The use of 4-methylumbelliferyl and other chromophoric glycosides in the study of cellulolytic enzymes

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Received 6 October 1982

HPLC-analysis of the reaction products of a series of 4-methylumbelliferyl glycosides from cello-oligosaccharides, used as substrates of a cellobiohydrolase from *Trichoderma reesei*, proves the lack of specificity for terminal cellobiosyl groups. Also, different reaction patterns are observed for this CBHI and for an endocellulase, when acting on these same substrates. 4-Methylumbelliferyl β-D-lactoside is an unexpected substrate for CBHI, yielding only lactose and phenol as reaction products. The binding characteristics of *p*-nitrobenzyl 1-thio-β-D-lactoside for this enzyme are determined by a dia-filtration technique, yielding 1 binding site and an association constant of 4.0×10^4 M^{-1}.

Cellulase *4-Methylumbelliferyl glycoside Specificity* *Exo-cellobiohydrolase* *Endocellulase Binding*

1. INTRODUCTION

The study of the constituent enzymes of several cellulolytic complexes (e.g., from *Trichoderma reesei* QM 9414) has gained renewed interest. Some of these enzymes have been purified to homogeneity (e.g., two exocellobiohydrolases, endocellulases and a β-glucosidase) [1,2].

However, thorough specificity studies are lacking, undoubtedly due to the poor characterisation of the substrates used (unsoluble cellulose such as Avicel, filter paper, cotton).

This report emphasises the advantageous use of chromophoric dissacharide derivatives and a homologous series of 4-methylumbelliferyl glycosides of cellooligosaccharides [MeUmb β-(Glc)$_n$; n = 2–6] in the study of pure cellulolytic enzymes from *Trichoderma reesei*.

The exo-cellobiohydrolase I (CBHI) possesses a single binding site for *p*-nitrobenzyl 1-thio-β-D-lactoside, with an association constant K = (4.0 ± 0.4) 10^4 M^{-1} at 25°C. The 4-methylumbelliferyl β-D-lactoside [MeUmb β-Gal*p* β (1→4)Glc*p*] is characterised as a substrate, yielding the phenol and lactose as reaction products, and its steady-state reaction parameters (25°C) are compared with those from the corresponding cellobioside [MeUmb β-(Glc*p*)$_2$]. In contrast to the lactoside the latter substrate is attacked at both scissable bonds, as proved by HPLC analysis. A complicated action mode of the CBHI is also demonstrated for the other members of the MeUmb β-(Glc*p*)$_n$ series.

Endocellulase action on these same substrates yields HPLC patterns which prove preferential at-

Abbreviations: MeUmb β-Glc*p*, 4-methylumbelliferyl β-D-glucopyranoside; MeUmb β-(Glc*p*)$_n$ (n = 2–6), 4-methylumbelliferyl β-D-glycosides from cellobiose to cellohexaose); MeUmb β-Gal*p* β (1→4)Glc*p*, 4-methylumbelliferyl β-D-lactoside; CBH, 1,4-β-glucan cellobiohydrolase (EC 3.2.1.91); endocellulase, 1,4-β-glucan glucanohydrolase (EC 3.2.1.4); HPLC, high-pressure liquid chromatography

Published by Elsevier Biomedical Press
001457593/82/0000—0000/$2.75

tack at the penultimate bond near the reducing end.

2. MATERIALS AND METHODS

Peracetylated 4-methylumbelliferyl β-D-glycosides derived from the cello-oligosaccharides were prepared from a cellulose acetolysate, via the acetobromo-derivatives and a Koenig–Knorr type condensation reaction as described for the cellobioside [3]. The resulting syrupy mixture of 4-methylumbelliferyl glycosides was deacetylated conventionally and separated by gelfiltration on Biogel P-2 (Biorad) or Silicagel TSKHW-20 (Merck) with water as eluent. Alternatively, preparative HPLC was used (see further). Unsubstituted cello-oligosaccharides (cellotriose, cellopentaose) were obtained analogously from a cellulose acetolysate. The purity of the factions was proved by HPLC and the degree of polymerisation was determined by enzymic glucose assay [4] after total acid hydrolysis (0.1 N HCl, 100°C, 3 h). The concentration of the MeUmb β-(Glc*p*)$_n$ was determined using the molar absorption coefficient 13600 $M^{-1} \cdot cm^{-1}$ at 316 nm.

