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ORIGINAL PAPER

Microbial Enzymes-Lectins Interactions: Applications for Glycoproteins Purification

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Abstract

Bacteria from Bacillus species produce a large variety of extracellular enzymes, a lot of them providing biotechnological importance (a complex enzyme-product including α-amylase, protease, β-glucanases, necessary in beer industry is obtained in the culture media from Bacillus subtilis strains). Filamentous fungi, such as those belonging to Trichoderma reesei, produce a potent cellulosolytic enzymes–set able to achieve efficient hydrolysis of a highly crystallized cellulose and consequently of natural cellulose forms degradation resisting. This work present some experimental studies based on interaction between plant lectins and components of cellulosolytic systems produced by strains of Bacillus subtilis and Trichoderma reesei useful to improve the enzymes purification.

Keywords: microbial enzymes, *Raphanus sativus* and *Pharbitis purpurea* lectins, affinity chromatography, enzyme purification

Introduction

Industrial enzymology is an important segment of biotechnology, which allows yielding concomitantly economically useful practical results (which enable multiple utilization in detergents industry, food industry, medicine, pharmaceutical industry) and scientific data with fundamental significance.

Recent approaches in this area are focused on microbial cellulose degradation. Yielded the enzymes by biosynthesis in a sustainable profitable way comparing to their competitors, vegetal or animal extraction enzymes, is not the only problem to be solved, the other requirement the mood to separate and also their purification. Moreover, the results obtained with this type of enzymes could be extended in other cases. In this context, a special attention has been given to a distinct group of glycoproteins - the lectins, which can be used in purification of some biotechnological interest enzymes if they also are glycoproteins [1].

We have directed our attention to the β-glucanase type of enzymes produced by *Bacillus subtilis*, as well as to analogous enzymes synthesized by fungal strains belonging to *Trichoderma reesei*.

The aim of our work was to investigate the interaction between two plant lectins and components of cellulosolytic systems produced by strains of *B.subtilis* and *T.reesei* in order to improve the methodology of microbial glycoenzymes purification by affinity chromatography on immobilized lectins.

Materials and methods

Biological materials. Several *B.subtilis* and *T.reesei* strains from the collection of Faculty of Biotechnology were tested in our experiments. Finally, one bacterial strain designated as *B.subtilis 1.65* and a strain of *T.reesei* were used. The source of lectins was the seeds of *Raphanus sativus* or *Pharbitus purpurea* purified as described previously [2].

Culture media. In order to maintain *Bacillus* cultures and to select some new strains it has been used nutrient agar. For the cultivation of fungal strains was used malt extract 1% medium. A synthetic medium previously described by Ishaque and Kluepfel [3] supplemented with 1% cellulose (carboxymethylcellulose = CMC or Avicel PH101) was used. For enzyme purification, 100 ml of liquid synthetic medium with Avicel PH101 as carbon source were inoculated with fungal spore suspension or bacterial suspension and incubated at 30-32ºC for 4 days.

β-glucanase activities were determined on carboxymethylcellulose (CMC), crystalline cellulose (Avicel PH101) or 1% β-glucan from malt flour (prepared as described Jorgensen as substrate[4]. The assays were done at 45ºC and the substrate concentration was 10mg/ml (CMC or Avicel PH101). Reducing sugar formation was determined by the dinitrosalicilic acid (DNS) method of Miller at al. [5]. One international unit of activity (IU) equals 1μmol of reducing sugar produced/min. Protein was determined by the dye-binding assay of Bradford.

The glycoproteic nature of some components of celullolytic complex produced by selected microorganisms was proved by PAS reaction[6]. Based on the fact that components of microbial cellulase systems are glycoproteins, the β-glucanase-lectins interactions were determined by Ouchterlony double-diffusion technique [7].

