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Temperature effect on the properties of β-galactosidase entrapped in alginate matrix: an experimental research supported by molecular modeling

Introduction

Entrapment of enzymes in the gel network is one of the fundamental techniques of immobilization. The principle advantage of this procedure is the ability to retain not only individual enzymes but also multienzymatic systems and whole cells [*Avnir et al., 1994*]. In general., applicability of enzymes immobilized by entrapping method is rather high, particularly due to: low costs of the procedure, no changes in the substrate specificity and high catalytic activity maintained by such biocatalyst after immobilization [*Avnir et al., 1994; Brady and Jordaan, 2009*]. Nevertheless, effective application of entrapped enzymes is limited to the biotransformation of low molecular weight compounds. One example of such a reaction with significant practical importance is the hydrolysis of lactose to glucose and galactose catalyzed by β-galactosidase [*Haider and Husain, 2009;Vieira et al., 2013*].

Besides pH and ionic strength, one of the fundamental physicochemical factor having significantly impact on the activity and stability of each enzyme is temperature of microenvironment [*Iyer and Ananthanarayan, 2008; Miłek and Wójcik, 2009*]. Furthermore, in case of biocatalysts entrapped in a gel network, this parameter could greatly influence both type and strength of interactions generated in the enzyme-carrier system and the properties of the hydrogel matrix itself used for immobilization. Since, during experiment it is able to measure only the rate of obtained product, the insight into molecular structure of calcium alginate matrix and its cooperation with enzyme, substrate and product could result in increasing understanding in this fairly complicated system. The calcium alginate matrix has been studied before by means of molecular dynamics and ab initio [*Plazinski and Drach, 2013; Stewart et al., 2014*] mainly concentrating on studies of the nature of binding between calcium ions and alginate units. However the change of the calcium alginate net topology and the cooperativity of alginate units with calcium ions in the function of increasing temperature of the net has never been investigated before.

In order to fully understand the effect of environmental conditions (e.g. temperature) on activity properties of immobilized enzyme (β-galactosidase), the current study was separated into two limiting processes: (i) the activity of the enzyme itself and (ii) the changes of structural properties of hydrogel network Therefore two main aims were defined. First of all – determination of the temperature profile of activity and stability of β-galactosidase immobilized in a alginate-Ca^{2+} matrix under experimental study; and the second one – preliminary modeling the changes generated in alginate-Ca^{2+} structure with respect to the temperature using molecular dynamics approach.

Experimental

Materials

Alginic acid sodium salt, β-galactosidase from *Kluyveromyces lactis* (*Sigma-Aldrich*, Germany); glucose oxy dst reagent (*Alpha diagnostics*, Poland); lactose, glucose, calcium chloride and other chemicals of analytical grade (*POCh*, Poland).

Methods

Immobilization of β-galactosidase was performed using entrapment method. Briefly, hydrogel particles with enclosed enzyme were received by dropping β-galactosidase solution prepared in 1.5% sodium alginate (0.1 M Tris-HCl buffer, *pH* 7.0) into cooled solution of calcium chloride (2.0%). Then the beads were left for 2h at 4°C in order to achieve suitable degree of cross-linking. After this time, particles with immobilized enzyme were rinsed twice with distilled water and 0.1 M Tris-HCl buffer (*pH* 7.0). Entrapped β-galactosidase retains 68% of initial activity of its native native form.

Catalytic activity of free and immobilized β-galactosidase was determined using enzymatic activity assay based on detection of glucose concentration in the reaction mixture. Enzyme activity was measured in 0.1 M Tris-HCl buffer (*pH* 7.0) using lactose (50 $g \cdot L^{-1}$) as a substrate. The reaction rate was determined spectrophotometrically at 500 nm. In case of β-galactosidase the activity unit of (1 U) was defined as an amount of enzyme with catalyze hydrolysis of lactose causing production of 1 μg of glucose per min.

The activity of alginate entrapped enzyme was measured in batch reactor (150 rpm). Firstly immobilized *β*-galactosidase (50÷200 particles) was suspended in 0.1 M Tris-HCl buffer (*pH* 7.0), placed into the reactor with temperature maintained at given value. Then preheated lactose was added (end concentration of 50 $g \cdot L^{-1}$) and several probes (for determination of produced glucose) were taken from the reactor at specified time intervals.

