DECOMPOSITION OF CELLULOSE BY THE FUNGUS *CURVULARIA LUNATA* WAKKAR—III: PROPERTIES OF CELLULOLYTIC ENZYME

G.M. VERMA, R. K. VERMA, D. D. SAHGAL AND P. K. VIJAYARAGHAVAN*
Defence Research Laboratory (Stores), Kanpur

ABSTRACT

THE various properties of cellulolytic enzyme system elaborated by the fungus *Curvularia lunata* have been studied from the point of view of finding out the mechanism of cellulose decomposition by this fungus. Evidence of the possibility of presence of —SH group in the enzyme molecule has been obtained. An *inter se* independent multienzymes system has been found to be elaborated by this fungus.

INTRODUCTION

Previous two communications[1,2] dealt with preliminary studies relating to the optimum activity of the cellulolytic enzyme, elaborated by the fungus *Curvularia lunata* Wakker and the nutritional requirements from the point of view of secretion of active cellulolytic enzyme[2]. This paper deals with purification, stimulation and inhibition of the enzymatic system and identification of end products of cellulose decomposition. The purpose of this investigation was to understand the mechanism of decomposition of cellulose by the fungus *Curvularia lunata* Wakker.

EXPERIMENTAL PROCEDURE

Culture medium—Modified Omeliansky's medium[3] containing ammonium chloride in place of ammonium sulphate was used in these studies. Cellulose substrate used was Cellofas B (i.e., sodium salt of carboxymethyl cellulose with degree of substitution 0·45 to 0·55).

Assessment of enzyme activity—The details of preperation of medium, inoculation, separation of extracellular enzyme and assessment of enzymatic activity were essentially the same as given in previous communications [1,2]. Besides reducing sugar estimation, fall in viscosity of solution of Cellofas B by viscometer method according to Thomas[4] and hydrolysis of celluabiose into glucose were also used for the assessment of cellulolytic activity of the enzyme.

Effect of the enzyme on cotton dosootie was assessed *in vitro* as well as *in vivo* by the following methods:

(*a*) *in vitro*—Four dosootie pieces 3″×11″ each were incubated with 375 ml of metabolic liquor obtained from a two week old culture of the fungus, 125 ml of citrate buffer pH 5·2 and 140 ml of 0·10 cysteine solution at 37°C for 96 hours. A control experiment was also carried out with another set of four dosootie pieces incubated with medium cysteine and buffer (in the same ratio v/v). Dosootie pieces were then washed in running distilled water for 10 to 15 minutes and their breaking strength was determined. After drying, samples were conditioned at

* Now at Defence Food Research Laboratory, Mysore.

65-70% R.H. and 70°F for 48 hours and were broken warpwise 2″×7″ between grips in a Goodbrand Horizontal Cloth Testing machine, in which the test piece was stretched at a constant rate of 18″ per minute.

(*b*) *in vivo*—Twelve one litre flasks containing 3″×11″ dosootie pieces, each supported on inclined glass rods in modified Omeliansky's medium containing 0·1% cysteine solution in buffer pH 5·2, were sterilized at 15 lbs/sq. in. steam pressure for 20 minutes. Six out of these were inoculated with the fungus. The pieces were removed after 2 weeks' growth and washed in running distilled water for 15 minutes. The breaking strength was determined in the same way as described under (*a*) above.

For the measurements of fall in viscosity of solution of Cellofas B, 5 ml of metabolic liquor at different periods of growth was incubated with 5 ml of buffer pH 5·0 and 5 ml of 1·5% Cellofas B at 30°C in viscometer No. 1. The time of flow was noted and sepecific viscosity calculated.

Effect of ion-exchange resins—25 ml. of the metabolic liquor was passed through a 25 cm. long column of cation exchange resin Zeocarb 225 and then through anion exchange resin Deacidite FF at the rate of ten drops/min. The volume of the collected liquor was made to 50 ml by addition of sterile distilled water. Solid content in both the treated and untreated metabolic liquors was determined by drying these at 80°C under slight vacuum to constant weight.

