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ИММОБИЛИЗАЦИЯ ИНВЕРТАЗЫ НА НЕЙЛОНЕ-6, АКТИВИРОВАННОМ СОЛЯНОЙ КИСЛОТОЙ В ПРИСУТСТВИИ ГЛУТАРОВОГО АЛЬДЕГИДА В КАЧЕСТВЕ СШИВАЮЩЕГО АГЕНТА

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Цель работы – ковалентная иммобилизация инвертазы на нейлоне-6, активированном глутаровым альдегидом. Данный метод модификации носителя был выбран из-за того, что нейлон-6 содержит функциональные карбоксильные (–COOH) и амино ($–NH_2$)-группы. Глутаровый альдегид связывался с нейлоном-6 и инвертазой через амино-группы. Диски нейлона-6 диаметром 30 мм и толщиной 2 мм получали методом экструзионного прессования. Диски имели непористую, но отчетливо шероховатую поверхность. Диски обрабатывали соляной кислотой в различных концентрациях и активировали глутаровым альдегидом. В среднем 1 г диска содержал 0,58 мг иммобилизованной инвертазы, выход иммобилизованной инвертазы лежал в диапазоне от 57 до 60%. Активность инвертазы составляла 51–64% от исходной непосредственно после иммобилизации, 23–43 и 18–31% соответственно через 3 и 6 мес. хранения при 4°C. Иммобилизованную инвертазу использовали в 12 реакционных циклах без уменьшения активности.

INTRODUCTION

Immobilization of enzymes provides a promising technology in the area of enzymology, particularly in the case of enzymatic electrodes, achieving a major development in analytical instrumentation and industrial applications [1, 2]. Invertase (beta--fructofuranoside fructohydrolase, EC 3.2.1.26) is a catalyst for sucrose hydrolysis [3]. Its application in the industrial processes requires immobilization for its recovery and easy separation. The enzyme immobilization technology offers technical and economical advantages such as longer half-life to the immobilized form than in the soluble form [4, 5]. Also the immobilized enzyme process can be adapted to the preferred continuous processes, such as fixed or fluidized bed reactors, in which it is possible to use higher enzyme dosage per volume of reactor than in the soluble enzyme process. This contributes to high reaction rates and consequently, small reactor sizes [3, 6–8].

ifferent polymeric supports have been investigated for enzyme immobilization due to the fact that they can have several functional groups and can be easily modified chemically [9, 10]. These polymeric materials ought to fulfill many conditions, both of physical and chemical nature. First, their surface structure must be strong enough to withstand an enhanced pressure [11, 13]. In addition, the polymer material must be resistant chemically, thermally and biologically. The above conditions are well fulfilled by many aliphatic and aromatic polycaprolactames (*i.e.*, the widely accessible aliphatic nylon-6 as the base material for preparation of immobilized enzymes). Nylon is readily available in many physical forms, such as powder, pellets, tubes, membranes, discs, and more, which offer various surface/weight ratios and are economical [10]. The important advantage of this polymer is its strong hydrophylicity. Polycaprolactame has been used as polymeric support for the enzyme immobilization, by methods that involve the formation of covalent bonds, the micro-encapsulation and absorption [10]. Since nylon has only few free end groups for covalent attachment of enzyme molecules, it must be pretreated to generate potentially reactive centers [10].

The objective of this work was to immobilize invertase by covalent bonding on glutaraldehyde activated nylon-6 in form of discs. The discs of nylon-6 were treated with HCl at different concentrations and activated with glutaraldehyde. The efficiency of the immobilization process and stability of the immobilized enzyme was studied.

MATERIALS AND METHODS

Nylon-6 pellets were used as the basic material for the preparation of discs, which are modified chemically for invertase immobilization. Some physical properties of the initial polymer nylon-6 manufactured by Akra RP

Table 1

Physical properties of nylon-6 used for disc preparation

Properties	Mean Value
Density (g/cm^3)	1.149
Melting temperature, ºC	231.22
Molecular weight g/mol	$31.1935_{\times}10^3$
Humidity, %	0.62
Resistance to the impact, Kg/mm	0.882
Elongation, %	240.0
Crystallization temperature, ºC	170.88
Resistance to the flex, Kg/cm^2	658.0

15-IAA, are presented in the Table 1. The commercial yeast invertase (EC 3.2.1.26, grade V, essentially free of lactose and α-galactosidase), was purchased from Sigma-Aldrich Chemical Co. It had an activity of 30–50 U/mg. One Unit hydrolyzed 0.001 mmole of sucrose to invert sugar per min at pH 4.5 at 55°C. All other chemicals used to modify the nylon-6 discs were of analytical grade and were purchased from J.T. Baker (USA).

