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Glycan modification of a thermostable recombinant (1-3,1-4)-β-glucanase secreted from *Saccharomyces cerevisiae* is determined by strain and culture conditions

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High level biosynthesis and secretion of the thermostable hybrid (1-3,1-4)-β-glucanase H(A16-M) has been achieved in *Saccharomyces cerevisiae* by means of the yeast vacuolar endoprotease B promoter ($PRB1_p$) and the *Bacillus macerans* (1-3,1-4)-β-glucanase signal peptide. The N-glycans present on the yeast-secreted H(A16-M), denoted H(A16-M)-Y, were released by endoglycosidase H, and identified by proton NMR spectroscopy to be a homologous series of $Man_{8\text{-}13}GlcNAc_2$, although only traces of $Man_9GlcNAc_2$ were found. Therefore, processing of N-glycans on H(A16-M)-Y is similar to that on homologous proteins. Most of the N-glycans (88%) were neutral while the remainder were charged due to phosphorylation. Site-directed mutagenesis of Asn to Gln in two of the N-glycosylation sequons, and subsequent analysis of the N-glycans on the yeast-secreted proteins together with analysis of the N-glycans from the individual sites of H(A16-M)-Y suggest the presence of steric hindrance to glycan modification by the glycans themselves. H(A16-M)-Y produced under control of either the yeast protease B or the yeast 3′-phosphoglycerate kinase promoter, each in two different *Saccharomyces* strains revealed a dependence of N-glycan profile on both strain and culture conditions. The extent of O-glycosylation was found to be nine mannose units per H(A16-M)-Y molecule. An attempt to identify the linkage-sites for the O-glycans by amino acid sequencing failed, suggesting non-stoichiometric or heterogeneous O-glycosylation. The possible modes in which N-glycans might contribute to resistance of H(A16-M)-Y to irreversible thermal denaturation are discussed with respect to structural information available for H(A16-M)-Y.

Keywords: glycosylation, *Saccharomyces cerevisiae*, heterologous, glucanase, thermostability

Abbreviations: AMY, *B. amyloliquefaciens* (1-3,1-4)-β-glucanase; MAC, *B. macerans* (1-3,1-4)-β-glucanase H(A16-M), H(A36-M), H(A78-M),H(A107-M) and H(A152-M), hybrid (1-3,1-4)-β-glucanases containing 16, 36, 78, 107 and 152 N-terminal amino acids, respectively, derived from AMY with the remaining amino acids derived from MAC; similar enzyme abbreviations followed by Y, e.g. H(A16-M)-Y, denote the enzymes secreted from yeast cells; PCR, polymerase chain reaction; PGK_p, yeast 3′-phosphoglycerate kinase promoter; $PRB1_p$, yeast protease B promoter; LB, Luria-Bertani medium; SC, minimal medium; CNBr, cyanogen bromide; Endo H_f, endoglycosidase H fusion protein; PNGase F, peptide:N-glycosidase F; HPAEC; high pH anion exchange chromatography; HVE, high voltage paper electrophoresis; CPY, yeast carboxypeptidase Y.

Introduction

The yeast *Saccharomyces cerevisiae* is often used for expression of heterologous genes [1–4]. An important feature of homologous and heterologous yeast extracellular proteins transported through the secretory apparatus is possible N-glycosylation at Asn residues of the sequence Asn-Xaa-Ser/Thr, where Xaa is any amino acid except Pro [5, 6], or O-glycosylation at Ser or Thr residues [7, 8]. Although environmental conditions and cell-type have been reported to influence glycosylation of proteins synthesized in mammalian cells [9–11], no extensive analysis have been performed with yeast despite the fact that proteins secreted from yeast exhibit heterogeneity in the glycan profile. This has been found both for homologous proteins such as invertase [12] and carboxypeptidase Y (CPY) [13] and for heterologous proteins such as human insulin-like growth factor I [14], murine and human granulocyte macrophage colony-stimulating factor [15] and human alpha-

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1-antitrypsin [16]. Similarly, glycosylation was found for *S. cerevisiae*-secreted barley (1-3,1-4)-β-glucanase [17] and for a series of bacterial hybrid (1-3,1-4)-β-glucanases [18, 19]. The latter were derived from *B. amyloliquefaciens* (1-3,1-4)-β-glucanase (AMY) [20] and *Bacillus macerans* (1-3,1-4)-β-glucanase (MAC) [21]. Concomitant N- and O-glycosylation of individual hybrid enzymes by *S. cerevisiae* was observed to alter the thermostability, either enhancing or reducing their resistance to irreversible thermal inactivation [19]. One highly thermostable hybrid (1-3,1-4)-β-glucanase, H(A16-M), consisting of the first 16 N-terminal amino acid residues derived from mature AMY and the remaining C-terminal segment from MAC, displayed superior resistance towards irreversible thermoinactivation [22]. Secretion of H(A16-M) as the glycoprotein H(A16-M)-Y from *S. cerevisiae* resulted in a 1.6-times increase in the half-life of the enzyme at 70°C, pH 6.0 [19]. This enhancement of thermostability was the highest observed in the hybrid enzymes series. However, for the parental AMY enzyme the enhancement was 7.6-fold. The crystal structure of H(A16-M) has been determined to a 2 Å resolution [23] and a calcium tightly bound to the protein was found to be important for the enzymic stability [24].

Yeast cells are normally cultivated in shake-flask or fermentor. While much attention has been focused on the yield of expressed recombinant proteins, there is little information with respect to factors determining the glycosylation of these. As H(A16-M)-Y was found to be N-glycosylated at two sites, we have chosen this enzyme to analyse in detail how the physical environment of the cells influences the glycan patterns of an enzyme. In line with this, we have analysed whether the glycosylation is site-dependent or influenced by the glycan profile at a neighbouring site.

Methods

Organisms, media, transformation procedures and growth conditions

Genetic transformations of E. coli strain DH5α (Life Technologies, DK) were done according to a published procedure [25]. Transformants were incubated on solid Luria-Bertani medium (LB) [26] containing 0.1% (w/v) lichenan and 100 mg l^{-1} ampicillin. Genetic transformation of S. cerevisiae DBY 746 [27] and C79-994 [28] were carried out by electroporation [29]. Transformants were incubated on solid minimal medium (SC) [30] without Leu, containing 0.1% (w/v) lichenan. Staining with Congo red revealed clearing zones around colonies secreting active recombinant (1-3,1-4)-glucanase. Cultivation in 1 l SC without Leu was at 30°C for 4 days. For fermentation the 1 l culture was transferred to 10 l SC in a 20 l aerated fermentor and grown at 30°C, 400 rpm and 90% oxygen saturation for 3–4 days. This culture was used as inoculum for a 100 l fermentation in SC without Leu in a 150 l draft tube fermentor with the dimensions height:diameter = 3:1. The cells were cultured for 4 days at 30°C, 500 rpm with an airflow of 150 l min^{-1}.