4-Methylumbelliferyl β-D-lactoside (MeUmb β-Gal*p* (1→4) Glc*p*) was synthesized as in [5] and the *p*-nitrobenzyl 1-thio-β-D-glycosides derived from cellobiose and lactose were prepared following [6]. The physical characteristics of all compounds newly synthesized will be published elsewhere.

The cellobiohydrolase I (CBHI) from *Trichoderma reesei* QM 9414 was purified from a commercial cellulase preparation (Celluclast™, type N from Novo Industries, København) by successive DEAE–Sepharose chromatography and isoelectric focusing [7]. Its purity was demonstrated by analytical isoelectric focusing [7]. The absorption coefficient at 280 nm (ϵ = 1.42 $mg^{-1} \cdot cm^2$) and relative molecular mass (M_r 48800) are as in [8].

1,4-β-Glucan glucanohydrolase from *Trichoderma reesei* QM 9414 was a gift from Dr R.D. Brown jr (Gainesville FL).

Enzymic assays are at 25°C and pH 5.0 (0.05 M sodium acetate buffer). Neither of the enzymes hydrolysed MeUmb β-Glc*p* (absence of β-glucosidase activity). HPLC (Waters Assoc. Model M-45 pump) was performed on a μPorasil column (0.39 × 30 cm) (Waters Assoc.); a R_{sil} column (1 × 25 cm) (Alltech) was used for preparative HPLC. Isocratic elution (flow 1.5 ml . min^{-1}) was either with 13% water in acetonitrile (for the lower-M_r glycosides) or with 17% (for the higher-M_r oligomers). Separation of unsubstituted cello-oligosaccharides was achieved by reversed-phase chromatography on Bondapak C-18 (0.39 × 30 cm) (Waters Assoc.), using 0.02 M sodium-acetate buffer (pH 5.0) as eluent (flow 0.5 ml . min^{-1}). The 4-methylumbelliferyl derivatives were detected at 313 nm, the unsubstituted cello-oligosaccharides with a refractive index detector.

Equilibrium binding studies (25 ± 2°C) by the diafiltration technique [9] were performed with an Amicon Model 8MC dialysis cell. The enzyme sample (in 6 ml 0.05 M sodium acetate buffer, 0.1 M KCl, pH 5.0) was stirred and a ligand stock solution (in the same buffer) added continuously under N_2-pressure (3 kg . cm^{-2}). A PM-10 filter (Amicon) was used and the filtrate was collected in ~ 0.6 ml fractions. The results were computed as in [9].

RESULTS AND DISCUSSION

3.1. Steady-state reaction parameters and binding characteristics of some low-M_r substrates and ligands of CBHI

The kinetic constants of cellotriose, MeUmB β-(Glc*p*)$_2$ and MeUmb β-Gal*p* β(1→4)Glc*p* are gathered in table 1. Catalytic efficiency of the CBHI for these substrates is low and, as revealed by qualitative HPLC analysis, the breakdown of the cellobioside is complicated by the formation of small amounts of MeUmb β-Glc*p* (fig. 2). On the other hand, the fluorogenic lactoside, an unexpected substrate of this enzyme, yields solely the phenol and lactose as reaction products. *p*-Nitrobenzyl 1-thio-β-D-lactoside is an excellent competitive inhibitor ($1/K_i$ = (3.4 ± 0.8) × 10^4 M^{-1} at 25°C), whereas the corresponding cellobioside is slowly degraded at the holosidic bond. The use of the 1-thio-lactoside in forced dialysis experiments (diafiltration) allowed the determination of the binding parameters of this low-M_r ligand to the CBHI (fig. 1A). The combined data from binding and blank experiments yield linear Scatchard plots (fig. 1B). These indicate 1.1 ± 0.1 binding sites per M_r = 48800 and an association constant of (4.0 ± 0.4) × 10^4 M^{-1} for *p*-nitrobenzyl 1-thio-β-D-lactoside, in excellent agreement with the above inhibition constant.

