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Differences in the thermostabilities of barley (1→3,1→4)-β-glucanases are only partly determined by *N*-glycosylation

Danny N.P. Doan and Geoffrey B. Fincher

Department of Biochemistry, La Trobe University, Bundoora, Victoria 3083, Australia

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Barley (1→3,1→4)-β-glucan 4-glucanohydrolase (EC 3.2.1.73) isoenzyme EII carries 4% by weight carbohydrate and is more stable at elevated temperatures than isoenzyme EI, which has no associated carbohydrate. The relationship between carbohydrate content and thermostability has been investigated by treatment of the two isoenzymes with *N*-glycopeptidase F (EC 3.5.1.52). Removal of carbohydrate from isoenzyme EII results in a decrease in the enzyme's thermostability, but treatment of isoenzyme EI with the *N*-glycopeptidase F has no effect. In addition, removal of a single *N*-glycosylation site in isoenzyme EII (Asn^{190}-Ala-Ser) by site-directed mutagenesis of the corresponding cDNA led to a reduction in thermostability, while the introduction of this site into isoenzyme EI enhanced stability. We conclude that *N*-glycosylation of Asn^{190} enhances the stability of isoenzyme EII at elevated temperatures, but that other factors related to their primary structures also contribute to the differences in thermostabilities of the barley (1→3,1→4)-β-glucanases.

Baculovirus; (1→3,1→4)-β-Glucanase; *N*-Glycosylation; Protein engineering; Thermostability

1. INTRODUCTION

The (1→3,1→4)-β-glucanases (EC 3.2.1.73) of barley (*Hordeum vulgare*) catalyse the hydrolysis of cell wall (1→3,1→4)-β-glucans and are detected in germinating grain and in growing vegetative tissues [1–4]. There are two (1→3,1→4)-β-glucanase isoenzymes, designated isoenzymes EI and EII, in barley and these are encoded by separate genes [2,5]. The genes and cDNAs for both isoenzymes have been characterized and reveal that the mature enzymes share more than 92% positional identity at the amino acid level [2,6–8].

Although the substrate specificities of the two isoenzymes are identical, they have different isoelectric points, different apparent M_r values on gel electrophoresis and carry different amounts of associated carbohydrate [5,9]. Thus, isoenzyme EII has approximately 4% by weight carbohydrate and an apparent M_r of 32,000, while isoenzyme EI has no associated carbohydrate (P.B. Høj, personal communication) and an apparent M_r of 30,000 [10,11]. The detection of *N*-acetylglucosamine residues during amino acid analysis of isoenzyme EII suggested that at least part of the carbohydrate was *N*-linked [5]. Isoenzyme EII has one potential *N*-glycosylation site (Asn^{190}-Ala-Ser) [2,6], whereas at the same position in isoenzyme EI the sequence is Thr^{190}-Ala-Ser; the amino acid substitution arises from a C⇌A nucleotide substitution in position 2 of the Asn/Thr codon [2]. In addition, both isoenzymes have an Asn^{178}-Pro-Ser sequence, which is unlikely to be glycosylated because of the distorting influence of the proline residue [12].

Another important difference between the barley (1→3,1→4)-β-glucanase isoenzymes is the much greater stability of isoenzyme EII at elevated temperatures, either in the purified form or in unpurified extracts of germinated grain [5,13–14]. In view of the commercial importance of (1→3,1→4)-β-glucanase thermostability in the malting and brewing industries [15,16] and the possibility that the different glycosylation patterns of the two isoenzymes might be responsible for the observed differences in the stabilities of isoenzymes EI and EII, we have used two approaches to examine the effects of *N*-glycosylation on thermostability. Firstly, carbohydrate has been removed from isoenzyme EII with *N*-glycopeptidase F, an enzyme which specifically hydrolyzes the linkage between asparagine and the first *N*-acetylglucosamine residue of *N*-linked carbohydrate chains [17,18] and releases the entire carbohydrate moiety from the glycoprotein. Secondly, the two isoenzymes have been modified by oligonucleotide-based, site-directed mutagenesis of the (1→3,1→4)-β-glucanase cDNAs [2], using the polymerase chain reaction (PCR). Thus, a potential *N*-glycosylation site (Asn^{190}-Ala-Ser) has been engineered into isoenzyme EI and the Asn^{190}-Ala-Ser site of isoenzyme EII has been converted to Thr^{190}-Ala-Ser, thereby removing the *N*-glycosylation site from isoenzyme EII. Mutant isoenzymes EI and EII

Correspondence address: G.B. Fincher, Department of Biochemistry, La Trobe University, Bundoora, Victoria 3083, Australia. Fax: (61) (3) 4792467.

were purified following expression of the modified cDNAs in insect cells. Measurement of the thermostabilities of the deglycosylated enzymes and the mutant isoforms indicated that although *N*-glycosylation patterns of barley (1→3,1→4)-β-glucanases contribute to the differences in their thermostabilities, additional factors are involved.