Plasmid constructions

Isolation of plasmid DNA from E. coli, separation of DNA in 1% (w/v) agarose and digestion with restriction endonucleases were carried out using standard techniques. DNA fragments of interest were recovered from agarose gels using Prep-A-Gene (BioRad, DK). Construction of the gene encoding H(A16-M) [31] and cloning of the gene in the yeast expression vector pMA91 [32], placing the gene under control of the constitutive 3′-phosphoglycerate kinase promoter (PGK_p), have been described [19]. The B. macerans (1-3,1-4)-β-glucanase signal peptide was used to direct secretion.

Plasmid pMK4G1 [33] containing the *S. cerevisiae* protease B (*PRB*1) gene was used as template for amplifying by polymerase chain reaction (PCR) the PRB*1* promoter sequence fused to a segment encoding the N-proximal part of the *B. macerans* (1-3,1-4)-β-glucanase signal sequence. The 5′ primer (5′-GGG GAA TCG TAG AGT TTG CAC AAT T GC TCG TA-3′) contained an *Eco* RI recognition sequence (double underlined) linked to a sequence corresponding to bases 608–631 of the *PRB*1 sequence (EMBL Data Libary, accession number M18097), while the 3′ primer (5′-AAT GTG GTC ACC AGT GTA AAA CAG GAC TTC TTC TTC ATC TTT GCT TGT TAG AAT TAG GTT TAG TTT GTT TTT TTA TTG GCGA-3′) links the (1-3,1-4)-β-glucanase gene sequence (underlined) – including a *Bst* EII recognition sequence (double underlined) – to the 5′ untranslated sequence upstream of the translational start codon of PRB*1*. The resulting amplified fragment was digested with *Eco* RI-*Bst* EII and exchanged with the corresponding fragment of plasmid pGEMMA-pMMH(A16-M) consisting of the small *Hin* dIII fragment of pMMH(A16-M) [19] cloned in pGEM-7zf(+) (Promega, Bie & Berntsen, DK) linerized with *Hin* dIII. In the resulting plasmid, pB1-H(A16-M), the gene for (1-3,1-4)-β-glucanase H(A16-M) is cloned between the PRB*1* promoter ($PRB1_p$) and the terminator sequence of the yeast *PGK* gene [32]. A partial *Eco* RI digestion of pMA91 [32] liberated a fragment with the *S. cerevisiae* 2μ-*Leu*2 sequences. This was finally cloned in pB1-MH(A16-M) linerized with *Eco* RI giving the yeast expression plasmid pB1-L-MH(A16-M). Introduction of the base mutations corresponding to the changes $Asn_{31} \rightarrow$ Gln and $Asn_{185} \rightarrow$ Gln in H(A16-M) was carried out according to a previously described method [34] using two 33-bases long oligonucleotides complementary to the template DNA apart from the bases to be changed. The template was the *Bgl* II-*Bam* HI fragment of plasmid pUC13-MH(A16-M) containing the sequence encoding the *B. macerans* (1-3,1-4)-β-glucanase signal peptide in frame with the gene encoding H(A16-M). The sequence of the outer 5′ primer contained the recognition sequence for *Bgl* II (with an additional five-base extension) upstream of a sequence identical to the *B. macerans* (1-3,1-4)-β-glucanase signal peptide.

Similarly, the outer 3′ primer contained the recognition sequence for *Bam* HI (with an additional five-base extension) upstream of a sequence specifying the 3′ end of the coding region of H(A16-M). The two primary amplification products were purified following separation in a 1% agarose gel, combined, and the annealed molecule amplified using the outer 5′ and 3′ primers. The resulting product was digested with Bgl II/Bam HI and cloned into the Bgl II-site of the yeast expression vector pMA91 resulting in plasmids pMM-H(A16-M) N31Q and pMM-H(A16-M)-N185Q. A double mutant, pMM-H(A16-M)-N31Q-N185Q, was constructed as described above except that pMM-H(A16-M)-N31Q was used as the template in the first PCR with the mutagenic primers introducing the N185Q mutation. Introduction of the mutations were verified by DNA sequencing on a 373A DNA Sequencer (Applied Biosystems, Perkin Elmer, DK) according to the manufacturer's recommendations.

Protein purification, protein cleavage and polypeptide purification

(1-3,1-4)-β-glucanase secreted from yeast grown in 1 l cultures containing SC without Leu was purified as previously described [19]. For purification from the 100 l fermentor culture, a Pellicon ultrafiltration unit (Millipore) equipped with 10 kDa cut off filters was used for concentration and diafiltration of the culture supernatant. The proteins from this were fractionated by gel filtration chromatography using Sepharose S200 HR (Pharmacia, DK) packed in a column with the dimensions 5 × 100 cm. Protein was eluted with a flow of 100 ml h–1. The elution profile from the gelfiltration resembled that of the purification of (1-3,1-4)-β-glucanase from the 1 l culture. Protein samples were prepared and separated in 8–18% (w/v) acrylamide ExcelGel gels (Pharmacia, DK). After electrophoresis the gels were stained with Coomassie Brilliant Blue R. Met-Xaa bonds (Xaa being any amino acid) of 20 mg H(A16-M)-Y were cleaved by incubation at room temperature overnight with 220 mg cyanogen bromide (CNBr) in 70 % (v/v) formic acid. CNBr was removed by flushing nitrogen through the reaction mixture before loading onto a Sephacryl S100 HR (Pharmacia) gel filtration column (2.6 × 100 cm) equilibrated with 10 % (v/v) acetic acid. The peptides were eluted with the equilibration buffer at a flow rate of 35 ml h–1. N-terminal sequencing was performed as previously described [19].

Quantification of O-linked carbohydrate

Five mg of purified glycoprotein was digested with PNGase F (New England Biolabs, Medinova, DK) according to the instructions of the supplier. The deglycosylated enzyme was purified using Sephacryl S200 HR (Pharmacia, DK) as described above. The exact protein concentration was determined by hydrolysis of a small portion of the protein in 6 N HCl at 110°C for 24 h in evacuated and sealed tubes and analysed on an Alpha Plus amino acid analyser (Pharmacia, DK). Quantitation of Gly residues was used to determine the protein concentration. The concentration was confirmed by measuring Ala and Leu. Carbohydrate O-linked to the protein was quantitatively measured by the phenol/sulphuric acid assay [35] using mannose as a standard. Accordingly, the amount of O-linked carbohydrate is a measure of the number of mannose units per protein molecule.

