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Characteristics of Enzymes Produced by *Ruminococcus flavefaciens* which Degrade Plant Cell Walls

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Enzyme preparations active against crystalline cellulose, marble-milled filter paper, carboxymethylcellulose (CM-cellulose), hemicellulose and xylan were obtained from cultures of *Ruminococcus flavefaciens*. These preparations also contained swelling factor, pectin methylesterase, pectin lyase and low levels of aryl β-glucosidase and aryl β-xylosidase. CM-cellulase and xylanase activities were present in a high molecular weight complex, but substrate competition studies showed that different active sites were probably responsible for each activity. Analysis of products and viscosity changes during enzymic hydrolysis of CM-cellulose and xylan indicated that the most active enzymes were of the exo-1,4-β-glycosidase type. A variety of reducing sugars were released from cell walls of *Lolium perenne* (perennial ryegrass) by enzyme preparations.

INTRODUCTION

Ruminococcus flavefaciens is one of the few species of rumen bacteria capable of degrading the cellulose, hemicellulose and pectin present in plant cell walls. Based on work with fungal enzymes, Reese *et al.* (1950) suggested that most cellulases comprised a C_x enzyme, which degraded soluble cellulose, and a C_1 component, which by its synergistic activity enabled C_x to degrade insoluble cellulose. More recent work has shown C_1 to be hydrolytic and cellulases are now described in terms of their exo- or endo-1,4-β-glucanase activity (Wood & McCrae, 1975). Exo- and endo-1,4-β-glucanases have been detected in various species of soil or aquatic bacteria (King, 1963; Suzuki *et al.*, 1969), but the cellulase components of the rumen bacterium *R. albus* have a non-hydrolytic 'affinity factor' as well as hydrolytic activity (Leatherwood, 1969). Smith *et al.* (1973) also considered that the cellulase of *R. albus* contained more than one component because of the effect of oxygen on the activity and adsorption and desorption characteristics of the enzyme. Studies on the cellulase of *R. flavefaciens* are limited to the immunochemical investigation of Sharma (1968) and the mode of action of this enzyme has not been reported.

Hemicellulases degrade linear and branched polymers of xylose, arabinose, galactose and mannose by exo- and endo-hydrolytic mechanisms (Dekker & Richards, 1976), but the degradation of pectin involves pectin methylesterases, which release methanol from polymethylgalacturonic acids (pectin), and pectin lyases and polygalacturonases, which cleave the glycosidic bonds (Rombouts, 1972). Degradation of hemicellulose and pectin by growing cultures of several species of rumen bacteria including *R. flavefaciens* has been described (Gradel & Dehority, 1972; Dehority, 1973) but the enzymes responsible for these activities have not been characterized.

In the paper we describe some properties of the cellulases, hemicellulases and pectinases produced by *R. flavefaciens*. A preliminary report of this work has appeared previously (Pettipher & Latham, 1976).

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METHODS

Organism and culture media. Ruminococcus flavefaciens, strain 67, was maintained on RUM10 agar (Latham *et al.*, 1978). A modification of this medium, basal medium BM10, was prepared by omitting cellobiose, haemin and agar, adding $FeSO_4.7H_2O$ and $CoCl_2.6H_2O$ (0·0001 %, w/v, each) and replacing the volatile fatty-acid mixture with 1 % (v/v) of a branched fatty-acid mixture containing 1 % (v/v) isovaleric, isobutyric and DL-α-methyl-*n*-butyric acids and 1 % (w/v) phenylacetic acid. Cellobiose, or Whatman no. 1 filter paper which had been marble-milled in water for 72 h (MMFP), were added as sole energy sources at a concentration of 0·1 % (w/v) to give complete media.

Enzyme preparations. Supernatants from 17 h BM10-cellobiose or 72 h BM10-MMFP cultures were freeze-dried and the solids were dissolved in cold (8 °C) 0·02 M-2-(*N*-morpholino)ethanesulphonic acid (MES) buffer (pH 6·3) to give a 10-fold concentration with respect to the original supernatant. The solution was made 50 % saturated with ammonium sulphate, centrifuged at 35000 *g* for 1 h and the precipitate was resuspended in and dialysed overnight against MES buffer (0·02 M, pH 6·3) before being stored at −20 °C. All enzyme studies were conducted using these crude enzyme preparations unless otherwise indicated.