2. EXPERIMENTAL

2.1. *Materials*

Barley grains (*Hordeum vulgare* cv. Clipper) were obtained from the Victorian Institute of Dryland Agriculture, Horsham, Victoria, Australia. Barley (1→3,1→4)-β-glucan substrate was purchased from Biocon (Victoria, Australia). Bio-Gel P-60 was from Bio-Rad Laboratories (Richmond, CA, USA). Chloramphenicol, neomycin, nystatin and penicillin were from Sigma Chemical Co. (St. Louis, MO, USA). Coomassie protein assay reagent was obtained from Pierce (Rockford, IL, USA). DEAE-Cellulose DE-52 was from Whatman Ltd (Maidstone, UK). CM-Sepharose CL-6B and the electrophoretic calibration kit were from Pharmacia-LKB (Uppsala, Sweden). *N*-Glycopeptidase F (PNGase F) isolated from *Flavobacterium meningosepticum* was purchased from Boehringer Mannheim GmbH (Germany). *Spodoptera frugiperda* (*Sf*9) cell culture, plasmid pVL1393 and the baculovirus *Ac*NPV were generously provided by Dr. M. Summers (Texas A&M University). *Taq* polymerase and dNTPs (dATP, dCTP, dGTP and dTTP) were from Promega Corporation (Madison, WI, USA). All restriction endonucleases were from Bethesda Research Laboratories and Life Technologies Inc. (MD, USA). Oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer.

2.2. *Treatment of (1→3,1→4)-β-glucanases with N-glycopeptidase F*

Barley (1→3,1→4)-β-glucanase isoenzymes EI and EII were purified from germinated grain according to the method of Woodward and Fincher [5]. Purified isoenzymes (10 μg) were incubated at 37°C with 0.2 unit *N*-glycopeptidase F (specific activity 25,000 U/mg) in 20 μl 50 mM sodium acetate buffer, pH 5.5 for 2, 4 and 16 h. Controls contained either (1→3,1→4)-β-glucanase alone, or *N*-glycopeptidase F alone and were incubated for 16 h. All samples were frozen at −20°C for 24 h prior to the heat stability assay to ensure a constant thermal history [9].

2.3. *Assay of (1→3,1→4)-β-glucanase activity*

(1→3,1→4)-β-Glucanase activity was measured viscometrically in an Ostwald viscometer (Cannon 100, Cannon Instrument Co., State College, PA, USA), using 0.5% w/v barley β-glucan in 50 mM sodium acetate buffer, pH 5.5 as substrate [5]. One unit of activity is defined as the change in the reciprocal of the specific viscosity (η_{sp}) per min and specific activity is expressed as $(\Delta 1/\eta_{sp})$/min/mg protein [5]. Protein was estimated by the Coomassie blue method [19] or by absorbance at 280 nm.

2.4. *Construction of plasmid vectors and mutant cDNAs*

Isoenzyme EI and EII cDNA inserts were excised from plasmids pS5 and pA3 [2] with *Eco*RI, ligated into *Eco*RI-cut pVL1393 plasmid and cloned in *E. coli* MC1061. Before high levels of expression could be obtained in the insect cell-baculovirus system, it was necessary to modify the cDNAs at both their 5′ and 3′ ends. Thus, the modified cDNAs have identical 5′-untranslated regions of 17 bp, both have the leader peptide sequence corresponding to isoenzyme EI and both have isoenzyme EII 3′-untranslated region [11]. The respective coding regions of the mature isoenzymes EI and EII were unchanged, except for a single amino acid substitution near the COOH terminus of isoenzyme EI (Ser^{305}→Asn^{305}). The modified cDNA constructs were designated pVL-EI and pVL-EII, respectively [11].

Site-directed mutagenesis of glycosylation site regions of the (1→3,1→4)-β-glucanase cDNAs was effected using the PCR-based megaprimer method [20]. Four oligonucleotides were prepared for the site-directed mutagenesis. Oligonucleotides 1 and 3 were based on nucleotide sequences of the isoenzyme EII cDNA, corresponding to amino acid sequences 133–140 and 222–215, respectively. Although these oligonucleotides are not identical to the sequence of the isoenzyme EI cDNA (3 mismatches in oligonucleotide 1, and 1 mismatch in oligonucleotide 3), they were also used successfully for the construction of the mutant isoenzyme EI cDNA. Oligonucleotides 2 and 4 were based on sequences of isoenzyme EI cDNA and isoenzyme EII cDNAs but contained a single base substitution that led to the introduction of an *N*-glycosylation site in isoenzyme EI (Thr^{190}-Ala-Ser→Asn^{190}-Ala-Ser) and the removal of the *N*-glycosylation site from isoenzyme EII (Asn^{190}-Ala-Ser→Thr^{190}-Ala-Ser). In an attempt to minimise nucleotide sequence errors introduced during the PCR [21], a 152 bp *Apa*I/*Kpn*I fragment containing the Asn^{190}/Thr^{190} region was excised from the total PCR product in each case, ligated back into the same sites of the corresponding pVL-EI or pVL-EII plasmids and checked by sequence analysis. The plasmids containing the mutant cDNAs were designated pVL-EIm and pVL-EIIm.

