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STUDIES ON FUNGISTATIC ACTIVITY OF *BACILLUS COAGULANS* AGAINST *TRICHOTHECIUM ROSEUM* AND CHARACTERICTICS OF THE BACTERIAL METABOLITES

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Abstract: A characteristics of three extracellular metabolites produced by *Bacillus coagulans* strain that were supposed to be responsible for its fungistatic activity against *Trichothecium roseum* has been done by TLC, GC-MS and Western blotting methods. It was shown that these compounds of molecular weights as 41, 45 and 65 kDa secreted to the medium by bacteria had glycoprotein properties and did not become inactive when boiled for 20 min., as characteristic for glycoproteins.

Key words: alkaloid-rich lupin, compost straw, *Bacillus coagulans*, *Trichothecium roseum*, antagonism, active metabolites

INTRODUCTION

Phytopathogenic fungi cause at least 80% of plant diseases. They are mostly controlled using synthetic fungicides but their traces lasting in the environment for a long time cause its pollution. So, there have been performed numerous studies on the new strategies in plant protection, e.g. synthesis of the new, safe compounds, isolation of the natural ones as well as using biological methods (Olszak et al. 2000).

Biological methods utilizing an antagonism phenomenon seem to be a promising direction in an ecological plant protection however, they have not been used in a wide practice so far due to, among others, high requirements imposed on antagonistic microorganisms (Saniewska 2000). Generally, bacteria of *Bacillus* genus are antagonists of many pathogenic fungi (Pussey 1989) and produce a lot of substances that can act against pests and pathogens e.g. enzymes such as chitinases, xylanases, catalases, mannases and glucanases or antibiotics. These enzymes are able to the lysis of cell walls of e.g. *Basidomycetes* and *Oomycetes* (Kim and Kim 1993; Mavingui and Heulin 1994). Nevertheless, the most important fungistatic activity seems to be interactions between antibiotics produced by bacteria and cell walls and membranes of the pathogen (Vanittankom and Loefer 1986). Antibiotics that are synthesized out of ribosome by well known *Bacillus subtilis* strains include iturins (Maget-Dana and Peypoux 1994), surfactine (Kluge et al. 1988), fengicine and plipastatine (Vanittankom and Loefer 1986; Moyne et al. 2001).

Composts obtained from plant material are more and more of interest. A resistance of plants cultivated on them to various pathogens can be caused mainly by two factors: activity of chemical compounds (natural products) existing in the material or generated during compost processing, and/or presence of microorganisms antagonistic to pathogens. Moreover, composts induced systemic resistance of plants against some pathogens including fungi (Orlikowski and Wolski 2000).

From among bacteria existed in the composts of alkaloid-rich lupin straw, *Bacillus coagulans* and *B. circulans* strains, much less described than *B. subtilis*, have showed the strongest suppressive effect on fungi such as *Trichothecium roseum*, and *Fusarium oxysporum*. It was also shown that addition of 10% of bitter lupin extract to the straw increased fungiastic activity of the compost (Gulewicz and Trojanowska 1995; Gulewicz et al. 1997).

The main aim of these studies was to demonstrate a fungistatic activity of *B. coagulans* strains against pathogenic fungus *T. roseum*, localization of responsible metabolites by fractionation and ultrafiltration of the post-culture supernatant as well as characterize the active compounds, using thin layer chromatography, GC-MS and Western blotting.

MATERIALS AND METHODS

Chemicals

Chemicals of following companies were used for presented experiments: Coomassie Briliant Blue G-250, APS (Bio-Rad), peptobak, agar (BTL), PEG 20000, Tris (Fluka), $(NH_4)_2SO_4$, β-mercaptoethanol (Merck), $MgSO_4 \times 7H_2O$, KH_2PO_4, $NaNO_3$, $FeSO_4 \times 7H_2O$, NaH_2PO_4, Na_2HPO_4, isopropanol, methanol, ethanol, n-butanol, glycerol, ethyl acetate, acetic acid, *o*-phosphoric acid, SDS, chloronaphtol, glucose, saccharose (POCH – Gliwice, Poland), glycine, acrylamide, bromophenol blue, Tween 20 (Sigma), TEMED (Serva), NaCl, KCl (Standard). All chemicals were of the analytical grade.

