Different effects of *N*-glycosylation on the thermostability of highly homologous bacterial (1,3-1,4)- β -glucanases secreted from yeast

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Genes encoding *Bacillus amyloliquefaciens* (1,3-1,4)- β -glucanase (AMY), *B. macerans* (1,3-1,4)- β -glucanase (MAC), and a series of hybrid enzymes containing N-terminal sequence segments of different length derived from AMY with the remaining C-terminal segment derived from MAC, were expressed in *Saccharomyces cerevisiae*. The cells secreted active enzyme into the medium. While the quantity of *N*-glycan linked to the different enzymes was similar, pronounced differences in thermostability were observed when the glycosylated enzymes were compared with the unglycosylated counterparts produced in *Escherichia coli*. Glycosylated AMY and hybrid enzyme H(A16-M), consisting of 16 N-terminal amino acids derived from AMY with the remaining C-terminal segment from MAC, exhibited a 7.5- and 1.6-fold increase in half-life at 70 °C, pH 6.0. N-terminal sequencing established that only two out of three sites for potential *N*-glycosylation of H(A16-M) secreted from yeast were actually glycosylated. Removal of *N*-glycans by endoglycosidase H and peptide:*N*-glycosidase F from H(A16-M) resulted in a 16- and 133-fold decrease of thermostability, demonstrating that *N*-glycans are a major determinant for the resistance of this enzyme to thermal inactivation. Glycosylated MAC and hybrid enzymes H(A36-M), H(A107-M) and H(A152-M) had increased thermostability but hybrid enzyme H(A78-M) was less thermostable. *N*-Glycosylation thus changes thermostability of (1,3-1,4)- β -glucanases with similar primary structure in a variable, so far unpredictable way.

Keywords: (1,3-1,4)- β -glucanases, thermostability, *N*-glycosylation, hybrid enzymes, yeast

INTRODUCTION

Enzymic inactivation by thermal energy is an important characteristic related to the stability of an enzyme in solution. Accordingly there has been considerable interest in improving the thermostability of proteins in order to widen their range of application (reviewed by Nosoh & Sekiguchi, 1990; Fágáin & O'Kennedy, 1991). The effect of glycosylation on thermostability has been investigated for several proteins. Recombinant non-glycosylated

erythropoietin produced in *Escherichia coli* was found to be less resistant to unfolding than the normally glycosylated enzyme synthesized in mammalian cells (Narhi *et al.*, 1991). It was concluded that carbohydrates play an important role in stabilizing the protein. Glycosylated forms of two bovine pancreatic RNases unfold in guanidinium chloride with a slower rate than their deglycosylated counterparts, while both forms of porcine RNase unfold with similar kinetics (Grafl *et al.*, 1987). More recently, synthetic aminoglucose-based carbohydrate monomers linked to α -chymotrypsin, trypsin and subtilisin have been shown to stabilize the proteins (Hill *et al.*, 1991; Wang *et al.*, 1992). For all three enzymes a marked increase in thermostability was observed. Despite such observations, the molecular basis for glycan-protection against protein denaturation and inactivation require further elucidation.

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; Endo H, endoglycosidase H; PNGase F, peptide:*N*-glycosidase F.

On proteins secreted by yeast, similar to the situation in higher eukaryotes, *N*-linked glycosylation may occur at Asn residues of the sequence Asn-Xxx-Ser/Thr, Xxx not being Pro (Roitsch & Lehle, 1989), while *O*-linked glycosylation may occur at either Ser or Thr (Lehle & Bause, 1984; Wilson *et al.*, 1991). The synthesis of yeast *N*-glycans has been studied extensively (reviewed by Kukuruzinska *et al.*, 1987; Tanner & Lehle, 1987). An *N*-glycan core consisting of two *N*-acetylglucosamine (GlcNAc), nine mannose (Man) and three glucose (Glc) units is synthesized in the endoplasmic reticulum, and thereafter attached to the polypeptide. The core is then modified by removal of the three Glc units and one Man unit. In the Golgi further modifications may take place by stepwise addition of Man units, resulting in a high-mannose-type *N*-glycan consisting of two GlcNAc and 8–14 Man units. Synthesis of heterologous proteins in yeast is often accompanied by glycosylation of the products, when they are secreted. This was also observed for *Bacillus macerans* (1,3-1,4)- β -glucanase (Olsen & Thomsen, 1991) and barley (1,3-1,4)- β -glucanase (Olsen & Thomsen, 1989).