2.5. *Selection of recombinant viruses*

For transfections, approximately 2×10^6 cells were placed in 25 ml flasks and co-transfected with 1 μg wild-type *Ac*NPV DNA and 25 μg recombinant transfer vector containing the four (1→3,1→4)-β-glucanase cDNAs. Recombinant virus containing the appropriate cDNA was selected by a combination of limiting dilution, using DNA dot-blot hybridization or enzymic activity, and plaque purification [11,22].

2.6. *Purification of (1→3,1→4)-β-glucanases from Sf9 cell cultures*

*Sf*9 cells (5×10^7 cells) were infected with 10 ml recombinant viral solution (≅ 10^8 pfu/ml) for 1 h at room temperature and incubated essentially as described previously [11,22]. Medium was collected and centrifuged at 1,500 × *g* for 5 min to remove cell debris. Purification of the (1→3,1→4)-β-glucanase isoenzymes from the cell culture medium was carried out using fractional precipitation with ammonium sulphate, DEAE-Cellulose, CM-Sepharose and Bio-Gel P-60 gel filtration chromatography [5]. The wild-type enzymes expressed in the insect cells are designated expEI and expEII, while the mutant enzymes are referred to as expEIm and expEIIm (cf. [11]). Purification of expressed (1→3,1→4)-β-glucanase isoenzymes EII and expEIm included a final ConA-Sepharose affinity chromatography step [11].

2.7. *SDS-PAGE*

Protein samples were analyzed by electrophoresis on 12.5% SDS-polyacrylamide gels [23]. Gels were stained with Coomassie brilliant blue R-250 prepared in 20% (v/v) ethanol and 7% (v/v) glacial acetic acid at room temperature for 1 h, and destained in 20% (v/v) ethanol and 7 % (v/v) glacial acetic acid [10]. Molecular weight marker proteins were phosphorylase b (M_r 94,000), bovine serum albumin (M_r 67,000), ovalbumin (M_r 43,000), carbonic anhydrase (M_r 30,000), soybean trypsin inhibitor (M_r 20,100) and α-lactalbumin (M_r 14,400) (Electrophoretic Calibration Kit, Pharmacia-LKB, Sweden).

2.8. *Sequence analyses*

The dideoxynucleotide chain termination method [24] was used to sequence the boundary regions of the various plasmid constructs and to confirm the sequences of the *N*-glycosylation site regions of the mutant cDNAs after site-directed mutagenesis. Automated sequence analyses of the NH_2-terminal amino acids of purified enzymes were performed in an Applied Biosystems model 470A gas-liquid phase sequencer using trifluoroacetic acid conversion chemistry [25].

2.9. *Heat stability assay*

Aliquots of 1 μg deglycosylated or mutant isoenzymes were diluted to 50 μl with 50 mM sodium acetate buffer, pH 5.5 and incubated at temperatures ranging from 40°C to 60°C for 15 min [9]. Samples incubated at 0°C were used as controls. Residual enzyme activity was determined viscometrically with 550 μl (1→3,1→4)-β-glucan substrate.

3. RESULTS

3.1. *Treatment of (1→3,1→4)-β-glucanases with N-glycopeptidase F*

Isoenzymes EI and EII purified from germinated grain had specific activities of 313 U/mg and 415 U/mg, respectively. The identity of each isoenzyme was determined on the basis of electrophoretic mobility and NH_2-terminal amino acid sequence analysis. Isoenzyme EI has an apparent M_r of approx. 30,000 and isoenzyme EII of approx. 32,000 (Fig. 1 cf [26]).

Treatment with *N*-glycopeptidase F had no apparent effect on the mobility of isoenzyme EI during SDS-PAGE, but two bands were observed with isoenzyme EII following treatment with *N*-glycopeptidase F for 2 h or longer (Fig. 1). The mobility of the upper band in the treated isoenzyme EII preparation corresponds to the untreated isoenzyme EII while the lower band has a mobility similar to that of isoenzyme EI. Prolonged incubation of isoenzyme EII in the presence of *N*-glycopeptidase F did not increase the intensity of the lower molecular weight band. It should be noted that this lower band is not *N*-glycopeptidase F because no protein bands could be detected in control samples containing an equivalent amount of *N*-glycopeptidase F (approx. 2 ng per lane) (result not shown). These observations show that the protein with the lower apparent M_r in the *N*-glycopeptidase F digests of isoenzyme EII represents a modified form of isoenzyme EII generated by *N*-glycopeptidase F action (Fig. 1).