Aqueous solvents were prepared using deionized, millipored water (Simplicity, Millipore).

Microorganisms

Bacterial strain of *B. coagulans* no. 6 with high antifungal activity isolated from lupin compost was used (Gulewicz and Trojanowska 1995; Gulewicz et al. 1997). This strain was kept on nutrient agar slants until use.

Fungal indicator *T. roseum* strain BPR 671 was supported by the Collection of Pathogenic Microorganisms (BPR) of the Institute of Plant Protection, Poznań, Poland. These strains were kept on Czapek-Dox agar slants.

Media

Carrot medium: 10% carrot extract was made by boiling of carrot in water for 1 h. One liter of the medium contained 5 g of glucose, 1 g of peptobak and 1 g of NaCl.

Cultivation of bacteria

Cultivation of *B. coagulans* strain was carried out in a liquid carrot medium for 24 h at 37°C. Ten milliliters of the obtained culture was transferred into 90 mL of sterile carrot medium and incubated for 18 h at 37°C at the shaking speed of 100 strokes per minute. Number of colony forming units (CFU) was determined by dilution plating.

The final bacterial culture was centrifuged at 10000 × *g* for 10 min. and the supernatant so obtained was filtered with 0.22 μm filter (Millipore).

Evaluation of the fungistatic activity of *Bacillus coagulans* strain

A well developed fungal culture on a slant was rinsed with 10 mL of sterile water. The resulting fungal spore suspension after 7 days was mixed with Czapek-Dox medium in the proportion of 1:10 and poured onto Petri dishes (15 mL). The wells (10 mm in diameter) were cut out centrally in medium in each Petri dish. The plates were placed in an incubator at 30°C for 24 h. After this period 0.1 mL of 18 h bacterial culture or its supernatant was pipetted into the well. Antifungal activity was estimated as growth inhibition zones (mm). Dishes containing pure sterile carrot medium were used as the control.

Fractionation of post-culture supernatant using ammonium sulphate

Fractionation was performed according to Chambers and Rickwood (1993). Post-culture supernatant and the control were fractionated using ammonium sulphate of final concentrations at 0°C as follows: 20, 40, 60 and 80%.

Dialysis of proteins

Proteins were dialyzed using 0.02 M phosphate buffer, pH 7.0. Dialysis was performed overnight using dialysis bags (Spectra/Por) of 3.5 kDa. Samples were concentrated in the presence of PEG 20000 and then dried under diminished pressure over phosphorus pentaoxide.

Fractionation of proteins of post-culture supernatant by means of ultrafiltration

In order to obtaining of compounds of various molecular weights ultrafiltration of the post-culture supernatant and control was performed using filters of 1 and 10 kDa (Amicon YM1 and YM10) in apparatus for protein concentration (Amicon 8050).

Hydrolysis of fractions obtained after ultrafiltration procedure

Fractions of molecular weights higher than 10 kDa were dissolved in phosphate buffer of pH 7.0, divided into two equal parts and finally hydrolyzed using following conditions:

a) 20% HCl, 121°C, 1.5 atm, 3 h
b) 2% HCl, 105°C, 3 h

in order to obtain hydrolyzates of proteins and sugars, respectively.

Precipitation of proteins with acetone

Proteins occurring in fractions were precipitated with four volumes of 100% cold acetone, overnight at 4°C. Next day precipitate was centrifuged at 4°C, 12000 × *g* for 20 min. Proteins so obtained were washed twice with 80% acetone at –20°C for 45 min. and centrifuged at 4°C, 12000 × *g* for 20 min. Protein concentrations were determined by a Bradford method.

Determination of protein concentrations by a Bradford method

A Bradford reagent: Coomassie Briliant Blue of final concentration 1 mg/mL; 23.75% ethanol; 42.5% *o*–phosphoric acid.