Table 1

Steady-state kinetic data for some substrates of CBH I[a]

Substrate	Range (M)	K (M)	Turnover no. (s^{-1})
MeUmb β-(Glc$p$)$_2$[b]	10^{-5}–10^{-4}	$3.5\times10^{-5}\pm0.8\times10^{-5}$	0.002
MeUmb β-Gal$p\beta(1\rightarrow4)$Glc$p$[b]	10^{-4}–10^{-3}	$5.2\times10^{-4}\pm0.5\times10^{-4}$	0.04
Cellotriose[c]	10^{-3}–10^{-2}	$1.2\times10^{-2}\pm0.1\times10^{-2}$	0.26

[a] The data were obtained by plotting of the reciprocal initial velocities against the corresponding substrate concentrations (Hanes-plot); enzyme 0.05 μM (25°C, 0.05 M sodium acetate buffer, pH 5.0)

[b] Fluorimetric measurement (pH 10.0) of 4-methylumbelliferone (7-hydroxy-4-methylcoumarine) (excitation 335–400 nm, emission >435 nm)

[c] Glucose assayed with the glucose oxidase–peroxidase reagent [4]

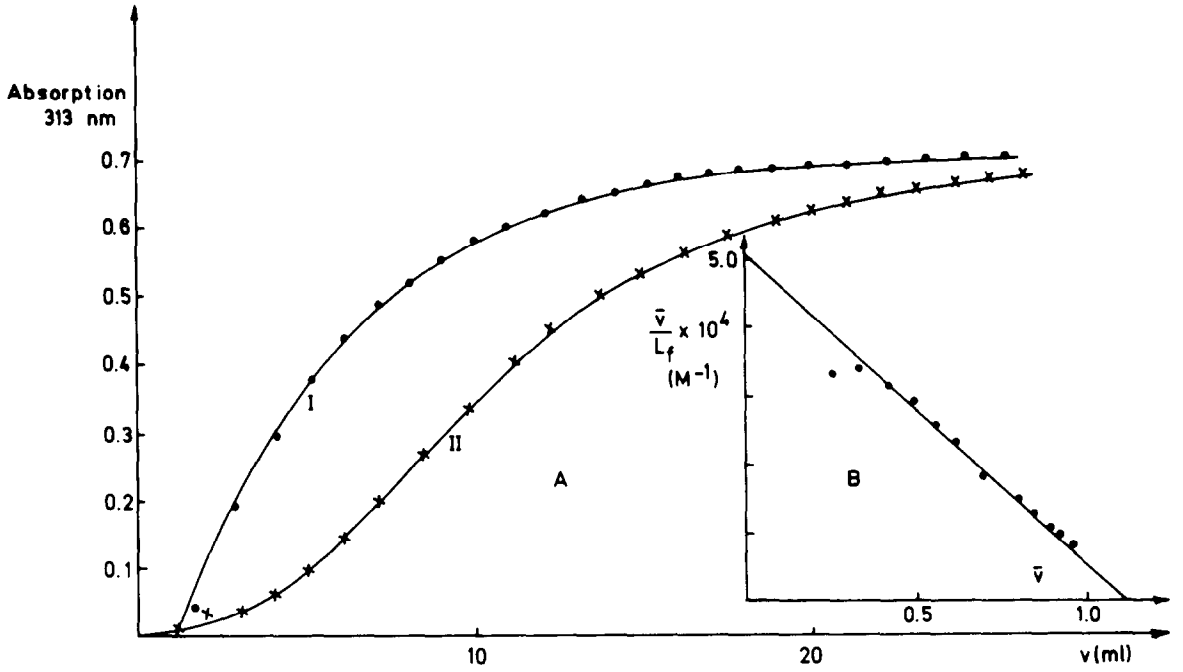

Fig. 1. Determination of binding constants of p-nitrobenzyl 1-thio-β-D-lactoside for CBH I by diafiltration [9]. (A) The filtrate is collected in fractions and the concentration of free ligand (L_f) is determined spectrophotometrically (313 nm). The ligand concentration in the stock solution is 200 μM. The amount of bound ligand is computed as in [9] from a blank (curve I) and a binding experiment (curve II). (B) Scatchard plot: $\bar{\nu}$ is the degree of saturation as calculated from the amount of bound ligand and protein (170 μM) in the dialysis cell.