Enzyme substrates. The following substrates were used to test for enzyme activity. Cellulase activity: carboxymethylcellulose DS 0·4 (CM-cellulose, Hercules Powder Co., London), marble-milled Whatman no. 1 filter paper (MMFP), Avicel PH-101 (Honeywell & Stein, Wallington, Surrey), Solka floc BW 200 (Brown Co., Berlin, N.H., U.S.A.) and scoured native cotton (Shirley Institute, Manchester). Hemicellulase activity: coniferous wood xylan (Dr K.-E. Eriksson, Swedish Forest Products Research Institute, Stockholm), larchwood xylan and oak sapwood hemicellulose. Pectinase activity: citrus pectin (degree of methoxylation 0·7, H. P. Bulmer, Hereford). Aryl β-glucosidase and aryl β-xylosidase activities were detected using *o*-nitrophenyl β-D-glucopyranoside (ONPG) and *p*-nitrophenyl β-D-xylopyranoside (PNPX), respectively. Mesophyll and crude cell wall preparations from leaves of *Lolium perenne* cv. s24 were provided by Dr P. J. Harris, Grassland Research Institute, Hurley, Berkshire.

Enzyme assays. Cellulase and hemicellulase activities were measured from the amount of reducing-sugar (Halliwell, 1961) released after incubation of enzyme with substrate. Activity against insoluble cellulose was assayed by incubating 1 ml enzyme with 1 ml 0·25 M-MES buffer pH 6·4, 1 ml of a 3 % (w/v) suspension of Avicel or MMFP in water, and 0·1 ml toluene. Activity against soluble cellulose and hemicellulose was assayed by incubating 1 ml enzyme with 1 ml 0·25 M-MES buffer pH 6·4 and 2 ml of a 0·75 % (w/v) or 0·25 % (w/v) solution of CM-cellulose or larchwood xylan, respectively. Optimum temperatures and times for these assays are given in Table 1. Reactions were stopped by briefly heating to 100 °C and residual insoluble substrate was sedimented by centrifugation. Controls were prepared by incubating enzyme, buffer and substrate separately and heating each to 100 °C before mixing. A unit (U) of activity was defined as that amount of enzyme which produced 1 μmol of reducing-sugar (equivalent to glucose or xylose, as appropriate) per minute under the conditions of assay.

Pectin lyase activity was detected by measuring the release of unsaturated uronides (Rombouts, 1972) after incubating enzyme with citrus pectin. Pectin methylesterase was detected by measuring the production of methanol (Carlsson, 1973) and by the lowering of pH in unbuffered assays.

Aryl β-glucosidase activity was estimated by the method of Wood (1968) using 0·25 M-MES buffer pH 6·4, and aryl β-xylosidase was detected similarly with PNPX as substrate. β-Glucosidase activity was measured under the same conditions using cellobiose as substrate.

Swelling factor activity was determined by the method of Nisizawa *et al.* (1966).

Mechanisms of enzymic hydrolysis. Changes in viscosity and concentration of reducing-sugar during enzymic hydrolysis of CM-cellulose or coniferous xylan were compared with measurements made during hydrolysis in 80 mM-phosphoric acid at 100 °C. Viscosity was measured by timing the rate of flow of water and of treated and untreated substrate solutions between two fixed points in a 1 ml bulb pipette at a constant temperature of 20 °C. Specific fluidity (ϕ_{sp}) was calculated as described by Nisizawa (1973).

Gel filtration chromatography. Enzyme preparations (1 ml) were eluted through Sephadex G-200 with MES buffer (0·02 M, pH 6·3) containing 0·2 M-NaCl to reduce non-specific adsorption. Fractions (0·55 ml) were assayed for protein (Lowry *et al.*, 1951) and enzyme activity and the columns were calibrated with standard proteins (Andrews, 1965).

Inhibition and activation studies. CM-cellulase and xylanase activities were assayed 5 min after mixing 1 ml of enzyme preparation with 1 ml of an aqueous solution (0·02 M) of one of the following reagents: mercuric chloride, iodoacetic acid, 4-chloromercuribenzoic acid and *N*-ethylmaleimide (thiol-blocking reagents), mercaptoethanol and cysteine.HCl (reducing agents) and EDTA (chelating reagent). The ability of divalent cations to overcome inhibition by EDTA was determined by adding 1 ml of 0·025 M aqueous solutions of either $FeSO_4$, $CoCl_2$, $MnSO_4$, $MgCl_2$, $ZnCl_2$ or $CaCl_2$ to the reaction mixture before assaying for enzyme activity. Solutions of cellobiose, glucose, xylose, arabinose and fructose (0·01 M final concentration) were also mixed with enzyme to determine their effect on activity.

