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METABOLISM OF THE POLYSACCHARIDES OF HUMAN DENTAL PLAQUE

PART II* PURIFICATION AND PROPERTIES OF *Cladosporium resinae* (1→3)-α-D-GLUCANASE, AND THE ENZYMIC HYDROLYSIS OF GLUCANS SYNTHESISED BY EXTRACELLULAR D-GLUCOSYLTRANSFERASES OF ORAL STREPTOCOCCI

GWEN J WALKER AND MARGARET D HARE†

Institute of Dental Research 2 Chalmers St Sydney N S W 2010 (Australia)

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ABSTRACT

Cladosporium resinae (1→3)-α-D-glucanase has been characterized as an endo-glucanase capable of completely hydrolysing insoluble (1→3)-α-D-glucans isolated from fungal cell-walls D-Glucose was the major product, but a small amount of nigerose was also produced The enzyme was specific for the hydrolysis of (1→3) bonds that occur in sequence, and nigerotetraose was the smallest substrate that was rapidly attacked Isolated (1→3)-α-D-glucosidic linkages that occur in mycodextran, isolichenin, dextrans, and oligosaccharides derived from dextran were not hydrolysed Insoluble glucans synthesised from sucrose by culture filtrates of *Streptococcus spp* were all hydrolysed to various limits, the range was 11–61% A soluble glucan, synthesised by an extracellular D-glucosyltransferase of *S mutans* OMZ176, was not a substrate, whereas insoluble glucans synthesised by a different D-glucosyltransferase, isolated from *S mutans* strains OMZ176 and K1-R, were extensively hydrolysed (84 and 92%, respectively) It is suggested that dextranase-CB, a bacterial endo-(1→6)-α-D-glucanase that does not release D-glucose from any substrate, could be used together with *C resinae* (1→3)-α-D-glucanase to determine the relative proportions of (1→6)-linked to (1→3)-linked sequences of D-glucose residues in the insoluble glucans produced by oral streptococci The simultaneous action of the two D-glucanases was highly effective in solubilizing the glucans

INTRODUCTION

(1→3)-α-D-Glucanases[1–4] (EC 3 2 1 59) are capable of hydrolysing (1→3)-α-D-glucans that are components of the cell wall of several species of fungi[5–10] Some (1→3)-α-D-glucanases[3 4 11 12] can also partially degrade the extracellular glucans

*For Part I, see Ref 11
†née Dewar

produced by *Streptococcus mutans* and *Streptococcus sanguis*, and by some strains of *Streptococcus salivarius* These polysaccharides are synthesised from sucrose by D-glucosyltransferases that are released into the culture fluids of D-glucose-grown streptococci[11–18] A glucan isolated in this way from *S mutans* OMZ176 was shown by methylation analysis[16] to contain ~50% of (1→3) linkages The polysaccharide was designated as "mutan"[17 18], so as to distinguish it from dextran, hitherto defined as a polysaccharide composed of a substantial, if not a major, proportion of (1→6)-linked α-D-glucopyranosyl residues

Enzymes specific for the hydrolysis of (1→3)-α-D-glucosidic linkages in polysaccharides were first isolated[1] from culture filtrates of *Trichoderma viride* QM6a and *Penicillium funiculosum* that had grown in a medium containing (1→3)-α-D-glucan (pseudonigeran) isolated from the cell walls of *Aspergillus niger* Purified *T viride* (1→3)-α-D-glucanase[2] was characterized as an endo-glucanase, and the enzyme displayed a broad specificity, hydrolysing such linear glucans as isolichenin and mycodextran, where (1→3)-α-D- and (1→4)-α-D-glucosidic linkages alternate, in addition to pseudonigeran, where (1→3)-α-D-glucosidic linkages occur in sequence[7 33] *T viride* QM6a grown on medium supplemented with mutan did not produce an enzyme capable of hydrolysing mutan[17], but *Trichoderma harzianum* OMZ779 grown under the same conditions produced a "mutanase" that, even after extensive purification, was able to hydrolyse mutan completely[3]

A survey[4] of over 200 micro-organisms revealed seven fungi that produced up to 10-fold higher yields of (1→3)-α-D-glucanase than could be obtained from either *T viride*, or *T harzianum*, when the cultures were grown in a simple salts medium[19] with (1→3)-α-D-glucan from *Polyporus betulinus* tramal tissue as carbon source Among the most active producers of extracellular (1→3)-α-D-glucanase was one organism, *Cladosporium resinae* QM7998, that was unique in its ability to produce (1→3)-α-D-glucanase in the absence of (1→3)-α-D-glucan Equivalent amounts of enzyme were produced whether *C resinae* was grown on starch, D-glucose, or (1→3)-α-D-glucan Partially purified *C resinae* (1→3)-α-D-glucanase was without action on mycodextran[20], but was reported[4] to hydrolyse mutan to the extent of 24% after prolonged incubation

A purified preparation of *C resinae* (1→3)-α-D-glucanase was subsequently incubated with glucans[11] produced from sucrose by twelve strains of *Streptococcus spp* isolated from human dental plaque The limit of hydrolysis of the insoluble *S mutans* glucans was in the range 27–47% Glucans from strains resembling *S sanguis* were more resistant to attack (9–18% hydrolysis), but their degradation resulted in greatly increased solubilization These results might be more easily interpreted if more were known about the mode of action and specificity of *C resinae* (1→3)-α-D-glucanase The limit of hydrolysis of pseudonigeran by endo-(1→3)-α-D-glucanases has not been reported

In this paper, we present a method for isolating *C resinae* (1→3)-α-D-glucanase that is electrophoretically homogeneous The use of polysaccharides and oligosaccharides having a known proportion and distribution of (1→3)-α-D-glucosidic

linkages has enabled the specificity of the enzyme to be defined The conditions necessary to hydrolyse fungal (1→3)-α-D-glucans to the limit have been established Under these conditions, the action of the enzyme has been tested on the glucans synthesised from sucrose by purified D-glucosyltransferases isolated from culture fluids of *S mutans*

