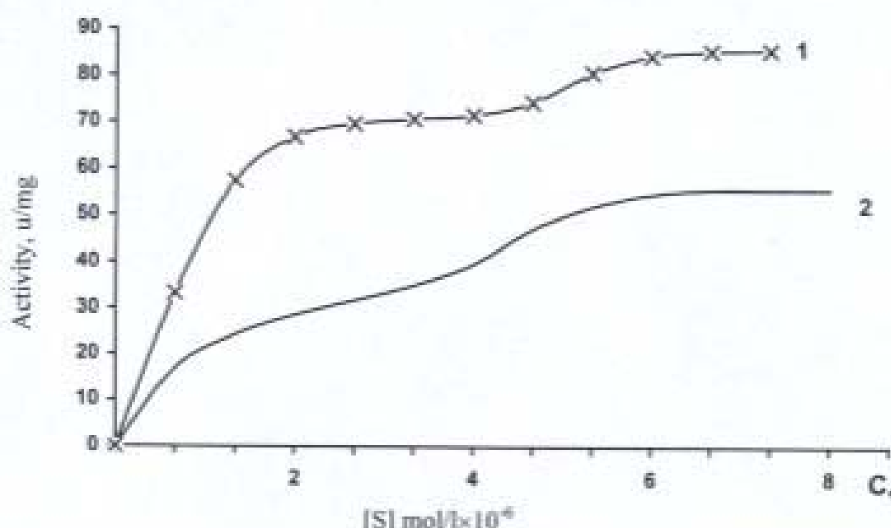


Fig.1. Dependence of catalytic activity of soluble (1) and immobilized on Duolit A 368 PR (2) glucoamylase on substrate concentration

It has been shown (Table 1) that immobilization of glucoamylase on ionite Duolit 368 PR by modified glutaralgehyde method leads to an increase of K_m and to decrease of V_{max}. Immobilized glucoamylase retained 45% of soluble enzyme activity.

Table 1. Values of kinetic parameters of starch hydrolysis, by soluble and immobilized enzyme on Duolit 368 PR glucoamylase

Enzyme	K_m, mol/l	V_{max}, mcmol/ml·min
Soluble glucoamylase	$7.6 \cdot 10^{-7}$	370
Immobilized glucoamylase	$1.0 \cdot 10^{-6}$	125

The obtained results showed that the dependence V(S) of the reactions of polymeric substrates hydrolysis, catalysed by inulase (Fig.2) and lipase (Fig.3) do not correspond to Michaelis kinetics and have more complex configuration than that for soluble ones. Sigma-like forms of the curves confirm that the quaternary structures of hydrolytic enzymes stay intact after the immobilization. Complication of

the kinetics of enzymatic catalysis proves that the glutaraldehyde immobilization causes some changes in conformation of separated subunits, influencing on cooperativity of interactions during substrate hydrolysis.

From this study it can be concluded that the covalent bonding of glucoamylase, inulase and lipase with the matrix of the support can lead to some changes in connections between enzyme subunits, caused by the modification of polypeptide chain conformation.

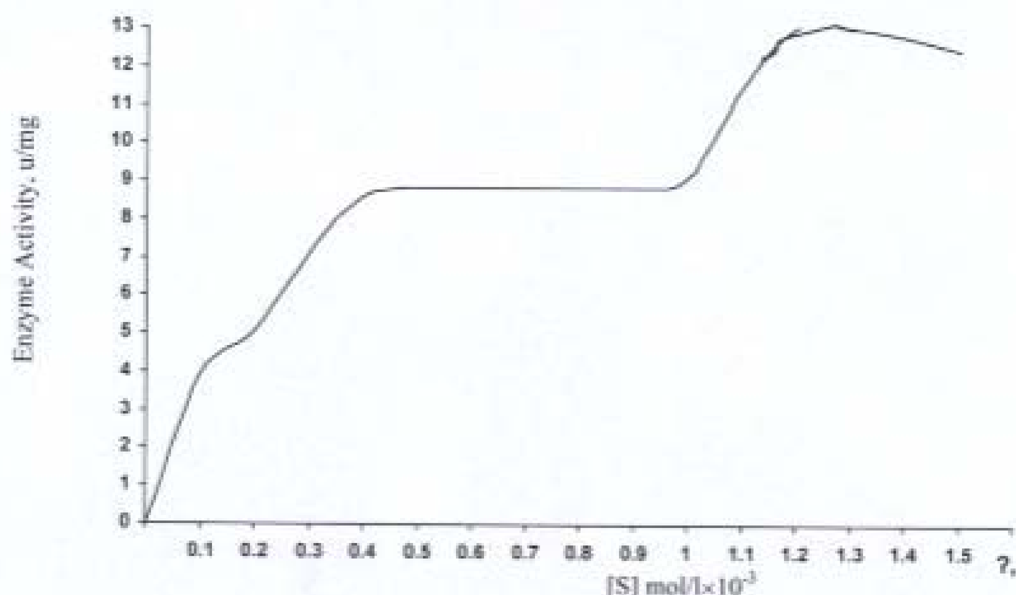


Fig. 2. Influence of substrate concentration on catalytic activity of immobilized on AV-17-2P inulase

A change in the microenvironment of protein molecules after the immobilization can influence its reactionary behaviour and local concentration of different substances, taking part in fermentative process of substrate hydrolysis.