Table 1. *Physical characteristics of cellulase, xylanase and aryl β-glucosidase produced by R. flavefaciens and other activities associated with plant cell wall degradation*

Substrate	Optimum pH	Optimum temp. (°C)	Time (h)	K_m* (mg ml^{-1})
Crystalline cellulose (Avicel)	6·4–6·6	39	20	
Marble-milled filter paper (MMFP)	6·4	39	20	
Carboxymethylcellulose (CM-cellulose)	6·4	45	0·75	0·432±0·053
Larchwood xylan	6·4	45	0·75	0·626±0·092
o-Nitrophenyl β-D-glucopyranoside (ONPG)	6·4	40	0·5	0·267±0·023

Other activities detected were: short fibre formation; swelling factor activity; pectin lyase; pectin methylesterase; aryl β-xylosidase.

* K_m values calculated by bilinear regression (Wilkinson, 1961).

Substrate competition experiment. The release of reducing-sugars from substrate mixtures containing increasing concentrations of CM-cellulose (0 to 4·04 mg ml^{-1}) and decreasing concentrations of larchwood xylan (1·35 to 0 mg ml^{-1}) was measured. These results were compared with theoretical predictions for CM-cellulase and xylanase activity originating from either the same or different active sites calculated by the method of Shikata & Nisizawa (1975) using K_m values previously determined for solutions of single substrates.

Identification of carbohydrates. Particle-free supernatants from assays and 20 h incubations of enzyme with *L. perenne* cell walls were concentrated 10-fold and the sugars present (up to tetrasaccharides) were separated on Whatman no. 1 chromatography paper using a descending butanol/pyridine/0·1 M-HCl (5:3:2, by vol.) solvent system. The carbohydrates were located by spraying with aniline (2·6%, v/v), orthophosphoric acid (1·2%, v/v) and acetic acid (40%, v/v, in acetone). Carbohydrates were also quantified by gas–liquid chromatography after silylation (Pierce Chemical Co., 1976).

RESULTS

Enzyme activities

Enzymes active against Avicel, MMFP, CM-cellulose, xylan, ONPG, PNPX and pectin were recovered from culture supernatants of *R. flavefaciens* (Table 1). The CM-cellulase and xylanase activities in the crude enzyme preparations were concentrated approximately 40-fold compared with those in the original culture supernatant, giving a final recovery of 50%. CM-cellulase activity was stable when stored aerobically at −20 °C but was unstable at higher temperatures, 50% of the original activity being lost after 36 h at 39 °C or only 3 h at 45 °C. Consequently, optimum temperatures were lower for assays requiring prolonged incubations (Table 1). CM-cellulose and larchwood xylan were actively hydrolysed. Aryl β-glucosidase and aryl β-xylosidase activities were weak and β-glucosidase activity was negligible. As a measure of the relative activity of the enzyme preparations towards the different substrates, the ratio of total reducing-sugar released from a test substrate to that released from MMFP under identical conditions was obtained. These values were: larchwood xylan, 9·9; CM-cellulose, 9·3; oak sapwood hemicellulose, 5·8; mesophyll cell walls, 3·4; MMFP, 1·0; Solka floc BW 200, 0·5; Avicel, 0·4; scoured native cotton, 0·03. Other activities detected were swelling factor (extent of swelling about 0·25), pectin lyase and pectin methylesterase. The formation of short fibres (Halliwell & Riaz, 1971) was also observed in cultures growing on MMFP.