Concentrations of proteins were determined by measurement of the absorbance at λ = 595 nm after adding a Bradford reagent. Absorbance of samples was referred to the calibration curve that was derived using BSA as a standard and concentration of proteins calculated.

Thin layer chromatography (TLC)

Chromatography was performed using Merck silica gel 60 F_{254} TLC plates. Chromatograms were run in following phases:

A) n-butanol : acetic acid : water (4:1:1 v/v) for protein/peptides separation, and
B) isopropanol : ethyl acetate : water (5:2:3 v/v) for sugars separation

After run completion, plates were dried and sprayed with 0.2% ninhydrine solution for proteins/peptides indication and 0.2% naphtorezorcine one as a sugar developer.

Electrophoresis (SDS-PAGE) of proteins

SDS-PAGE of proteins was performed according to a Laemmli (1970) method on polyacrylamide gel. Concentrating followed by separating gels, 4 and 16%, respectively were used. Electrophoreses were conducted in the buffer of the following composition: Tris, 3.03 g; glycine, 14.4 g; SDS, 1 g; water ad 1000 mL. Samples for the procedure (precipitated proteins) were dissolved in the buffer consisting of 0.25 *M* Tris/Cl^- (pH 6.8), 20% glycerol, 4% SDS, 10% β-mercaptoethanol, 0.0025% bromophenol blue and then boiled for 3 min. After centrifugation at 4000 × *g* for 3 min. samples were applied to the conditioned gel. Electrophoresis was performed by means of current of 10 mA followed by 15 mA for concentration and separation, respectively.

Immunodetection of glycoproteins by a Western blotting method

Proteins separated during one – dimension SDS-PAGE procedure were transferred to the nitrocellulose membrane by means of current of 400 mA at 4°C for

90 min. using the buffer containing CAPS, pH 11 (120 mL) and methanol (120 mL) in 1200 mL of water. In these conditions proteins were bound to the membrane. Resulted print of separated proteins was then incubated in the TBST buffer containing 1% of BSA in order to saturate other fragments of the membrane. Nitrocellulose print was then incubated for 1 h in TBST-BSA containing concanavalin A that bounds into oligosaccharide units. After washing with TBST the print was incubated with antibodies against concanavalin A in TBST-BSA overnight at room temperature. The membrane was then washed with TBST and incubated for 1 h with secondary antibodies coupled with peroxidase, directed against primary ones. The membrane was washed with TBST for 30 min. Detection of peroxidase was performed using the developer containing chloronaphtol (10 mg), methanol (4 mL) and 16 mL of TBS. Hydrogen peroxide was used as an initiator of the reaction.

RESULTS AND DISCUSSION

Subjected studies were performed according to the scheme presented in figure 1.

Fractionation of the supernatant proteins according to a Chambers and Rickwood method (1993), using ammonium sulphate gave fractions containing proteins of various molecular weights (MW) and different degree of saturation with $(NH_4)_2SO_4$ at 0°C, namely 20, 40, 60 and 80% that were marked as 20H, 40H, 60H and 80H, respectively. Similarly, four fractions for the controls prepared of the pure medium were obtained and marked as 20C, 40C, 60C, 80C, respectively. Table 1 has presented protein concentrations in the above fractions. As we can see concentration of proteins was lower than that of the control and in the cases of 60H and 80H containing proteins of higher molecular weights even drastically. The H/C rates for them equal 0.05 and 0.08, respectively. Nevertheless, the fungistatic activity was observed just for 60H and 80H fractions only however, it was at least four times lower than that of unprocessed supernatant S (Tab. 2). It seems to be clear that processing of the supernatant using $(NH_4)_2SO_4$ influenced negatively its biological activity. On the other hand such studies have shown that only compounds of relatively high molecular weights performed fungistatic activity.

