A C T A A L I M E N T A R I A P O L O N I C A

Vol. XVII/XLI/ No. 3 1991

EUGENIA PODGÓRSKA

ACTIVITY OF VARIOUSLY OBTAINED CELLULOLYTIC PREPARATIONS

Department of Food Technology and Storage
Agricultural Academy, Lublin

Key words: cellulytic preparation, *Fusarium sp.*, enzyme purification

Activities of cellulolytic preparations obtained with various methods from *Fusarium sp.* culture filtrates were compared. The highest total activity was displayed by ethanol-precipitated cellulolytic preparations regardless of the substrate. Hydrolysates obtained with preparations precipitated from four-days culture filtrates contained three times cellobiose than glucose, while hydrolysis with preparations from six-day filtrates yielded twice more glucose than cellobiose.

Results were compared of the activity of cellulolytic preparations obtained from *Fusarium sp.* culture filtrate concentrated to one-fifth of its original volume by precipitation with methanol, ethanol, acetone and ammonium sulfate. The obtained enzymatic protein was used to hydrolyze Whatman 1 paper, filter paper pre-digested partially with 85% phosphoric acid, carboxymethylcellulose sodium salt, rye straw, and deciduous tree sawdust. The highest cellulolytic activity was observed in ethanol-precipitated preparations, and the highest specific activity in preparations salted out with ammonium sulfate. Hydrolyzates obtained using preparations from four-day culture filtrates contained three times more cellobiose than glucose, while those from six-day cultures had twice as much glucose as cellobiose.

Various aspects of the synthesis of cellulolytic enzymes by Fusarium fungi were studied: the kinetics of their production [22], the optimal conditions of cellulases separation from the culture filtrate [16] and hydrolysis of pre-treated birch sawdust [23], among others.

The course and results of enzymatic hydrolysis of cellulose in lignocellulosic raw materials depend on the structure and chemical composition of these materials, on the manner of their pretreatment, the activity and composition of the cellulolytic complex, and on the methods and conditions of hydrolysis (such as temperature, substrate hydrolysis) [8], the presence of nitric or phosphoric compounds [9], and other factors. The optimization of enzymatic hydrolysis of cellulose must thus involve both the creation of the best conditions for the activity of the preparation that is being used, and the best preparation of the raw material.

The purpose of this research was to compare the effectiveness of cellulolytic preparations obtained by various methods from *Fusarium sp.* culture filtrate.

Enzymatic protein was accordingly precipitated with organic solvents and ammonium sulfate, and the results of enzymatic hydrolysis of several cellulosic substrates with the obtained preparations were studied.

MATERIAL AND METHODS

The experimental material was postculture filtrate obtained from cultures of the fungus *Fusarium sp.* from the collection of the Department of Food Technology and Storage Agricultural Academy, Lublin. Cultures were maintained for four and six days on Saunders medium [19] on a rotary shaker. The obtained postculture filtrate was concentrated to one fifth of its volume and enzymatic protein precipitated with methanol, ethanol, acetone and ammonium sulfate as follows:

1. methanol was used to precipitate protein over 6 h at –8°C at 3:1 methanol-to concentrated fluid ratio;
2. cellulases were educed with 96% ethanol over 1 h at -4°C at 2.5:1 alcohol-to-postculture filtrate ratio;
3. cellulases were also precipitated with acetone over 1 h at -10°C (3:1 acetone-to-postculture filtrate ratio);
4. enzymatic protein was precipitated with ammonium sulfate at +20°C by 50% salting out of the postculture filtrate.

After centrifugation, the separated enzymatic protein was dissolved in acetate buffer of pH 5.5 and used in enzymatic hydrolysis of the following substrates: Whatman 1 chromatographic paper, filter paper partially digested with 85% orthophosphoric acid, carboxymethylcellulose sodium salt (CMC Na), rye straw, and deciduous tree sawdust. The latter two substrates were comminuted and passed through a sieve (0.05 mm mesh). Next, 1% suspensions were prepared in 0.05 M citrate-sodium buffer (pH 5.5).

