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Journal of Chromatography B, 752 (2001) 349–368

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Characterization of cellobiohydrolase I (Cel7A) glycoforms from extracts of *Trichoderma reesei* using capillary isoelectric focusing and electrospray mass spectrometry

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Abstract

Capillary isoelectric focusing (CIEF) was used to profile the cellulase composition in complex fermentation samples of secreted proteins from *Trichoderma reesei*. The enzyme cellobiohydrolase I (CBH I, also referred to as Cel7A), a major component in these extracts, was purified from different strains and characterized using analytical methods such as CIEF, high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC–PAD), and capillary liquid chromatography–electrospray mass spectrometry (cLC–ESMS). ESMS was also used to monitor the extent of glycosylation in CBH I isolated from *T. reesei* strain RUT-C30 and two derivative mutant strains. Selective identification of tryptic N-linked glycopeptides was achieved using LC–ESMS on a quadrupole/time-of-flight instrument with a mixed scan function. The suspected glycopeptides were further analyzed by on-line tandem mass spectrometry to determine the nature of N-linked glycans and their attachment sites. This strategy enabled the identification of a high mannose glycan attached to Asn270 (predominantly $Man_8GlcNAc_2$) and single GlcNAc occupancy at Asn45 and Asn384 with some site heterogeneity depending on strains and fermentation conditions. The linker region of CBH I was shown to be extensively glycosylated with di-, and tri-saccharides at Thr and Ser residues as indicated by MALDI-TOF and HPAEC–PAD experiments. Additional heterogeneity was noted in the CBH I linker peptide of RUT-C30 strain with the presence of a phosphorylated di-saccharide.

Keywords: Enzymes; Cellobiohydrolase I

1. Introduction

Trichoderma reesei is a filamentous fungus capable of effectively hydrolyzing crystalline cellulose to glucose. Its efficient secretory ability makes it a useful organism for the large-scale production of enzymes for a variety of applications in the pulp and paper, textiles and animal feeds industries as well as a target for the development of recombinant strains for heterologous protein production [1–3]. The cellulolytic enzyme system of *T. reesei* consists of two major cellobiohydrolases (CBH) and several endoglucanases (EG), together with small amounts of β-glucosidase [4]. Synergism exists between the

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0378-4347/01/$ – see front matter
PII: S0378-4347(00)00373-X

cellobiohydrolases and endoglucanases to carry out the degradation of cellulose [5,6]. In general, the cellobiohydrolases and endoglucanases show significant N- and/or O-linked glycosylation. With the exception of EG III, these cellulases comprise a catalytic domain (core) and a cellulose-binding domain (CBD) connected via a linker peptide which is heavily glycosylated at Ser and Thr residues. The role of glycosylation is believed to be related to the secretion of these extracellular enzymes, to provide sufficient spatial separation between the core and CBD domains and to protect the linker peptide against proteolysis [7,8]. Differences in biological roles between N- and O-linked glycosylation in *T. reesei* have been studied to some extent, and it has been reported that only the O-linked glycosylation is necessary for the secretion of EG I and EG II [9].

Among the cellulolytic enzymes, the CBH I comprises up to 60% of the total protein secreted by *T. reesei* [10]. CBH I contains four putative N-linked sites located in the CBH I core (positions 45, 64, 270 and 384, Fig. 1) of which three of these sites, Asn45, Asn270 and Asn384, have been shown to be glycosylated [10–12]. The presence of mammalian high-mannose-type N-linked glycans has been reported in the CBH I from *T. reesei* RUT-C30 strain by M. Maras et al. [13]. They found that the high mannose-type glycans are heterogeneous, ranging from the simple $Man_5GlcNAc_2$ to the most complex configuration $ManP\text{-}GlcMAn_7GlcNAc_2$. In detailed structur-

pESACTLQSET	HPPLTWQKCS	SGGTCTQQTG	SVVIDANWRW	THAT(N)[a]SSTNC 50
YDGNTWSSTL	CPD(N)ETCAKN	CCLDGAAYAS	TYGVTTSGNS	LSIGFVTQSA 100
QKNVGARLYL	MASDTTYQEF	TLLGNEFSFD	VDVSQLPCGL	NGALYFVSMD 150
ADGGVSKYPT	NTAGAKYGTG	YCDSQCPRDL	KFINGQANVE	GWEPSSNNAN 200
TGIGGHGSCC	SEMDIWEANS	ISEALTPHPC	TTVGQEICEG	DGCGGTYSDN 250
RYGGTCDPDG	CDWNPYRLG(N)[b]	TSFYGPGSSF	TLDTTKKLTV	VTQFETSGAI 300
NRYYVQNGVT	FQQPNAELGS	YSGNELNDDY	CTAEEAEFGG	SSFSDKGGLT 350
QFKKATSGGM	VLVMSLWDDY	YANMLWLDST	YPT(N)[c]ETSSTP	GAVRGSCSTS 400
SGVPAQVESQ	SPNAKVTFSN	IKFGPIGSTG	NPSGGN*PPGG*	*NPPGTTTTRR* 450
PATTTGSSPG	***P*TQSHYGQCG**	**GIGYSGPTVC**	**ASGTTCQVLN**	**PYYSQCL** 497

Catalytic Domain (Core) – [1 – 436]
Linker Domain – [437 – 461]
Cellulose-Binding Domain (CBD) – [462 – 497]
(N) Putative N-glycosylation sites
[a] GlcNAc; [b] GlcNAc or $Man_8GlcNAc_2$; [c] GlcNAc
pE denotes N-terminal pyroglutamic acid
↑ Papain cleavage site

Fig. 1. Amino acid sequence of CBH I from *T. reesei*. The structure consists of the CBH I catalytic domain and the cellulose-binding domain connected via a linker peptide. The four putative N-linked glycosylation sites are circled. Inset indicates glycan and site occupancy found in the present investigation.

al investigations, Klarskov et al. [12] and Harrison et al. [11] have characterized the sites of N-linked glycosylation in CBH I from *T. reesei* strain QM9414 [12] and strain ALKO2877, a derivative of strain QM9414 from which the genes for the major endoglucanases have been deleted [11]. In each study, selective identification of glycosylated residues revealed the presence of single GlcNAc in three putative N-linked sites located in the catalytic domain. The O-linked glycans, on the other hand, are located mainly in the linker domain, and the glycosylation consists of one to three mannoses [11]. In addition, the linker region of this CBH I was found to be partially sulfated [11]. These two glycosylation modifications have not been reported previously for CBH I from other strains from *T. reesei*.

In the present study, we investigated the analytical potential of CIEF to probe the complex heterogeneity of cellulase extracts obtained from different strains of *T. reesei*. Cellulases all have different isoelectric points (p*I*) which confer to them physico-chemical properties ideally suited to this mode of separation. This technique, first introduced by Hjertén and Zhu in 1985 [14], combines the high resolving power of conventional IEF performed in slab gels with the efficient dissipation of Joule heat due to the separation in small diameter capillaries [15]. Separation is performed usually with capillary of reduced electroosmotic flow (EOF) in a two step process: focusing of analytes according to their p*I* values in a pH gradient created by carrier ampholyte, followed by hydrodynamic or chemical mobilization. Single-step CIEF has also been described using uncoated or dynamically coated capillaries, in which dynamic focusing is performed simultaneously with mobilization [16,17]. This technique provides resolution of proteins differing only by 0.05 p*I* units with an enhancement of concentration detection limit ranging from 50–100 fold compared to conventional zone electrophoresis [18]. The application of CIEF has been demonstrated previously for clinical samples [19,20] and for the quantitation of hemoglobins and its variants [21,22]. However, precise quantitation measurements often require the use of internal standard to account for variation in migration time and sample buffers. The difficulty in analyzing crude cellulase samples is further compounded by the natural heterogeneity of these glycoproteins as recently described by Medve and co-workers for CBH I, CBH II and EG II using fast protein liquid chromatography (FPLC) and IEF separation of the resulting fractions [23].

In the present report, electrospray mass spectrometry (ESMS) was used to monitor the extent of glycosylation in the native CBH I purified by ion-exchange chromatography from three related *T. reesei* strains. The identification of N-linked glycosylation sites in tryptic peptides from CBH I was achieved using LC–ESMS on a quadrupole/time-of-flight instrument with a mixed scan function enabling alternate acquisition of $m/z<400$ at high orifice voltage and $m/z>400$ at low orifice voltage [24,25]. The structures of the oligosaccharides and the peptide backbone were determined by on-line tandem mass spectrometry. Papain digestion of CBHI followed by MALDI-TOF analysis of the resulting proteolytic fragment revealed an extensively glycosylated linker peptide. The extent of this heterogeneity was monitored by HPAEC–PAD analysis of the hydrazinolysis products with subsequent characterization using ESMS. An abbreviated account of this work was presented in an earlier communication [26].

2. Experimental

2.1. Reagents and materials

All cellulase fermentation samples and purified cellulolytic enzyme standards were provided by Iogen Corporation (Ottawa, ON, Canada). For CBH I purification, the DEAE sepharose (diethylaminoethyl weak anion-exchanger) was obtained from Amersham Pharmacia Biotech (Amersham Place, Buckinghamshire, UK) and packed into a poly-prep chromatography column purchased from Bio-Rad (Hercules, CA, USA). Protein concentration and desalting was achieved using Ultrafree-4 centrifugal filter units, Biomax-5 from Millipore (Bedford, MA, USA). Micro-dialysis was carried out using 2000 Da and 5000 Da molecular weight cut-off (MWCO) membranes from AmiKa Corporation (Columbia, MD, USA). Sodium phosphate monobasic ($NaH_2PO_4 \cdot H_2O$) and dibasic ($Na_2HPO_4 \cdot 7H_2O$)

were from BDH (Toronto, ON, Canada). The DL-dithiothreitol and iodoacetamide were from Sigma (St. Louis, MO, USA) while β-mercaptoethanol and the ammonium bicarbonate were obtained from BDH (Toronto, ON, Canada) and from Fisher Scientific (New Jersey, NY, USA), respectively. Sequencing-grade trypsin was purchased from Promega (Madison, WI, USA), lyophilized papain powder was from Sigma (St. Louis, MO, USA), and both the calf intestinal alkaline phosphatase (CIP) and PNGase F were from New England BioLabs (Beverly, MA, USA). Wide range pH 3–10 and narrow range pH 3–6 carrier ampholytes were obtained from Beckman (Fullerton, CA, USA) and from SERVA Electrophoresis GmbH (Heidelberg, Germany), respectively.

