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Tel: (018) 23-953 Fax: (018) 24-488

INFLUENCE OF THE LOW IONIC STRENGTHS ON THE IMMOBILIZATION OF DEXTRANSUCRASE

Ž. Petronijević*, S. Jovanović and R. Nešić*****

* Faculty of Technology, Durmitorska 19, 16000 Leskovac, Yugoslavia,
** Faculty of Technology and Metallurgy, P.O. Box 494, 11001 Belgrade, Yugoslavia
*** "ZDRAVLJE", Pharmaceutical and Chemical Works, Vlajkova 199, 16000 Leskovac,Yugoslavia

Abstract. Influence of low ionic strengths (75 mM and 20 mM) on the immobilization process of dextransucrase enzyme on the hydrophobic carriers phenoxyacetyl and cinnamoyl cellulose has been tested. It has been established that the lowering of ionic strength leads to the decrease in quantities of bound proteins and to the low values for the immobilized activity. Immobilization process efficiency is also substantially decreased.

1. Introduction

The dextransucrase (sucrose: 1,6-α-D-glucan 6-α-D-glucosyl-transferase, EC 2.4.1.5) catalyses the reaction of the dextran production, glucan of a great molar mass in which the glucosyl units are linked first of all (about 95 per cent) by α-1,6 glycoside bonds. The important application of both dextran and its derivatives have been found in the pharmaceutical, food and textile industry, fine chemical industry, paper industry, agriculture, and in many other areas [1-4].

The commercial production of dextran is still achieved batchwise, with the bacteria *Leuconostoc mesenteroides* and *Leuconostoc dextranicum*, through cultivation on liquid media and through precipitation of the obtained dextran after the finished fermentation. The use of the dextransucrase enzyme, instead of the bacteria, would prevent the use of sucrose on the growth of the biomass; the use of the immobilized dextransucrase would enable the more economical production of dextran in a heterogeneous, first of all, continual process, with great possibilities of automation [5].

The dextransucrase from *L. mesenteroides B-512 F* strain is an enzyme with isoelectric point 4.1, optimum pH in the range 5.0-5.5, the optimal temperature for the activity 30°C, Michaelis constant for sucrose Km=12-16 mM and the molar mass of 158 and 177 kDa [6,7]. The immobilization process is successful if the enzyme is linked to the carrier by sufficiently strong

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bonds and if one obtains a preparation of the immobilized enzyme of sufficient stability for practical application. So far the dextransucrase has been immobilized through covalent bonds by means of glutaraldehyde on Bio-Gel P-2 [8,9], by means of alkylamine porous silica [10-12] and by means of ionic bonds with DEAE-cellulose, DEAE-Sephadex and SP-Sephadex [12]. The high values for the immobilized activity (up to 40 u/g) were obtained through the use of greater quantities of the highly purified enzyme solution and if the immobilization is performed along with the addition of maltose for the purpose of releasing the dextransucrase from the enzyme-dextranosyle complex [10]. However, until now all the tested methods of immobilization through covalent and ionic bonds lead to the significant denaturation of enzyme and to the low immobilization efficiency. The use of cellulose esters of the exactly defined hydrophobicity as a carrier for immobilization can ensure the high immobilization efficiency, whereas the size of immobilized activity depends also on the initial activity of the used enzyme solution [13-16]. The high immobilized activities, greater than 40 u/g, have been obtained with phenoxyacetyl, cinnamoyl and benzoyl cellulose as hydrophobic carriers, through the use of the unpurified solution of extracellular dextransucrase, whereas the present dextran and the unreacted sucrose act on the enzyme in a stabilising way [14-16]. Study of influence of the temperature and the treatment time on the immobilization process [15,16] showed that the lowering of the temperature leads to decreased quantity of the bound proteins (the weakening of hydrophobic interaction) and to increase of immobilization efficiency. The enzyme solutions of ionic strength of about 470 mM in the cited papers have been used, and the immobilization itself was performed under these conditions. The aim of this paper was to test the influence of the ionic strengths lower than 470 mM on the immobilization process of dextransucrase on phenoxyacetyl and cinnamoyl cellulose as carriers.

