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Improvement of bacterial β-glucanase thermostability by glycosylation

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The relationship between enzyme stability and glycosylation was examined for two different *Bacillus* (1,3–1,4)-β-glucanases following expression of the corresponding genes in *Escherichia coli* and in *Saccharomyces cerevisiae*. Both of the (1,3–1,4)-β-glucanases secreted from yeast cells were glycosylated and a pronounced difference in the type and extent of glycosylation was observed. Thermostability analysis of the glycosylated enzymes and their unglycosylated counterparts synthesized by *E. coli* disclosed a substantially higher thermotolerance of the glycosylated enzymes. At 70 °C the half-life of the glycosylated form of *B. macerans* (1,3–1,4)-β-glucanase was 26 min, as compared to 10 min for the unglycosylated form of the enzyme. Using the same conditions, the half-life of the *B. amyloliquefaciens–B. macerans* hybrid (1,3–1,4)-β-glucanase was 5 min for the unglycosylated enzyme and about 100 min when the enzyme was glycosylated.

Introduction

Many proteins exhibit changes in conformation and stability following substitution of individual amino acid residues. In recent years several attempts have been made to utilize such alterations for construction of improved versions of biologically active proteins to widen their range of applications. Introduction of changes that lead to increased protein thermostability have attracted much interest (reviewed by Nosoh & Sekiguchi, 1990). Among the approaches used to accomplish this are (i) introduction of disulphide bonds, (ii) substitution of specific residues in order to increase α-helical stability, and (iii) substitution of residues involved in non-covalent interactions and folding patterns. Although there is circumstantial evidence that glycan moieties contribute to enhanced protein stability and may protect some proteins against proteolytic attack (Olden *et al.*, 1985; Gu *et al.*, 1989), the effect on thermostability of adding glycan groups to normally unglycosylated enzymes has not been systematically investigated. In yeast, similar to the situation in higher eukaryotes, *N*-linked glycosylation may occur at the Asn residue of the sequence Asn-Xxx-Ser/Thr while *O*-linked glycosylation occurs at either Ser or Thr (Innis, 1989). In the work described here, *Bacillus* (1,3–1,4)-β-glucanases (EC 3.2.1.73) that catalyse the cleavage of (1,4)-β-linkages of 3-*O*-substituted β-D-glucanopyranosyl residues were used as a paradigm to investigate the influence of glycosylation on enzyme thermostability. The (1,3–1,4)-β-glucanase gene from *B. macerans* and a *B. amyloliquefaciens–B. macerans* hybrid (1,3–1,4)-β-glucanase gene were expressed in both *Escherichia coli* and *Saccharomyces cerevisiae* host cells. Biochemical characterization of the mature enzymes purified from the cell culture supernatants demonstrated the beneficial effect of the addition of glycan groups to these prokaryotic enzymes when synthesized in a eukaryotic host capable of performing glycosylation.

Abbreviations: AMY, *B. amyloliquefaciens* (1,3–1,4)-β-glucanase; H1, *B. amyloliquifaciens–B. macerans* hybrid (1,3–1,4)-β-glucanase; MAC, *B. macerans* (1,3–1,4)-β-glucanase; endo H, endo-β-*N*-acetylglucosaminidase H; IEF, isoelectric focusing; PCR, polymerase chain reaction; TMS, per-*O*-trimethylsilyl.

Methods

Organisms, media, growth conditions and transformation procedures. E. coli DH5α [F⁻ λ-*recA1 endA1 hsdR17* (r_K^- m_K^+), Δ(*lacZYA–argF*)U169 φ80d*lacZ*ΔM15 *supE44 thi-1 gyrA96 relA1*] was supplied by Gibco-BRL. Transformants were incubated on solid medium containing 0·2% (w/v) lichenan. Staining with 0·1% (w/v) Congo Red revealed a clearing zone around colonies secreting recombinant (1,3–1,4)-β-glucanase. Transformants were grown at 37 °C for 3 d in standard minimal medium supplemented with 50 mg ampicillin l^{-1}. Genetic transformations were done according to the procedure of Golub (1988).

