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Studies on Cellulolytic Enzymes I

The Use of 3,4-Dinitrophenyl Glycosides as Substrates

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The syntheses of 3,4-dinitrophenyl β-D-glucoside, β-cellobioside, β-cellotrioside, and β-cellotetraoside and their use to monitor the purification of two enzymes from a crude commercial cellulase preparation from *Trichoderma viride* are described. The enzymes isolated are an *endo*-β-1,4-D-glucan glucanohydrolase (EI) of molecular weight ca. 12 000 which catalysed the release of 3,4-dinitrophenol from 3,4-dinitrophenol-β-cellotetraoside, and an enzyme of molecular weight about 76 000 which catalysed the hydrolysis of 3,4-dinitrophenyl β-D-glucoside (EII) and is probably a cellobiase or *exo*-β-1,4-D-glucan glucohydrolase. Kinetic parameters are reported for the hydrolyses of 3,4-dinitrophenyl β-cellobioside, β-cellotrioside, and β-cellotetraoside catalysed by enzyme EI. In the presence of cellotriose, cellotetraose, or cellopentaose 3,4-dinitrophenyl β-D-glucoside underwent induced hydrolyses by EI. Similar but faster induced hydrolyses were shown by 3,4-dinitrophenyl β-D-xyloside and 3,4-dinitrophenyl β-D-6-deoxyglucoside; 3,4-dinitrophenyl 6-chloro-6-deoxy-β-D-glucoside and 3,4-dinitrophenyl 6-*O*-methyl-β-D-glucoside underwent slower induced hydrolyses than the glucoside. *p*-Nitrophenyl β-D-glucoside also underwent an induced hydrolysis in the presence of cellopentaose and the enzyme EI, but *p*-nitrophenyl 2-deoxy-β-D-glucoside did not. These results are discussed and compared with the results obtained previously on induced hydrolyses found with lysozyme. Kinetic parameters are reported for the hydrolysis of 3,4-dinitrophenyl and *p*-nitrophenyl β-D-glucosides catalysed by the enzyme EII. 3,4-Dinitrophenyl 6-deoxy-β-D-glucoside, β-D-xyloside, 6-chloro-6-deoxy-β-D-glucoside, 6-*O*-methyl-β-D-glucoside and *p*-nitrophenyl-β-D-galactopyranoside and 2-deoxy-β-D-glucopyranoside were hydrolysed 10^2 to 10^3 times slower by EII than the corresponding glucosides, but 3,4-dinitrophenyl 2-acetamido-2-deoxy-β-D-glucoside was only hydrolysed about 25 times slower than 3,4-dinitrophenyl β-D-glucoside. The significance of these results is discussed. EII catalysed the release of 3,4-dinitrophenol from 3,4-dinitrophenyl β-cellobioside, β-cellobioside, and β-cellotetraoside, but these reactions showed induction periods which are consistent with stepwise removal of glucose residues from the oligosaccharide chains before release of the phenol.

INTRODUCTION

Several types of substrates have been used for cellulolytic enzymes (*1*, *2*), but all of these have some disadvantages (*3*, *4*). Although the ultimate efficiency of a cellulase must be measured by its ability to degrade crystalline cellulose, this and noncrystalline cellulose are not good substrates for many types of investigation, especially mechanistic ones, since they are insoluble, do not have a sufficiently well-defined structure, and have many similar but slightly different points of attack. Carboxymethylcellulose[1] and

[1] Abbreviations used: Glc, D-glucose; GlcNAc, *N*-acetyl-D-glucosamine; DNP, dinitrophenyl; PNP, *p*-nitrophenyl; CMC, carboxymethylcellulose; HEW, hens' egg white.

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hydroxyethylcellulose have the last two of these disadvantages and in addition pose the problem of "what is the effect of the substituent?" Thus, with some enzymes it may be small and with others large, but in general this is not known. The use of cello-oligosaccharides (*5*) or modified cello-oligosaccharides (*6*, *7*) overcomes some of these problems. These substrates are water soluble and have a well-defined structure, but they are usually attacked at more than one position by the enzyme, and the most easily used analytical method (determination of reducing power) does not distinguish between the different reactions which take place. It has recently been shown (*8*, *9*) that 3,4-dinitrophenyl tri-*N*-acetyl-β-chitotrioside, $(GlcNAc)_3$ 3,4-DNP, and 3,4-dinitrophenyl tetra-*N*-acetyl-β-chitotetraoside, $(GlcNAc)_4$ 3,4-DNP, are useful substrates for the investigation of lysozyme, and it was therefore decided to investigate the use of 3,4-dinitrophenyl glycosides of β-D-glucose and β-cello-oligosaccharides, $(Glc)_n$ 3,4-DNP, $n = 1,4$, as substrates for cellulolytic enzymes. Although these may undergo attack at more than one position, measurement of the formation of 3,4-dinitrophenol by uv spectrophotometry measures just one process. Since at zero time only the substrate originally introduced is present, the initial rate of formation of 3,4-dinitrophenol is the rate of cleavage of one bond of one well-characterized substrate. The disadvantages of this type of substrate are (i) that if binding of the "aglycone portion" of the natural substrate is important, the effect of this on the catalytic efficiency will be lacking with 3,4-dinitrophenyl glycosides since a 3,4-dinitrophenyl group would be bound differently, from the β-D-glucan chain or possibly not at all, and (ii) the modes of fission which do not involve release of 3,4-dinitrophenol are not so easily measured as that which does. However, this should be no more difficult than the determination of the different modes of fission of other chemically modified cello-oligosaccharides (*3*, *6*, *7*).

Several workers have previously used *p*-nitrophenyl β-cellobioside as a substrate for *endo*-β-1,4-glucanohydrolases (*10–15*), but this work has usually been restricted to an investigation of the relative amounts of cleavage at the two possible positions. There has been one report of the use of *p*-nitrophenyl β-cellotetraoside (*16*), but the use of this substrate was not developed. The advantages of using a 3,4-dinitrophenyl glycoside rather than a *p*-nitrophenyl one are that the proportion of cleavage of the C_1–OAr bond should be greater and that the concentration of the anion of the liberated phenol is higher at acidic pHs which makes the kinetic measurements easier, as the difference in uv spectrum between the glycoside and the phenolate ion is greater than between that of the glycoside and unionized phenol.

PREPARATION OF SUBSTRATES

A mixture of acetylated cello-oligosaccharides was prepared by acetolysis of cellulose. The components were separated on a silica column in a similar manner to that used for the separation of acetylated chitin oligosaccharides (*9*). The acetylated cello-oligosaccharides were less polar than the acetylated chitin oligosaccharides. This difference meant that the solvent composition used for the chromatography had to be controlled more carefully; but the greater solubility of the acetylated cello-oligosaccharides and their derivatives meant that there were no problems of the

solubility of certain intermediates, as encountered when working with acetylated chitin oligosaccharides (*9*). The acetylated cello-oligosaccharides were converted to the 3,4-dinitrophenyl glycosides as outlined under Experimental.

PURIFICATION OF ENZYMES

Several crude cellulase preparations were examined for their activity towards the 3,4-dinitrophenyl glycosides. Since it was intended to isolate an enzyme with a high activity towards 3,4-dinitrophenyl β-cellotetraoside, the crude enzyme with the highest ratio of activity towards $(Glc)_4$ 3,4-DNP was used, i.e., that from *Trichoderma viride* supplied by BDH. This crude enzyme also hydrolysed $(Glc)_4$ 3,4-DNP faster than $(Glc)_2$ 3,4-DNP (cf. Fig. 3). The enzyme was purified by successive chromatography on DEAE–Sephadex A-25 and Sephadex G-75 (twice) as described under Experimental. The residual β-glucosidase activity was then removed by affinity chromatography. The resultant enzyme, EI, catalysed the release of 3,4-dinitrophenol 133 times faster from $(Glc)_4$ 3,4-DNP than from $(Glc)_1$ 3,4-DNP under the standard assay conditions (Fig. 11). A second enzyme, EII, with a high activity towards $(Glc)_1$ 3,4-DNP was also isolated.

PROPERTIES OF ENZYME EI

This enzyme causes a rapid decrease in the viscosity of CMC (cf. Fig. 15) and is therefore most likely an *endo*-β-1,4-D-glucan glucanohydrolase. Its molecular weight is ca. 12 000 on the basis of gel filtration, and it is therefore very similar to or identical with the CMCase of Selby and Maitland (*17*) and the endoglucanase I of Berghem *et al.* (*18*). It also catalyses the hydrolysis of xylan; and so the possibility cannot, at present, be excluded that it is really an endoxylanase with the ability to hydrolyse β-1,4-D-glucans, but for the time being this will be assumed not to be so. An enzyme which was considered to be a xylanase was isolated from "Meicellase," a crude commercial cellulase (*19*), but its activity towards cellulose and CMC was not reported. The ability of enzymes designated cellulases to catalyse the hydrolysis of xylan has been noted before by several workers (*20–27*). Clearly it is not a simple problem to decide whether such an enzyme is really a cellulase or a xylanase. Presumably this could only be decided by determining which polysaccharide is its inducer.

Enzyme EI catalyses the release of 3,4-dinitrophenol for all the glycosides $(Glc)_n$ 3,4-DNP with $n = 1,4$. The glucoside ($n = 1$) reacts 133 times more slowly than the cellotetraoside ($n = 4$) under the standard assay conditions, and we cannot be certain that this slight activity does not arise from an impurity. However, repassage of the EI through an affinity column for β-D-glucosidases did not remove this activity, which suggests that it is a property of the β-1,4-D-glucan glucanohydrolase itself.