MATERIALS AND METHODS

Carbohydrates — (1→3)-α-D-Glucan (pseudonigeran) was isolated from the mycelia of *Aspergillus niger* NRRL326 as described by Hasegawa *et al* [2] The fungus was grown in submerged culture in a 10-litre fermentor at the University of New South Wales, by courtesy of Professor B J Ralph Samples of pseudonigeran and *Polyporus betulinus* glucan were kindly supplied by Dr E T Reese (1→3)-α-D-Glucan from *Polyporus tumulosus*[6] was provided by Dr V J Bender and by Dr D C Ellwood, and *Phytophora infestans* glucan[8] was a gift from Dr T Miyozaki Mutan[18] was synthesised from sucrose by the mixture of D-glucosyltransferases present in culture fluids of *Streptococcus mutans* OMZ176 A sample of mutan was also generously provided by Professor B Guggenheim Insoluble glucans[11] from other strains of *S mutans*, *Streptococcus sanguis*, and *Streptococcus salivarius* were similarly prepared by incubating cell-free filtrates with sucrose (4%) Isolichenin was a generous gift from Dr J R Turvey, and mycodextran was purchased from Sturge Co Ltd *Streptococcus mutans* K1-R (1→3)-α-D-glucan was prepared by the action on sucrose of a purified D-glucosyltransferase[21] isolated from culture filtrates *S mutans* OMZ176 (1→3)-α-D-glucan and *S mutans* OMZ176 soluble glucan were similarly synthesised from sucrose by the action of D-glucosyltransferase-I and D-glucosyltransferase-S, respectively The separation of these two D-glucosyltransferases is described below A mixed-linkage, insoluble glucan was also synthesised from sucrose by the combined action of D-glucosyltransferase-S (0 043 i u) and D-glucosyltransferase-I (0 004 i u)

Nigerose (N_2) and nigerose oligosaccharides (N_3 and N_4) were isolated from a partial, acid hydrolysate of pseudonigeran The polysaccharide (500 mg) was heated with 90% formic acid (7 ml) for 10 min at 100° Water (7 ml) was then added, and heating was continued for 3 h Formic acid was removed by evaporation, and the residue was heated with 0 25M H_2SO_4 (12 ml) for 15 min at 100° After neutralization with $BaCO_3$, and removal of $BaSO_4$, the supernatant solution was concentrated, and the products were separated by preparative chromatography on Whatman No 3MM paper A plot of the logarithm of the partition function against chain length was linear, showing that the oligosaccharides formed a homologous series Authentic samples of nigerose and nigerotriose were also kindly provided by Dr I R Johnston

Dextrans from *Leuconostoc mesenteroides* strains NRRL B-512(F) and B-1355 (fractions S and L) were gifts from Dr A Jeanes The tetrasaccharide *O*-α-glucopyranosyl-(1→3)-*O*-α-D-glucopyranosyl-(1→6)-*O*-α-D-glucopyranosyl-(1→6)-α-D-glucopyranose (3^3-α-D-glucosylisomaltotriose) and the branched heptasaccharide

3^3-α-D-glucosylisomaltohexaose (B_7), which was possibly in admixture with 3^3-α-isomaltosylisomaltopentaose, were isolated[22] from the reaction products of *Penicillium funiculosum* dextranase on dextran B-512(F)

Total carbohydrate was estimated with a cysteine–sulphuric acid reagent[23] Reducing sugars were determined by the method of Nelson[24], and D-glucose was assayed with the D-glucose oxidase reagent as modified by Dahlqvist[25] Separations of glucose, isomaltosaccharides, nigerosaccharides, and branched oligosaccharides were made by paper chromatography on Whatman No 3MM paper with ethyl acetate–pyridine–water (10 4 3), detection was effected with alkaline silver nitrate[26]

Bacteria — *Streptococcus mutans* strains OMZ176, K1-R, and B13 were obtained from Professor B Guggenheim, Dr R J Fitzgerald, and Dr S Edwardsson, respectively, and *Streptococcus sanguis* 804 was from Professor J Carlsson The micro-organisms were grown overnight in a Microferm fermentor (New Brunswick Scientific Co), with the pH controlled at 6 0, under the conditions described previously[11]

Separation of D-*glucosyltransferase-S and* D-*glucosyltransferase-I from culture fluids of* S mutans *OMZ176* — After removing the bacteria by centrifugation at 2° for 10 min at 4,000 *g*, the cell-free supernatant solution (2 l) was concentrated to 38 ml in a hollow-fibre device (DC2, Amicon Corp Lexington, Mass , U S A) The concentrate was dialysed against 50mM sodium citrate buffer (pH 6 5), and then ammonium sulphate was added to give 50% saturation The precipitated protein was dissolved in 50 mM potassium phosphate buffer (pH 6 5), dialysed overnight against the same buffer, and then applied to a column (42 × 3 cm) of hydroxylapatite (Bio-Gel HTP) prepared in the same buffer The column was eluted with a linear gradient of potassium phosphate (pH 6 5), and the peak fractions of D-glucosyltransferase-S, dextranase, and D-glucosyltransferase-I were eluted with 0 07, 0 095, and 0 40M buffer, respectively The combined fractions of D-glucosyltransferase-S were then readsorbed onto hydroxylapatite in 50mM buffer, and by elution with a shallower gradient, the enzyme was obtained free from dextranase The combined fractions of the two D-glucosyltransferases were dialysed against 50mM sodium citrate buffer (pH 6 5) Incubation of D-glucosyltransferase-S with sucrose produced a soluble glucan, whereas glucosyltransferase-I synthesised an insoluble glucan, later shown to be a (1→3)-α-D-glucan The soluble glucan was precipitated and washed with 60% ethanol, dissolved in water, reprecipitated and washed with 75% ethanol, and dissolved in water, and the solution was then freeze-dried The insoluble glucan was washed (× 6) with water, and freeze-dried

Fungi — Cultures of *Cladosporium resinae* QM7998 and *Trichoderma viride* QM9123, and acetone precipitates of (1→3)-α-D-glucanase from culture filtrates of the fungi, were kindly provided by Dr E T Reese

Growth of C resinae *and* T viride — The culture conditions that provided optimal yields of fungal (1→3)-α-D-glucanase were thoroughly examined by Reese *et al* [4], and our growth conditions were based on their recommendations *C resinae* QM7998 spores ($10–15 \times 10^6$) were inoculated into *Trichoderma viride* salts medium[19]

(100 ml) containing a carbon source (0 5 g) and Tween 80 (0 1%) Production of extracellular enzyme capable of hydrolysing mutan was not increased when starch as carbon source was replaced with mutan, or with insoluble glucans from other strains[11] of streptococci isolated from human dental plaque Either starch or *P betulinus* glucan was therefore provided, and the flasks (500 ml) were shaken at 90 strokes/min in a New Brunswick incubator-shaker at 29° The pH rose to 6 5 within 5 days, and remained steady thereafter The mycelia were harvested after 14 days, when enzyme activity in the medium was usually at a maximum