2. Experimental

Materials

Avicel PH 102 (microcrystalline cellulose with the average particle size of 100 μ) was purchased from Selectchemie AG (ZÏrich). Merthiolate and the bovine serum albumin (BSA) are products of the Sigma Chemical Co. All other chemicals are of p.a. quality.

The dextransucrase enzyme solution was obtained through the cultivation of the bacteria *Luconostoc mesenteroides B-512 F* in the Chemap fermentor, as described in the literature [10,17], along with the maintaining of pH in the range 6.6-6.7 and along with the additional removal of the cells by means of a separator. The pH of the enzyme solution was adjusted to 5.2, preserved with 0.005% merthiolate and maintained at $+4^0$C up to the use.

The carriers for the enzyme immobilization, phenoxyacetyl cellulose (PC 27) and cinnamoyl cellulose (CC 43), with the acylation degree of 1.69 meq/g and 2.67 meq/g, respectively, were

sinthesized from Avicel PH 102 and from chloride of the corresponding acid according to the Butler method [18].

Methodes

- Preparation of enzyme solution of low ionic strength

A. Dilution method: Dilute 5 ml of the enzyme solution with the corresponding quantity of the redistilled water in order to diminish the ionic strength from the initial 470 mM to a) 75 mM and b) 20 mM (Table 1). Adjusting in the diluted solutions up to same concentration of $CaCl_2$ and that of merthiolate, 3 mM and 0.005%, respectively, as in the starting enzyme solution has been done.

B. Dialysis method: The dialysis of 500 ml of the enzyme solution was performed with plate HD(TM) dialyser "Medial", Model S11, Zdravlje, Leskovac, against the 20 mM acetate buffer of pH 5.2 containing 3 mM $CaCl_2$ and 0.005% merthiolate (6 times à 500 ml of the buffer; the flow rate 10 ml/min). The dialysis was monitored by measuring the conductivity, reducing sugars and the activity of dextransucrase in the enzyme solution (Table 2).

- Immobilization of the dextransucrase

Shake occasionally the samples à 0.2 g (dry weight) of the carriers PC 27 and CC 43 for 10 to 15 minutes with 5 ml of the following solutions: 95%, 70%, 45% ethanol, and 10 mM acetate buffer (pH 5.6) with 25% ethanol, respectively. At the end equilibrate the carriers with 5 ml of acetate buffer (pH 5.6) each of the same ionic strength and of the same concentration of $CaCl_2$ and merthiolate as the enzyme solutions in the process of immobilization. The samples of carriers are treated (in the course of the immobilization process) 60 minutes at 23^0C and pH 5.6 with: a) 5 ml of the undiluted enzyme solution of the ionic strength 470 mM, b) 31.33 ml of enzyme solution obtained by diluting 5 ml of the starting enzyme solution up to ionic strength of 75 mM, and c) 117.5 ml of the enzyme solution obtained by diluting 5 ml of the starting enzyme solution up to the ionic strength of 75 mM (Table 3). After the 60-minute treatment of the carriers with the enzyme solution, remove the precipitates with immobilized enzyme by centrifuging, transfer them to the small cuvettes and rinse them four times with 5 ml, each of the buffer of the corresponding concentration. In the starting solutions of enzymes, supernatants and postimmobilization wash solutions the enzyme activity and protein content has been determined. The immobili zed activity of dextransucrase in the precipitate of the immobilized enzyme has been determined.