The values of k_{cat}/K_m for the cellobioside, cellotrioside, and cellotetraoside are given in Table 1. They increase about twofold on going from the cellobioside to the trioside, but only slightly on going from the trioside to the tetraoside. This observation suggests that there are three binding subsites for the glycone portion of the substrate

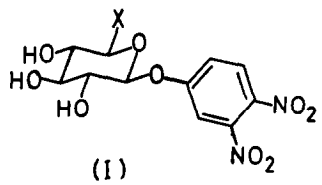
TABLE 1

KINETIC PARAMETERS FOR THE HYDROLYSIS OF 3,4-DINITROPHENYL GLYCOSIDES CATALYSED BY ENZYME EI AT 40°C in ACETATE BUFFER, pH 5.02, $I = 0.1\ M$

	$(Glc)_2$ 3,4-DNP	$(Glc)_3$ 3,4-DNP	$(Glc)_4$ 3,4-DNP
$10^2\ k_{cat}(sec^{-1})$	9.7	20.3	21.7
$10^4\ K_m(M)$	6.02	6.08	5.13
$k_{cat}/K_m(M^{-1}\ sec^{-1})$	161	334	423

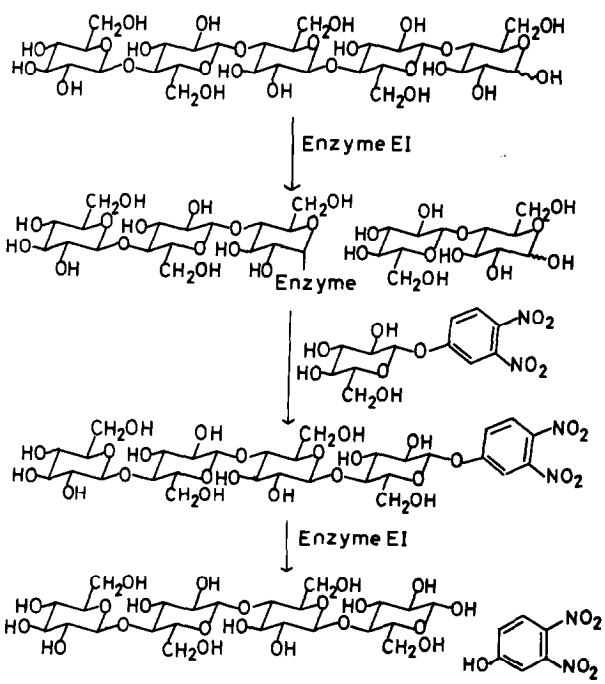
(see Fig. 1). This situation contrasts with that obtaining for HEW lysozyme, in which case the value of k_{cat}/K_m increases about 30-fold on going from $(GlcNAc)_3$ 3,4-DNP to $(GlcNAc)_4$ 3,4-DNP (*8*, *9*), consistent with the presence of four binding subsites for the glycone portion of the substrate (*28*).

As described under Experimental the enzyme EI carries out an induced hydrolysis of 3,4-dinitrophenyl β-D-glucoside (I, $X = CH_2OH$) in the presence of cellotriose,



(I)

cellotetraose, and cellopentaose (Table 11). This behaviour is similar to that found with HEW lysozyme and aryl 2-acetamido-2-deoxy-β-D-glucosides in the presence of β-1,4-linked oligosaccharides of *N*-acetyl-D-glucosamine (*29*, *9*), and presumably the reaction



Scheme 1

involves a similar pathway (Scheme 1). The enzyme first catalyses fission of the cello-oligosaccharide; and the resulting intermediate, either a carbonium ion or a glycosyl enzyme, is captured by the 3,4-dinitrophenyl β-D-glucoside. The enzyme then catalyses release of the phenol from the aryl oligosaccharide-glycoside it has synthesized. The behaviour on increasing the chain length of the added oligosaccharide is also similar to that found with lysozyme (*9*). On going from $(Glc)_3$ to $(Glc)_4$ to $(Glc)_5$ the induction period for the release of 3,4-dinitrophenol decreases and the rate of release increases. However, with variations of the structure of the added aryl glycoside, the behaviour is quite different from that found with lysozyme. With lysozyme, if a glycoside is used in which H, CH_3, CH_2F, or CH_2Cl has replaced the CH_2OH group of 3,4-dinitrophenyl 2-acetamido-2-deoxy-β-D-glucoside, no induced hydrolysis is found (*9*). However, with the enzyme EI the induced hydrolysis is faster if 3,4-dinitrophenyl β-D-xyloside (I, X = H) or 6-deoxy-β-D-glucoside (I = CH_3) is used instead of 3,4-dinitrophenyl β-D-glucoside (I, $X = CH_2OH$); and a detectable hydrolysis is found with the 6-chloro-6-deoxy- and 6-*O*-methyl- compounds (I, $X = CH_2Cl$ and CH_2OCH_3) (see Table 12). Since the measured rates of release of 3,4-dinitrophenol must be complex functions of several rate and equilibrium constants, it is difficult to put a precise interpretation on these results; but they strongly suggest that for substrates $(Glc)_n$ 3,4-DNP the CH_2OH group of the glucosyl residue at which bond fission occurs does not have an energetically favourable interaction with the enzyme EI in the transition state. The greater rate of release of 3,4-dinitrophenol from the xyloside and 6-deoxyglucoside may arise from the greater electron releasing ability of H and CH_3 compared to CH_2OH, which leads to a more rapid fission of the glycosyl–aryloxy bond. The slower release of the phenol from the 6-chloro and 6-*O*-methyl compounds may arise from a steric interaction between the chloro- and methoxy- groups and the enzyme and from the greater electron-withdrawing ability of Cl ($\sigma_I = 0.47$) compared to HO ($\sigma_I = 0.25$) (*30*).

The effect of the hydroxyl group at C-2 was investigated by comparing *p*-nitrophenyl 2-deoxy-β-D-glucoside with *p*-nitrophenyl β-D-glucoside (Table 13). The latter behaves similarly to 3,4-dinitrophenyl β-D-glucoside (Table 12), but no induced hydrolysis could be detected with the 2-deoxy- compound. This binding suggests that with the substrates $(Glc)_nAr$ there is an energetically favourable interaction in the transition state between the enzyme and the hydroxyl group at C-2 of the glucosyl residue at which bond fission occurs. This behaviour is again different from that found with lysozyme, as here the induced hydrolysis is faster with *p*-nitrophenyl 2-deoxy-β-D-glucoside than with *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucoside (*28*).

From these results the partial picture of the active site shown in Fig. 1 emerges. In this, a system of labeling of the binding subsites is used that follows that used by Berger and Schechter for Papain (*31*). Subsites which bind the glycone residues (G) of the substrate are labelled S_1, S_2, S_3, etc., and those which bind the aglycone residues (A) are labelled $S_1'S_2'$, etc. Bond fission occurs between the residues bound in subsites S_1 and S_1'. This system of nomenclature is preferred to that used by Phillips and co-workers for lysozyme (*28*), which when applied to other enzymes lead to a variation in the labels of the subsites binding the residues at which bond fission occurs. With enzyme E_1 there appears to be three subsites, S_1, S_2, and S_3, which bind the aglycone residues. S_4 if it exists at all must be a very weak binding site as the difference in k_{cat}/K_m

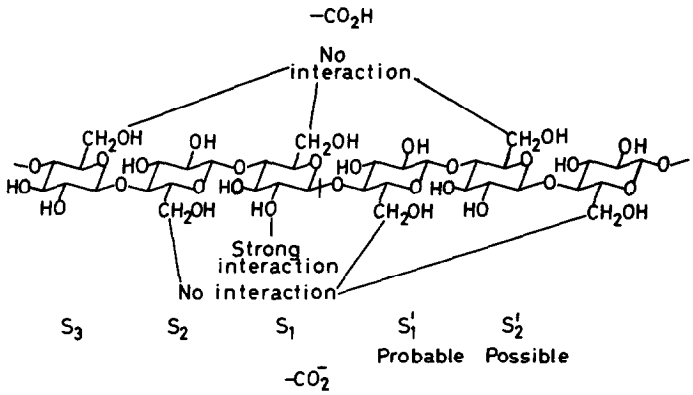


FIG. 1. Proposed interactions between a β-1,4-D-glucan and the enzyme EI in the transition state for hydrolysis.

for the hydrolysis of $(Glc)_3$ 3,4-DNP and $(Glc)_4$ 3,4-DNP is small. The number of binding subsites for the aglycone portion of the substrate is at present unknown; but it seems likely that there is at least one (S_1'), since transglycosylation occurs with aryl β-D-glucosides ($6 \times 10^{-3}M$), which must therefore compete for an intermediate more efficiently than water. The fact that 3,4-dinitrophenyl β-D-xyloside and 6-deoxy-D-glucoside also undergo transglycosylation suggests that there is not an energetically favourable interaction between the CH_2OH group of residue A and subsite S_1'. Indeed the fact that xylan is a very good substrate for EI suggests that none of the CH_2OH groups of a cellulose-type substrate makes an energetically favourable interaction with the enzyme in the transition state.

It is possible that there is a second subsite for the aglycone portion of the substrate S_2. The evidence for this is that the induction period for induced hydrolyses is shorter with $(Glc)_5$ than with $(Glc)_4$. This observation suggests that $(Glc)_5$ is hydrolysed faster than $(Glc)_4$; and hence if there are three binding sites, S_1, S_2, and S_3, for the glycone portion, there must be two for the aglycone, S_1', S_2'.

This partial picture of the active site may be compared with that constructed on the basis of our earlier work (*9*) and that of others (*28*) for HEW lysozyme (Fig. 2).

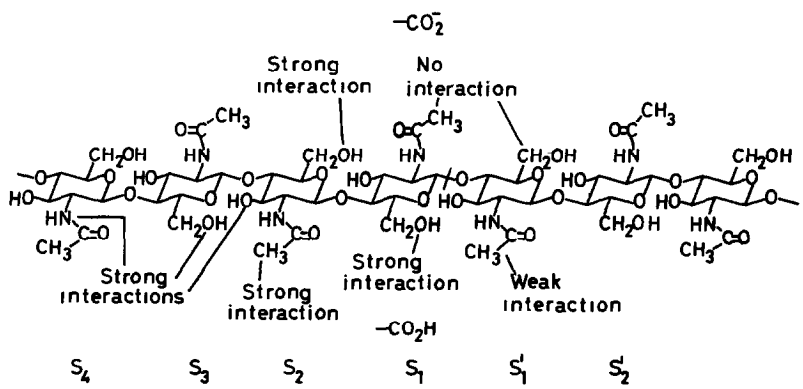


FIG. 2. Proposed interactions between chitin and HEW lysozyme in the transition state for hydrolysis.

The important differences that emerge so far are that lysozyme has four subsites which bind residues in the glycone portion of the substrate and that there is an energetically important interaction ($\geqslant$4.3 kcal mol^{-1}) between the CH_2OH group of residue G_1 and subsite S_1 in the transition state. However, the 2-acetamido group of residue G_1 does not appear to make an important interaction, since *p*-nitrophenyl 2-deoxy-β-D-glucoside undergoes a rapid induced hydrolyses (*29*).

