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Highly efficient immobilization of endo-1,3-β-D-glucanases (laminarinases) from marine mollusks in novel hybrid polysaccharide-silica nanocomposites with regulated composition

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Abstract

A novel immobilizing method developed previously by ourselves was successfully used to entrap endo-1,3-β-D-glucanases (laminarinases) separated from marine bivalvia *Spisula sacchalinensis* (glucanase L_{IV}) and *Chlamys albidus* (glucanase L_o) into hybrid polysaccharide-silica nanocomposite materials by means of the sol–gel processing. Its main advantage over the current immobilizing procedures is that the entrapment conditions are dictated by the enzymes, but not the processing. It was shown that both the 1,3-β-D-glucanases retained or even had sometimes an increased activity after the immobilization. At the same time, their characteristics (optimal pH, temperature and ionic strength) noticeably were not changed. They provided a depth of hydrolysis of laminaran comparable with that caused by free enzymes in solutions. Furthermore, glucanase L_o retained its glucanosyl transferase activity, affording an enzymatic synthesis of biologically active 1,3;1,6-β-D-glucan, called translam, from the initially inactive laminaran. It was also demonstrated that the laminarinase entrapment into the hybrid nanocomposites led to a prominent increase of thermal and long-term stability that was particular striking in a case of such a labile enzyme as the glucanase L_o. By varying the nanomaterial composition, its influence on the glucanase activity was found that differed for the studied enzymes.

Keywords: Laminaran; Glucanase; Immobilization; Sol–gel technology; Silica nanocomposite; Biocatalyst

1. Introduction

The sol–gel technology is presently believed to be one of the most promising approaches for the immobilization of enzymes [1–10]. Its main advantage lies in the fact that the entrapment of proteins proceeds without formation of covalent linkages between biomolecules and matrix. As a result, the enzymes are in their intact state after the immobilization. This is the reason why they hold functionality that is supplemented by a substantial increase in their long-term and thermal stability.

Although the sol–gel derived materials can be fabricated on the base of oxides of various metals including alumina, titania and zirconia, the entrapment of enzymes is usually performed by using silica because of its better biocompatibility. For the gel fabrication, a starting compound is needed that is called a precursor. The silica-based materials are usually synthesized with the help of tetramethoxy- or tetraethoxysilane. Because of their poor solubility in water, an organic solvent is added and, in addition, alcohol is evolved in the course of hydrolysis. To trigger the sol–gel processes, a catalyst – acid or alkaline – is introduced in the reaction mixture [11,12]. The organic solvent(s) and acidification or alkalization may account for a denaturation of enzymes that sets considerable restrictions on their immobilization [2,5,7–9]. Generally, the sol–gel technology is applicable with success to stable enzymes such as for instance lipases [6,10,13,14].

A partial decrease of the negative effect of sol–gel processes is attained when they are performed through two stages

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[2,4,15,16]. A precursor is initially hydrolyzed in acidic media in which a sol is formed. Then a pH of solution is shifted in acidic region and an enzyme is added. A following jellification provides an entrapment of protein macromolecules into a three-dimensional silica network.

Gill and Ballesteros [17] to improve the compatibility of sol–gel technique to entrapped proteins performed a transesterification of initially prepared oligosilicates by various polyols. The authors also used two-stage procedure that was complicated by the chemical treatment of intermediate silica sol. Enzymes were introduced into a prejelled system. Then it was transferred to a gel at near neutral pH region even in the absence of a catalyst.

The detrimental effect of an alcohol evolved during the tetraethoxysilane hydrolysis and condensation is suggested in Ref. [18] to eliminate through ethanol removal by gentle vacuum evaporation from initially prepared silica sol. When horseradish peroxidase was entrapped into an alcohol-free gel, it demonstrated an increased enzymatic activity in comparison with an enzyme immobilized by the common route. Here the two-stage procedure was also complicated with alcohol removal.