2.2. Culture and growth conditions

Fermentation Conditions for *Trichoderma reesei* strains: Strains RUT-C30, Iogen-M4 and Iogen-B13 were grown in 10 l fermentation vessels at 28°C for 5–6 days essentially as described by Mandels and Reese [27] for optimal growth and production of cellulase by QM6a. The growth medium typically contains: 1.4 g/l $(NH_4)_2SO_4$, 2 g/l KH_2PO_4, 0.3 g/l $CaCl_2$, 0.3 g/l $MgSO_4$, 5 mg/l $FeSO_4 \cdot 7H_2O$, 1.6 mg/l $MnSO_4 \cdot H_2O$, 1.4 g/l $ZnSO_4 \cdot 7H_2O$, 2 g/l $CoCl_2$, 4.2% corn steep liquor adjusted to pH 4.0 or pH 5.0. The carbon source for the fermentations comprises 5 g/l glucose plus 10 g/l Solka floc or other carbon source which can be sterilized separately as an aqueous solution at pH 2 to 7 and added to the remaining media.

2.3. CBH I purification

The intact CBH I from fermentation samples produced by RUT-C30 and two derivative strains, Iogen-M4 and Iogen-B13, was fractionated in several steps using ion-exchange chromatography with DEAE sepharose gel [28]. A low salt phosphate buffer (50 m*M* sodium phosphate, pH 6.0, 25 m*M* NaCl) was used for column equilibration. Approximately 10 mg of cellulase extract was loaded onto the column packed with 3 ml bed volume. After elution of the unretained components, CBH I was eluted in a phosphate high salt buffer (50 m*M* sodium phosphate, pH 6.0, 300 m*M* NaCl). The DEAE sepharose was then washed with five column volumes of 1.0 *M* NaOH, followed by ten column volumes of de-ionized water and stored in 20% ethanol. The enriched CBH I fraction was desalted and concentrated by using the Ultrafree-4 centrifugal filter unit, on a refrigerated swinging-bucket centrifuge (Sorvall RT6000B, Du Pont Instruments, Newtown, Connecticut, USA).

2.4. Purification of CBH I papain digestion products

The proteolytic fragments obtained from papain digestion of CBH I (Section 2.6) were separated using gel-filtration chromatography on a Pharmacia Superdex 75 HR 10/30 (high resolution, ID 10 mm×30 cm in length) column as described previously [29]. The column was initially equilibrated with 50 m*M* ammonium carbonate pH 5.0, 150 m*M* NaCl and the flow-rate was maintained at 0.5 ml/min by a Pharmacia fast protein liquid chromatographic (FPLC) system. Approximately 1 mg of CBH 1 papain digest in 200 μl final volume was introduced into the column via an injection port. The elution of individual components was monitored by an on-line UV detector with wavelength set at 280 nm. Initially, a sharp peak of CBH I core was eluted, followed by a broad peak of the combined CBH I linker and CBD domains of weaker absorbance. The collected components were concentrated and desalted by the Ultrafree-4 centrifugal filter unit, and subsequently diluted with deionized water to the desired concentration.

2.5. CIEF–UV

All CIEF experiments were conducted on a Beckman PACE 5000 system. Separations were carried out using a Beckman eCAP neutral coated capillary (ID 50 μm×OD 365 μm, 27 cm in length). The cellulase standards or cellulase extracts were dissolved to their final concentration in an aqueous solution consisting of 3% blended carrier ampholyte [Beckman ampholyte:Servalyt (3:1, v/v)]. The analyte (inlet) was 10 m*M* phosphoric acid and a catholyte of 20 m*M* NaOH was used at the capillary outlet. A voltage of 13.5 kV was used to focus the

analyte band for a duration of 10 min followed by cathodic mobilization using a buffer of methanol–water–acetic acid (50:49:1, v/v/v) [22]. During focusing and mobilization, the voltage was maintained at a field strength of 13.5 kV (500 V/cm). The detector was set at 280 nm.

2.6. CBH I enzymatic digestion

Tryptic digests (typically 500 μg of CBH I) were performed on reduced and alkylated proteins previously denatured in 50 μl of 6.0 *M* guanidine/HCl, 0.1 *M* Tris–HCl, 1 m*M* EDTA, pH 8.5. Argon gas was gently blown over the solution surface for 10 min. Reduction was carried out by the addition of 50 μl of 4 m*M* DL-dithiothreitol, and the solution was then incubated for 2 h at 37°C. Alkylation was performed by adding 40 μl of 50 m*M* iodoacetamide dropwise to the mixture, followed by incubation in the darkness for 1 h at 37°C. The excess iodoacetamide was quenched by adding 1 μl of β-mercaptoethanol and subsequently dialyzed against 500 ml of 50 m*M* ammonium bicarbonate for a minimum of 24 h with periodic change of dialysis buffer. Sample solution was then lyophilized and redissolved in 0.1 *M* ammonium bicarbonate, 0.1 m*M* calcium chloride, pH 8. Tryptic digestion was carried out at 37°C for 12 h using a substrate-to-enzyme ratio of 25:1 (w/w).

The papain digestion of CBH I was conducted according to a procedure described previously [11]. Briefly, the lyophilized papain powder was re-suspended in 200 m*M* K_2HPO_4, 2 m*M* EDTA, 5 m*M* L-cysteine, pH 7.0 to a concentration of 2 mg/ml. An aliquot of 8 μl of this enzyme suspension was added to 200 μl of 5 mg/ml CBH I dissolved in 100 m*M* ammonium acetate, pH 5 equivalent to a substrate-to-enzyme ratio of 60:1 (w/w). The mixture was incubated at 37°C for 16 h. The resulting digest was purified using gel filtration chromatography described in Section 2.4.

The alkaline phosphatase incubation was performed on purified CBH I linker and CBD domains from RUT-C30. Approximately 100 μg of CBH I linker plus CBD was dissolved in 20 μl of 1X NE Buffer 3 also supplied by New England BioLabs, followed by the addition of 10 units of CIP. The mixture was incubated at 37°C and small aliquot was pipetted out periodically to monitor the dephosphorylation activities by MALDI-TOF mass spectral analysis. No further structural change was detected after 3 h.

All PNGase F incubations were performed on approximately 20 μg of the original tryptic digests of CBH I to which were added 500 U endoglycosidase corresponding to an equivalent of substrate-to-enzyme ratio of 70:1. The digestion was allowed to proceed for 24 h at 37°C in 50 m*M* ammonium bicarbonate, pH 8 [30].

2.7. Mass spectrometry

MALDI-TOF mass spectra were acquired on a PerSeptive Biosystems Elite-STR (Framingham, MA, USA) linear or reflectron positive ion mode equipped with delayed extraction technology. An aliquot of the tryptic digest (1 μl) was mixed with 2 μl of the MALDI matrix, 2,5-dihydroxybenzoic acid (2,5-DHB), originally prepared as 0.2 *M* in 50:50 methanol–deionized water. A 0.5 μl aliquot of this final solution was applied on the MALDI target and allowed to evaporate at room temperature prior to mass spectral analysis. The singly-protonated ions of a peptide standard mixture (PerSeptive Biosystems) were used to calibrate the mass spectrometer (external calibration) and reflectron mode was applied to enhance mass resolution. In papain digest analysis, a 1 μl of sample with typical concentration of 1 mg/ml was mixed with 10 μl of sinapinic acid (SA) matrix solution, prepared as 10 mg/ml in a mixture of methanol–acetonitrile–water (1:1:1, v/v/v), and an aliquot of 0.5 μl was deposited on the MALDI plate. External mass calibration was carried out using the singly-protonated ions of insulin and apo-myoglobin and the mass spectra were acquired using linear mode.

ESMS experiments were acquired using either a Micromass Q-TOF (Manchester, UK) or a PE/Sciex Q-Star (Thornhill, ON, Canada) hybrid quadrupole/time-of-flight instrument. Flow injection analyses were obtained by dissolving the CBH I preparation in 50% acetonitrile (0.2% acetic acid). Approximately 1 μl of this solution was injected into a stream of 50% acetonitrile (0.2% acetic acid) introduced at a flow-rate of 5 μl/min to the mass spectrometer.

An HPLC HP1100 (Hewlett-Packard, Palo Alto,

CA) was used for on-line cLC–ESMS experiments. Chromatographic separations were achieved on a 15 cm×0.32 mm PepMap capillary column (LC Packings, San Francisco CA) using a linear gradient elution of 5–95% acetonitrile (0.2% HCOOH) in 35 min. A 100:1 flow splitter was mounted before the injector such that only 3–4 μl/min flow-rate was introduced in the capillary column. The sample was manually injected via a Rheodyne six-port valve (Rohnert Park, California, USA) fitted with a 5 μl sample loop. Conventional mass spectra were obtained by operating the quadrupole in a RF-only mode while a pusher electrode was pulsed to transfer all ions to the time-of-flight analyzer. Mass spectra were acquired using stepped orifice-voltage scanning similar to that described previously [24,25]. In the present work, the sampling orifice voltage was maintained at 100 V during low mass scanning (m/z 150–400) and 30 V during high mass scanning (m/z 400–2000). In MS–MS experiments, glycopeptide precursor ions were selected by the first quadrupole while a pusher electrode was pulsed to transfer fragment ions formed in the R.F.-only hexapole cell to the time-of-flight analyzer. Mass spectral resolution was typically 4000–5000. A scan duration of 1 s and 2 s was set for conventional and MS–MS mass spectral acquisition, respectively. Collisional activation was performed using argon collision gas with a 25 V offset between the DC voltage of the entrance quadrupole and the R.F.-only hexapole cell. Data were acquired and processed in the Mass Lynx Window NT based data system.