Preparation of nylon-6 discs

The powdered nylon-6 pellets were first dried at 30°C for 24 hours. Polycaprolactame discs were manufactured in extruder (ake). The operating conditions of injection were: melt temperature of nylon-6 (T_m) was 231.2°C, applied pressure (P) was 80 psi (lb/in^2) and the time of filling was 2 min. The diameter and thickness of obtained discs were evaluated using a micrometer Model 3 ial Comp (USA).

Nylon Activation by Partial Acid Hydrolysis

Partial acid hydrolysis of polymeric material was performed to increase the quantity of amino groups available for interaction with glutaraldehyde. Initially a range of HCl concentrations from 0.05 M to 4 M was tested and the hydrolysis condition which maintained the discs integrity was selected. Hydrolysis was carried out using 10 ml of 1 and 2 M HCl for each disc, at 20 and 30°C for 10, 20 and 30 minutes. The acid was then removed, and the discs were washed with distilled water to neutral pH. In order to evaluate the maximum amount of carboxyl groups formed after the acid hydrolysis, a disc (2.3 g of nylon) was shaken in 10 ml of 0.1156 M sodium hydroxide solution for 2 h at room temperature by using a shaker IK-VIBRAX-VXR. The decrease in alkalinity or the residual concentration of sodium hydroxide in the filtrate was determined by titrating with standard 0.1 N HCl using phenolphthalein as an indicator. The amount of free functional groups was expressed as mEq NaOH, which was calculated as difference between initial mEq NaOH (1.156 mEq) and residual NaOH solution.

Chemical Modification of Nylon-6

The chemical activation of nylon-6 was performed by reaction with glutaraldehyde [5]. Each partially hydrolyzed nylon-6 disc as well as the control disc without hydrolysis were activated by treating them with 10 ml of 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 8.0, for 3 h at 40°C. After this treatment, the glutaraldehyde solution was removed and the nylon-6 discs were washed several times with 0.1 M sodium phosphate buffer pH 7.8 Activated nylon-6 discs were immediately used for invertase immobilization.

Enzyme Immobilization

To immobilize invertase on the activated discs, 10 ml of 1 mg/ml invertase solution in 0.1 M sodium phosphate buffer pH 7.0 was incubated with 1 disc (2.3 g of nylon) for 12 h at 37°C [5, 14]. The immobilization time was chosen from the results of assay carried out for 0.05, 1, 2, 3, 4, 5, 6 and 12 h using the discs activated with 1 M HCl at 20°C for 20 min. Similar discs were used for another assay with different invertase concentrations from 0.05 to 1.2 mg/ml.

Unbound enzymes were removed by filtration and several washings with distilled water. A similar treatment was carried out with no activated discs and the discs activated with glutaraldehyde but without hydrolysis to compare its adsorption behavior for invertase with that of discs activated by the described techniques in order to validate the application of the activation procedures of this study.

The protein content of the initial enzyme solution as well as the residual solutions obtained after washing was determined by means of absorbance at A_{280}. The percent of enzyme immobilized was calculated from the results obtained from the difference in the absorbance between the initial and the residual solutions.

The activity of the free enzyme and the immobilized enzyme were determined. The enzyme preparations were stored at 4°C in dry state. Storage stability was studied by means of spectrophotometric technique.

Invertase Activity Measurements

The activity of the free and immobilized enzyme was measured spectrophotometrically by means of quantification of glucose formed during the hydrolysis of 0.35 mM sucrose in phosphate buffer at pH 7.0, at 37°C after 1 h [15]. The reaction mixture contained 10 ml of sucrose solution and the disc with immobilized

invertase or 10 ml of enzyme solution adding sucrose to maintain the above sucrose concentration. The activity was measured using initial enzyme solution, residual solution and solutions obtained after washing, as well as with immobilized enzyme [15]. Glucose concentration was measured by means of the reaction with *o*-toluidine [16] monitoring the absorbance at 630 nm in a spectrophotometer (Model 340, Spectronic 20).

The stability of the immobilized invertase after 3 and 6 months of storage at 4°C was determined by activity measurements. For comparison, the activity of same amount of free invertase in solution was determined under similar conditions. Moreover the activity of immobilized invertase was studied for several cycles to determine the number of applications that can be used for the sucrose hydrolysis.