We demonstrated in our recent article [19] that the negative effect of the sol–gel processing on the immobilized enzyme was reduced to minimum when a novel silica precursor, tetrakis(2-hydroxyethyl) orthosilicate (THEOS) was applied. THEOS has a decided advantage over current precursors that is in its complete water solubility [20]. In addition, the hydrolysis occurs with the evolution of ethylene glycol instead of an alcohol that is better compatible with proteins than the latter. An important point is also that we applied hybrid polysaccharide-silica nanocomposite materials developed in Refs. [21–23]. The polysaccharides fulfill the dual function of catalyzing the sol–gel processes and serving as a template for silica generated in situ. Their catalytic effect provided an opportunity to perform processing at any desired pH value of aqueous solution through one stage. When THEOS was used as the precursor, an enzyme, but not the sol–gel processes dictated the immobilization conditions [19].

The aim of this study was to extend our method for the immobilization of a highly labile enzymes, endo-1,3-β-D-glucanases (laminarinases). Their properties and specificity in the immobilized state as well as effect of matrix composition on their activity are detailed in this article.

The endo-1,3-β-D-glucanases (EC3.2.1.6) from marine mollusks *Spisula sacchalinensis* and *Chlamys albidus* belonging to *O*-glycoside hydrolyses (EC3.2.1) is of great interest to biotechnological applications [24–26]. These enzymes, known first as hydrolases, catalyze, along with hydrolytic, also transglycosylation and glucanosyl transferase reactions running simultaneously and practically with almost equal efficiency. Their transglycosylation activity provides synthesis of poorly available 1,3- and 1,3;1,6-β-D-glucooligosaccharides and glycosides as well as a branched 1,3;1,6-β-D-glucans. One of the β-D-glucans, called translam, in contrast to the initial laminaran possesses documented immonostimulating and anticancer activities [26–28]. It is an analog of well-known schizophillan that is extracted from yeasts. The latter possesses side effect on patients that is related to its high molecular weight. The translam, as a low molecular weight polysaccharide, has been found to be more suited for the same biomedical applications [29,30].

2. Experimental

2.1. Materials

Tetrakis(2-hydroxyethyl) orthosilicate was synthesized as described in Ref. [31]. Xanthan and locust bean gum (LBG) were obtained from Fluka, cationic derivative of hydrohyethylcellulose (cat-HEC), from Hoechst AG. The latter contains glycidyl-trimethylammonium chloride (-CH_2-CHOH-CH_2-$N^+(CH_3)_3Cl^-$) as a cationic group. Laminaran was separated from the brown seaweeds *Laminaria cichorioides* in accordance with the method in Ref. [32]. 1,3-β-D-Glucanases L_{IV} and L_o, hereinafter mentioned as glucanase L_{IV} or L_o, from marine bivalvia *S. sacchalinensis* and *Ch. albidus*, respectively, were separated and purified as in [33,34].

2.2. Enzyme immobilization

The entrapment of enzymes in the hybrid polysaccharide-silica nanocomposites was performed in accordance with the procedure detailed in Ref. [19]. 0.5 ml of a buffered solution of enzyme was thoroughly admixed with a prejelled aqueous solution (2.5 ml) containing THEOS and one of the three polysaccharides. Before and after the admixing, the solutions were placed at 3–5 °C in a refrigerator. Fabricated hydrogels with entrapped enzymes were not dried. They were used without any additional treatment.

2.3. Main analytical procedures

The total and reducing sugars were assayed by phenol-sulfuric acid [35] and Nelson's [36] methods, respectively. The molecular weight of polysaccharides was determined by a gel-permeation FPLC on a column (1.5 cm × 30 cm) with Superdex 75 HR 10/30 (Amersham Pharmacia Biotech AB). The elution was carried out with 0.1 M sodium phosphate buffer (pH 7.4) containing 0.15 M NaCl at the rate of 0.4 ml/h. Dextrans of various molecular weight (10, 20, 40 and 80 kDa) were used as standards.

The liquid chromatography of sugars was performed by means of a JEOL-JLC-6AH automatic analyzer. A solution with pH 5.2 containing 0.05 M sodium acetate and 0.2 M sodium chloride was passed through a column (0.9 cm × 90 cm) filled by biogel P-2 at the rate of 7–9 ml/h. The sugars were determined by orcin-sulfuric acid reagent.

^{13}C NMR spectra were obtained in D_2O at 60 °C at 62.9 MHz by a Bruker WM-250 spectrometer. The analyzed aqueous solutions were freeze-dried or evaporated under vacuum at 40 °C and then the dried polysaccharides were dissolved in D_2O. The amount of β-1,3- and β-1,6-glycosidic linkages was found from

integral intensities of resonances at 61.4 and 69.4, 70.3 and 68.8 as well as 103 and 83 ppm, respectively.