Above observations were confirmed by fractionation of the supernatant proteins by ultrafiltration method using filters of 1 kDa and 10 kDa. After filtration following fractions marked as: 1H (compounds of MW up to ca. 1 kDa), 2H (compounds of MW above 1 kDa), 3H (compounds of MW up to 10 kDa), 4H (compounds of MW above 10 kDa) and 5H one (compounds of MW from 1 to 10 kDa) were obtained. Similar set to above was made for the control i.e. pure culture medium without bacteria, and fractions 1C, 2C, 3C, 4C and 5C, respectively were resulted. Fungistatic activity of fractions that obtained and unprocessed post-culture supernatant, H_0 as well as pure, not fractionated medium, C_0 was studied. The activity was confirmed for 2H and 4H by determination of inhibition zone that equaled ca. 45 mm (Fig. 2), similarly to H_0 one. These fractions also contained compounds of MW higher than 10 kDa.

Properties and characteristics of compounds that could be responsible for fungistatic activity were then established.

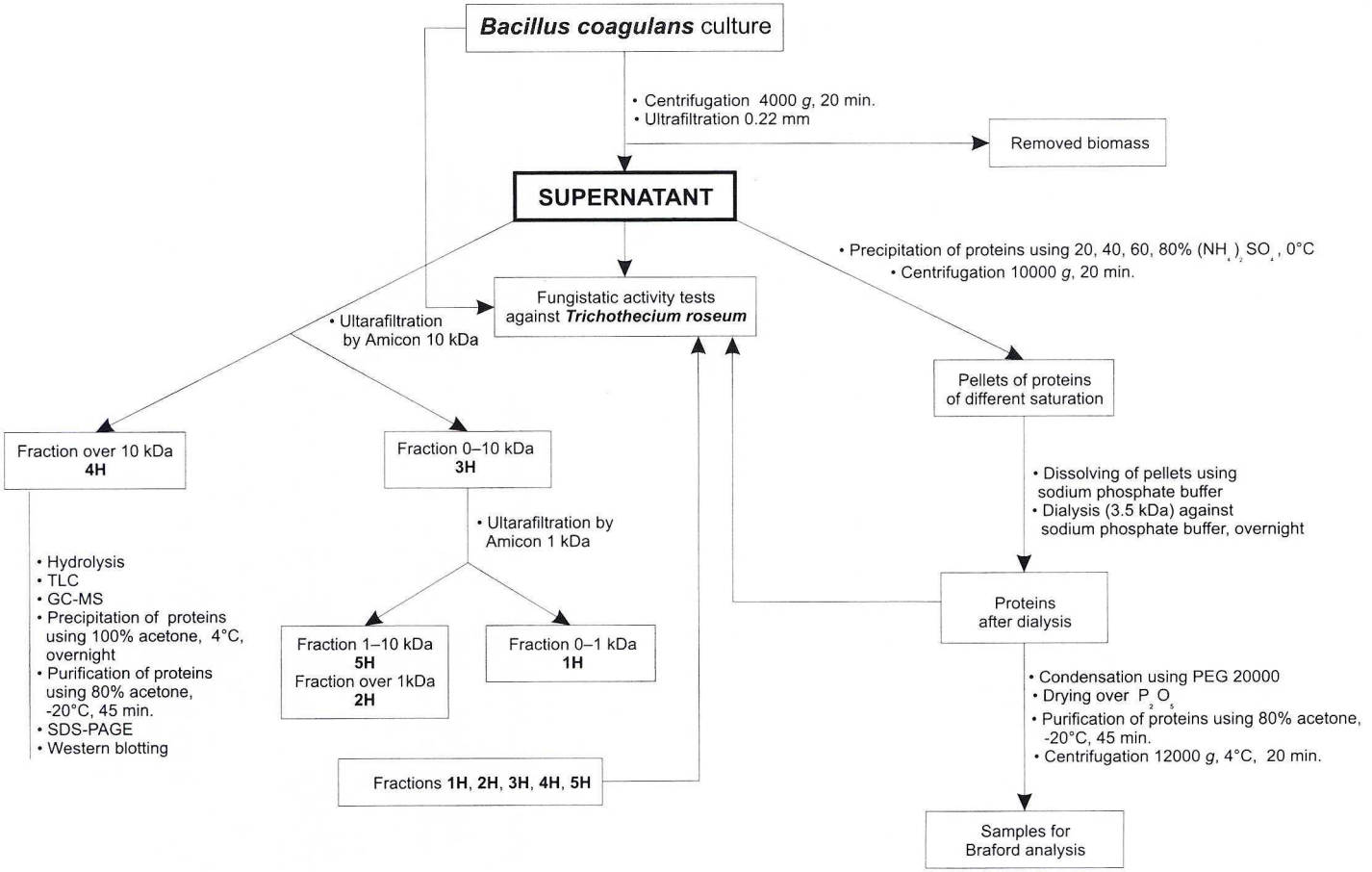

Fig. 1. Scheme of the studies on determination of compounds responsible for fungistatic activity of *Bacillus coagulans* to *Trichothecium roseum*

Table 1. Content of protein in particular fractions of the post-culture supernatant and pure medium

Sample	20		40		60		80	
	H	C	H	C	H	C	H	C
Protein concentration [mg/100mL]	2.05	2.49	2.31	3.85	2.38	41.40	3.75	48.90
% protein H/C	0.82		0.6		0.05		0.08	

Table 2. Inhibition zones (mm) for *Thrichothecium roseum* growth caused by particular protein fractions

Sample	S*	20		40		60		80	
		H	C	H	C	H	C	H	C
Inhibition zone	45	0	0	0	0	8	0	11	0

*post-culture supernatant without fractionation

Table 3. Relation between time of boiling of fractions and diameters of inhibition zones for *Thrichothecium roseum*