S. cerevisiae DBY746 (Jackson *et al.*, 1986) transformants secreting recombinant *Bacillus* (1,3–1,4)-β-glucanase were grown at 30 °C for 4 d on solid SC medium (Petersen *et al.*, 1983) without leucine and containing 0·2% (w/v) lichenan. Staining with Congo Red was as described above for *E. coli* transformants. Yeast cells were grown at

30 °C for 4 d in SC medium without leucine. Genetic transformations were done according to Ito *et al.* (1983), using lithium acetate to induce competence.

Preparation and analysis of DNA. Plasmid DNA (miniprep DNA) was isolated from *E. coli* as described by Hattori & Sakaki (1986). Standard techniques for DNA isolation and agarose gel electrophoresis were used (Sambrook *et al.*, 1989). Plasmid DNA was digested with appropriate restriction endonucleases, and the resulting fragments were separated on 1% (w/v) agarose gels. The fragments of interest were excised and the DNA recovered from the agarose by using Geneclean (Bio101) according to the manufacturer's recommendations. DNA sequencing was performed by the procedure of Zhang *et al.* (1988), except that miniprep DNA was used as template.

Plasmid constructions. Plasmids pUC13-M and pUC13-H1, encoding *B. macerans* (MAC) (1,3–1,4)-β-glucanase and a *B. amyloliquefaciens–B. macerans* hybrid (H1) (1,3–1,4)-β-glucanase, respectively, have been described (Borriss *et al.*, 1989). The (1,3–1,4)-β-glucanase genes were excised from pUC13-M as a *DraI–Hin*dIII fragment and from pUC13-H1 as a *BsmI–SphI* fragment. Following T4 DNA polymerase treatment to produce blunt ends, the DNA fragments were cloned into the filled-in *Bgl*II site downstream from the phosphoglycerate kinase promoter of the yeast expression vector pMA91 (Mellor *et al.*, 1983). In order to obtain efficient secretion of the hybrid enzyme from yeast cells, the *B. amyloliquefaciens* (AMY) (1,3–1,4)-β-glucanase signal peptide coding region was exchanged with the corresponding region from the MAC (1,3–1,4)-β-glucanase gene. A synthetic 99 bp oligonucleotide encoding the complete MAC (1,3–1,4)-β-glucanase signal peptide plus five NH_2-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, was cloned into pUC13 linearized with *Sma*I. The small *Bam*HI–*Hin*dIII fragment was exchanged with the 680 bp *Sau*3AI–*Hin*dIII hybrid gene fragment of pUC13-H1 giving plasmid pUC13-MH1. This plasmid was digested with *Bgl*II/*Bam*HI and the small fragment containing the (1,3–1,4)-β-glucanase gene was cloned into pMA91 linearized with *Bgl*II.

Site-directed mutagenesis. Oligonucleotide site-directed mutagenesis using PCR was performed essentially as described by Kamman *et al.* (1989) and outlined in Fig. 1. The MAC (1,3–1,4)-β-glucanase promoter and coding region of pUC13-M served as template. The 5′ amplification primer used was a 17 base M13/pUC direct sequencing primer. The 3′ amplification primer, 27 bases long with one base mismatch to the template, was used to introduce an AAC codon for amino acid no. 62, thereby causing the substitution of a tyrosyl residue by an asparaginyl residue in the synthesized heterologous protein. The amplified 409 bp fragment was mixed with a partially overlapping 784 bp *Dra*I–*Hin*dIII fragment of plasmid pUC13-M and a second PCR was carried out using the M13/pUC direct sequencing primer and a 27 base primer complementary to the 3′ non-coding region of the gene and including the *Hin*dIII site. Following *Eco*RI/*Hin*dIII digestion and agarose gel electrophoresis the amplified and mutated gene was isolated as an 860 bp fragment and cloned into pUC13 digested with the same enzymes. The resulting plasmid was named pUC13-M-Y62N, and the mutation was confirmed by DNA sequence analysis. No other mutations were found. For expression in yeast the (1,3–1,4)-β-glucanase coding region was excised with *Dra*I/*Hin*dIII, treated with T4 DNA polymerase and cloned into the filled-in *Bgl*II site of pMA91.

