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Purification of (1 → 3)-β-glucan endohydrolase isoenzyme II from germinated barley and determination of its primary structure from a cDNA clone

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Abstract

A (1 → 3)-β-D-glucan 3-glucanohydrolase (EC 3.2.1.39) of apparent M_r 32000, designated GII, has been purified from germinated barley grain and characterized. The isoenzyme is resolved from a previously purified isoenzyme (GI) on the basis of differences in their isoelectric points; (1 → 3)-β-glucanases GI and GII have pI values of 8.6 and ≥ 10.0, respectively. Comparison of the sequences of their 40 NH_2-terminal amino acids reveals 68% positional identity. A 1265 nucleotide pair cDNA encoding (1 → 3)-β-glucanase isoenzyme GII has been isolated from a library prepared with mRNA of 2-day germinated barley scutella. Nucleotide sequence analysis of the cDNA has enabled the complete primary structure of the 306 amino acid (1 → 3)-β-glucanase to be deduced, together with that of a putative NH_2-terminal signal peptide of 28 amino acid residues. The (1 → 3)-β-glucanase cDNA is characterized by a high (G + C) content, which reflects a strong bias for the use of G or C in the wobble base position of codons. The amino acid sequence of the (1 → 3)-β-glucanase shows highly conserved internal domains and 52% overall positional identity with barley (1 → 3, 1 → 4)-β-glucanase isoenzyme EII, an enzyme of related but quite distinct substrate specificity. Thus, the (1 → 3)-β-glucanases, which may provide a degree of protection against microbial invasion of germinated barley grain through their ability to degrade fungal cell wall polysaccharides, appear to share a common evolutionary origin with the (1 → 3, 1 → 4)-β-glucanases, which function to depolymerize endosperm cell walls in the germinated grain.

Introduction

Three major classes of β-glucan endohydrolases have been detected in germinated barley grains. These include (1 → 3, 1 → 4)-β-D-glucan 4-glucanohydrolase (EC 3.2.1.73) [39], (1 → 4)-β-D-glucan 4-glucanohydrolase (cellulase, EC 3.2.1.4) [22] and (1 → 3)-β-D-glucan 3-glucanohydrolase (EC 3.2.1.39) [2, 21]. Levels of cellulase are low and originate predominantly from

The nucleotide sequence reported in this article has been submitted to the EMBL/GenBank Data Bank with accession number M23548.

commensal microflora associated with the grain [22]. The component of cellulase which is of plant origin may participate in the degradation of cellulose, a minor component of endosperm cell walls [13, 22]. The $(1 \rightarrow 3, 1 \rightarrow 4)$-$\beta$-glucanases are primarily responsible for the depolymerization of $(1 \rightarrow 3, 1 \rightarrow 4)$-$\beta$-glucan, which constitutes about 70% of starchy endosperm cell walls, during endosperm mobilization [14].

$(1 \rightarrow 3)$-β-Glucanase activity is relatively high in germinating barley [2] but its physiological function remains to be demonstrated. The activity is located in the embryo of ungerminated grain but increases significantly in the endosperm during germination and is enhanced by gibberellic acid [2]. Isolated barley aleurone layers are also capable of secreting $(1 \rightarrow 3)$-β-glucanase [35].

A $(1 \rightarrow 3)$-β-glucanase has recently been purified from extracts of germinated barley [21]. The enzyme has an apparent M_r 32000, a pI of 8.6, and is designated GI. In the present work we have purified and characterized a second $(1 \rightarrow 3)$-β-glucanase, designated GII, of similar molecular weight but higher isoelectric point. A cDNA encoding the $(1 \rightarrow 3)$-β-glucanase isoenzyme GII has been isolated from a cDNA library prepared using mRNA from scutella of 2-day germinated barley. The primary sequence of the enzyme has been deduced from the cDNA and compared with amino acid sequences of other endo-β-glucanases from germinating barley grain.

Materials and methods

Plant material

Barley (*Hordeum vulgare* L. cv. Clipper) grains were surface-sterilized with 0.2% (w/v) silver nitrate for 20 min, rinsed with 0.5 M NaCl and sterile water, and immersed for 24 h in sterile water containing 100 μg/ml neomycin, 10 μg/ml chloramphenicol, 100 units/ml penicillin G and 100 units/ml nystatin. Grains were adjusted to 44% moisture content with the antibiotic solution and germinated in the dark at 25 °C for 5 days [21]. For mRNA isolation, grains were treated as described, except that after 2 days germination at 15 °C, scutella were excised and frozen in liquid nitrogen [28].