Affinity chromatography on immobilized lectins

Lectins extracted from *Raphanus sativus* seeds were immobilized with 25% glutaraldehyde and used for cellulase purification [8]. 20ml of culture filtrate were applied on immobilized lectins (batch technique) and the mixture was incubated for 5h at 4ºC. After centrifugation, the sediment was washed with distilled water (3 times). The enzymes were eluted with 50mM glycine-HCl buffer, pH 3.0 containing 0.5M NaCl.

SDS-PAGE was performed in 8% polyacrylamide gels in the presence of 0.1% SDS by the method of Laemmli [9]. When the separated proteins were to be stained for enzyme activity, the separating gel contains cellulose as substrate and the steps in detection of cellulase activity were performed as described Schlochtermeyer et al. [10].

Results and Discussions

Our previous studies regarding the interactions between enzymes produced by different strains of *Streptomyces sp.* and some plant lectins allowed the elaboration of a specific protocol, which was applied for the microorganisms investigated in this work [2].

Lectins were purified according a method described previously[6].

Following double diffusion in agarose gel, precipitation lines were highlighted implicitly interactions between culture media compounds from *Bacillus subtilis* and partly purified lectinic mixture from seeds of *Raphanus sativus*. Based on these results, the next step in our study was the identification of the glycoproteins, which interact with the lectins.

It is well known that, both categories of microorganisms used in this study, *Bacillus subtilis* and *Trichoderma reesei* are capable to synthesis a very large range of hydrolytic enzymes [11]. After highlighting the precipitation lines as a result of interaction between culture filtrates and lectinic extracts, equal volumes of lectinic extract and culture filtrate were mixed, incubated over night at 4°C and after centrifugation two phases were separated: supernatant (Sup) and sediment (Sed). Both phases were subjected to further studies concerning the identification of microbial glycoproteins involved in the interaction with *Raphanus* lectins.

Lectins- *Bacillus subtilis* enzymes interactions

In case of the interaction between *Bacillus subtilis* enzymes and *Raphanus sativus* lectins, results reveal that amylolytic activity of the culture filtrate was entirely recovered in Sup phase, therefore this β-glucanase enzyme is not involved in the interaction with lectins.

On the other hand, β-glucanase activity (using 1% β-glucan from malt flour as substrate) determined in Sup phase represents approximately 50% from global activity of the culture medium submitted to interaction. Correlating these results with the fact that in resulted precipitate (Sed) can be recovered only β-glucanase activity, we could conclude that only β-glucanase interacts with a lectinic part of the extract from *Raphanus sativus* seeds.

The investigation of control samples showed the surprising presence of a low β-glucanase activity (6U/mL/min) in the lectinic crude extract.

The survey of global β-glucanase activity from *Bacillus subtilis* culture medium + lectinic extract was estimated as 21U/mL/min in Sup + Sed, comparing to 22U/mL/min in controls separately investigated, that suiting to a 95% recovery proportion.

Assuming that the origin of entire β-glucanase activity detected in the precipitate is from the culture medium, that means the enzymatic activity detected in the Sup of lectinic extract represent the unbound enzyme (the lectin-enzyme ratio is favourable to the enzyme).

The confirmation of this hypothesis has been materialized through a distinct series of experiments in which culture medium/lectinic extract ratio has been varied, the bulk of lectinic extract being maintained constant and varying the amount of culture medium.

Results revealed that for small-sized amounts of culture filtrate it was recovered in supernatant the same enzyme activity as in *Raphanus* extract: it means that microbial enzyme is involved in the affinity interaction. The best output with a view to obtaining maximum amount of precipitated enzyme results for a volumetric ratio culture medium/lectinic extract of 4/5 (**Table 1**).

Table 1. Variation of precipitation efficiency of β-glucanase from culture filtrate by modifying filtrate/lectinic extract ratio

Culture filtrate (ml)	Lectinic extract (ml)	β-glucanase in supernatant (U/mL/min)	Efficiency of binding (%)
0.1	0.5	6	100
0.2	0.5	6	100
0.3	0.5	6	100
0.4	0.5	6	100
0.5	0.5	8.6	90
0.6	0.5	9.8	60

The treatment with glutaraldehyde of the lectinic extract from *Raphanus* resulted in a insoluble proteinaceous precipitate, which have been used in the subsequent steps of microbial glycoenzymes purification.

