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Kinetic studies of β-galactosidase immobilized in chitosan/xanthan multilayers

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A study of the kinetic parameters of β-galactosidase produced from *Aspergillus niger* was carried out in the present work. This enzyme was immobilized in chitosan and xanthan polyelectrolyte multilayers (PEMs) deposited by dip coating method on corona charged polylactic acid pads. The enzyme activity showed a temperature optimum at 50 °C and a pH optimum at 5.0. The effects of lactose concentrations on the initial velocity of the enzyme reaction were also compared and Michaelis-Menten constans were calculated at 53.4 mmol of lactose. These results gave insights for further optimization of transgalactosydase reactions in order to obtain lactulose and other specific galactooligosaccharides having pronounced bioactive properties.

Keywords: β-galactosidase, lactose, kinetics, transgalactosylation, multilayers

INTRODUCTION

β-Galactosidases (EC 3.2.1.23) also known as lactases are enzymes belonging to glycoside hydrolase families 1, 2, 35, 42 and 59 (GH1, GH2, GH35, GH42 and GH59) [1]. These enzymes catalyze the hydrolysis of terminal non-reducing β-D-galactose residues in β-D-galactoside substrates. In the case of lactose as a substrate the products of hydrolysis – namely glucose and galactose are transferred to water. When the initial concentration of lactose is more that 15%, acceptor of galactose residue can be another carbohydrate presents in the reaction mixture – for example lactose, fructose or lactulose, and an transgalactosylation reaction is observed, resulting in production of galactooligosaccharides (GalOSs) with a different degree of polymerization (DP) [2, 3]. Known microbial sources of β-galactosidases are bacteria (*Bacillus*, *Lactobacillus*, *Bifidobacterium*, *Escherichia*), yeasts (*Kluyveromyces*, *Sterigmatomyces*) and fungal producers (*Aspergillus*) [3, 4]. Among these producers, the commercial preparations from *Kluyveromyces lactis* (Lactozym 3000L and Maxilact LGX 5000), *Aspergillus oryzae* (Enzeco® Fungal Lactase) and *Bacillus circulans* (Biolacta – Daiwa Kasei and Biolactase) are applied mainly for the hydrolysis of lactose in dairy industry concerning lactose intolerance in significant part of human populations [5]. On the other hand the GalOSs synthesized by β-galactosidases contain variety of bonds including β-(1→4), β-(1→6) and β-(1→3), which determines their application as food additives with significant prebiotic potential and other beneficial effects [3, 4]. The amount and structure of the synthesized GalOSs are significantly affected by the enzyme source, the acceptor molecules and the reaction conditions (substrate concentration, pH and temperature) [6-8].

The review [9] sheded light on the strategic advantages of the microscale promising technology for the development and realization of biocatalytic processes and subsequent product recovery steps.

The immobilization of enzymes into polymer matrices is widely used in biotechnology. This technique makes it possible to increase the functional efficiency of enzyme, enhance the reproducibility of the processes, improve the process control and ensure stable supply of the products in the market [10]. The basic idea of enzyme immobilization is to entrap the protein in a semi-permeable support material, which prevents the enzyme from leaving while allowing substrates, products, and co-factors to pass through [11]. The main requirements for the immobilizing matrix are that the material should be non-degradable and compatible with the enzymes. The immobilization process should be mild enough and not to denature the enzyme during preparation [12].

One of the suitable and mostly used natural polymers for enzyme immobilization is chitosan. It is characterized as biocompatible, nontoxic, physiologically inert, and hydrophilic, offers the unique characteristic of a remarkable affinity to proteins and has been widely applied in medicine and biological research. Furthermore, chitosan is positively charged in acidic aqueous solutions and its charge density is high [13].

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The effect of the support size on the properties of enzyme immobilization was investigated by using chitosan macroparticles and nanoparticles in [14]. β-Galactosidase was used as a model enzyme. It was found that the different sizes and porosities of the particles modify the enzymatic load, activity, and thermal stability of the immobilized biocatalysts. The biocatalysts thermal stability was improved for macroparticles, especially under reactive conditions (presence of lactose) in comparison with the free enzyme. Besides, both preparations could be reused for 50 repeated batches in the lactose hydrolysis without any outstanding loss of enzyme activity.

