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EFFECTIVENESS OF CELLULASES OF *ARMILLARIELLA MELLEA* (FR. EX. WAHL) P. KARSTEN IN RELEASING PROTEINS FROM THE ALEURONIC LAYER IN BARLEY AND OATS BRAN

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A highly active preparation of cellulases from an edible fungus *Arm. mellea* (Fr. ex. Wahl) P. Karsten was obtained. Its hydrolitic capability to decompose cellulose in cell membranes of the aleuronic layer of barley and oats bran was determined. Application of successive treatment of the bran's initial processing with pronase and then to the cellulases obtained from *Arm. mellea* rendered it possible to extract proteins almost completely from barley bran, and somewhat below that level from oats bran.

INTRODUCTION

In a number of earlier publications [3, 4, 5, 8, 9, 10] results were presented on obtaining and application of cellulases of fungus *Trichoderma lignorum* (Tode) Harz 155 in hydrolysis of cellular membranes of the aleuronic layer in cereals. The method used was effective and making possible to 'recover' from the thick-walled cells of the aleuronic layer in cereals almost their entire protein contents. Fundamental assumptions of the method are confirmed by Saunders [17].

Also other studies [6, 7] upon cellulolytic properties of *Arm. mellea* proved that the fungus when cultivated on a medium with high content of cellulose causes a very strong hydrolysis into monosaccharides with an accompanying process of protein biosynthesis. The fungus is also commonly known among phitopathologists as a strong patogenic factor in roots and trunks of trees. In some special conditions of culture, as Manka [13] reports, it also has saprophytic properties.

Due to a growing interest in obtaining and application of cellulases in food and fodder industries, studies have been undertaken to receive and to assess the activity of the complex of cellulases from *Ar. mellea* and its usefulness in releasing aleuronic proteins from barley and oats brans.

The aim of the project was to obtain a preparation of highly active cellulases from cereal brans as cellulose substrates in the media, and also to provide its characteristics; to determine their effect on the degree of enzymatic decomposition of cellulose in barley and oats brans; extraction of edible proteins from the aleuronic layers in the cereals by successive application of pronase and cellulase enzymes.

THE EXPERIMENT

1. MATERIALS

The experiment was carried out with brans of barley and oats obtained in 1977 from a Polish milling plant at Wągrowiec. Table 1 gives the chemical characteristic of the brans. The investigated organism was *Arm. mellea* fungus obtained from the Museum of Forest Preservation Institute, Phitopathology Department, Agricultural University of Poznań. It had been isolated on a pine at Rakownia District of Forestry, also a part of the university.

Table 1. Chemical characteristics of the raw materials in percentage of dry mass

Components	Barley bran	Oats bran
Dry mass	92.42	91.29
Total protein (N × 6.25)	16.83	14.97
Reducing sugars as glucose	2.59	1.95
Cellulose	10.46	13.51
Fats	4.24	8.17
Starch	45.66	33.61
Ash	4.17	3.38

2. METHODS

The dry mass was determined with the drying method, nitrogen with Kjeldahl method, reducing sugars — Somogyi-Nelson [18], decreasing quantity and isolation of cellulose — Scharrer-Kurschner after Kamer and Ginkel [11] lipids — after Soxhlet; starch was marked polarimetrically, and ash was determined by burning at 920° for six hours. Cellulolytic activity of the post-culture liquids was determined as follows:

Activity of glucanohydrolase β-1.4 glucane (F. C. No. 3.2.1.4.):

— endo-β-1.4-glucanase (endocarboxymethylocellulase by the viscosimetric method in ratio of 1% solution KMC-Na as the substrate. Measurements of reduction in viscosity were performed on the Oswald viscosimeter on the basis of the method suggested by Reese et al [15] and by the Consultations Protocol S.F.V. [14];

— exo-1.4-β-glucanase (exo-carboxymethylocellulase) by the reduction method on the basis of Reese et al. [15, 16] in ratio of 0.5% solution of sodium salt KMC-Na as the substrate;

— general activity of cellullolitic enzymes was determined with Chrapkowska's method [2] in ratio of cellulose isolated from brans of barley and oats as substrates. It was given in mg glucose (g enzyme proteins) determined with Lowry's method [12]. Isolation of the aleuronic layer in the investigated materials was done with the pronase method reported by Chrapkowska [1]. The proteins of enzymes in post-cultivation liquids was determined with Lowry's method [12] using beef albumin as a standard.