Enzymic release of N-glycans

Purified native glycoprotein was digested with recombinant endoglycosidase H (Endo H_f, New England BioLabs, Medinova, DK) according to the instructions of the supplier. For preparative use, 50 mg H(A16-M)-Y in 2 ml 50 mM sodium acetate buffer, pH 6.0 was incubated with 5 UB units Endo H_f 37°C for 1 h. Upon one further addition of 5 UB units of Endo H_f the protein was incubated at 37°C for 2 h.

High pH anion exchange chromatography (HPAEC)

Endo H_f-released N-glycans were fractionated by gradient elution of a CarboPac PA100 column (4 × 250 mm), using the Dionex Chromatograph and Pulsed Electrochemical Detector. Stock solutions were 1 M sodium hydroxide, prepared by dilution of 12.5 M sodium hydroxide solution (BDH, Bie & Berntsen, DK) and 1 M sodium acetate (Merck, Struers, DK) filtered through a 0.5 μm Duralon filter (Millipore, DK). Water of resistance greater than 18 MΩ cm^{-1} was used as diluent. The gradient starting concentration was 10 mM sodium acetate and 50 mM sodium hydroxide. Successive 20 μl volumes of the preparative scale Endo H_f digest of H(A16-M)-Y were injected after clarification by centrifugation (10 000 × **g**, 5 min). The glycans were eluted with a complex gradient of sodium acetate from 10 mM to 150 mM in 50 mM sodium hydroxide at a flow rate of 1 ml min^{-1} over 45 min, partially desalted by a post-column ion exchange cartridge and collected at 1 min intervals. Appropriate fractions from successive runs were pooled and desalted first by chromatography on a column packed with 2 ml AG 50–X12 (H^+) cation exchange resin (BioRad, DK), and then by gel filtration on a column (26 × 350 mm) packed with BioGel P2 (BioRad, DK) and equilibrated with water. Isolated glycan fractions were finally freeze-dried twice from 99.996% deuterium oxide (Cambridge Isotope Laboratories, UK). Fractionation of analytical scale digests was carried out with the same gradient of sodium acetate in 100 mM sodium hydroxide. $Man_8GlcNAc_2$ (Oxford GlycoSystems, Kebo, DK) was treated with Endo H_f prior to injection on the CarboPac PA100 column.

Determination of N-glycan phosphorylation

N-glycans were released from a 0.5 mg salt-free lyophilized sample of pure H(A16-M)-Y by treatment with anhydrous hydrazine for 4 h at 90°C, following the protocol of the N-glycan release kit (Oxford GlycoSystems, Kebo, DK). After separation from protein degradation products, the N-glycans were labelled with tritium by reduction with sodium borotritide in 50 mM sodium hydroxide-boric acid, pH 11.0 as

described [36]. Neutral and anionic glycans were separated using paper high voltage electrophoresis (HVE) in pyridine:acetic acid:water 3:1:387 at pH 5.4 [36]. Liquid scintillation counting of eluted glycans was used to determine the proportion of neutral to charged glycans. Phosphorylated N-glycans were rendered neutral by treatment with 50% aqueous hydrofluoric acid at 4°C [37].

Proton NMR spectroscopy Oligosaccharide containing fractions were dissolved in 0.5 ml deuterium oxide. Spectra for ^1H were recorded at 500 MHz, 27°C, with a Bruker AM-500 spectrometer using 5 mm tubes. The ^1H resonances were measured relative to internal acetone (2.225 ppm, HDO 4.75 ppm and 27°C).

Results

For the 100 l fermentation, *S. cerevisiae* strain C79–994 was used and the expression of the gene encoding the hybrid (1-3,1-4)-β-glucanase H(A16-M) was directed by $PRB1_p$ [33, 38]. The *B. macerans* (1-3,1-4)-β-glucanase signal peptide [20] was used to direct secretion of mature enzyme. The fermentation resulted in a yield of 50 mg secreted H(A16-M)-Y per l medium, a five-fold increase compared with shake-flask cultures. Hyperglycosylated H(A16-M)-Y was separated from the remaining (1-3,1-4)-β-glucanase during gel filtration as previously reported [19] and not further characterized. Enzymes isolated for N- and O-glycosylation analysis were thus only core-glycosylated. N-linked glycans were released for purification and analysis by preparative scale digestion of H(A16-M)-Y with Endo H_f. Release of N-glycans was 90% complete as estimated from the relative proportions of glycosylated and deglycosylated protein following analysis by SDS-PAGE (data not shown). The released N-glycans were fractionated by high pH anion exchange chromatography (Fig. 1). Elution in 50 mM NaOH was chosen to compress the separation and allow collection of the whole glycan-profile in 10 fractions (A–J), most of which contained more than one species, as judged by the occurrence of multiple peaks in the spectrum (Fig. 1). Upon desalting, freeze drying and dissolving in D_2O, the glycans were analysed by ^1H NMR spectroscopy. Fractions A–C contained no glycans, fractions D–H gave spectra characteristic of *S. cerevisiae* N-glycans (Fig. 2) [12], while fractions I and J contained partially degraded protein. The chemical shifts of the anomeric protons (C1–H) and the C2 protons (C2–H) on the glycosyl residues of the glycans in each fraction (Table 1) were used to identify the different structures present (Fig. 2) by reference to previously published data [12, 39]. Since one-dimensional ^1H NMR cannot always be used to distinguish between different posi-

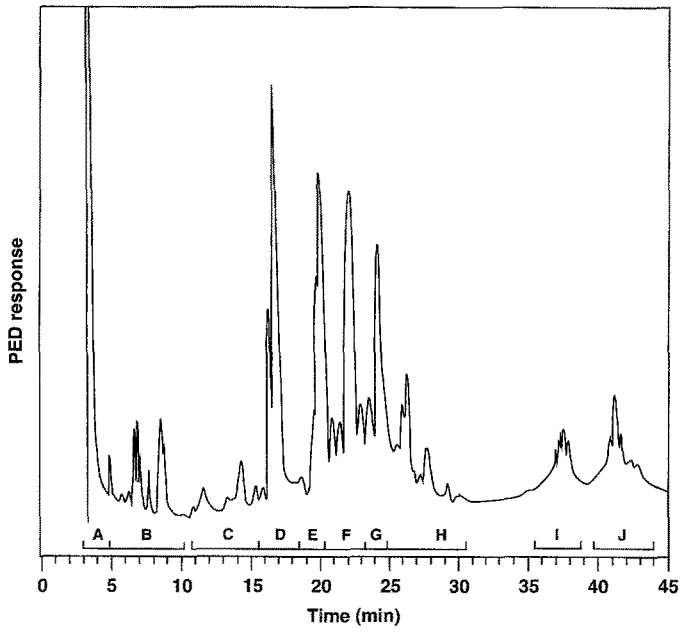

Figure 1. Separation of the products of preparative Endo H_f-treatment of H(A16-M)-Y by HPAEC. 20 µl of the clarified digest was injected on the CarboPac PA100 column and eluted in 50 mM NaOH with a gradient of sodium acetate from 10 to 150 mM over 45 min. Fractions A to I were collected and analysed by ^1H NMR.