2.8. Automated hydrazinolysis

Pools of glycans were released from 2 mg of each glycoprotein using the Glycoprep 1000 glycan release and preparation system (Oxford GlycoSciences, UK) for automated hydrazinolysis. Each glycoprotein was processed using a N+O program (95°C, 5 h) mode for optimal release and isolation of N- and O-linked oligosaccharides. At the end of each processing run, the intact and unreduced pools of glycans were immediately filtered through a 0.2 μm nylon membrane and dried by centrifugal vacuum evaporation at room temperature.

2.9. HPAEC–PAD

The dried glycans released by automated hydrazinolysis were dissolved in deionized water and aliquots were analyzed by HPAEC–PAD using a DX-300 chromatograph (Dionex Corporation, Sunnyvale, CA) equipped with a PA-100 analytical column (4×250 mm). Chromatographic conditions typically used a linear gradient of 5–230 m*M* sodium acetate (100 m*M* sodium hydroxide) in 45 min with a flow-rate of 1 ml/min. Approximately 10% of the glycan pool was injected on the column. All data processing and peak integration was performed using the AI-450 chromatography software (Dionex). To further determine the structure of released oligosaccharides, a carbohydrate membrane desalter (CMD) from Dionex was introduced after the PAD detection cell thereby allowing direct fraction collection. The CMD used 0.15 *M* trifluoroacetic acid (TFA) to generate H^+ ions required for cation-exchange. The desalted fractions were subjected to mass spectral analysis.

3. Results and discussion

3.1. CIEF–UV analysis of cellulase extracts

The main cellulases from *T. reesei* are acidic glycoproteins (p*I*<5.5) and this property is exploited advantageously for their purification using ion-exchange chromatography [23,27]. The most abundant cellobiohydrolases and endoglucanases have distinct p*I* values which make them ideal analytes for separation based on isoelectric focusing. The analysis of cellulase standards together with crude secreted protein samples from three different *T. reesei* strains (RUT-C30, Iogen-M4 and Iogen-B13) is shown in Fig. 2. In this application, a wide range ampholyte 3–10 was blended with a Servalyt 3–7 buffer to enhance the resolution of the cellulases in the acidic region of the pH gradient. The electropherogram shown in Fig. 2a shows the separation of six cellulase standards at a concentration level of 120 μg/ml each. In most instances, the glycoproteins were focused into narrow peaks with no discrete separation of their corresponding glycoforms. The

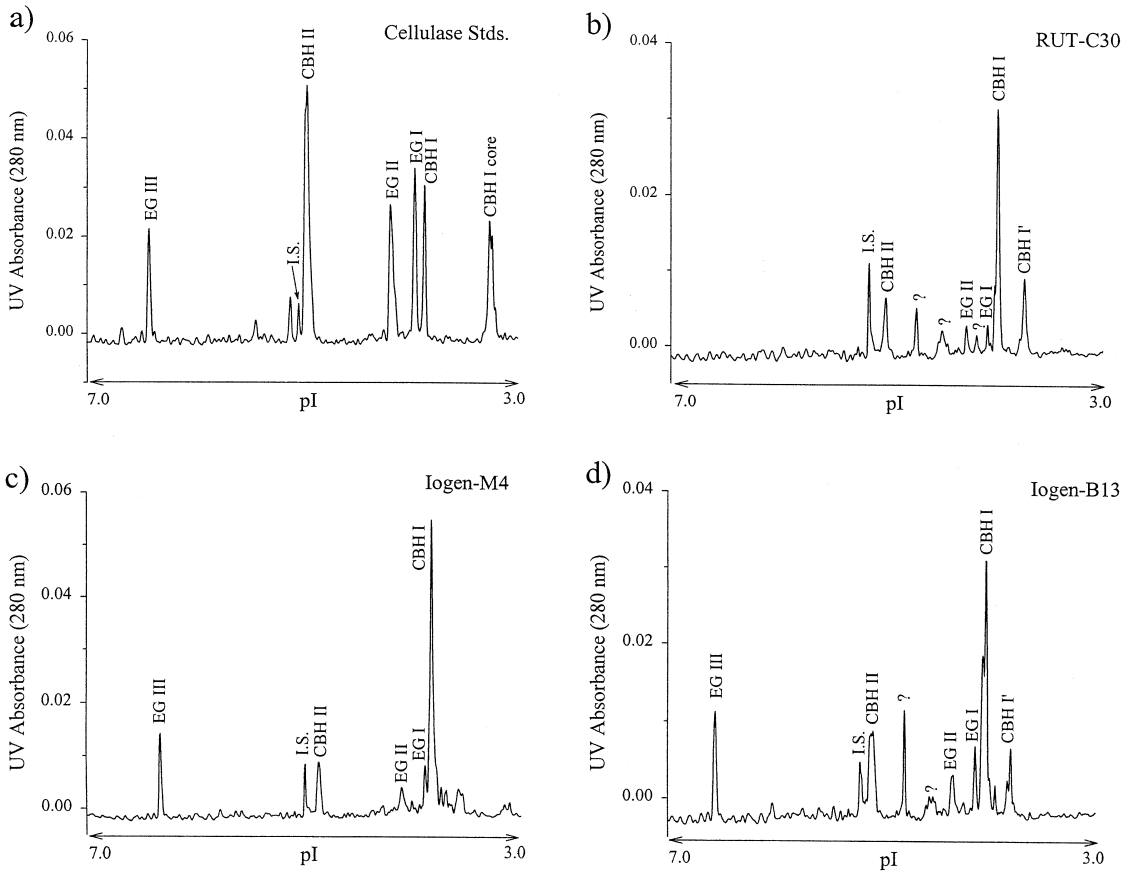

Fig. 2. CIEF analysis of cellulase standards and fermentation samples from *T. reesei*. (a) mixture of 6 cellulase standards; protein conc.: 120 μg/ml each except for EG=30 μg/ml (b) RUT-C30, protein conc.: 480 μg/ml; (c) Iogen-M4, protein conc.: 578 μg/ml; (d) Iogen-B13, protein conc.: 531 μg/ml. All samples were spiked with the internal standard (I.S.) 50 μg/ml β-lactoglobulin A. The CBH I' indicated on (b) and (d) is likely due to the phosphate modifications in the linker region. See experimental for conditions.

limit of detection of the CIEF technique was investigated using serial dilutions ranging from 1 to 500 μg/ml of each cellulase.

In all cases good linearity, with a correlation coefficient $r^2=0.982–0.999$ was found over analyte concentrations ranging from 10 μg/ml to 200 μg/ml. Low μg/ml detection limits were achieved for all cellulase standards. CIEF analyses of the same samples spiked with chemical markers obtained separately also provided a convenient means of determining their p*I* values. It is noteworthy that the observed p*I* for most cellulases are generally lower than that calculated from their corresponding amino acid sequences. This suggests possible changes in acidity due to the effects of tertiary structure and protein glycosylation (Table 1). One source for the relatively high concentration detection limit observed in Fig. 2 is the instability of the chemical noise at 280 nm presumably associated with the carrier ampholyte. The performance of several carrier ampholytes was evaluated. The carrier ampholyte, pharmalyte offered a stable baseline although lower resolution was generally obtained for these acidic proteins. A blended ampholyte mixture of the Beckman ampholyte 3–10 with the Servalyt 3–7 was found to yield a reasonable compromise in terms of resolution and sensitivity, and was thus used in all CIEF experiments.

Table 1
Distribution of cellulases in secreted protein extract from different strains of *T. reesei*

Cellulases	RUT-C30[a]	Iogen-M4[a]	Iogen-B13[a]	p*I*		LOD	r^2
	(μg/ml)	(μg/ml)	(μg/ml)	Obs.	Calc.[b]	(μg/ml)	
CBH I	169.3±12.1 (87.9)	135.5±16.3 (76.8)	174.5±21.9 (74.5)	3.82	4.30	6	0.941
CBH I core	n.o.	n.o.	n.o.	<3.0	4.17	5	0.999
CBH II	8.1±0.2 (4.2)	9.6±0.8 (5.5)	21.2±2.5 (9.1)	5.20	5.12	24	0.987
EG I	7.0±0.6 (3.6)	9.5±1.1 (5.4)	14.5±1.7 (6.2)	3.88	4.51	9	0.989
EG II	8.2±0.6 (4.2)	10.8±0.9 (6.2)	13.8±2.4 (5.9)	4.19	4.73	9	0.982
EG III	n.o.	10.8±0.4 (6.2)	9.9±1.7 (4.3)	6.79	6.49	4	0.995

[a] Total protein concentration was 480, 578 and 531 μg/ml for RUT-C30, Iogen-M4 and Iogen-B13, respectively (based on Bradford assay). Numbers in parentheses correspond to relative percentage of the different cellulases in the corresponding protein extract ($n=5$) using β-lactoglobulin A as an internal standard.
[b] p*I* values obtained from ProMaC 1.1.1 program (urs.roethlisberger@roche.com). n.o.: not observed.