Enzyme purification

Homogenization of the grain (3 kg), collection of the 20 to 40% (w/v) ammonium sulphate fraction, desalting and chromatography on Procion Blue MX4GD (Imperial Chemical Industries, Australia Operation Pty. Ltd., Melbourne) were performed at 4 °C as described previously [21]. Fractions containing $(1 \rightarrow 3)$-β-glucanase activity were pooled and dialysed against 10 mM 4-morphine-propanesulphonic acid (MOPS) buffer, pH 7.0, for 16 h. Precipitated material was removed by centrifugation and the supernatant concentrated by ultrafiltration on a YM-10 membrane (Amicon Corporation, Danvers, MA, USA). The concentrated enzyme extract was applied to a 1 cm × 28 cm CM-Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden) column equilibrated with 10 mM MOPS, pH 7.0. Following removal of unbound proteins by washing with equilibration buffer, $(1 \rightarrow 3)$-β-glucanases were eluted by applying a 800 ml 0–200 mM NaCl gradient in the same buffer. Two peaks of $(1 \rightarrow 3)$-β-glucanase activity, designated GI and GII, were purified further. The purification of GI has been described previously [21]. Fractions containing $(1 \rightarrow 3)$-β-glucanase isoenzyme GII were pooled, concentrated by ultrafiltration to 5 ml as previously described and applied to a 2.5 cm × 75 cm BioGel P-60 column (100–200 mesh, BioRad, Richmond, CA, USA) equilibrated in 25 mM sodium acetate, pH 5.0 and eluted at a flow rate of 15 ml/h. Fractions containing $(1 \rightarrow 3)$-β-glucanase were examined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Appropriate fractions were combined and rechromatographed on the BioGel P-60 column to yield 300 μg purified isoenzyme GII.

Enzyme activity was determined using laminarin (from *Laminaria digitata*; Sigma, St. Louis, MO, USA) as a substrate [21]. SDS-PAGE was performed on 12.5% polyacrylamide gels [16] and protein estimated [5] using ovalbumin

(Sigma) as a standard. The apparent isoelectric points, substrate specificities and action patterns of purified enzymes were estimated as described previously [21], except that reducing sugars were detected on thin-layer plates with aniline-diphenylamine reagent [9]. Reduced pneumococcal polysaccharide RSIII was generously provided by Professor B.A. Stone. Purified $(1 \rightarrow 3, 1 \rightarrow 4)$-$\beta$-glucanase isoenzymes EI and EII were obtained as described by Woodward & Fincher [39].

Amino acid sequencing and analyses

Automated amino acid sequence analysis was performed in an Applied Biosystems model 470A gas-liquid phase sequencer using trifluoroacetic acid conversion chemistry [19]. Phenylhydantoin derivates were identified by HPLC on a Zorbax C8 reversed-phase column (Du Pont, Wilmington, DE, USA) eluted with a discontinuous acetonitrile gradient [41]. For amino acid compositional analysis, protein was hydrolysed for 24 h at 110 °C in evacuated, sealed tubes containing 6 M HCl and 5 mM phenol. Amino acids were analysed in a Beckman model 6300 amino acid analyser.

Isolation of cDNAs encoding $(1 \rightarrow 3)$-β-glucanase

Total mRNA isolated from homogenates of excised scutella using the guanidinium chloride procedure was fractionated on poly(U)-Sepharose 4B (Pharmacia LKB Biotechnology Inc.) [28]. The poly(A)-enriched RNA was used as a template to synthesize cDNA by the ribonuclease H method [11, 18]. The cDNA population was ligated into the *Eco* RI site of bacteriophage λgt10 and cloned in *Escherichia coli* NM514 cells [11].

The cDNA library was screened by hybridization of nitrocellulose filter plaque replicas [25] with a 734 nucleotide pair *Hinf* I fragment of the barley $(1 \rightarrow 3, 1 \rightarrow 4)$-$\beta$-glucanase cDNA [15] labelled to a specific activity of approximately 10^9 cpm/μg with deoxycytidine 5′-[α-^{32}P]triphosphate, using random sequence hexanucleotides [12]. Hybridization was for 16 h at 68 °C in 6 × standard saline citrate (1 × SSC is 0.15 M NaCl, 15 mM sodium citrate, pH 7.0), 1 × Denhart's solution [10], 5% dextran sulphate, together with 100 μg/ml sheared herring sperm DNA (Boeringer Mannheim, West Germany) and labelled cDNA probe which had been heated at 100 °C for 10 min. Filters were washed in 6 × SSC and 0.1% SDS for 1 h at room temperature and finally for 20 min at 68 °C. Autoradiography was performed with Kodak XAR X-ray film and Du Pont intensifying screens at −70 °C for 16 h.