Table 1. ^1H NMR intensities and assignment to structural isomers of the glycans in fractions D to H (Fig. 1).

Residue no.	*Residue name*	*Frac. D*	*Frac. E*	*Frac. F*	*Frac. G*	*Frac.H*
C1-H						
5	$3i^2$	5.340	5.343	5.341	5.348	5.342
8	$2i^2$	5.303	5.309	5.306	5.306	5.305
1	α-GN	5.245	5.247	5.241	5.246	5.243
6	$6i^2$	5.144	5.149	5.145	5.144	5.144
12	$6i^2$		5.149	5.145	5.144	5.144
14, 15, 16	3t-O-2			5.145	5.144	5.144
7	3t	5.102	5.115	5.110	5.114	5.117
7	3t	5.073	5.086	5.080	5.086	8.086
13 } α, β	2t-O-6		5.066	5.063	5.065	
13 } α, β	2t-O-6		5.049	5.049	5.050	
11	$2t^1$	5.040	5.049			
9	$2t^u$	5.040	5.043	5.036		
13	3t-O-2				5.050	5.050
11	$2i^1$			5.036	5.038	5.038
9	$2i^u$				5.030	5.033
4	6i	4.869	4.876	4.869	4.875	4.875
C2-H						
14, 15, 16,	3-O-2i			4.222	4.225	4.226
4	6i	4.143	4.149	4.149	4.150	4.149
8	$2i^2$	4.104	4.113	4.112	4.113	4.114
5	3i	4.089	4.094	4.094	4.098	4.097
7, 9, 11, 13-16	2t, 3t	4.067	4.073	4.069	4.072	4.071
3	4i	4.235	4.166	4.168	4.163	4.163
6, 12	$6i^2$	4.027	4.023	4.019	4.021	4.021
NAc-CH_3		2.040	2.044	2.040	2.043	2.044

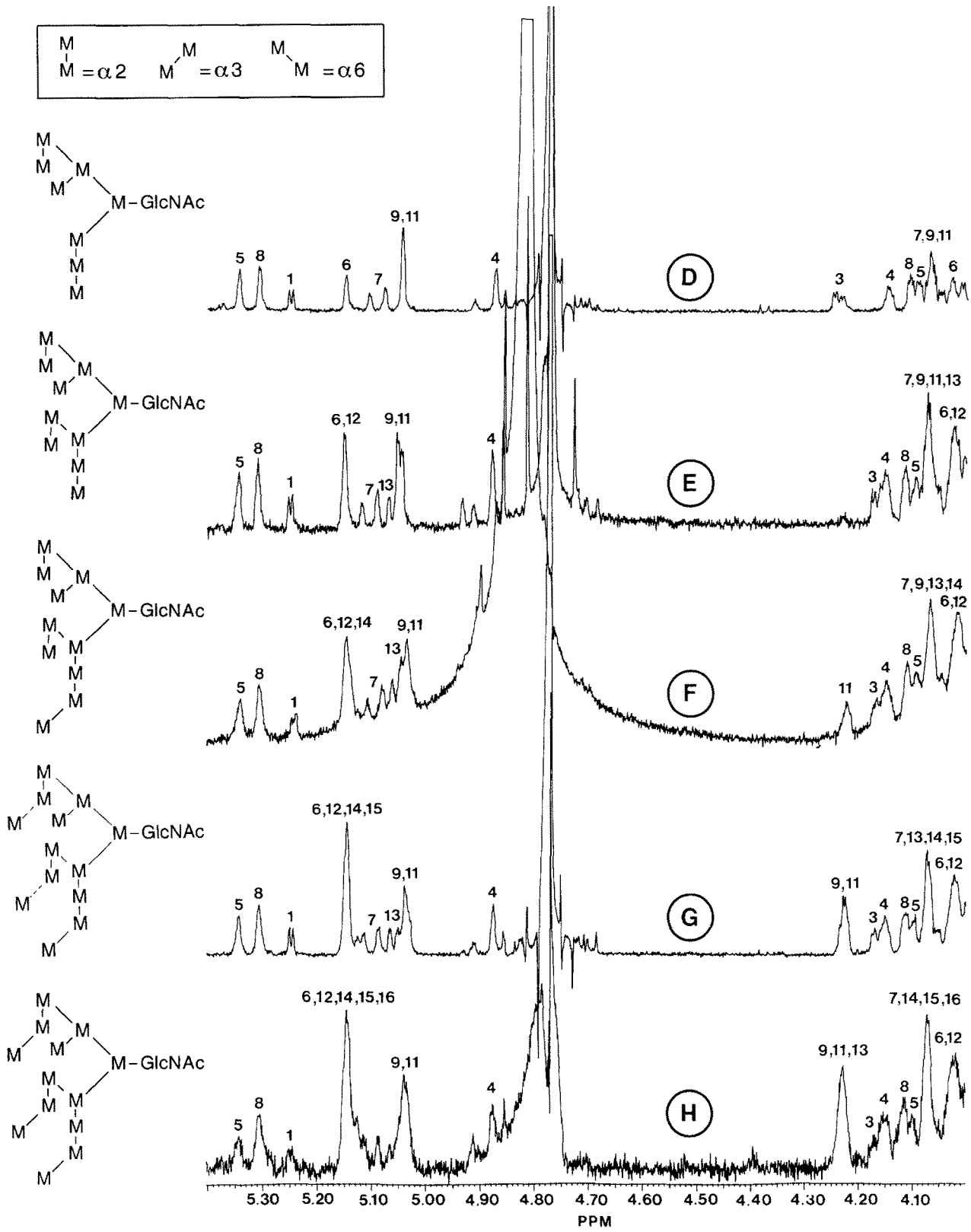

Figure 2. 500-Mhz one dimensional ^{1}H NMR spectra of the glycans in pool D-H (Fig. 1) recorded at 27°C and structure of the major glycans in each pool. Peak assignments and integrations are summarized in Table 1. Proposed structure of the major glycans are given on basis of previous published results [12, 39]. The dashed lines in the $Man_{12}GlcNAc$ structure signifies doubt as to which mannose is added to $Man_{11}GlcNAc$ to give $Man_{12}GlcNAc$.