In the present case, CIEF analyses using the optimized blended ampholyte system provided a rapid analytical tool to determine the relative distribution of individual enzymes in crude cellulase fermentation samples and offered a viable alternative to slab IEF gel. In order to obtain good reproducibility, an internal standard (β-lactoglobulin A) was spiked in all samples to correct for instrumental variations between subsequent runs. Individual response factors were first calculated in a preliminary CIEF experiment. These values were then used to determine the concentration of each cellulase in their respective extracts (Table 1). Among the six standards, EG III had the lowest response factor possibly related to its relatively small molecular mass. In all cases data presented in Table 1 correspond to the cellulase concentrations based on peak area ratios obtained from five consecutive injections. The relative proportion of individual cellulase is indicated in parentheses. For CBH I, the intra-day % RSD ranged from 0.3% to 3%. This is in agreement with the reproducibility evaluation of CIEF reported by Tang et al. [31]. However, the % RSD becomes larger for other cellulases of lower concentration thus reflecting the larger influence of the noise level on the corresponding measurement.

Consistent with previous investigations [10,32], the results displayed in Table 1 clearly indicate that CBH I is the major cellulase observed in the secreted protein from *T. reesei*. On a relative weight basis, its presence accounts for more than 50% of the total protein secreted by three strains examined. RUT-C30 showed the highest relative proportion of CBH I with a values of 87.9% of the total secreted cellulase complement. In the fermentation samples from Iogen-B13 and RUTC 30 a component migrating between CBH I and CBH I core was observed at approximately p*I* 3.6. Separate experiments suggested that this protein corresponded to a phosphorylated isoform of CBH I (discussed below). CBH II and EG II were the next most prominent enzymes found in these extracts though their relative proportions were generally below 10% of the corresponding total protein. Interestingly, no significant level of EG III was observed in RUT-C30 in contrast to Iogen-M4 and Iogen-B13 which showed a relative abundance of at least 5% in each case. Other peaks of unknown identity were also observed (Fig. 2) but could not be assigned to any of the known cellulases.

3.2. Profiling microheterogeneity in purified CBH I using electrospray mass spectrometry

The *cbh 1* gene from *T. reesei* strain L27 has been sequenced by Shoemaker et al. [33] and the corresponding amino acid sequence is available in the Swiss-Prot database (http://www.expasy.ch/sprot).

The protein sequence of CBH I from the strains used in the present investigation differs from that of CBH I from strain L27 by replacement of an arginine at position 442 by two prolines (Fig. 1). As a result, the present CBH I has 497 amino acid residues and an average molecular mass of 52205.8 Da accounting for an N-terminal pyroglutamic acid and twelve disulfide bridges. Interestingly, the same mutation at position 442 has also been reported for the CBH I sequence from strain ALKO2877 [11]. Fig. 1 also shows the positions of the four putative N-linked glycosylation sites.

Flow injection analysis of intact CBH I purified from the three strains provided a unique method to assess their corresponding glycoform distributions. The mass spectra and reconstructed molecular mass profiles of CBH I of RUT-C30, Iogen-M4 and Iogen-B13 are shown in Fig. 3a–d. As an example, the mass spectrum of CBH I from Iogen-M4 (Fig. 3a) displayed a multiply-charged ion envelope extend from m/z 3000–4000 from which the molecular mass profile can be obtained (Fig. 3b). The CBH I from Iogen-M4 (Fig. 3b) also showed a relatively narrow distribution of glycoforms each spaced by 162 Da (hexose) extending from 55 000–57 000 Da. The reconstructed molecular mass profiles for CBH I from each of the three strains are compared in Fig. 3b–d. Based on these molecular distributions, the relative proportion of glycan in CBH I (with respect to the protein mass) from RUT-C30, Iogen-M4 and Iogen-B13 ranged from 8.2–11.1%, 5.7–9.1%, and 4.7–11.1%, respectively. In contrast, CBH I from Iogen-B13 showed more significant heterogeneity as reflected by spacing of 162 Da (hexose) for the lower mass glycoforms (<56 000 Da) whereas a series of doublet spaced by 162 and 203 Da (hexose and *N*-acetyl-hexosamine) was noted for the higher molecular mass glycoforms (56 000–57 000 Da). The CBH I from RUT-C30 showed the highest molecular mass distribution of all three strains examined. Interestingly, an extra series of peaks spaced by 80 Da was found embedded between the hexose spacing in the RUT-C30 sample. This unusual glycoform pattern, was previously reported as sulfation of the linker peptide of CBH I from strain ALK02877 [11], but subsequent experiments enabled the identification of a phosphorylated saccharide based on HPAEC–PAD analyses (described below).

3.3. LC–MS and LC–MS–MS analyses of tryptic peptides from CBH I

The tryptic digest of the reduced and alkylated CBH I from the three *T. reesei* strains were subjected to on-line cLC–ESMS. In order to facilitate the identification of tryptic glycopeptides on the quadrupole/time-of-flight instrument, a mixed scan function [24,25] was used to promote the in-source formation of selected oxonium ions under high orifice voltage conditions (100 V), while enabling detection of multiply-protonated ions using low orifice voltage (30 V). The extracted ion electropherogram for the characteristic oxonium di-saccharide ion Hex-HexNAc at m/z 366 is shown in Fig. 4 together with the total ion chromatogram (TIC) for m/z 400–2000.

The peptide masses derived from the extracted mass spectra were matched against those predicted from the theoretical digest of CBH I (Fig. 1) as calculated by the ProMaC 1.1.1 program (urs.roethlisberger@roche.com). The observed tryptic peptides are presented in Table 2 in ascending order of molecular masses together with their peak number and sequence assignment. It is noteworthy that hydrophobic peptides of masses above 4000 Da were, in general, not eluted from the C_{18} reversed-phase column under the present chromatographic conditions or were not efficiently solubilized prior to injection. This was the case for a number of tryptic peptides from the cellulose binding domain (462–497) and linker (437–461) regions. As a result, the sequence coverage obtained by cLC–ESMS was only 53%. However, MALDI-TOF analysis of the corresponding tryptic digest confirmed the presence of additional peptides including those corresponding to amino acids 108–157, 182–251 and a relatively large and heterogenous peptide at approximately 10 kDa corresponding to the combined linker and CBD domains. The combined results from both MALDI and cLC–ESMS analyses provided to close to 92% coverage of the entire CBH I sequence. More importantly, the present cLC–ESMS analysis enabled the identification of potential glycopeptides from the RUT-C30 and Iogen-B13 CBH I samples such as peak 20 in Table 2. The extracted mass spectrum of the corresponding peak showed an abundant $[M+3H]^{3+}$ ion at m/z 1232.3 consistent

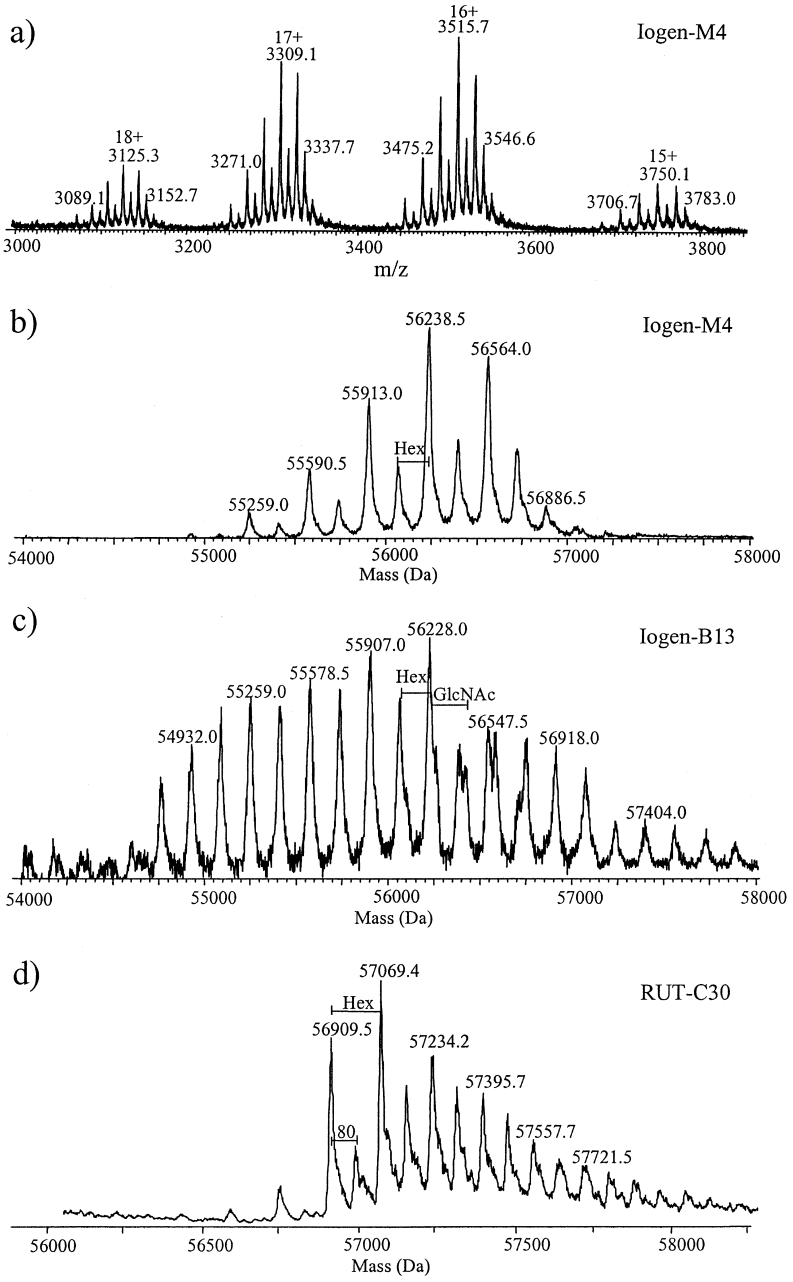

Fig. 3. ESMS mass spectrum of CBH I for (a) Iogen-M4 and reconstructed molecular mass profiles of CBH I for (b) Iogen-M4; (c) Iogen-B13; and (d) RUT-C30. Conditions: flow injection of 1 μg of CBH I in a flow of 50% aqueous acetonitrile (0.2% acetic acid) introduced at 4 μl/min to the mass spectrometer.