End products of cellulose decomposition were identified by circular paper chromatography. Irrigation solvent used was pyridine-water—n. butanol in the ratio of 1·0 : 1·5 : 3·0 by volume. Aniline hydrogen phthalate solution in water saturated n-butanol was used as developer.

RESULTS

Stimulation of the enzyme

(*a*) *By Cysteine*—The effect of addition of small amounts of cysteine to the metabolic liquor on the cellulase activity was studied. The results are given in Table I below:

TABLE 1

STIMULATION OF ENZYME BY CYSTEINE

No.	Concentration of cysteine (mgm per 10 ml of enzyme extract)	Cellulase activity (% hydrolysis of cellulose)	
		cellulose powder	cuprammonium cellulose
1	0	6·8	3·0
2	2	11·3	6·2
3	4	27·0	16·3
4	6	6·5	5·6
5	8	5·0	4·8

The above results show that the addition of cysteine leads to considerable activation of the enzyme, the maximum effect being at 4 mgm/10 ml of metabolic liquor. Higher concentrations of cysteine do not seem to have any appreciable effect.

(*b*) *By different substrates*—Comparative efficiency of cellulose acetates with different degrees of substitution, ethyl methyl ether of cellulose (Cellofas A), Cellofas B and cellulose powder (obtained from little paper) as substrates was studied by incubating the metabolic liquor with these substates at pH 5·0 for 96 hours. Reducing sugars were assessed at the end of incubation. The results are summarised in Table 2.

TABLE 2

STIMULATION OF ENZYME BY DIFFERENT SUBSTRATES

Expt. No.		Substrate for enzyme activity	Cellulase activity (% hydrolysis of cellulose): Period of growth in days					
			6	10	12	15	19	22
1.	(*i*)	Cellulose acetate (acetic acid yield 18·3%).	1·6	0·49	0·95	0·58	0·66	0·54
	(*ii*)	Cellulose acetate (ascetic acid yield 34·6%).	1·7	0·93	0·85	0·53	0·61	0·15
	(*iii*)	Cellulose acetate (acetic acid yield 48·4%).	2·0	0·56	0·93	0·72	0·47	0·15
	(*iv*)	Cellofas B	4·0	5·80	11·20	10·6	4·7	2·8
2.	(*i*)	Cellulose powder	1·0	1·3	1·6	1·5	1·6	3·3
	(*ii*)	Hydrocellulose	1·5	1·0	1·4	2·0	1·2	2·3
	(*iii*)	Cellofas A	3·6	3·8	5·7	2·4	4·3	4·8
	(*iv*)	Cellofas B	6·4	5·4	11·0	10·2	6·5	8·0

It is evident from the data given above that the use of Cellofas B results in higher rate of hydrolysis than the use of other substrates. In subsequent experiments Cellofas B has been used as a substrate in place of cuprammonium cellulose.

Effect of cellulolytic enzyme on cotton dosootie—The loss in breaking strength of the fabric during *in vitro* and *in vivo* studies was determined. The results are given in Tables 3 and 4 :

TABLE 3

EFFECT OF CELLULOLYTIC ENZYME ON DOSOOTIE—*in Uitro*

No.	Breaking strength (pounds)		% Loss in breaking strength
	Control	Experimental	
1	170	165	
2	165	165	
3	160	155	
4	160	145	
Average	163	158	3

TABLE 4

EFFECT OF CELLULOLYTIC ENZYME ON DOSOOTIE—*in vivo*

No.	Breaking strength (pound)		% loss in breaking strength
	Control	Experimental	
1	170	105	
2	170	85	
3	170	85	
4	175	100	
5	175	95	
Average	173	90	48

It is clear from the results that *in vivo* cellulolytic activity is greater than the *in vitro* one. In the latter it is practically negligible.

Effect of enzyme on the fall in viscosity of Cellofas B—The percentage fall in specific viscosity of Cellofas B at different periods of incubation with metabolic liquor obtained at various periods of growth is given in Table 5:

TABLE 5

EFFECT OF ENZYME ON THE FALL IN VISCOSITY OF CMC

No.	Age of culture (days)	% fall in viscosity in 5 hours	
		Control (autoclaved enzyme)	Experimental
1	6	6·0	20·0
2	10	5·8	31·4
3	12	6·4	33·9
4	15	4·6	28·1
5	19	9·8	28·0
6	22	9·2	23·5

Results show that the maximum fall in viscosity is observed with metabolic liquor obtained at about 10—15 days of growth.