PROPERTIES OF ENZYME EII

This enzyme was isolated by the purification of fractions which hydrolysed 3,4-dinitrophenyl β-D-glucoside. However, it is probably not a β-glucosidase and should be more correctly designated a cellobiase or *exo*-β-1,4-D-glucan glucohydrolase (*32*).

TABLE 2

KINETIC PARAMETERS FOR THE HYDROLYSIS OF 3,4-DINITROPHENYL β-D-GLUCOSIDE BY ENZYME EII AT 40°C IN ACETATE BUFFER, pH 5.02, I=0.1 M

k_{cat} (sec^{-1})	$10^4 K_m$ (M)	k_{cat}/K_m (M^{-1} sec^{-1})
0.217	5.13	423

The enzyme does not catalyse the hydrolysis of xylan. It has a molecular weight of about 74 400 which is similar to that reported for the exoglucanase of Li *et al.* (*33*) and the cellobiase of Gong *et al.* (*34*). These enzymes also catalysed the hydrolysis of aryl β-D-glucosides and our enzyme EII may be identical to one or both of them. Enzyme EII does, however, appear to be different from the cellobiase of Maguire, which has a molecular weight of 45 000 (*35*). The values of k_{cat} and K_m are given in Table 2. Normally the rate of hydrolysis of a glycoside catalysed by a glycosidase increases with increasing electron withdrawing power in the aglycone and decreasing pK_a of the released alcohol or phenol (*36*). Therefore a β-D-glucosidase would be expected to catalyse the fission of 3,4-dinitrophenyl β-D-glucoside more rapidly than that of cellobiose or other cello-oligosaccharides. A cellobiase, however, should have a binding subsite for the aglycone glucose residue and an *exo*-β-1,4-D-glucan glucohydrolase should have a subsite for several aglycone glucose residues (*32*). Therefore if an enzyme catalyses the hydrolysis of cellobiose and other cello-oligosaccharides almost as fast or faster than 3,4-dinitrophenyl β-D-glucoside, it is probably a cellobiase or an *exo*-β-1,4-D-glucan glucohydrolase. The enzyme EII catalyses the hydrolysis of CMC with a slow decrease in viscosity (Fig. 15) and therefore is acting as an exoenzyme. It also catalyses the release of 3,4-dinitrophenol from $(Glc)_n$ 3,4-DNP with $n = 2$, 3, 4 but with short induction periods which increase as n increases. The occurrence of induction periods which increase with increasing chain length is consistent with the enzyme removing glucose residues from the ends of the oligo-

saccharide chains before release of the 3,4-dinitrophenol. However, the fact that they are short suggests that the difference in the rate of cleavage of the oligosaccharide chain is similar to that for cleavage of the 3,4-dinitrophenoxy group of 3,4-dinitrophenyl β-D-glucoside. Enzyme EII is therefore most likely a cellobiase or and *exo*-β-1,4-D-glucan glucohydrolase, but a more definite assignment of its aglycone specificity must await further investigations.

The glycone specificity of EII was investigated by studying the hydrolysis of modified aryl β-D-glucosides (Table 3). Most of these react very slowly, and so a high enzyme concentration was used. The figures obtained are of course upper limits for the rates of reaction catalysed by EII, since some of the small rates observed with the modified

TABLE 3

RATE OF HYDROLYSIS OF ARYL GLYCOSIDES (1.2×10^{-4} *M*) IN THE PRESENCE OF ENZYME EII (2.3×10^{-4} *M*) AT 40°C IN ACETATE BUFFER, pH 5.02, $I = 0.1$ *M*

Glycoside	$10^8 \times$ Rate (M sec^{-1})	Percentage of glucoside
3,4-Dinitrophenyl β-D-glycosides		
Glucoside	1485	100
2-Acetamido-2-deoxyglucoside	59.1	3.97
6-Deoxyglucoside	8.66	0.58
Xyloside	1.52	0.10
6-Chloro-6-deoxyglucoside	1.04	0.07
6-*O*-Methylglucoside	0.23	0.01
p-Nitrophenyl β-D-glycosides		
Glucoside	1153	100
Galactoside	4.33	0.37
2-Deoxyglucoside	0	0

substrates may be the result of catalysis by an impurity. Replacement of the CH_2OH group of 3,4-dinitrophenyl β-D-glucoside by CH_3, H, CH_2Cl, or CH_2OMe lead to a large rate decrease, which suggests that the 6-hydroxy group makes an energetically favourable interaction with the enzyme in the transition state. Replacement of the 2-hydroxy group of *p*-nitrophenyl β-D-glucoside by hydrogen also leads to a large rate decrease, which indicates that this group also makes an energetically important interaction with the enzyme in the transition state. *p*-Nitrophenyl β-D-galactoside is hydrolysed 266 times more slowly than *p*-nitrophenyl β-D-glucoside. Either the 4-hydroxy group of the latter makes an important interaction with the enzyme or the axial hydroxy group of the galactoside makes an unfavourable steric interaction with the enzyme.

The specificity of EII is slightly different from that found with almond-emulsin β-glucosidase, for which replacement of the 6-hydroxyl group by hydrogen causes less than a 10-fold rate decrease (*37*). With emulsin on going from phenyl and methyl β-D-glucoside to the corresponding xylosides there is a large rate decrease as found here. The present effect of replacing the 6-hydroxyl by methoxyl or chloro is also similar to that observed with emulsin (*37*) and other β-D-glucosidases (*37*, *38*).

EXPERIMENTAL

General Methods

Melting points were determined on a Kofler–Reichert hot-stage melting point apparatus.

Infrared spectra were measured using a Perkin–Elmer 257 spectrophotometer and ultraviolet spectra using a Pye–Unicam SP 800 spectrophotometer. Proton magnetic resonance spectra were measured on a Varian T-60, Varian HA-100, or Varian XL-100 spectrometer.

Elemental analyses were determined by Mrs. W. Harkness, University of Glasgow.

Preparation of Peracetylated Cellotriose, Cellotetraose, and Cellohexaose

A mixture of peracetylated cello-oligosaccharides was prepared from Whatman cellulose powder using a method similar to that described by Wolfrom and his

TABLE 4

YIELDS AND PROPERTIES OF PERACETYLATED α-CELLO-OLIGOSACCHARIDES

	Yield (g)	Melting point (°C) (lit. *39*)	$[\alpha]_d$ (lit. *39*) (°)
Cellobiose	0.3	229 (225–226)	39.8 (39.6)
Cellotriose	1.3	223–225 (223–224)	21.1 (22.6)
Cellotetraose	1.6	230–232 (230–234)	10.6 (13.4)
Cellopentaose	0.8	235–238 (240–241)	7.0 (4.17)
Cellohexaose	0.5	—	—

co-workers (*39*). Cellobiose octoacetate was selectively removed by recrystallization from methanol and the resulting mixture of higher oligomers was separated on a silica column similar to that used by Ballardie *et al.* (*9*). The mixture was applied to the column in 30:70 (v/v) chloroform–carbon tetrachloride and eluted with the same mixture to which 1% of methanol had been added. The distribution of the products and their properties are shown in Table 4. The proton nmr spectra of all the acetates showed a one-proton signal with $\delta 6.24$ and $J = 4$Hz which is characteristic of an α-acetate. The presumed peracetylated cellohexaose was not characterized.

Preparation of Cello-oligosaccharides

These were prepared from the corresponding peracetates by deacetylation with sodium methoxide in methanol. The cellotetraose and cellopentaose crystallized directly from the reaction solution and the cellotriose was obtained after neutralization with Amberlite 1R 120(H^+), evaporation of the methanol, and recrystallization from 1/1 ethanol/water.

Cellotriose: mp 207–213°C, lit. (*39*) 206–209°C, $[\alpha]_d$ +30.5° (H_2O, *c* 0.28), lit. (*39*) +35°.

Cellotetraose: mp 245–251°C, lit. (*39*) 252–253°C; $[\alpha]_d$ +14.4° (H_2O, *c* 0.19), lit. (*39*) +8.4 → +16.5°.

Cellopentaose: mp 262°C, lit. (*39*) 266–268°C; $[\alpha]_d$ +11° (H_2O, *c* 0.11), lit. (*39*) +8 → +11°.

3,4-Dinitrophenyl Decaacetyl-β-cellotrioside

Peracetyl-cellotriose was added to a mixture of dichloromethane (5 ml) and 40% hydrogen bromide in acetic acid and left at room temperature for 2 h. The solution was poured into ice water (10 ml) and extracted with chloroform. The chloroform layer was washed with sodium bicarbonate solution, dried, and the solvent was removed to give a pale yellow syrup which solidified on trituration with ether, yield 0.35 g, 85%. The acetobromcellotriose was allowed to react immediately with potassium carbonate (0.5 g) and 3,4-dinitrophenol (0.2 g) in dry acetone. After 5 days the solution was poured into ice water (50 ml) and extracted four times with 50-ml portions of chloroform. The solution was dried ($MgSO_4$) and the solvent evaporated to yield a pale yellow syrup. This would not crystallise and tlc analysis showed that the aryl glucoside and cellobioside were present as impurities. The 3,4-dinitrophenyl decaacetyl-β-cellotrioside was separated by preparative tlc, eluting with 2% methanol in 30:70 chloroform:carbon tetrachloride and crystallized from ethanol. Yield 0.15 g, 40%, mp 135–139°C; ir (Nujol) $\nu = 1755$, C=O, 1615 cm^{-1}, aromatic; nmr ($CDCl_3$) δ8.02 (d, $J = 8.5$ Hz, 1H), 7.42 (d, $J = 2.5$ Hz, 1H), 7.26 (q, $J = 8.5$, 2.5 Hz, 1H), 5.45–3.60 (m, 21H), 2.26–1.90 (10s, 30H); uv, $\lambda_{max} = 282$ nm, $\log \varepsilon = 3.80$ (methanol), $[\alpha]_d = -22.2°$ (*c* 0.216, $CHCl_3$).

Anal. Calcd for $C_{14}H_{54}N_2O_{30}$: C, 48.44; H, 4.99; N, 2.57. Found: C, 48.25; H, 5.03; N, 2.28.

