Improvement of bacterial \( \beta \)-glucanase thermostability by glycosylation

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The relationship between enzyme stability and glycosylation was examined for two different \textit{Bacillus} (1,3-1,4)-\( \beta \)-glucanases following expression of the corresponding genes in \textit{Escherichia coli} and in \textit{Saccharomyces cerevisiae}. Both of the (1,3-1,4)-\( \beta \)-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 \textit{E. coli} disclosed a substantially higher thermostolerance of the glycosylated enzymes. At 70 °C the half-life of the glycosylated form of \textit{B. macerans} (1,3-1,4)-\( \beta \)-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 \textit{B. amyloliquefaciens}-\textit{B. macerans} hybrid (1,3-1,4)-\( \beta \)-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 \( \alpha \)-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 \textit{et al.}, 1985; Gu \textit{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, \textit{Bacillus} (1,3-1,4)-\( \beta \)-glucanases (EC 3.2.1.73) that catalyse the cleavage of (1,4)-\( \beta \)-linkages of 3-O-substituted \( \beta \)-D-glucanopyranosyl residues were used as a paradigm to investigate the influence of glycosylation on enzyme thermostability. The (1,3-1,4)-\( \beta \)-glucanase gene from \textit{B. macerans} and a \textit{B. amyloliquefaciens-} \textit{B. macerans} hybrid (1,3-1,4)-\( \beta \)-glucanase gene were expressed in both \textit{Escherichia coli} and \textit{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.

Methods

\textit{Organisms, media, growth conditions and transformation procedures.} \textit{E. coli} DH5\( \alpha \) [\( \phi^{\text{recA1 endA1 hsdR17 (rK^{-} mK^{+}) , M(lacZYA-argF)U169 \delta80dlacZAM15 supE44 thi-1 gyrA96 relA1}} \) 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)-\( \beta \)-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).

\textit{S. cerevisiae} DBY746 (Jackson \textit{et al.}, 1986) transformants secreting recombinant \textit{Bacillus} (1,3-1,4)-\( \beta \)-glucanase were grown at 30 °C for 4 d on solid SC medium (Petersen \textit{et al.}, 1983) without leucine and containing 0.2% (w/v) lichenan. Staining with Congo Red was as described above for \textit{E. coli} transformants. Yeast cells were grown at

Abbreviations: AMY, \textit{B. amyloliquefaciens} (1,3-1,4)-\( \beta \)-glucanase; H1, \textit{B. amyloliquefaciens-} \textit{B. macerans} hybrid (1,3-1,4)-\( \beta \)-glucanase; MAC, \textit{B. macerans} (1,3-1,4)-\( \beta \)-glucanase; endo H, endo-\( \beta \)-N-acetylglucosaminidase H; IEF, isoelectric focusing; PCR, polymerase chain reaction; TMS, per-O-trimethylsilyl.
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. amylophilaequicus-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-HindIII 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 BglII 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. amylophilaequicus (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 NH₂-terminal amino acids of mature AMY (1,3-1,4)-β-glucanase and containing a BglII and a BamHI site at the 5' and 3' end, respectively, was cloned into pUC13 linearized with Smal. The small BamHI–HindIII fragment was excised with the 680 bp Smal–HindIII hybrid gene fragment of pUC13-H1 giving plasmid pUC13-MH1. This plasmid was digested with BglII/BamHI and the small fragment containing the (1,3-1,4)-β-glucanase gene was cloned into pMA91 linearized with BglII.

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 base primer complementary to the 3' end of the gene and including a HindIII recognition sequence, and the resulting fragments were excised and the DNA recovered from agarose by using GeneClean (Bio101) according to the manufacturer’s recommendations. PCR analysis was performed in parallel with an aliquot of a standard monosaccharide composition. A 1.2 mg sample of enzyme was treated with Endo H digestion. Purified glycoproteins were digested with endo H (Boehringer Mannheim) according to the manufacturer’s recommendations.

PAGE and isoelectric focusing. Samples for SDS-PAGE were prepared as described by Borriss et al. (1989) and separated on 5–18% (w/v) acrylamide gels. Proteins were stained with Coomassie Blue R250.

Analysis of monosaccharide composition. A 1.2 mg sample of enzyme was assayed 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 (scyll-o-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. 3a). Automated NH2-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 NH2-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 NH2-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 peptide is crucial for high-level secretion of foreign proteins (Olsen & Thomsen, 1989), the DNA segment encoding the B. amylopliquefaciens 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 NH2-terminal amino acid 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 NH2-terminus of the secreted protein. The (1,3-1,4)-β-glucanases synthesized by yeast were analysed by SDS-PAGE (Fig. 3a). 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-acetylglycosamine linked to the Asn residue (Maley et al., 1989). After this treatment the enzymes were analysed by SDS-PAGE (Fig. 3a). 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-acetylglycosamine 1.0, mannose 4.9, galactose 2.1, glucose 5.5. The relative content of mannose and N-acetylglycosamine 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 NH₂-terminus) and lane 8 (blocked NH₂-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.

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 NH₂-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 Asn⁶⁴-Arg⁶⁵-Ser⁶⁶ when compared to the sequence Tyr⁶²-Arg⁶³-Ser⁶⁴ 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 Tyr⁶²→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. 3a). 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. 3b). 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 Tyr62→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.

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 Kᵣ 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. 5b). 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. 6a). 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. 6b) 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
(1,3-1,4)-β-glucanase secreted from incubated at 70 °C in than 10%.

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-CaCl₂. 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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References


