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Chemical Modification of a Cellulase from *Aspergillus niger*

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N-Bromosuccinimide completely inactivated the cellulase, and titration experiments showed that oxidation of one tryptophan residue per cellulase molecule coincided with 100% inactivation. CM-cellulose protected the enzyme from inactivation by *N*-bromosuccinimide. The cellulase was inhibited by active benzyl halides, and reaction with 2-hydroxy-5-nitrobenzyl bromide resulted in the incorporation of 2.3 hydroxy-5-nitrobenzyl groups per enzyme molecule; one tryptophan residue was shown to be essential for activity. Diazocarbonyl compounds in the presence of Cu^{2+} ions inhibited the enzyme. The pH-dependence of inactivation was consistent with the reaction occurring with a protonated carboxyl group. Carbodi-imide inhibited the cellulase, and kinetic analysis indicated that there was an average of 1 mol of carbodi-imide binding to the cellulase during inactivation. Treatment of the cellulase with diethyl pyrocarbonate resulted in the modification of two out of the four histidine residues present in the cellulase. The modified enzyme retained 40% of its original activity. Inhibition of cellulase activity by the metal ions Ag^{+} and Hg^{2+} was ascribed to interaction with tryptophan residues, rather than with thiol groups.

Although the synergism of the components of fungal cellulase systems has been the subject of intensive investigation (Halliwell & Riaz, 1971; Berghem *et al.*, 1975; Streamer *et al.*, 1975), the mechanism of action of cellulases has received scant attention. Pettersson (1968) and Eriksson & Pettersson (1968) have shown that tryptophan and possibly histidine are involved at the active site of *Penicillium notatum* cellulase. Legler & Bause (1973) have implicated a carboxyl group in the catalytic process of cellulases from an *Oxyporus* and two *Aspergillus* species. In a number of enzymes the essential catalytic and substrate-binding residues have been identified through chemical modification of the protein (Means & Feeney, 1971; Sigman & Mooser, 1975). In a previous paper (Hurst *et al.*, 1977) evidence was presented for the involvement of carboxyl groups during catalysis of a cellulase from *Aspergillus niger*. The present paper describes experiments designed to investigate the role of carboxyl, tryptophan, imidazole and tyrosine residues in the catalytic mechanism of cellulase.

Experimental

Materials

Cellulase (EC 3.2.1.4) from *A. niger* was purified from cellulase type II (Sigma, St. Louis, MO, U.S.A.) (Hurst *et al.*, 1977). The final preparation was homogeneous in the ultracentrifuge, on SDS†/polyacrylamide-gel electrophoresis and Bio-Gel P-60 gel filtration. The protein concentration of the purified enzyme was 9 mg/ml.

CM-cellulose 7LIXP was from Hercules Inc., Wilmington, DE, U.S.A. *p*-Hydroxybenzoic acid hydrazide was purchased from Fluka A.G., Buchs, Switzerland. *N*-Acetylimidazole was from K & K Laboratories Inc., Plainview, NY, U.S.A. All other chemicals were obtained from Sigma.

Methods

Organic syntheses. Methyl diazoacetate was prepared from glycine methyl ester hydrochloride by the method of Searle (1956). The concentration of methyl diazoacetate in the yellow oily product was determined by using $\varepsilon = 1.77 \times 10^4$ litre·mol^{-1}·cm^{-1} (Wilcox, 1967). Diazoacetamide was synthesized by ammonolysis of methyl diazoacetate (Wilcox, 1967). The yield of yellow crystals from 40 g of methyl diazoacetate was 2.3 g, m.p. 114–116°C. Diazoacetylglycine ethyl ester was synthesized from glycylglycine ethyl ester by the procedure of Riehm & Scheraga (1965). Dimethyl-(2-hydroxy-5-nitrobenzyl)-sulphonium bromide was prepared from 2-hydroxy-5-nitrobenzyl bromide and dimethyl sulphide as described by Horton & Tucker (1970).

Assay of cellulase and determination of protein. Cellulase activity was determined by the *p*-hydroxy-

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† Abbreviations: SDS, sodium dodecyl sulphate; Mes, 4-morpholine-ethanesulphonic acid; Tes, 2-{[2-hydroxy-1, 1-bis(hydroxymethyl)ethyl]amino}ethanesulphonic acid.

benzoic acid hydrazide reagent as previously described (Hurst *et al.*, 1977). Protein concentration was measured by the Eggstein & Kreutz (1967) modification of the method of Lowry *et al.* (1951), with crystalline bovine serum albumin as standard.