Boiling time [min]	Inhibition zone [mm]					
	2C	2H	4C	4H	C_0	H_0
0	0	44	0	43	0	45
5	0	41	0	40	0	42
20	0	40	0	40	0	42

An effect of the temperature on biological activity of the supernatant fractions 2H, 4H, H_0 compared to the controls 2C, 4C and C_0 has been shown in table 3. Samples were then heated at 100°C for 0, 5 and 20 min. Next samples were injected to cut wells in inoculated medium and then inhibition zones was measured after 7 days. There was no influence of heating on fungistatic activity of fractions studied. Stability of compounds in high temperature suggested that they could be of the glycoprotein character. That derivatives, complexes of proteins and carbohydrates of MW from a few to dozens kDa have been known to be resistant at boiling temperature for 15–30 min. (Kozak et al. 1978; Davey and Richardson 1981).

To prove that post-culture supernatants contained glycoproteins, active fraction 4H was applied onto TLC plates and chromatograms developed in mobile phases for sugars and aminoacids/peptides (Fig. 3), as described in Materials and Methods. As we can see there was a big difference between fraction 4H and the control one, 4C. They differred in content of high molecular weight sugars that in our chromatography conditions were placed at a starting point (Fig. 3A). Differences were also noticeable in content of glucose and saccharose that in chromatograms constituted one spot but based on the color of spots fraction 4H did not contained saccharose contrary to 4C one. Figure 3B has illustrated differences in content of aminoacids and peptides. Control fraction 4C contained much more of them that 4H one. Both fractions contained high molecular weight peptides that were located at the starting point of the chromatogram.