All assays were done in triplicate and by using 5 discs for each determination.

RESULTS AND DISCUSSION

The polycaprolactame discs used in the present study for the immobilization of invertase were manufactured form powdered pellets of nylon-6. The high values of mechanical properties of this material are observed (Table 1). The melting temperature was important to make the physical modification to manufacture nylon-6 discs. Each discs obtained from this material was 30 mm of diameter () and 2 mm of thickness (E). It weighed 2.3 g with an available surface area (A) of 16.018 cm^2 and the surface/weight ratio (R) was 6.96 $cm^2 \cdot g^{-1}$. The nylon-6 discs had a nonporous but reasonably rough external surface.

It is known that nylon-6 has only few free end groups which could be used for covalent attachment of enzyme molecules. The titration of the untreated discs demonstrated the presence of 0.06 mEq NaOH of carboxyl groups per gram of support. In order to increase the amount of potentially reactive centers the polymeric material was subjected to partial acid hydrolysis. This approach is based on the partial hydrolysis of amide (−CO−NH−) groups that led to the increase in the yield of free amino and carboxyl groups on the polymer surface. Both the carboxyl and amino groups of the partially hydrolyzed nylon can be used for the direct attachment of the enzyme molecules [15]. The greater number of carboxyl groups corresponds to major number of amino groups that can react with glutaraldehyde. However, the acid concentration, reaction temperature as well as the duration of reaction can influence not only the group concentration, but also the mechanical properties of material. It may be dissociated completely or partially and the appearance can be changed significantly. Several preliminary assays showed

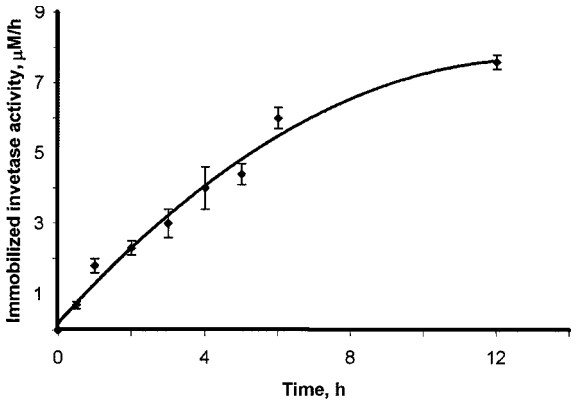

Fig. 1. Activity of invertase immobilized on nylon-6 discs as the function of immobilization time

that the partial hydrolysis must be carried out on the mild condition to avoid the material loss of nylon-6. It was observed that the hydrolysis with 0.5 M HCl for 20, 30 and 45 min did not increase the concentration of carboxyl groups. However, the use of 3 and 4 M HCl for 10, 20, 30 or 45 min at 15, 20, 25°C led to significant increase in the amount of −COOH groups, but the nilon-6 discs lost their mechanical resistance. The numerous assays with different hydrolysis conditions allowed the selection of 1 or 2 M as the acid concentration, 20 or 25°C as appropriate temperature and 20 or 30 min as the best reaction time. Practically under this condition the nylon-6 discs maintained their integrity and demonstrated a considerable increase in the carboxyl free groups.

Study of invertase immobilization kinetics was carried out using nylon-6 discs activated with 1 M HCl at 20°C for 20 min according to Onyezili [5]. The activity of the invertase immobilized on discs by cross-linking with glutaraldehyde was measured at different times of incubation with invertase (Fig. 1) and was expressed as a concentration of reaction product (glucose) formed after 1 h of enzymatic sucrose hydrolysis [11]. The major activity was observed after 12 h of incubation, and it was 51.4% of the activity of free invertase used for immobilization. This incubation time was used on the next assays for invertase immobilization on nylon-6 discs pretreated with HCl under different conditions.