with a tryptic peptide of 3693.9 Da (inset Fig. 4c). The cLC–MS analysis of this tryptic fragment of CBH I from both RUT-C30 and Iogen-B13 that was subsequently incubated with PNGase F indicated that this suspected glycopeptide was deglycosylated resulting in a later eluting tryptic peptide of 1992.6 Da

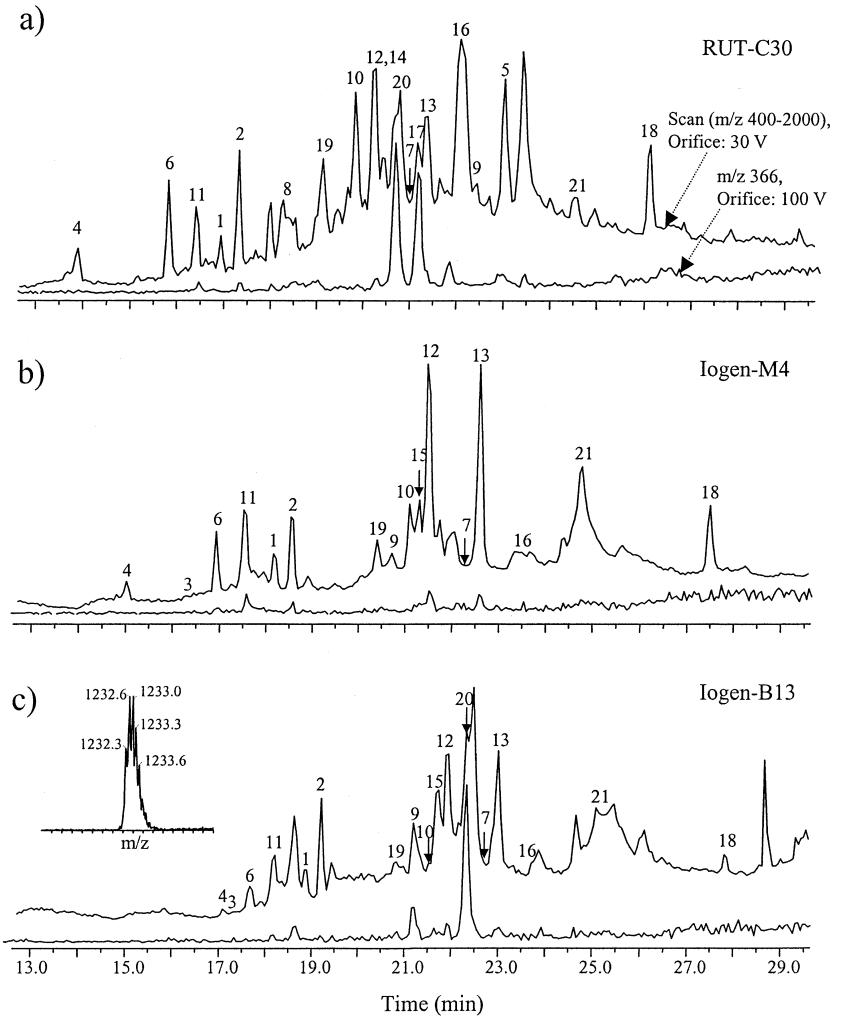

Fig. 4. cLC–ESMS analysis of tryptic digests of CBH I for the three investigated strains. (a) RUT-C30; (b) Iogen-M4; (c) Iogen-B13. Inset in Fig. 4c corresponds to the expanded region of the triply charged ion from peak 20. The number and corresponding amino acid sequences are shown in Table 2. Separation conditions: Pepmap C_{18} reversed-phase column (15 cm×350 μm I.D.), linear gradient of 5–95% (acetonitrile, 0.2% formic acid) in 40 min; sheath liquid of (50% methanol, 1% acetic acid) operating at a flow-rate of 1.5 μl/min.

(data not shown). The molecular mass of this deglycosylated peptide was consistent with the mass predicted for the tryptic fragment 268–286.

The product ion of *m/z* 1232.3 corresponding to peak 20 for Iogen-B13 CBH I (Fig. 4c) is presented in Fig. 5a and shows fragment ions associated with cleavages of glycosidic and peptide bonds. For example, a series of doubly-charged fragment ions at *m/z* 996.9, 1098.4, 1200.0, 1281.5, 1363.0 and 1443.4 corresponded to loss of hexose (Man) and hexosamine (GlcNAc) residues supporting the proposal of a high mannose N-linked glycan. The identity of the corresponding tryptic peptide was assigned to 268–286 based on the observation of a series of y-type fragment ions (Fig. 5a). The site of N-linked attachment was assigned to Asn270 (consensus sequence Asn-Thr-Ser) and comprised a predominant $Man_8GlcNAc_2$ glycan with minor Man_5-Man_9 glycoforms. This result is consistent with previous reports of the release of these other high mannose-type N-linked glycans from the CBH I from RUT-C30 [13].

Table 2
Assignment of tryptic peptides of CBH I from *T. reesei*

Peak no.	Residues	Assignment	$Mass_{mono.}$ (Da)	CBH I (tryptic masses)[a]		
				RUT-C30	Iogen-M4	Iogen-B13
1	347–353	GGLTQFK	749.4	749.4	749.3	749.3
2	416–422	VTFSNIK	807.4	807.4	807.3	807.3
3	347–354	GGLTQFKK	877.5	–	877.4	877.4
4	158–166	YPTNTAGAK	921.5	921.4	921.4	921.4
5	355–364	ATSGGMVLVM	964.5	964.4	–	–
6	167–178	YGTGYCDSQCPR	1462.6	1462.4	1462.5	1462.4
7	288–302	LTVVTQFETSGAINR	1634.9	1634.6	1634.6	1634.6
8*	5–18	TLQSETHPPLTWQK	1664.9	1664.7	–	–
9	287–302	KLTVVTQFETSGAINR	1763.0	1762.8	1762.8	1762.8
10	252–267	YGGTCDPDGCDWNPYR	1931.7	1931.6	1931.4	1931.4
11	395–415	GSCSTSSGVPAQVESQSPNAK	2076.9	2076.9	2076.6	2076.9
12*	1–18	pESACTLQSETHPPLTWQK	2094.0	2093.8	2093.7	2093.7
13*	268–286	LGNTSFYGPGSSFTLDTTK + GlcNAc	2195.0	2194.8	2194.8	2194.8
14	19–39	CSSGGTCTQQTGSVVIDANWR	2283.0	2283.4	–	–
15*	268–287	LGNTSFYGPGSSFTLDTTKK + GlcNAc	2323.1	–	2322.9	2322.9
16*	374–394	MLWLDSTYPTNETSSTPGAVR + GlcNAc	2528.2	2528.1	2528.1	2528.1
17*	268–280	LGNTSFYGPGSSF + $Man_8GlcNAc_2$	3035.2	3034.8	–	–
18*	365–394	SLWDDYYANMLWL-DSTYPTNETSSTPGAVR + GlcNAc	3655.6	3655.2	3654.9	3655.2
19*	40–69	WTHATNSSTNCYDGNTWSSTLCPDNETCAK + GlcNAc	3680.5	3680.4	3680.0	3680.4
20*	268–286	LGNTSFYGPGSSFTLDTTK + $Man_8GlcNAc_2$	3694.5	3694.2	–	3693.9
21	303–346	YYVQNGVTFQQPNAELGSYSGNEL-NDDYCTAEEAEFGGSSFSDK	4887.1	4887.6	4888.0	4887.6

[a] Refers to ^{12}C monoisotopic component, cysteine is carbamidomethylated, asterisk indicated tryptic peptides subjected to MS–MS analyses.

Interestingly, a later eluting peak 13 in Table 2, with a molecular mass 2194.8 Da was suspected to contain a single GlcNAc residue at the same glycosylation site. The product ion spectrum of the corresponding $[M+3H]^{3+}$ ion at m/z 732.6 is presented in Fig. 5b, and shows a series of *y*-type fragment ions consistent with that expected for tryptic peptide 268–286. An abundant fragment ion at m/z 204 (oxonium ion of GlcNAc) with no detectable signal at m/z 163 (oxonium ion of Man) further supported this proposal. The relative proportion of Asn270 with high mannose to that with single GlcNAc was determined by using the integrated peak area. For CBH I from RUT-C30, the ratio of $Man_8GlcNAc_2$ relative to single GlcNAc at Asn270 was calculated to be 7:1, while in Iogen-B13, this ratio was found to be 1:2. In contrast, Iogen-M4 only contained a single GlcNAc at Asn270.

The detection of a N-linked glycopeptide bearing a single GlcNAc residue at Asn270 was not totally unexpected. Indeed, both Klarskov et al. [12] and Harrison et al. [11] have reported that the catalytic core domain of CBH I contains three N-linked glycans each consisting of a single GlcNAc. In the present work, CBH I was isolated from *T. reesei* strains of different lineage to that of QM9414 and ALKO2877 (a derivative of QM9414) and variability in site occupancy or glycan heterogeneity could be expected. Alternately, the growth conditions under which CBH I was produced by the various strains could explain some of the glycan heterogeneity at Asn270 as well as the other *N*-glycosylation sites.