Enzyme purifications. The cell-free culture fluid from transformed *E. coli* cells secreting recombinant (1,3–1,4)-β-glucanase was concentrated 40-fold by ultrafiltration to 100 ml, dialysed against 20 mM-sodium acetate (pH 5·0), 1 mM-$CaCl_2$, and applied to a CM-Sepharose CL-4B column (10 cm × 2·6 cm). Bound proteins were eluted with 50 mM-sodium acetate (pH 5·0), 50 mM-NaCl, 5 mM-$CaCl_2$. The MAC (1,3–1,4)-β-glucanase was eluted in one activity peak, whereas the H1

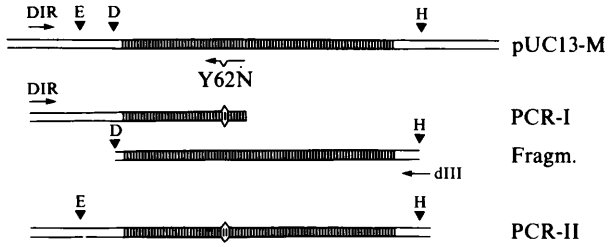

Fig. 1. Site-directed mutagenesis. Plasmid pUC13-M containing the MAC (1,3–1,4)-β-glucanase gene cloned in pUC13 served as template for a PCR reaction with an M13/pUC direct sequencing primer (DIR) and a mutagenic primer (Y62N). The resulting product (PCR-I) was mixed with an overlapping gene fragment (Fragm.). A second PCR using DIR and a primer (dIII) complementary to the 3′ end of the gene and including a *Hin*dIII recognition sequence gave rise to product PCR-II which was digested with *Eco*RI/*Hin*dIII and cloned in pUC13. All of four sequenced clones contained the mutation. E, D and H denote positions of *Eco*RI, *Dra*I and *Hin*dIII restriction sites, respectively. The coding region of the gene is schematically shown with vertical lines.

(1,3–1,4)-β-glucanase was eluted in two separate activity peaks. Proteins were then concentrated by ultrafiltration and further purified by gel filtration on a Sephacryl S-200HR column (70 cm × 2·5 cm). Fractions containing pure (1,3–1,4)-β-glucanase, as determined by SDS-PAGE, were used for further analysis. The yield of purified enzyme was 5–25 mg (1 culture medium)$^{-1}$.

Recombinant (1,3–1,4)-β-glucanase secreted by yeast cells was purified as described by Olsen & Thomsen (1989). The yield of purified enzyme was 2–5 mg (1 culture medium)$^{-1}$.

The purified enzymes were subjected to automated NH_2-terminal sequence analysis (Borriss *et al.*, 1989). Protein concentration was determined using the method described by Bradford (1976).

Enzyme assays and kinetic analyses. All assays were performed in duplicate. For determination of temperature optima for activity, 10 min incubations were carried out at temperatures over the range 25–85 °C. The enzymes were dissolved in 50 mM-sodium acetate (pH 6·0), 5 mM-$CaCl_2$ in the presence of 0·005 mg bovine serum albumin ml^{-1}. (1,3–1,4)-β-Glucanase activity was determined by measuring the amount of reducing sugars released due to enzymic hydrolysis of lichenan (Miller, 1959). One unit of enzyme activity is defined as the enzyme quantity producing 1 μmol reducing sugar (as glucose equivalents) min^{-1} at 50 °C under the conditions described above.

For determination of thermal stability, enzyme preparations in 50 mM-sodium acetate (pH 6·0), 10 mM-$CaCl_2$, were incubated for 60 min at 70 °C. The enzyme concentration was 0·1 mg ml^{-1}. Samples of 0·1 ml were taken periodically and immediately diluted into 0·4 ml ice-cold incubation buffer. From these dilutions, the percentage of remaining enzyme activity relative to the amount at 0 min was determined (McCleary, 1988).

Endo H digestion. Purified glycoproteins were digested with endo H (Boehringer Mannheim) according to the manufacturer's recommendations.

PAGE and electrofocusing. Samples for SDS-PAGE were prepared as described by Borriss *et al.* (1989) and separated on 8–18% (w/v) acrylamide gels. Proteins were stained with Coomassie Blue R250.

IEF was performed on agarose isoelectric focusing gels (FMC Corp., IsoGel; pH 3–10) according to the manufacturer's recommendations. Proteins were stained with Coomassie Blue R250.