Before experiments the activity of native and immobilized enzyme (measured at 310 K) was unified approximately to 1 500 U per mL of applied preparation. Then, the effect of temperature on the activity of free and entrapped enzyme was determined by measuring the activity of tested preparation (*pH* 7.0) at 277–333 K.

Next, research of thermal inactivation of immobilized β-galactosidase were performed at 298, 303, 310 and 313 K. Experiment at each temperature was carried out in well-mixed reactor filled with appropriate volume of preheated buffer (0.1 M Tris-HCl, *pH* 7.0). The inactivation process was started by adding the alginate-Ca^{2+} beads with entrapped enzyme (from 50 to 200 items) to reactor. After a specified period of time immobilized β-galascosidase particles were separated, rinsed with cold buffer and left at 4°C until the activity measurements. The inactivation kinetics was described as a first-order irreversible process and according to it rate constants (k) were calculated for each temperature. Then, temperature dependence of obtained k values was expressed using *Arrhenius* equation:

$$\ln k = \ln A - \frac{E_a}{RT}, \tag{1}$$

where:
k – rate constant at individual temperature [h^{-1}]
A – pre-exponential factor [h^{-1}]
E_a – inactivation energy [$J \cdot mol^{-1}$]
R – universal gas constant [$J \cdot mol^{-1} \cdot K^{-1}$]
T – temperature [K]

To fully understand the structural changes in calcium alginate system molecular dynamics technique was employed. For this purpose standard *Verlet's* algorithm as implemented in *Biovia Materials Studio 7.0* package was used. The CVFF forcefield (*Consistent Valence Forcefield*) was applied for modeling covalent interactions within alginate subunits and interactions between alginate and calcium and sodium ions.

Results and discussion

At first, typical experimental research associated with study the effect of temperature on activity and stability of β-galactosidase entrapped within alginate-Ca^{2+} matrix was carried out. They included: determination of temperature in which enzyme activity achieves the maximum value; followed by estimation of parameters of thermal inactivation, which consequently leads to the final information about thermostability of given biocatalyst.

Results obtained during study of microenvironment temperature effect on the catalytic activity both for immobilized and native en-

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zyme are presented in Figure 1. They indicate that, despite the fact that enzyme entrapped in the gel network does not change its temperature optimum. Nevertheless the immobilized biocatalyst retains more than 95% of the maximum activity in a broader temperature range. These results could be summarized as follows: applied immobilization method does not alter the catalytic properties of entrapped enzyme and provides a favorable microenvironment conditions for effective application of β-galactosidase.

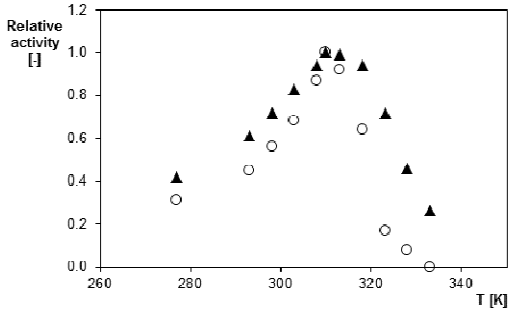

Fig.1. Effect of temperature on relative activity at given temperature for free (○) and (▲) immobilized β-galactosidase. The highest activity values were set as 100%.

Next, activity profiles of inactivation were determined at several temperatures for immobilized biocatalyst. Kinetics of this process was described by first-order irreversible model. Values of inactivation rate constants (k) and half-life of immobilized enzyme ($t_{1/2}$) are presented in Tab. 1. According to obtained results it could be assumed that, compared to literature reports [*Zhou and Chen, 2001; Taqieddin and Amiji, 2004*], β-galactosidase preparation obtained in current study seems to be quite stable.

Tab. 1. Inactivation rate constants (*k*) and half-life of β-galactosidase immobilized within alginate-Ca^{2+} matrix

Temperature T [K]	Inactivation rate constant k [h^{-1}]	Half-life of entrapped enzyme $t_{1/2}$ [h]
298	0.0102	49.8
303	0.0139	36.1
310	0.0255	20.1
313	0.0398	12.9

The temperature dependence of rate constants can be used to evaluate inactivation energy according to the *Arrhenius* equation (Fig.2). In this case, energy of thermal inactivation for β-galactosidase entrapped within alginate-Ca^{2+} matrix was determined. It is worth