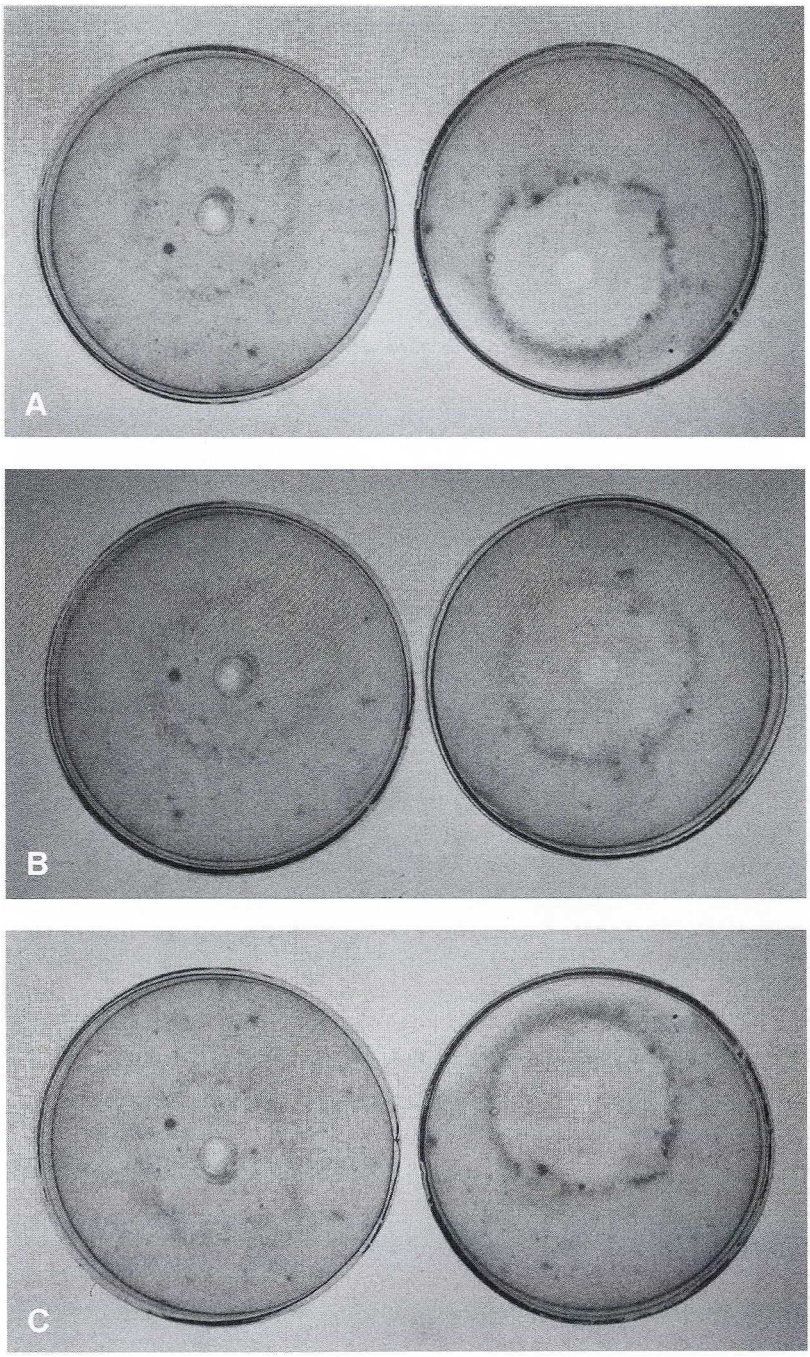

Fig. 2. Inhibition zones of growth of *Trichothecium roseum* by: supernatant H_0, fraction 2H and fraction 4H (right A, B and C, respectively) compared to: C_0, 2C and 4C (left A, B and C, respectively)