Interestingly, the tryptic peptide for amino acids 355–394 containing a putative N-link site at Asn384 was not observed in any of these tryptic digests. Rather, a truncated glycopeptide for amino acids 365–394 bearing a single GlcNAc of mass 3655.2 Da (peak 18 in Table 2) was detected and subsequent tandem mass spectrometry experiments confirmed this assignment (data not shown). The tryptic peptide corresponding to amino acids 40–69 was observed as peak 19 (Table 2) and its measured molecular mass,

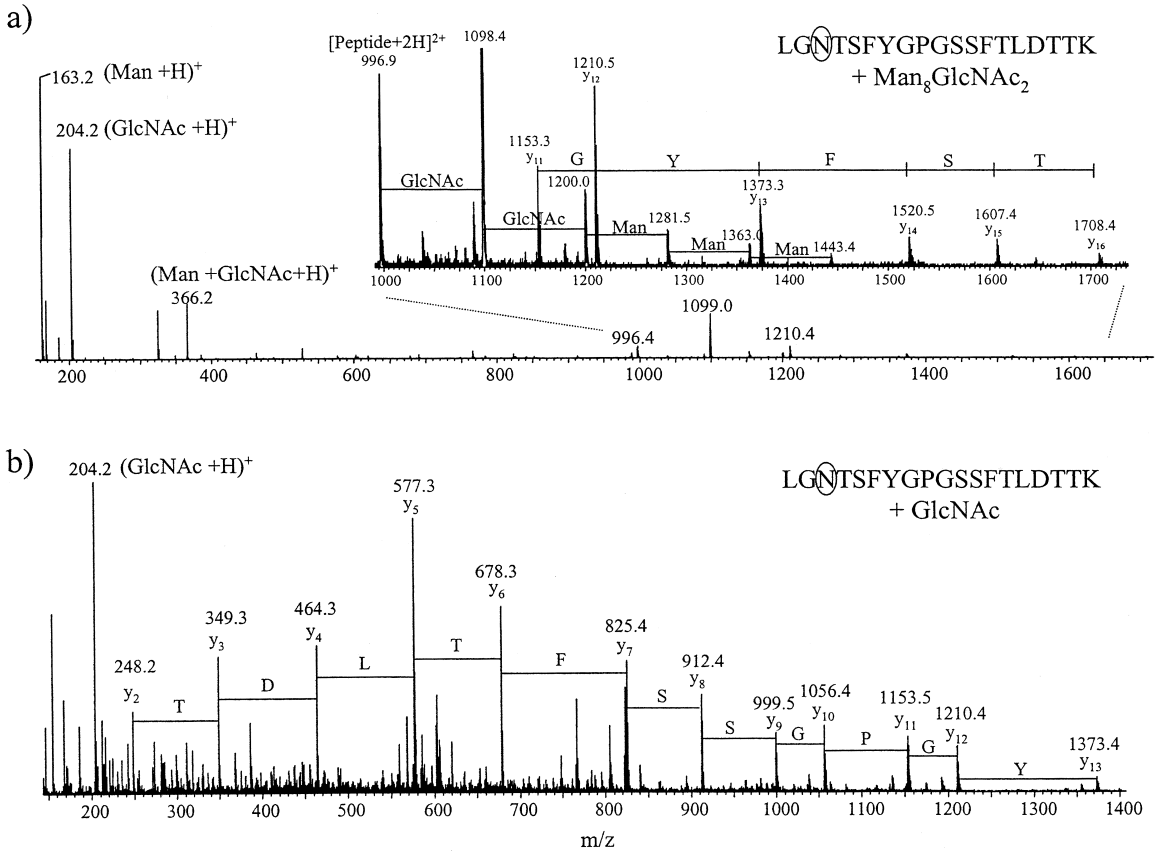

Fig. 5. MS–MS spectra of N-linked glycopeptides of CBH I tryptic digest. (a) Product ion scan of m/z 1232.3 (peak 20 in Table 2) confirming the presence of high mannose $Man_8GlcNAc_2$ on Asn270; (b) Product ion scan of m/z 732.6 (peak 13 in Table 2) confirming the presence of a single GlcNAc on Asn270. Conditions as for Fig. 4 except collision energy (laboratory frame of reference) of 90 eV for (a) and 75 eV for (b).

3680.4 Da suggested the incorporation of a single GlcNAc on either Asn45 or Asn64. The first generation of product ion from the corresponding $[M+3H]^{3+}$ at m/z 1227.8 (Fig. 6a) shows a prominent oxonium ion at m/z 204 together with a number of structurally-related y-type fragment ions. As indicated no single GlcNAc was found on the first $y_1 - y_{10}$ fragment ions suggesting that glycosylation occurs on Asn45. This was confirmed by selecting the precursor ion at m/z 914.3 corresponding to the singly-charged fragment ion b_6 plus GlcNAc formed in the orifice/skimmer region of the Q-TOF instrument by raising the orifice voltage to approximately 120 V (Fig. 6b). The second generation fragment ions thus obtained showed a characteristic fragment at m/z 711.2 resulting from the loss of a single GlcNAc residue from the precursor ion along with a number of consecutive *b*-type fragment ions from which the glycosylation site could be unambiguously assigned. Based on these tandem mass spectrometric data Asn45 comprised a single GlcNAc residue while Asn64 was not glycosylated which is consistent with previous reports [11,12].

The variability in $Man_8GlcNAc_2$:GlcNac ratios observed for Asn270 in CBH I produced by the three related *T. reesei* strains in the present study is unusual and may reflect differences in the activities of trimming glycosidases (such as glucosidase II, α-1,2-mannosidase or endoglycosidase H) produced by the different strains or under different growth conditions. Most notably, Iogen-M4, whose CBH I contained only GlcNAc at this position, was grown

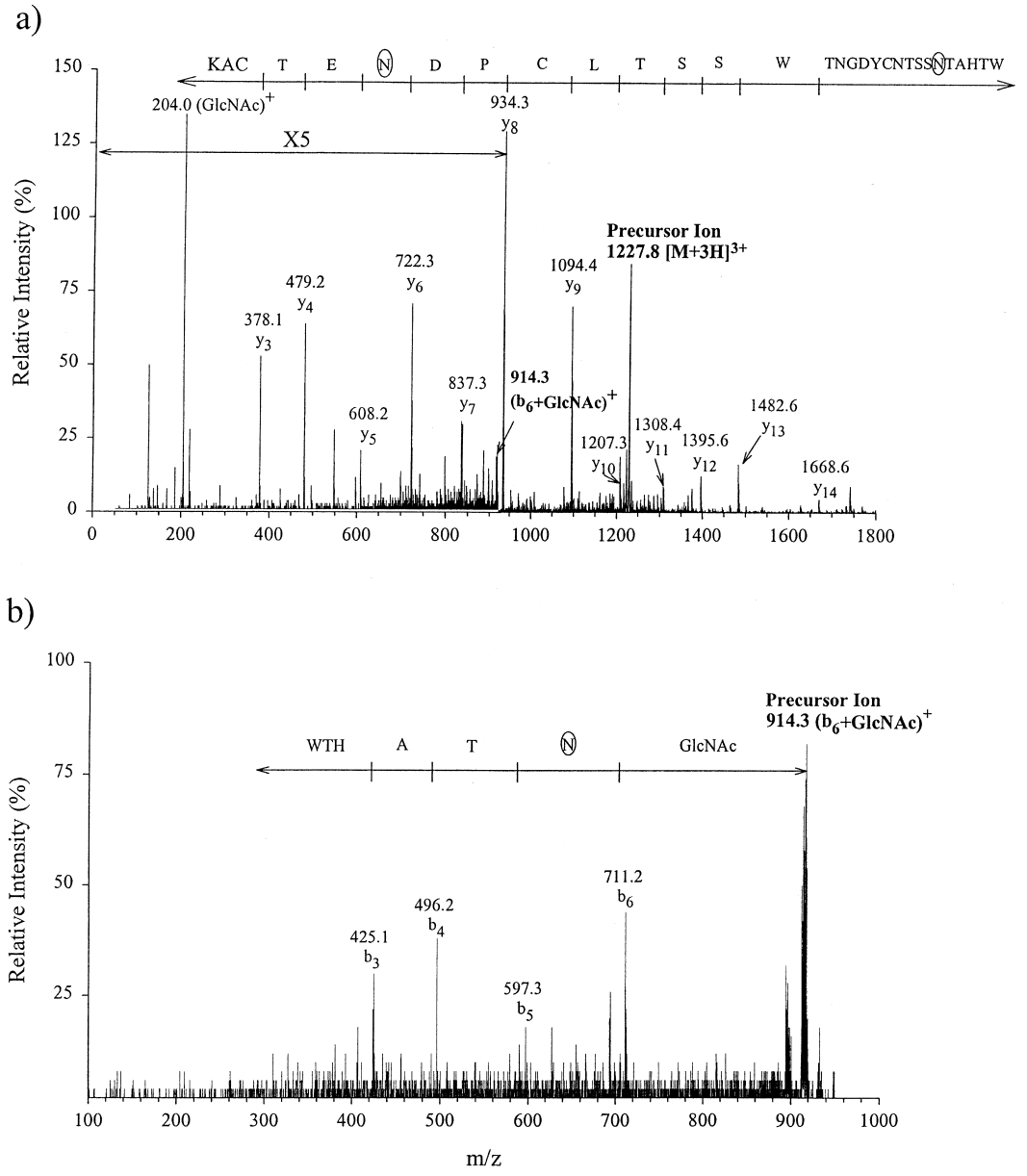

Fig. 6. MS–MS spectra of CBH I tryptic peak 19 from Iogen-M4 (a) First generation product of m/z 1227.8 at an orifice voltage of 50 V; (b) second generation product ion of m/z 914.3 formed at an orifice voltage of 120 V. Conditions as for Fig. 4 except collision energy (laboratory frame of reference) of 75 eV for (a) and 50 eV for (b).

at pH 5 while both RUT-C30 and Iogen-B13, whose CBH I showed some $Man_8GlcNAc_2$ at Asn270, were grown at pH 4. The partial deglycosylation at Asn270 may be a direct consequence of steric hindrance at this site whereas glycans appended to residues Asn45 and Asn384 would be more directly exposed to the outer protein core as predicted from the three dimensional crystal structure of CBH I [34,35]. Further experiments to verify this possibility would be warranted.