- Determination of dextransucrase activity in solution

Incubate 1 ml of the enzyme solution (diluted up to 0.07-0.30 units of activity) for 30 minutes at 30^0C with 1 ml of the sucrose solution (200 mg/ml) in 0.15 M acetate buffer (pH 5.2). Interrupt the reaction by keeping the test tubes with the reaction mixture for 3 minutes in the boiling water bath. After cooling, determine reducing sugarsin the reaction mixture. At the same

time perform the blank assays to determine the already present reducing sugars in the enzyme sample as well as the quantity of reducing sugars produced under the given conditions, from sucrose without any enzyme's action [14].

The unit of the dextransucrase activity (u) represents a quantity of enzyme producing the quantity of reducing sugars equivalent to 1 μmol fructose per minute.

- Determination of the activity of immobilized dextransucrase

Samples of 0.01 g (dry substance) of carrier with immobilized enzyme in the form of a suspension (50mg/ml) in 0.5M acetate buffer (pH 5.2) containing 5 mM $CaCl_2$ and 0.005% merthiolate was incubated with 1 ml of 20% sucrose solution at 30^0C for 15 minutes. Reaction were stoped and the reducing sugars determined as described previously.

- Other analytical methods

The reducing sugars were determined by modified ferricyanide method [19] with fructose as a standard. The protein content in the solution was determined according to Bradford [20] with the use of BSA as a standard.

3. Results and discussion

The ionic strength of the starting enzyme solution was calculated on the basis of the well-known concentrations of all the components making its composition; it makes 470 mM at pH 5.6 [14]. The results of determination of dextransucrase activity, reducing sugars, proteins and conductivity in the enzyme solution in the course of obtaining the enzyme solution of the lowered ionic strength are shown in the Table I and II. The advantage of the dialysis as a method of lowering the ionic strength of the enzyme is in the fact that no lowering of the enzyme concentration occurs. The advantage of the dilution method is in the simplicity, precision and the rate of performance, while the loss of activity is, approximately, the same.

Table I. Lowering of the ionic strength of the enzyme solution by dilution

Ionic strength	mM	470	75	20
Volume of enzyme solution	ml	5	31.33	117.5
Protein content	μg/ml	100	16.3	4.3
Activity	u/ml u	0.61 3.05	0.08 2.51	0.02 2.35
Activity lost by dilution	%	--	17.84	22.94

Table II. Lowering of the ionic strength of the enzyme solution by dialysis

		Before dialysis	After dialysis
Conductivity	S	>0.1	0.22 a)
Reducing sugars	mg/ml	1.82	0.16
Activity	u/ml	2.21	1.80
	%	100	81.4

a) The conductivity of buffer with which the dialysis was performed was 0.013 S.

The immobilization of dextransucrase was performed with the enzyme solutions of ionic strength of 470 mM, and with the solutions of 75 mM and 20 mM obtained by diluting the starting solution. The results of immobilization are shown in the Table III.

It can be seen that the lowering of the ionic strength causes the increase in the residual activity and residual proteins in the supernatant, as well as the total residual activity and the total residual proteins in the solution after immobilization. It can be seen that the lowering of the ionic strength causes the significant reduction in the quantities of the bound proteins and the great reduction of immobilized activity. The obtained results confirm the earlier given explanations that the immobilization in this case occurs first of all through the hydrophobic interactions of the proteins with the carriers [13-16].

It is well-known that the lowering of the ionic strength causes the reduction of the hydrophobic interactions [21-23]. The total residual activity is, on the whole, less than 50%, implying the significant enzyme denaturation. The immobilization efficiency, representing fraction of immobilized activity within the total loss of activity of the enzyme from the solution, is also rather slender at the low ionic strengths.

A conclusion may be drawn that the diminution in the ionic strength of the enzyme solution not only, in itself, causes the loss of activity of about 20%, but also, because of the reduction in the hydrophobic interactions, it causes the low values of the immobilized activity and the low immobilization efficiency. One can expect that immobilization of dextransucrase at higher ionic strengths and with great starting activities of the enzyme solution will yield better results of the immobilized activity and ensure the greater immobilization efficiency.