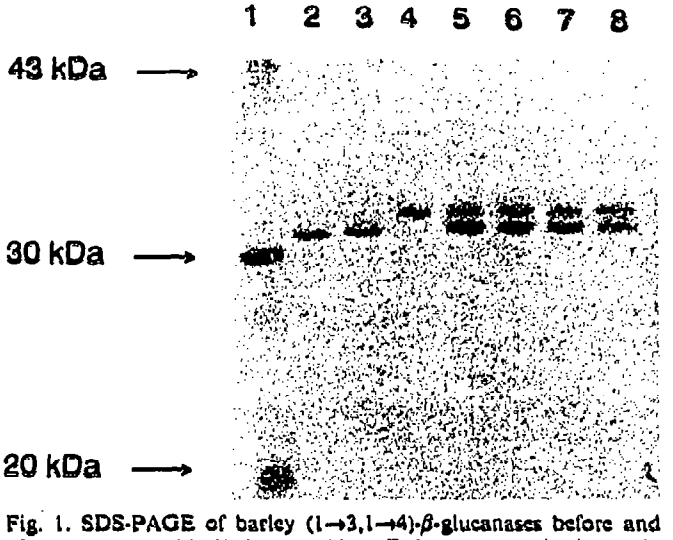

Fig. 1. SDS-PAGE of barley (1→3,1→4)-β-glucanases before and after treatment with *N*-glycopeptidase F. Lane 1, standard protein markers; lane 2, untreated isoenzyme EI (incubated in buffer without *N*-glycopeptidase F for 16 h); lane 3, *N*-glycopeptidase F-treated isoenzyme EI (incubated with *N*-glycopeptidase F for 16 h); lane 4, untreated isoenzyme EII (16 h); lane 5, *N*-glycopeptidase F-treated isoenzyme EII (2 h treatment); lane 6, *N*-glycopeptidase F-treated isoenzyme EII (4 h treatment); lane 7, *N*-glycopeptidase F-treated isoenzyme EII (8 h treatment); lane 8, *N*-glycopeptidase F-treated isoenzyme EII (16 h treatment).

3.2. *Thermostability of N-glycopeptidase F-treated enzymes*

(1→3,1→4)-β-Glucanase isoenzyme EI was relatively unstable at elevated temperatures, with 50% activity remaining after incubation at 47°C for 15 min and complete inactivation occurring at 55°C (Fig. 2). The temperatures at which 50% initial activity is retained are hereafter referred to as the 't_{50}' values. The t_{50} value of 47°C for isoenzyme EI is somewhat higher than the value of 37°C determined by Woodward and Fincher [9], possibly because freshly prepared enzyme was used in the experiments described here. Isoenzyme EI samples treated with and without *N*-glycopeptidase F have similar thermostability profiles (Fig. 2).

Isoenzyme EII was relatively more heat stable than isoenzyme EI (Fig. 2), with a t_{50} value of 52°C; this may be compared to a value of 45°C obtained previously [9]. Isoenzyme EII was completely inactivated at 60°C (Fig. 2). Treatment with *N*-glycopeptidase F reduces its thermostability to a t_{50} value of approximately 49°C (Fig. 2). However, *N*-glycopeptidase F-treated isoenzyme EII was still significantly more stable than isoenzyme EI at elevated temperatures (Fig. 2). The *N*-glycopeptidase F treatments and thermostability experiments were repeated three times and results almost identical to those shown in Fig. 2 were obtained.

3.3. *Site-directed mutagenesis of cDNAs*

The megaprimer PCR products produced from pVL-EI and pVL-EII cDNAs were of the expected size and when the *ApaI/KpnI* fragments were re-introduced into the original plasmids, nucleotide sequence analysis indicated that a single base substitution (C→A) had been generated in the second position of the codon corresponding to amino acid 190 of isoenzyme EI and hence

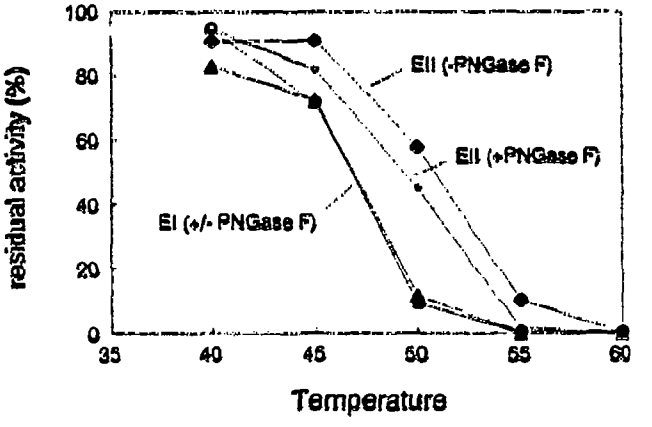

Fig. 2. Thermostability of barley (1→3,1→4)-β-glucanase isoenzymes before and after *N*-glycopeptidase F (PNGase F) treatment. Residual enzyme activity was measured after 15 min at temperatures in the range 40–60°C. Triangles indicate untreated isoenzyme EI, large circles indicate *N*-glycopeptidase F-treated isoenzyme EI, squares indicate untreated isoenzyme EII, small circles indicate *N*-glycopeptidase F-treated isoenzyme EII.