Reaction of chemical inhibitors with cellulase. The effect of a number of chemical inhibitors on cellulase activity was tested (Table 1). All incubations were at 25°C for 30min. Controls were performed without enzyme, but containing inhibitor to check for any subsequent interference in the assay. Reaction procedures for iodoacetate, iodoacetamide, *N*-ethylmaleimide, *p*-hydroxymercuribenzoate were: 0.2ml of cellulase (25μg/ml), 0.4ml of inhibitor solution in buffer or water, and appropriate buffer to 1ml; incubated, then quenched by addition of 1ml of 20mM-cysteine; after 5min 0.1ml samples were removed and assayed as described above. Reaction with *N*-bromosuccinimide was performed in a similar manner, except that the reaction was quenched with 1ml of 10mM-tryptophan in acetate buffer (0.1M-acetic acid/NaOH, pH4.0). Reaction with *N*-acetylimidazole was by incubating 0.1ml of cellulase (25μg/ml), 0.5ml of inhibitor in buffer and 0.4ml of buffer; the reaction was stopped by adjusting to pH4 by the addition of 0.1ml of 1M-acetic acid, and then 0.1ml samples were removed for assay. The incubation mixture with tetranitromethane contained 0.1ml of cellulase (25μg/ml), 0.89ml of buffer and 0.01ml of tetranitromethane in ethanol, and was stopped after incubation by the addition of 1M-acetic acid as above. Reaction with methyl diazoacetate was performed by incubating 0.1ml of cellulase (25μg/ml), 0.05ml of cupric acetate, 0.25ml of buffer, 0.5ml of water and 0.1ml of methyl diazoacetate in ethanol; then 0.1ml samples were assayed for activity.

Reaction of cellulase with active benzyl halide. In the reaction with dimethyl-(2-hydroxy-5-nitrobenzyl)-sulphonium bromide each mixture contained 0.1ml of cellulase (0.9mg/ml), 0.1ml of 0.2M buffer and 0.1ml of reagent solution in water to give the final concentrations indicated (Fig. 1*a*). In the reaction with 2-hydroxy-5-nitrobenzyl bromide, reaction mixtures contained 0.05ml of cellulase (1mg/ml), 0.13ml of 0.2M-sodium acetate buffer, pH4.0, and 0.02ml of 2-hydroxy-5-nitrobenzyl bromide in dioxan to give the final concentrations indicated (Fig. 1*b*). Each reaction was left to proceed at room temperature (22°C) for 15min; samples (10μl) were removed and diluted in 1ml of 10mM-tryptophan in 0.1M-sodium acetate buffer. Samples (0.1ml) were removed and assayed for residual activity. Appropriate controls were performed to allow for background A_{420}.

Titration of cellulase with 2-hydroxy-5-nitrobenzyl bromide. Reaction mixtures contained 0.5ml of cellulase (1mg/ml), 0.45ml of 0.2M-sodium acetate buffer, pH4.0, and 0.05ml of 2-hydroxy-5-nitrobenzyl bromide in dioxan. The reaction was allowed to proceed for 10min at room temperature, then duplicated samples (10μl) were removed and diluted in 2ml of acetate buffer; the residual activity of these diluted fractions was then determined. The remaining mixtures of 2-hydroxy-5-nitrobenzyl bromide-treated protein were denatured by adding 0.1ml of a solution containing 1% (w/v) SDS and 1% (v/v) mercaptoethanol. The samples were exhaustively dialysed against 0.1M-$NaHCO_3$/NaOH buffer, pH10.5, containing 0.01% SDS, to remove excess of 2-hydroxy-5-nitrobenzyl alcohol.

Reaction of N-bromosuccinimide with cellulase. To 1ml of a cellulase solution (0.83mg/ml) in 0.02M-acetate buffer, pH4.0, successive portions (5μl) of 5mM-*N*-bromosuccinimide solutions were added, and the decrease in A_{280} was measured on a Cary 118 spectrophotometer. Samples (5μl) were removed after each addition of *N*-bromosuccinimide and diluted in 1.5ml of the acetate buffer containing 10mM-tryptophan; portions (0.1ml) of these diluted samples were assayed for activity.

The reaction mixture used for protection of cellulase against *N*-bromosuccinimide inactivation was 0.2ml of cellulase solution (26μg/ml, in 0.1M-sodium acetate buffer, pH4.0), 0.3ml of the 0.1M-acetate buffer containing CM-cellulose (40mg/ml) and 0.5ml of *N*-bromosuccinimide solutions to give the concentrations indicated. The mixtures were left to react for 5min and then quenched by the addition of 1ml of 10mM-tryptophan in the sodium acetate buffer. Samples (0.1ml) were assayed for activity.