Affinity chromatography has been carried out by batch technique. To the insoluble lectinic polymer were added 10 ml microbial culture filtrate and incubated overnight in refrigerator. The various enzymatic activities of the culture filtrate are presented in (**Table 2**).

Table 2. The level of different enzymes produced by *B.subtilis* B36

Total protein (mg/ml)	**Proteolytic activity (U/ml/min)**	**α-amylase (U/ml/min)**	**β-glucanase (U/ml/min)**
38	7.85	382	43.7

After centrifugation, the level of protease, α-amylase and β-glucanase in the resulted supernatant has been analysed and the results are shown in (**Table 3**).

Table 3. Enzymatic activities in the supernatant obtained after interaction of microbial proteins and immobilized lectins

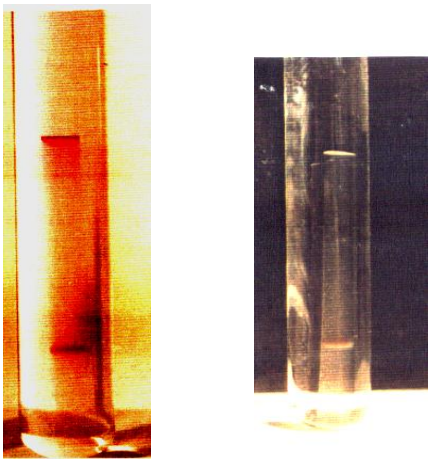
Total protein (mg/ml)	**Proteolytic activity (U/ml/min)**	**α-amylase (U/ml/min)**	**β-glucanase (U/ml/min)**
28.5	7.85	379.5	2

Separately, the sediment resulted after centrifugation was washed with distilled water in order to remove contaminating proteins and the enzymes we were interested in have been eluted with glycocol-HCl buffer, pH 3.0, containing 0.5M NaCl (**Table 4**)

Table 4. Enzymatic activities in the sample obtained after the elution of the bound enzymes (after interaction of microbial proteins and immobilized lectins)

Total protein (mg/ml)	Proteolytic activity (U/ml/min)	α-amylase (U/ml/min)	β-glucanase (U/ml/min)
4	0	0	38

Supernatant resulted includes only β-glucanase and it was used in the subsequent stages for the characterization of the enzyme. The polyacrilamide gel electrophoresis of the enzyme obtained with this method shown the presence of a single proteic band, of glycoproteic type with β-glucanase activity.



a b

Figure 1. PAGE pattern of the enzyme preparation obtained by affinity chromatography on immobilized lectins a) Schiff reagent (PAS technique); b) gel detection of enzymatic activity.

Since a lot of studies demonstrated glycoenzymes stability by binding various chemical agents to the glucidic region of the molecules [12], in this work we examined the consequence of coupling the lectin with bacterial β-glucanase. The interaction enzyme-lectins was conducted in centrifuge test tubes for 24 h. The precipitate enzyme-lectin was rendered soluble in acetic acid – sodium acetate buffer, pH 5.0 and the β-glucanasic activity was determined. The values obtained were higher than those resulted for the culture medium submitted to the interaction discussed in the present paper and also than those registered for the purified enzyme.

The conclusion resulted from our experimental data is that the lectin is bound at the glucidic region of the enzyme, forming an extremely active supermolecular aggregate and inducing some changes in the conformation and function of to the enzyme.

Lectins – *Trichoderma reesei* enzymes interaction

It is known that *Trichoderma sp.* produced different glycoenzymes, including β-glucanase [13]; for this reason we tested different types of plant lectins for interaction with fungal culture filtrate. The first group of lectins included those with glucose/manose, specificity since the literature data reporting the glycoproteic nature of fungi cellulases suggested the existence of these oses into the prosthetic group [14, 15].