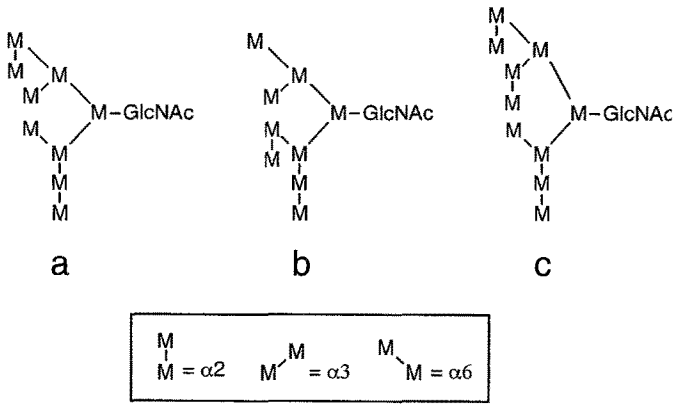

Figure 3. Glycan structures found on yeast secreted proteins by Trimble and Atkinson [39]. a and b are $Man_9GlcNac$ isomers published in [39], Table II, b and c, respectively. c is the $Man_{10}GlcNac$ structure given in [39], Table III, b. The structures are redrawn from reference 39.

tional isomers, particularly those substituted with α3-mannose [13], the major isomeric structures present were also identified by comparison of the HPAEC retention times of the glycans liberated from H(A16-M)-Y with those of Endo H_f-treated CPY. The same sodium acetate gradient was used as in the preparative separation, but in 100 mM NaOH, which gave a higher resolution. The retention times of $Man_8GlcNAc$ to $Man_{14}GlcNAc$ of CPY-derived glycans are shown by arrows in Figs 4I, 5I and 6I. Since only one major structure was identified in each of fractions E–H by ^1H NMR analysis, multiple peaks probably represent different positional isomers of individual structures. Figure 2 presents the major glycan isomers of H(A16-M)-Y. They are a homologous series of high-mannose type glycans ranging from $Man_8GlcNAc$ to $Man_{13}GlcNAc$, with the exception of $Man_9GlcNAc$. The ^1H NMR gave minor resonances at 4.910 ppm in spectrums D and E and at 4.930 ppm in spectrum E (Fig. 2), indicating the presence of $Man_9GlcNAc$ structures previously published [39] (Fig. 3 a and b). However, the resonance at 4.930 ppm in spectrum E could also derive from a $Man_{10}GlcNAc$ structure described by Trimble and Atkinson [39] (Fig. 3c). The $Man_9GlcNAc$ structure shown in Fig 3a is part of the core filling pathway while structure c is the result of alternate core filling [39]. The $Man_{8\text{-}13}GlcNAc$ structures identified are identical to those found previously [13] with the exception of $Man_9GlcNAc$. The $Man_9GlcNAc$ reported by Ballou *et al.* [13] is also not found by Trimble and Atkinson [39] although it might have been present in their Man_{9b} pool.

The ratio of neutral to charged N-glycans was determined by paper high voltage electrophoresis (HVE) of hydrazine-released and tritium-labelled N-glycans from H(A16-M)-Y (profile not shown). Eighty-eight per cent of the radioactivity of the total glycan pool was eluted in the neutral fraction remaining at the origin, while the rest was negatively charged

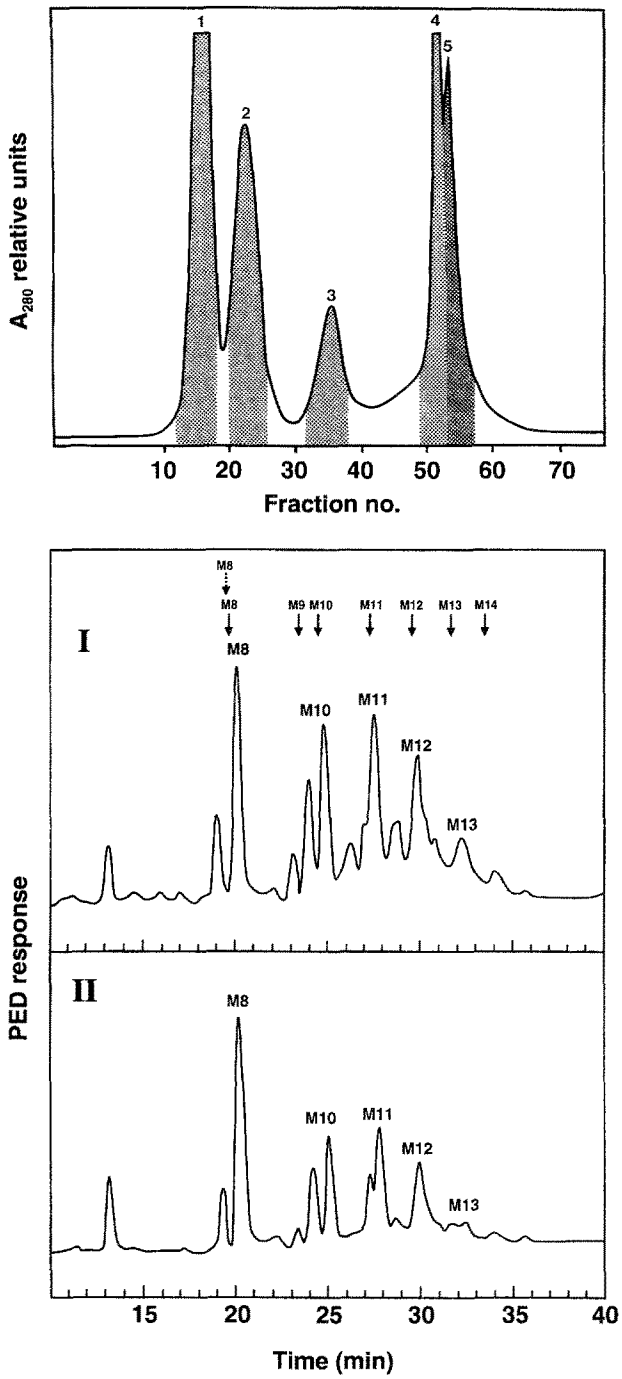

Figure 4. Separation by gel filtration chromatography of peptides generated by CNBr-cleavage of H(A16-M)-Y (top). The peptides were eluted with 10 %(v/v) acetic acid with a flow rate of 35 ml h^{-1} and pools 1–5 collected. HPAEC profiles of Endo H_f-released glycans on the peptides in pool 2 (profile I) and 3 (profile II). The glycans corresponding to the individual peaks are identified by reference to Fig. 5 profile IV. The Endo H_f-treated $Man_8GlcNAc$ standard is indicated with a dashed arrow. Retention times of the N-glycans from yeast CPY are indicated with arrows.