Reaction of diazo compounds with cellulase. Reaction mixtures contained: 0.05ml of cellulase (1mg/ml, in water), 115μl of 0.2M-sodium acetate buffer, pH5.0, 25μl of cupric acetate (at the final concentration given in Fig. 4) and 0.01ml of 50mM-diazoacetylglycine ethyl ester in ethanol. The mixture was left to react at room temperature for the time indicated then 10μl samples were removed and diluted into 1ml of 0.1M-sodium acetate buffer, pH4.0, and the residual activity was determined. The influence of pH on the rate of inactivation was tested by using different buffers and 50mM-cupric acetate (Fig. 4).

Inhibition of cellulase with metal ions. Cellulase solutions (0.5ml, 20μg/ml) were incubated at 25°C for 5min with 2ml of 10mM-metal salt solution. Then 3ml of CM-cellulose (20mg/ml, in 0.2M-sodium acetate buffer, pH4.0) was added and the viscosity of the mixture determined at the times indicated with an Ostwald viscometer. The specific viscosity at zero time was determined by substituting buffer for the buffered enzyme solution. Efflux times through the viscometer were measured at room temperature (20°C).

Ethoxycarbonylation studies. In a cuvette 0.5ml of

cellulase (1 mg/ml) was added to 0.45 ml of 20 mM-Mes/NaOH buffer, pH 6.0 at 25°C. The A_{242} was measured, then at zero time 0.05 ml of diethyl pyrocarbonate (diluted approx. 1 : 10 in ethanol) was added to give a final concentration of 30 mM. The change in A_{242} was noted and from this the number of histidine residues modified was calculated by using $\varepsilon = 3.9 \times 10^3$ litre·mol^{-1}·cm^{-1} for the N^{α}-ethoxycarbonylhistidine derivative (Choong *et al.*, 1977). At the same time samples (10 μl) were removed and diluted in 2 ml of 0.1 M-sodium acetate buffer, pH 4.0. Residual activity was measured and an allowance was made for the decrease in the initial absorbance of the enzyme solution on addition of the ethanolic diethyl pyrocarbonate solution.

Results

The probable importance of particular functional groups for the activity of the cellulase was explored by the use of reagents with restricted amino acid specificity. The results of the initial inhibitor studies are shown in Table 1. *N*-Ethylmaleimide and *p*-hydroxymercuribenzoate were not inhibitory, indicating that thiol groups are not involved [amino acid analysis (Hurst *et al.*, 1977) revealed the absence of free thiol groups from the cellulase]. Iodoacetamide and iodoacetate were not effective, suggesting a non-critical role for imidazole, methionine or amino groups. *N*-Bromosuccinimide was a potent inhibitor of cellulase activity, suggesting a crucial role for tryptophan or imidazole residues. Reaction of cellulase with tetranitromethane failed to give significant inhibition, and by using a spectrophotometric analysis (Riordan *et al.*, 1966) the number of modified tyrosine residues per cellulase molecule was 0.92 at 60 mM-tetranitromethane and 250 μg of cellulase/ml. Methyl diazoacetate with Cu^{2+} greatly decreased cellulase activity. In view of the above results the involvement of tryptophan, carboxyl and imidazole groups in cellulase activity was further investigated.

Reactions of cellulase with active benzyl halides

2-Hydroxy-5-nitrobenzyl bromide was a potent inhibitor of cellulase activity (Fig. 1*b*), and in contrast with the water-soluble sulphonium derivative (Fig. 1*a*), the enzyme inactivation was linear with increasing inhibitor concentration. The reaction of these benzyl halides with proteins may be quantified (Loudon & Koshland, 1970). Fig. 2 shows the relationship between 2-hydroxy-5-nitrobenzyl incorporation and loss of activity. Total inactivation was obtained with 2.3 2-hydroxy-5-nitrobenzyl groups/enzyme molecule. Extrapolation of the linear part of the curve, however, corresponds to 1 2-hydroxy-5-nitrobenzyl group/molecule of cellulase and 100% inactivation.

Reaction of cellulase with N-bromosuccinimide

The effect of *N*-bromosuccinimide on the enzyme activity and number of tryptophan residues oxidized is shown in Fig. 3(*a*) as a function of the amount of *N*-bromosuccinimide added. Complete inhibition occurred at a low molar excess of reagent and corresponds to the destruction of 1.0 tryptophan residue per enzyme molecule. This end point coincided with the development of turbidity.

In separate experiments, with dilute enzyme solutions, inactivation occurred at slightly higher molar ratios of *N*-bromosuccinimide to cellulase

Table 1. *Effect of chemical inhibitors on cellulase*

The experimental details are as described under 'Methods'.