The results of protein immobilization are given in Table 2. The applied hydrolysis conditions (HCl concentration, temperature and hydrolysis reaction time) are described. The amount of free functional groups is expressed as mEq NaOH/gram of support, defined by titration measurements. It was higher on discs treated with 1 M HCl at 20 and 25°C for 20 or 30 min. The values obtained for other treatments were approximately 2-fold less. The results were comparable with data report-

Table 2

Yield of invertase protein immobilized on activated nylon-6

Hydrolysis condition HCl(M)/T(°C)/t(min)	(mEq NaOH/g of support)	A_{280} on residual invertase solution	Yield of protein immobilized (%)
Nylon without hydrolysis and glutaraldehyde treatments	0.06+/–0.02	1.343+/–0.021	19.8
Nylon without hydrolysis but with glutaraldehyde treatment	0.11+/–0.03	0.933+/–0.012	44.3
1/20/20	1.034+/–0.03	0.703+/–0.009	58.0
1/20/30	0.946+/–0.02	0.700+/–0.010	58.2
1/25/10	0.485+/–0.04	0.720+/–0.014	57.0
1/25/30	0.484+/–0.04	0.670+/–0.020	60.0
2/20/30	0.484+/–0.03	0.688+/–0.007	58.9
2/25/30	0.486+/–0.02	0.703+/–0.005	58.0

*A_{280}=1.675 corresponded to initial invertase solution and was used as 100%.

ed earlier for invertase (EC 3.2.1.26) immobilized inside the modified nylon tubes, which showed that between 4 and 20% of the protein exposed were immobilized [17].

It was demonstrated that the use of glutaraldehyde as well as the hydrolysis treatment led to an increase in the immobilization of protein on discs (Table 2). The amount of immobilized protein increased by 2-fold due to glutaraldehyde treatment and approximately by 2.9-fold by means of HCl pretreatment in comparison with control without treatments. The difference between different HCl treatments was not very significant. It was in the range of 57 to 60.0% of immobilized protein. It was not correlated with the number of titrated carboxyl groups. Considering the initial invertase quantity was 1 mg/ml×10 ml = 1 mg, it was calculated that approximately from 0.57 mg to 0.60 mg of invertase per disc or from 0.247 mg to 0.261 mg of invertase per g of support was immobilized. The quantity of immobilized invertase obtained in this study was greater than the data reported earlier [17].

The activities of the immobilized invertase were defined and compared with that of free invertase (Table 3). It was recorded that the glutaraldehyde as well as the hydrolysis treatment led to an increase in the activity of immobilized invertase (Table 3). The activity increased by 2.8-fold due to glutaraldehyde treatment and approximately by 3.1-fold by means of HCl pretreatment in comparison with control without treatments. The differences between HCl treatments were on range from 41.2% to 64.2%, i.e. approximately at 2-fold less than the activity of free invertase. The lesser activity of immobilized invertase was partially related to the incomplete protein immobilization (Table 2) as well as to inactivation of enzyme during the chemical reaction with cross-linking reagent and active groups of nylon-6 [5, 8]. According to the results, in some case the activity of the immobilized invertase was higher with a smaller quantity of immobilized protein and this might be re-

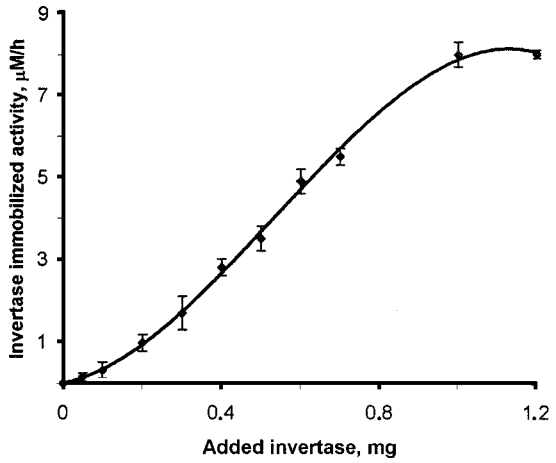

Fig. 2. Activity of immobilized invertase as the function of invertase concentration

Table 3

Activity of invertase immobilized on activated nylon-6

Hydrolysis condition HCl(M)/T(∘C)/t(min)	Activity (μM glucose/ 1h)	Activity of immobilized invertase (%)
Nylon without hydrolysis and glutaraldehyde treatments	2.5+/–0.1	16.8
Nylon without hydrolysis but with glutaraldehyde treatment	7.01+/–0.2	47.3
1/20/20	7.6+/–0.1	51.4
1/20/30	9.5+/–0.2	64.2
1/25/10	8.0+/–0.1	54.1
1/25/30	7.1+/–0.1	48.0
2/20/30	6.1+/–0.2	41.2
2/25/30	8.3+/–0.1	56.1

*14.8 mM/h corresponded to initial invertase solution and was used as 100%.

lated to some space effect and that some active enzyme center were available to react with the sucrose [11].