T viride was grown under similar conditions, with *P betulinus* glucan as carbon source

Purification of C resinae *(1→3)-α-D-glucanase* — All operations were carried out at 1–4° Mycelium and cell debris were removed by centrifugation at 34,000 *g* for 20 min

Step 1 Cold acetone (1 volume) was added dropwise to the cell-free filtrate (150 ml), and, after 1 h, the precipitate was removed by centrifugation for 10 min at 34,000 *g* A second volume of acetone was added to the supernatant solution, and the precipitate was likewise collected after 1 h The two precipitates were dissolved in 5 ml of 20mM sodium phosphate buffer (pH 6 0), and dialysed against the same buffer

Step 2 The enzyme, which was mainly recovered in the 50–66% acetone fraction, was applied to a column (50 × 3 cm) packed with DEAE-Sephadex (A-50) that had been washed with the same buffer The column was eluted with a linear gradient of sodium chloride at 11 ml/h (1→3)-α-D-Glucanase activity was eluted with 0 11M sodium chloride Enzymes having activity towards mycodextran and nigerose oligosaccharides were removed in this step, being eluted with 0 22 and 0 26M sodium chloride, respectively (Fig 1)

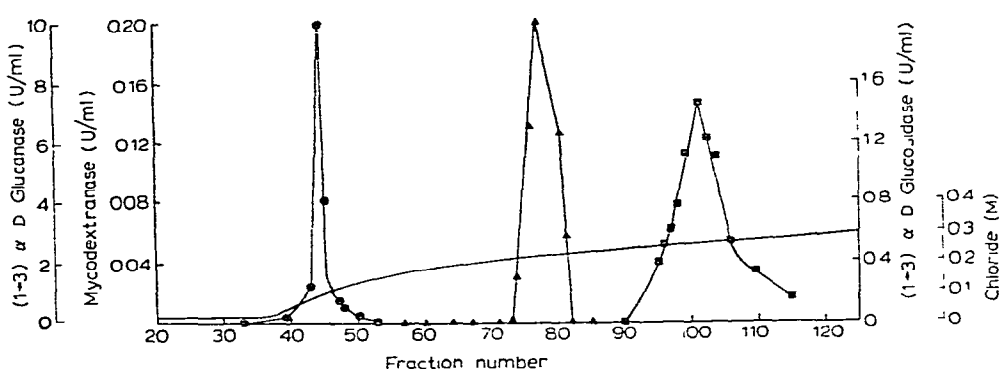

Fig 1 Chromatography of *C resinae* (1→3)-α-D-glucanase on DEAE-Sephadex (A-50) The fraction (44, 6 ml) containing peak activity was prepared for isoelectric focusing ●, (1→3)-α-D-glucanase ▲, mycodextranase, ■, (1→3)-α-D-glucosidase, ———, sodium chloride gradient β-D-Glucanase, active towards laminarin, was eluted in fractions 58–75 and 95–105, amyloglucosidase with activity towards glycogen and maltose, was eluted in fractions 56–62 and 72–82 (not shown)

Step 3 Traces of amyloglucosidase having activity towards glycogen, pullulan, and isomaltosaccharides were removed by isoelectric focusing in an LKB column (110 ml) with a density gradient of glycerol and a pH gradient of 3–6 Peak fractions of (1→3)-α-D-glucanase from step 2 were dialysed against 2M urea containing glycine (1%), and then focused for 96 h with a terminal voltage of 600 V The column was then drained, and fractions devoid of amyloglucosidase were combined A small peak of (1→3)-β-D-glucanase was also separated from (1→3)-α-D-glucanase in this step

Step 4 The enzyme was separated from ampholytes, glycerol, urea, and glycine by gel filtration on Bio-Gel P-10 in 50mM sodium citrate buffer (pH 6 0) A summary of the purification is given in Table I

The isoelectric point of (1→3)-α-D-glucanase was at pH 4 2 The enzyme was homogeneous by the criterion of disc gel-electrophoresis[27]

TABLE I

PURIFICATION OF (1→3)-α-D-GLUCANASE

Purification step	*Enzyme recovery*		*Specific activity (Units/mg of protein)*	*Purification*
	(Units)	*(%)*		
Cell-free filtrate	300	100	7 1	
Acetone fractionation	210	70	11 0	1 6
DEAE-Sephadex chromatography	171	57	28 5	4 0
Isoelectric focusing[a]	43	14[b]	102	14 4

[a]Only the peak fractions from the previous step (51 units specific activity, 45) were taken for isoelectric focusing [b]Recovery in this step was 84%

Determination of (1→3)-α-D-glucanase activity — The activity digest (1 0 ml) contained mutan (7 mg), 10mM sodium citrate buffer (pH 4 5), and enzyme After incubation at 50° for 15 min and 30 min, samples were withdrawn into ice-cold centrifuge tubes, and centrifuged in a cold room at 2,000 *g* for 5 min D-Glucose oxidase reagent (0 75 ml) was added to a portion (0 05 ml) of the supernatant solution, and the mixture (1 0 ml) was incubated for 1 h at 35° One unit of enzyme is defined as the amount that liberates 1 μmol of D-glucose per min in the activity digest The rate of the reaction was not increased by shaking the digests

Determination of mycodextranase activity — The activity digest (1 0 ml) contained mycodextran (5 mg), 10mM sodium citrate buffer (pH 4 5), and enzyme After incubation for 2 h at 40°, a sample was centrifuged at 2°, and a portion (0 25 ml) of the supernatant solution was withdrawn into copper reagent for the determination of reducing power

Determination of activity towards nigerose and isomaltotriose — The activity digests (0 25 ml) contained nigerose (50 μg) or isomaltotriose (250 μg), 10mM sodium citrate (pH 4 5), and enzyme After 30 min at 50°, portions were withdrawn for the

determination of D-glucose with D-glucose oxidase reagent Isomaltotriose digests were also incubated at pH 6 0 and 35° for 5 days when it was necessary to ensure that (1→3)-α-D-glucanase was free from enzymes having activity towards the products of dextranase

Dextranases — *Penicillium funiculosum* QM474 dextranase was induced by shaking the organism for 12 days at 30° in *T viride* salts medium, supplemented with oral dextrans[28] The mycelium was removed by centrifugation, and the clear, supernatant solution was dialysed against 5mM sodium citrate buffer (pH 6 0) and then freeze-dried