2.4. Enzyme activity determination

The reaction mixture was prepared by adding 100 μl of the enzyme solution or 100 mg of gel with the immobilized enzyme as well as 1 ml of substrate (laminaran) solution (5 mg ml^{-1}) into a solution buffered by 0.05 M sodium succinate. It was incubated for 1 h at 25 °C or 37 °C in a case of glucanase L_o or L_{IV}, respectively. Where the immobilized enzymes were examined, the incubation time was increased up to 4 h. The hydrolytic activity was estimated by determining the concentration of reducing sugars released from substrate by means of the Nelson's method [36]. The enzyme unit (U) corresponded to the enzyme quantity that catalyzed the formation of 1 μM of glucose for 1 min.

2.5. Characterization of products of the enzymatic reaction

An aliquot taken from the mixture within definite time intervals was boiled to stop the reaction and then analyzed by the JEOL-JLC-6AH liquid chromatograph. In parallel with the product analysis, their total yield was determined by determining the concentration of reducing sugars.

2.6. Characterization of enzymes

The results are summarized in Table 1.

2.7. Optimum pH Value

The enzymatic reaction was performed as described in the previous section, but the reaction media contained 0.2 M succinate and phosphate buffers. It allowed changing pH in the range between 4.4 and 7.4.

2.8. Optimum temperature

One of the studied enzymes was incubated with laminaran as described above at a temperature ranging from 20 to 70 °C. Then the concentration of reducing sugars was determined.

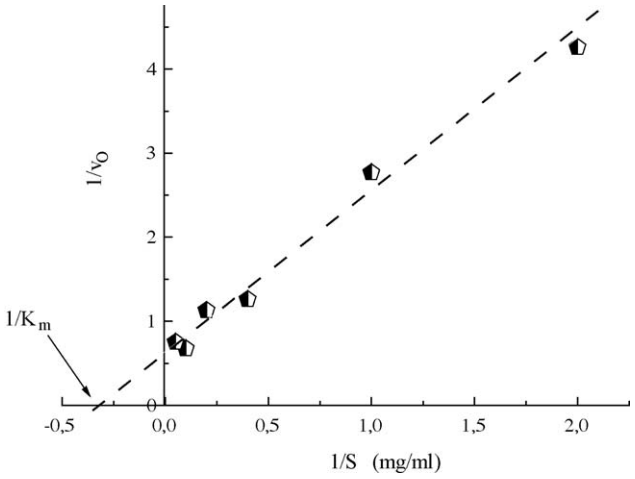

Fig. 1. An initial hydrolysis rate by 1,3-β-D-glucanase from *S. sachalinensis* vs. the concentration of laminaran from *L. cichorioides*. The plot is used to determine the Michaelis–Menten constant (a K_m value).

2.9. Thermal stability

An aliquot of enzyme solution (100 μl) or 100 mg of gel with immobilized glucanase L_o was kept at 12, 25 or 37 °C. The time of exposure varied from 10 min to 6 h. A solution containing 1 mg of laminaran, 0.05 M succinate buffer and 0.1 M sodium chloride (pH 5.2) was then added. The mixture was incubated at 25 °C for 1 or 4 h to determine residual enzymatic activity for the enzyme, respectively, in the solution or immobilized state.

2.10. Michaelis–Menten constant (K_m)

It was determined for the immobilized glucanase L_o. The enzymatic reaction was carried out in the above-described reaction mixture with a substrate concentration varied from 0.5 to 20 mg/ml at 25 °C for 4 and 6 h. A K_m value was calculated by the method Lineweaver–Burk. It is illustrated by Fig. 1 in which the initial transformation rate is given as a function of substrate concentration.