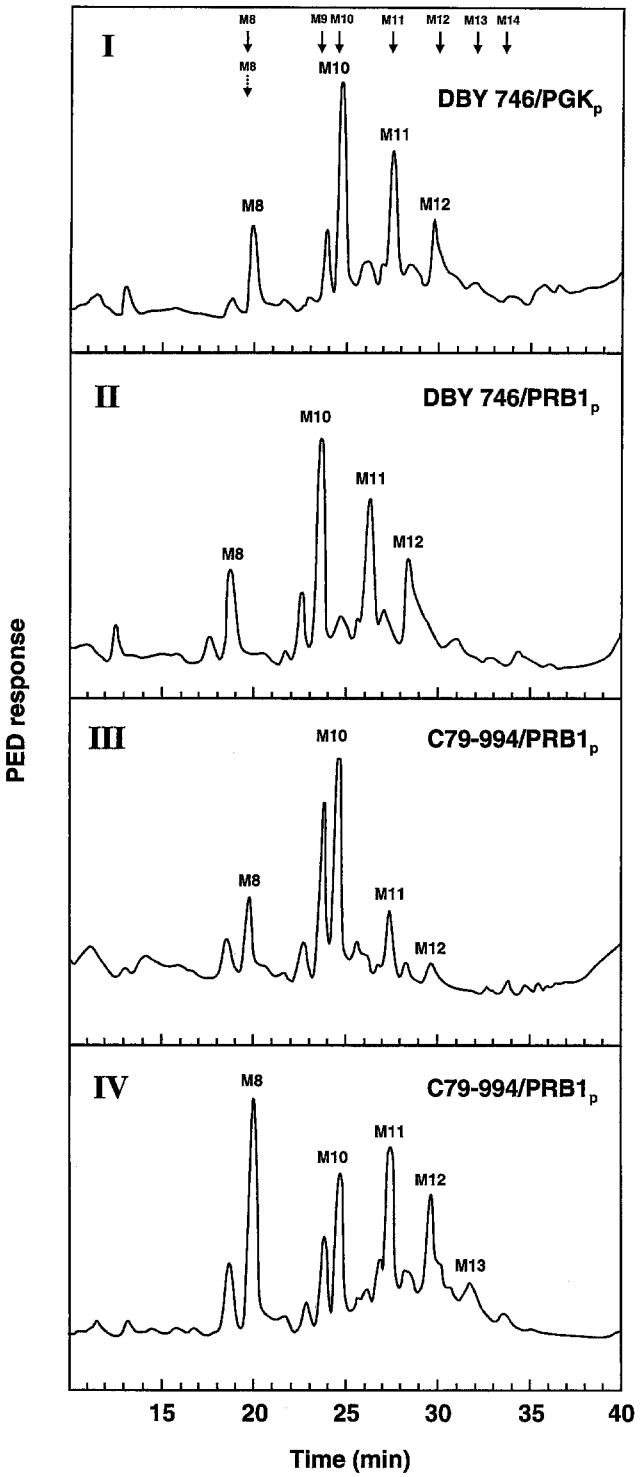

Figure 5. HPAEC profiles of Endo H_f-released N-glycans from H(A16-M)-Y produced under different conditions. Profiles I-III are of N-glycans on H(A16-M)-Y produced in small scale shake flasks while profile IV is from N-glycans on H(A16-M)-Y produced in 100 l fermentor. *S. cerevisiae* strains and promoter used to control expression of H(A16-M)-Y are noted on each profile. The glycans corresponding to the individual peaks are identified by reference to profile IV. The Endo H_f-treated $Man_8GlcNAc$ standard is indicated with a dashed arrow. Retention times of the N-glycans from yeast CPY are indicated with arrows

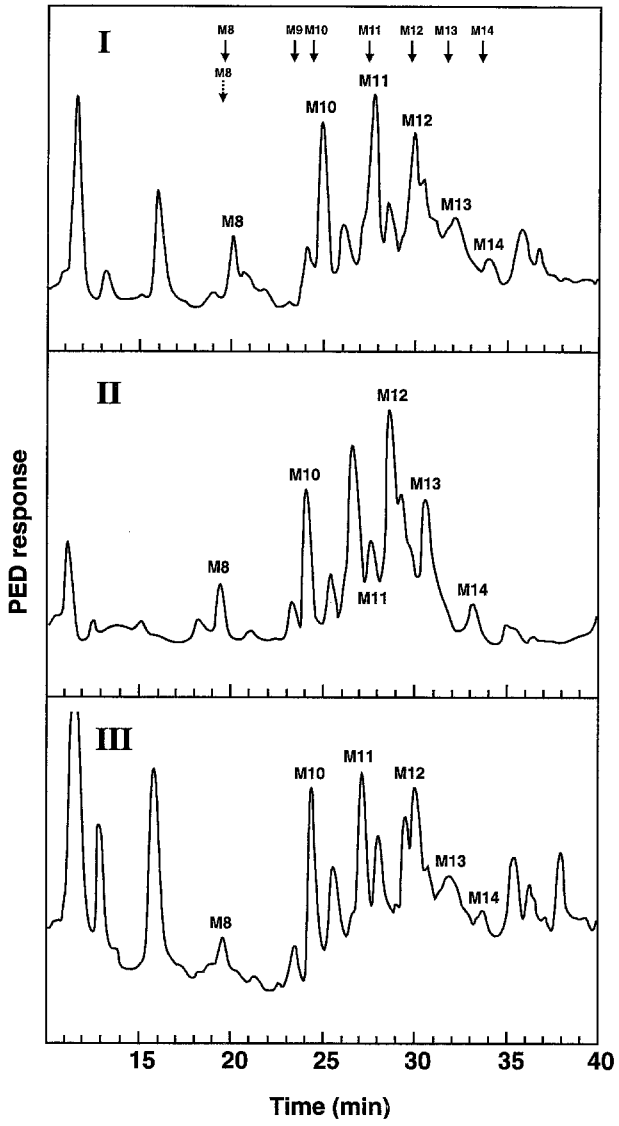

Figure 6. HPAEC profiles of N-glycans released by Endo H_f from H(A16-M)-N31Q-Y (profile I), H(A16-M)-N185Q-Y (profile II) and H(A16-M)-N31Q-N185Q-Y (profile III) produced in DBY 746 under control of PGK_p. The individual peaks are identified by reference to profile IV in Fig. 5. $Man_8GlcNAc$ standard is indicated with a dashed arrow. Retention times of the N-glycans from yeast CPY are indicated with arrows.

at pH 5.4 and migrated away from the origin as a single peak. The anionic charge was due to phosphorylation of the N-glycans since mild acid hydrolysis with hydrofluoric acid, known to remove phosphate groups [37], resulted in exclusively neutral N-glycans on paper HVE.