In [15] the suitability of spray drying as a method for the preparation of cross-linked chitosan microparticles with immobilizes laccase, and the influence of process parameters, composition and cross-linking method on the properties of the resulting microparticles and the activity of the immobilized enzyme were investigated.

Alginate–chitosan core-shell microcapsules were prepared in order to develop a biocompatible matrix for enzyme immobilization, where the protein is retained either in a liquid or solid core and the shell allows permeability control over substrates and products. In [16] alginate–chitosan core-shell microcapsules were prepared as a novel biocompatible matrix system for β-galactosidase enzyme immobilization where the catalyst is confined to either a liquid or solid core and the transport properties of the substrate and product are dictated by the permeability of the shell. Thus the biological agent is protected in the inner biocompatible alginate core and the outer chitosan shell dictates the transport properties.

The method of surface modification by layer-by-layer (LbL) polyelectrolyte multilayers allows very precise control and changes in a wide range of the carrier's physicochemical properties — thickness, charge, hydrophilic–hydrophobic balance [17]. Such multilayer coatings with included functional components (nanoparticles, enzymes, and dyes) may be used in microelectronics, optics, biotechnology, and pharmacy [18]. The LbL technique is based on a sequential deposition of oppositely charged polyelectrolytes from their solutions via electrostatic interactions. The assembly is based on spontaneous adsorptions, no stoichiometric control is necessary to maintain surface functionality, and the assembled films have good thermal and mechanical stability [19]. In [20] the immobilization of α-chymotrypsin on the surface of boron silicate glass microspheres is conducted via the technique of multilayer adsorption of polyelectrolytes. It is shown that the enzyme is adsorbed on both positively and negatively charged surfaces and its activity is partially preserved relative to that in solution. It was established that the activity of the enzyme depends on the number of polyectrolyte layers preliminarily adsorbed on glass microspheres and on the charge of the surface. The activity of α-chymotrypsin adsorbed on the negatively charged surface is four times higher than the activity of this enzyme adsorbed on a positively charged surface.

The aim of the present work is to describe the immobilization of the β-galactosidase enzym in chitosan/xanthan multylayers deposited on corona charged polylactic acid pads. Emphasis was focused on the effect of the number and the sequence of the multilayers on the immobilized enzyme functional properties.

MATERIALS AND METHODS

Enzyme activity

A commercial fungal β-galactosidase (from *Aspergillus niger*) was used in the current kinetic studies. One unit of β-galactosidase activity is defined as the amount of enzyme catalyzing the release of 1 μmol min^{-1} ortho-nitrophenol at 37 °C and pH 5.0. The influence of the substrate concentration on the initial velocity of the enzyme reaction was studied at a range 0.01 M – 1.30 M lactose. β-Galactosidase activity was studied in the presence of lactose – 1%, 5%, and 10% and mixtures of chitosan (0.1%) and lactose (1%, 5%, and 10%). One unit of β-galactosidase activity is defined as the amount of enzyme catalyzing the release of 1 μmol min^{-1} glucose or fructose at 37 °C and pH 5.0. The concentrations of the released glucose and fructose were determined enzymatically [21]. Protein concentration was assayed by the method of Bradford [22]. All the analyses were performed at least in triplicate. Programmable scientific calculation "CITIZEN" SRP-45N and SigmaPlot 12.0 (Systat Software, Inc) were used for data analysis.

Samples preparation

Biodegradable pads were prepared from polylactic acid (PLA) with ester end groups and intrinsic viscosity 0.55 - 0.75 dL/g purchased from Lactel Absorbable Polymers (USA). The PLA pads were cast from 2% w/v PLA solution in chloroform and dried at 35 °C for 48 hours. Then the PLA pads were kept for 24 hours in an excicator, at room

temperature, and 54% relative humidity (RH). Before the deposition process, the pads were charged in a corona discharge system, consisting of a corona electrode (needle), a grounded plate electrode, and a metal grid placed between them. Positive or negative 5 kV voltage was applied to the corona electrode. 1 kV voltage of the same polarity as that of the corona electrode was applied to the grid. The samples were charged under standard room conditions (T = 21÷23 °C and RH = 50-60%) for one minute. The polylactic acid electret properties are described in [23].