Chemical	Inhibitor concn. in reaction mixture (mM)	Residual activity	Incubation buffer
Iodoacetate	10	100	0.1 M-Sodium citrate, pH 6.0
	10	100	0.1 M-Tris/HCl, pH 7.5
Iodoacetamide	10	100	0.1 M-Sodium citrate, pH 6.0
	10	100	0.1 M-Tris/HCl, pH 7.5
N-Ethylmaleimide	10	100	0.1 M-Sodium citrate, pH 6.0
	10	100	0.1 M-Tris/HCl, pH 7.5
N-Bromosuccinimide	0.001	95	0.1 M-Sodium acetate, pH 4.0
	0.005	0	0.1 M-Sodium acetate, pH 4.0
p-Hydroxymercuribenzoate	0.5	100	0.1 M-Imidazole/HCl, pH 7.0
Tetranitromethane	8.4	86	0.05 M-Tris/HCl, pH 8.0
N-Acetylimidazole	5	79	0.05 M-Sodium barbital, pH 7.5
Methyl diazoacetate, Cu^{2+}	1, 0.5	26	0.2 M-Sodium acetate, pH 5.0
Methyl diazoacetate	1	100	0.2 M-Sodium acetate, pH 5.0
Cu^{2+}	0.5	100	0.2 M-Sodium acetate, pH 5.0

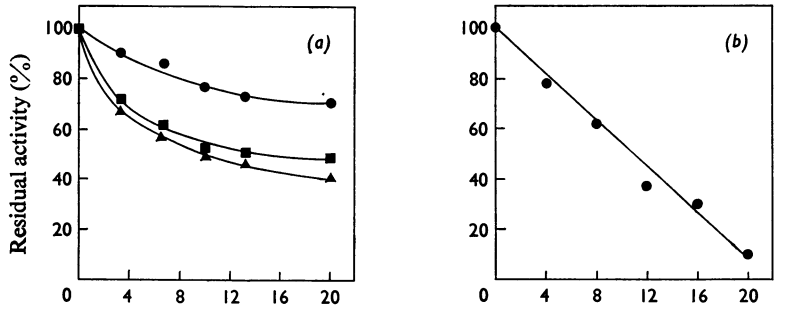

Fig. 1. *Effect of active benzyl halides on cellulase activity*

(*a*) Dimethyl (2-hydroxy-5-nitrobenzyl)sulphonium bromide. Points are the means of duplicate experiments. Reaction buffers were: ●, 0.2M-glycine/HCl, pH3.0; ■, 0.2M-sodium acetate, pH4.0; ▲, 0.2M-sodium succinate, pH6.0. (*b*) 2-Hydroxy-5-nitrobenzyl bromide. Points are the means of duplicate experiments and the reaction was in 0.2M-sodium acetate buffer, pH4.0.

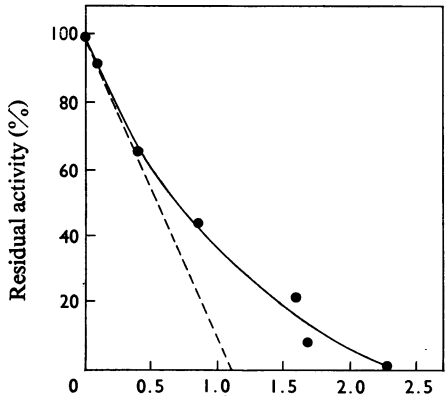

Fig. 2. *Titration of cellulase with 2-hydroxy-5-nitrobenzyl bromide*

The concentration of 2-hydroxy-5-nitrobenzyl groups was estimated from the A_{410} (Loudon & Koshland, 1970) and protein concentration was determined as described under 'Methods.'

(Fig. 3*b*). When 1% (w/v) CM-cellulose was added to this reaction system, before addition of *N*-bromosuccinimide, complete protection from inactivation was achieved. Low enzyme concentrations were used to approach substrate saturation of the enzyme.

Reaction of cellulase with diazoacetyl compounds

Methyl diazoacetate, diazoacetamide and diazoacetylglycine ethyl ester were prepared as described under 'Methods' and tested as inhibitors of cellulase in the absence and presence of Cu^{2+} ions at pH5.0.

Table 2. *Reaction of diazoacetyl compounds with cellulase*

Reaction mixtures contained: 0.05ml of cellulase (1mg/ml), 115μl of 0.2M-sodium acetate buffer, pH5.0, 25μl of 24mM-cupric acetate, 0.01ml of diazo compound (2.5mM in ethanol). Controls were run without Cu^{2+} and/or diazo compound. The mixtures were incubated at 20°C for 30min; samples (20μl) were removed and diluted with 2ml of 0.1M-sodium acetate buffer, pH4.0. Samples (0.1ml) were then assayed for activity. Values are the means of duplicate determinations.