2.11. Synthesis of translam

Laminaran (3 g) was dissolved in 300 ml of aqueous solution (pH 5.2) containing 0.05 M sodium succinate and 0.1 M

Table 1
Characteristics of endo-1,3-β-D-glucanases in solution and immobilized state

Enzyme source	Type of hydrolyzing bond	Mw (kDa)	Location	K_m (mg/ml)	Optimum conditions		
					pH	T (°C)	NaCl (M)
Glucanase L_o. *Ch. albidus*	Glc. β-1 → 3	20[a]/38[b]	Solution[c]	0.7	4.6	≤30	0.1–0.25
			Gel	4.0	4.6	37	
Glucanase L_{IV}. *S. sachalinensis*	Glc. β-1 → 3	22[a]/39[b]	Solution[c]	0.25	5.6	45	0.01–0.3
			Gel	3.0	5.6	50	

[a] Determined by the gel filtration (SDS-PAGE).
[b] Determined by the electrophoresis.
[c] Data from Refs. [33,34].

sodium chloride. Then 30 g of a gel with immobilized glucanase L_o was added. It was synthesized by mixing 10 wt.% THEOS and 0.3 wt.% xanthan. The laminaran solution with the immobilized enzyme was left at 25 °C for 5 days. After the incubation, the gel was separated from the supernatant by the centrifugation and washed three times by a Na succinate buffer. The combined supernatant and washing solutions were passed trough a column (3 cm × 20 cm) with Polychrome-1 (polytetraftorethylene). The elution was made first by water and then by water containing 2.5 and 5% ethanol so long as a negative reaction toward the sugar presence was not obtained in each case. This allowed eluting salts, glucose and glucooligosaccharides. Fractions of 1,3- and 1,6-β-D-glucans having the molecular weight of 3–8 kDa were eluted with the help of 7.5 and 10% ethanol, respectively, as well as translam, with 15% ethanol. The every elution was accomplished when the negative reaction toward the sugar presence was obtained. To regenerate the column, a 1 l of 70% ethanol and water were passed successively through it.

2.12. Scanning electron microscopy (SEM)

Micrographs were taken by means of a FE-SEM Leo 1530 electron microscope. To prepare samples for observations, a polysaccharide-silica gel was frozen by the liquid nitrogen, then it was cleft and a platinum layer was evaporated on the fresh gel surface to cover it.

3. Results and discussion

The hybrid polysaccharide-silica nanocomposites represent a novel type of immobilizing materials that demonstrated excellent compatibility with enzymes [19]. The immobilization proceeds through entrapment of protein macromolecules into a network created by silica that synthesized as the result of sol–gel processes. The processing is started where a precursor (silane) is brought into contact with water. This leads first to a hydrolysis in accordance with a reaction:

$$\mathrm{Si(\text{-}OR)_4} + n\mathrm{H_2O} \rightarrow \mathrm{(HO\text{-})_n Si(\text{-}OR)_{4-n}} + n\mathrm{HO\text{-}R}, \quad (1)$$

and then the produced sylanol (Si-OH) groups are involved in condensation reactions:

$$2\mathrm{(HO\text{-})_n Si(\text{-}OR)_{4-n}} \rightarrow \mathrm{(OH)_{n-1}(RO\text{-})_{4-n}Si\text{-}O\text{-}Si(\text{-}OR)_{4-n}(OH)_{n-1}} + \mathrm{H_2O} \quad (2)$$

or:

$$\mathrm{Si(\text{-}OR)_4} + \mathrm{(HO\text{-})_n Si(\text{-}OR)_{4-n}} \rightarrow \mathrm{(RO\text{-})_3 Si\text{-}O\text{-}Si(\text{-}OR)_{4-n}(OH)_{n-1}} + \mathrm{HO\text{-}R}, \quad (3)$$

where R is a hydrocarbon radical and $n \leq 4$. The sol–gel transition takes place if a catalyst is introduced into the reaction media [11,12]. This role in systems with THEOS is taken by polysaccharides. As shown in Refs. [21–23], their macromolecules serve as nucleating centers for precipitated silica owing to a formation of hydrogen bonds between hydroxyl groups and sylanols. The silica templating by polysaccharides has a great consequence that consists in regulating the structure of hybrid nanocomposites. Fig. 2 demonstrates the pictures of gels obtained by a SEM. One may see a network from crossed fibrils. They represent carbohydrate macromolecules covered by silica. The observed structure is typical of these hybrid materials fabricated by the sol–gel processing in polysaccharide solutions [19,21,22].