Genes encoding *Bacillus macerans* (1,3-1,4)- β -glucanase (MAC) [Borriss *et al.*, 1990; GenBank accession no. X55959], *Bacillus amyloliquefaciens* (1,3-1,4)- β -glucanase (AMY) [Hofemeister *et al.*, 1986; GenBank accession no. M15674] and five hybrid enzymes thereof, H(A16-M), H(A36-M), H(A78-M), H(A107-M) and H(A152-M), were expressed in *E. coli*, and the enzymes purified and examined by Olsen *et al.* (1991). SDS-PAGE showed a single protein band of molecular mass 24 kDa. Despite the high primary sequence homology, the enzymes exhibited more than threefold differences in specific activity at 50 °C, pH 6.0. The temperature for optimal activity at pH 6.0 was 55 °C for AMY, H(A107-M) and H(A152-M), and 65 °C for the remaining (1,3-1,4)- β -glucanases when measured over a period of 10 min. *pI* ranged from 5.6 for H(A152-M) to 8.8 for AMY. The enzymes also exhibited significant differences in resistance to irreversible thermal inactivation when incubated at 70 °C, pH 6.0. AMY and MAC retained 3–5% activity after 60 min, H(A16-M), H(A36-M) and H(A78-M) retained 90%, 22% and 5% activity, respectively, while H(A107-M) and H(A152-M) were completely inactivated. These highly homologous enzymes are very similar with respect to sequons for *N*-glycosylation. Previous results have shown that resistance to thermal inactivation is enhanced for the glycosylated forms of MAC and H(A107-M) (Olsen & Thomsen, 1991). It was of interest to determine whether the parental and the series of hybrid enzymes are stabilized to the same degree upon *N*-glycosylation or if a systematic variation can be detected. To investigate this, the parental and hybrid genes have been expressed in yeast cells, active enzymes purified, and their enzymic thermostabilities compared before and after deglycosylation.

METHODS

Organisms, media, transformation procedures and growth conditions. *E. coli* DH5 α was supplied by Gibco-BRL. Genetic

transformations were done according to the procedure of Golub (1988). Transformants were incubated on solid LB medium containing 0.1% (w/v) lichenan and 100 mg ampicillin l⁻¹. Staining with 0.1% (w/v) Congo Red revealed a clearing zone around colonies secreting active recombinant (1,3-1,4)- β -glucanase. Cultivation of transformed *E. coli* cells for purification of (1,3-1,4)- β -glucanase was as described by Olsen *et al.* (1991). Genetic transformation of *Saccharomyces cerevisiae* strain DBY 746 (Mortimer & Contopoulou, 1991) was carried out by electroporation (Becker & Guarente, 1992). Transformants secreting recombinant (1,3-1,4)- β -glucanase were grown at 30 °C for 4 d on solid SC medium (Petersen *et al.*, 1983) without leucine, containing 0.1% (w/v) lichenan. Staining with Congo Red was as described above. In order to purify (1,3-1,4)- β -glucanase secreted from *S. cerevisiae* the cells were first inoculated in 4 × 5 ml SC medium without leucine and incubated at 30 °C with shaking for 4 d before transfer to 1 litre of medium in a 3 litre conical flask. The cultures were then incubated at 30 °C with shaking for 4–5 d.

Preparation and analysis of DNA. 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 (Bio-Rad). DNA sequencing was performed on a 373A DNA sequencer (Applied Biosystems) according to the manufacturer's recommendations. The polymerase chain reaction (PCR) technique was used according to Innis & Gelfand (1990).

Plasmid constructions. Construction and cloning of the genes encoding AMY, MAC and the hybrid (1,3-1,4)- β -glucanases have been described (Borriss *et al.*, 1989; Olsen *et al.*, 1991). The DNA sequence encoding the *B. macerans* (1,3-1,4)- β -glucanase signal peptide was fused in-frame with the sequence encoding either MAC or H1 [redesignated H(A107-M)] before cloning in the yeast expression vector pMA91 (Mellor *et al.*, 1983) as described previously (Olsen & Thomsen, 1991). For the present study, yeast DBY 746 cells were retransformed with the plasmid constructs. The genes encoding H(A16-M), H(A36-M), H(A78-M) and H(A152-M) were excised from pTZ19R-H(AX-M), X = 16, 36, 78 and 152 (Olsen *et al.*, 1991), using *BsmI*–*HindIII*. Following incubation with T4 DNA polymerase to produce blunt ends, the fragments were digested with *Sau3AI*, which cleaves 11 bp downstream of the codon for the amino-terminal residue of the mature (1,3-1,4)- β -glucanases. These fragments were cloned into pUC-OliMAC (Fig. 1a) digested with *BamHI*–*HindIII* in order to obtain the DNA fragment encoding the *B. macerans* (1,3-1,4)- β -glucanase signal sequence in-frame with the hybrid genes. From the resulting plasmids the genes were excised with *BglII*–*SphI* before ligation with the large *BglII*–*SphI* fragment of pUC13-MH1 (Olsen & Thomsen, 1991). This replaces the H1 gene with the desired gene and introduces a *BamHI* site at the 3' end of the gene encoding (1,3-1,4)- β -glucanase. The small *BglII*–*BamHI* fragments from these plasmids were individually cloned into the *BglII* site of the yeast expression vector pMA91 giving pMM-H(AX-M), X = 16, 36, 78 and 152 (Fig. 1b).