Results of enzymatic hydrolysis were expressed as mg of reducing sugars obtained during 1 h of hydrolysis by 1 cm^3 of concentrated postculture fluid. For this reason, reducing sugars content was determined by the Somogyi-Nelson method [20]. Specific activity of the studied cellulolytic preparations was gauged by determining their protein content by Lowry's method [11].

Thin layer chromatography was used to identify hydrolysis products appearing during the treatment of the various substrates with cellulolytic preparations obtained with various solvents and ammonium sulfate from the postculture solution. Plates coated with silica gel (Kieselgel G) were twice developed in a 1:1:2 isopropanol-water-ethyl acetate system for 1 h over a 12 cm path. Stains on the plates were developed with 50% sulfuric acid solution and the separated saccharides determined quantitatively with a densitometer [21].

Also determined was aryl-β-glucosidase activity after four and six days of *Fusarium sp.* culture [22].

Cellulolytic activities were determined according to Mandels et al. [12].

RESULTS AND DISCUSSION

Figures for cellulolytic and specific activities of enzymatic preparations precipitated with ethanol, methanol, acetone and ammonium sulfate in the case of orthophosphoric acid-treated cellulose as substrate are presented in Table 1.

Table 1. Enzymatic activity of cellulolytic preparations in filter paper pretreated with orthophosphoric acid

Enzymatic protein precipitated with	Total activity*		Specific activity**	
	culture duration (days)			
	4	6	4	6
Ethanol	17.8	23.3	13.3	8.3
Methanol	16.8	19.5	16.5	11.3
Acetone	15.2	17.5	18.6	13.2
Ammonium sulfate	15.0	15.5	20.0	20.7

*Expressed as mg reducing sugars/cm³ postculture fluid/h
** Expressed as mg reducing sugars/mg protein

The highest activity was demonstrated by ethanol-precipitated preparations from both the four- and the six-days cultures. The cellulolytic activity of enzymatic preparations in the medium increased with the increase of fungus culture duration, the exception here being the preparation salted out with ammonium sulfate, whose total activity was the same after four and six days of culture. The highest specific activity expressed as mg of reducing sugars liberated from the substrate during 1 h by 1 mg of protein was exhibited by preparation precipitated with ammonium sulfate.

Table 2. Products of enzymatic decomposition of cellulose of paper pretreated with orthophosphoric acid

Enzymatic protein precipitated with	Cellobiose content (mg/cm³)		Glucose content (mg/cm³)	
	culture duration (days)			
	4	6	4	6
Ethanol	11.5	8.5	4.8	13.4
Methanol	12.6	5.0	3.3	13.0
Acetone	11.0	5.5	2.9	10.5
Ammonium sulfate	12.5	4.5	2.0	9.5

Thin layer chromatography revealed the presence of cellobiose and glucose among products of hydrolysis of filter paper pretreated with 85% orthophosphoric acid. In the case of enzymatic preparations from four-day fungus cultures the content of cellobiose was about three times that of glucose, while in preparations from six-day cultures there was about twice more glucose than cellobiose (Table 2).

The highest cellulolytic activity in the case of carboxymethylcellulose sodium salt (CMC Na) was displayed by preparations precipitated by ethanol from both the four- and six-day culture filtrates, while the highest specific activity was exhibited by ammonium sulfate-precipitated preparations (Table 3). Hydrolyzates obtained as a result of treatment with preparations from four-day cultures contained about 2.5 times more cellobiose than glucose, while those due to preparations from six-day cultures had about two times more glucose than cellobiose (Table 4).