Compound	Residual activity (%) Without Cu^{2+}	With Cu^{2+} (3mM)
Methyl diazoacetate	96	26
Diazoacetamide	100	45
Diazoacetylglycine ethyl ester	84	28

Table 2 shows that in the absence of Cu^{2+} diazo compounds were poor inhibitors of cellulase. In the presence of Cu^{2+}, however, inhibition by all three reagents was markedly increased. For subsequent experiments diazoacetylglycine ethyl ester was the reagent of choice, since it is a stable recrystallized solid (cf. liquid methyl diazoacetate and hygroscopic diazoacetamide). The influence of the concentration of Cu^{2+} ions on the rate of inactivation of the enzyme is shown in Fig. 4(*a*). Increasing amounts of Cu^{2+} ions eliminated the lag phase observed in the absence of Cu^{2+} or with low concentrations of Cu^{2+}. In a separate experiment, in which the cellulase and Cu^{2+} (3mM) were preincubated for 5min before the addition of the diazoacetyl glycine ethyl ester, the rate of inactivation was indistinguishable from the

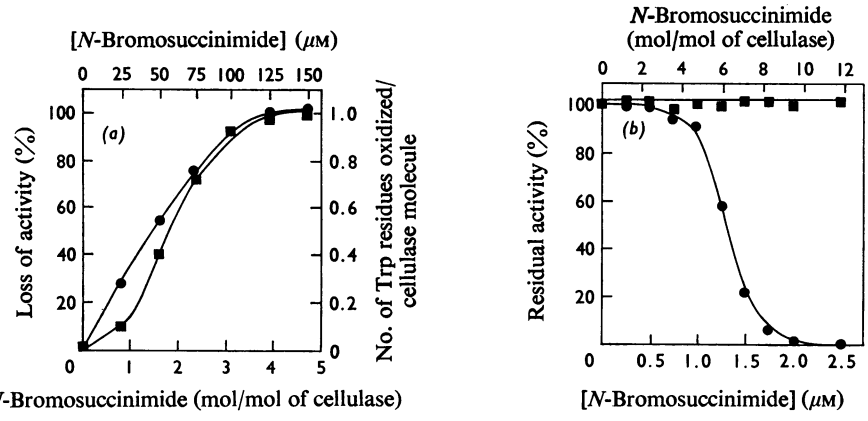

Fig. 3. *Effect of N-bromosuccinimide on cellulase activity*

(*a*) Titration of cellulase with *N*-bromosuccinimide. The number of tryptophan residues oxidized (*n*, ●) was estimated from the relationship:

$$n = \frac{1.31 \times \text{change in } A_{280}}{5500 \times \text{molarity of cellulase}}$$

(Spande & Witkop, 1967); ■, residual activity. (*b*) Protection of cellulase by CM-cellulose from *N*-bromosuccinimide inactivation. ●, Control; ■, 0.3 ml of buffer replaced by 0.3 ml of CM-cellulose (40 mg/ml, in the acetate buffer, pH 4.0). Points are the means of duplicate determinations.

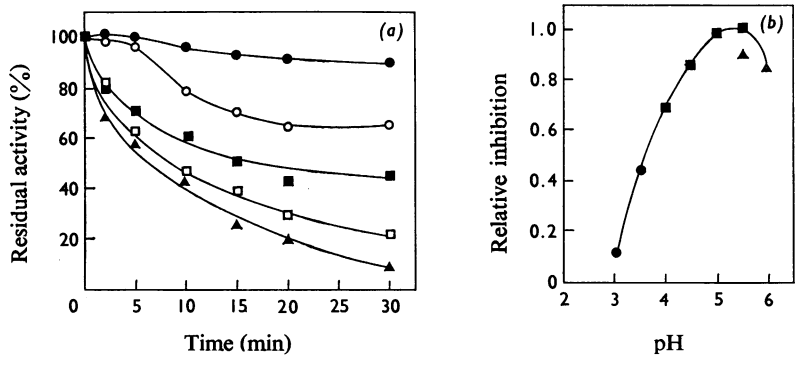

Fig. 4. *Effect of diazo compound on cellulase activity*

(*a*) Influence of Cu^{2+} concentration. Residual activity was measured with 0.1 ml samples from diluted reaction mixtures. Final Cu^{2+} concentrations were: ●, 0; ○, 0.75 mM; ■, 1.5 mM; □, 3 mM; ▲, 6 mM. (*b*) Influence of pH. The mixture was left to react at room temperature for 30 min, then residual activity was measured as in (*a*). Buffers used were: ●, sodium formate, pH 3.0, 3.5; ■, sodium acetate, pH 4.0, 4.5, 5.0, 5.5; ▲, sodium succinate pH 5.5, 6.0. Points are the means of duplicate determinations.

inactivation rate obtained when Cu^{2+} and diazo compound were added to the cellulase simultaneously. The extent of inactivation of cellulase by diazoacetylglycine ethyl ester and Cu^{2+} as a function of pH is shown in Fig. 4(*b*). Inactivation by the diazo compound and Cu^{2+} was not decreased by the presence of cellotriose, cellopentaose or lichenin at a concentration of 1 mg/ml. Further, cellopentaose (1 mg/ml) did not protect the enzyme from inactivation by diazoacetylglycine ethyl ester in the absence of Cu^{2+}.