In order to clone the gene encoding AMY in-frame with the DNA fragment encoding *B. macerans* (1,3-1,4)- β -glucanase signal peptide, the fragment spanning the region between nucleotides 271 to 813 of the gene encoding H2 (Borriss *et al.*, 1989) was amplified using PCR with primers M-US, which is identical to nucleotides 271–303 of the sense strand of gene H2, and A-H1-LS, which has a *BamHI* recognition site followed by bases identical to nucleotide 813–781 of the antisense strand of gene H2 (Fig. 2). The amplified DNA fragment encoding the C-

Fig. 1. (a) Diagram of the pUC-OliMAC vector. It was constructed by Olsen & Thomsen (1991) using a synthetic 99 bp oligonucleotide encoding the MAC (1,3-1,4)- β -glucanase signal peptide plus five NH₂-terminal amino acids of mature AMY (1,3-1,4)- β -glucanase and containing a *Bgl*II and a *Bam*HI site at the 5' and 3' end, respectively. This oligonucleotide was cloned into pUC13 linearized with *Sma*I. (b) Diagram of the yeast expression plasmid pMM-H(AX-M), X = 16, 36, 78 and 152. The DNA fragment coding for AMY and H(AX-M) is indicated. Shaded regions indicate the gene encoding the *B. macerans* (1,3-1,4)- β -glucanase signal peptide.

terminal half of the mature AMY was digested with *Eco*RV-*Bam*HI and cloned into *Eco*RV-*Bam*HI-digested pUC13-MH1 (Olsen & Thomsen, 1991), which provided the gene encoding the fusion of the MAC signal peptide with the N-terminal half of AMY. This plasmid was digested with *Bgl*II-*Bam*HI and the small fragment containing the DNA fragment encoding the *macerans* signal peptide fused with the gene encoding AMY was isolated. This fragment was cloned into the *Bgl*II site downstream of the PGK promoter of the expression vector pMA91.

Protein purification. (1,3-1,4)- β -Glucanase secreted from *E. coli* cells was purified as described by Olsen *et al.* (1991). The 1 litre of cell-free yeast culture fluid containing recombinant (1,3-1,4)- β -glucanase was passed through a 1.2 μ m filter and concentrated 20-fold to 50 ml by ultrafiltration using a Minitan unit (Millipore) equipped with filters allowing passage of molecules smaller than 10 kDa. Further ultrafiltration to 5 ml was accomplished using an ultrafiltration cell (Amicon) equipped with a filter allowing passage of molecules smaller than 6 kDa. The concentrated sample was applied to a Sephacryl S200 HR (Pharmacia) gel filtration column (2.6 cm $\times$ 100 cm) equilibrated with 20 mM sodium acetate, pH 5.0, 1 mM CaCl₂. The proteins were eluted with the equilibration buffer at a flow rate of 30 ml h⁻¹. (1,3-1,4)- β -Glucanase was eluted after 65–75% of

Fig. 2. Diagram of plasmid pUC19-H2 (Borriess *et al.*, 1989) used to amplify the DNA fragment encoding the C-terminal half of the mature AMY in order to construct a fusion gene coding for the mature part of AMY (1,3-1,4)- β -glucanase with a signal peptide originating from the corresponding *B. macerans* enzyme. The region spanning nucleotides 271–813 was amplified using primers M-US and A-H1-LS. Nucleotide numbering is from Borriess *et al.* (1989).

the total bed volume had been applied. In this fraction active (1,3-1,4)- β -glucanase of 30 kDa and 33 kDa, respectively, was identified after SDS-PAGE. Since the isoforms could not be further separated, a mixture of the two forms was used for the analysis.

Enzyme assay and inactivation analysis. All assays were performed in triplicate. Determination of (1,3-1,4)- β -glucanase activity was performed using a kit for the assay of malt β -D-glucanase (MegaZyme) essentially according to the manufacturer's recommendations, except that 1–25 μ l of sample containing (1,3-1,4)- β -glucanase was incubated in 200 μ l 50 mM MES, pH 6.0, 5 mM CaCl₂ and 200 μ l Azo-barley glucan substrate. The reaction was stopped by addition of 1 ml precipitant [0.30 M sodium acetate, 0.02 M zinc acetate, pH 5.0, 80% (v/v) 2-methoxyethanol]. Thermal stability of the (1,3-1,4)- β -glucanases was determined by measuring the rate of irreversible inactivation at 70 °C for up to 9 h. Enzyme preparations of 100 μ l each containing 10 μ g purified enzyme were incubated in 50 mM MES, pH 6.0, 5 mM CaCl₂. Aliquots were withdrawn periodically and placed on ice. The percentage of remaining enzymic activity relative to 0 min was calculated.

Endo H and PNGase F digestion. Purified native or denatured glycoproteins were digested with endoglycosidase H (Endo H) or peptide: N-glycosidase F (PNGase F) (New England BioLabs) according to the description given by the supplier. For preparative use, 5 mg of purified glycoprotein was digested with either Endo H, or PNGase F in a total volume of 1 ml. Deglycosylated protein was then purified by gel filtration as described above.

SDS-PAGE. Samples were prepared and separated on premade 8–18% (w/v) acrylamide ExcelGel gels (Pharmacia). After electrophoresis the gels were stained with Coomassie Brilliant Blue R (Sigma).

N-terminal sequencing. This was performed using an Applied Biosystems Sequencer (model 470A) equipped with an on-line phenylthiohydantoin (PTH) amino acid analyser (model 120A); 50–100 pmol of polypeptide was used for each analysis.