3,4-Dinitrophenyl-β-Cellotrioside

The deca-acetyl derivative (0.1 g) was deacetylated by Zemplen's method (*40*) to give a pale yellow syrup product which was dissolved in water (0.1 ml) and passed through a column of Sephadex G-15 (1.6 × 20 cm). The uv absorption of the effluent was monitored at 282 nm and the fraction which absorbed at this wavelength was freeze dried to give a white amorphous powder. Yield 54 mg, 88%, mp 165–167°C; ir (KBr) no acetates, 3600–3100, OH; 1615 cm^{-1}, aromatic; nmr (D_2O), δ8.16 (d, $J = 8.5$ Hz, 1H), 7.62 (d, $J = 2.5$ Hz, 1H), 7.44 (q, $J = 8.5$, 2.5 Hz, 1H), 5.28 (d, $J = 7$ Hz, 1H), 4.54 (d, $J = 8$ Hz, 1H), 4.46 (d, $J = 8$ Hz, 1H), 4.10–3.16 (m, 18H); uv, $\lambda_{max} = 282$ nm, $\log \varepsilon = 3.75$ (water, pH 5); $[\alpha]_d = -23.4°$ (*c* 0.095, water).

Anal. Calcd for $C_{24}H_{34}N_2O_{20}$: C, 43.00; H, 5.11; N, 4.18. Found: C, 43.22; H, 5.37; N, 4.20.

3,4-Dinitrophenyl Trideca-O-acetyl-β-cellotetraoside

This was prepared via the acetobromo derivative in a way similar to that used for the corresponding cellotrioside. The crude product was purified on a column (2 × 40 cm) packed with dry silica. The sample was applied in a minimum volume of 30/70 chloroform/carbon tetrachloride and was eluted with this solvent containing 2% methanol. Yield 0.25 g, 53%, mp 140–146°C; ir (Nujol) γ1755, C=O; 1615 cm^{-1}, aromatic; nmr ($CDCl_3$), δ8.00 (d, $J = 8.5$ Hz), 7.42 (d, $J = 2.5$ Hz), 7.26 (q, $J = 8.5$, 2.5 Hz), 5.44–3.58 (m), 2.26–1.92 (CH_3 singlets); uv, $\lambda_{max} = 284$ nm, $\log \varepsilon = 3.8$ (methanol), $[\alpha]_d^{20} = -19.2°$ (*c* 0.240, $CHCl_3$).

Anal. Calcd for $C_{56}H_{70}N_2O_{38}$: C, 48.77; H, 5.12; N, 2.03. Found: C, 48.40; H, 5.10, N, 2.50.

3,4-Dinitrophenyl β-cellotetraoside

The acetyl derivative (0.2 g) was deacetylated by Zemplen's method (*40*) and the crude glycoside was passed down a column of Sephadex G-15 as for the cellotrioside. The resulting solution was freeze dried to give an amorphous white powder. Yield 0.104 g, 86%, mp 183–187°C (dec); ir (KBr) no acetates, γ3600–3100, O–H; 1615 cm^{-1}, aromatic; nmr (D_2O), δ8.16 (d, J = 8.5 Hz, 1H), 7.61 (d, J = 2.5 Hz, 1H), 7.44 (q, J 8.5, 2.5 Hz, 1H), 5.28 (d, J = 7 Hz, 1H), 4.50 (m, 3H), 4.10–3.12 (m, 24H); uv, λ_{max} = 283 nm, log ε = 3.75 (water, pH 5), $[\alpha]_d = -14.4°$ (*c* 0.16, water).

Anal. Calcd for $C_{30}H_{44}N_2O_{25}$: C, 43.27; H, 5.33; N, 3.37. Found: C, 43.12; H, 5.57; N, 3.26.

3,4-Dinitrophenyl Hepta-O-acetyl-β-cellobioside

Acetobromo-cellobiose (*41*) (3.5 g) was converted into the 3,4-dinitrophenyl glycoside as described for the cellobioside. It was recrystallized from 50:50 methanol: ethanol. Yield 2.1 g, 52.3%, mp 240–242°C; $[\alpha]_d^{20} = -37°$ (*c* 0.24, $CHCl_3$). The ir and nmr spectra were consistent with the assigned structure.

Anal. Calcd for $C_{32}H_{38}N_2O_{22}$: C, 47.89; H, 4.77; N, 3.49. Found: C, 47.95; H, 4.97; N, 3.30.

3,4-Dinitrophenyl β-Cellobioside

The hepta-*O*-acetyl derivative (1 g) was deacetylated by Zemplen's method (*40*), and the product was recrystallized from 1:4 methanol:water. Yield 0.58, mp 187–192°C, $[\alpha]_d^{20} = -76°$ (*c* 0.235, H_2O); ir (KBr) no acetates present, γ3600–3100, OH; 1615 cm^{-1}, aromatic; nmr (pyridine, d_5), δ8.00 (d, J = 8.5 Hz, 1H), 7.75 (d, J = 2.5 Hz, 1H), 7.42 (q, J = 8.5, 2.5 Hz, 1H), 6.70 (s, 7 OH), 5.74 (d, J = 8 Hz, 1H), 5.16 (d, J = 8 Hz, 1H), 4.70–3.85 (m, 12H); uv, λ_{max} = 284 nm, log ε = 3.8 (water, pH 5).

3,4-Dinitrophenyl Tetra-O-acetyl-β-D-glucopyranoside

Acetobromoglucose (3.5 g) was converted into the 3,4-dinitrophenyl glycoside as described for the cellotrioside. The resulting syrup readily solidified and was recrystallized from methanol. Yield 2.9 g, 66%, mp 169–171°C, $[\alpha]_d^{20} = -42.3°$ (*c* 0.46, $CHCl_3$). The ir and nmr spectra were consistent with the assigned structure.

Anal. Calcd for $C_{20}H_{22}N_2O_{14}$: C, 46.70; H, 4.31; N, 5.45. Found: C, 46.50; H, 4.52; N, 5.25.

3,4-Dinitrophenyl-β-D-Glucopyranoside

The tetra-*O*-acetyl derivative (1 g) was deacetylated by Zemplen's method (*40*) and the product was recrystallized from a 1:4 mixture of methanol and water. Yield 0.6 g, 90%, mp 105–109°C; ir (KBr) no acetates present; γ3600–3100 (OH), 1615 cm^{-1}, aromatic; nmr (pyridine-d_5), δ8.04 (d, J = 8.5 Hz, 1H), 7.79 (d, j = 2.5 Hz, 1H), 7.48 (q, j = 8.5, 2.5 Hz, 1H), 5.78 (d, J = 8 Hz, 1H), 4.68–4.05 (m, 6H); uv, λ_{max} = 282 nm, log ε = 3.8 (water, pH 5), $[\alpha]_d^{20} = -67°$ (*c* 0.1, water).

Anal. Calcd for $C_{12}H_{14}N_2O_{10}$, H_2O: C, 39.56; H, 4.43; N, 7.69. Found: C, 39.09; H, 4.72; N, 7.61.

Modified 3,4-Dinitrophenyl β-D-Glucopyranosides

The *O*-acetylated 3,4-dinitrophenyl β-D-glycopyranosides from 6-deoxy-D-glucose (Koch–Light), 6-*O*-methyl-D-glucose (*42*), 6-chloro-6-deoxy-D-glucose (*42*), and D-xylose (Koch–Light) were prepared via the acetobromo derivatives. They all had nmr and ir spectra consistent with the assigned structure. Other properties are given in Table 5. They were deacetylated by Zemplen's method (*40*). The 6-deoxy-, 6-chloro-6-deoxy-, and the 6-*O*-methylglucosides were recrystallized from ethanol and the xyloside from methanol. The pmr spectra (pyridine-d_5) all showed signals characteristic

TABLE 5

PROPERTIES OF 3,4-DINITROPHENYL 2,3,4-TRI-*O*-ACETYL-β-D-GLYCOPYRANOSIDES

Parent sugar	Melting point (°C)	$[\alpha]_d$ ($CHCl_3$) (°)	Analyses					
			Found			Calc		
			C	H	N	C	H	N
6-Deoxy-D-glucose	176–178	−83.5	47.50	4.41	6.44	47.38	4.42	6.14
6-*O*-Methyl-D-glucose	201–204	−42.0	47.02	4.54	5.50	46.91	4.56	5.78
6-Chloro-6-deoxy-D-glucose[a]	177–181	−69.3	44.20	3.88	5.99	44.04	3.90	5.72
D-Xylose	181–183	−116.4	46.22	4.20	6.60	46.15	4.10	6.35

[a] Cl, calcd, 7.23; found: 7.24.

TABLE 6

PROPERTIES OF 3,4-DINITROPHENYL β-D-GLYCOPYRANOSIDES

Parent sugar	Melting point (°C)	$[\alpha]_d$ (H_2O) (°)	Analyses					
			Found			Calc		
			C	H	N	C	H	N
6-Deoxy-D-glucose[a]	95–98	−215	41.62	4.75	8.19	41.39	4.63	8.04
6-*O*-Methyl-D-glucose[a]	148–150	−111.4	41.57	4.74	6.99	41.27	4.79	7.43
6-Chloro-6-deoxy-D-glucose[b]	135–139	−40.9	37.47	4.53	6.96	37.65	4.23	7.32
D-Xylose	142–144	−70.5	41.78	3.87	8.55	41.77	3.82	8.88

[a] Isolated as the monohydrate.
[b] Cl, Calcd 9.26; found: 9.68.

of the 3,4-dinitrophenyl group and of an anomeric proton (δ ca 5.75, $J = 8$ Hz). The uv spectra all showed a peak with $\lambda_{max} = 284$ nm, $\log \varepsilon =$ ca. 3.8. Their properties are given in Table 6.

3,4-Dinitrophenyl 2-Acetamido-2-Deoxy-β-D-Glucoside

This was prepared by the standard method (*9*) by Mr. M. Cuthbert, mp 138–139°C.

Anal. Calcd for $C_8H_{17}N_3O_{10}$: C, 43.42; H, 4.42; N, 10.85. Found: C, 43.02; H, 4.59; N, 10.79.

4-Nitrophenyl β-D-Glucopyranoside

This was synthesized via acetobromoglucose, mp 164–165°C [lit. (*43*), 164–165°C].