Dextranase-CB, a product of Calbiochem, Los Angeles, was described previously[29] This bacterial dextranase required the presence of at least five consecutive (1→6)-α-D-glucosidic linkages before rapid attack could occur The main products from dextran B-512(F) were isomaltotriose and isomaltotetraose, with a smaller amount of isomaltopentaose and traces of isomaltose Isomaltose oligosaccharides having d p >5 were hydrolysed mainly at linkages 3 and 4 (counting from the reducing end) D-Glucose was not a product of hydrolysis of oligosaccharides or dextran The enzyme had no action on pseudonigeran

Limit of enzymic hydrolysis and solubilization of insoluble glucans — Because of the insoluble nature of the substrates and the consequent difficulty of obtaining a true aliquot, several digests for each glucan were prepared in centrifuge tubes, and each digest was sampled only once A determination of total carbohydrate in the supernatant solutions gave the extent of solubilization of the substrates by each enzyme, the glucans did not dissolve when controls were incubated without enzyme Toluene was added to all digests in order to prevent the growth of bacteria

(*a*) *C resinae* (1→3)-α-D-glucanase (0 23 unit) was incubated for a total of 10 days with polysaccharide (1 mg) in 50mM sodium citrate buffer (pH 6 0, 0 29 ml) at 35° At each time-interval, one digest from each set was centrifuged, and portions of the supernatant solution were withdrawn for the determination of D-glucose, reducing sugars, and total carbohydrate More enzyme (0 12 unit) was added after 5 days

(*b*) Dextranase-CB (0 022 unit) was incubated for 5 days with polysaccharide (1 mg) as in (*a*) The release of reducing sugars was expressed as apparent conversion into isomaltotriose Under these conditions, the hydrolysis of dextran B-512(F) was 94%, and *S sanguis* 804 insoluble glucan was degraded to the limit (62%) A higher concentration (up to 20-fold) of enzyme produced no further hydrolysis

(*c*) The combined action of *C resinae* (1→3)-α-D-glucanase (0 23 unit) and dextranase-CB (0 022 unit) was followed in digests (0 29 ml) incubated under the same conditions as for (*a*) and (*b*) Duplicate digests were incubated for 5 and 10 days, respectively Reducing sugar not accounted for as D-glucose and nigerose, was expressed as isomaltotriose, and the hydrolysis (%) was calculated from the sum of D-glucose, nigerose, and apparent isomaltotriose

RESULTS

The ability of *C resinae* (1→3)-α-D-glucanase to hydrolyse mutan did not alter during the various steps in the purification of the enzyme Cell-free filtrate, eluate from DEAE-Sephadex, and the final pool of enzyme from the isoelectric focusing step all hydrolysed mutan to the same limit (40% as glucose), and the extent of solubilization was 50% Therefore, *C resinae* did not produce extracellular enzymes that supplemented the action of (1→3)-α-D-glucanase on mutan Addition of a *Penicillium funiculosum* dextranase-preparation to a digest in which mutan had been hydrolysed to the limit with *C resinae* cell-free filtrate (Fig 2) resulted in a rapid increase in hydrolysis Enzyme precipitated from culture filtrates of *T viride* QM9123 with acetone (1 vol) hydrolysed mutan to a limit of 53%, and its solubilization was 70% This organism does not produce dextranase when grown with *P betulinus* glucan as carbon source[4]

The rate of hydrolysis of mutan by purified *C resinae* (1→3)-α-D-glucanase was compared with that of the essentially linear (1→3)-α-D-glucan, pseudonigeran During the initial stages of the reaction, the relative activity towards pseudonigeran was 6-fold greater than that towards mutan (Fig 3) Finally, pseudonigeran was completely hydrolysed and solubilized The incubation conditions (pH 6 0 and 35°), which were different from those used for activity digests of short duration, provided for optimal stability of the enzyme over the long period necessary to hydrolyse (1→3)-α-D-glucans There was no loss in soluble-enzyme activity after incubation for 4 days, showing that adsorption to the insoluble substrate did not occur Incubation with higher concentrations of enzyme did not accelerate the reaction

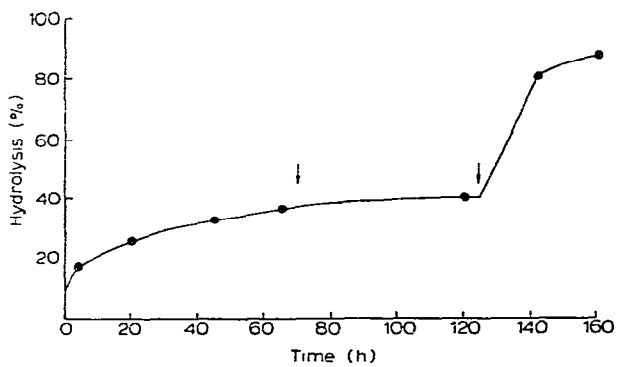

Fig 2 Hydrolysis of mutan with culture filtrate of *C resinae*, followed by that of *P funiculosum* Each digest (0 5 ml) contained mutan (1 mg), sodium citrate buffer (pH 6, 50 mM), and dialysed *C resinae* cell-free filtrate containing 0 5 unit of (1→3)-α-D-glucanase activity, more enzyme (0 25 unit) was added at 70 h (↓) After 125 h (↓), freeze-dried cell-free filtrate of *P funiculosum* (33 μg, containing 0 07 unit of dextranase and 0 05 milliunit of (1→3)-α-D-glucanase) was added to the remaining digests Incubation was at 35°

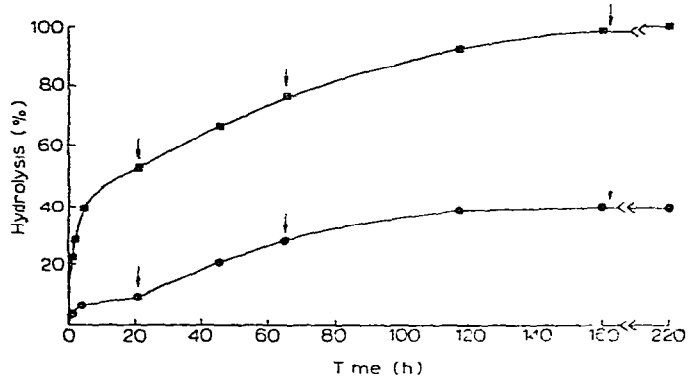

Fig 3 Limit of hydrolysis of pseudonigeran (■) and mutan (●) by a purified preparation of *C resinae* (1→3)-α-D-glucanase Digests (0 5 ml) containing substrate (1 mg), sodium citrate buffer (pH 6, 30mM), and enzyme (0 23 unit) were incubated at 35°, and more enzyme (0 12 unit) was added at 22 h, 66 h, and 160 h Several digests were sampled during the first hour (not shown) so as to compare the relative rates of hydrolysis