Protein cleavage and polypeptide purification. In order to cleave Asn-Gly bonds, 5 mg purified H(A16-M)-Y was incubated at room temperature for 3 h in 2 M hydroxylamine, 6 M guanidinium hydrochloride, pH 9.0. The reaction mixture was

then applied to a Biogel P10 (Bio-Rad) gel filtration column (0.9 cm × 90 cm) equilibrated with 10% (v/v) acetic acid. The peptides were eluted with the equilibration buffer at a flow rate of 8.5 ml h⁻¹. The eluate was monitored at 280 nm; relevant peaks were pooled, freeze-dried and their N-terminal sequences determined. Specific cleavage after Arg was accomplished with activated clostripain (Boehringer Mannheim) on reduced and vinylpyridinated protein in 6 M urea at 37 °C overnight with a substrate:enzyme ratio of 100:1. Separation of peptides was accomplished on a Biogel P30 (Bio-Rad) gel filtration column (1.5 cm × 90 cm) equilibrated with 30% (v/v) acetic acid. The smallest peptides were further purified by HPLC using a linear gradient in acetonitrile from 0 to 60% (v/v) in 1 h. In order to cleave Met-Xxx bonds, Xxx being any amino acid, 55 mg CNBr was added to 5 mg purified H(A16-M)-Y in 70% (v/v) formic acid. After incubation at room temperature overnight, CNBr was removed by flushing nitrogen through the reaction mixture. Separation and analysis was as described for hydroxylamine-digested H(A16-M)-Y.

RESULTS AND DISCUSSION

The complete series of genes encoding *B. amyloliquefaciens* (1,3-1,4)- β -glucanase, *B. macerans* (1,3-1,4)- β -glucanase and the five hybrid genes thereof have now been expressed in yeast cells using the 3'-phosphoglycerate kinase (PGK) promoter (Mellor *et al.*, 1983). With the *B. macerans* (1,3-1,4)- β -glucanase signal peptide, secretion of glycosylated active (1,3-1,4)- β -glucanase and correct processing of the pre-protein was achieved. Bacterial signal peptides have previously been shown to function in yeast, e.g. the signal peptide of *E. coli* β -lactamase (Hollenberg *et al.*, 1983). It has been found that the yeast signal peptidase can recognize and cleave off the *B. macerans* (1,3-1,4)- β -glucanase signal peptide but not that of the *B. amyloliquefaciens* pre-enzyme (Olsen & Thomsen, 1991). This is not surprising since there is little primary amino acid sequence identity between the two signal peptides. Purification of the enzymes by gel filtration showed that active (1,3-1,4)- β -glucanase was eluted after applying buffer corresponding to 65–75% of the total gel volume. Calculated on enzymic activity and depending on the individual preparations, 5–50% of the secreted (1,3-1,4)- β -glucanase was hyperglycosylated (data not shown). It was eluted from the gel filtration column in the void volume (35–45% of the total gel volume) and thus well separated from the remaining (1,3-1,4)- β -glucanase. When the transformed yeast was propagated in conical flasks, the individual yeast enzyme preparations revealed two (1,3-1,4)- β -glucanases with molecular masses of 30 kDa and 33 kDa after SDS-PAGE, as shown for H(A16-M)-Y in Fig. 3, lane 5. Sequence analysis of N-terminal amino acids revealed that a sample of H(A16-M)-Y with a mixture of the 30 kDa and 33 kDa forms consists of proteins with two different sequences. One sequence corresponds to signal peptide processing as observed for the (1,3-1,4)- β -glucanases secreted from *E. coli*, whereas the other N-terminal sequence shows signal peptidase cleavage at position -2 adding Leu-Ala to the amino-terminus of the mature enzyme (see Fig. 5). Both cleavages occur after an Ala residue, which is often observed for signal peptide cleavage in yeast (Hitzeman *et al.*, 1990). Whether two additional amino acids at the N-terminus of

the mature enzyme influence enzyme thermostability is not yet clear. However, since the four N-terminal amino acids of H(A16-M) secreted from *E. coli* are without contact to the rest of the protein (Keitel *et al.*, 1993, Fig. 2), it is likely that an extension with two N-terminal

Fig. 3. SDS-PAGE analysis of hybrid (1,3-1,4)- β -glucanase in an 8–18% gradient gel. Proteins were stained with Coomassie Blue R. The positions of the molecular mass markers are indicated to the left. Lane 1, PNGase F-treated H(A16-M)-Y; lanes 2 and 3, Endo H_t-treated H(A16-M)-Y (expressed in yeast); lane 4, H(A16-M) (expressed in *E. coli*); lane 5, H(A16-M)-Y. The proteins were either denatured (+) or not denatured (–) prior to Endo H_t treatment. Endo H_t (60 kDa) is detected in lanes 2 and 3, while PNGase F (35 kDa) is detected in lane 1.