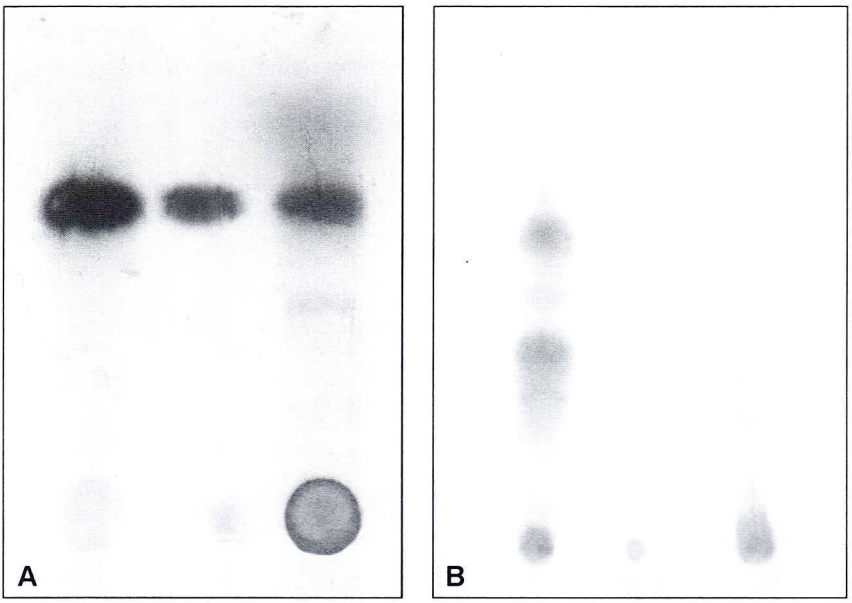

Fig. 3. Chromatograms of fraction 4H of post-culture supernatant, A – developed for sugar detection, B – developed for aminiacid/peptide detection. From the left: 4C, glucose, 4H

Figure 4 has presented chromatograms of hydrolyzates of spots that were located at the starting point of chromatograms presented in figures 3A and 3B. It has been shown that compounds at the starting point were of high molecular weight and during hydrolysis degraded to low molecular weight ones i.e. simple sugars and aminoacids.

Table 4 has compared percentage of particular aminoacids in the total pool based on the GC-MS analysis of fractions 4H and 4C after hydrolysis. There were differences between these fractions namely, methionine was not found in fraction 4C contrary to 4H one. Moreover, percentage of lysine was also significantly different and equaled 13.0 and 4.9% for 4H and 4C, respectively. Above results supported an evidence that in the supernatants appeared new glycoprotein complexes of different composition from that of the control. However above tests did not disclose character and largeness of the complexes.

This problem was resolved by immunodetection test of the proteins by Western blotting method of fraction 4H and control one, 4C. The test showed (Fig. 5) that fraction 4H derived from post-culture supernatant contained three glycoproteins of MW ca. 41, 45, and 65 kDa that were not present in pure medium represented by fraction 4C. Control fraction contained only one glycoprotein of MW 68 kDa that was also present in the supernatant fraction. Therefore, complexes of MW 41, 45 and 65 kDa had to be products generated during *B. coagulans* culturing. Approximate masses of glycoproteins were established using regression factor, $r = 0.8049$ (calculated on the base of retention factors, R_f) and have been presented in table 5.

Fungistatic activity appeared only in fractions containing above glycoproteins contrary to the control ones that did not perform any such activity. Moreover,

glycoproteins have been known to be quite temperature resistant and our supernatant had this property. It has been known that sugar part of these complexes makes membrane transport easier so, helps in biological activity.

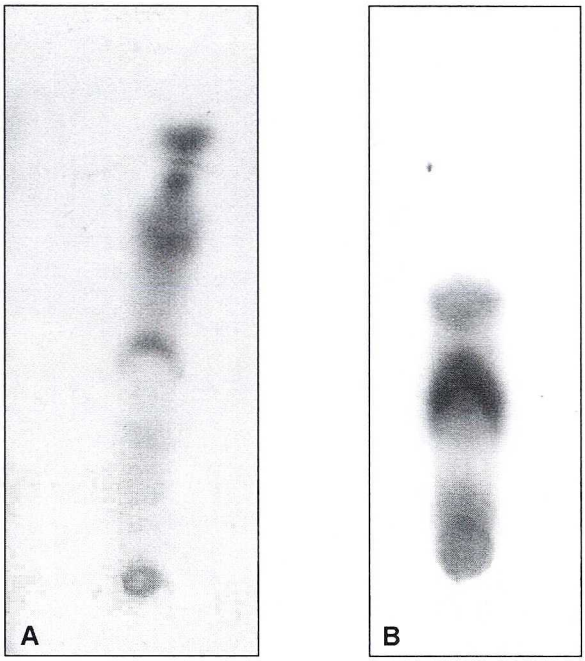

Fig. 4. Chromatograms of hydrolysates of spots from fraction 4H located at the start of chromatogram (see Fig. 3 A, B), that contained high-molecular compounds: A – sugar hydrolysate, B – peptide hydrolysate

Table 4. Percentage of particular aminoacids identified in fractions 4C and 4H in the total pool