Limit of hydrolysis of D-*glucans from fungi, sea-weed, and moss* — The glucans were incubated for 5 and 10 days under the standard conditions for hydrolysis Although, in most cases, the hydrolysis was completed within 5 days, more enzyme was routinely added at 5 days, and the incubation continued for a total of 10 days The results (Table II) showed that only the glucans that contained (1→3)-α-D-glucosidic linkages exclusively were hydrolysed The release of reducing sugar was slightly higher than that of D-glucose, and analysis of the products by paper chromatography showed that nigerose (R_{Glc} 0 85) was present in addition to the major product, D-glucose The extra reducing-power was therefore expressed as disaccharide, and the limit of hydrolysis in Table II was obtained from the sum of D-glucose and nigerose

TABLE II

ACTION OF (1→3)-α-D-GLUCANASE ON VARIOUS D-GLUCANS OF KNOWN STRUCTURE

Source	*Main linkage(s)*	*Limit of hydrolysis* (%)
Aspergillus niger (pseudonigeran)	α-(1→3)	100
Aspergillus niger (mycodextran)	α-(1→3) and α-(1→4)	0
Iceland moss (isolichenin)	α-(1→3) and α-(1→4)	0
Laminaria digitata (laminarin)	β-(1→3)	0
Polyporus tumulosus	α-(1→3)	93
Phytophora infestans	α-(1→3)	100

Limit of hydrolysis of bacterial α-D-*glucans* — Glucans from *Leuconostoc mesenteroides* and *Streptococcus spp* were incubated with *C resinae* (1→3)-α-D-

glucanase under the same conditions as the fungal cell-wall (1→3)-α-D-glucans The streptococcal glucans were all hydrolysed to various extents, and the values for the limit of hydrolysis of glucans from *S mutans* OMZ176 and *S sanguis* 804 were in agreement with their known content[30] of (1→3)-linked α-D-glucose residues (Table III) The leuconostoc dextrans, on the other hand, were totally resistant to hydrolysis, despite the presence of 40% of (1→3)-linked α-D-glucose residues in one sample (strain B-1355, fraction S)

TABLE III

ACTION OF (1→3)-α-D-GLUCANASE ON BACTERIAL α-D-GLUCANS[a]

Micro-organism	*Strain*	*(1→3)-linkages*[b] (%)	*Limit of hydrolysis* (%)
Leuconostoc mesenteroides	B-512(F)	0	0
	B-1355(S)	40	0
	B-1355(L)	—	0
Streptococcus mutans	OMZ176	49	40
	K1-R	—	49
	B13	—	61
Streptococcus sanguis	804	18	16
Streptococcus salivarius	ATCC13419	—	40

[a]Synthesised from sucrose by cell-free filtrates [b]Determination of 2,4,6-tri-*O*-Me-Glc by methylation analysis[30]

TABLE IV

ACTION OF DEXTRANASE-CB AND (1→3)-α-D-GLUCANASE ON *S mutans* α-D-GLUCANS

Strain	*Enzyme*	*Glucan*	*Limit of hydrolysis and solubilization*[a] (%)		
			C resinae *(1→3)*-α-D-*glucanase*	*Dextranase-CB*	*(1→3)*-α-D-*Glucanase+ dextranase*
OMZ176	CFF[b]	Mutan	40 (51)	22 (46)	51 (92)
	GTF[c]-I	(1→3)-α-D-Glucan	84 (100)	4 (7)	96 (100)
	GTF-S	Soluble glucan	0	15	22
	GTF-I+S	Mixed glucan	31 (39)	11 (62)	39 (86)
K1-R	CFF	Crude glucan	49 (54)	5 (21)	65 (94)
	GTF-I	(1→3)-α-D-Glucan	92 (100)	4 (17)	96 (100)
B13	CFF	Crude glucan	61 (81)	3 (21)	68 (93)

[a]Values in parentheses refer to solubilization [b]CFF, cell-free filtrate [c]GTF, D-glucosyltransferase

Limit of hydrolysis of glucans synthesised by isolated D-*glucosyltransferases and cell-free filtrates of* S mutans — The action of *C resinae* (1→3)-α-D-glucanase on the insoluble glucans synthesised by D-glucosyltransferases-I of *S mutans* K1-R and

OMZ176 was compared with that on insoluble glucans synthesised by untreated cell-free filtrates of *S mutans* strains (Table IV) The extent of hydrolysis of the former was high (92 and 84%, respectively) and their solubilization was complete, thus indicating that they were essentially (1→3)-α-D-glucans Glucose and a trace of nigerose were the products seen from both glucans By contrast, *S mutans* OMZ176 soluble glucan was completely resistant to hydrolysis, the "mixed" glucan gave an intermediate result, similar to that of mutan

The extent of hydrolysis by dextranase-CB also showed marked differences, being very low (~4%) for insoluble glucans of strains K1-R and B13, and for (1→3)-α-D-glucans of K1-R and OMZ176, whereas the hydrolysis of soluble glucan, "mixed glucan", and mutan from strain OMZ176 was in the range 11–22% The simultaneous action of dextranase-CB and (1→3)-α-D-glucanase resulted in extensive solubilization of all the insoluble glucans Analysis by paper chromatography indicated that the extent of hydrolysis by dextranase-CB was being underestimated when calculated as isomaltotriose, for the soluble glucan gave small spots, and most of the crude glucans (mutans) gave large spots, corresponding to branched tetra- and penta-saccharides (R_{Glc} 0 25 and 0 13, respectively), as well as isomaltotriose (R_{Glc} 0 35) and isomaltotetraose (R_{Glc} 0 19) The exception were *S mutans* B13 and K1-R crude glucans, hydrolysis of these glucans by the two glucanases, acting together, gave similar products to those seen on paper chromatograms of digests containing (1→3)-α-D-glucanase alone Glucose was the major product, with traces of nigerose, isomaltosaccharides were not visible

Mechanism of action of (1→3)-α-D-*glucanase* — *S mutans* K1-R (1→3)-α-D-glucan, synthesised from sucrose with D-glucosyltransferase-I, was selected as the substrate for a study of the action of purified *C resinae* (1→3)-α-D-glucanase on a *S mutans* (1→3)-α-D-glucan