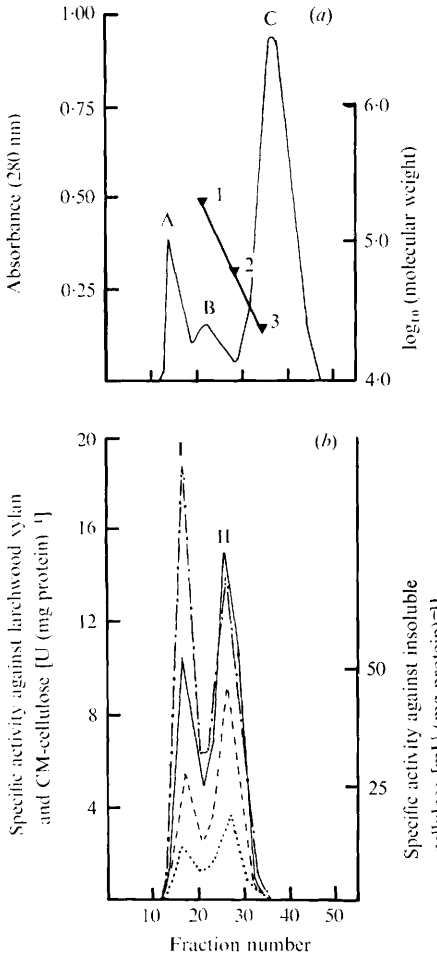

Fig. 1

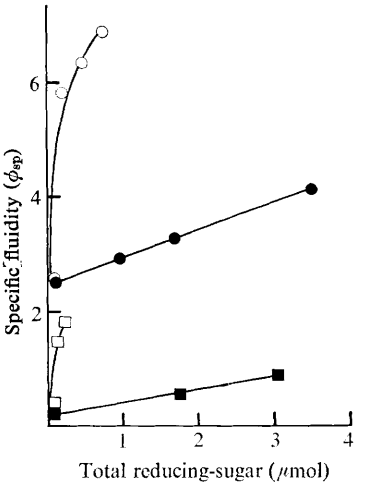

Fig. 2

Fig. 1. Elution of protein and enzymes following gel filtration on Sephadex G-200 of preparations obtained from supernatants of *R. flavefaciens* cultures grown on MMFP. (*a*) Protein precipitated from culture supernatants by ammonium sulphate. Molecular weight standards were: 1, γ-globulin; 2, bovine serum albumin; 3, chymotrypsinogen. (*b*) Specific activity against larchwood xylan (—·—), CM-cellulose (——), MMFP (– – –) and Avicel (····).

Fig. 2. Increase in specific fluidity and total reducing-sugar during the hydrolysis of coniferous xylan by phosphoric acid (○) and enzyme preparation (●) and during the hydrolysis of CM-cellulose by phosphoric acid (□) and enzyme preparation (■). The enzyme preparations were obtained from supernatants of *R. flavefaciens* cultures grown on MMFP.

Gel filtration

Protein from crude enzyme preparations was eluted in three peaks A, B and C (Fig. 1*a*) of which C, by comparison with preparations from uninoculated media, was derived from medium components. Activity with CM-cellulose, Avicel and MMFP as substrates was eluted as two major peaks from Sephadex G-200 (Fig. 1*b*). Peak I was excluded from the gel and thus had a molecular weight greater than 800000 whereas peak II (molecular weight range from 25000 to 214000) had a maximum specific activity coinciding with a molecular weight of 89000. Avicel- and MMFP-hydrolysing activities were weak compared with CM-cellulase activity. Xylanase was eluted in a similar manner to CM-cellulase except that more activity was present in peak I. A significant proportion (about 10%) of both activities

Table 2. *Observed and predicted reaction velocities of R. flavefaciens enzyme preparation with various mixtures of CM-cellulose and larchwood xylan*

Substrate mixture		Reducing-sugars released (μmol)		
		Observed	Predicted*	
CM-cellulose (mg ml^{-1})	Xylan (mg ml^{-1})		Different active site	Same active site
4·62	0	17·66	NPV†	NPV
4·04	0·19	22·13	19·74	15·95
3·46	0·39	23·30	21·86	15·73
2·89	0·58	23·94	23·13	15·45
2·31	0·77	24·27	23·80	15·09
1·73	0·96	23·24	23·92	14·06
1·15	1·15	20·48	23·37	13·98
0·58	1·35	18·05	21·13	13·01
0	1·54	16·19	NPV	NPV

* Predicted values calculated according to the method of Shikata & Nisizawa (1975) based on competitive and non-competitive inhibition between substrates.

† NPV, No predicted value.

was excluded from Sepharose 4B, indicating that these enzymes were present in material having a molecular weight greater than 3×10^6. Pectin lyase was eluted from Sephadex G-200 in peaks I and II but aryl β-glucosidase was eluted in peak II only.

Inhibition and activation

CM-cellulase and xylanase activities were reduced by treatment with thiol-blocking reagents but were stimulated by reducing agents. Divalent cations were required by CM-cellulase since activity was reduced after treatment with EDTA but restored by addition of Ca^{2+} or Mg^{2+}. Neither these, nor any other divalent cations tested, overcame the inhibition of xylanase activity by EDTA. None of the sugars tested had any effect on CM-cellulase or xylanase activity.

