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STUDIES ON SOME PROPERTIES OF *IN-VITRO* CELLULOLYTIC ENZYMES OF *HELMINTHOSPORIUM APATTARNAE* DESH. & DESH.

by M. P. GHEWANDE* *and* K. B. DESHPANDE,

Plant Pathology Laboratory, Botany Department, Marathwada University, Aurangabad 431 004

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An attempt has been made to study some properties of *in-vitro* cellulolytic enzymes (C_X and C_2) of *Helminthosporium apattarnae* Desh. & Desh. The optimum *p*H for C_X and C_2 enzyme was found to be at 4·6 and 7·6 respectively. Temperature optimum for both the enzymes was 25°C. The C_X enzyme was more thermosensitive than C_2 enzyme. In both cases the activity was not lost completely even after heating for 5 min at 75°C. There was gradual reduction in C_X activity with increasing dilutions while C_2 activity was not reduced at 40 % dilutions. The activity of C_X and C_2 enzyme was decreased during dialysis by 5 times and 2 times respectively over the control. The activity of both the enzymes was neither restored by addition of any salt nor by adding autoclaved crude enzyme preparations. C_X activity was comparable with control when acetone and ethanol were used as precipitants, while it was reduced in case of lead acetate. As regards C_2 enzyme activity, it was reduced in case of ethanol and lead acetate, and was comparable with control when acetone was used.

INTRODUCTION

Microbiological decomposition of cellulosic materials takes place through a family of enzymes collectively called cellulases. The pathogen—*Helminthosporium apattarnae* Desh. & Desh. isolated from severe leaf spot of *Cynodon dactylon* Pers. has been observed to decompose cellulosic substrates (cotton, filter paper, cellulose powder and carboxymethyl cellulose — CMC) in culture and produced active cellulolytic enzymes (Ghewande, 1973). The present trend of investigation appears to be confined largely to the study of production of cellulases by different fungi. Although studies on production of cellulases by a few species of *Helminthosporium* have been carried out by some workers (Gilligan & Reese, 1954; Flannigan 1970; Vidhyasekaran, Parambaramani & Govindswamy, 1971), no attempt has been yet made to study properties of cellulase enzymes of fungi in general and of *Helminthosporium* in particular. In view of these facts and of the economic importance of cellulolytic enzymes, it was thought fit to investigate some properties of *in-vitro* cellulases (C_X and C_2) of *H. apattarnae*.

**Present Address:* Scientist (Plant Pathology), Directorate of Oilseeds Research (I.C.A.R.), Rajendranagar, Hyderabad—500 030.

Materials and Methods

The pathogen—*Helminthosporium apattarnae* Desh. & Desh. isolated from leaf spot of *Cynodon dactylon* Pers. was cultivated in the medium containing cellulose powder, 1 %; $Ca(NO_3)_2$, 0.25 %, KH_2PO_4; 0.1 %; $MgSO_4.7H_2O$, 0.05%. Twenty-five ml aliquot of the medium was taken in 250 ml Erlenmeyer conical flasks, autoclaved at 15 lbs/inch^2 for 20 min and inoculated with 5 drops of spore suspension (98 spores/ml medium) prepared from 6-day old potato dextrose agar slope cultures by adding 10 ml of sterile distilled water. The fungus was allowed to grow for 10 days at 25±1°C. Later the contents of the flasks were filtered and the filtrate was centrifuged at 5,000 rpm for 10 min to make it cell free. The *p*H of the supernatant was noted by a *p*H meter and it was then used as crude enzyme solution to assay cellulase (C_x and C_2) activity by estimating reduction in viscosity of carboxymethyl cellulose (CMC—0.5 %) and loss of coherence of filter paper discs respectively (Ghewande & Deshpande, 1976).

Results and Discussion

Effect of p*H*

The substrate and culture filtrate were adjusted to different *p*H values by adding N/10 HCl or NaOH. It was then assayed for cellulase activity and results are presented in Table I.

Table I

Effect of p*H on cellulase (C_x and C_2) activity*

*p*H range	Cellulase activity: % viscosity loss after (min) 5	10	20	30	R. T. (min)
2·6	40	41	43	43	35
3·6	31	41	48	53	38
4·6	37	52	65	71	29
5·6	33	48	59	67	18
6·6	32	44	56	62	17
7·6	12	23	39	44	16
8·6	16	22	34	40	20
9·6	11	20	28	37	23

The data clearly indicate that the optimum *p*H for viscosity reducing cellulase (C_x) lies at *p*H 4.6. In the case of filter paper macerating cellulase (C_2), the optimum *p*H appears to be 7.6. Results similar to those obtained in this work regarding C_x enzyme were reported for *Aspergillus fumigatus* and *A. niger* by Loginova & Tashpulatov (1965), for *Trichoderma koningi* by Toyama (1953) and for *Diplodia zeae* by BeMiller, Tegtoneier & Pappelis (1968). On the contrary, Whitney, Chapman & Heale (1969) observed three components of *Verticillium albo-atrum* cellulase, i.e. A, B and C, showing activity over a wide range of *p*H, A and B had

two optima, A at *p*H 4.6 and 7.5, and B at *p*H 5.0 and above 8.0, whereas C had a single optimum at *p*H 8.0. However, Nehemiah (1976) reported *p*H 7.6 as optimum for three components of cellulase (C_x, C_2 and C_1) enzyme of *Alternaria brassicae*. Sampathnarayanan & Shanmugasundaram (1970) also reported two *p*H optima, i.e. 5.4-6.0, 8.0-8.4 for *Fusarium vasinfectum* cellulase activity. Although not reported, possibly they were dealing with two components of cellulase complex of *F. vasinfectum*.