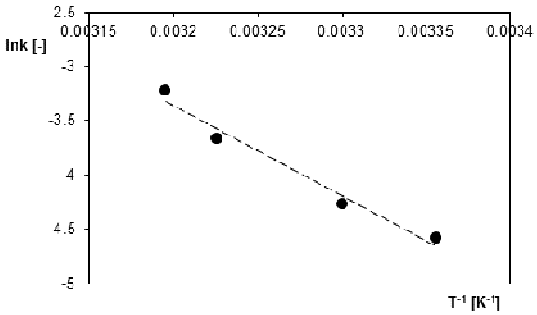

Fig.2. *Arrhenius* plot for the first-order rate constants obtained experimentally for inactivation of β-galactosidase entrapped in alginate Ca^{2+} matrix.

mention, that energy value obtained in this study (68.8 $kJ \cdot mol^{-1}$) is approximately twice lower than for free form of this enzyme described in literature [*Matioli et al.,2001*]. This phenomenon could be explain by the fact that entrapment of this enzyme preserves its tertiary structure, and protects it from conformational changes, that could be affected by microenvironmental conditions in case of non-immobilized enzyme. Increased resistance to activity loss at variety of temperature values confirmed the feasibility and effectiveness of immobilization of β-galactosidase in alginate-Ca^{2+} network. Thereby indicating the possibility of increasing the operational stability of the biocatalyst.

Molecular modeling approach

The overall reaction catalyzed by the enzyme immobilized within hydrogel matrix is fairly complicated and requires detailed analysis on atomic level. There are several unanswered questions having great impact on the detailed understanding of this process. The most important ones include what are the transport properties of the alginate-Ca^{2+} network, the mechanism of ion selectivity in the process of alginate cross-linking – specifically why only divalent metal ions take part in the process and what is the mechanism and the conditions for ions removal resulting in disorganization of the 3-dimensional alginate structure. To fully address above mentioned problems detailed analysis on atomistic level is necessary, henceforth molecular modeling approach was employed.

Alginate is linear copolymer of β-D-mannuronate (M) and α-L-guluronate (G) residues connected by 1,4-linkage arranged in linear chains. Carboxyl moieties both in G and M residues enable to bind selected group of ions inducing hydrogel creation. In the most common case calcium ions are bound to G residues forming so called *egg-box* model. Recent theoretical and experimental results [*Liang-bin, 2007; Plazinski and Drach, 2013*] confirm this type of interactions together with some other bounding sites of metal ions in the alginate chain.

The main aim of this part of research was to give some qualitative picture of the influence of temperature on the pore diameter between alginate chains bonded with interactions mediated by Ca^{2+} ions. In this case, molecular modeling seems to be natural choice of investigating of topological factors due to difficulties in obtaining them in experimental way. In this paper we present preliminary results obtained for simpler example of alginate-Ca^{2+} structure, that include two chains of 4 G units each bound by two calcium ions (Fig. 3).

First of all, two blocks of above described alginate matrix were modeled.

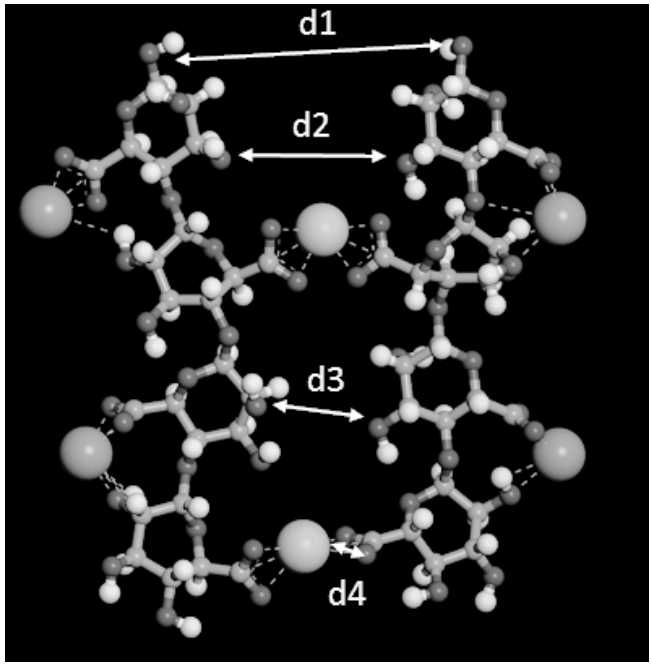

Fig.3. Alginate dimer with four G units each bound by two Ca^{2+} ions (middle large bright spheres). Four Na^{+} (outer large dark spheres) are also added in order to preserve charge neutrality of the system. During the simulation distances (d1, d2, d3 and d4) between oxygen atoms (dark small spheres) were measured in order to study its structural properties. Grey small spheres indicate carbon atoms and bright small spheres hydrogen atoms.