Effect of ion exchange resin treatment on enzyme activity—Equivalent quantities of treated and untreated metabolic liquors were incubated with substrate and buffer and the reducing sugars produced were estimated after 96 hours incubation at 37°C. The results are given in Table 6:

TABLE 6

PURIFICATION OF THE ENZYME BY ION-EXCHANGE RESINS

Nature of enzyme	Cellulase activity (% hydrolysis of cellulose)	Total solids gms/100 ml. of soln.
Metabolic liquor (as such) ..	3·2	2·35
Metabolic liquor after passing through resins.	5·2	0·12

The above results show that the activity of the enzyme has increased considerably after passing the metabolic liquor through the ion-exchange resins.

End products of cellulose decomposition—Free reducing sugars and the cellulolytic activity were determined in the metabolic liquor after 15 days' growth (*i*) in the beginning, (*ii*) after storage at 5°C for 10 days and one month. The results are given in Table 7.

TABLE 7

CHROMATOGRAPHIC IDENTIFICATION OF SUGARS IN THE METABOLIC LIQUOR

Period of storage of extract at 5° c (days)	Free reducing sugars/ 5ml. expressed as glucose	Cellulase activity (% hydrolysis of cellulose)		Nature of chromatogram of the extract	
		per 5 ml	per 20 ml	No. of rings	Rf values
0	0·27	15·8	19·2	2	·44 ·35
10	0·27	9·2	13·1	2	·44 ·35
30	0·27	2·5	5·0	—	—

It may be observed that the extract contains two reducing sugars. Table 8 below gives the Rf values for some of the known sugars chromatographed under similar conditions.

TABLE 8.

RF VALUES OF VARIOUS KNOWN SUGARS

No.	Sugars	Rf values
1	Xylose	·52
2	Glucose	·45
3	Galactose	·41
4	Cellobiose	·37
5	Cellotriose	·23
6	Cellotetraose	·18
7	Cellohexaose	·09

It is evident that the two sugars indentified are glucose and cellobiose.

Effect of inhibitors on cellulase activity—(*a*) *Diethyl malonate*—The metabolic liquor was incubated with substrate, buffer and different concentrations of diethyl malonate (up to the extent of 2·0% i.e., saturation limit). The amount of reducing sugars produced was assessed. The results are given in Table 9;

TABLE 9

INHIBITION OF ENZYME BY DIETHYL MALONATE

Concentration of diethyl malonate(%)	Cellulase activity (% hydrolysis of cellulose)		Cellobiase activity (% hydrolysis of cellobiose)	
	Expt. I	Expt. II	Expt. I	Expt. II
0·0	16·1	11·3	15·0	9·2
0·5	8·2		27·4	14·1
1·0	5·4	..	26·0	14·0
1·5	3·8	6·1	25·3	12·3
2·0	1·3	1·4	20·7	9·5

The results show that there is a gradual decrease in cellulase activity with increasing concentration of the ester whereas the cellobiase activity increases (particularly up to 1·0% concentration of the ester).

(*b*) *Inorganic Inhibitors*—The metabolic liquor obtained after 12 days' growth of the fungus was incubated with Cellofas B, buffer and different concentrations of the following inorganic inhibitors:—

(*a*) Potassium cyanide (KCN)

(*b*) Mercuric chloride ($HgCl_2$)

(*c*) Copper sulphate ($CuSO_4.5H_2O$

(*d*) Sodium fluoride (NaF)

The enzyme activity was assessed by (*i*) measuring the amount of reducing sugars produced from Cellofas B, (*ii*) determining the reducing sugars produced from cellobiose and (*iii*) measuring the fall in viscosity of Cellofas B. The results are given in Table 10.