Fig. 4. Diagram of the parental and the hybrid (1,3-1,4)- β -glucanases used in this study. Potential N-glycosylation sites are indicated (●) and the amino acid position shown. MAC consists of 212 amino acids while the other (1,3-1,4)- β -glucanases have two additional amino acids at the N-terminal end. White and shaded regions illustrate AMY- and MAC-derived sequences, respectively. Amino acid homology between MAC and AMY is illustrated at the bottom with solid regions indicating amino acid identity. Amino acid numbering is from the N-terminal amino acid for the mature *E. coli* secreted H(A16-M) (see Fig. 5).

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      -10          ▼ ▼          10
MKKKSCFTLVTTFAESLIFSVALAQTGGSFPEPFNSYNS
      GGVFXXTWRA (seq1)          50
GTWEKADGYSNGGVFNCTWRANNVNFTNDGKLKGLTSSA
      ANNVNFTN (seq2)
YMKFDCAEYRSTNIYGYGLYEVSMKPAKNTGIVSSFITYT
      130
GPAHGTQWDEIDIEFLGKDTTKVQFNYYTNGVGGHEKVIS
      170
LGFDASKGFHTYAFDQWPGYIKWYVDGVLKHTATANIPST
      NLWXTG (seq3)          210
PGKIMMNLWNGTGVDDWLGSYNGANPLYAEYDWVKYTSN

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Fig. 5. Amino acid sequence of H(A16-M)-Y. Sequence 1 (seq1) was determined from the peptide obtained after incubation of H(A16-M)-Y in 2 M hydroxylamine; seq2 is the N-terminal sequence of a peptide purified after clostripain cleavage and seq3 after cyanogen bromide cleavage. [X] indicates absence of an eluting amino acid derivative in the sequencing cycle. The underlined sequence represents the amino acid sequence of *B. macerans* (1,3,1,4)- β -glucanase signal peptide. Signal peptidase cleavage sites are marked (▼). Amino acid numbers are given for the mature enzyme secreted from *E. coli* as described by Olsen *et al.* (1991).

residues will not significantly influence stability or activity of the enzyme.

Glycosylation sites

MAC, H(A16-M) and H(A36-M) have three sequons for potential *N*-glycosylation, while H(A78-M), H(A107-M), H(A152-M) and AMY have four (Fig. 4). In order to investigate which of the potential *N*-glycosylation sites of H(A16-M)-Y are actually glycosylated, peptides each containing one of the three potentially *N*-glycosylated Asn residues were produced by specific cleavage of the purified protein. Since a glycosylated Asn is not extracted from the sequencing support due to its hydrophilic nature, no signal is expected in that particular sequencing cycle when the peptide is N-terminally sequenced. H(A16-M)-Y was digested using either hydroxylamine, clostripain or cyanogen bromide. Hydroxylamine specifically hydrolyses Asn-Gly bonds and the peptide spanning Gly-27 to Asn-125 was purified by gel filtration. Ten sequencing cycles could be performed when this peptide was N-terminally sequenced (Fig. 5, seq1); cycle 5 gave no signal, showing that Asn-31 is actually glycosylated. Furthermore, cycle 6 did not give any signal. This shows that Cys-32 forms a S-S bond to another Cys in the protein, which can only be Cys-61 since there are only two Cys residues in H(A16-M)-Y. This is in line with results obtained by Keitel *et al.* (1993), who found a S-S bond between Cys-32 and Cys-61 in the crystal structure of H(A16-M). Upon reduction of the S-S bond followed by clostripain cleavage after Arg residues, three peptides

were produced and the peptide spanning Ala-36 to Arg-65 was purified. Eight N-terminal sequencing cycles (Fig. 5, Seq2) showed no glycosylation of Asn-40. Treatment of H(A16-M)-Y with cyanogen bromide, cleaving after Met residues, resulted in three peptides. The one spanning Asn-182 to Asn-214 was purified and six N-terminal sequencing cycles were performed (Fig. 5, Seq3), revealing glycosylation of Asn-185. In summary it can be concluded that Asn-31 and Asn-185 of the 30/33 kDa forms of H(A16-M)-Y are actually glycosylated while Asn-40 is not. PNGase F digestion of the 30/33-kDa H(A16-M)-Y forms reduced the molecular mass to 24 kDa, similar to that of H(A16-M) (Fig. 3, lanes 1 and 4), revealing that the difference between the 30 kDa and the 33 kDa forms is primarily due to heterogeneous *N*-glycosylation. Whether the glycosylation patterns of the other (1,3,1,4)- β -glucanases are similar to that of H(A16-M)-Y is a subject for further investigation. However, introduction of the substitution Tyr-62 $\rightarrow$ Asn in MAC-Y in order to introduce a site for potential glycosylation had no effect on the molecular mass of the recombinant enzyme (Olsen & Thomsen, 1991). Thus, the available data suggest that Asn-64 of H(A78-M)-Y, H(A107-M)-Y, H(A152-M)-Y and AMY-Y is not glycosylated.