The configuration of D-glucose released by (1→3)-α-D-glucanase was determined with D-glucose oxidase (β-D-glucose oxygen 1-oxidoreductase) (1→3)-α-D-Glucan (7 mg) was incubated with enzyme (0 016 unit) at pH 6 and room temperature for 7 5 min The digest was centrifuged, and then one portion of the supernatant solution was incubated with D-glucose oxidase reagent, and another portion was treated with 4% aqueous sodium carbonate before addition of D-glucose oxidase The rate of oxidation at room temperature was followed on the spectrophotometer (Fig 4) The mutarotated sample was oxidized more slowly than the untreated sample indicating that the enzyme had released β-D-glucose

The degree of polymerization of the soluble products of the reaction was determined during the very early stages of the hydrolysis When the extent of hydrolysis of (1→3)-α-D-glucan was 0 7%, 1 4%, and 2 7%, the d p of the products, calculated from the ratio of solubilized carbohydrate reducing equivalents[31], was 1 9, 1 2, and 1 1, respectively

(1→3)-α-D-Glucans of *S mutans* strains OMZ176 and K1-R were treated with periodate and borohydride, so as to modify the D-glucose residue at the non-reducing end of the chains[32] The rate of hydrolysis of the modified glucans, determined in

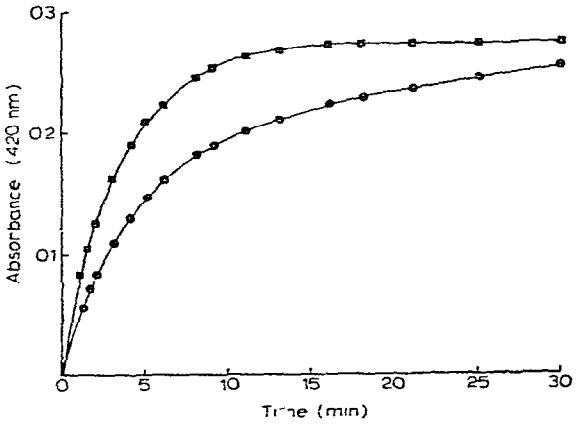

Fig 4 Determination of the configuration of D-glucose released by (1→3)-α-D-glucanase The rate of oxidation of an untreated sample (■) was compared with that of a digest sample that had been mutarotated (●) before addition of D-glucose oxidase

standard activity digests, was 80–85% of that of the untreated glucans The limit of hydrolysis of the modified glucans from strains OMZ176 and K1-R was 87 and 93%, respectively

The affinity of *C resinae* (1→3)-α-D-glucanase for K1-R (1→3)-α-D-glucan and for mutan was compared by incubating the enzyme with a range of substrate concentrations (1 5 to 30 mg per ml) Lineweaver–Burk plots gave a K_m value of 33 g/l for mutan and 2 4 g/l for (1→3)-α-D-glucan

Hydrolysis of oligosaccharides — Digests (0 5 ml) containing nigerose oligosaccharides and isomaltose oligosaccharides (0 75 μmol) were incubated with enzyme (0 4 unit) at pH 4 5 and 40° The rate of release of D-glucose from nigerotetraose was initially high, but later became closer to that of nigerotriose (Fig 5) Isomaltose,

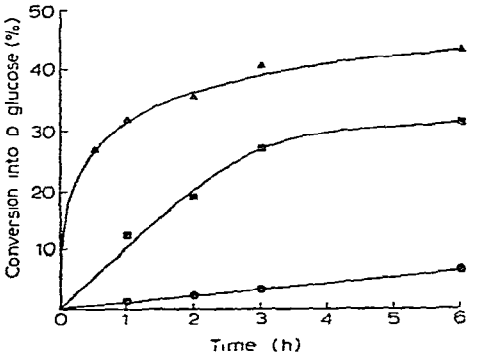

Fig 5 Relative rate of hydrolysis of nigerose (●), nigerotriose (■), and nigerotetraose (▲) by (1→3)-α-D-glucanase

isomaltotriose, 3^3-α-D-glucosylisomaltotriose, and the branched heptasaccharide B_7 were not hydrolysed during incubation under these conditions for 24 h Nigerose was hydrolysed at the rate of 1% per hour in this experiment, and the disaccharide should therefore not have persisted among the products of digestion of (1→3)-α-D-glucans Inhibition of its hydrolysis in the presence of a large excess of D-glucose could explain the survival of small amounts of nigerose after exhaustive hydrolysis of (1→3)-α-D-glucans

The value of K_m for nigerotetraose was $0\,64 \times 10^{-3}$M (Fig 6) This low value illustrates the affinity of the enzyme for soluble oligosaccharides of the nigerose series, and explains why these products were never observed in samples taken for p c during the hydrolysis of (1→3)-α-D-glucans

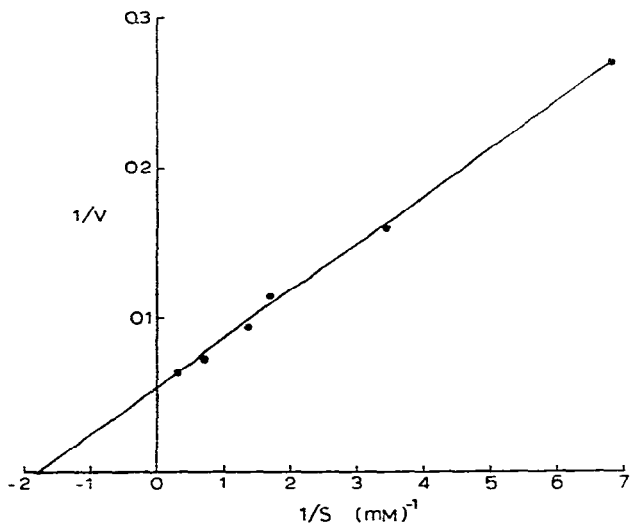

Fig 6 Lineweaver–Burk plot of the action of (1→3)-α-D-glucanase on nigerotetraose Digests (0 25 ml) containing enzyme (0 008 unit), sodium citrate buffer (20mM) and a range of substrate concentrations were incubated at 40° After 15 min samples were withdrawn for the specific determination of D-glucose