Analysis of monosaccharide composition. A 1·2 mg sample of enzyme was analysed in parallel with an aliquot of a standard monosaccharide

mixture and a blank sample essentially as described by White & Kennedy (1989) and Parekh *et al.* (1987). The procedure involves the use of anhydrous methanolic HCl to liberate monosaccharides as the 1-*O*-methyl derivatives followed by *N*-acetylation of any available amino group and, as the final step, the conversion of individual monosaccharides into TMS-methyl glycosides. The TMS-methyl glycosides were analysed by mass spectrometry in conjunction with gas–liquid chromatography, and compared with standard reference TMS-methyl glycosides (see Fig. 4). Quantification of the individual TMS-methyl glycosides was achieved by using an internal standard (scyllo-inositol), and from the relative molar response factors of individual TMS-methyl glycosides as calculated from the standard monosaccharide mixture.

Results and Discussion

Expression and glycosylation

The MAC (1,3–1,4)-β-glucanase gene and the gene encoding the hybrid H1 (1,3–1,4)-β-glucanase (Fig. 2) were both expressed in *E. coli* cells using their *Bacillus* promoters (Borriss *et al.*, 1989). Analysis by SDS-PAGE, showed that both of the secreted enzymes had an apparent molecular mass of about 24 kDa (Fig. 3*a*). Automated NH_2-terminal amino acid sequencing revealed that the H1 (1,3–1,4)-β-glucanase which was eluted first from the CM-Sepharose column contained a blocked terminus, probably due to a non-enzymic conversion from Gln to pyroglutamic acid (Yuuki *et al.*, 1989). The NH_2-terminal residue of the enzyme eluted in the second peak was Gln followed by eight amino acids, Thr-Gly-Gly-Ser-Phe-Phe-Glu-Pro, identical to the NH_2-terminal residues of AMY (1,3–1,4)-β-glucanase. Sequencing of the purified MAC (1,3–1,4)-β-glucanase revealed sequences identical to the native *B. macerans* enzyme. Accordingly, the heterologous *Bacillus* signal peptides were recognized and correctly processed by the *E. coli* cells.

Similarly, the two (1,3–1,4)-β-glucanase genes were expressed in yeast cells under the control of the phosphoglycerate kinase promoter (Mellor *et al.*, 1983). Correctly processed MAC (1,3–1,4)-β-glucanase was obtained from the culture medium, but the yield of H1 (1,3–1,4)-β-glucanase was too low for purification and analysis. Since it has been shown that specific and correct cleavage by the yeast signal peptidase is crucial for high-level secretion of foreign proteins (Olsen & Thomsen, 1989), the DNA segment encoding the *B. amyloliquefaciens* signal peptide of the H1 (1,3–1,4)-β-glucanase gene was replaced by a synthetic oligonucleotide encoding the secretion signal sequence of MAC (1,3–1,4)-β-glucanase. When expressed in yeast cells this manipulated gene directed synthesis and secretion of H1 (1,3–1,4)-β-glucanase in a quantity similar to the amount of enzyme obtained with the *B. macerans*-derived gene. Analysis showed that the NH_2-terminal amino acid

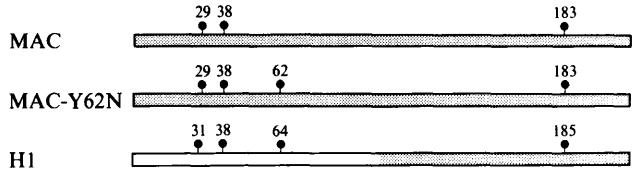

Fig. 2. Schematic representation of the enzymes used in this study. The *Bacillus macerans* (1,3–1,4)-β-glucanase (MAC) and a Tyr^{62}→Asn mutant enzyme thereof (MAC-Y62N) both consist of 212 amino acids whereas the hybrid enzyme (H1) consists of 214 amino acids, 107 from AMY (1,3–1,4)-β-glucanase and 107 from MAC (1,3–1,4)-β-glucanase. The enzymes are displayed horizontally with the left and right ends corresponding to the NH_2- and COOH-termini of the mature enzymes. The stippled portions of the proteins denote amino acid sequences derived from MAC whereas the white part of the H1 enzyme originates from AMY. The location of potential signals for Asn-linked glycosylation (Asn-Xxx-Ser/Thr) are marked with filled circles and correspond to the residues Asn^{29}, Asn^{38}, Asn^{183} of MAC, Asn^{31}, Asn^{38}, Asn^{64}, Asn^{185} of H1, and Asn^{29}, Asn^{38}, Asn^{62}, Asn^{183} of MAC-Y62N. The signal sequence of all enzymes was from the MAC (1,3–1,4)-β-glucanase.