Table 10

Inhibition of enzymes by inorganic inhibitors

Inhibitor	Concentration of inhibitor (%)	Cellolose activity (% hydrolysis of Cellulose)		Cellobiase activity (% hydrolysis of cellobiose)		% fall viscosity in 4½ hours	
		Expt. I	Expt. II	Expt. I	Expt. II	Expt. I	Expt. II
KCN	0·0	13·1	8·3	43·5	26·0	8·8	37·0
	0·005	11·8	7·6	40·3	18·0	9·5	35·8
	0·05	10·8	6·5	22·3	14·3	8·7 (2·1)	35·8 (2·0)
$HgCl_2$	0·0	13·1	8·3	43·5	26·0	14·5	9·5
	0·005	9·2	7·7	8·9	24·3	12·0	9·0
	0·05	0·7	3·0	28·5	16·7	10·2 (1·6)	7·1 (3·2)
$CuSO_4 \cdot 5\ H_2O$ ..	0·0	8·8	10·0	21·0	28·5	27·4	37·3
	0·005	9·5	10·9	21·3	28·0	22·3	34·6
	0·05	11·6	12·7	17·3	27·5	26·0 (1·4)	35·5 (20·3)
NaF	0·0	8·8	10·0	21·0	28·5	21·3	27·6
	0·005	10·1	13·6	22·5	24·0	22·3	24·3
	0·05	4·1	8·3	25·7	32·0	22·0 (nil)	26·6 (nil)

N.B.—The viscosity figures within brackets are those for the control obtained with the autoclaved metabolic liquor.

The results in Table 10 show that the action of inhibitor varies with the type of reaction involved.

DISCUSSION

Stimulation of the enzyme by cysteine—The addition of cysteine in small quantities (0·04% w/v) increases the enzyme activity about fourfold though further increase does not affect much.

The use of cysteine as an activator is well known. Sulphydryl groups have been known to be essential for the action of many enzymes. Inactivation by oxidants and reactivation by reductants such as cysteine and reduced glutathione may in many cases be due to oxidation of —SH groups or reduction of —S—S— linkage. The cellulolytic enzyme from Helix[5] however, has been reported to be inhibited by cysteine.

Effect of substitution of cellulose on cellulolytic enzyme—Whereas cellulose acetate at various degrees of substitution resists the hydrolysis by the enzyme, ethyl methyl ether (Cell of as A) and particularly sodium carboxymethyl derivative (Cellofas B) stimulated greater cellulolytic activity than to be cellulosic substrates such as hydrocellulose and cellulose powder. CMC has also been reported to be a good substrate for the cellulolytic enzymes from other organisms.[4,6] Cellulose triacetate has been found to be resistant to *in vivo* decomposition by various cellulose destroying fungi by Burkholder *et al* and by Siu *et al*[7], *8*.

Effect on breaking strength of dosootie—Though the enzyme is active on sodium salt of carboxymethyl cellulose, the effect seems to be negligible on cotton dosootie. Similar results have been reported by Thomas[4] and other workers in the field.[9,10] *In vivo* studies, however, show that the fungus does possess a high cellulolytic capacity.

Fall in viscosity of CMC (sodium salt)—The enzyme is quite active as a liquefier. This reaction may be analogous to the liquefying factor in the diastase.[11] The fall in viscosity is much more in early stage of enzymatic digestion than the production of reducing sugars — Whereas the fall in viscosity of Cellofas B in about 5 hours *is as* much as 20—25%, the reducing sugars produced by the enzyme on cuprammonium cellulose in the same period is almost negligible[1]. Moreover, the elaboration of enzyme as measured by fall in viscosity of Cellofas B is maximum at about 12—15 days growth whereas that of the enzyme as measured by production of reducing sugars from cuprammonium cellulose it is maximum at about 15—20 days of growth of the fungus[11]. These facts indicate that fall in viscosity width is a measure of fall in degree of polymerisation[12] of cellulose molecule precedes liberation of glucose from the chain.

Thomas[4] and Levinson and Reese[13] have previously reported that the viscosity of CMC falls as a result of cellulolytic action of the enzyme.