DISCUSSION

Investigations[33] into the structure of *A niger* NRRL326 (1→3)-α-D-glucan (pseudonigeran) by the techniques of periodate oxidation, Smith degradation, methylation analysis, and partial hydrolysis with acid, indicated that pseudonigeran is a linear glucan containing at least 98% of α-(1→3)-linked D-glucose residues The remaining 2% were α-(1→4)-linked D-glucose residues, possibly derived from traces of nigeran (mycodextran) which is associated with (1→3)-α-D-glucan in fungal cell-walls Similar studies[6] revealed that an alkali-soluble α-D-glucan from *Polyporus tumulosus* cell-wall is also an essentially unbranched polymer of (1→3)-linked D-

glucose residues The d p of native pseudonigeran was reported[2] to be 700, whereas pseudonigeran, purified by an additional acetylation step, had[33] a d p of 330 Our preparation of pseudonigeran was completely converted into D-glucose and nigerose by the purified (1→3)-α-D-glucanase from *Cladosporium resinae* (1→3)-α-D-Glucans extracted from cell walls of *Polyporus tumulosus*, *Polyporus betulinus*, and *Phytophora infestans* were also extensively hydrolysed (Table II)

Chromatography on DEAE-Sephadex permitted the separation of three *C resinae* enzymes having activity towards substrates containing (1→3)-α-D-glucosidic linkages (Fig 1) The three enzymes were specific for the hydrolysis of (1→3)-α-D-glucan, mycodextran, and nigerose oligosaccharides, respectively The (1→3)-α-D-glucanase was further purified by isoelectric focusing, and its action was then tested on various glucans and oligosaccharides under the same conditions of enzyme concentration and reaction time that were required to achieve complete hydrolysis of (1→3)-α-D-glucans

Nigerotetraose (N_4) was the smallest nigerose oligosaccharide that was rapidly attacked by (1→3)-α-D-glucanase The initial products of the reaction, D-glucose and nigerotriose (R_{Glc} 0 72) seen on paper chromatograms, indicated that the inner linkage in N_4 was not hydrolysed, and that nigerose is produced later from secondary attack on nigerotriose The rate of reaction with N_4 fell sharply after the release of one molar proportion of glucose Nigerotriose was more slowly attacked, and the hydrolysis of nigerose was extremely slow (Fig 5), some preparations of (1→3)-α-D-glucanase were without detectable action on nigerose[11]

The results with nigerose oligosaccharides indicated that *C resinae* (1→3)-α-D-glucanase required substrates having two or more consecutive (1→3)-α-D-glucosidic linkages In accord with this view was the inability of the enzyme to hydrolyse mycodextran, in which (1→3)-linked α-D-glucose residues alternate with (1→4)-linked α-D-glucose residues, or to have any action on *L mesenteroides* NRRL B-1355 dextran (fraction S, with 40% of (1→3)-α-D-glucosidic linkages), in which the (1→3)-α-linkages alternate with (1→6)-α-D-glucosidic linkages Isolichenin, a linear glucan which consists of sequences where either one or two (1→3)-α-linked D-glucose residues alternate with adjacent (1→4)-α-linked residues, was also totally resistant to *C resinae* (1→3)-α-D-glucanase The enzyme had no action on the single (1→3)-α-D-glucosidic linkage at the non-reducing end of 3^3-α-D-glucosylisomaltotriose, or on the (1→3)-α-D-glucosidic branch-linkage in 3^3-α-D-glucosylisomaltohexaose *L mesenteroides* NRRL B-512(F) dextran [with 5% of (1→3)-α-D-glucosidic branch-linkages] and *L mesenteroides* NRRL B-1355 [fraction L, with 12% of (1→3)-α-D-glucosidic branch-linkages] were totally resistant to (1→3)-α-D-glucanase (Table III) These results show that the enzyme was free from all traces of enzymes, *e g*, amyloglucosidase, which have a limited ability to attack (1→6)-α-D-glucosidic linkages in oligosaccharides and dextran

The complete hydrolysis of linear (1→3)-α-D-glucans of fungal origin, and the total resistance of dextrans containing (1→3)-α-D-glucosidic branch linkages or isolated (1→3)-α bonds within the main chain, indicated the suitability of *C resinae*

(1→3)-α-D-glucanase for revealing consecutive (1→3)-α-D-glucosidic linkages in glucans The polysaccharide defined as mutan, synthesised by culture filtrates of *S mutans* OMZ176, was already known to be hydrolysed to the limit of ~40%, as glucose[11 20] Direct adsorption of protein from cell-free filtrates of *S mutans* OMZ176 on to hydroxylapatite had previously[18] led to the elution of two peaks of D-glucosyltransferases, each of which yielded multiple D-glucosyltransferase peaks on subsequent isoelectric focusing All of these D-glucosyltransferases synthesised insoluble glucan In the present work, where chromatography on hydroxylapatite was preceded by ammonium sulphate fractionation two D-glucosyltransferases were also obtained The first of these, D-glucosyltransferase-S, converted sucrose into a soluble glucan which was not a substrate for *C resinae* (1→3)-α-D-glucanase The second D-glucosyltransferase synthesised an insoluble glucan which was 84% hydrolysed by the (1→3)-α-D-glucanase (Table IV) A D-glucosyltransferase of *S mutans* K1-R, isolated by a similar procedure, synthesised an insoluble glucan which was hydrolysed to the extent of 92% These two insoluble polysaccharides can therefore be designated as (1→3)-α-D-glucans The limit of hydrolysis of the insoluble glucans from both strains of *S mutans* was raised to 96% when *C resinae* (1→3)-α-D-glucanase acted in concert with a bacterial dextranase (Calbiochem) Dextranase-CB, when acting alone, gave 4% hydrolysis of each insoluble glucan, and small amounts of IM_3 and IM_4 were seen on paper chromatograms of the reaction products This result indicates the presence of consecutive (1→6)-α-linked D-glucose residues (perhaps an average of 16 per molecule of mol wt [16] ~70,000), which could represent the original primer within each molecule Stimulation of synthesis of *S mutans* 6715 glucan was reported[34] to occur with isomaltosaccharides of d p >8 and primer efficiency increased with chain length, the saccharide with d p 23 being as effective as dextran