Reaction of cellulase with carbodi-imide

The water-soluble carbodi-imide 1-cyclohexyl-3-(2-morpholinoethyl)carbodi-imide methotoluene-*p*-sulphonate was tested as an inhibitor of cellulase activity. The reaction was carried out at pH 6.0 in the absence of added nucleophile. Semi-logarithmic plots of enzymic activity as a function of time of inactivation by the carbodi-imide at various concentrations of inhibitor are linear until the loss of activity exceeds 90% (Fig. 5). These data indicate that

the inactivation process exhibits pseudo-first-order kinetics with respect to time at any fixed concentration of carbodi-imide. The same data, when replotted (Fig. 6*a*) as the rate of inactivation as a function of inhibitor concentration, show that the inactivation is first order with respect to the carbodi-imide concentration used in this experiment. Applying the analysis described by Levy *et al.* (1963), a plot of logarithim k (k is the pseudo-first-order rate constant for enzyme inactivation) against logarithim [carbodi-imide] yielded a slope of 1, which indicates that an average of at least 1 molecule of inhibitor binds 1 molecule of cellulase during inactivation.

Since the carbodi-imide might potentially react with functional groups other than carboxyl groups (Carraway & Koshland, 1972), a study on the effect of the H^+ concentration on the rate of inactivation was undertaken (Fig. 7). The rate of inactivation of the cellulase was markedly pH-dependent, with maximum inactivation occurring at pH 5.5–6.0.

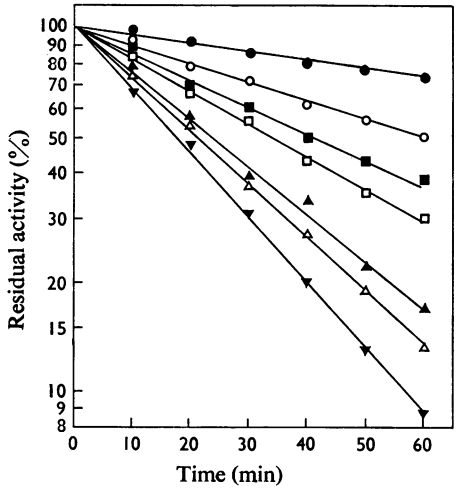

Fig. 5. *Effect of carbodi-imide on cellulase activity*
Reaction mixtures contained: 0.05ml of cellulase (1mg/ml), 0.10ml of Mes buffer, pH6.0, and 0.05ml of the carbodi-imide in buffer. Samples (10μl) were removed from the reaction mixture (25°C) at the times indicated and diluted into 1ml of 0.1M-sodium acetate buffer, pH4.0. From these diluted samples 0.1ml portions were used for assaying residual activity. Final carbodi-imide concns. were: ●, 5mM; ○, 10mM; ■, 17.5mM; □, 25mM; ▲, 35mM; △, 40mM; ▼, 50mM.

Reaction of cellulase with diethyl pyrocarbonate

The enzyme (20μM) was treated with 30mM-diethyl pyrocarbonate at pH6.0 (Fig. 8). The increase in A_{242} was used to calculate the number of histidine residues modified. One histidine residue reacted rapidly, and a second was modified slowly. Concomitant with the modification was a gradual decrease in activity, but the enzyme retained about 30% activity when two histidine residues were acylated per molecule; non-specific amino-group acylation is likely to be occurring (Means & Feeney, 1971).

Effect of metal ions on cellulase activity

Cellulase activity was measured in the presence of various metal salts, after a preincubation with the appropriate metal salt. A viscometric assay was used because of metal ion interference of the *p*-hydroxybenzoic acid hydrazide reducing-sugar method. Fig. 9 shows that at a final metal ion concentration of 3.6mM, Ag^+ completely inhibited the enzyme; Hg^{2+} gave partial inhibition, and Pb^{2+} resulted in a slight decrease in activity, which was gradually regained with increasing hydrolysis time.