Table 3. Results of hydrolysis of CMC Na by preparations isolated from filtrates with various methods

Enzymatic protein precipitated with	Total activity *		Specific activity **	
	culture duration (days)			
	4	6	4	6
Ethanol	15.0	25.0	11.2	8.9
Methanol	13.2	15.8	12.9	9.0
Acetone	12.1	12.0	14.8	9.1
Ammonium sulfate	11.8	9.6	15.7	12.8

* Expressed as mg reducing sugars/cm³ postculture fluid/h
** Expressed as mg reducing sugars/mg protein

Table 4. Products of enzymatic decomposition of CMC Na by various preparations

Enzymatic protein precipitated with	Content (mg/cm³) of		Proportion (per cent of reducing sugars) of	
	cellobiose	glucose	cellobiose	glucose
	Four-day culture			
Ethanol	9.7	4.2	69.8	30.2
Methanol	7.9	4.3	64.7	35.3
Acetone	8.7	2.3	79.0	21.0
Ammonium sulfate	7.7	2.6	74.7	25.3
	Six-day culture			
Ethanol	7.1	12.0	37.2	62.8
Methanol	4.2	10.8	28.0	72.0
Acetone	3.7	7.0	34.6	65.4
Ammonium sulfate	2.8	6.7	29.5	70.5

Maximum specific activity in the case Whatman 1 chromatographic paper as substrate was displayed by preparations precipitated with ammonium sulfate from four- and six-day postculture filtrates (Table 5). As in the hydrolyzates of the previous substrates about 2.5 times more cellobiose than glucose was obtained by treatment with preparations form the four-day cultures, and about 2.5 times more glucose than cellobiose when hydrolysis was done with preparations from six-day cultures (Table 6).

Table 5. Enzymatic activity of cellulolytic preparations in Whatman 1 chromatographic paper

Enzymatic protein precipitated with	Total activity *		Specific activity **	
	culture duration (days)			
	4	6	4	6
Ethanol	12.1	9.4	9.0	3.4
Methanol	12.0	5.0	11.7	2.8
Acetone	10.5	4.8	12.8	3.6
Ammonium sulfate	10.4	3.4	13.8	4.6

* Expressed as mg reducing sugars/cm³ postculture fluid/h
** Expressed as mg reducing sugars/mg protein

Table 6. Enzymatic products of hydrolysis of cellulose in Whatman 1 chromatographic paper by various preparations

Enzymatic protein precipitated with	Content (mg/cm³) of		Proportion (per cent of reducing sugars) of	
	cellobiose	glucose	cellobiose	glucose
	Four-day culture			
Ethanol	7.8	3.4	69.6	30.4
Methanol	7.2	3.9	64.9	35.1
Acetone	7.6	2.0	79.2	20.8
Ammonium sulfate	6.8	2.3	74.7	25.3
	Six-day culture			
Ethanol	3.5	4.6	43.2	56.8
Methanol	1.3	2.9	30.9	69.1
Acetone	0.9	2.4	27.3	72.7
Ammonium sulfate	0.7	2.4	22.6	77.4

Cellulose in rye straw and deciduous tree sawdust was best hydrolyzed by the preparation precipitated with ethanol from the six-day culture filtrate, although the hydrolysis yield in this case was lower than in the case of paper and CMC. The hydrolysis of sawdust was particularly slow and ineffective, this being due to the

specific structure of wood [23, 25]. The highest specific activity was exhibited by enzymatic preparations precipitated with ammonium sulfate and acetone (Table 7 and 8). Saccharides content was not determined in the hydrolyzates given the poor results of hydrolysis of the straw and sawdust which were not treated prior to treatment with enzymes.