Nucleotide sequence analysis

After plaque purification, phage DNA was isolated from positive cDNA clones [11], cDNA inserts were excised with *Eco* RI and subcloned into M13mp19, and nucleotide sequence determined by the dideoxynucleotide chain termination procedure [31]. Appropriate *Sau*3AI and *Taq* I fragments of the cDNA were recovered from agarose gels with Geneclean (BIO 101) and subcloned into M13mp19 cut with *Bam* HI, *Acc* I, *Bam* HI and *Acc* I, or *Bam* HI and *Eco* RI prior to sequencing.

Results

Purification of $(1 \rightarrow 3)$-β-glucanase isoenzymes

Ion exchange chromatography of grain extracts on CM-Sepharose revealed three distinct peaks of $(1 \rightarrow 3)$-β-glucanase activity (Fig. 1). The first, small peak which eluted during washing was not examined in detail here but may correspond to an exo-$(1 \rightarrow 3)(4)$-β-glucanase present in germinated barley (unpublished data). Two major peaks, designated GI and GII, were resolved during elution of proteins in the NaCl gradient (Fig. 1). The $(1 \rightarrow 3)$-β-glucanase in peak GI has been purified and characterized in earlier work [21]. Proteins in peak GII were further purified by gel filtration chromatography on BioGel P-60. The

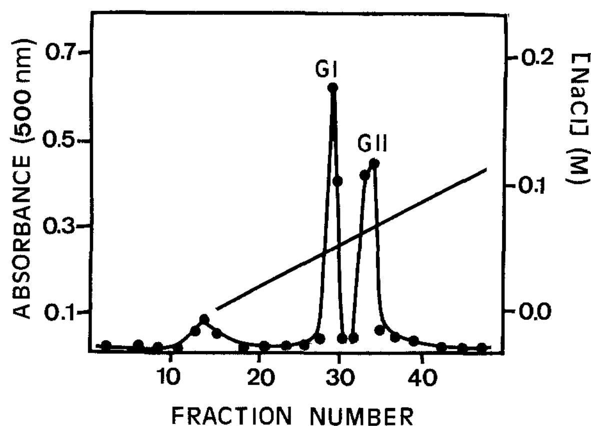

Fig. 1. Elution of (1 → 3)-β-glucanase isoenzymes GI and GII from CM-Sepharose with a NaCl gradient (solid line). Activity (●) on laminarin was monitored by a colorimetric reducing sugar assay (absorbance at 500 nm).

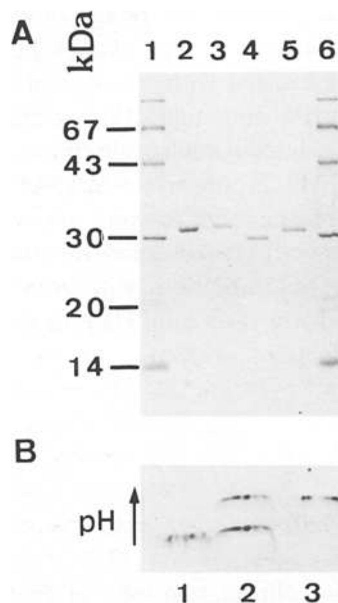

Fig. 2. A. SDS-polyacrylamide gel electrophoresis of purified barley endo-β-glucanases. Lanes 1 and 6, standard proteins (M_r 67000, bovine serum albumin; M_r 43000, ovalbumin; M_r 30000, carbonic anhydrase; M_r 20000, trypsin inhibitor; M_r 14400, α-lactalbumin); lane 2, 3 μg (1 → 3)-β-glucanase isoenzyme GI; lane 3, 1.5 μg (1 → 3)-β-glucanase isoenzyme GII; lane 4, 2 μg (1 → 3, 1 → 4)-β-glucanase isoenzyme EI; lane 5, 2 μg (1 → 3, 1 → 4)-β-glucanase EII. B. Non-equilibrium isoelectric focusing of (1 → 3)-β-glucanase isoenzymes GI (3 μg) (lane 1) and GII (3 μg) (lane 3). Both isoenzymes were loaded in lane 2. When isoelectric focusing is complete, the pI of isoenzyme GI is found to be 8.6 [21] and isoenzyme GII greater than or equal to 10.0 (data not shown).

final preparations of (1 → 3)-β-glucanases GI and GII are compared with purified (1 → 3, 1 → 4)-β-glucanase isoenzymes (designated EI and EII) in Figure 2a. The apparent M_r values for (1 → 3)-β-glucanases GI and GII are approximately 32000, although isoenzyme GII consistently migrates slightly more slowly than isoenzyme GI in SDS-polyacrylamide gels (Fig. 2a). The isoelectric points of (1 → 3)-β-glucanases GI and GII are 8.6 and approximately 10, respectively (Fig. 2b); these differences in pI values are consistent with the elution behaviour of the isoenzymes on CM-Sepharose (Fig. 1).