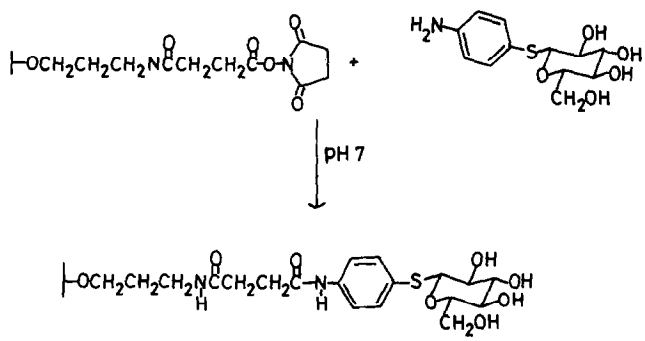
4-Nitrophenyl 2-deoxy-β-D-Glucoside

This was prepared by Mr. M. Cuthbert, mp 165–166°C [lit. (*44*) 167–169°C].

Affinity Column

4-Aminophenyl β-D-thioglucopyranoside was prepared as described by Shah and Bahl for the thiogalactopyranoside (*45*); mp 144–145°C [lit. (*46*) 147–148°C, $[\alpha]_d$ = −43° (1/1 methanol/water, *c* 0.14), lit. (*46*) −35.9°/methanol].

Sixty milligrams (210 μ*M*) was dissolved in 25 ml 0.1 *M* phosphate buffer, pH 7. This solution was added to Affi-Gel 10 (Bio-Rad) (1 g) at 4°C and shaken for 24 h at room temperature. The slurry was poured into a column (1.6 × 20 cm) and washed with 1 *M* sodium chloride solution until the absorbance of the effluent was zero at 260 nm. This indicates removal of the *N*-hydroxysuccinimide released during coupling. The reaction which takes place is shown in Scheme 2.



Scheme 2

Purification of the Enzymes

Commercial cellulase from *Trichoderma viride* (BDH) was extracted with 0.06 *M* acetate buffer, pH 5 (1 g in 30 ml). The extract was filtered through a sintered glass filter. The activity of this initial solution against several cellulolytic substrates is shown in Table 7. The release of 3,4-dinitrophenol from 3,4-dinitrophenyl glycosides is shown in Fig. 3. The crude enzyme is clearly rich in β-glucosidase activity. The release of the phenol from $(Glc)_2$ 3,4-DNP and $(Glc)_4$ 3,4-DNP shows an induction period. This presumably occurs because the β-glucosidase removes glucose units from the nonreducing end of the cello-oligosaccharide chain. Only with (Glc) 3,4-DNP is there

TABLE 7

ACTIVITIES OF FRACTIONS FROM THE CRUDE CELLULASE AFTER CHROMATOGRAPHY ON SEPHADEX A-25[a]

Substrate	Crude	FI	FII	FIII	FIV	FV	FVI	FVII
Avicel	1.48	0.91	0.60	0.66	0.08	0.44	0.17	0
CMC	0.39	0.60	1.74	3.61	0.51	0.22	0.23	0
$(Glc)_1$ 3,4-DNP	1.77	5.88	6.00	2.67	0	0	0.40	0
$(Glc)_4$ 3,4-DNP	0.10	3.68	1.25	0	0	0	0.24	0

[a] Numbers are units as defined in the text at an enzyme concentration with absorbance 1 at 280 nm.

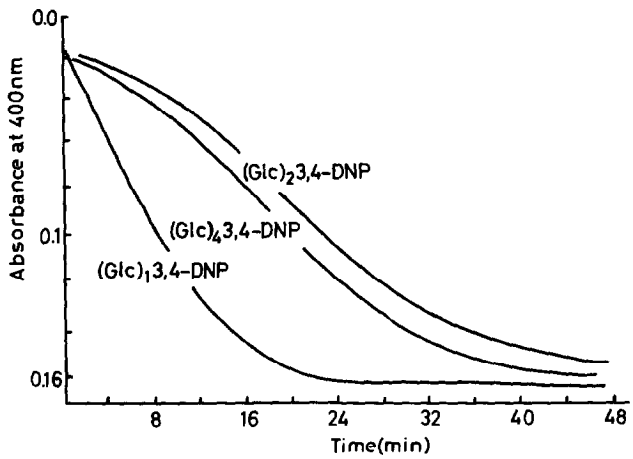


FIG. 3. Graphs of absorbance at 400 nm against time for the reactions of 3,4-dinitrophenyl glycosides with the crude cellulase. The assay was conducted as described in the text.

no discernible induction period. If only β-glucosidases were present the phenol should be released more rapidly from $(Glc)_2$ 3,4-DNP than from $(Glc)_4$ 3,4-DNP but this is not so. Therefore another enzyme (or enzymes) must be present which requires more than two glucose units for optimum activity.

Chromatography on DEAE–Sephadex A-25

The plot of absorbance at 280 nm versus effluent volume is shown in Fig. 4. The crude cellulase solution (25 ml) was applied to a column (2.6 × 70 cm) of DEAE–Sephadex A-25 (Pharmacia) equilibrated with 0.06 *M* acetate buffer, pH 5. The column was eluted at a rate of 1 ml/min with the same buffer. A salt gradient was applied as shown in Fig. 4 to elute the more tightly bound enzymes. Seven fractions, FI to FVII,

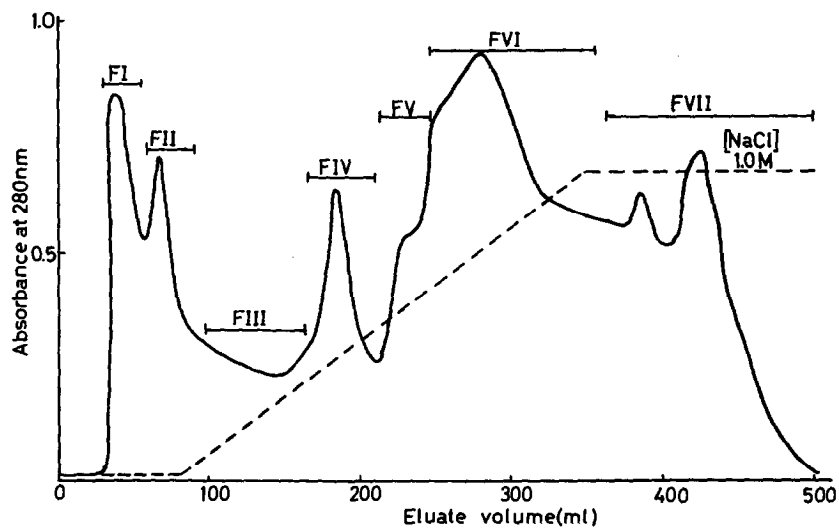


FIG. 4. Graph of absorbance at 280 nm against eluate volume on passage of the crude cellulase through a column of Sephadex A-25.

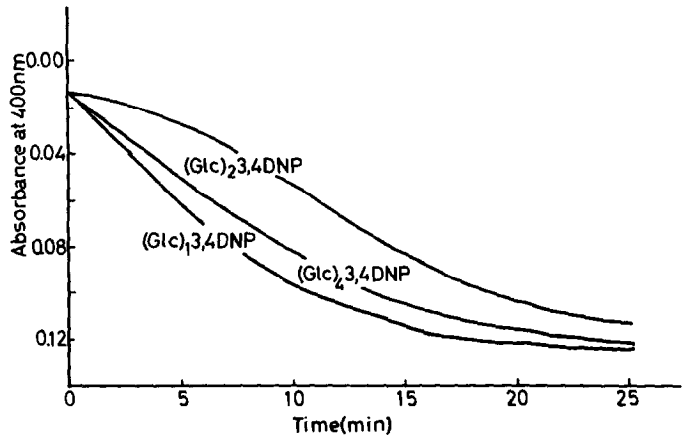


FIG. 5. Graphs of absorbance at 400 nm against time for the reactions of 3,4-dinitrophenyl glycosides with fraction FI. The assay was conducted as described in the text.

were collected as shown in Fig. 4 and were assayed against various cellulolytic substrates (Table 7).

Fraction FI showed the highest ratio of activity for $(Glc)_4$ 3,4-DNP to $(Glc)_1$ 3,4-DNP and was investigated further. From the graph of absorbance against time (Fig. 5) it can be seen that $(Glc)_2$ 3,4-DNP still shows an induction period but that $(Glc)_4$ 3,4-DNP does not. The most active enzyme is still a β-glucosidase.

Chromatography on Sephadex G-75

Fraction FI was lyophilized and the lyophilized powder dissolved in 10 ml of 0.06 *M* acetate buffer, pH 5. This was applied to a column of Sephadex G-75 (Pharmacia). Five fractions FIA to FIE were collected as shown in Fig. 6. The assays towards

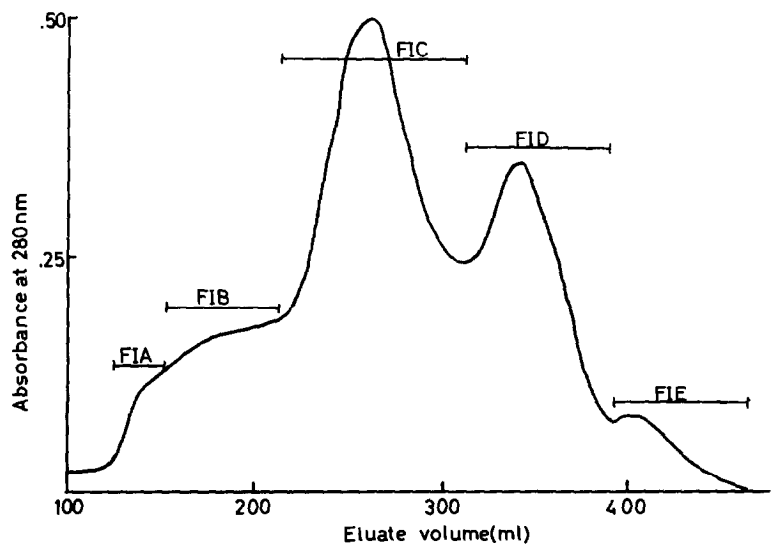


FIG. 6. Graph of absorbance at 280 nm against eluate volume on passage of fraction FI through a column of Sephadex G-75.