Thus, at least in the case of the lactose derivatives, the presence of a restricted binding mode or area on the CBHI can be suggested, whereas for the MeUmb β-(Glc$p$)$_2$ hydrolysis two productive complexes could exist.

3.2. *The use of MeUmb β-(Glc$p$)$_n$ (n = 2–6) in a qualitative HPLC study of the action of CBHI and endocellulase*

With the unsubstituted cello-oligosaccharides as substrates of CBHI it was shown by reversed-phase

HPLC that glucose and cellobiose were formed from cellotriose, whereas cellopentaose yielded considerable amounts of cellotetraose (not shown). The findings prompted us to investigate the action of the title enzymes on MeUmb(Glc*p*)$_n$. The presence of the chromophoric group improved considerably the detection (313 nm) during HPLC analysis. With both enzymes the reaction rates increase with the degree of polymerisation of these substrates. This could readily be judged from the peak heights in the degradation patterns (fig. 2) valid for ~50% substrate consumption.

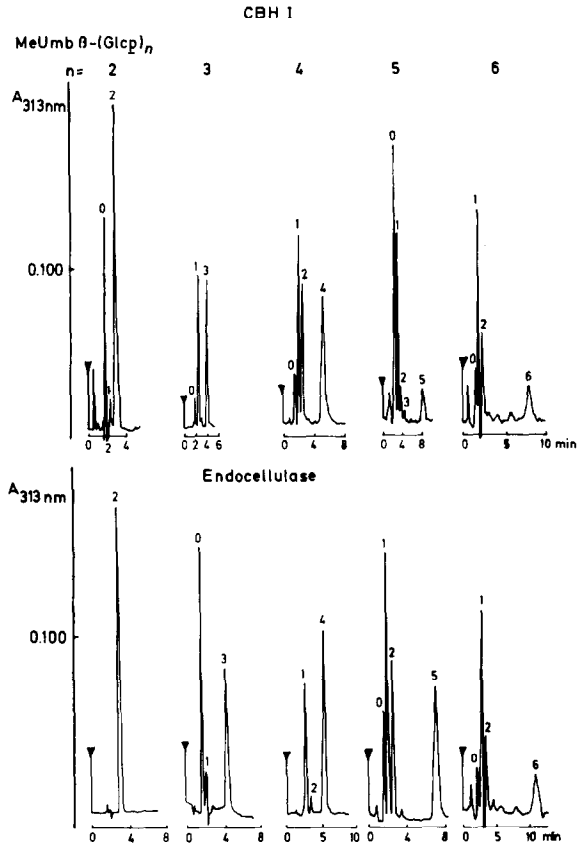

Fig. 2. HPLC analysis (μPorasil column) of the degradation of MeUmb β-(Glc*p*)$_n$ ($n=2$–6) by CBH I and by endocellulase. Sample volume was 20 μl and substrate concentrations were between 500 μM (for the bioside) and 10 μM (for the hexaoside); enzyme was ~0.05 μM (pH 5.0, 25°C). Reaction times were <30 min. Peak identification: (0) 4-methylumbelliferone; (1) MeUmb β-Glc*p*; (2) MeUmb β-(Glc*p*)$_2$; (3) MeUmb β-(Glc*p*)$_3$; (4) MeUmb β-(Glc*p*)$_4$; (5) MeUmb β-(Glc*p*)$_5$; (6) MeUmb β-(Glc*p*)$_6$.