Interaction lectins – enzymes has been proceeded by double diffusion in agarose gel, (Ouchterlony technique), taking under consideration purified lectins from *Pisum sativum*, *Lens culinaris*, *Con A*, several isolectins from *Vicia* sort and *Pharbitis purpurea*. Excepting the lectin from *Pharbitis* in which case the precipitation lines come out after diffusion vanished during the phases of washing and staining, no interaction has been observed, although such interactions had been cited in literature, at least in the case of *Con A* [1]. Reaction in the presence of *Pharbitis* lectin could be explained through the possibility that the affinity super-molecular aggregate dissociate into the physiologic serum during washing phases. Lack of interaction during the experiment carried-out might be due to the fact that the specific glucide is bound into glycoenzyme structure in a way that hinders the lectin in its recognition. The explanation – a quantity-related one – might be the following: despite of high enzymatic activity, the culture filtrate features low proteic content and that might cause the lack of interaction evidence (precipitation line).

Subsequent experiment stage consisted in estimating through the same pathway the interaction with lectins of various specificities, N-acetylglucosamine and N-acetylgalactose, namely lectins from tomatoes, potatoes, castor-oil plant, soybean, *Datura inoxia*, *Sophora japonica*.

Results obtained were alike but no precipitation lines were registered. The new lectins, less known or featured, have been the subject for the interaction with enzymes from *Trichoderma reesei* culture medium. These lectins appeared as partly purified extracts obtained from seeds yielded by roumanian flora: *Geum urbanum* (common bennet), *Calendula officinalis* (marigold), *Papaver somniferum* (poppy) and *Raphanus sativus* (radish), nevertheless showing an indisputable lectinic activity. Precipitation lines have been observed only in the case of lectinic extract from *Raphanus sativus* (**Figure 2**).

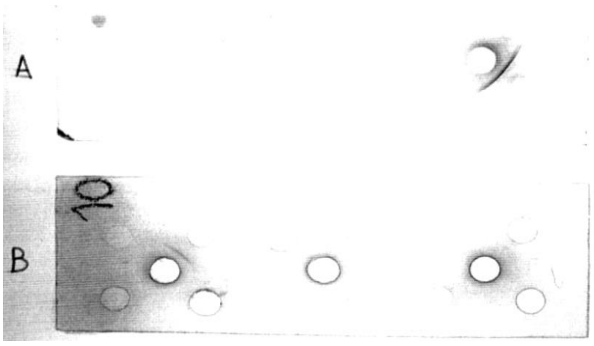


Figure 2. Interactions between *T.reesei* culture filtrate and *Pharbitis* lectins or *Raphanus* lectins. Culture filtrate was applied into the central hole; the other holes formed are for applying lectins with various specificities.

Assuming that compounds precipitated onto the agarose gel ought to precipitate also into the test-tube, the interaction has been accomplished, consequently being identified the nature of microbial compounds in accordance with the following diagram:

3 ml *Trichoderma* culture filtrate
+
3 ml lectinic extract
↓
overnight incubation at 4^0C meanwhile a precipitate is formed
↓
centrifugation at 4000 rpm
↙ ↘
precipitate supernatant
↓
resuspended in 3 ml phosphate – citric acid buffer, pH 4.8

The samples obtained following this scheme were tested for cellulosolytic activity comparing with the activity of the same type of enzymes in the culture filtrate (used as control), the results being presented in (**Table 5**).