The fungus *Arm. mellea* was cultivated on an agar-plum medium at about 23° [13]. The experimental culture was a submerged fermentation on the medium and under the conditions given by Chrapkowska et al. [6] on Universal Shaker 327 type of equipment (amplitude: -3, frequency of vibration -5) over 14 days at ca 25° and pH 4.7 in two variants of the experiment — with barley bran and the oats bran (5%). The post cultivation liquids were separated by centrifugation and stored in a refrigerator.

3. DESCRIPTION OF EXPERIMENTATION

The obtained post-cultivation liquids (characteristics are given in Table 2) were used as raw enzymatic preparations to treat directly the barley and oats brans, and to do the same after a prior isolation of their aleuronic layers by means of a complex of proteolytic enzymes, that is, pronase. Every substrate was treated with each of the specific preparations. After the direct treatment, 10 g bran ground in an electric mill was supplemented with 25 ml post-cultivation liquid, 50 ml H_2O (distilled), and 2 ml solution of merthillate as an antiseptic (2.5 mg/500 ml H_2O), pH was brought up to 5, and all was heated at 37°C for 36 hours. The samples were agitated every several hours. After completion of hydrolysis enzymes were inactivated at 90°C for 10 minutes. The extracts were supplemented to 200 ml and in 10 combined samples contents of protein, reducing sugars were determined. The same was done with cellulose content in sediments. The results are given in Table 3.

When treating brans with cellulase preparations after a prior treatment with pronase, 25 g ground bran was mixed with 20 ml buffer Tris

Table 2. Characteristics of cellulolytic activity of post-cultivation liquids from fungus Arm. mellea (Fr. ex. Wahl) P. Karsten

Activity of the cellulose complex	Experimental culture			
	Barley bran 5%		Oats bran 5%	
Enzyme proteins; mg/ml post-cultivation liquid	0.197		0.252	
Activity of endo-1.4-βglucanase (endo-KMC-ase)*) in ratio of 1% KMC-Na as substrate J/g enzyme proteins	73,034		18,700	
Activity of exo-1.4-βglucanase**) (exo-KMC-ase) in ratio of 0.5% KMC-Na as substrate	Glucose; mg/g enzyme proteins	J-units	Glucose; mg/g enzyme proteins	J-units
	55,837	0.0179	55,552	0.018
Total cellulolytic***) activity in ratio of isolated cellulose of bran as substrate	447,439	0.0022	114,262	0.0087

*) J: the unit of activity of endo-1.4-βglucanase (endo-KMC-ase) is a volume of protein which lowers viscosity of 0.15 g KMC-Na by 25% over 20 min. at 30°C and pH 4.7

**) J: the unit of activity of exo-1.4-βglucanase (exo-KMC-ase) is a volume of enzyme proteins which produces 1 mg glucose within 2 hrs at 37°C and pH 4.7

***) J: the unit of total cellulolitic activity is a volume of enzyme proteins which produces 1 mg glucose from the specific substrate — cellulose isolated from bran within 36 hrs at 37°C and pH 5.

Table 3. Effects of hydrolitic activity of raw celluloses preparation Arm. mellea (Fr. ex. Wahl) P. Karsten in direct application to barley and oats bran

Bran	Extractable protein; N × 6.25		Reducing sugars as glucose		Decrease of cellulose %	Increase of reducing sugars in ratio of cellulose in 100 g dry mass sample; %
	mg/100 bran dry mass	Extracted in ratio of the initial contents; %	mg/100 bran dry mass	Increase %		
Barley	7,702.21	45.77	3,522.59	36.01	17.04	33.68
Oats	4,513.75	30.15	2,150.74	10.29	7.33	15.92

(pH 7.4); pronase was added in 5, 10, 15, and 20 mg portions solved in 20 ml buffer Tris. Following proper agitation and addition of 2 ml merthiolate the samples were kept at 37°C for 48 hours, and were agitated every several hours.

