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

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Genes encoding Bacillus amyloliquefaciens (1,3-1,4)-\(\beta\)-glucanase (AMY), B. macerans (1,3-1,4)-\(\beta\)-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 thermostolerance 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 16-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)-\(\beta\)-glucanases with similar primary structure in a variable, so far unpredictable way.

**Keywords**: (1,3-1,4)-\(\beta\)-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; Fagin & 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 \(\alpha\)-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)-\(\beta\)-glucanase; MAC, B. macerans (1,3-1,4)-\(\beta\)-glucanase; H(A16-M), H(A36-M), H(A78-M), H(A107-M) and H(A152-M), hybrid (1,3-1,4)-\(\beta\)-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 & Lehlé, 1989), while O-linked glycosylation may occur at either Ser or Thr (Lehlé & Bause, 1984; Wilson et al., 1991). The synthesis of yeast N-glycans has been studied extensively (reviewed by Kukuruzinska et al., 1987; Tanner & Lehlé, 1987). An N-glycan core consisting of two N-acetylglucosamine (GlcNAc), one 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. X55939], *Bacillus amylobacterium* (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* DH5α was supplied by Gibco-BRL. Genetic transformations were done according to the procedure of Golin (1988). Transformants were incubated on solid LB medium containing 0.1% (w/v) lichenan and 100 μg 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 & Contopoulos, 1991) was carried out by electroporation (Becker & Guerente, 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.

**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 (Mellott 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), H(A16-H), H(A36-H), H(A78-H) and H(A152-H) were excised from pTZ19R-H(AX-M), H(A16-M) and H(A36-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 SnaAI, which cleaves 11 bp downstream of the codon for the amino-terminus 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 *BglI*–SpolI after ligation with the large *BglI*–Spol 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 *BglI*–BamHI fragments from these plasmids were individually cloned into the *BglI* 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 nucleotides 813–781 of the antisense strand of gene H2 (Fig. 2). The amplified DNA fragment encoding the C-
terminal half of the mature AMY was digested with EcoRV-
BamHI and cloned into EcoRV-BamHI-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 BglII-BamHI 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 BglII site down-
stream 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 µm filter and concentrated
20-fold to 50 ml by ultrafiltration using a Minitan unit
(Pharmacia) equipped with filters allowing passage of molecules
smaller than 6 kDa. Further ultrafiltration to 5 ml was accom-
plished 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 x 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
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 manufac-
turer's recommendations, except that 1–25 µl of sample containing
(1,3-1,4)-β-glucanase was incubated in 200 µl
50 mM MES, pH 6.0, 5 mM CaCl₂ and 200 µl Azo-barley glu-
can substrate. The reaction was stopped by addition of 1 ml of
precipitant [0.30 M sodium acetate, 0.02 M zinc acetate, pH 5.0,
80% (v/v) 2-mercaptoethanol]. 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 µl each containing 10 µg 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 Bio-
Labs) 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
Ecosystems 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 incu-
bated 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 (95 cm × 90 cm) equilibrated with 10% (v/v) acetic acid. The 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 (15 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 peptide 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 the 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 Hf-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 Hf treatment. Endo Hf (60 kDa) is detected in lanes 2 and 3, while PNGase F (35 kDa) is detected in lane 1.](image-url)

![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).](image-url)
N-Glycosylation and thermostability of β-glucanase

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→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 µg 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)-Y 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 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. 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 or PNGase F. Endo H 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 (Fig. 3, lane 3). Similarly, the N-glycans were also partially resistant to cleavage by Endo H 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 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- 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 μg of the purified deglycosylated H(A16-M)-Y (Fig. 8). Deglycosylation of H(A16-M)-Y using either Endo H or PNGase F caused a marked
N-Glycosylation and thermostability of β-glucanase

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 analyzed. 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 glycylated 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. 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 preprotein 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 analyzed. 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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