Polyelectrolyte multilayers deposition

Xanthan gum and chitosan (low molecular weight) were purchased from Sigma-Aldrich. They were used without further purification or characterization. The layer-by-layer (LbL) deposition technique was applied for multilayer build-up. For the LbL assembly 0.1% w/v chitosan and 0.05% w/v xanthan solutions in acetate buffer (pH 5 and ionic strength 0.1 M) were prepared. 1 g/L β-galactosidase was dissolved in the chitosan solution just before the deposition process. The deposition was done by the dip-coating process. The first built-up layer always possesses opposite to the pad electric charge. A slide stainer (Poly Stainer IUL, Spain) was used with the following program: 15 min dipping process – adsorption from the first polyelectrolyte solution, 5 min washing step in the acetate buffer (pH 5 and ionic strength 0.1 M), 15 min dipping process - adsorption from the second polyelectrolyte molecules of opposite charge; 5 min washing in the same acetate buffer. The procedure was repeated until obtaining the desired numbers of even layers (8, 14 or 20 xanthan/chitosan or chitosan/xanthan). After the deposition of the last layer the film was dried in hot air. The produced PEMs structures were stored in an exicator at 55% RH.

RESULTS AND DISCUSSION

Effect of type of PEMs on immobilization and enzyme activity

The effect of the initial concentration of lactose on the enzyme activity of β-galactosidase was studied. Enzyme activity was measured under various immobilization procedures in different types of PEMs. Chitosan was selected as a material for polyelectrolyte multilayer preparation for several reasons - cationic biopolymer with intra- and intermolecular hydrogen bonding ability; unique approach to modify the surface of chitosan by anionic biopolymer xanthan to improve biocompatibility; and the possibility to prepare chitosan membranes with controlled pore size and density. The results of the enzyme activity measuring are shown in Table 1. The enzyme activity of immobilized β-galactosidase was compared with that of free enzyme using o-nitrophenol-β-D-galactopyranoside (ONPG) as a substrate. As it is shown in Table 1, the highest activity was determined for positively charged PLA pad with 14 multilayers. The immobilization efficiency indicated that significant amount of the enzyme was bond to the positively charged PLA pad with 14 multilayers of xanthan/chitosan. On the contrary, immobilization efficiency is almost 3 times less in negatively charged PLA pad with 14 multilayers of chitosan/xanthan. Obviously, in order to achieve a good enzyme activity the optimal configuration is 14 multilayers with consecutive xanthan/chitosan layer deposition. We have to note that the order of layer deposition is important. For the PEMs with 8 layers and with 14 layers we observed better enzyme activities with consecutive deposition of xanthan/chitosan layers. The enzyme activity was very low for the 20-layer PEMs.

Table 1. β-Galactosidase activity in different types of PEMs.

Type of PEMs	Enzyme activity (U/ml)
Positively charged PLA pad with 8 multilayers	0.0191
Negatively charged PLA pad with 8 multilayers	0.0104
Positively charged PLA pad with 14 multilayers	0.0557
Negatively charged PLA pad with 14 multilayers	0.0194
Positively charged PLA pad with 20 multilayers	0.0010
Negatively charged PLA pad with 20 multilayers	0.0011

Effect of lactose concentration in 0.1% chitosan solution on enzyme activity

The operational stability of the studied enzyme was evaluated by the hydrolysis of buffered lactose solutions (1%; 5%; 10% w/v; pH 5.0) at 37 °C.

Lactose hydrolysis was performed at varying concentrations of lactose and with an enzyme concentration of 1 U/ml. As illustrated in Table 2, there is not any inhibition effect of 0.1% chitosan in 1% lactose.

Table 2. Lactose hydrolysis preformed with β-galactosidase

a) Concentration of lactose – 1%

Time (h)	Glucose (mg/ml)	
	1.0% Lactose	**1.0% Lactose in 0.1% Chitosan**
0	0.00	0.00
0.5	0.00	0.00
1.0	0.06	0.74
1.5	0.26	1.01
2.0	0.66	1.33
2.5	1.28	1.52
3.0	1.33	1.86

b) Concentration of lactose – 5 %

Time (h)	Glucose (mg/ml)	
	5.0% Lactose	**5.0% Lactose in 0.1% Chitosan**
0	0.00	0.00
0.5	0.38	0.28
1.0	1.63	1.85
1.5	1.84	1.93
2.0	3.12	2.29
2.5	4.09	2.66
3.0	4.19	3.67

c) Concentration of lactose – 10%

Time (h)	Glucose (mg/ml)	
	10.0% Lactose	**10.0% Lactose in 0.1% Chitosan**
0	0.00	0.00
0.5	0.79	1.40
1.0	1.77	2.06
1.5	2.68	2.98
2.0	4.09	4.47
2.5	4.76	5.01
3.0	5.68	5.50