TABLE 8

ACTIVITIES OF FRACTIONS FROM FRACTION FI AFTER CHROMATOGRAPHY ON SEPHADEX G-75[a]

Substrate	FI	FIA	FIB	FIC	FID	FIE
Avicel	0.91	0.09	0.34	0.11	0	0
CMC	0.60	3.86	0.92	0.49	2.66	0.02
$(Glc)_1$ 3,4-DNP	5.88	39.0	9.54	1.01	1.00	0
$(Glc)_4$ 3,4-DNP	3.68	0.48	1.37	6.91	0.88	0

[a] Numbers are units as defined in the text at an enzyme concentration with absorbance 1 at 280 nm.

various substrates are shown in Table 8. Fraction FIC showed the highest ratio of activities for $(Glc)_4$ 3,4-DNP to $(Glc)_1$ 3,4-DNP. The plots of absorbance versus time for the aryl glycosides are shown in Fig. 7. The phenol is now released more rapidly from $(Glc)_4$ 3,4-DNP than from $(Glc)_1$ 3,4-DNP.

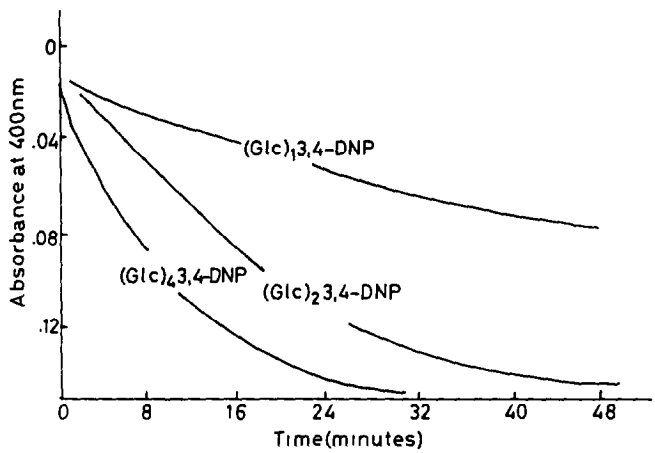


FIG. 7. Graphs of absorbance at 400 nm against time for the reactions of 3,4-dinitrophenyl glycosides with fraction FIC. The assay was conducted as described in the text.

Rechromatography on Sephadex G-75

Fraction FIC was lyophilized and the lyophilized powder was dissolved in 10 ml of 0.06 *M* acetate buffer, pH 5. This was applied to the same Sephadex G-75 column as before. Fractions of 10 ml were collected and labelled FICi to FICvii (Fig. 8).

TABLE 9

RATIO OF ACTIVITY TOWARDS 3,4-DINITROPHENYL β-CELLOTETRAOSIDE TO ACTIVITY TOWARDS 3,4-DINITROPHENYL β-D-GLUCOSIDE OF FRACTIONS OBTAINED ON RECHROMATOGRAPHY OF FRACTION FIC ON SEPHADEX G-75

	FICi	FICii	FICiii	FICiv	FICv	FICvi	FICvii
Ratios	0.94	1.79	9.98	18.1	9.50	6.92	3.45

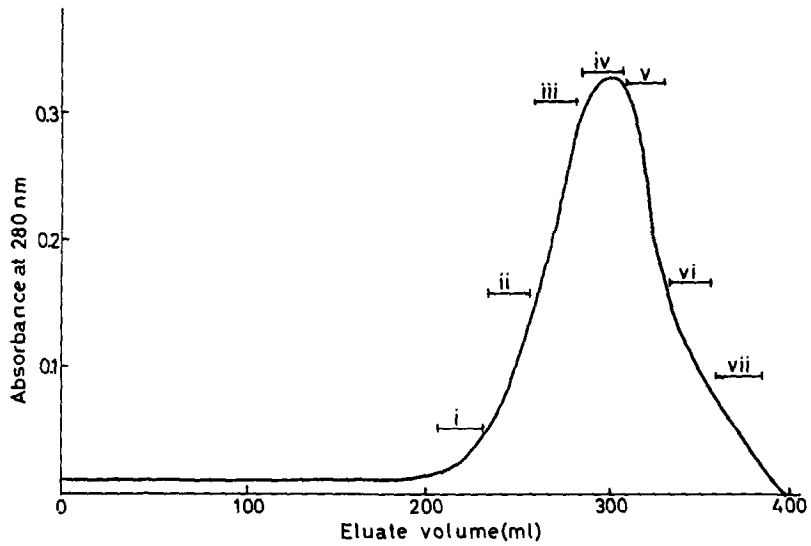


FIG. 8. Graph of absorbance at 280 nm against eluate volume on repassage of fraction FIC through a column of Sephadex G-75.

The ratios of the activities for $(Glc)_4$ 3,4-DNP to $(Glc)_1$ 3,4-DNP are shown in Table 9. The highest ratio, 18.1, was shown by fraction FICiv.

Affinity Chromatography

Fraction FICiv was purified further by removing the β-glucosidase by affinity chromatography. The affinity column used was prepared as described above. To check that the column was specific for β-glucosidases a sample of fraction FI was passed through the column. The column was equilibrated with 0.06 *M* acetate buffer, pH5, and eluted with buffer, 0.2 *M* D-glucose, pH 5, and 0.2 *M* cellobiose, pH 5 (Fig. 9). The D-glucose solution was given time to come to anomeric equilibrium before being applied to the column, but it did not elute the β-D-glucoside. Elution of the

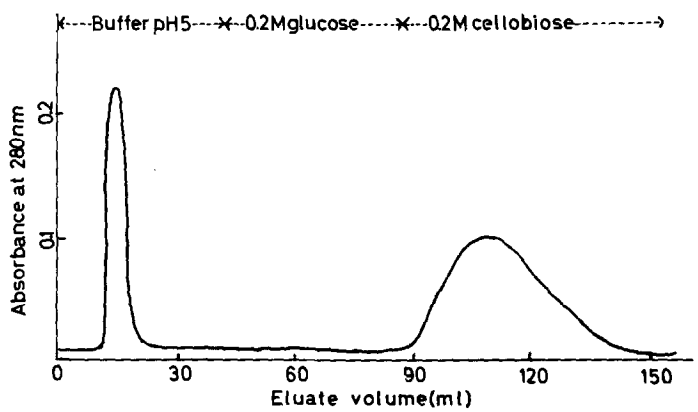


FIG. 9. Graph of absorbance at 280 nm against eluate volume on passage of fraction FI through the affinity column.

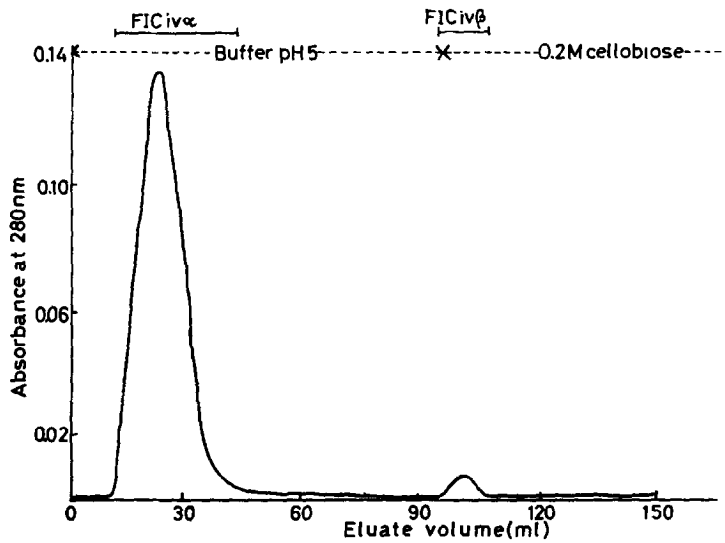


FIG. 10. Graph of absorbance at 280 nm against eluate volume on passage of fraction FICiv through the affinity column.

column with cellobiose solution caused gradual release of the enzyme as measured by its absorbance at 280 nm. When the column was eluted with 1 *M* sodium chloride, pH 5, instead of cellobiose solution the enzyme was released from the column in a small volume.

Fraction FICiv (10 ml) was applied to the column and eluted with the acetate buffer. The majority of the protein passed through the column and, on elution with 0.2 *M* cellobiose, a small amount of *β*-glucosidase was released (Fig. 10). The enzyme fractions were labelled FICiv*α* and FICiv*β* respectively and fraction FICiv*α* was designated enzyme EI. Its activity towards $(Glc)_4$ 3,4-DNP was about 100-fold greater

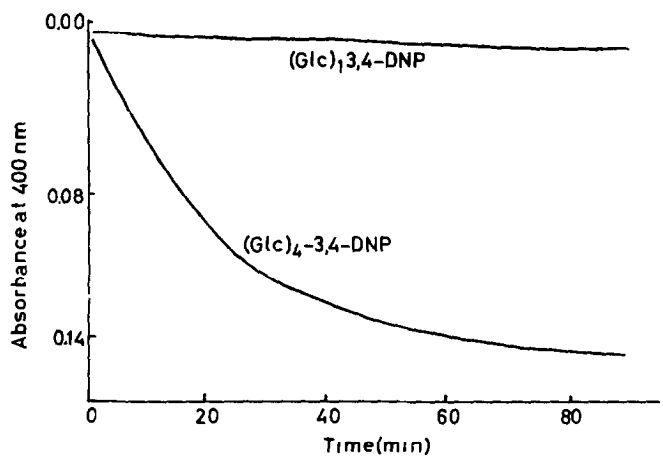


FIG. 11. Graphs of absorbance at 400 nm against time for the reactions of 3,4-dinitrophenyl glycosides with fraction FICiv*α* (EI). The assay was conducted as described in the text.

TABLE 10

ACTIVITIES OF ENZYME EI (FICivα) AND EII[a]

Substrate	Activity	
	EI	EII
Avicel	0	0
CMC	0.68	4.06
$(Glc)_1$ 3,4-DNP	0.078	44.0
$(Glc)_4$ 3,4-DNP	10.4	0

[a] Numbers are units as defined in the text at an enzyme concentration with absorbance 1 at 280 nm.

than the crude enzyme (Table 10) and it catalysed the release of 3,4-dinitrophenol from $(Glc)_4$ 3,4-DNP 133 times faster than from $(Glc)_1$ 3,4-DNP under the standard assay conditions (Fig. 11).