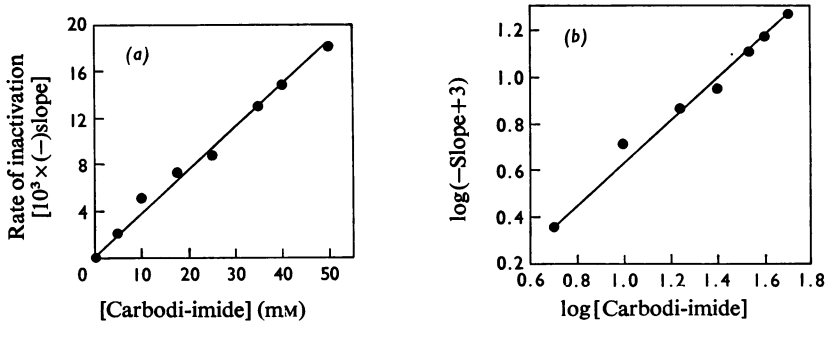

Fig. 6. *Kinetic analysis of cellulase inactivation by carbodi-imide*
(*a*) The pseudo-first-order kinetics of cellulase inactivation with respect to carbodi-imide. The rate of inactivation (slope) was obtained from the data in Fig. 5 and was plotted against carbodi-imide concentration. (*b*) Determination of the order of the reaction between cellulase with respect to carbodi-imide. The data of (*a*) are replotted in logarithmic form.

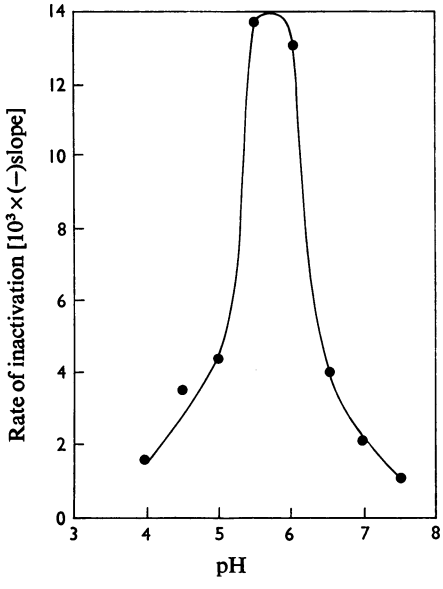

Fig. 7. *Influence of pH on cellulase inactivation by carbodi-imide*
Reaction mixtures contained: 0.05ml of cellulase (1mg/ml), 0.10ml of appropriate buffer and 0.05ml of 35mM-carbodi-imide. Samples (10μl) were taken at 10min intervals and treated as in Fig. 5. The rate of inactivation was calculated from the slopes of plots of log (% residual activity) versus time. Buffers were: 0.2M-pyridine/HCl, pH4.0, 4.5, 5.0; 0.1M-Mes/NaOH, pH5.5, 6.0, 6.5; 0.1M-Tes/NaOH, pH7.0, 7.5.

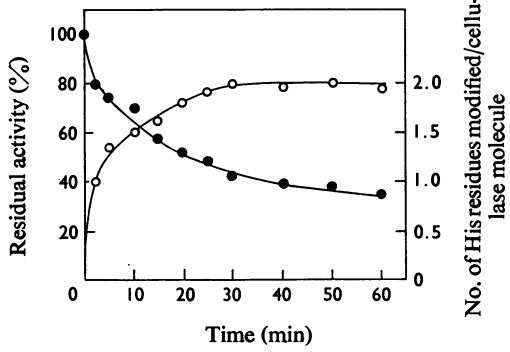

Fig. 8. *Effect of diethyl pyrocarbonate on cellulase activity*
Residual activity after diethyl pyrocarbonate treatment was determined at the end of the experiment as described under 'Methods'. ●, Residual activity; ○, number of histidine residues modified per cellulase molecule (calculated as described under 'Methods').

Discussion

Of the six tryptophan residues present in this *A. niger* cellulase (Hurst *et al.*, 1977) it was found that

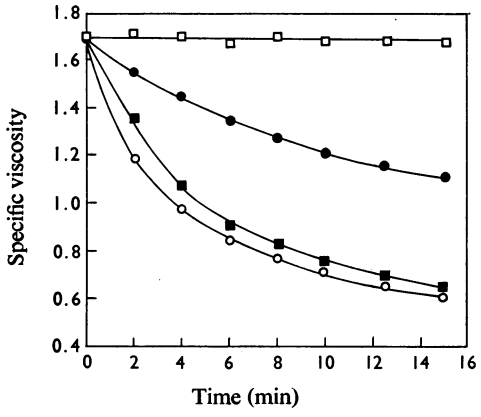

Fig. 9. *Effect of metal ions on cellulase activity*
The effect of metal ions on cellulase activity was tested as described in the text. Metal salt solutions were: □, $AgNO_3$; ●, $HgCl_2$; ■, $Pb(NO_3)_2$; ○, control, no metal salt. The following metal salts gave results indistinguishable from the control: $MgCl_2$, $MnCl_2$, $ZnSO_4$, cupric acetate, $CoCl_2$, $NiCl_2$, $CaCl_2$, $FeSO_4$. Controls, containing metal ion but no enzyme, were performed and the points are the means of duplicate determinations.