The highest value of enzyme hydrolysis from the tested concentrations of lactose was measured in the presence of 1% of this sugar (19% of initial concentration). When the enzyme reactions were performed in the presence of increasing concentration of lactose (from 5% to 10%) a light decrease of hydrolyzing reaction was observed in the presence of 0.1% chitosan (88% of total substrate by 5% lactose and 96% of total substrate by 10% lactose).

Effect of type of multilayer on lactose hydrolysis with immobilized β-galactosidase

On the other hand lactose hydrolysis performed with 1 U/ml β-galactosidase in the presence of 0.1 % chitosan resulted in 19% of lactose conversion in 3h reaction time by 1% lactose concentration. When the lactose concentration was increased, its conversion was decreased to 7.3% by 5% initial lactose concentration and 5.5% by 10% initial lactose concentration. From these results, it can be concluded that the β-galactosidase studied shows good operational stability in the hydrolysis of lactose in presence of 0.1% chitosan and under the conditions stated above. It was necessary to set the experimental conditions at which the diffusion of substrates in the PEMs of the support does not modify the rate of the hydrolysis reaction. The immobilization of β-galactosidase on PEMs was based mainly on absorption. It has been reported that protein adsorption on a surface follows a three step diffusion, attachment and reconfirmation regime.

Lactose hydrolysis was performed at different types of PEMs and with different enzyme activity. From the results in Table 3, the maximum of glucose concentration was detected for the positively charged PLA pads with 20 multilayers at 3h of incubation.

Table 3. Lactose hydrolysis preformed with immobilized β-galactosidase in PEMs

Type of PEMs	Glucose (mg/ml)		
	1 h	**2 h**	**3 h**
Positively charged PLA pad with 8 multilayers	0.1	0.1	0.21
Negatively charged PLA pad with 8 multilayers	0	0.21	0.21
Positively charged PLA pad with 14 multilayers	0.1	0.21	0.39
Negatively charged PLA pad with 14 multilayers	0	0.20	0.28
Positively charged PLA pad with 20 multilayers	0	0.11	0.80
Negatively charged PLA pad with 20 multilayers	0.1	0.28	0.36

For the tested PEMs, the highest value of enzyme hydrolysis was measured for the ones with 14 and 20 multilayers with xanthan/chitosan consecutive deposition. Obviously, the order of layer deposition is significant.

When comparing the activity of the immobilized enzyme with that of the free enzyme, a similar inhibition has been observed due to galactose in the activity of the immobilized enzyme and a decrease in the activation energy of Michaelis–Menten constant of the immobilized enzyme compared to that of the free enzyme at 37 °C. The activity of the

enzyme after immobilization was in all cases equal or higher than 50% of the activity of the free enzyme (data not show).

In the present work, we have studied the influence of different concentrations of lactose on the initial velocity of β-galactosidase reaction. Such kind of kinetic information for lactose hydrolysis by β-galactosidases is scarce in the literature, despite of the fact that this disaccharide can use by transgalactosylation reaction or used in many studies as a donor / acceptor of galactose / glucose units during the synthesis of GalOSs having specific structures and bioactive properties [24-26].

From studies performed by other authors it is known that the galactosyltransferase reaction for synthesis of GalOSs is favored when the lactose concentration in the reaction mixture is more than 15%. Under these conditions the content of free water is low and the transfer of galactose moiety of lactose to suitable acceptor molecules is more likely to occur [27, 28]. It is necessary to perform additional studies concerning the distribution, composition and yield of the obtained transgalactosylation products during the reactions with increasing concentrations of lactose and fructose molecules.

CONCLUSION

The current study is the first time when the influence of chitosan on the initial velocities of reaction catalyzed by β-galactosidase is investigated. The studied immobilizing enzyme in PEMs showed substantial differences in its affinity to the lactose concentration. The obtained results will be further applied and extended in experiments dealing with the direction and optimization of the GalOSs production.

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