Effect of treatment of metabolic liquor with ion-exchange resins—Whereas te earlier efforts to purify the enzyme were unsuccessful, the treatment with ion-exchange resins increased the activity about thirty-fold for the same solid content. Obviously the mineral constituents have been removed completely by this method. Also it shows that the enzyme molecule does not depend upon nor does it include any metallic ions for its cellulolytic activity. The stimulatory effect of Mg^{2} on elaboration of enzyme by the fungus, therefore, indicates a stimulation *in vivo*. If a metal is at all a part of the enzyme molecule, it must be in the unionisable form.

End products of cellulose hydrolysis—The chromatographic analysis shows that the metabolic liquor seems to contain both glucose and cellobiose. This has been widely reported in the literature[4,14] Saunders *et al*,[15] however, did not find cellobiase in the metabolic filtrate from *Myrothecium verrucaria*.

Inhibition studies—Diethyl malonate inhibits the enzyme activity on cellulose but not on cellobiose. The similar results with respect to cellulase have been reported by Misra and Vijayaraghavan[16] on termite gut enzyme. Malonic acid is known to be a competitive inhibitor[17] for succinic dehydrogenase system but its role as an inhibitor for cellulolytic enzyme is not clear.

For the inorganic inhibitors the behaviour of each of these is different for the three reactions i.e.

(*a*) hydrolysis of Cellofas B to reducing sugars,

(*b*) fall in viscosity of Cellofas B

and (*c*) hydrolysis of cellobiose to glucose.

Mercuric chloride inhibited the cellulase activity (reaction a) completely. This has also been found by Grassmann *et al*[18] on cellulase from *Aspergillus oryzae*. The partial inhibition is observed on cellobiase activity, (reaction c) whereas inhibition of the 'liquefying' factor (reaction b) is not observed. Similarly potassium cyanide does not inhibit any reaction excepting the cellobiose hydrolysis (reaction c).

Copper sulphate usually known to be an excellent inhibitor for various fungal enzymes[19] has apparently no inhibitory action on any of the three reactions. This may mean the absence of —SH group as an essential part of the enzyme molecule.

Sodium fluoride does not affect viscosity of Cellofas B or the cellobiase activity but affects the hydrolysis of Cellofas B to reducing sugars. All these inorganic inhibitors were found by Thomas[4] to be active inhibitors for the enzyme from *Stachybotrys atra*.

Mechanism of cellulolytic degradation—It has been observed that in the initial stages of enzyme action (first 8 hours) there is little formation of reducing sugar.[1] During this period, however, the enzyme action leads to an appreciable fall in the viscosity of CMC (often amounting to 25% fall). It would appear that the initial enzyme action is confined to transforming the cellulosic substrate to an easily hydrolysable form. This may possibly involve the removal of groups that bind neighbouring cellulose chains together. That such groups may be present in Cellofas A and Cellofas B is borne out by the observation of Chamberlain and Khera[20] that the cross linkages in the native cellulose are intact in the rayon filaments. If the assumption[14] that CMC molecule is devoid of cross linkages present in the native cellulose is taken into consideration, it can be easily stated from the above data that primary action of the enzyme is such as to cause cleavage in the cellulose molecule at about the middle of the chain so that whereas the degree of polymerisation is halved, the amount of reducing sugars produced is nil.

Further hydrolysis to glucose has been reported[21, 22] to take place through the intermediate formation of cellobiose, though this has never been above controversy. Some light is thrown on this hypothesis by the observations obtained during studies on enzyme inhibition given above. Mercuric chloride inhibits the cellobiase activity only partially but the hydrolysis of CMC is almost completely inhibited. Further, potassium cyanide

inhibits the cellobiase activity partially but not the cellulase activity. It would, therefore, appear that the enzyme complex taking part in the assimilation of cellulose by the fungus has at least three components, i.e.,

(*i*) which causes 'liquefaction' or initial reduction in the viscosity and fall in degree of polymerisation of cellulose molecule,

(*ii*) which hydrolyses the reduced chain to reducing sugars, and

(*iii*) which breaks up cellobiose to glucose.

These three components may be independent from each other for their respective functions. The results indicate the independence of component (*ii*) from the component (*iii*) though the evidence is not conclusive. More work on this aspect with other cellulolytic fungi is in hand.

A similar conclusion from other considerations have been drawn by Levinson *et al*[23] and other workers[24,25 26].

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