Table 7. Activity of cellulases of various preparations in rye straw

Enzymatic protein precipitated with	Cellulolytic activity *	Specific activity **
	Four-day culture	
Ethanol	1.18	0.76
Methanol	1.09	1.07
Acetone	1.00	1.21
Ammonium sulfate	0.95	1.30
	Six-day culture	
Ethanol	1.60	0.60
Methanol	1.37	0.78
Acetone	1.26	0.96
Ammonium sulfate	0.90	1.20

* Expressed as mg reducing sugars/cm³ concentrated postculture fluid/h
** Expressed as mg reducing sugars/mg protein

Table 8. Enzymatic activity of various cellulolytic preparations in deciduous tree sawdust

Enzymatic protein precipitated with	Cellulolytic activity *	Specific activity **
	Four-day culture	
Ethanol	0.72	0.53
Methanol	0.61	0.60
Acetone	0.53	0.64
Ammonium sulfate	0.50	0.66
	Six-day culture	
Ethanol	0.81	0.29
Methanol	0.75	0.43
Acetone	0.69	0.52
Ammonium sulfate	0.45	0.60

* Expressed as mg reducing sugars/cm³ concentrated postculture fluid/h
** Expressed as mg reducing sugars/mg protein

The activity of aryl-β-glucosidase in preparations precipitated with ethanol, methanol and acetone from four-day culture filtrates was similar (Table 9), differences appearing only in preparations from six-day culture filtrates. Most

active were preparations educed with ethanol and acetone. The activity of β-glucosidase of preparations precipitated with ammonium sulfate was lowest regardless of culture duration.

Table 9. Activity of aryl-β-glucosidase of various cellulolytic preparations from various stages of *Fusarium sp.* culture

Protein precipitated with	β-glucosidase activity *	
	four-day culture	six-day culture
Ethanol	4.13	11.39
Methanol	4.50	6.88
Acetone	4.75	11.13
Ammonium sulfate	1.75	2.18

* Expressed as mg reducing sugars/cm³ of concentrated postculture fluid/h

Comparisons of results of enzymatic hydrolysis of substrates such as filter paper treated with orthophosphoric acid, carboxymethylcellulose sodium salt, Whatman 1 chromatographic paper, rye straw and deciduous tree sawdust show that the largest amounts of reducing substances were obtained during enzymatic hydrolysis of cellulose treated with phosphoric acid. The substrates least susceptible to the action of cellulolytic enzymes were straw and sawdust which were only comminuted and not subjected to any chemical pretreatment. The remaining substrates were either pure cellulose or materials suitably prepared by treatment with 85% H_3PO_4 and these turned out to be more susceptible to enzymes contained in cellulolytic preparations. According to a number of publications [6, 24], the susceptibility of lignocellulosic structures to enzymatic degradation increases after removal of hemicelluloses following treatment with acid solutions or high temperature, or removal of lignins by treatment with hydrogen peroxide or lye solutions.

Treatment of raw extract with organic solvents or ammonium sulfate leads to enzymatic activity stabilization and also partly purifies the preparations. This is the reason why in the experiments reported here the highest specific activity was displayed by the preparation obtained by salting out with ammonium sulfate (given its small protein content), a slightly lower one by the acetone- and methanol-precipitated preparations, and the lowest by the ethanol-precipitated preparation which, however, boasted the highest total activity. Longinowa et al.[10] also found that cellulases obtained by salting out with ammonium sulfate to 30-50% saturation were more active than ethyl alcohol-precipitated cellulases, but that at the same time the cellobiase activity was lower. Pork and Bezborodov [17] demonstrated that the activity of acetone-precipitated cellulolytic preparations was on the average three times higher than of preparations precipitated with ethanol and methanol. Other authors recommend the use of ethanol denatured from 3% acetone [18] or isopropanol [5] since the preparations they

thus obtained were more active than those obtained by salting out with ammonium sulfate. Odegaard et al. [15] suggest the addition of quaternary butanol to a water solution of enzymes containing ammonium sulfate of 40% saturation. They found exo- and endoglucanases of the *T. reesei* complex on the boundary of solvents phases. Differences in enzymatic activities of preparation obtained by precipitation with organic solvents and ammonium sulfate depended on the kind of culture and technique of precipitation.