Only Asn_{31} and Asn_{185}, but not Asn_{40}, of H(A16-M)-Y is glycosylated [19]. For analysis of site-specific N-glycan heterogeneity, peptides were produced from H(A16-M)-Y by CNBr-cleavage, hereby separating the Asn residues glycosylated. After separation of the peptides by gel chromatography, five peptide-pools were collected (Fig. 4) and identified by N-terminal sequencing. Pools 1 and 2 were found to contain

peptides derived from the internal and N-terminal part of H(A16-M)-Y respectively. The latter containing Asn_{31}. Pool 5 contained randomly cleaved protein, while pools 3 and 4 both contained peptide derived from the C-terminal part including Asn_{185}. N-terminal sequencing revealed that pool 3 contained N-glycosylated peptide and pool 4 contained non-glycosylated peptide. From integration of the absorbance peaks at 280 nm (Fig. 4), the ratio between glycosylated and non-glycosylated peptide was estimated to be 2:3. The N-glycans from the peptides in pools 2 and 3 (Fig. 3) were released by Endo H_f and analysed by HPAEC. The heterogeneity observed for analysis of a mixture of N-glycans linked to Asn_{31} and Asn_{185} of H(A16-M)-Y (Fig. 5 profile IV) was also observed when N-glycans from the two sites were analysed individually (Fig. 4 profiles I and II). However, $Man_8GlcNAc$ was the predominant glycan at Asn_{185}.

O-linked glycosylation of H(A16-M)-Y was determined to be nine mannose molecules per polypeptide. In order to account for possible interference of the polypeptide in the phenol/sulfuric acid assay [40], the assay was performed on E. *coli*-secreted H(A16-M) using the same protein concentration as for PNGase F-treated H(A16-M)-Y. No interference could be detected. Since O-glycans on proteins secreted from *S. cerevisiae* cells generally consists of one to five mannose units [41] it may be assumed that 2–9 Ser/Thr residues of H(A16-M)-Y are O-glycosylated. To determine which of 34 sites for potential O-glycosylation sites are actually glycosylated, the peptides in pools 1, 2 and 4 (Fig. 4) were sequenced for 26, 39 and 33 cycles respectively, the latter covering the entire sequence of the peptide. In total 18 of the Ser/Thr residues were sequenced. Phenol/sulphuric acid assays of the peptides revealed carbohydrate in pools 1 and 4. This could only derive from O-linked mannose since no N-linked carbohydrate was present in either pool. However, amino acid sequencing failed to detect any specific O-glycosylated sites.

To investigate the effect of production conditions on the final glycan profile of yeast-secreted H(A16-M)-Y, glycosylation profiles were obtained by HPAEC for H(A16-M)-Y secreted from two *S. cerevisiae* strains DBY 746 and C79-994, grown in 1 l of SC medium without Leu in shake flasks. Two plasmid constructs were used, one where the gene encoding H(A16-M)-Y was under control of a strong promoter ($PRB1_P$), and one where it was under control of PGK_p. The *B. macerans* (1-3,1-4)-β-glucanase signal peptide directed secretion. Strain/promoter combinations DBY 746/PGK_p, DBY 746/$PRB1_p$ and C79-994/$PRB1_p$ were grown under identical conditions. Secreted H(A16-M)-Y was purified, N-glycans were released by digestion with Endo H_f and analysed by HPAEC. The glycan profiles were similar (Fig. 5) although the C79-994/$PRB1_p$ combination exhibited N-glycans with differences in the proportions of the various glycans compared with the two other combinations. This was attributed to the different strains used. The difference in retention time of approximately 1 min for the glycan profile from the DBY 746/$PRB1_p$ combination compared with the other glycan profiles (Fig. 5, profile II) could not be explained. None of the three profiles has $Man_8GlcNAc$ as the dominant structure as is the case for C79-994/$PRB1_p$ grown in a 100 l fermentor (Fig. 5, profile IV). This profile is therefore associated with H(A16-M)-Y produced in the fermentor. No difference in resistance to irreversible thermal inactivation at 70°C could be detected for H(A16-M)-Y produced under the four production conditions.

A series of glycosylation mutants of H(A16-M)-Y was constructed by site-directed mutagenesis using PCR to analyse the effect of site-specific glycosylation interdependence during enzymic synthesis. The code for Asn_{31} and Asn_{185} was changed to that for Gln. The sites were mutated individually or H(A16-M)-N185Q and H(A16-M)-N31Q-N185Q, leaving 2, 2 and 1 sites for potential N-glycosylation, respectively. Following expression using the DBY 746/PGK_p strain/promoter combination, active N-glycosylated (1-3,1-4)-β-glucanase was secreted and thereafter purified by gel filtration. Most of the N-glycans were released by Endo H_f and identified by HPAEC (Fig. 6). However, the retention times of some of the glycans did not correspond to any of those of glycans identified by NMR. These unidentified glycans were assumed to derive from alternate core filling [39]. A larger proportion of the N-glycans on H(A16-M)-N31Q-N185Q-Y (Fig. 6, profile III) than on H(A16-M)-N185Q-Y (Fig. 6, profile II) had been modified by alternate core filling as determined by the relative sizes of the peaks in the HPAEC profiles. The proportion of glycans on H(A16-M)-N31Q-Y modified by alternate core filling was in between the two (Fig. 6 profile I).

The glycosylation profiles of the (1-3,1-4)-β-glucanases MAC-Y, H(A36-M)-Y and H(A78-M)-Y [19] resembled that of H(A16-M)-Y closely when expressed in DBY 746 propagated in shake flask cultures (data not shown). In contrast, the glycan profiles for H(A107-M)-Y, H(A152-M)-Y and AMY-Y [19] differed from the above group and from each other. The glycans present in the latter group were not identifiable by comparison with either CPY or H(A16-M)-Y N-glycan profiles. Increased resistance of the N-glycans to Endo H_f cleavage was observed for the series of hybrid (1-3,1-4)-β-glucanases as the proportion of AMY-derived sequence increased.

Discussion

In *S. cerevisiae* core glycosylation is believed to occur cotranslocationally as nascent proteins are translocated into the lumen of the rough endoplasmic reticulum (RER) [42]. In this step, the presynthesized core oligosaccharide, $Glc_3Man_9GlcNAc_2$, is transferred from the lipid carrier dolicol to an Asn residue in the nacent peptide chain [41]. Rapid trimming to $Man_8GlcNAc$ $t_{\frac{1}{2}}=3$ min [43], is also carried out in the RER. After translocation of the glycoprotein to the Golgi, most of the glycans are modified in a global processing pathway giving $Man_{9\text{-}13}GlcNAc_2$ [39]. The glycan structures from this pathway were all found on H(A16-M)-Y, thereby suggesting similar

recognition and processing events of homologous and heterologous proteins in yeast. The first mannose added to $Man_8GlcNAc_2$ in the Golgi is α-1,6-linked, the only monosaccharide attached to that linkage type in the core filling pathway [39]. Transit of proteins through ER and Golgi has been estimated to be around 5–10 min at 30°C [44–47]. α-1,6-Mannosyltranferase is present only in the early compartments of Golgi where protein transit probably takes 1–2 min [48, 49]. As the transferase is relatively inefficient ($t_{\frac{1}{2}}$ = 10–20 min at 30°C) [43] the small quantities of $Man_9GlcNAc_2$ detected on H(A16-M)-Y from fermentor grown cultures, suggest that only a fraction of the glycans attached to H(A16-M)-Y becomes modified by the addition of α-1,6-linked mannose at high-level expression of the glucanase. Further evidence for the influence of culture conditions and expression level on the N-glycan profile comes from the presence of relatively more glycan molecules with only RER-specific modifications, $Man_8GlcNAc_2$ [41], on H(A16-M)-Y from yeast grown in the fermentor (Fig. 5, profile IV) compared with the shake flask (Fig. 5, profile III). Accordingly it appears that several components of the Golgi become saturated with glycoprotein to be modified when H(A16-M)-Y is produced at a high level.