sequence of H1 (1,3–1,4)-β-glucanase was identical to that of native AMY (1,3–1,4)-β-glucanase, with no indication of heterogeneity at the NH_2-terminus of the secreted protein. The (1,3–1,4)-β-glucanases synthesized by yeast were analysed by SDS-PAGE (Fig. 3*a*). MAC (1,3–1,4)-β-glucanase had an apparent molecular mass of 33 kDa and appeared as a slightly heterogeneous product while the H1 (1,3–1,4)-β-glucanase migrated as a sharp band corresponding to a 24 kDa polypeptide like native *Bacillus* (1,3–1,4)-β-glucanases. Both enzymes obtained from yeast were treated with endo H, which cleaves high-mannose, Asn-linked carbohydrate moieties from glycoproteins, leaving only an *N*-acetylglucosamine linked to the Asn residue (Maley *et al.*, 1989). After this treatment the enzymes were analysed by SDS-PAGE (Fig. 3*a*). MAC (1,3–1,4)-β-glucanase migrated as a distinct band with an apparent molecular mass of about 24 kDa and was indistinguishable from the enzymes obtained from *E. coli*, indicating that the increase in molecular mass of the yeast-secreted enzyme was caused by heterogeneous *N*-glycosylation.

Endo H treatment of H1 (1,3–1,4)-β-glucanase had no effect on the apparent molecular mass, but preliminary analysis of the enzymic properties indicated strongly that the H1 (1,3–1,4)-β-glucanase from yeast differed from the enzyme synthesized by *E. coli*. It was anticipated that this difference might be caused by addition of glycan moieties to the hydroxyl groups of Ser and Thr residues. H1 (1,3–1,4)-β-glucanase secreted by yeast was therefore subjected to an analysis for the identification of monosaccharides (Fig. 4). The relative molar content of monosaccharides was: *N*-acetylglucosamine 1·0, mannose 4·9, galactose 2·1, glucose 5·5. The relative content of mannose and *N*-acetylglucosamine indicated the

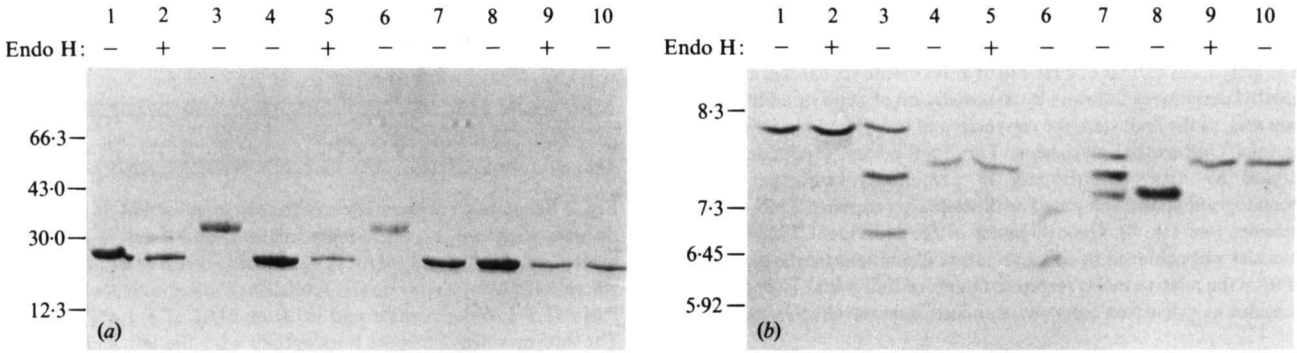

Fig. 3. Analysis of recombinant (1,3–1,4)-β-glucanases. (*a*) Analysis by SDS-PAGE on 8–18% acrylamide gradient gels. Proteins were stained with Coomassie Blue R250. The positions of the molecular mass markers in kDa are indicated to the left. The purified MAC (1,3–1,4)-β-glucanase secreted from *E. coli* (lane 1) and from yeast (lanes 2 and 3), MAC-Y62N (1,3–1,4)-β-glucanase secreted from *E. coli* (lane 4) and from yeast (lanes 5 and 6), and H1 (1,3–1,4)-β-glucanase secreted from *E. coli* [lane 7 (unblocked NH_2-terminus) and lane 8 (blocked NH_2-terminus)] and yeast (lanes 9 and 10) were either treated (+) or not treated (−) with endo H. (*b*) Isoelectric point. IEF was performed on agarose isoelectric focusing gels. Samples were applied in the same order as in (*a*). The positions of the pI markers are indicated to the left.