It is noteworthy that the network inside the polysaccharide-silica nanocomposites is not dense. Its density is dependent on the carbohydrate concentration. As seen from comparison of Fig. 2A and B, a decrease of the content of cat-HEC results in a looser network. However, its mesh size is much as several microns even at the largest carbohydrate concentration used in our experiments (Fig. 2B). This allows the penetration of large macromolecules into the hybrid nanocomposites. It is of great importance to enzymatic processes with the endo-1,3-β-D-glucanases because laminarans have a molecular weight ranged from 3 to 20 kDa, and other 1,3-β-D-glucans, e.g., from yeast can be as much as 200–800 kDa. Our study demonstrated that laminarans could easily access the immobilized enzymes.

Results on the enzymatic activity glucanases L_o and L_{IV} determined with different time are presented in Tables 2 and 3,

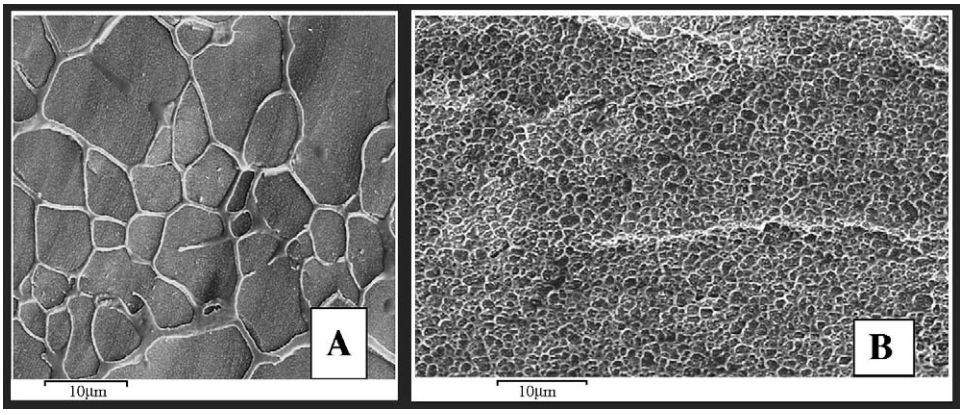

Fig. 2. SEM micrographs of gel synthesized in an aqueous solution containing 10 wt.% THEOS and (A) 0.5 or (B) 1.8 wt.% of cat-HEC.

Table 2
Enzymatic activity of 1,3-β-D-glucanase L_o from *Chlamys albidus* in different polysaccharide-silica matrices examined at various intervals after the immobilization

#	Gel composition (wt.%)			Activity U × 10^{-2}/0.1 g of gel[a]			
	THEOS concentration	Polysaccharide		Storage time (days)			
		Name	Concentration	1	50	90	120
1	10%	LBG	1.3	3.1	4.1	4.6	0.3
2			1.0	3.0	4.8	2.9	1.0
3			0.3	4.7	5.1	2.8	2.2
4		Xanthan	1.7	2.8	2.8	2.1	2.0
5			1.0	2.8	2.3	1.7	2.4
6			0.3	6.2	3.3	2.9	3.2
7		Cat-HEC	1.3	0.5	0	0	nd
8			1.0	4.0	2.5	1.7	1.9
9			0.3	7.4	7.6	2.7	3.1
10	20%	LBG	1.0	2.3	3.6	3.0	0.4
11		Xanthan	1.0	3.0	1.6	2.2	1.9
12		Cat-HEC	1.0	4.3	3.9	2.6	2.6
Control (in solution)	–	–	–	0	nd	nd	nd

[a] μmol Glc/h.

respectively. It is immediately apparent from their examination that (i) both enzymes retained the activity after the immobilization and (ii) the entrapment into the nanocomposite matrix led to a substantial increase of the long-term stability. They were active within 120 days of their testing. The effect is most obvious for glucanase L_o because it lost its activity in solution within one hour. Though free glucanase L_{IV} has a longer/better stability, nevertheless the stabilizing effect of immobilization is also in evidence when one compares the enzyme life times in the free and immobilized states.

A further examination of the results makes it apparent that the enzymatic activity of glucanase L_o (Table 2) depends on the hybrid material composition. One may see a high sensitivity to the concentration and nature of polysaccharide inside the nanocomposite. The concentration effect is most obvious in a case of cat-HEC. An increase of its content in a silica matrix from 0.3 to 1.3 wt.% (#7–9) led to a decrease in the enzymatic activity by a factor of ca. 15. Similar, but less expressed changes take place in the nanocomposites with LBG (#1–3) and xanthan (#4–6). In addition, the glucanase in matrices with increased amount of polysaccharides are not so stable. No activity, for instance, was found in the nanocomposite with 1.3 wt.% of cat-HEC (#7) after 50 days and its sharp decrease towards the end of the examination may be seen in the sample with 1.3 wt.% of xanthan (#4).