Effect of Heat Treatment

Ten ml aliquots of the crude culture filtrates were heated in test tubes kept in the water bath for 5 min at different temperatures ranging from 25-75°C. Test tubes were supported in the waterbath at a particular temperature before adding the culture filtrate. A thermometer was placed inside the tube. The time required for the culture filtrate to reach a particular temperature was also noted. After treatment, each sample was assayed for cellulase activity at optimum *p*H. Results are given in Table II.

TABLE II

Effect of heat treatment on cellulase (C_x and C_2) activity at optimum p*H*

Temperature	Cellulase activity % viscosity loss after (min) at *p*H 4·6R.				T. (min) at *p*H 7·6
°C	5	10	20	30	
25	37	52	65	71	16
35	8	10	14	17	31
45	8	10	14	15	47
55	0	5	5	5	48
65	0	5	5	3	50
75	0	0	3	3	54
Autoclaved enzyme	—	—	—	—	—

— = No activity

The results indicated that the temperature optimum for activity of both the enzymes lies at 25°C. There was steep loss in C_x enzyme activity beyond 25°C, and thereafter gradual loss of enzyme activity occurred. Even at 75°C slight activity (C_x) was exhibited. Autoclaved enzyme solution did not show any activity. Similar results regarding optimum temperature for C_x enzyme were also obtained for *Fusarium lateritium f. cajani* by Singh (1968), for *Rhizoctonia solani* by Bateman (1964), for *Aspergillus, quuidrilineatus, A. nidulans, A. flavus, A. variecolor* and *A. niger* by Prasad and Bilgrami (1963) and for *Alternaria brassicae* cellulase (C_1, C_x and C_2) by Nehemiah (1976). However, different results regarding cellulase in general are reported by Akai (1951), Thomas (1956), Jothianandan and Shanmugasundaram (1968), BeMiller *et al.* (1968), Toyama *et al.* (1970) and Jensen (1971)

for *Helminthosporium oryzae* 35°C), *Trichoderma koningi* (40°C), *Piricularia oryzae* (35° and 45°C), *Diplodia zeae* (45°C), *A. niger* (60°C) and *Stereum gausapatum* (50°C) respectively.

Effect of dilution

Different quantities of distilled water were added to the active crude enzyme solution to give a series of dilutions with concentration of active enzyme ranging from 100, 80, 60, 40, 20 and 10%. These diluted samples were assayed for cellulase activity at optimum *p*H and temperature and results are summarized in Table III.

TABLE III

Effect of dilution on cellulase (C_x and C_2) activity at optimum p*H and temperature (25°C)*

Enzyme concentration %	Cellulase activity: % viscosity loss after (min) at *p*H 4·6				R.T. (min) at *p*H 7·6
	5	10	20	30	
100	37	52	65	71	16
80	17	27	41	48	17
60	13	22	34	41	19
40	4	18	29	37	32
20	7	12	18	25	35
10	6	9	9	12	52

It is clear from the results that viscosity reducing activity shows steep fall at first dilution and later showed gradual reduction. In the case of filter paper disintegrating activity, in spite of dilution at 60% (40% concentration) the activity did not show any reduction. Later with further dilution there was gradual reduction. Even at 10% concentration of enzyme solution, activity of both the enzymes was not lost completely. These results regarding concentration/activity relationship of C_x activity agree with those reported for *Aspergillus oryzae* (Jermyn, 1952), *Myrothecium verrucaria* and Actinomycetes (Levinson & Reese, 1950), *Trichoderma koningi* (Iwasaki, 1964), buffalo rumen microorganisms (Anand Gandhi, 1971) and *Alternaria brassicae* cellulase (C_1, C_x and C_2) (Nehemiah, 1976).

Effect of dialysis

Crude enzyme solution (25 ml) was placed in a cellophane bag, suspended in a beaker and was dialysed against running tap water for 24 hr at 26±1°C. Then the *p*H of dialysed solution was noted. It was found that the *p*H of dialysed solution changed into alkaline, i.e. from 6.8 to 7.7. The colour of solution did not change. The *p*H of dialysed solution was adjusted at optimum *p*H. Salts, two monovalent and two bivalent, were added at 10^{-4}M concentrations in dialysed solution. In other treatment undialysed autoclaved enzyme solution was also added and then assayed for cellulase activity. Results are reported in Table IV.