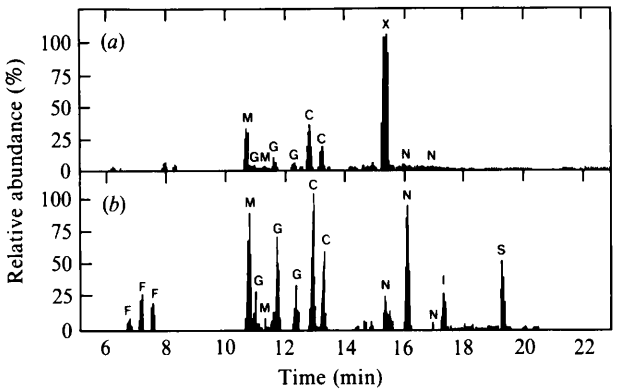

Fig. 4. Monosaccharide composition analysis of yeast-secreted H1 (1,3–1,4)-β-glucanase. The GLC profile for the TMS-methyl glycosides derived from the standard monosaccharides mannose (M), galactose (G), glucose (C), *N*-acetylglucosamine (N), fucose (F), scyllo inositol (I), and sialic acid (S) is shown in (*b*), and the comparable chromatogram of the TMS-methyl glycosides from the glycoprotein H1 is shown in (*a*). The monosaccharides in the eluted peak fractions were identified by mass spectrometry (not shown). The majority of the material eluted at 15·4 min (labelled X) was found not to be saccharide.

presence of oligomannose or hybrid-type oligosaccharides in H1 (1,3–1,4)-β-glucanase from yeast, whereas the presence of galactose opens the possibility of either *O*-linked or hybrid-type oligosaccharides. The presence of glucose is most likely due to non-covalently associated glucose originating from the culture medium, but may also indicate the presence of glucose-containing oligosaccharide chains. Since it has been suggested that in yeast the addition of an *N*-linked oligosaccharide typically corresponds to an increase in apparent molecular mass of about 3 kDa (Melnick *et al.*, 1990), it is most likely that the carbohydrates found in H1 (1,3–1,4)-β-glucanase from yeast represent *O*-linked oligosaccharide side chains. However, detection of *N*-acetylglucosamine may be an indication of low-molecular-mass *N*-glycosyl groups conjugated to the peptide chain.

The primary structures of the MAC- and H1 (1,3–1,4)-β-glucanases are highly homologous, with the COOH-terminal halves of the enzymes being identical. In the NH_2-terminal halves 71% of the homologous positions are occupied by identical amino acids. The H1 (1,3–1,4)-β-glucanase has an additional *N*-glycosylation consensus sequence Asn^{64}-Arg^{65}-Ser^{66} when compared to the sequence Tyr^{62}-Arg^{63}-Ser^{64} in the MAC (1,3–1,4)-β-glucanase (Fig. 2). To determine whether the absence of one glycosylation site may influence processing or lack of processing of other glycosylation sites, a mutation was introduced in the MAC (1,3–1,4)-β-glucanase gene causing a Tyr^{62}→Asn amino acid substitution. The mutated gene was expressed in yeast cells. In SDS-PAGE the mutant enzyme MAC-Y62N (1,3–1,4)-β-glucanase co-migrated with MAC (1,3–1,4)-β-glucanase with an apparent molecular mass of 33 kDa, showing that both enzymes were glycosylated to the same extent (Fig. 3*a*). This suggests that, at least in the mutant enzyme, not all of the potential sites for *N*-glycosylation are utilized. Like those of MAC (1,3–1,4)-β-glucanase, the glycosyl groups of MAC-Y62N (1,3–1,4)-β-glucanase were endo H sensitive. Comparison of the glycosylation pattern of H1 (1,3–1,4)-β-glucanase with that of MAC- and MAC-Y62N (1,3–1,4)-β-glucanases suggests that varying folding and tertiary structures of the polypeptides during passage through the yeast secretory apparatus mediate the observed differences. This is supported by the recent finding that the effect of protein environment is crucial in determining the relative activities of glycan-processing enzymes in yeast cells (Flores-Carreon *et al.*, 1990). Conformational analysis of the (1,3–1,4)-β-glucanases may disclose the structural basis for the observed differences in glycosylation.