In addition, the predominant high mannose-type glycan from RUT-C30 CBH I in the present study was found to be $Man_8GlcNAc_2$ while Maras et al. [13] previously showed that the predominant N-linked glycans from RUT-C30 CBH I were $GlcMan_8GlcNAc_2$, $GlcMan_7GlcNAc_2$ and $ManP\text{-}GlcMan_7GlcNAc_2$. These differences in glycosylations are possibly explained by the culture conditions under which RUT-C30 was grown. In the present study the enriched medium was used for CBH I production by RUT-C30 and could have induced higher glucosidase activity while the minimal medium used in the previous study may have led to higher levels of mannosylphosphotransferase activity. The observation of this type of activity in *Trichoderma* is not totally unexpected since mannosyl phosphorylation of N- and O-linked glycans has been observed from a number of *S. cerevisiae* strains [36] and has been shown to occur in response to cellular stress resulting from environmental changes or growth phase [37].

3.4. MALDI-TOF analysis of CBH I linker domain

Purification of the peptide fragment from papain proteolysis of CBH I using gel filtration chromatographic purification revealed a highly heterogeneous glycopeptide of 8000–9500 Da comprising the linker and the cellulose-binding domain. MALDI-TOF analyses of these samples (Fig. 7) showed a complex profile displaying an incremental number of hexose residues. Degeneracy in the papain digest resulted in peak doublets spaced by 57 Da corresponding to cleavage on either side of Gly439. In addition to this, the glycopeptide obtained from papain digestion of CBH I from RUT-C30 (Fig. 7a) showed a third set of peaks separated by 80 Da from the major and minor series described above. This mass spacing was also noted in the intact CBH I and suggested the presence of a single sulfate or phosphate group on the linker. Incubation of this glycopeptide with alkaline phosphatase (Fig. 7b) successfully removed the phosphate group on most linker glycoforms except for the larger oligomer series above m/z 8700. This observation possibly suggests that extensive glycosylation of the linker might impart steric hindrance preventing the catalytic activity of the phosphatase. This result along with purified saccharides from HPAEC–PAD experiments (described below) indicates that the linker from RUT-C30 is phosphorylated. The presence of a phosphate group on the linker and not a sulfate as reported previously for strain ALK02877 [11] might reflect the different lineage of the strains from which the CBH I was isolated.

From the mass of the glycopeptide observed in Fig. 7 it was possible to deduce the number of hexose residues appended to the linker region. In the case of RUT-C30 the major glycoform series corresponded to the mass of the C-terminal peptide (M: 5834.4 Da) containing 16–21 hexose residues. Similar glycoform distributions were observed for the linker of Iogen-M4 (Fig. 7c) and Iogen-B13 (Fig. 7d) where the number of hexose varied from 15–24 and 11–24, respectively.

3.5. HPAEC–PAD analysis of oligosaccharides released by hydrazinolysis

The chromatograms corresponding to oligosaccharides released from the hydrazinolysis of CBH I at 95°C are shown in Fig. 8 for the three different strains. Under the present chromatographic conditions, the small neutral di- and tri-saccharides eluted before 10 min whereas higher oligomer such as $GlcNAc_2Man_{5-9}$ were observed between 15 and 20 min in Fig. 8. As indicated, CBH I from Iogen-M4 and Iogen-B13 contained a high proportion of di- and tri-saccharides presumably located on the linker region as reported previously by Harrison et al. [11]. CBH I from RUT-C30 contained predominantly disaccharides and a high mannose glycan ($GlcNAc_2Man_8$) on Asn270 as indicated in Fig. 8a. Interestingly, additional components eluting between 38–44 min were observed mainly in the RUT-C30 strain. These extra peaks were presumed to be strongly retained anionic species. Confirmation of the identity of some of these peaks was achieved by conducting on-line desalting of the separated components followed by ESMS analyses.

The mass spectral analysis of peak 10 from the HPAEC–PAD separation of glycans from CBH I of RUT-C30 is shown in Fig. 9. The conventional negative ion ESMS spectrum (Fig. 9a) of the desalted sample showed a prominent $[M-H]^-$ ion at m/z 421. Accurate mass measurements on this ion

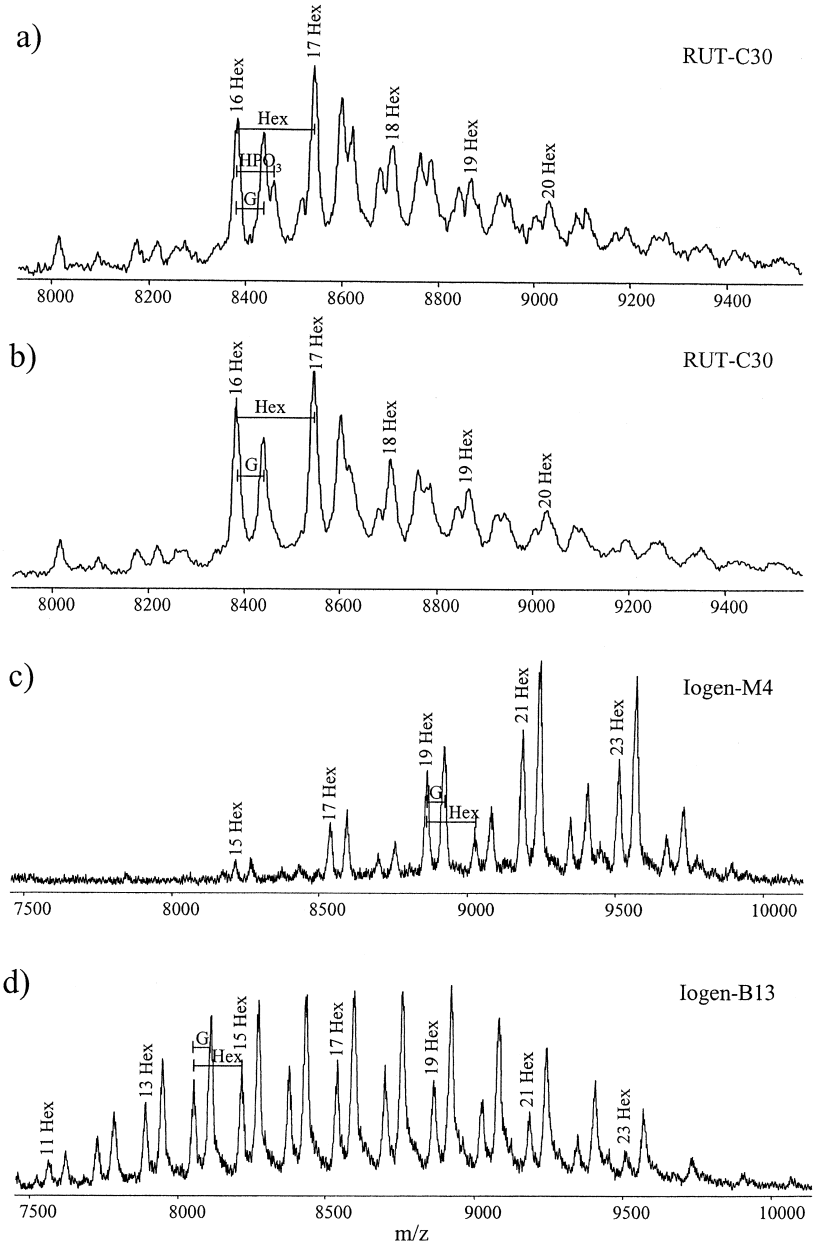

Fig. 7. MALDI-TOF analysis of the linker domain following papain digestion of CBH I. (a) RUT-C30; (b) RUT-C30 after alkaline phosphatase treatment; (c) Iogen-M4 and (d) Iogen-B13 (G = glycine).

(m/z 421.080) were consistent with a phosphorylated di-hexose ($[M-H]^-_{calc}$: 421.075) compared to a sulfate di-saccharide ($[M-H]^-_{calc}$: 421.065). The product ion spectrum of the corresponding singly-deprotonated precursor (Fig. 9b) indicated several fragment ions corresponding to losses of water and cleavage of glycosidic bonds including diagnostic fragments ions at m/z 96.965 and 78.963 supporting the presence of a phosphate group. The mass of this component together with its characteristic fragmenta-