One conceivable reason for the decrease in the enzymatic activity with increasing polysaccharide content inside the hybrid nanocomposite is the increase of network density. This is illustrated in Fig. 2A and B. The decrease of mesh size can cause a mass-transfer limitation for the substrate and reaction products in pores. This explanation, however, does not provide an insight into the decrease in the stability of enzymes when the polysaccharide concentration inside the silica matrix is increased.

A correlation of the initial activity of glucanase L_o in hybrid nanocomposites fabricated with various polysaccharides (Table 2) reveals that it is maximal in the presence of 0.3 wt.% of cat-HEC (#9). There is an activity decrease as one passes to materials with the same content of xanthan (#6) and LBG (#3). Over the testing period the yield of products decreased in most cases to half of its value. It seems reasonable to mention that the activity levels for hybrid materials with various polysaccharides (#3, 6, 9) approached each other at the end of the examination period. This fact means that the polysaccharides play likely a notable role at the stage of the enzyme entrapment. In due

Table 3
Enzymatic activity of 1,3-β-D-glucanase L_{IV} from *Chlamys albidus* in different polysaccharide-silica matrices examined at various intervals after the immobilization

#	Gel composition (wt.%)			Activity U × 10^{-2}/0.1 g gel[a]				
	THEOS concentration	Polysaccharide		Time of storage (days)				
		Name	Concentration	5	10	50	90	120
13	10	LBG	1.5	34.6	36.6	28.1	25.6	26.4
14			0.28	31.9	27.8	28.2	23.7	21.2
Control (in solution)	–	–	–	31	24	5.5	nd	nd
15[b]	10	LBG	0.25	9.4	14.5	12.2	7.6	9.8
Control (in solution)	–	–	–	10	3	1	nd	nd

[a] μmol Glc/h.
[b] Concentration of enzyme is three times smaller then that in the gels of #13 and 14.

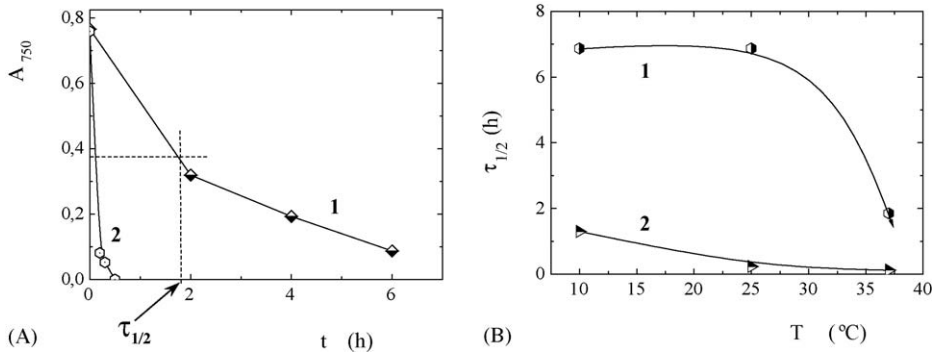

Fig. 3. (A) Residual enzymatic activity of glucanase L_o in the immobilized state (1) and solution (2) vs. the time of incubation at 37 °C. The dashed lines show as a half-life time of enzyme was determined. (B) Half-life times of glucanase L_o in the immobilized state (1) and solution (2) vs. the incubation temperature.

course, there are restructuring processes in the sol–gel derived materials causing their maturation [11,12] that can attenuate the carbohydrate influence on the immobilized enzyme.

Table 2 includes also data on glucanase L_o entrapped into hybrid nanocomposites containing the doubled silica amount (#10–12). Their comparison with the above-considered results reveals that the variation of content of inorganic component did not have a notable effect on the enzymatic activity. This is valid for all the studied polysaccharides.

The data on glucanase L_{IV} examination are presented in Table 3. They allow one to assume that the enzyme was entrapped only into LBG-silica nanocomposites. This is based on a previous study that was described yet in [19]. It was found that the LBG-containing silica matrix was most convenient for the enzyme functioning, but the effect of the polysaccharide concentration was not revealed. This point was under study in this work.