Thermostability of the enzymes

Stability against irreversible thermal inactivation was determined by incubating 10 μ g purified (1,3,1,4)- β -glucanase at 70 °C and by following the residual enzymic activity with time (Fig. 6). H(A16-M)-Y was the only hybrid (1,3,1,4)- β -glucanase exhibiting a stability superior to both of the parental (1,3,1,4)- β -glucanases. The inactivation half-life for H(A36-M)-Y was intermediate between the parental enzymes, whereas H(A107-M)-Y and H(A152-M)-Y exhibited the same inactivation characteristics as MAC-Y. H(A78-M)-Y was a yeast-secreted (1,3,1,4)- β -glucanase which was less stable than the two yeast-secreted parental enzymes. In agreement with the results given by Olsen *et al.* (1991), H(A16-M) and H(A36-M) were more thermostable than both parental (1,3,1,4)- β -glucanases, while H(A107-M) and H(A152-M) were less thermostable. H(A78-M) exhibited inactivation characteristics similar to MAC. H(A107-M)-Y displayed different inactivation kinetics from those previously described (Olsen & Thomsen, 1991), which might be due to the fact that the enzyme preparation used in the present study was derived from retransformed yeast cells with a different genotype than those used previously.

Inactivation half-lives of the (1,3,1,4)- β -glucanases incubated at 70 °C are shown in Fig. 7. It is evident that AMY-Y and H(A16-M)-Y exhibit a significant increase in resistance to irreversible thermal denaturation compared to the non-glycosylated AMY and H(A16-M). The increase in half-life is 7.5- and 1.6-fold, respectively. In contrast, H(A78-M)-Y is less stable than H(A78-M), with a 3.3-fold decrease in half-life. The thermostability of MAC-Y, H(A36-M)-Y, H(A107-M)-Y and H(A152-M)-Y is enhanced 1.2- to 1.4-fold over their counterparts secreted from *E. coli*.

Fig. 6. Irreversible thermal denaturation of parental and hybrid (1,3-1,4)- β -glucanases as a function of time. Enzymic activity relative to 0 min is shown. Samples of MAC-Y (□), H(A16-M)-Y (■), H(A36-M)-Y (○), H(A78-M)-Y (●), H(A107-M)-Y (△), H(A152-M)-Y (▲) and AMY-Y (▽) were incubated at 70 °C. Samples were withdrawn at intervals up to 60 min. Individual points represent the mean of three independent measurements differing by less than 10%.

Fig. 8. Irreversible thermal inactivation of H(A16-M)-Y (■), and H(A16-M)-Y deglycosylated using either Endo H_f (●) or PNGase F (▲). Enzymic activity relative to 0 min is plotted against time. Enzyme preparations were incubated at 70 °C and samples were withdrawn at intervals for up to 60 min. Individual points represent the mean of three independent measurements differing by less than 10%.

Fig. 7. Illustration of the calculated half-lives at 70 °C of the parental and hybrid (1,3-1,4)- β -glucanases secreted from *E. coli* and yeast (signified by '-Y').

Role of *N*-glycans in thermostability

To further investigate the role of *N*-linked glycans in enhancing the resistance of proteins to irreversible thermal inactivation, carbohydrates were enzymically

removed from H(A16-M)-Y using either Endo H_f or PNGase F. Endo H_f cleaves between the two GlcNAc residues of the *N*-glycan core, leaving one GlcNAc residue attached to the Asn residue (Robbins *et al.*, 1984; Maley *et al.*, 1989). Approximately 10% of the *N*-glycans attached to H(A16-M)-Y were resistant to Endo H_f (Fig. 3, lane 3). Similarly, the *N*-glycans were also partially resistant to cleavage by Endo H_f using denatured H(A16-M)-Y (Fig. 3, lane 2). These results suggest that it is not the folding of the amino acid backbone that makes some of the *N*-glycans inaccessible to Endo H_f but rather the composition of the *N*-glycan. PNGase F cleaves between the inner GlcNAc and the Asn, thus completely removing the *N*-glycan (Plummer *et al.*, 1984; Tarentino *et al.*, 1990). In this reaction the side chain of Asn is deamidated, giving Asp and thus altering the amino acid sequence and charge of the enzyme. Complete removal of *N*-glycans from H(A16-M)-Y was accomplished using PNGase F (Fig. 3, lane 1).

Both deglycosylated (1,3-1,4)- β -glucanases were purified using gel filtration chromatography. Examination of the purified enzymes by SDS-PAGE revealed that both the Endo H_f- and PNGase F-deglycosylated H(A16-M)-Y consisted of a single protein band of molecular mass 24 kDa, similar to the unglycosylated counterpart secreted from *E. coli*. Stability against irreversible thermal inactivation was determined by measuring residual activity after incubation at 70 °C of 10 μ g of the purified deglycosylated H(A16-M)-Y (Fig. 8). Deglycosylation of H(A16-M)-Y using either Endo H_f or PNGase F caused a marked

decrease in thermostability of the enzyme, reducing the inactivation half-life from 400 min to 25 min and 3 min, respectively. This is very significantly lower than the half-life of the unglycosylated H(A16-M) secreted from *E. coli* (250 min). The reduced thermostability of H(A16-M)-Y deglycosylated with PNGase F may be explained by the substitutions Asn-31 $\rightarrow$ Asp and Asn-185 $\rightarrow$ Asp, resulting in more acidic regions of the enzyme surface leading to destabilization. This hypothesis is in accordance with results of Tomazic & Klivanov (1988) showing that thermoinactivation of *Bacillus licheniformis* α -amylase was caused by deamidation of Asn and Gln residues. Since deglycosylation of H(A16-M)-Y with Endo H_f leaves the amino acid composition unchanged, the decrease in thermostability is due to removal of the N-linked carbohydrates. This implies that removal of N-glycans either yields a different, less stable, conformation of the polypeptide or that additional post-translational modifications occur during the secretion process in yeast, which as such are detrimental to stability and become apparent upon removal of the glycans.