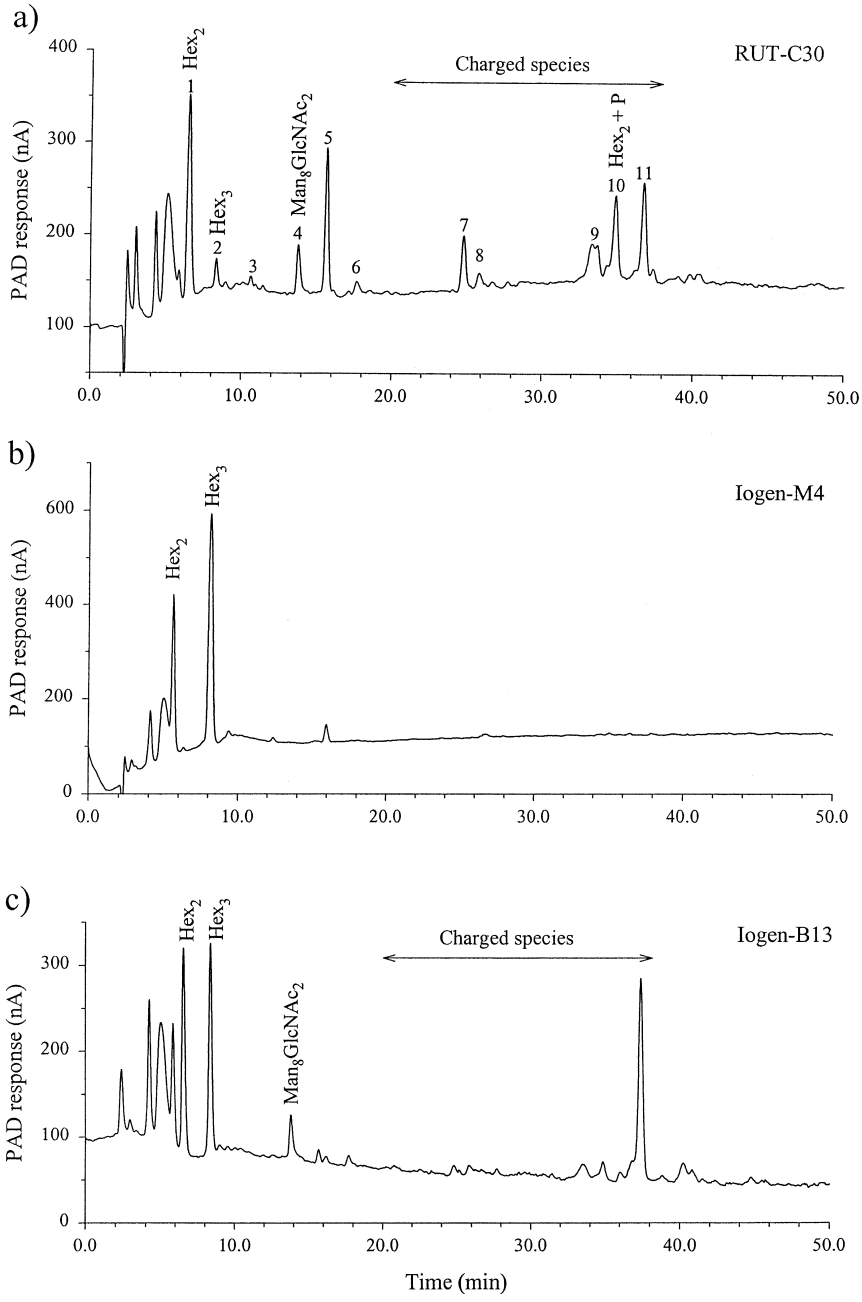

Fig. 8. HPAEC–PAD analysis of oligosaccharides released from automated hydrazinolysis (N+O linked) of CBH I from the investigated strains. (a) RUT-C30; (b) Iogen-M4 and (c) Iogen-B13. (p=phosphate).

tion patterns were consistent with a phosphorylated dihexose glycan. Based on the analysis of the papain digest of CBH I from RUT-C30 (Section 3.4), this unusual carbohydrate was assigned as a phosphorylated di-saccharide on the linker glycopeptide. Mannosyl phosphorylation on O-linked glycans was

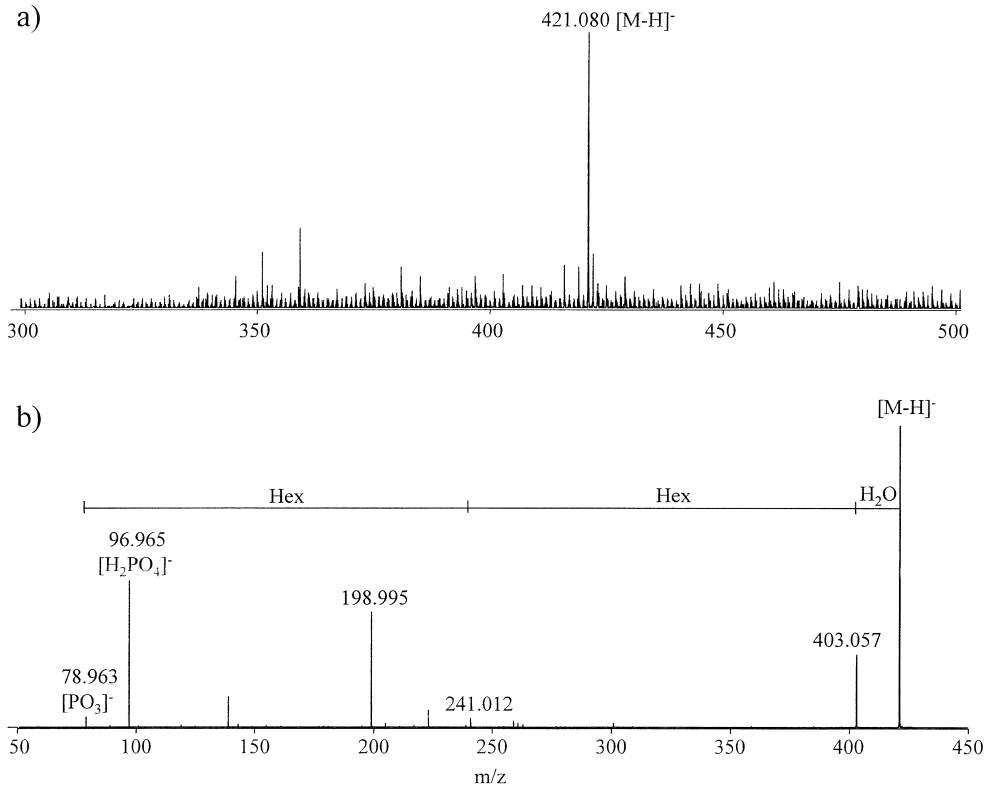

Fig. 9. ESMS analysis (quadrupole/time-of-flight) of fraction 10 from HPAEC–PAD purification of oligosaccharides derived from CBH I (Fig. 8). (a) Negative ion ESMS mass spectrum and (b) product ion spectrum of m/z 421.1 obtained at a collision energy of 40 eV (laboratory frame of reference).

also described for mannotriose oligosaccharides in mannosyltransferase mutants (*mnn 1*) strains of *S. cerevisiae* [38]. The occurrence and significance of phosphorylated O-linked glycans in CBH I from *T. reesei* is not yet understood. However, recent report from Jigami and Odani suggests a number of functions for mannosyl transferase in yeast including the supply of GMP for sugar nucleotide transport in the Golgi, cross-linking of mannoproteins to β-glucan and cellular stress responses [37]. The relationship between biological functions of secreted cellulases and the mannosylphosphate transfer would merit further investigation.

4. Conclusion

The present report described the application of mass spectrometry and CIEF for the analysis of crude cellulase fermentation samples produced by *T. reesei* strain RUTC30 and two of its derivative strains. CIEF analysis of secreted proteins from *T. reesei* provided a rapid means of determining the concentration and proportion of different cellulase components in complex mixtures in addition to identifying unexpected components. The profile of N- and O-linked glycans appended to the protein backbone of CBH I was obtained by flow injection mass spectrometry on a hybrid quadrupole time-of-flight instrument and highlighted the presence of unexpected patterns including the variability in N-linked glycans and phosphorylated residues on the O-linked glycans in the linker regions of the CBH I from strain RUT-C30.

The precise identification of N-linked glycans on four putative sites was achieved using cLC–ESMS and tandem mass spectrometry. A high/low orifice stepping function was incorporated as part of the

mass spectral acquisition enabled the identification of glycopeptides from the complex tryptic digests of CBH I from the three different *T. reesei* strains. The suspected glycopeptides were further subjected to MS–MS to confirm the nature of glycans and their attachment sites. A single GlcNAc was found at Asn45 and Asn384 in CBH I from all strains while Asn270 predominantly contained a high mannose oligosaccharide, $Man_8GlcNAc_2$, in CBH I from RUT-C30, a mixture of $Man_8GlcNAc_2$ and GlcNAc in CBH I from Iogen-B13, and a single GlcNAc in CBH I from Iogen-M4. No N-linked glycans were observed at Asn65 in any of the CBH I samples. The presence of single GlcNAc residues at Asn45 and Asn384 and the heterogeneity in the glycan population at Asn270 may reflect some endoglycosidase H activity in *T. reesei* whose accessibility to the N-linked glycan at Asn270 may be limited by steric hindrance.

The linker region of all three *T. reesei* strains examined showed an heterogenous population of di- and tri-mannosyl saccharides as indicated from the HPAEC–PAD profiles of the hydrazinolysis products of CBH I. The molecular mass profiles of CBH I from RUT-C30 showed additional heterogeneity in the form of a phosphorylated glycan. Alkaline phosphatase treatment of the linker-CBD peptide together with mass spectral analyses of HPAEC–PAD fractions of acidic oligosaccharides supported the presence of a phosphorylated di-saccharide on the linker region. Further investigations will be required to address the significance and functional relationship of the phosphate transfer to CBH I.

5. Nomenclature

CBH I	cellobiohydrolase I
CIEF	Capillary isoelectric focusing
HPAEC–PAD	high-performance anion-exchange chromatography with pulsed amperometric detection
cLC–ESMS	capillary liquid chromatography–electrospray mass spectrometry
TIC	total ion chromatogram
MALDI-TOF	matrix-assisted laser desorption ionization time-of-flight
MS–MS	tandem mass spectrometry

Acknowledgements

J.P.M.H. acknowledges the Fonds pour la Formation de Chercheurs et l'Aide à la Recherche (FCAR) for a graduate student scholarship. The authors would like to thank Iogen for their financial support, Micromass for access to a Q-TOF mass spectrometer, G. Giroux (Iogen) for technical assistance, and Dr. R. MacKenzie and T. Hirama (IBS, NRC) for their assistance in the purification of the cellulases.

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