The results of Table 2 and Table 3 suggested that the applied invertase concentration was near to the point of saturation of support with the protein. To verify this hypothesis, an assay with different enzyme quantities was carried out. It was observed the activity increased with the concentration of invertase up to 1.0 mg. However, there was no difference in the activity between 1.0 and 1.2 mg of enzyme (Fig. 2). These results demonstrated the saturation of nylon-6 disc with the applied enzyme.

Similar conclusion may be obtained from simple modeling calculations. It was supposed that surface area and porosity of nylon-6 disc determined the amount of enzyme that may be immobilized. In this study it was demonstrated that at less as 0.58 mg of enzyme was immobilized on one disc. Using the molecular weight of invertase $M_w = 135000$ a [17], the quantity of immobilized invertase was calculated as 4.29×10^{-9} mole. It is 2.59×10^{15} molecules of invertase. Considering the dimensional size of the nylon disc as cylinder, the surface area of both sides is calculated as 1.6×10^{17} Å^2. Considering spherical model of enzyme molecule and radius of protein as 50 Å, area of the superficial projection of this molecule is 7850 Å^2 and for the mole-

Table 4

Storage stability (%) of immobilized and free invertase at 4° C

Hydrolysis condition HCl(M)/T(°C)/t(min)	Storage stability of immobilized and non-immobilized invertase, %			
	1 week	1 month	3 months	6 months
Free Invertase	12	5	0	0
Nylon without hydrolysis and glutaraldehyde treatments	97	36	12	4
Nylon without hydrolysis but with glutaraldehyde treatment	69	40	36	18
1/20/20	58	30	23	18
1/20/30	62	46	33	30
1/25/10	50	45	42	30
1/25/30	88	69	43	30
2/20/30	81	70	38	20
2/25/30	40	40	31	31

*Enzyme activity at the beginning of assay was used as 100%. The initial activities are presented in Table 3

cules immobilized on nylon disc it is 2.03×10^{19} Å^2, that is 126 times more than the available surface. It is demonstrated that the surface of support is practically occupied by the immobilized enzyme. The molecules may have the space problem to perform its activity. Moreover, the change in the amount of the active support groups after certain concentration is not the limiting factor of enzyme immobilization and activity. This theoretical approach confirmed the experimental results obtained in this study.

One of the advantages of immobilized enzymes in comparison with free soluble enzymes its reusability. The immobilized enzyme can be easily separated from the reaction mixture and reused for more cycles of enzymatic reaction. In the present study it was demonstrated that all preparations of invertase cross-linked on the nylon discs pretreated o untreated with HCl maintained their activity for 12 cycles of enzymatic reaction, and beyond that the activity decreased but was detectable up to 21st cycle, while the enzyme immobilized on discs without glutaraldehyde lost their activity after seven cycles, which might be related to the wash out of enzyme due to the weak forces of attraction with support. The high storage stability of immobilized enzyme was also observed in this study (Table 4). The free enzyme solution was inactivated after 1 month of storage, while the immobilized preparations were active for 6 months at 4°C. The behavior of different immobilized preparations was variable (Table 4). In general, the enzyme immobilized on the HCl treated discs was more stable than the enzyme from untreated discs during their storage for 3 and 6 months, but after one week the major activity was detected on untreated discs.

Thus, the present study demonstrated that the nylon-6 discs pretreated with HCl are a good carrier system for invertase immobilization by means of cross-linking with glutaraldehyde, which provided reusability of immobilized enzyme and its high storage stability.

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INVERTASE IMMOBILIZATION ON NYLON-6 ACTIVATED BY HYDROCHLORIC ACID IN THE PRESENCE OF GLUTARALDEHYDE AS CROSS-LINKER

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The objective of this work was to immobilize invertase on glutaraldehyde activated nylon-6 by covalent bonding. This method of nylon-6 modification was chosen due to its chemical structure containing the functional carboxyl (–COOH) and amine (–NH_2) groups. Amine groups bind with glutaraldehyde, which in turn binds with invertase. Nylon-6 discs were produced by extrusion (the disk diameter (D) was 30 mm and the thickness (E) was 2 mm). The discs had nonporous but fairly rough surfaces. The discs were treated with different concentrations of HCl and activated with glutaraldehyde. On an average, 0.58 mg of invertase was immobilized onto each gram of a disc. The percent yield of immobilization of the invertase varied between 57 and 60%. Immediately after immobilization 51–64% activity was retained, 23–43% after 3 months and 18–31% after 6 months of storage at 4°C. The immobilized invertase was used in 12 cycles of reaction without a decrease in activity.