The presence or absence of N-linked oligosaccharides at specific sites in a glycoprotein is determined during the process of core glycosylation in the RER. Expression of H(A16-M)-Y produced two major glycoforms, where Asn_{185} of H(A16-M)-Y is glycosylated in only 40% of the protein molecules while Asn_{31} is fully glycosylated. This demonstrates inefficient glycosylation at amino acid position 185, suggesting that the site is temporarily inaccessible to the oligosaccharyl transferase in the RER. Accordingly, it can be speculated that folding of the polypeptide masks the epitope for glycan addition, because Asn_{185} is close to the carboxyl terminus of the polypeptide chain where the probability of non-stoichiometric glycosylation of Asn is higher [6]. Alternatively, high-level expression of H(A16-M)-Y saturates the glycosylation apparatus giving heterogenous glycan profiles as found for overexpressed homologous carboxypeptidase Y [50] and Protease A [51]. The small quantitative differences between the glycan profiles of Asn_{31} and Asn_{185}, for example the extent to which $Man_8GlcNAc_2$ predominates, may be attributed to the influence of the folded polypeptide chain in the neighbourhood of the glycosylated Asn on Golgi processing [52]. By analysis of the crystal structure of *E. coli*-secreted H(A16-M) [23] it is observed that Asn_{31} and Asn_{185} are situated in neighbouring positions on the surface of the folded polypeptide. Provided that glycosylation does not substantially alter the folding of H(A16-M)-Y, it might be suggested that the structures themselves give steric hindrance for modifications introduced by enzymes residing in the Golgi. This is supported by data showing flexible N-glycans occupying a comparatively large solution volume in comparison to a folded polypeptide chain [9, 53]. Introduction of the mutations $Asn_{31} \rightarrow$ Gln and $Asn_{185} \rightarrow$Gln gave rise to N-glycosylated glucanase. Therefore, N-glycosylation of Asn_{31} prevents glycosylation of the neighbouring site at Asn_{40}. The hypothesis of steric hindrance to glycan modelling by the glycans themselves is also supported by the analysis of the N-glycans from the mutant forms of H(A16-M)-Y. The double mutant with only Asn_{40} N-glycosylated gave the most complex series of N-glycans while H(A16-M)-N31Q-Y had the second most complex N-glycans. In the latter case probably both Asn_{40} and Asn_{185} are glycosylated but Asn_{40} is situated further away in space from Asn_{185} on the folded polypeptide than Asn_{31} thus giving rise to a lower degree of steric hindrance.

In addition to N-linked glycosylation, O-glycosylation is a characteristic modification of yeast-secreted proteins [41]. As H(A16-M)-Y retained a number of monosaccharide units after N-deglycosylation with PNGase F, these were assumed to be O-linked mannose units. However, failure of protein sequencing to detect by substituted amino acid residues in peptides obtained by cyanogen bromide cleavage, suggests that O-glycosylation of particular Ser and/or Thr residues might be non-stoichiometric. Alternatively, O-glycosylation is heterogeneous with respect to linkage position on the molecule. Proline in the vicinity of Ser and Thr appears to predispose these amino acid residues to O-glycosylation [7]. Since H(A16-M)-Y contains the sequence Pro_{171}-Ser-Thr-Pro, it might provide a starting point for more rigorous investigation of factors determining O-glycosylation.

Glycosylation can influence many properties of proteins, including stability against irreversible thermal inactivation [19]. The resistance of H(A16-M) to irreversible thermal denaturation was increased following glycosylation. In quantitative terms the half-life of the enzyme incubated at 70°C and pH 6.0 was 240 min in the non-glycosylated form and 400 min when glycosylated [19]. The determinants of thermostability of the non-glycosylated H(A16-M) have been shown to be conferred by eight amino proximal residues of H(A16-M) [54]. It appears from the crystal structure of H(A16-M) that N-glycosylation of Asn_{31} and Asn_{185} are unlikely to contribute to the hydrogen bonding which stabilizes the N- and C-terminal portions of the protein (assuming no major perturbation of protein folding by glycosylation), and that the effect of glycosylation must be exerted at some other point. Compared with unglycosylated enzyme, thermal inactivation of glycosylated H(A16-M) reduces visible aggregation of the polypeptide. The significant contribution of glycosylation to the resistance of H(A16-M)-Y to irreversible thermal inactivation may therefore be due to the prevention of polypeptide aggregation, possibly by glycan-polypeptide interactions whereby the folded protein is stabilized or the number of possible conformations of the unfolded polypeptide is reduced [11, 52]. The glycan-protein interactions might also protect the protein in solution against deamidation of Asn and Gln residues, hydrolysis of polypeptide bonds adjacent to Asp residues and destruction of disulfide linkages during heating, effects reported to result in irreversible inactivation [55, 56]. If the degree of glycan-polypeptide interaction is important for stabilization of the glycoprotein this would mean that both glycan

structure/charge and structure of the folded polypeptide play an important role. This would explain that although MAC-Y, H(A36-M)-Y and H(A78-M)-Y have glycosylation profiles very similar to that of H(A16-M)-Y produced in shake flask culture, none showed the marked enhancement of thermostability on glycosylation of H(A16-M)-Y [19]. The folding of the molecule in the remaining hybrids, H(A107-M)-Y, H(A152-M)-Y and AMY-Y, may be judged from the altered glycosylation profile to have altered juxtaposition of the glycosylated sites and presumably also the factors determining thermodynamic stability. Hence no inference can be drawn from the present study as to the factors which confer improved thermostability on glycosylated AMY-Y, but not on glycosylated H(A107-M)-Y and H(A152-M)-Y compared to the non-glycosylated counterparts.

In conclusion, even detailed knowledge of the N-glycan structures of H(A16-M)-Y coupled with the solution of the polypeptide crystal structure of H(A16-M) is inadequate to allow precise assigment of the stabilizing interactions between glycan and polypeptide or between glycan and solvent.

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