After 24 hours 20 ml portions of buffer Tris (pH 7.4) were added. Whe the hydrolysis was finished the samples were combined, four at a time,

liquids were separated by centrifugation, supplemented to a given volume. In clear extracts proteins and reducing sugars were determined (taking into account the initial content in bran) as well as cellulose in the sediments. The results are given in Table 4.

Table 4. Effects of different doses of pronase on barley and oats bran

Bran	Pronase; mg	Protein content, N × 6.25		Content of reducing sugars as glucose in extracts	
		mg/100 dry mass bran	Extraction in ratio of the initial content; %	mg/100 dry mass bran	Release; %
Barley	5	3692.64	21.94	1162.77	39.42
	20	5138.10	30.53	1707.80	67.90
Oats	5	1895.32	12.66	497.81	25.55
	20	4122.81	27.54	490.89	45.69

The centrifuged sediments from samples of the isolated aleuronic layer of barley and oats brans were subjected to hydrolytic activity of raw cellulolytic preparations under the conditions and with parameters analogous to those present during the direct treatment. The results are given in Table 5.

RESULTS AND DISCUSSION

The obtained relatively high results of the activity of the cellulases preparation (given in Table 2) prove that the choice of the fungus as a producer of cellulolytic enzymes was correct, even though the species is still underestimated in the literature in the subject. The cultivation was conducted on two substrates with an addition of 5% barley and oats brans to media, which also were two different sources of carbon; they showed a very high substrate specifity of the enzymes under investigation. Higher activity of the complex of cellullolytic enzymes of *Arm. mellea* on barley oats in regard to cellulose isolated from this bran is a good evidence of relationship between enzymes in the substrate.

Earlier studies by Chrapkowska [2] on isolated substrates of cellulose from barley and oats and their hydrolysis by cellulases of *Trichoderma lignorum* (Tode) Harz 155 as well as cellulases of *Trichothecium roseum*, showed a reversed usefulness in which different fractions of the isolated cellulose from oats bran were more vulnerable to hydrolysis than fraction of cellulose from barley bran.

Table 5. Results of hydrolytic effect of raw preparations of *Arm. mellea* (Fr. ex. Wahl) P. Karsten on the pronase — isolated aleuronic layer in barley and oats bran

Type of substrate	Pronase; mg	Extractable protein N × 6.25		Sugars obtained as glucose		Loss of cellulose %	Increment of reducing sugars in ratio of cellulose in 100 g dry mass sample; %
		mg/100 g dry mass bran	Successive extraction in ratio of the initial content; %	mg/100 g dry mass bran	Increment after successive activity of enzymes; %		
Isolated aleuronic layer in barley	5	11,998.59	93.23	3967.45	173.91	80.26	39.49
	20	11,385.98	98.18	4793.64	220.39	80.75	62.16
Isolated aleuronic layer in oats	5	6,530.38	56.28	2098.16	133.12	87.03	19.22
	20	8,301.50	82.99	2668.71	162.03	89.33	23.38

The results given in Table 3 indicate that decomposition of the cell membrane cellulose in barley and oats brans permitted a relative high release of the aleuronic layer with simultaneous accumulation of large quantities of reducing sugars. Differences in the decomposition of cellulose and an increase of released proteins and sugars between barley and oats brans were observed. The complex of cellulases from *Arm. mellea* was also more active on the non-isolated cellulose of barley bran as the substrate than on the cellulose from oats bran.

Table 4 presents the effect of different selected doses of pronase applied during isolation of the aleuronic layer (5 mg and 20 mg) which produced the lowest and the highest degrees of release of proteins and sugars.

Increased levels of pronase used for digesting the proteins of a part of albumin of barley and oats brans caused a clear increase of the isolated proteins in the two cereals. No major increase in the quantity of reducing sugars was observed. It can be surmised that in this manner it is possible to isolate the aleuronic layer from the albumin layer both in barley and in oats brans. The experiment is a supporting evidence to the method used by Chrapkowska [1] in isolating the aleuronic layer of wheat bran.