It is significant that the glucanase L_{IV} in our previous study [19] was taken for the entrapment in small concentrations comparable to its content in the living cells. Nevertheless, the enzyme even at the minute concentration held its activity over more than 5 months. In this work (Table 3) the 1,3-β-D-glucanase L_{IV} was taken in the concentration which was 20 times greater than that in our previous study. Here, it demonstrated the activity over 4 months for which it was examined.

It may be seen from Table 3 that the polysaccharide inside the silica matrix had only minor effect on the enzyme activity. There is a small increase in the activity when increasing the LBG concentration (#13–14) that is opposite to the trend mentioned for glucanase L_o (Table 2).

The comparison of both glucanases functioning in the immobilized state makes it obvious that they exhibit various sensitivity to the composition of hybrid nanocomposites. The glucanase L_{IV} demonstrated the most activity in LBG-silica materials [19], whereas it was the worst matrix for glucanase L_o (#1–3, Table 2). With increasing the concentration of polysaccharide inside the nanocomposite, one can observe also the differences in their behavior. This implies the enzyme specificity to the matrix composition in which they were entrapped. It is particular remarkable that the differences are obvious even in the case of such rather similar enzymes as those considered in the article.

A further study of glucanases was performed to characterize some details of their functioning in the immobilized state. Results are presented in Table 1. There are optimal temperature and pH values for both glucanases. When comparing them with similar parameters determined for the free enzymes in solution, one does not find notable differences. This signifies that the glucanase entrapment into the hybrid matrix did not lead to a significant change in the conditions at which they demonstrate the maximum activity. It is only necessary to add that there was extending of temperature and pH optimal regions.

The immobilization gave prominent rise to the temperature stability of glucanase L_o in comparison with that in the solution. This is illustrated by Fig. 3A and B. The former demonstrates a time dependence of the concentration of sugars released in the course of an enzymatic hydrolysis at 37 °C. The latter represents the half-life times $\tau_{1/2}$ of enzyme at various temperatures. It was determined as shown in Fig. 3A on the example of enzymatic reaction performed at 37 °C. The presented data makes it apparent that the immobilized enzyme possesses a better stability than that being in the solution at the same conditions.

This result is in line with observations of other researchers (see, e.g., Refs. [2,4,37]). The entrapment of protein macromolecules with the help of sol–gel technique provides their stabilization because of close fit into silica matrix that restricts their unfolding and denaturation.

Kinetics of the product build-up in the course of enzymatic reaction was examined in a case of glucanase L_{IV} immobilized in various hybrid nanocomposites (#13–15, Table 3, results are not shown). It was found that the laminaran hydrolysis proceeded up to ca. 50%. This is comparable with the hydrolytic activity of free enzyme in solution [38]. Furthermore, there was also close similarity in product composition. All these facts demonstrate that the activity of glucanase was not changed significantly after the entrapment into the polysaccharide-silica matrix.

Michaelis–Menten constant values are given in Table 1. As seen, K_m are equal to 3 and 4 mg/ml, respectively, for the immobilized glucanases L_{IV} and L_o, These values are noticeably greater than K_m found for the free enzymes in solutions [25].

Of great importance is the question whether the glucanase L_o retained its ability to catalyze the glucanosyl transferase reaction after the immobilization. To elucidate it, we performed the enzy-

Table 4
Characteristics of laminaran and product (translam) synthesized by means of immobilized 1,3-β-D-glucanase L_o (details are given in Section 2)

Glucan	Yield (%)[a]	Mw (kDa)	Bond		Chemical shifts in the ^{13}C NMR-spectra						
			Type	Content (%)	Carbon atom of the monosaccharide residue						
					C1	C2	C3	C4	C5	C6	C6 (mannitol)
Laminaran	–	5	1 → 3	90	103.6	74.4	85.8 86.1	69.4	76.8	62.0	64.4
			1 → 6	10	103.9	74.4	76.8	70.8	75.7	70.0	–
Translam	10	8	1 → 3	75	103.4	74.4	85.6 85.9 86.1	69.4	76.8	62.0	–
			1 → 6	25	103.7	74.4	76.8	70.8	75.7 76.0	70.1	–

[a] Yield in reference to the initial amount of laminaran.

matic process resulting in the synthesis of branched 1,3;1,6-β-D-glucan, called translam, at conditions which were previously determined in Ref. [26]. It was found that maximum yield was reached when a degree of laminaran conversion was 7–10%. This was taken into account where immobilized glucanase L_o was examined. The enzymatic reaction was accomplished when about 7% of laminaran was hydrolyzed. It is obvious from Fig. 4. One may see there a dependence of the substrate hydrolysis on the time of solution treatment.