Mechanisms and products of enzymic hydrolysis

The experimentally determined values of reducing-sugar released from the substrate competition experiments agreed closely with theoretical values predicted for CM-cellulase and xylanase activities originating from different active sites (Table 2). More reducing-sugar was released for a given rise in specific fluidity during the enzymic hydrolysis of CM-cellulose and coniferous xylan than during hydrolysis by phosphoric acid (Fig. 2), with the major products of enzymic CM-cellulose hydrolysis being cellobiose, cellotriose and glucose in the ratio 6·2:5·8:1·0. Cellobiose was the only product of enzymic hydrolysis of Avicel and MMFP. Xylobiose, xylotriose and xylose were released from larchwood xylan in the ratio 4·5:3·0:1·0. Xylotetraose was also detected. Using *L. perenne* cell walls as substrate, 7% (w/w) was released as soluble reducing-sugar of which 50% comprised hexose and pentose monomers. The major sugars released were glucose, galactose, cellobiose, arabinose, xylose with trace amounts of oligosaccharides. Unsaturated uronides were also released as a result of pectin lyase activity.

DISCUSSION

Ruminococcus flavefaciens produces a complex array of enzymes active against cellulose, hemicellulose and pectin which aid it in the digestion of plant cell walls. A significant proportion of the CM-cellulase, xylanase and pectin lyase activity was associated with high molecular weight material which may have been derived from the bacterial cell wall. High

molecular weight fractions containing cellulase were found in *R. albus* (Leatherwood, 1969) but the optimum temperature for cellulase activity in this organism (Smith *et al.*, 1973) was higher than that observed for *R. flavefaciens*. The requirement of the *R. flavefaciens* enzymes for intact thiol groups and divalent cations is likely to be met by the anaerobic conditions and mineral concentrations encountered *in vivo*.

In common with many other cellulolytic bacteria and fungi, the cellulases of *R. flavefaciens* were most active towards the less ordered cellulose substrates. The production of mainly cellobiose and cellotriose from CM-cellulose, and xylobiose and xylotriose from xylan, suggested that the most active enzymes of *R. flavefaciens* were exo-1,4-β-glycosidases which preferentially hydrolysed the second and third β-glycosidic bonds from the end of the polymers. This was substantiated by the rapid release of reducing-sugar, relative to the increase in specific fluidity, that occurred during enzymic hydrolysis of CM-cellulose and xylan. The slight increase in specific fluidity indicated the presence of weak endo-1,4-β-glycosidases which may have been responsible for the ability of the enzyme preparations to swell cotton fibres (Wood & McCrae, 1978). The small amounts of glucose and xylose released may reflect the low levels of aryl β-glucosidase and aryl β-xylosidase activity. Production of cellobiose as the sole product from enzymic hydrolysis of MMFP and Avicel may be due to the accessibility of the enzyme being restricted to alternate, sterically identical, β-glycosidic bonds in the insoluble substrates (Hungate, 1966).

A proportion of the total cellulase and xylanase activity in the brown-rot fungus *Trichoderma viride* is due to the action of a single enzyme (Toda *et al.*, 1971) and our results indicate that, although the CM-cellulase and xylanase activities of *R. flavefaciens* originate in different active sites, the sites may be present in the same protein complex. Furthermore, the distribution of specific activity towards CM-cellulose, MMFP and Avicel following gel filtration suggests that the same protein complex is responsible for the hydrolysis of both soluble and insoluble cellulose.

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REFERENCES

ANDREWS, P. (1965). The gel filtration behaviour of proteins related to their molecular weights over a wide range. *Biochemical Journal* **96**, 595–606.

CARLSSON, J. (1973). Simplified gas chromatographic procedure for identification of bacterial metabolic products. *Applied Microbiology* **25**, 287–289.

DEHORITY, B. A. (1973). Hemicellulose degradation by rumen bacteria. *Federation Proceedings* **32**, 1819–1825.

DEKKER, R. F. H. & RICHARDS, G. N. (1976). Hemicellulases: their occurrence, purification, properties and mode of action. *Advances in Carbohydrate Chemistry and Biochemistry* **32**, 277–352.

GRADEL, C. M. & DEHORITY, B. A. (1972). Fermentation of isolated pectin and pectin from intact forages by pure cultures of rumen bacteria. *Applied Microbiology* **23**, 332–340.