Enzymes purification may be greatly improved by ultrafiltration using membranes. To apply this process, however, one needs to know the molecular mass of the enzyme which is to be separated from he other proteins and various accompanying substance in order to correctly select the type of membrane. The conditions of separation must be right, for otherwise there may occur losses of activity, of up to 65%, as a result of destruction of the tertiary structure of enzymatic proteins. In such a case, however, the specific activity of lipolytic enzymes was 2.5 times higher than of the preparation obtained by salting out with ammonium sulfate, and 6.8 times higher than of acetone-precipitated protein [1].

In this research the products of enzymatic hydrolysis were cellobiose and glucose, since the used methods of determinations failed to reveal the presence of other combinations. Quantitative determinations of those saccharides showed that in hydrolyzates produced by enzymatic protein from four-day culture filtrates the amount of cellobiose was about three times that of glucose. In hydrolyzates produced by cellulases from six-day cultures, there was about twice as much glucose than cellobiose.These differences in mutual proportions of glucose and cellobiose are due to the compositon of the enzymatic complex from various stages of *Fusarium sp.* cultures. There were analogous changes in the activity of aryl-β-glucosidase: in preparations from four-day cultures this enzyme was 20-60% lower than in preparations form six-day cultures. As the time of culture increased, part of the mycelium underwent lysis and some of the β-glucosidase connected with the cell wall passed to the solution and then to the enzymatic preparation as a result of enzymes precipitation. Accordingly, the hydrolyzates of cellulosic substrates obtained with preparations from six-day culture filtrates contained more glucose than hydrolyzates due to preparations from four-day cultures [7].

These findings are confirmed by the results of Beldman et al. [2, 3]. Determining products of hydrolysis of Avicel cellulose and H_3PO_4-treated cellulose by endo- and exo-glucanases isolated from a commercial cellulases preparation obtained from *Trichoderma viride*, they identified, using HPLC, mainly cellobiose and glucose, and in several cases cellotriose. As hydrolysis time increased from 2 to 21 h, so did the content of glucose.

CONCLUSIONS

1. The highest total activity was exhibited by ethanol-precipitated cellulolytic preparations, regardless of the kind of substrate.

2. Enzymatic protein precipitated with ammonium sulfate was found to have the highest specific activity in the case of all substrates used in the experiments.

3. Of all the substrates used in this research the one most susceptible to cellulase treatment was filter paper pretreated with orthophosphoric acid.

4. Hydrolysates obtained with preparations precipitated from four-day culture filtrates contained about three times more cellobiose than glucose, while in those obtained with preparations from six-day culture filtrates there was about two times more glucose than cellobiose.

5. Cellulolytic preparations from six-day *Fusarium sp.* cultures contained more β-glucosidase than preparations obtained from four-day cultures of this fungus.