Concluding remarks

The results presented show that although highly homologous *Bacillus* (1,3-1,4)- β -glucanases secreted from yeast cells exhibit similar quantitative N-glycan decoration the effects on resistance to irreversible thermoinactivation are diverse. This suggests that the effects of glycosylation on thermostability do not follow simple predictable rules. In order to investigate whether the different effects of N-glycosylation are due to differences in glycan structure, the glycans from the individual (1,3-1,4)- β -glucanases will have to be released and analysed. Yeast N-glycans may be phosphorylated (Hernandez *et al.*, 1992), which may influence thermostability. If it turns out that the structure and/or charge of the N-glycans are the major determinants for altered thermostability, the different hybrids offer the possibility to investigate whether the structure of the N-glycans is determined by the amino acid sequence around the glycosylated Asn. If so, N-glycans attached to Asn-185 are expected to be very similar for all the (1,3-1,4)- β -glucanases described here since this region has an identical amino acid sequence (Fig. 4). N-Glycans attached to Asn-residues within the first 70 N-terminal amino acids may vary since the similarity between MAC and AMY in this region is low (Fig. 4). However, the effect of N-glycans on thermostability may also be dependent on the N-glycan-peptide interaction. N-Glycans are attached during translocation of the pre-protein into the endoplasmic reticulum and folding of the mature protein (Kukuruzinska *et al.*, 1987), and the N-glycan may influence folding of the protein. For a molecular explanation of the observed differential aspects of N-glycosylation on thermostability, the three-dimensional structure of the (1,3-1,4)- β -glucanases described here will have to be analysed. In this connection it is of interest that the three glycosylation sequons of H(A16-M) are located on the surface of the protein (Keitel *et al.*, 1993).

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REFERENCES

- Becker, D. M. & Guarente, L. (1992). Protocols for high-efficiency yeast transformation. In *Guide to Electroporation and Electrofusion*, pp. 501–505. Edited by D. C. Chang, B. M. Chassy, J. A. Saunders & A. E. Sowers. San Diego: Academic Press.
- Borriss, R., Olsen, O., Thomsen, K. K. & von Wettstein, D. (1989). Hybrid *Bacillus endo*(1-3,1-4)- β -glucanases: construction of recombinant genes and molecular properties of the gene products. *Carlsberg Res Commun* **54**, 41–54.
- Borriss, R., Buettner, K. & Maentsaelae, P. (1990). Structure of the beta-1,3-1,4-glucanase gene of *Bacillus macerans*: homologies to other beta-glucanases. *Mol & Gen Genet* **222**, 278–283.
- Fágáin, Ó. C. & O'Kennedy, R. (1991). Functionally-stabilized proteins – a review. *Biotechnol Adv* **9**, 351–409.
- Golub, E. I. (1988). 'One minute' transformation of competent *E. coli* by plasmid DNA. *Nucleic Acids Res* **16**, 1641.
- Grafl, R., Lang, K., Vogl, H. & Schmidt, F. X. (1987). The mechanism of folding of pancreatic ribonucleases is independent of the presence of covalently linked carbohydrate. *J Biol Chem* **262**, 10624–10629.
- Hernandez, L. M., Olivero, I., Alvarado, E. & Larriba, G. (1992). Oligosaccharide structure of the major exoglucanase secreted by *Saccharomyces cerevisiae*. *Biochemistry* **31**, 9823–9831.
- Hill, T. G., Wang, P., Huston, M. E., Wartchow, T. G., Oehler, L. M., Smith, M. B., Bednarski, M. D. & Callstrom, M. R. (1991). Carbohydrate protein conjugates (CPC): the design of new materials to stabilize enzymes. *Tetrahedron Lett* **32**, 6823–6826.
- Hitzeman, R. A., Chen, C. Y., Dowbenko, D. J., Renz, M. E., Liu, C., Pai, R., Simpson, N. J., Kohr, W. J., Singh, A., Chisholm, V., Hamilton, R. & Chang, C. N. (1990). Use of heterologous and homologous signal sequences for secretion of heterologous proteins from yeast. *Methods Enzymol* **185**, 421–436.
- Hofemeister, J., Kurtz, A., Borriss, R. & Knowles, J. (1986). The β -glucanase gene from *Bacillus amyloliquefaciens* shows extensive homology with that of *Bacillus subtilis*. *Gene* **49**, 177–187.
- Hollenberg, C. P., Roggenkamp, R., Erhart, K., Breuning, K. & Reipen, G. (1983). The expression of bacterial β -lactamase and its applications to gene technology in yeast. In *Gene Expression in Yeast* (Proceedings of The Alko Yeast Symposium June 1–3, Helsinki, Finland), pp. 73–90. Edited by M. Korhola & E. Väisänen. Helsinki: Alko.
- Innis, M. A. & Gelfand, D. H. (1990). A guide to methods and applications. In *PCR Protocols*, pp. 3–12. Edited by M. A. Innis, D. H. Gelfand, J. J. Sninsky & T. J. White. London: Academic Press.
- Keitel, T., Simon, O., Borriss, R. & Heinemann, U. (1993). Molecular and active-site structure of a *Bacillus* (1-3,1-4)- β -glucanase. *Proc Natl Acad Sci USA* **90**, 5287–5291.
- Kukuruzinska, M. A., Berg, M. L. E. & Jackson, B. J. (1987). Protein glycosylation in yeast. *Ann Rev Biochem* **56**, 915–944.
- Lehle, L. & Bause, E. (1984). Primary structural requirements for N- and O-glycosylation of yeast mannoproteins. *Biochim Biophys Acta* **799**, 246–251.
- Maley, F., Trimble, R. B., Tarentino, A. L. & Plummer, T. H. (1989). Characterization of glycoproteins and their associated oligosac-