Purification of Enzyme with β-Glucosidase Activity

Fraction FIA was lyophilized and the lyophilized powder was dissolved in acetate buffer, pH 5 (5 ml). This was applied to a column (2.6 × 70 cm) of Sephadex G-100 (Pharmacia) equilibrated with the same buffer (Fig. 12). This main fraction was designated enzyme EII. Its activities towards various cellulolytic substrates are shown in Fig. 13 and Table 10. Its activity towards $(Glc)_1$ 3,4-DNP was about 25 times greater than that of the crude enzyme.

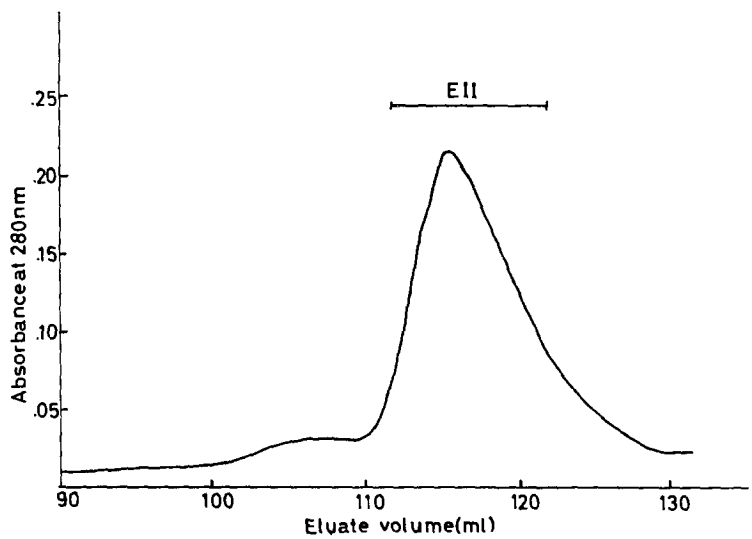


FIG. 12. Graph of absorbance at 280 nm against eluate volume on passage of fraction FIA through a column of Sephadex G-100.

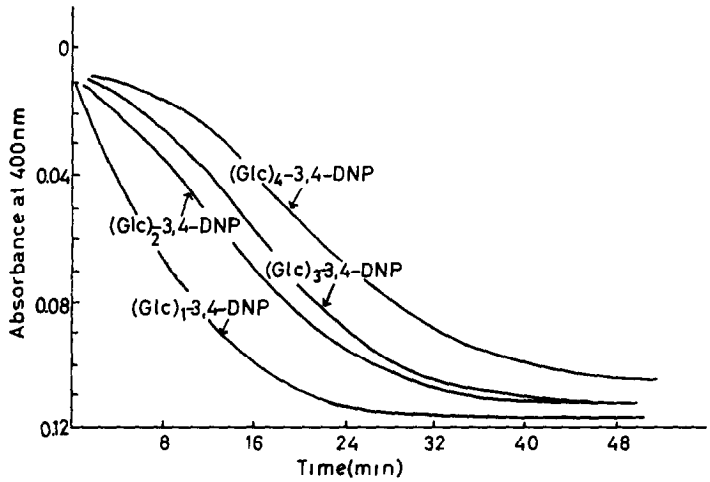


FIG. 13. Graphs of absorbance at 400 nm against time for the reactions of 3,4-dinitrophenyl glycosides with enzyme EII. The assay was conducted as described in the text.

Enzyme purity

SDS–gel electrophoresis. The purified enzymes were subjected to electrophoresis in polyacrylamide gel (7.5%, w/v) in the presence of sodium dodecyl sulphate (SDS), pH 6.6. The precast gel was obtained from Bio-Rad and measured 5.5 × 100 mm. The protein was mixed with glycerol, SDS buffer, and bromophenol blue (Bio-Rad). The sample (100 μl) was applied to the top of the gel. Electrophoresis was conducted at a constant current of 12 mA per gel for about 3 h. The gel was stained with Coomassie brilliant blue (Bio-Rad) and destained by standing in 7.5% acetic acid, 5% methanol. Both enzymes EI and EII gave a single band.

Molecular sieve chromatography. Both enzymes behaved as single proteins on passage through Sephadex G-75 and Sephadex G-100 columns (2.6 × 70 cm).

Determination of molecular weights. A column (2.6 × 70 cm) of Sephadex G-100 was equilibrated with 0.06 *M* acetate buffer, pH 5. The void volume (V_0) of the column was estimated with Blue Dextran 2000 (MW 2 000 000, Pharmacia). The column was calibrated with three marker proteins (Sigma); cytochrome *c* (MW 12 4000), trypsin (MW 23 000), and ovalbumin (MW 45 000).

A few milligrams of the marker proteins were dissolved in 2.0 ml of the purified enzyme solution, applied to the column, and eluted with the same buffer at a rate of 1 ml/5 min. The protein was located in the effluent by their absorbance at 280 nm. The effluent volume (V_e) was calculated from the midpoint of the peak. The molecular weights of the enzymes EI and EII were estimated to be ca. 12 000 and ca. 74 400 respectively (Fig. 14).

Determination of extinction coefficients. These were determined by desalting a solution of the enzyme on a Sephadex G-15 column (1.6 × 4.0 cm), lyophilizing, and measuring the absorbance of a measured concentration of the enzyme using the estimated molecular weight. The extinction coefficients of EI and EII were 17 800 and 21 000 M^{-1} cm^{-1}.

Activity towards xylan and amylose. Both enzymes were incubated with a solution

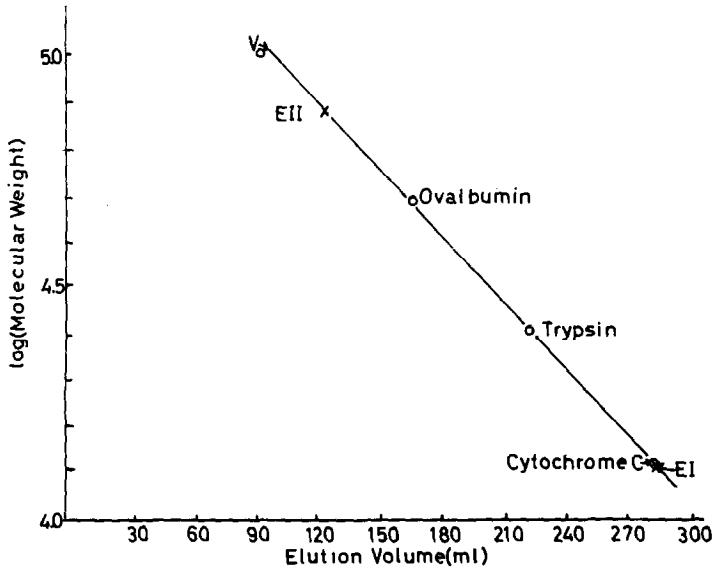


FIG. 14. Plot of log (molecular weight) against elution volume used for the determination of the molecular weights of enzymes EI and EII.

of 1% xylan (ex larch sawdust, Koch–Light) and 1% amylose (Koch–Light). Neither enzyme produced any increase in reducing power with amylose over a period of 2 h but the xylan was hydrolysed by the enzyme EI approximately as rapidly as carboxymethylcellulose. Enzyme EII had no effect on the xylan.

Randomness of CMC-saccharifying activity. Solutions of EI and EII with the same activity towards CMC as determined by the reducing method were assayed visco-

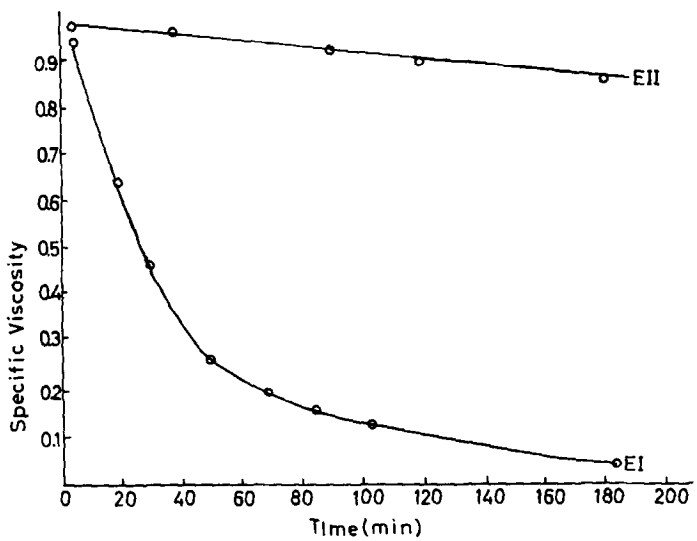


FIG. 15. Graphs of specific viscosity versus time on hydrolysis of carboxymethylcellulose in the presence of enzymes EI and EII. The assays were carried out as described in the text and the activities of each enzyme as determined by the rate of increase of reducing power were both 0.56 units (as defined in the text).

metrically. The decrease in viscosity with time is given in Fig. 15. EI produces a rapid decrease in viscosity which indicates a random cleavage of CMC whereas EII causes only a slow change in viscosity which indicates endwise attack. Therefore EI is an endoenzyme and EII an exoenzyme.

Enzyme Assays

Activity towards carboxymethylcellulose. This was determined on a 1% solution of CMC sodium salt, low viscosity, degree of substitution 0.7–0.8 (BDH) in a 0.1 *M* citrate buffer, pH 5, which contained 0.1 mg/ml glucose. The formation of reducing sugar was determined as described by Miller *et al.* (*47*) and converted to the equivalent amount of glucose by a calibration curve. The activities given in Tables 7, 8, and 10 are the number of micrograms of glucose equivalents produced per minute by the enzyme being assayed, at a concentration with an absorbance of 1 at 280 nm.

Viscometric assays were carried out with the same solution of CMC, sodium salt in a Ubbelohde viscometer.

Activity towards avicel. A reaction mixture which contained Avicel (FMC Corporation) (70 mg), 0.1 *M* acetate buffer, pH 5, (70 mg) and the enzyme solution (1 ml). The mixture was incubated at 30°C for 7 days and then filtered. The amount of reducing sugar was determined (*47*) and converted to glucose equivalents. The activities given in Tables 7, 8, and 10 are micrograms of glucose equivalents produced per day by an enzyme solution with absorbance 1 at 280 nm.