Upon IEF the amino-terminal blocked form of H1 (1,3–1,4)-β-glucanase appeared as a single stainable protein band whereas the unblocked form was separated into three different isoforms (Fig. 3*b*). One of the isoforms had the same pI as the blocked enzyme. H1 (1,3–1,4)-β-glucanase secreted by yeast was seen as a single band after IEF with a pI value higher than the blocked form secreted by *E. coli*. Endo H treatment of H1 (1,3–1,4)-β-glucanase did not alter the pI of the enzyme, also suggesting that the carbohydrates are *O*-linked oligosaccharide chains. *E. coli*-secreted MAC (1,3–1,4)-β-glucanase and the mutant MAC-Y62N (1,3–1,4)-β-glucanase both migrated to a single position during IEF. The mutant enzyme had a lower pI value than the wild-type, which is in agreement with the $Tyr^{62}\rightarrow Asn$ substitution. IEF of the corresponding enzymes secreted from yeast cells revealed three different and distinct glycoforms (same polypeptide, but oligosaccharides differing in either sequence or disposition). The presence of distinct glycoforms is consistent with the notion that attached glycan groups may alter the physical character of a protein, and it indicates that there are differences either in the number of *N*-glycosylation sites utilized or in the extent of glycan decoration at each site (Parekh *et al.*, 1987). Treatment of the glycosylated enzymes with endo H converted the three glycoforms to single bands displaying pI values similar to the values observed for the corresponding enzymes from *E. coli*.

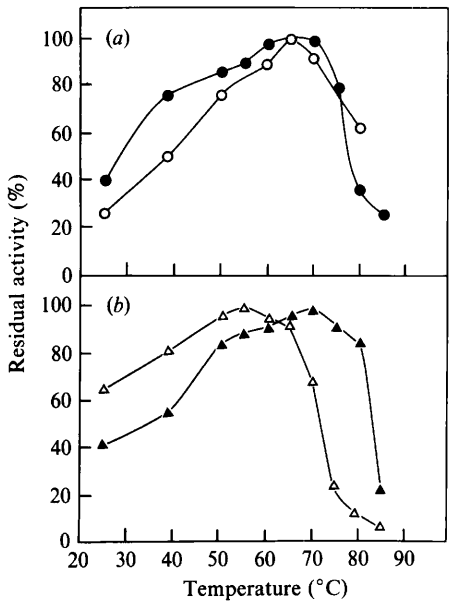

Fig. 5. Temperature dependence of activity. (1,3–1,4)-β-Glucanases secreted from *E. coli* and from yeast were incubated for 10 min at the indicated temperatures. (1,3–1,4)-β-Glucanase activity was determined by measuring the amount of reducing sugars released due to enzymic hydrolysis of lichenan. The percentage of enzyme activity relative to the activity at the optimal temperature was calculated for MAC (1,3–1,4)-β-glucanases (*a*) and H1 (1,3–1,4)-β-glucanases (*b*) secreted from *E. coli* (open symbols) and from yeast (filled symbols). Each point represents the mean of two independent measurements. The variation between pairs of measurements was always less than 10%.

Biochemical characterization and thermostability

Experiments with purified (1,3–1,4)-β-glucanase synthesized by *E. coli* and yeast showed that glycosylation may cause changes in enzyme properties. The specific activities at 50 °C were reduced from 1180 to 450 units mg^{-1} for the MAC (1,3–1,4)-β-glucanase and from 3690 to 1940 units mg^{-1} for H1 (1,3–1,4)-β-glucanase. This is not surprising, since the enzymes are normally unglycosylated, and have evolved in organisms without a glycosylation apparatus. The glycosylated enzymes showed no differences with respect to pH optimum and K_m when compared to the non-glycosylated enzyme counterparts (not shown).