For the CBHI none of these substrates gives the expected pattern of reaction products. MeUmb β-(Glc*p*)$_2$, in contrast to MeUmb β-Gal*p* β (1 → 4)Glc*p*, is attacked at both glycosidic bonds. For MeUmb(Glc*p*)$_3$ and MeUmb β-(Glc*p*)$_5$ simultaneous monitoring of the substrate disappearance (HPLC) and phenol production (fluorescence) proves that both run concurrently, without any evidence for a lag-period (not shown). Thus 4-methylumbelliferone production probably results from direct enzymic attack at the heterosidic bond of these substrates. This anomaly in the alleged specificity of the CBHI becomes even more prominent in the case of the higher cellodextrin derivatives: with MeUmb β-(Glc*p*)$_4$, the glucoside becomes an important reaction product and the cellobioside is formed with MeUmb β-(Glc*p*)$_5$. For this last substrate, as well as for MeUmb β-(Glc*p*)$_6$, only spurious amounts of intermediate products, such as MeUmb β-(Glc*p*)$_3$ or MeUmb β-(Glc*p*)$_4$ can be observed.

All these facts point to a serious lack of specificity of the CBHI, at least for this class of soluble substrates.

The endocellulase has no action on MeUmb β-(Glc*p*)$_2$ or MeUmb β-Gal*p* β-(1 → 4)Glc*p*. MeUmb β-(Glc*p*)$_3$ yields predominantly free phenol (and cellotriose), in contrast to the action of CBHI on the same substrate. For the higher members of the series MeUmb β-Glc*p* becomes the main reaction product, which points to a preferred scission at the penultimate glycosidic bond near the reducing end. In fact, random action seems less obvious for this endocellulase than for the exo-enzyme (CBHI). For both enzymes, and at the low substrate concentrations used, no evidence (HPLC) for the formation of transfer products could be obtained.

In conclusion, our results with MeUmb β-(Glc*p*)$_n$ indicate complex action patterns for both CBHI and endocellulase from *Trichoderma reesei*. Only with the lactosides and CBHI were straightforward kinetic and binding data obtained.

With the higher oligomer derivatives a complete description of the action pattern should probably involve multiple binding modes of the substrates with the active sites of the enzymes.

ACKNOWLEDGEMENT

M.C. is indebted to NATO and to the Belgian NFWO for financial support.

REFERENCES

[1] Fägerstam, L.G. (1981) Acta Universitatis Upsaliensis, Dissertations from the Faculty of Science. 610 abst.
[2] Gritzali, M. and Brown, R.D. jr (1979) Adv. Chem. Ser. 181, 237–260.
[3] Constantzas, N. and Kocourek, J. (1959) Coll. Czech. Chem. Commun. 24, 1099–1111.
[4] Lloyd, J.B. and Whelan, W.J. (1969) Anal. Biochem. 30, 467–469.
[5] De Boeck, H., Matta, K.L., Claeyssens, M., Sharon, N. and Loontiens, F. (1982) Eur. J. Biochem. in press.
[6] Claeyssens, M., Kersters-Hilderson, H., Van Wauwe, J.P. and De Bruyne, C.K. (1970) FEBS Lett. 11, 336–338.
[7] Fägerstam, L.G. and Pettersson, L.G. (1979) FEBS Lett. 98, 363–367.
[8] Gum, E.K. and Brown, R.D. jr (1976) Biochim. Biophys. Acta 446, 371–386.
[9] Yap, W. and Schaffer, R. (1977) Clin. Chem. 23, 986–989.