Further studies on (1→3)-α-D-glucanase were carried out with *S mutans* K1-R (1→3)-α-D-glucan This substrate was chosen because of its extensive hydrolysis and its low K_m value, furthermore, the glucan originates from a well-studied cariogenic strain of *S mutans* The enzyme was considered to be an endo-glucanase according to two criteria Modification of the glucose residue at the non-reducing terminal by periodate oxidation, followed by borohydride reduction[32], had little influence on the rate of attack and did not alter the limit of hydrolysis of (1→3)-α-D-glucan In addition, use was made of a sensitive technique[31] that can distinguish between the action of exo- and endo-glucanases on insoluble substrates A concave curve, indicative of an endo-glucanase, was obtained by plotting the average d p of the products in the reaction mixture against the release of glucose The average d p (1 9) at 0 7% hydrolysis quickly fell to 1 1, and thereafter the extent of hydrolysis determined by reducing power (expressed as D-glucose) was close to that determined with D-glucose oxidase Clearly, the concentration of enzyme used to degrade the insoluble polysaccharide rapidly hydrolysed the solubilized fragments to glucose No oligosaccharides other than small spots of nigerose were ever seen in samples taken for analysis by p c at various stages in the hydrolysis of bacterial or fungal

(1→3)-α-D-glucans The endo-action of *C resinae* (1→3)-α-D-glucanase is thus supported by the same evidence that finally characterized *T viride* (1→3)-α-D-glucanase[2] as an endo-glucanase

Ebisu *et al*[35] proposed that mutan is a highly branched D-glucan having a main chain containing long sequences of (1→3)-α-linked D-glucose residues, possibly flanked by (1→6) linkages, but with most of the (1→6)-linked D-glucose residues located in side chains An endo-mechanism would permit *C resinae* (1→3)-α-D-glucanase to hydrolyse sequences of (1→3)-α-D-glucosidic linkages in mutan, whether they occur within the molecule or as external chains Since no evidence was found for (1→3)-linked D-glucose residues at the non-reducing terminals of the side chain in mutan[35], the polysaccharide would be resistant to hydrolysis by an exo-(1→3)-α-D-glucanase

Dextranase-CB, an endo-(1→6)-α-D-glucanase, should hydrolyse unbranched chains of (1→6)-α-linked D-glucose residues, regardless of their position However, the insoluble glucans that were not fully hydrolysed by *C resinae* (1→3)-α-D-glucanase were still not completely hydrolysed when dextranase-CB was also present, although all the glucans were solubilized to the extent of 86% or more (Table IV) The incomplete hydrolysis could be explained by a ramified structure of (1→3)-α-D-glucan chains intersected by (1→6)-linked glucan chains The requirement of dextranase-CB for 6 consecutive (1→6)-linked D-glucose residues[29] was not a limiting factor, for *P fumiculosum* dextranase, which will hydrolyse isomaltotriose, hydrolysed mutan to the same extent[12] (20–22%) Short side-chains consisting of only one or two (1→6)-linked D-glucose residues would not be hydrolysed by endo-dextranases

We can assume that soluble glucan does not contain long sequences of (1→3)-linked D-glucose residues The limited hydrolysis by dextranase-CB (15%), and by *S mutans* endo-dextranase[21] (17%), indicates the presence of branch points or isolated (1→3) bonds in the main chain, soluble glucan was totally resistant to exo-dextranases[36] A chemical and enzymic analysis of the structure of soluble glucan is now in progress

The value of the particular combination of endo-glucanases, dextranase-CB with *C resinae* (1→3)-α-D-glucanase, chosen for this work, lies in the ease of distinguishing between their reaction products A specific determination of D-glucose indicates the hydrolysis of (1→3) bonds, with an accuracy of $\succ 90\%$ Addition of a specific (1→3)-α-D-glucosidase[20] would be necessary to hydrolyse the minor product nigerose, and so give a complete conversion into D-glucose The release of other reducing sugars would then represent the hydrolysis of (1→6)-α-D-glucosidic linkages by dextranase-CB Since dextranase-CB has no action on pseudonigeran, and *C resinae* (1→3)-α-D-glucanase does not hydrolyse (1→6)-α-D-glucans, the two glucanases can contribute to the structural analysis of polysaccharides by providing values for the relative proportions of (1→3)- and (1→6)-α-linked sequences of D-glucose residues The results in Table III indicate that insoluble glucans from five strains of *Streptococcus spp* contain different proportions of sequential (1→3)-α-linked D-glucose residues When combined with earlier results[11] obtained with

C resinae (1→3)-α-D-glucanase, the enzymic hydrolyses reveal a range of 11–61% of susceptible (1→3)-α-D-glucosidic linkages in the insoluble glucans synthesised from sucrose by *Streptococcus spp* The proportion of (1→3)-α-linked D-glucosyl residues in each glucan will depend on the relative activities of the D-glucosyltransferases involved, and although these activities will change with the conditions of growth of each organism, the results illustrate the existence of strain and species differences, because each micro-organism was grown in the same way Mutan preparations from two laboratories were hydrolysed to the same extent (40%) by *C resinae* (1→3)-α-D-glucanase Thus, the synthesis of a particular insoluble glucan can be reproduced when the *S mutans* strain is grown under standard conditions

T viride (1→3)-α-D-glucanase[2] has a wider specificity than *C resinae* (1→3)-α-D-glucanase, and also degrades mutan more extensively The *T viride* enzyme may hydrolyse certain isolated (1→3) bonds, or those adjacent to (1→6) bonds, that are resistant to the *C resinae* enzyme The (1→3)-α-D-glucanase of *T harzianum*[3], another inducible endo-glucanase, was reported to solubilize and hydrolyse mutan completely An enzyme of such broad specificity could not be used to distinguish between the different α-D-glucosidic linkages in *S mutans* glucan Other inducible glucanases, produced for example by *Penicillium lilacinum*, *P funiculosum*, and *Spicaria violacea*, are also unsuitable for exploring the structure of streptococcal glucans that contain substantial amounts of both (1→3)- and (1→6)-α-linked D-glucose residues This is because these fungi produce (1→3)- and (1→6)-α-D-glucanases together, their proportions being dependent on the growth conditions, in particular the nature of the inducer Therefore, unpurified, commercial dextranases of unspecified origin should not be used in attempts to distinguish between the various glucans synthesised by D-glucosyltransferases of *S mutans* strains The conflicting results published for the limit of hydrolysis and solubilization of mutan by glucanohydrolase preparations from *P funiculosum*[12 17 37] and *T harzianum*[3 12] could be due to the difficulty of isolating specific glucanases from these organisms Constitutive enzymes having narrow specificities could provide more consistent results Recently, another constitutive (1→3)-α-D-glucanase, from *Flavobacterium sp* EK-14[38], was reported to give a limited, specific degradation of mutan, the products of the hydrolysis (11%, as glucose) being oligosaccharides of the nigerose series The extent of solubilization of mutan (~50%) was similar to that reported here for the constitutive endo-(1→3)-α-D-glucanase of *C resinae*

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