TABLE IV

Effect of dialysis on cellulase (C_x and C_2) activity at optimum pH and temperature

Treatment	Cellulase activity: % viscosity loss after (min) at *p*H 4·6				R.T. (min) at *p*H 7·6
	5	10	20	30	
Crude enzyme	37	52	65	71	16
Dialysed enzyme	12	13	14	14	28
Dialysed enzyme + undialysed auto claved enzyme	2	7	7	7	35
Dialysed enzyme + $CaCl_2$	4	5	5	5	33
Dialysed enzyme + $MgCl_2$	3	3	3	4	39
Dialysed enzyme + KCl	2	4	8	9	42
Dialysed enzyme + NaCl	2	6	7	7	42

The data show that culture filtrate after dialysis lost viscosity reducing activity 5 times over the control, whereas two times in the case of loss of coherence activity. It was neither restored by the addition of Ca^{++}, Mg^{++}, KCl or NaCl at 10^{-4}M nor was restored by adding non-dialysed autoclaved enzyme preparation to dialysed solution. Similarly, loss of activity on dialysis was also reported for *Rhizoctonia solani* (C_x enzyme *in-vivo*) (Bateman, 1964) and for *Alternaria brassicae* cellulase in-vitro (C_x and C_2) (Nehemiah, 1976) regarding the addition of salts. However, Singh (1968) showed in the case of *Fusarium lateritium* f. *cajani* that loss of activity on dialysis could be restored only by the addition of potassium chloride. Heath & wood (1971) reported that the results for cellulase (C_x *in-vivo*) of *Aschochyta pisi* and *Mycosphaerella pinodes* were inconclusive, in some extracts there was an increase, in others a decrease after dialysis.

Effect of precipitation

Acetone, elhanol and lead acetate at 100, 100 and 1% concentration respectively were precooled overnight at 0°C. On the second day morning 25 ml of enzyme solution was mixed with 25 ml of the precipitants and kept at 0°C for overnight to settle the precipitates. The solutions containing the precipitates were centrifuged at 5,000 rpm for 10 min in the graduated glass tubes. The supernatant was discarded. The last trace of precipitant was removed by placing the tubes under vacuum for 30 min. Each precipitate was dissolved in distilled water corresponding to half the volume of the original enzyme solution. After allowing the mixture to stand for 2 hr at laboratory temperature 25-30°C, it was centrifuged to remove the insoluble residue. As a result of precipitation, initial *p*H of the culture filtrate (6.8) drifted to 7.0. 7.0 and 5.5 respectively. The *p*H of each supernatant solution was adjusted by adding N/10 HCl or NaOH at optimum values. Then the enzyme solutions were assayed for cellulase activity and results are shown in Table V.

TABLE V

Effect of precipitation on cellulase (C_x and C_2) activity at optimum pH and temperature

Precipitants	Cellulase activity				
	% viscosity loss after (min) at *p*H 4·6				R.T. (min) at *p*H 7·6
	5	10	20	30	
Acetone	26	46	63	76	19
Ethanol	28	53	65	75	32
Lead acetate	36	40	47	53	35
Crude enzyme (Untreated)	37	52	65	71	16

The data show that precipitation showed comparable C_x activity with control, when acetone and ethanol were used as precipitants; while lead acetate strongly reduced it. The C_2 enzyme activity was reduced to a greater extent with ethanol and lead acetate and was comparable with control in the case of acetone. This result as regards precipitation of cellulase by ethanol agrees with the results reported for *Myrothecium verrucaria* (Kooiman, 1956) where the activity of the ethanol precipitated enzyme was the same as that of the untreated enzyme. Thomas (1956) reported that at 80% saturation with ammonium sulphate approximately half of the total activity was precipitated and further addition of it decreased the yield of enzyme from *Stachybotrys atra*. As regards reduction in activity of enzymes (C_1, C_x and C_2), Nehemiah (1976) also obtained reduction in activity for *Alternaria brassicae*. Contrary to these results are reports recorded for *Trichoderma viride* by Toyama (1960) and for *Myrothecium verrucaria* by Roth (1956).

Effects of various treatments on C_x and C_2 enzyme activity indicate that they can be differentiated on the basis of their different responses. Maximum activity of C_x occurred at acidic *p*H (4.6) and that of C_2 at alkaline (7.6) indicating that they are two different components of the same cellulase complex. As regards optimum temperature and heat treatment, these enzymes behave similarly, this may be due to the fact that they are the members of the same family. Although they are thermostable, C_x appears to be more thermosensitive than C_2 enzyme. Enzyme concentration/activity relationship indicates that C_2 enzyme is more powerful than C_x enzyme as C_2 activity was not reduced even at 60% dilution. However, at highest dilution (90%) they showed similarity. Reduction in activity of both the enzymes during dialysis might be due to the loss of some low molecular substances other than salts tested as the activity was not restored by the salts. This needs further investigation by analysis of chemicals which are associated with cellulase enzymes and are responsible for enzyme stimulation. Partial purification by means of acetone, ethanol and lead acetate also proved that these are two different components of the same cellulase complex. Differentiation into various components of cellulase complex may be further tried by adopting other different physical methods like electrophoresis thechniques, column chromatography and ultracentrifugation.

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