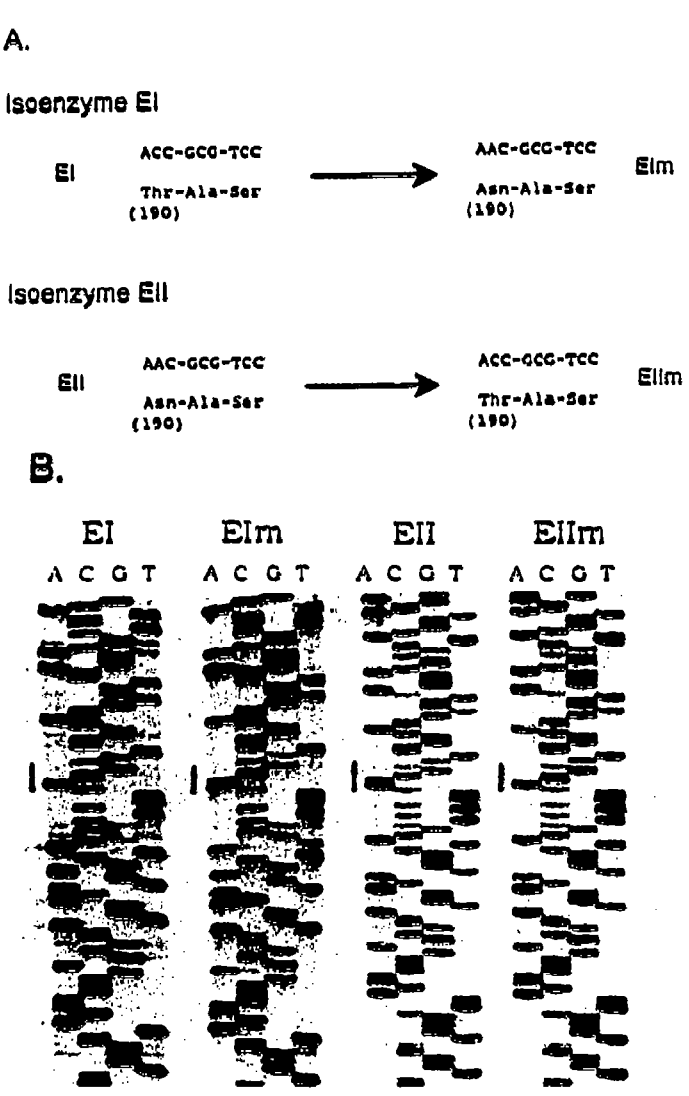

Fig. 3. Site-directed mutagenesis of cDNAs encoding barley (1→3,1→4)-β-glucanase isoenzymes EI and EII. A. Nucleotide and corresponding amino acid sequences in cDNAs for wild-type and mutant isoenzymes EI and EIm, and for isoenzymes EII and EIIm. B. Nucleotide sequence gels for (1→3,1→4)-β-glucanase isoenzymes; sidelines show positions of the codon (ACC) for Thr^{190} in isoenzymes EI and EIIm, and of the the codon (AAC) for Asn^{190} in isoenzymes EII and EIm.

that the *N*-glycosylation site (Asn^{190}-Ala-Ser) had been successfully introduced into the mutant isoenzyme EIm cDNA (Fig. 3). No sequence errors were detected in the isoenzyme EIm cDNA.

Similarly, sequence analysis confirmed the presence of the reverse substitution (A→C) in the second position of codon 190 of isoenzyme EII, indicating that the *N*-glycosylation site of the original cDNA had been removed from the mutant isoenzyme EIIm cDNA (Fig. 3). A second base substitution of C to T was detected in the wobble base position of codon 186 (Fig. 3). However, this substitution does not affect the Tyr residue at position 186, which is encoded by both TAC (isoenzyme EII cDNA) and TAT (isoenzyme EIIm cDNA).

Table 1

Purification of (1→3,1→4)-β-glucanases following expression of cDNAs in *Sf*9 insect cells

(1→3,1→4)-β-Glucanase isoenzyme	Specific activity (U/mg protein)
expEI	307
expEIm-u	754
expEIm-b	704
expEII-u	683
expEII-b	680
expEIIm	213