Activity towards 3,4-dinitrophenyl glycosides. Stock solutions (2×10^{-3} *M*) of $(Glc)_n$ 3,4-DNP, $n = 1$–4, were made up in 0.1 *M* acetate buffer, pH 5. The enzyme solution to be assayed (0.2 ml) was added 2.3 ml of 0.1 *M* acetate buffer, pH 5, in a 10-mm uv cell. The stock solution of the 3,4-dinitrophenyl glycoside (0.1 ml) was added and the graph of absorbance against time was obtained at 20 to 25°C in a Cecil CE212 spectrophotometer. The activities quoted in Tables 7–10 are the number of micromoles of 3,4-dinitrophenol produced per minute by an enzyme solution with absorbance 1 at 280 nm extrapolated to zero time.

Kinetic Measurements

General. Kinetic measurements with the aryl glycosides were carried out in a Cary 16 spectrophotometer operating on line with a Digico Micro 16P minicomputer as described previously (*9*).

pH optima. The rate of release of 3,4-dinitrophenol was measured at 400 nm from a solution which contained EI (1.04×10^{-7} *M*) and $(Glc)_4$ 3,4-DNP (2.38×10^{-4} *M*). The rate of change of absorbance with time was converted into the rate of formation of the phenol by using the independently determined extinction coefficient at each pH. The pH optimum is between 4.5 and 5.5 (Fig. 16).

Similar experiments were carried out with EII (2.3×10^{-8} *M*) and $(Glc)_1$ 3,4-DNP (1.21×10^{-4} *M*). The pH optimum was again found to be between pH 4.5 and 5.5 (Fig. 17).

Temperature optima. The effect of temperature on the rate of hydrolysis of $(Glc)_4$ 3,4-DNP (2.38×10^{-4} *M*) catalysed by EI (1.04×10^{-7} *M*) in 0.1 *M* acetate buffer (pH 5.02) was determined in the Cary 16 spectrophotometer. The optimum temperature was 60°C (Fig. 18). No loss of activity was observed after 24 h at 40°C. Similar

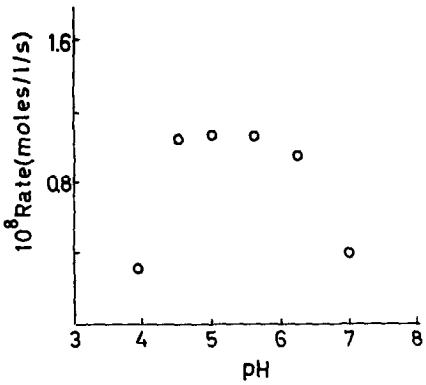


FIG. 16. Plot of initial rate of release of 3,4-dinitrophenyl from 3,4-dinitrophenyl β-cellotetraoside (2.38×10^{-4} *M*) against pH in the presence of EI (1.04×10^{-7} *M*) at 40°C.

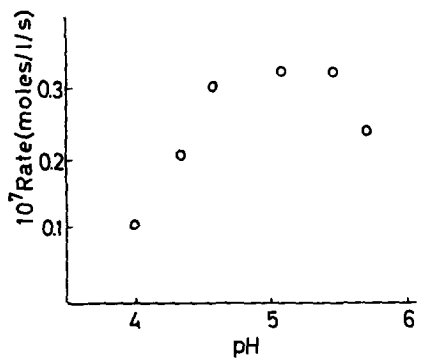


FIG. 17. Plot of initial rate of release of 3,4-dinitrophenol from 3,4-dinitrophenyl β-D-glucoside (1.21×10^{-4} *M*) against pH in the presence of EII (2.3×10^{-8} *M*) at 40°C.

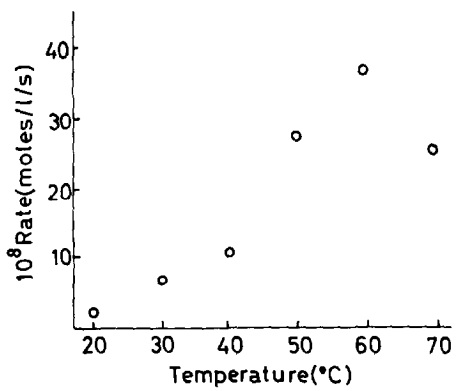


FIG. 18. Plot of initial rate of release of 3,4-dinitrophenol from 3,4-dinitrophenyl β-cellotetraoside (2.38×10^{-4} *M*) against temperature in the presence of EI (1.04×10^{-7} *M*) at pH 5.02.

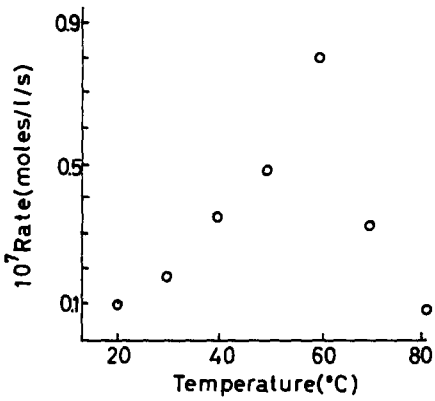


FIG. 19. Plot of initial rate of release of 3,4-dinitrophenol from 3,4-dinitrophenyl β-cellotetraoside (1.21×10^{-4} *M*) against pH in the presence of EII (2.3×10^{-8} *M*) at pH 5.02.

experiments were carried out with EII (2.3×10^{-8} *M*) and $(Glc)_1$ 3,4-DNP (1.21×10^{-4} *M*) (Fig. 19).

Induced Hydrolyses

Experiments were carried out to see if EI possessed any transglycosylating properties. Earlier work by Okada and Nisizawa (*14*) has already demonstrated transglycosylating properties of two cellulolytic enzymes from *Trichoderma viride.*

Cellotriose, cellotetraose, or cellopentaose and 3,4-dinitrophenyl β-D-glucoside were dissolved in an acetate buffer, pH 5.02, $I = 0.1$ *M* (23 ml) in a uv cell and a solution of the enzyme (200 μl) was added. The final concentrations are given in Table 11. The change in absorbance at 400 nm and 20°C was recorded. Any release of 3,4-dinitrophenol was taken as an indicator that transglycosylation was occurring since under the conditions used the glycoside was hydrolysed very slowly by the enzyme. The rate of release of 3,4-dinitrophenol after various time intervals and the corresponding percentage reaction are given in Table 11.

TABLE 11

RATE OF FORMATION OF 3,4-DINITROPHENOL DURING INCUBATION OF CELLO-OLIGOSACCHARIDES (6×10^{-3} *M*) AND 3,4-DINITROPHENYL β-D-GLUCOSIDE (1.20×10^{-4} *M*) WITH ENZYME EI (4.16×10^{-6} *M*)

	Cellotriose		Cellotetraose		Cellopentaose	
Time (min)	10^{10} Rate (*M* liter^{-1} sec^{-1})	Reaction (%)	10^{10} Rate (*M* liter^{-1} sec^{-1})	Reaction (%)	10^{10} Rate (*M* liter^{-1} sec^{-1})	Reaction (%)
5		0	0	0	0	0
10	0	0	0	0	3.03	0.02
20	0	0	2.75	0.30	3.87	0.46
30	2.69	0.02	3.14	0.46	4.54	0.61
60	2.72	0.61	3.69	0.78	4.95	0.91
120	2.75	1.50	4.04	1.68	5.02	2.58

The results show that as the chain length of the oligosaccharide increases the induction period before hydrolysis begins is reduced and the rate of release of phenol increases. Cellopentaose was therefore used in the subsequent experiments with modified aryl glucosides to investigate the features which make the aryl oligosaccharides substrates for the enzyme. The concentration of the aryl glycosides were increased by a factor of 5. The results are given in Tables 12 and 13.

TABLE 12

RATE OF FORMATION OF 3,4-DINITROPHENOL DURING INCUBATION OF CELLOPENTAOSE (5.7×10^{-3} *M*) AND 3,4-DINITROPHENYL β-D-GLYCOSIDES (6.0×10^{-4} *M*) WITH ENZYME EI (4.16×10^{-6} *M*)

	Glycoside		Xyloside		6-Deoxyglucoside	
Time (min)	10^{10} Rate (M liter^{-1} sec^{-1})	Reaction (%)	10^{10} Rate (M liter^{-1} sec^{-1})	Reaction (%)	10^{10} Rate (M liter^{-1} sec^{-1})	Reaction (%)
5	0	0	0	0	0	0
30	6.06	0.17	10.4	0.40	14.5	0.37
60	8.48	0.40	12.1	0.80	20.6	0.97
90	10.90	0.73	12.1	1.17	22.5	1.67
	6-Chloro-6-Deoxyglucoside		6-*O*-Methylglucoside			
45	0	0	0	0		
60	2.20	0.23	3.03	0.13		
120	3.89	0.40	3.03	0.27		

TABLE 13

RATE OF FORMATION OF *p*-NITROPHENOL DURING INCUBATION OF CELLOPENTAOSE (5.7×10^{-3} *M*) AND *p*-NITROPHENYL β-D-GLYCOSIDES (6×10^{-4} *M*) WITH ENZYME EI (4.16×10^{-6} *M*) AT 20°C

	Glucoside		2-Deoxyglucoside	
Time (min)	10^{10} Rate (M liter^{-1} sec^{-1})	Reaction (%)	10^{10} Rate (M liter^{-1} sec^{-1})	Reaction (%)
5	0	0	0	0
30	4.58	0.13	0	0
60	6.67	0.03	0	0
120	10.33	0.90	0	0

The presence of aryl glycosides of higher oligosaccharides was demonstrated in the reaction mixture with the compound which released 3,4-dinitrophenol most slowly, 3,4-dinitrophenyl 6-chloro-6-deoxy-β-D-glucoside, by using a method similar to that described by Raftery and Rand-Meir in their investigation of lysozyme catalysed transglycosylation (*29*). The reaction mixture of 3,4-dinitrophenyl 6-chloro-6-deoxy-β-D-glucoside, cellopentaose, and EI after 2 h incubation at 20°C was passed through a column of Sephadex G-13 and the effluent was monitored at 282 nm. Between the peaks which corresponded to the enzyme and 3,4-dinitrophenyl 6-chloro-6-deoxy-β-D-glucoside two additional peaks were observed with elution volumes which corresponded to aryl glycosides with four and three sugar residues.

ACKNOWLEDGMENT

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