charides through the use of endoglycosidases. *Anal Biochem* **180**, 195–204.

Mellor, J., Dobson, M. J., Roberts, N. A., Tuite, M. F., Emtage, J. S., White, S., Lowe, P. A., Patel, T., Kingsman, A. J. & Kingsman, S. M. (1983). Efficient synthesis of enzymatically active calf chymosin in *Saccharomyces cerevisiae*. *Gene* **24**, 1–14.

Mortimer, R. K. & Contopoulou, R. (1991). In *Yeast Genetic Stock Center Catalogue*, 7th edn, p. 19. Berkeley: Department of Molecular and Cellular Biology, Division of Genetics, University of California.

Narhi, L. O., Arakawa, T., Aoki, K. H., Elmore, R., Rohde, M. F., Boone, T. & Strickland, T. W. (1991). The effect of carbohydrate on the structure and stability of erythropoietin. *J Biol Chem* **266**, 23022–23026.

Nosoh, Y. & Sekiguchi, T. (1990). Protein engineering for thermostability. *Trends Biotechnol* **8**, 16–20.

Olsen, O. & Thomsen, K. K. (1989). Processing and secretion of barley (1-3,1-4)- β -glucanase in yeast. *Carlsberg Res Commun* **54**, 29–39.

Olsen, O. & Thomsen, K. K. (1991). Improvement of bacterial β -glucanase thermostability by glycosylation. *J Gen Microbiol* **137**, 579–585.

Olsen, O., Borris, R., Simon, O. & Thomsen, K. K. (1991). Hybrid *Bacillus* (1-3,1-4)- β -glucanases: engineering thermostable enzymes by construction of hybrid genes. *Mol & Gen Genet* **225**, 177–185.

Petersen, J. G. L., Kielland-Brandt, M. C., Holmberg, S. & Nilsson-Tillgren, T. (1983). Mutational analysis of isoleucine-valine biosynthesis in *Saccharomyces cerevisiae*. Mapping of *ilv2* and *ilv5*. *Carlsberg Res Commun* **48**, 21–34.

Plummer, T. H., Elder, J. H., Alexander, S., Phelan, A. W. & Tarentino, A. L. (1984). Demonstration of peptide:N-glycosidase F activity in endo- β -N-acetylglucosaminidase F preparations. *J Biol Chem* **259**, 10700–10704.

Robbins, P. W., Trimble, R. B., Wirth, D. F., Hering, C., Maley, F., Maley, G. F., Das, R., Gibson, B. W., Royal, N. & Biemann, K. (1984). Primary structure of the *Streptomyces* enzyme endo- β -N-acetylglucosaminidase H. *J Biol Chem* **259**, 7577–7583.

Roitsch, T. & Lehle, L. (1989). Structural requirements for protein N-glycosylation. *Eur J Biochem* **181**, 525–529.

Tanner, W. & Lehle, L. (1987). Protein glycosylation in yeast. *Biochim Biophys Acta* **906**, 81–99.

Tarentino, A. L., Quinones, G., Trumble, A., Changchien, L., Duceman, B., Maley, F. & Plummer, T. H. (1990). Molecular cloning and amino acid sequence of Peptide-N⁴-(N-acetyl- β -D-glucosaminyl)asparagine amidase from *Flavobacterium meningosepticum*. *J Biol Chem* **265**, 6961–6966.

Tomazic, S. J. & Klibanov, A. M. (1988). Why is one *Bacillus* α -amylase more resistant against irreversible thermoinactivation than another? *J Biol Chem* **263**, 3092–3096.

Wang, P., Hill, T. G., Wartchow, C. A., Huston, M. E., Oehler, L. M., Smith, M. B., Bednarski, M. D. & Callstrom, M. R. (1992). New carbohydrate-based materials for the stabilization of proteins. *J Am Chem Soc* **114**, 378–380.

Wilson, I. B. H., Gavel, Y., von Heijne, G. (1991). Amino acid distributions around O-linked glycosylation sites. *Biochem J* **275**, 529–534.

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