3.4. *Expression and purification of mutant (1→3,1→4)-β-glucanases*

The specific activities of expressed (1→3,1→4)-β-glucanase isoenzyme expEIm, which includes the 'unbound' and 'bound' ConA-Sepharose fractions (designated isoenzymes expEIm-u and expEIm-b, respectively) and expressed isoenzyme expEIIm are compared with values obtained for the wild-type enzymes expEI, expEII-u and expEII-b [11] in Table 1. Generally, the enzymes containing the potential *N*-glycosylation sites had higher specific activities (Table 1). A yield of 238 μg expEIm-u and 179 μg expEIm-b was obtained from 230 ml *Sf*9 cell cultures (1 × 10^6 cells/ml). Similarly, 100 μg expEIIm was purified from 100 ml *Sf*9 cell cultures (1 × 10^6 cells/ml). The electrophoretic mobilities of the expressed (1→3,1→4)-β-glucanase isoenzymes are compared in Fig. 4. While single protein bands of M_r 30,000 were observed for isoenzymes expEI, expEII-u [11] and expEIm-u (Fig. 4), an additional band of apparent M_r 32,000 was present in purified preparations of isoenzymes expEII-b, expEIIm and expEIm-b (Fig. 4). Each of the expressed isoenzymes were specifically recognised by the corresponding monoclonal antibodies raised against barley isoenzymes EI and EII [26] in Western blot analyses (data not shown). This indicated that neither the *N*-glycosylation site regions of the enzymes nor the N-linked carbohydrate itself are components of the epitopes recognised by the monoclonal antibodies.

3.5. *NH_2-terminal amino acid sequences*

The NH_2-terminal sequences of purified preparations of the expressed (1→3,1→4)-β-glucanases were routinely checked, both for the presence of contaminating proteins and for incorrect processing of the enzymes in the *Sf*9 insect cells [11]. Despite exhaustive washing of the column prior to chromatography, low levels of a contaminating protein that corresponds in amino acid sequence to a small fragment of jackbean concanavalin A [27] were always detected in fractions purified by affinity chromatography on Con A-Sepharose (cf. [11]). No other secondary sequences were found, except in isoenzyme expEIIm, where an estimated 25% of the protein carried 8 residues of the signal peptide (see also [11]).

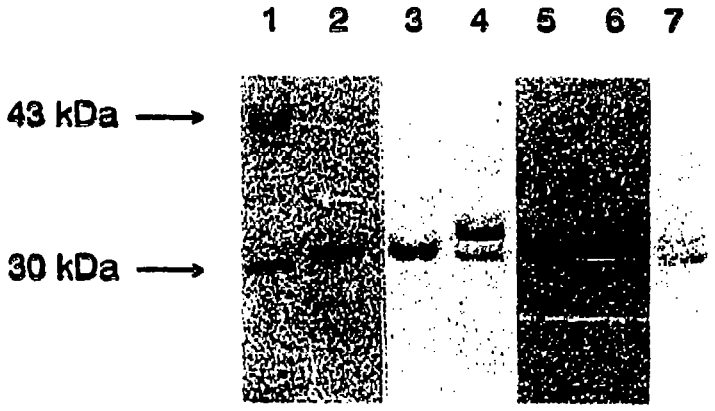

Fig. 4. SDS-PAGE of expressed (1→3,1→4)-β-glucanase isoenzymes purified from *Sf*9 insect cell cultures. Lanes 1, standard proteins; 2, expEI; 3, expEII-u; 4, expEII-b; 5, expEIm-u; 6, expEIm-b; 7, exp EIIm.

3.6. *Thermostability of mutant (1→3,1→4)-β-glucanases*

For both (1→3,1→4)-β-glucanases, the stabilities of expressed isoenzymes (Fig. 5) are very similar to those observed for the native enzymes purified from germinating barley grain (Fig. 2). However, the addition of an *N*-glycosylation site in the two mutant isoenzymes expEIm-b and expEIm-u (t_{50} = 46°C) results in a small but reproducible increase in thermostability compared with the expressed wild-type isoenzyme expEI (t_{50} = 45°C) (Fig. 5). All isoenzyme EI wild type and mutant isoforms are completely inactivated after 15 min at 52.5°C (Fig. 5). Conversely, removal of the *N*-glycosylation site in isoenzyme expEIIm (t_{50} = 49.5°C) results in a significant decrease of thermostability compared with the expressed wild-type isoenzymes expEII-u and expEII-b (t_{50} = 52°C) (Fig. 5).

4. DISCUSSION

Treatment of barley (1→3,1→4)-β-glucanase isoenzyme EI with *N*-glycopeptidase F causes no change in the electrophoretic mobility of the enzyme (Fig. 1), suggesting that this enzyme carries no accessible *N*-linked carbohydrate. On the other hand, *N*-glycopeptidase F treatment of isoenzyme EII leads to the appearance of two protein bands on SDS-PAGE (Fig. 1). The higher M_r band has the same electrophoretic mobility as native isoenzyme EII and presumably represents protein that is resistant to enzymic deglycosylation. The lower molecular weight band is probably deglycosylated isoenzyme EII. If this were so, the similarity in electrophoretic mobility of deglycosylated isoenzyme EII and native isoenzyme EI (Fig. 1) would suggest that differences in apparent M_r values observed for the two isoenzymes result from differences in carbohydrate content rather than from differences in the length of the polypeptide chains, as suggested elsewhere [5,7]. The absence of proteins of intermediate mobility would suggest that the *N*-glycopeptidase F is removing carbohydrate from one site only.