The gel permeation chromatography was used to examine the reaction products. Results are given in Fig. 5 as curve 1. There is also a dashed curve 2 demonstrating the result of examination of the initial laminaran. It is apparent that the products of the enzymatic reaction contain increased amounts of high-molecular weight derivatives. Their average molecular weight is equal to 8 kDa, while the laminaran was of 5 kDa (see Table 4). This means that the immobilized glucanase L_o catalyzed the transglycosylation reaction, providing a growth of carbohydrate macromolecule. To decide which kind of reaction took place, ^{13}C-spectra were taken by NMR spectroscopy. The results are summarized in Table 4, which also contains data on the initial laminaran. As followed from a comparison, the produced polysaccharide is more branched than the initial laminaran. Its characteristics are close to that of translam described in Ref. [26].

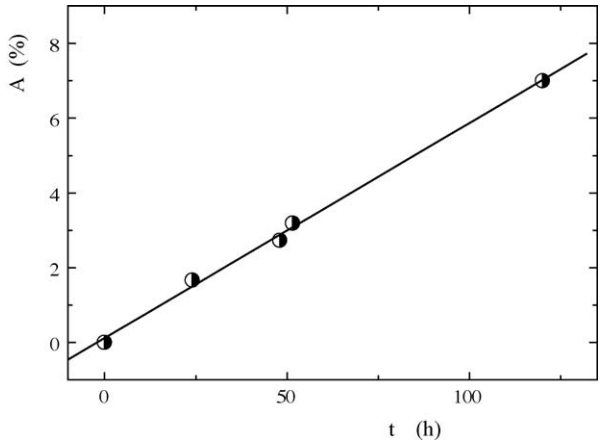

Fig. 4. Degree of hydrolysis of laminaran by 1,3-β-D-glucanase from *Ch. Albidus* vs. the time of treatment. The plot was used to determine the rate of polysaccharide transformation.

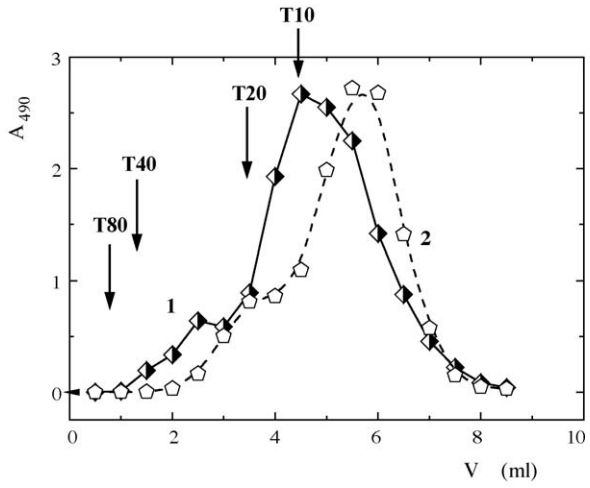

Fig. 5. Gel permeation chromatography of a product obtained after the incubation of laminaran with glucanase L_o (1) and initial laminaran from *L. cichorioides* (2). Details are given in Section 2.

4. Conclusions

The results presented in the article demonstrated that the 1,3-β-D-glucanases were successfully immobilized in the novel hybrid polysaccharide-silica nanocomposite materials. They had the maximal activity at conditions (pH, temperature and ionic strength) that were optimal for them in solutions before the entrapment. Furthermore, they provided an analogous completion of hydrolysis of substrate (laminaran) and synthesis (glucanase L_o) of what is assumed to be biologically active, branched 1,3;1,6-β-D-glucan, called translam. At the same time they retained or even had sometimes an increased activity, became more thermally stable and demonstrated prolonged long-term stability. These facts give evidence that the suggested immobilizing method is ideally suited for the entrapment of enzymes and development of biocatalyst for biotechnological applications.

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