LITERATURE

1. Bednarski W., Kowalewska J., Poznański S.: Postępy Mikrobiologii 1982, **21** (3/4), 171.
2. Beldman G., Voragen A.G.J., Rombouts F.M., Searle-van Leeuwen M.F., Pilnik W.: Biotechnol. Bioengin., 1987, 30, 251.
3. Beldman G., Searle-van Leeuwen M.F., Rombouts F.M., Voragen A.G.J.: Eur. J. Biochem., 1985, 146, 301.
4. Cing-Mars G., Howell J.: Biotechnol. Bioengin., 1977, 19, 377.
5. Galas E., Kubik C., Turkiewicz M., Zielińska M., Florianowicz T.: Przem. Ferment. i Owocowo-Warzywny 1986 (11-12), 20.
6. Gould J.M.: Biotechnol. Bioengin., 1985, 27, 896.
7. Kantham L.: Biotechnol. Bioengin., 1985, 27, 877.
8. Kundu A.B., Ghosh B.S., Ghosh B.L., Ghose S.N.: J. Ferment. Technol., 1983, 61, 185.
9. Levonen-Munoz E., Bone D.H., Daugulis A.J.: Eur. J. Appl. Microbiol. Biotechnol., 1983, 18, 120,
10. Loginowa Ł.G., Guzowa E.P., Ismaiłowa D. Ju., Burdienko Ł.G.: Prikład. Biochim. i Mikrobiol., 1978, **14**, (4), 485.
11. Lowry J.O.H., Rosenbrough N.J., Farr R.L., Randal R.J.: J. Biol. Chem., 1951, 193, 265.
12. Mandels M., Andreotti R., Roche C.: Biotechnol. Bioengin. Symp., 1976, 6, 21.
13. Milstein O., Vered Y., Sharma A., Gressel J., Flowers H.M.: Biotechnol. Bioengin., 1986, 28, 381.
14. Moloney A.P., Coughlan M.P.: Biotechnol. Bioengin., 1983, 25, 271.
15. Odegaard B.H., Anderson P.C., Lovrien R.E.: J. Applied Biochem., 1984, 6, 156.
16. Podgórska E., Bujak S.: Ann. Universitatis M.C.- Skłodowska, Lublin 1982, E, 37, 28.
17. Pork N.J., Biezborodow A.M.: Prikładn. Bioch. Mikrobiol., 1970, 6, 281.
18. Rogalski J., Szczodrak J., Ilczuk Z.: Acta Microbiologica Polonica 1983, 32, 4, 363.
19. Saunders P.R., Siu R.G.H., Genest R. N.: J. Biol. Chem., 1948, 174, 697.
20. Somogyi M.: J. Biol. Chem., 1952, 195, 19.
21. Sheikh S.R., Krischnamurti C.R., Kitts W.D.: J. Chromatography 1968, 38, 400.
22. Targoński Z.: Acta Aliment. Polonica 1984, **10**, (3-4), 301.
23. Targoński Z.: Enzyme Microbiol. Technol., 1985, 7, 126.
24. Targoński Z., Bujak S., Baraniak A.: Acta Microbiol. Polon., 1985, 34, 361.
25. Weimer P.J., Weston W.M.: Biotechnol. Bioengin., 1985, 27, 1540.

Manuscript received: March, 1988
Author address: 20-934 Lublin, Akademicka 13

E. Podgórska

AKTYWNOŚCI PREPARATÓW CELULOLITYCZNYCH OTRZYMANYCH RÓŻNYMI METODAMI

Katedra Technologii Przemysłu Rolno-Spożywczego i Przechowalnictwa.
Akademia Rolnicza, Lublin

Streszczenie

Celem pracy było porównanie wyników działania preparatów celulolitycznych otrzymanych różnymi metodami z filtratu hodowlanego *Fusarium sp.* Hodowle grzyba prowadzono przez 4 i 6 dób w podłożu Saundersa, na wytrząsarce rotacyjnej. Białko enzymatyczne wytrącano z filtratu 5-krotnie zagęszczonego przy użyciu: metanolu, etanolu, acetonu i siarczanu amonu. Wytrącone białko enzymatyczne stosowano do hydrolizy bibuły Whatman 1, filtracyjnej nadtrawionej 85% H_3PO_4, soli sodowej karboksymetylocelulozy, słomy żytniej i trocin z drzew liściastych.

Na podstawie przeprowadzonych oznaczeń stwierdzono, iż najwyższą aktywnością ogólną odznaczały się preparaty celulolityczne wytrącone etanolem, niezależnie od rodzaju użytego substratu. Białko enzymatyczne wytrącone siarczanem amonu wykazywało najwyższą aktywność właściwą wobec użytych w badaniach substratów. Bibuła filtracyjna traktowana uprzednio kw. o-fosforowym była najbardziej podatna na działanie celulaz. W hydrolizatach otrzymanych przy użyciu preparatów wytrąconych z 4-dobowych filtratów hodowlanych znajdowano 3-krotnie więcej celobiozy niż glukozy, natomiast w wyniku hydrolizy preparatami pochodzącymi z filtratów 6-dobowych hodowli uzyskiwano dwa razy więcej glukozy niż celobiozy, gdyż preparaty były bogatsze w β-glukozydazę od preparatów otrzymywanych z 4-dobowych hodowli tego grzyba.