Table 5. Activity of cellulases (C1 and Cx) and β-glucosidase from *T.reesei* culture filtrate and from the precipitates obtained after the interaction with lectins

	Control			Precipitate obtained after the interaction with the lectins from *Raphanus*			Precipitate obtained after the interaction with the lectins from *Pharbitis* sp.		
	C1	Cx	β-gluc	C1	Cx	β-gluc	C1	Cx	β-gluc
Enzymatic activity	2.8	200	1.55	3.5	-	-	3.9	2	2.25
% of enzymatic activity from precipitates comparing with control values	100	100	100	120.6	-	-	134.48	0.5	145.16

During the interaction with radish lectinic extract, in the precipitate was detected only cellobiohydrolase activity (C1 cellulase); therefore this enzyme has been one of the affinants and it can be separated from the other cellulololytic system components from the culture medium.

First step required for the obtaining of glycoproteins by affinity chromatography on immobilized lectins refers to lectins immobilization by glutaraldehyde. The insoluble proteic precipitate (resulted from the lectinic extract) was suspended in 5 ml fungal culture supernatant, stirring and then incubated overnight at 4ºC. After centrifugation the supernatant resulted in both variants (for each type of lectins, from *Raphanus* and *Pharbitis*) was examined for cellobiohydrolase and β-glucosidase activity. No activity was detected.

In case of *Raphanus* lectinic polymer, after affinity binding, several washes are carried out by recurrent centrifugation with physiologic serum and a final elution in citrate buffer pH 4.7. No washing was performed in case of *Pharbitis* lectinic polymer as the elution has been applied in two stages: in physiologic serum resulting the former eluted phase (E1) and then in citrate buffer pH 4.7 (E2).

Eluted bulk has been fixed to 5 ml so that an artificial dilution of the enzyme could not occur. Activity of the enzymes known as parts of the cellulosolytic system has been measured in each one of the eluted samples (**Table 6**).

Table 6. Enzymatic activity for the different samples containing *T.reesei* glycoproteins

	Total protein	C1 activity	Cx activity	β-glucanase activity (UE)
Culture filtrate	0.9	14.5	1000	7.75
Raphanus supernatant	0.61	0	1000	7.75
Raphanus eluted	0.21	16.5	-	-
Pharbitis supernatant	0.5	0	1000	0
Pharbitis eluted 1	0.12	0	0	8.6
Pharbitis eluted 2	0.21	15.8	0	0

Electrophoretic investigation of the samples revealed the purity as well as glycoproteic nature each one of them and the application of double diffusion technique revealed interaction both with total lectinic extract from *Raphanus* and the purified lectin with specificity for fucose (**Figure 3**).

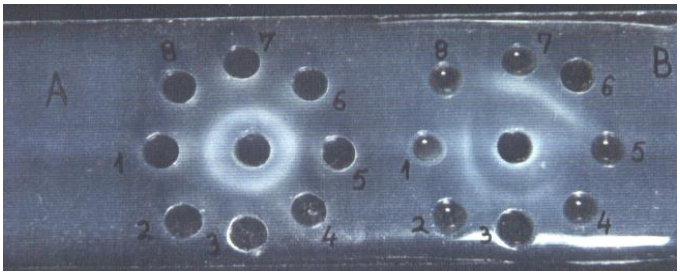


Figure 3. Interaction of total lectinic extract (A) with culture filtrate from *T.reesei* (1-4), *B.subtilis* (5) and the enzymes obtained after affinity chromatography on *Raphanus* lectins: from *T.reesei* (6-7) and *B.subtilis* (8). In B are presented the interactions of the same samples with one of the Raphanus lectins: that with fucose specificity.

As it can be seen in fig.3, only *T.reesei* culture filtrate and purified enzyme interact with both variants of lectins (total extract and purified lectin), instead the culture filtrate and purified β-glucanase from *B.subtilis* didn't recognized the lectin with fucose specificity. The conclusion in this case in that the β-glucanase from *B.subtilis* does not contain fucose in its glucidic region.

As a result of the experiments described above, the conclusion required is that it is possible to separate and purify microbial enzymes having biotechnological applications, such as β-1,3-1,4-glucanase from *B.subtilis*, cellobiohydrolase and β-glucosidase from *Trichoderma reesei* by affinity chromatography on immobilized lectins.

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