Aminoacid	Retention time		%	
	4C	4H	4C	4H
Alanine	8.64	8.67	11.5	8.0
Glycine	9.12	9.13	9.9	6.3
Threonine	10.65	10.67	4.7	3.6
Serine	11.06	11.08	6.9	4.9
Valine	11.49	11.51	4.7	4.2
Leucine	13.41	13.43	4.1	3.9
Isoleucine	13.60	13.70	1.6	1.3
Proline	16.67	16.68	5.3	6.0
Methionine	–	19.6	–	1.0
Aspartic acid	21.02	21.06	14.9	16.4
Phenylalanine	22.10	22.12	3.2	3.5
Glutaminic acid	24.00	24.03	28.3	24.9
Thyrosine	–	24.56	–	2.6
Lysine	24.89	24.95	4.9	13.4

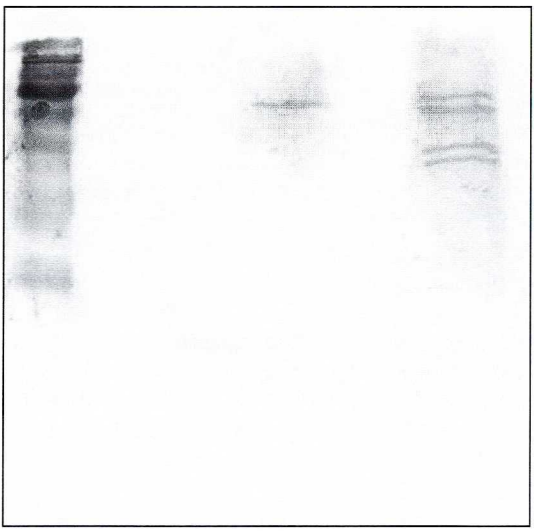

Fig. 5. Westren blotting of fraction 4H. From the left: markers, fraction 4C and 4H one

Table 5. Approximate masses of glycoproteins in fractions 4C and 4H

R_f	Fraction 4C MW [kDa]	Fraction 4H MW [kDa]
0.12	68.85	68.85
0.13	–	65.36
0.20	–	45.44
0.22	–	40.96

CONCLUSIONS

Bacteria *B. coagulans* strains isolated from composts of alkaloid-rich lupin straw and then cultured on the carrot medium secreted to the agar medium substances that perform fungistatic activity to fungus *T. roseum*. This activity was localized in fractions of post-culture supernatant containing high molecular weight compounds. Active fractions were relatively resistant when boiled for 20–30 min. as characteristic for glycoproteins. Glycoprotein character of the active compounds was confirmed by TLC and GC-MS analysis. Western blotting test showed that most active fraction, 4H contained four protein-carbohydrate complexes of approximate MW of 41, 45, 65 and 68 kDa contrary to the inactive control one, 4C where only the last compound was detected. GC-MS analyses showed differences between aminoacid composition of generated glycoproteins what concerned the content of methionine and tyrosine that were absent in the control. Also concentration of lysine in the active fraction was much higher than that of the control. It seems to be clear that there was dependence between glycoprotein content and fungistatic activity of post-culture supernatant. However, it is still not known which of three glycoproteins was responsible for the activity and whether it was an effect of one of them or synergism of two or three. Above is the subject of present studies.

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POLISH SUMMARY

BADANIA NAD AKTYWNOŚCIĄ FUNGISTATYCZNĄ *BACILLUS COAGULANS* PRZECIW *TRICHOTHECIUM ROSEUM* ORAZ CHARAKTERYSTYKA METABOLITÓW BAKTERYJNYCH

Na podstawie metod TLC, GC-MS i Western blotting scharakteryzowano 3 metabolity produkowane przez *Bacillus coagulans*, które były odpowiedzialne za fungistatyczną aktywność bakterii. Wykazano, że związki o masach cząsteczkowych 41, 45 i 65 kDa wydzielane przez bakterie mają właściwości glikoprotein. Frakcje, które je zawierały wykazywały aktywność fungistatyczną w stosunku do *Trichotheicium roseum* i nie traciły aktywności podczas gotowania przez 20 min., co jest charakterystyczne dla glikoprotein.