Visual comparison of band intensities in Fig. 1 indicates that the level of the smaller isoenzyme EII band in *N*-glycopeptidase F-treated samples increases to a maximum of approximately 60% of the mixture and that prolonged incubation does not release additional carbohydrate from the enzyme. It has previously been reported that *N*-glycopeptidase F treatment does not completely remove *N*-linked carbohydrate from native glycoproteins [17,18,28]. Complete deglycosylation can be achieved if the glycoprotein is denatured with detergent prior to *N*-glycopeptidase F treatment [18], but this was not pursued in the present work, where retention of enzymic activity was essential.

The apparent removal of carbohydrate from isoenzyme EII, as judged by the increase in the electrophoretic mobility of the enzyme preparation after *N*-glycopeptidase F treatment (Fig. 1), was associated with a reduction in the thermostability of isoenzyme EII to a point where the stability of the *N*-glycopeptidase F-treated preparation was placed between the native, untreated isoenzyme EI and EII preparations (Fig. 2). One might anticipate that the reduction in isoenzyme EII thermostability would be more pronounced if the deglycosylated form of the enzyme could be separated from the enzyme that appeared to be unaffected by the *N*-glycopeptidase F treatment. Attempts were made to resolve the two proteins by affinity chromatography of the *N*-glycopeptidase F digest of isoenzyme EII on Con A-Sepharose. However, since neither protein bound to the column no separation of glycosylated and deglycosylated forms could be achieved by this procedure (data not shown).

It appears therefore that *N*-glycopeptidase F treatment of barley (1→3,1→4)-β-glucanase isoenzyme EII has removed *N*-linked carbohydrate from some, but not all, of the enzyme molecules and that this is associated with a reduction in the thermostability of the enzyme.

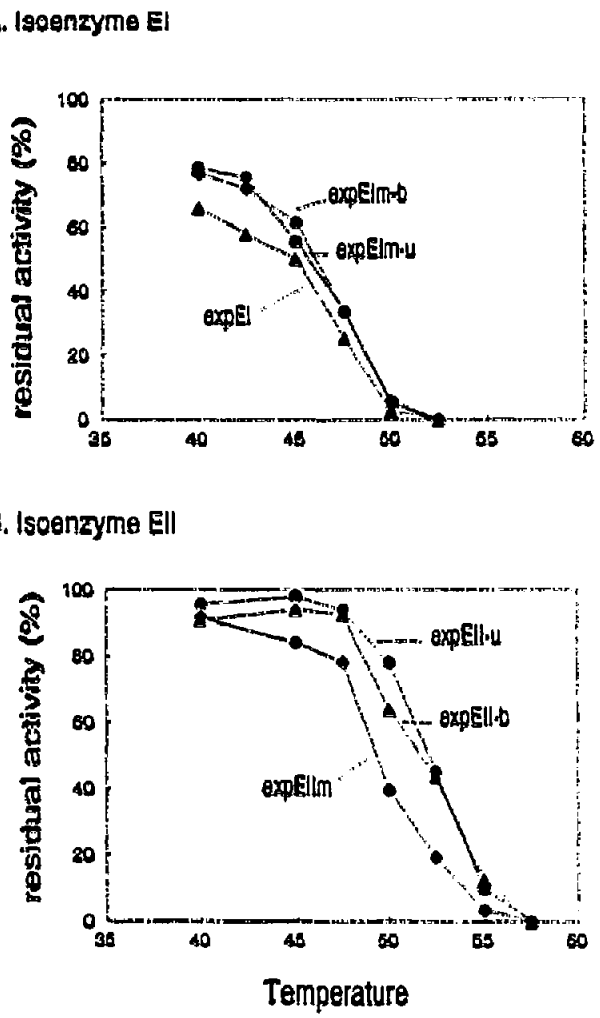

Fig. 5. Thermostabilities of purified (1→3,1→4)-β-glucanase isoenzymes expressed in *Sf*9 insect cells. A. Isoenzyme EI. Triangles indicate isoenzyme expEI, circles isoenzyme expEIm-u and squares isoenzyme expEIm-b. B. Isoenzyme EII. Squares indicate isoenzyme EIIm, circles isoenzyme expEII-u and triangles isoenzyme expEII-b.