HALLIWELL, G. (1961). The action of cellulolytic enzymes from *Myrothecium verrucaria*. *Biochemical Journal* **79**, 185–192.

HALLIWELL, G. & RIAZ, M. (1971). Interactions between components of the cellulase complex of *Trichoderma koningii* on native substrates. *Archiv für Mikrobiologie* **78**, 295–309.

HUNGATE, R. E. (1966). *The Rumen and its Microbes*. New York and London: Academic Press.

KING, K. W. (1963). Endwise degradation of cellulose. In *Enzymic Hydrolysis of Cellulose and Related Materials*, pp. 159–170. Edited by E. T. Reese. London: Pergamon Press.

LATHAM, M. J., BROOKER, B. E., PETTIPHER, G. L. & HARRIS, P. J. (1978). *Ruminococcus flavefaciens* cell coat and adhesion to cotton cellulose and to cell walls in leaves of perennial ryegrass (*Lolium perenne*). *Applied and Environmental Microbiology* **35**, 156–165.

LEATHERWOOD, J. M. (1969). Cellulase complex of *Ruminococcus* and a new mechanism for cellulose degradation. In *Cellulases and Their Applications*, Advances in Chemistry Series 95, pp. 53–57. Edited by R. F. Gould. Washington: American Chemical Society.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* **193**, 265–275.

NISIZAWA, K. (1973). Mode of action of cellulases. *Journal of Fermentation Technology* **51**, 267–304.

NISIZAWA, T., SUZUKI, H. & NISIZAWA, K. (1966). Swelling factor activity of *Trichoderma* cellulase for absorbent cotton. *Journal of Fermentation Technology* **44**, 659–668.

PETTIPHER, G. L. & LATHAM, M. J. (1976). Extracellular cellulases and xylanases of *Ruminococcus*

flavefaciens. Proceedings of the Society for General Microbiology **4**, 42.

PIERCE CHEMICAL CO. (1976). In *General Catalog*, 1976–1977, pp. 242–244. Rockford, Ill., U.S.A.: Pierce Chemical Co.

REESE, E. T., SIU, R. G. H. & LEVINSON, H. S. (1950). Biological degradation of soluble cellulose and its relationship to the mechanism of cellulose hydrolysis. *Journal of Bacteriology* **59**, 485–497.

ROMBOUTS, F. M. (1972). *Occurrence and properties of bacterial pectate lyases*. Ph.D. thesis, Wageningen Agricultural University, The Netherlands.

SHARMA, M. (1968). *Immunochemical studies of cellulases from several strains of Ruminococcus flavefaciens*. Ph.D. thesis, North Carolina State University, U.S.A.

SHIKATA, S. & NISIZAWA, K. (1975). Purification and properties of an exo-cellulase component of novel type from *Trichoderma viride*. *Journal of Biochemistry* **78**, 499–512.

SMITH, W. R., YU, I. & HUNGATE, R. E. (1973). Factors affecting cellulolysis by *Ruminococcus albus*. *Journal of Bacteriology* **114**, 729–737.

SUZUKI, H., YAMANE, K. & NISIZAWA, K. (1969). Extracellular and cell-bound cellulase components of bacteria. In *Cellulases and Their Applications*, Advances in Chemistry Series 95, pp. 60–82. Edited by R. F. Gould. Washington: American Chemical Society.

TODA, S., SUZUKI, H. & NISIZAWA, K. (1971). Some enzymic properties and substrate specificities of *Trichoderma* cellulases with special reference to their activity towards xylan. *Journal of Fermentation Technology* **49**, 499–521.

WILKINSON, G. N. (1961). Statistical estimations in enzyme kinetics. *Biochemical Journal* **80**, 324–332.

WOOD, T. M. (1968). Cellulolytic enzyme system of *Trichoderma koningii*. Separation of components attacking native cotton. *Biochemical Journal* **109**, 217–227.

WOOD, T. M. & MCCRAE, S. I. (1975). The cellulase complex of *Trichoderma koningii*. In *Symposium on Enzymatic Hydrolysis of Cellulose*, pp. 231–254. Edited by M. Bailey, T.-M. Enari & M. Linko. Helsinki: The Finnish National Fund for Research and Development (SITRA).

WOOD, T. M. & MCCRAE, S. I. (1978). The cellulase of *Trichoderma koningii*. Purification and properties of some endoglucanase components with special reference to their action on cellulose when acting alone and in synergism with the cellobiohydrolase. *Biochemical Journal* **171**, 61–72.