Table III. The influence of dilution on the immobilization of dextransucrase a,b)

Carrier			PC 27				CC 43	
Ionic strength		mM	470	75	20	470	75	20
Supernatant after immobilization	activity	u/ml	0.173	0.028	0.011	0.096	0.020	0.010
		u	0.867	0.890	1.245	0.481	0.618	1.188
		%	28.44	35.54	52.38	15.80	24.70	50.00
	protein	μg/ml	18.1	9.5	3.6	21.2	11.1	3.5
		μg	90.5	297.4	423.0	106.0	347.8	411.3
		%	18.10	58.23	83.72	21.20	68.10	81.40
First wash	activity	u/ml	0.013	0.009	0.0011	0.0048	0.0031	0.0006
		u	0.063	0.043	0.0053	0.024	0.0156	0.0029
		%	2.05	1.73	0.22	0.79	0.62	0.12
	protein	μg/ml	2.3	1.8	1.5	1.5	1.3	1.0
		μg	11.5	9.0	7.5	7.5	6.5	5.0
		%	2.30	1.76	1.48	1.50	1.27	0.99
Second wash	activity	u/ml	0.010	0.0014	0.0004	0.0070	0.0005	0.0002
		u	0.051	0.0072	0.0021	0.035	0.0026	0.0011
		%	1.66	0.29	0.09	1.15	0.11	0.046
	protein	μg/ml	1.0	0.8	0.4	0.5	0.5	0.2
		μg	5.0	4.0	2.0	2.5	2.5	1.0
		%	1.00	0.78	0.40	0.50	0.49	0.20
Third wash	activity	u/ml	0.0023	0.0003	0.0003	0	0.0001	0.0001
		u	0.0117	0.0013	0.0013	0	0.0007	0.0007
		%	0.39	0.05	0.06	0	0.03	0.030
	protein	μg/ml	0.2	0.2	0.1	0.2	0.1	0.1
		μg	1.0	1.0	0.5	1.0	0.5	0.5
		%	0.20	0.20	0.10	0.20	0.10	0.10
Total remaining in solution	activity	u	0.991	0.942	1.253	0.541	0.638	1.193
		%	32.49	37.58	53.32	17.74	25.45	50.77
	protein	μg	108.0	311.4	433.0	117.0	357.3	417.8
		%	21.60	60.98	85.70	23.40	69.97	82.68
At carrier	immobilized activity	u/g	1.779	0.302	0.170	1.482	0.154	0.096
		u	0.356	0.060	0.034	0.296	0.031	0.019
		%	11.67	2.39	1.45	9.70	1.24	0.81
	protein	μg	392.0	199.3	72.3	383.0	153.4	87.5
		%	78.4	39.0	14.3	76.6	30.0	17.3
		mg/g	1.96	1.00	0.36	1.92	0.77	0.44
Disappearance of activity from solution		%	67.51	62.42	46.68	82.26	74.55	49.23
Total preserved activity		u	1.35	1.00	1.29	0.84	0.67	1.21
		%	44.16	39.98	54.77	27.44	26.69	51.57
Immobilization efficiency		%	17.29	3.84	3.10	11.80	1.66	1.64

a) The starting values both for the activity and the protein content in the enzyme solutions are given in the Table 1.

b) All results are mean values.

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UTICAJ NISKIH JONSKIH JAČINA NA IMOBILIZACIJU DEKSTRANSAHARAZE

Ž. Petronijević, S. Jovanović and R. Nešić

U radu je ispitivan uticaj niskih jonskih jačina (75 mM i 20 mM) na proces imobilizacije enzima dekstransaharaze na hidrofobnim nosačima fenoksiacetil- i cinamoil-celulozi. Nađeno je da sniženje jonske jačine dovodi do smanjenja količine vezanih proteina i do niskih vrednosti za imobilisanu aktivnost. Efikasnost procesa imobilizacije se pri ovome takođe jako smanjuje.