oxidation of one tryptophan residue with *N*-bromosuccinimide caused complete inactivation of the enzyme. Fig. 3(*a*) shows that there is a correlation between the loss of activity and the number of tryptophan residues modified. The danger of splitting peptide bonds is minimal at the low excess of reagent (5mol/mol of protein) used in this study (Spande & Witkop, 1967). CM-cellulose completely protected the cellulase from *N*-bromosuccinimide inactivation under near-substrate-saturating conditions (Fig. 3*b*), and this is further evidence for the crucial involvement of the tryptophan residue at the active site. Inactivation of cellulase with 2-hydroxy-5-nitrobenzyl bromide indicated that one tryptophan residue is essential for activity (Fig. 2).

Diazoacetyl compounds have been found to inactivate the cellulase, and the reactions are markedly facilitated by Cu^{2+} ions (Table 2, Fig. 4). The results indicate the formation of a reactive complex between the diazo compound and Cu^{2+} ions, before the inactivation of the enzyme. The slow inactivation in the absence of added Cu^{2+} could be due to trace amounts of Cu^{2+} in the reaction mixture. Reaction at protonated carboxyl groups is proposed, since diazo compounds show a preference for protonated carboxyl groups (Doescher & Wilcox, 1961). The pH profile of cellulase inactivation by diazoacetylglycine ethyl ester (Fig. 4*b*) can be explained by reaction of the diazo ester with two carboxyl groups in or near the active site in a manner similar to that proposed for pepsin (Lundblad & Stein, 1969; Bayliss *et al.*, 1969;

Fry *et al.*, 1970). From Fig. 4(*b*) the ionized carboxyl group would have pK4.0–4.5 and the reactive protonated group pK about 5.5. The participation in the catalytic action of cellulase of a (carboxyl) group still protonated in the range pH5.0–5.5 is required by the kinetic experiments previously described (Hurst *et al.*, 1977).

Kinetic analysis of the effect of carbodi-imide on the cellulase indicated an average of at least 1 molecule of carbodi-imide binding to the enzyme during inactivation (Fig. 6). The effect of pH on the rate of inactivation of cellulase by this carbodi-imide was examined, since carbodi-imides might potentially react with functional groups other than carboxyls. The pH-dependence of inactivation observed (Fig. 7) precludes the involvement of histidine and amino groups. Experiments with the more selective tyrosine reagents discount the carbodi-imide reaction with tyrosine. The reaction of thiol groups need not be considered, in the light of the absence of free thiol groups from the cellulase.

The observed increase in inactivation rate in the range pH4.0–5.5 (Fig. 7) indicates ionization of a reactive carboxyl group. The p*K* value of 5.0–5.5 is close to the value required for the protonated carboxyl group that is proposed to react with the diazo compounds. The profile might have been expected to reach a plateau at pH6, but the rapid fall above pH6 could be a result of either a pH-induced enzyme conformational change (Hurst *et al.*, 1977) or the requirement for protons for the esterification (Hoare & Koshland, 1967). Reaction with either *N*-acetylimidazole or diethyl pyrocarbonate required a large molar excess of reagent to bring about significant inhibition, and it is concluded that neither tyrosine nor histidine residues have a crucial role in the mechanism of action of the cellulase.

Of those metal ions tested, Ag^+ completely inhibited the enzyme and Hg^{2+} gave partial inhibition (Fig. 9). Heavy-metal ions are generally thought to inactivate enzymes by forming covalent salts with cysteine, histidine or possibly carboxyl residues (Dixon & Webb, 1964). Since primary-structure analysis has shown that the cellulase is devoid of free thiol groups and disulphide linkages, interaction with thiol groups can be discounted. With Hg^{2+} at least, the observed inhibition could arise from the interaction with tryptophan residues (Eriksson & Pettersson, 1968).

Lysozyme is the most thoroughly studied polysaccharidase. X-ray crystallography, chemical modification and enzyme kinetics have revealed that tryptophan and aspartic residues participate in binding, whereas glutamic and aspartic residues function in catalysis (for reviews see Osserman *et al.*, 1974). The results obtained in the present investigation are consistent with a mechanism of action of the cellulase similar to that established for lysozyme. It is postulated that both tryptophan and carboxyl residues are involved in substrate binding. The catalytic amino acids are believed to be a carboxylate anion (pK4.0–4.5) and a protonated carboxyl group (pK5.0–5.5), acting in a manner similar to that observed in lysozyme.

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