When a comparison was made between the effect of successive activities of the two complexes of enzymes (pronase and cellulase) and the direct treatment of the investigated brans with the cellulase preparation (Table 5), it was observed that the former treatment releases barley bran proteins almost 100% and 83% in oats bran. At the same time the effect of cellulases induced a very high level of decomposition of cellulose. The degree of cellulose decomposition in the aleuronic layer of oats (89.33%) is higher than that of barley (80.75%). This is a reverse order as compared with the direct effect of the cellulase preparation on brans. As a result of combined activity of the two complexes of enzymes the raw brans are transformed into a product that can be fully assimilated. The aminoacid composition of the proteins released from the aleuronic layer is the subject of the second part of the investigations and shall be published in due time.

CONCLUSIONS

The experiment and the results following from it indicate the following:

1. Fungus *Arm. mellea* (Fr. ex. Wahl) P. Karsten is effective in obtaining an active complex of cellulases in submerged fermentation and with the parameters apllied here.

2. Direct application of cellulases onto barley and oats brans rendered

the following levels of release of proteins: barley — 45.77%, oats — 30.15%.

3. Direct activity of cellulases resulted in higher production of glucose in barley bran as compared with oats bran: 33.68% versus 15.92% in ratio of the cellulose content in the analysed samples.

4. The successive treatment with pronase and cellulases helped to obtain the maximum recovery of proteins from the aleuronic layer of the brans. In barley bran it was 98.18%; in oats bran — 82.99%.

5. There were clearly discernible differences as to the degree of decomposition of cellulose and accumulation of reducing sugars in the course of the successive treatment of barley and oats brans with pronase and cellulases in comparison with the direct effect of the cellulases complex. The decomposition reached 80.75% in barley bran and 89.33% in oats bran.

Increase in reducing sugars from the hydrolysed cellulose in the investigated samples was 62.16% (barley) and 23.38% (oats).

6. The obtained results point to the purposefulness of continuing of research regarding the use of the *Arm. mellea* (Fr. ex. Wahl) P. Karsten complex of cellulases in releasing the aleuronic layer of cereal brans. Besides, the fungs is commonly known, edible, non-toxic, which features make it a very useful element in the technology of fodders (feeds) and animal feeding.

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PRZYDATNOŚĆ CELULAZ GRZYBA *ARM. MELLEA* (FR. EX. WAHL) P. KARSTEN DLA UWOLNIENIA BIAŁEK WARSTWY ALEURONOWEJ OTRĄB Z ZIARNA JĘCZMIENIA I OWSA

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Streszczenie

Przeprowadzono badania nad otrzymaniem i charakterystyką preparatu celulaz z grzyba *Arm. mellea* (Fr. ex. Wahl) P. Karsten. Określono właściwości hydrolityczne tego kompleksu enzymów na stopień rozkładu błon komórkowych warstwy aleuronowej otrąb jęczmienia i owsa. Charakterystyka aktywności enzymów celulolitycznych preparatu wykazała aktywność (E.C. No. 3.2.1.4.) glukanohydrolazy β-1,4 glukanu:-endo- 1,4-β-glukanazy (endo-KMC-azy) 73034 j/g białka enzymów w przypadku hodowli grzyba z dodatkiem 5% otrąb jęczmiennych i 18700 mg glukozy/g białka z dodatkiem 5% otrąb owsianych; -egzo- 1,4-β-glukanazy (egzo-KMC-azy) 55837 mg glukozy/g białka w hodowli z dodatkiem 5% otrąb jęczmiennych oraz 55552 mg glukozy/g białka z dodatkiem otrąb owsianych. Aktywność ogólna w stosunku do wyizolowanej celulozy z otrąb jako substratu wynosiła 447439 mg glukozy/g białka w przypadku celulozy jęczmienia i 114262 mg glukozy/g białka z celulozy owsa. Ekstrakcja białek w wyniku działania bezpośredniego celulaz na otręby wynosiła 45,77% dla jęczmienia i 30,15% dla owsa. Zastosowanie pronazy do wstępnej obróbki otrąb i następnie działanie preparatami celulaz dało podwyższenie ilości wydobywanego białka do 98,18% dla jęczmienia i 82,99% dla owsa. Rozkład celulozy w tych próbkach wynosił dla otrąb jęczmiennych 80,75% i dla owsianych 89,33%, z równoczesnym przyrostem cukrów redukujących odpowiednio: 62,16% i 23,38% w stosunku do zawartości celulozy.