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This structure resembles typical *egg-box* model configuration. Additional four sodium ions were added to preserve neutrality of the system. The system was then optimized allowing ions to bound via ionic coupling to typical binding sites around carboxyl groups as indicated be dashed lines on Fig. 3.

In order to study the dynamics of the structural properties of this system in the function of temperature NVT simulations were performed. In this case, *Berendsen's* thermostat with 0.01 ps decay constant was used. The simulation was started from 50 K and equilibrated for 50 ps with 1 fs time step. After 50 ps the temperature was increased to 298 K and equilibrated for another 100 ps. Total simulation included 150 000 steps. Three characteristic distances evolution with temperature between oxygen atoms measured during simulation are presented in Fig. 3 and Tab. 2. They give qualitative information regarding the distance between alginate units in the function of increasing temperature. The distance between calcium ion and one of the bounding oxygen atom was also determined. The result of simulation is presented in Fig. 4

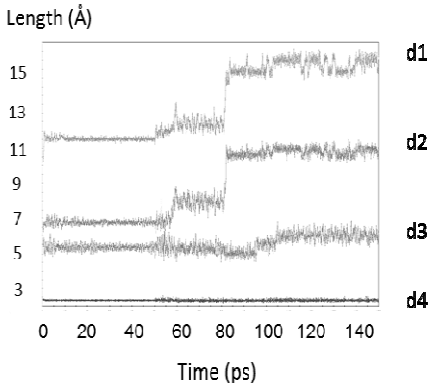

Fig.4. Temporal evolution d1, d2, d3 and d4 distances of Alginate dimer system with two Ca^{2+} and four Na^{+} ions. The system is initially equilibrated for 50 ps in 50 K. The temperature is then gradually increased up to 303 K and equilibrated for another 100 ps. Although the distance between Calcium ion and oxygen atom remains unchaged the inter dimer distance increased significantly with the increase of the temperature.

As one observe calcium ions have great impact on stabilizing alginate units geometry manifested by constant 2.38 Å distance between this type of divalent ion and carboxyl groups. However due to flexibility of glycosidic bond the inter alginate chains distance is increasing. This effect is greater with increasing distance from calcium ions.

The larger relative distance d1 and d2 (Fig. 3) is the result of the twist between first and second G unit. In fact with increasing temperature the alginate units separate starting from the top of our configuration in the zipper-like manner reducing Calcium-Oxygen coupling. The hypothesis is that this effect in presence of water molecules will be even greater due to 80 times larger dielectric constant than in vacuum.

Tab.2. Inter dimer distance changed due to increase of temperature from 50 to 303 K. (The relative change is given in the brackets)

Temperature T[K]	Distance, d [Å] (Relative change, [%])			
	d4	d3	d2	d1
50	2,313	5,343	6,759	11,531
303	2,313 (0%)	6,003 (12%)	10,894 (61%)	15,95 (38 %)

This scenario will be studied in the following papers. The main purpose of next study will be determination of the dependence of the distance between alginate oligomers and the size of immobilized enzyme, which could clarify the stabilization phenomenon of entrapped enzyme.

Conclusions

Summing up the experimental study of temperature impact on the catalytic properties of β-galactosidase entrapped in alginate-Ca^{2+} matrix, it could be concluded that this is a proper method of immobilization, which enables preservation of the specific catalytic properties and improve thermal stability of enclosed biocatalyst.

Nevertheless taking into account that experimental research allows only for determination the rate of obtained product, the insight into molecular structure of calcium alginate matrix and its cooperation with enzyme, substrate and product could be valuable tool for increase of detailed understanding of this fairly complicated system.

Preliminary computational approach presented in this study showed that molecular modeling gives a valuable insight into dynamics of the structure of hydrogel chains and offers opportunity to study its transport properties having great effect on catalytic properties of the investigated system.

The combination of experimental results and computer simulation on molecular level provides additional insight greatly enhancing capabilities to control and optimize the process using immobilized biocatalyst.

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