Based on the specificity of *N*-glycopeptidase F and the amino acid sequence of the two (1→3,1→4)-β-glucanase isoenzymes deduced from cDNA clones [2], these results suggest that carbohydrate at the *N*-glycosylation consensus sequence (Asn^{190}-Ala-Ser), which is present in isoenzyme EII but not in isoenzyme EI [2], might be responsible for the higher thermostability of isoenzyme EII. To further test this possibility, 'reciprocal' mutant cDNAs of the two isoenzymes were constructed by PCR-based site-directed mutagenesis (Fig. 3). Thus, the *N*-glycosylation site of isoenzyme EII was removed and a new site was introduced into isoenzyme EI at the same position (Fig. 3). The mutant and wild-type cDNAs were successfully expressed in *Sf*9 insect cells using the baculovirus vector system [11,22] and active enzymes were purified from the culture medium. Two major products were detected in the purified enzyme preparations (Fig. 4) and these could be partially separated on ConA-Sepharose. The unbound forms have an apparent M_r of approx. 30,000 (e.g. expEIm-u and expEII-u) while the bound isoforms consist of two proteins with apparent M_r values of approx. 32,000 and 30,000 (e.g. expEIm-b and expEII-b). No significant differences in the thermostabilities of Con A-Sepharose-bound and -unbound forms are apparent (Fig. 5). This leads to the conclusion that both the Con A-Sepharose fractions of expEIm and expEII are glycosylated, but that mannose residues of the *N*-glycan chains of unbound forms are shielded or inaccessible to concanavalin A [29].

When the thermostabilities of the enzymes expressed from the wild-type and mutant cDNAs were compared, it was clear that the isoforms carrying the Asn^{190}-Ala-Ser sequence were significantly more thermostable than the corresponding isoenzymes that lacked the sequence (Fig. 5). Although the differences were relatively small (Fig. 5), they were highly reproducible and were consistent with the decreased thermostability observed when native barley isoenzyme EII was deglycosylated with *N*-glycopeptidase F (Fig. 2). Taken together, the results suggest that glycosylation of Asn^{190} is an important determinant of barley (1→3,1→4)-β-glucanase thermostability. Carbohydrate has previously been identified as a factor that can enhance protein stability, possibly by directing folding or by reinforcing intrachain crosslinking interactions [15,30].

However, it is also clear that glycosylation of the Asn^{190} residue is not the only factor that accounts for differences in the thermostability patterns between isoenzymes EI and EII (Fig. 5). Careful examination of Figs. 2 and 5 indicate that the differences in t_{50} values between isoenzymes with an Asn^{190}-Ala-Ser sequence and those without this sequence are approximately 1°C for the expressed forms of isoenzyme EI (Fig. 5A) and approximately 2.5°C for the wild-type and mutant forms of isoenzyme EII (Fig. 5B). In contrast, the difference in t_{50} values for the native isoenzymes EI and EII from germinating barley (approximately 4.5°C) is significantly larger (Fig. 2), indicating that enzymic characteristics other than *N*-glycosylation contribute to the observed differences between isoenzymes EI and EII. Other factors that might influence thermostability include *O*-linked glycosylation, although molecular mass determination by electrospray mass spectroscopy indicates that the native barley isoenzyme EI contains no carbohydrate at all (P.B. Høj, personal communication). In addition, incorrect processing of the signal peptides of expressed plant genes, leading to the presence of NH_2-terminal extensions on secreted proteins, can cause a decrease in enzyme stability [11]. Thus, the isoenzyme expEIIm preparation contained approximately 25% aberrantly processed enzyme; this may have contributed, in part, to its reduced thermostability (Fig.

5) and might explain the presence of two bands on SDS gels (Fig. 4).

However, the most likely additional explanation for the difference in (1→3,1→4)-β-glucanase thermostability lies in the primary structures of the enzymes. Although isoenzymes EI and EII both have 306 amino acids, there are 25 amino acid substitutions in the two enzymes [2] and changes to single amino acids, whether these lead to changes in charge, hydrophobicity or size of the amino acid residue, can dramatically alter protein stability [31–33].

The thermostability of the (1→3,1→4)-β-glucanases is of extreme importance during the utilization of barley in the malting and brewing industries. Malt quality, as measured by the 'malt extract' index, is highly correlated with the ability of the grain to rapidly synthesize high levels of the enzyme during germination [34]. High levels of (1→3,1→4)-β-glucanase are also desirable in the brewing process, where residual (1→3,1→4)-β-glucans in malt extracts can adversely affect wort and beer filtration and can contribute to the formation of certain hazes or precipitates in the final beer [16]. However, during malting and brewing, elevated temperatures can lead to rapid and extensive inactivation of the (1→3,1→4)-β-glucanases. Thus, less than 40% of the activity found in green malt survives commercial kilning protocols, in which the malted barley is dried at temperatures of up to 85°C [13,14]. Isoenzyme EI is completely destroyed under these conditions [14]. Moreover, any residual (1→3,1→4)-β-glucanase in the kiln-dried malt is quickly inactivated when malt extracts are heated to temperatures of up to 65°C in the mash tun [14]. The heat lability of the (1→3,1→4)-β-glucanases, coupled with their importance in alleviating problems caused by (1→3,1→4)-β-glucans in the brewery, has resulted in considerable interest in the potential for improving their thermostability [15]. It has been shown here that glycosylation of a Asn^{196} of barley (1→3,1→4)-β-glucanases contributes to increased enzyme stability. However, design of barley (1→3,1→4)-β-glucanases with greatly improved stabilities will require a more thorough understanding of the 3D structure of the enzymes.

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