In Fig. 5(*a*) the relationship between temperature and enzyme activity of the MAC (1,3–1,4)-β-glucanases is shown. Under the assay conditions described, the optimal temperature for enzyme activity was 65 °C for both the glycosylated and the unglycosylated form of the MAC (1,3–1,4)-β-glucanase but the unglycosylated form had a sharper temperature optimum. A much more pronounced difference was observed when H1 (1,3–1,4)-β-glucanases were analysed (Fig. 5*b*). The optimal temperature for enzyme activity was 55 °C for the enzyme obtained from *E. coli* but 70 °C from the enzyme from yeast cells. The temperature range for obtaining at least 80% of the optimal activity was 37–65 °C for the unglycosylated H1 (1,3–1,4)-β-glucanase but 50–80 °C for the glycosylated H1 (1,3–1,4)-β-glucanase.

Enzyme thermostability was examined by measuring the loss of enzyme activity as a function of incubation time at 70 °C and pH 6·0 (Fig. 6*a*). Under these conditions the enzymes underwent thermoinactivation which was independent of protein concentration. Assuming first-order kinetics during the first 30 min of inactivation (Fig. 6*b*) it was found that the half-life of unglycosylated MAC (1,3–1,4)-β-glucanase was about 10 min while glycosylation of the enzyme led to a half-life of 26 min. Again the effect of glycosylation was much more pronounced for H1 (1,3–1,4)-β-glucanase. The half-life of H1 (1,3–1,4)-β-glucanase from *E. coli* was about 5 min using the conditions mentioned above, whereas the half-life of glycosylated H1 (1,3–1,4)-β-glucanase was about 100 min. Since the observed increase of thermostability due to glycosylation was more pronounced for H1 (1,3–1,4)-β-glucanase, which is quantitatively less glycosylated than MAC (1,3–1,4)-β-glucanase, it is concluded that the effect of glycosylation is qualitative rather than

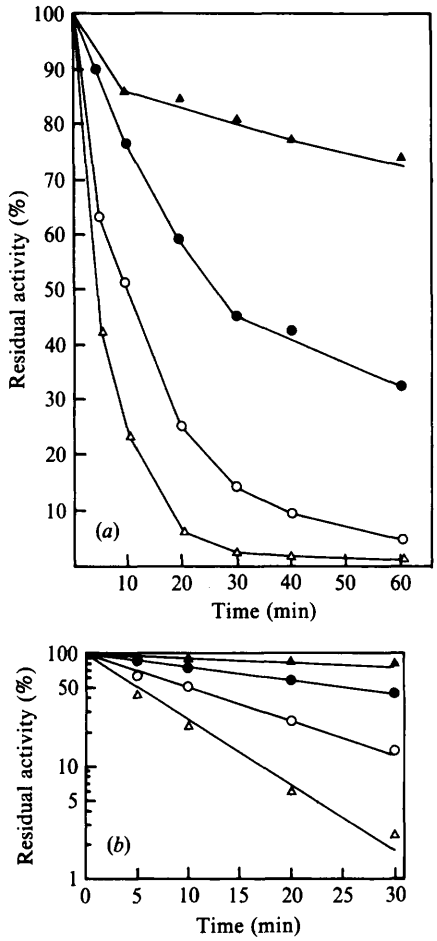

Fig. 6. Heat inactivation as a function of time. Samples of MAC (1,3–1,4)-β-glucanase secreted from *E. coli* (○) and yeast (●) and H1 (1,3–1,4)-β-glucanase secreted from *E. coli* (△) and yeast (▲) were incubated at 70 °C in 50 mM-sodium acetate (pH 6·0), 10 mM-$CaCl_2$. Samples were withdrawn at intervals up to 60 min. Individual points represent the mean of two independent measurements differing by less than 10%. (*a*) The percentage of remaining enzyme activity relative to the amount at 0 min was determined. (*b*) Semi-logarithmic plot of the data from (*a*).

quantitative. This result is supported by data showing that a bacterial cellulase synthesized in yeast was found to be heavily glycosylated and the thermostability was improved relative to the native form (Curry *et al.*, 1988), but the increase was moderate, i.e. of the same order as that observed for MAC (1,3–1,4)-β-glucanase.

The results presented thus illustrate that the position or type of glycosylation may be far more important for protein thermostability than the amount of glycan added.

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