Tables (1,2,3) explain determined kinetic and thermodynamic parameters of enzymatic polymeric substrates hydrolysis by soluble and immobilized hydrolases by graphic methods.

Table 2. Values of kinetic and thermodynamic parameters of inulin hydrolysis catalysed by soluble and immobilized on AV-17-2P inulase

Enzyme	K_m mol/l 10^{-4}	V_{max} µmol/mg·min	E_{akT} kJ/mol	ΔH kJ/mol	ΔS kJ/mol K
Soluble inulase	3.3	35.7	52.4	4.06	-51.5
Immobilized inulase	4.2	30.0	63.7	8.22	-173

Studying the dependence of reaction of tributirin hydrolysis rate on substrate concentration reveald that this dependence does not correspond to Michaelis kinetics because the inhibition by the substrate surplas occurs. It is connected first of all with the steric difficulties for lipolysis on the surface between water phase and lipid phase.

Ota, *et. al.* (5) have reported that the limiting stage of the lipolysis is the adsorption of the enzyme on the interphase lipid - water (5). Inhibition of immobilized lipase has been observed in lower concentrations of substrate (1,2 mol/l) than of soluble one (2,0 mol/l). It can be explained by diffusion difficulties in formation of interaction lipase – anionite matrix.

It has been suggested that the immobilization leads to an increase of E_{act}, ΔH of hydrolysis reactions of polymeric substrates compared with soluble enzymes, that can be caused by specific interactions of the support that impedes the transition of immobilized enzymes to catalytically active conformation under the substrate influence.

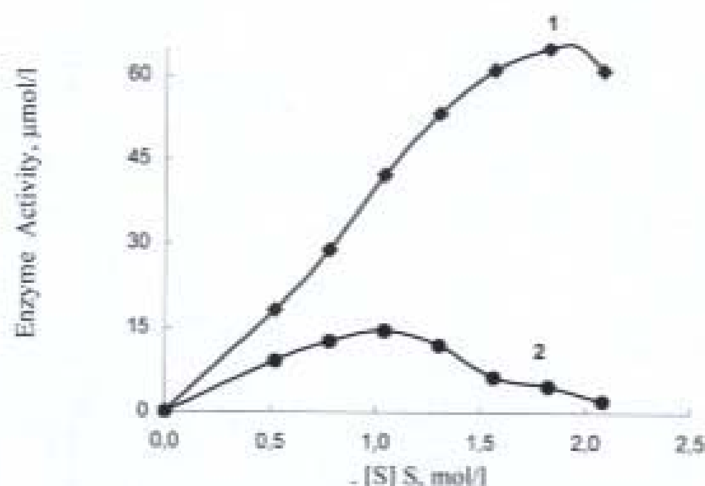


Fig. 3. Dependence of catalytic activity of soluble (-■-) and immobilized on AV-17-2P (-●-) lipase on substrate concentration

Negative ΔS values for hydrolysis reactions, catalysed by inulase (Table 2) and lipase (Table 3) confirm high rates and orderliness of the polymers substrates decomposition reaction. A decrease of ΔS after immobilization occurs, seemingly, due to more directed interaction between enzyme and substrate.

Table 3. Values of kinetic and thermodynamic parameters of tributirin hydrolysis reaction catalysed by soluble and immobilized lipase

Enzyme	K_m mol/l 10^{-4}	V_{max} µmol/mg-min	E_{akT} kJ/mol	ΔH kJ/mol	ΔS kJ/mol. K
Soluble lipase	3.22	71.42	52.4	2.62	-8.39
Immobilized lipase	1.56	37.04	63.7	4.01	-8.56

Analysis of experimental data allows to conclude that investigated hydrolytic enzymes have a complex multidomain structures, moreover the interaction between separate subunits is one of the ways of polymeric substrates hydrolysis rates regulation in vivo. Covalent immobilization of hydrolytic enzymes is accompanied by the fall of catalytic activity of enzymes, an increase of K_m, E_{act}, ΔH and a decrease of V_{max} and ΔS. These results showed that a chemical bond between hydrolase and polymeric support causes difficulties of transition of immobilized enzyme to the state of equilibrium and increases a duration of limiting stage of enzymatic catalysis.

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