Action pattern and substrate specificity

The action of purified (1 → 3)-β-glucanase isoenzyme GII on laminarin showed that the initial products of hydrolysis detected on thin-layer plates were oligosaccharides of greater than four glucosyl residues (Fig. 3A). After 1 h hydrolysis laminaritriose, laminaribiose and glucose were the major products, while after extended hydrolysis (16 h), only laminaribiose and glucose remained (Fig. 3A). This action pattern is characteristic of a polysaccharide endohydrolase. The purified enzyme does not hydrolyse barley (1 → 3, 1 → 4)-β-glucan, carboxymethylcellulose, the (1 → 6)-β-glucan pustulan, or the reduced pneumococcal polysaccharide RSIII (Fig. 3b). The RSIII polysaccharide is a β-glucan with alternating (1 → 4) and (1 → 3) linkages [1]. It may be concluded that the purified enzyme GII is a true (1 → 3)-β-glucan endohydrolase with a requirement for at least two contiguous (1 → 3)-β-glucosyl linkages in its substrate, and that it may be classified in the Enzyme Commission category EC 3.2.1.39.

NH_2-terminal amino acid sequence

The directly determined sequence of the 40 NH_2-terminal amino acids of (1 → 3)-β-glucanase isoenzyme GII is compared with isoenzyme GI [21] and the barley (1 → 3, 1 → 4)-β-glucanases EI and EII [40] in Figure 4. The (1 → 3)-β-glucanase

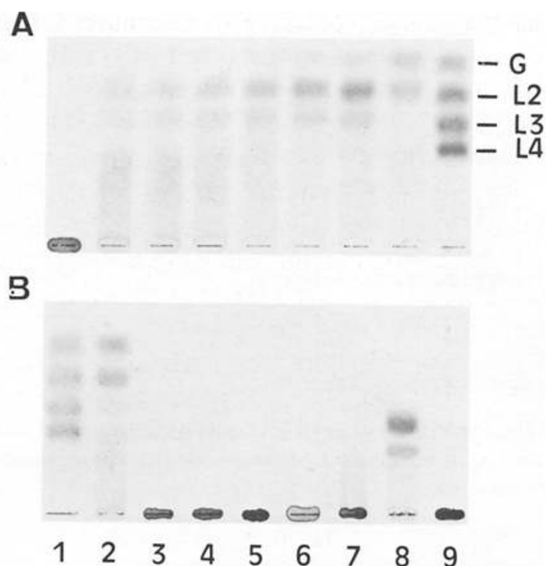

Fig. 3. Action pattern and substrate specificity of (1 → 3)-β-glucanase isoenzyme GII. A. Thin layer chromatography [21] of oligosaccharide products released when purified (1 → 3)-β-glucanase isoenzyme GII was incubated with laminarin for 0, 2, 5, 10, 20, 40, 60 and 900 min (lanes 1 to 8). Lane 9, standards (G, glucose; L2, laminaribiose; L3, laminaritriose; L4, laminaritetraose). B. Thin-layer chromatography of products of various polysaccharides incubated for 48 h at 40 °C with purified β-glucanases. In each case 6 μg purified enzyme was incubated with 0.5% (w/v) substrate in 50 mM sodium acetate buffer, pH 5.0 containing 0.7 mg/ml bovine serum albumin, except for the RSIII polysaccharide which was prepared at 0.3% (w/v). Laminaridextrin standards as described for Fig. 3A are in lane 1, followed by products of (1 → 3)-β-glucanase isoenzyme GII on laminarin (lane 2), on barley (1 → 3, 1 → 4)-β-glucan (lane 3), on carboxymethyl cellulose (lane 4), on reduced pneumococcal polysaccharide RSIII (lane 5), on pustulan (lane 6), and the products of (1 → 3, 1 → 4)-β-glucanase isoenzyme EII on laminarin (lane 7), on (1 → 3, 1 → 4)-β-glucan (lane 8), and on carboxymethyl cellulose (lane 9).

isoenzymes differ at their NH_2-terminal residue, but show an overall sequence identity of 68% for the first 40 residues. The differences in their sequences indicate that the two (1 → 3)-β-glucan endohydrolases are derived from separate genes, since barley is predominantly self-fertilizing and plants of established cultivars are essentially homozygous [40].

Characterization of a cDNA encoding (1 → 3)-β-glucanase GII

The scutellum cDNA library was screened with a (1 → 3, 1 → 4)-β-glucanase cDNA probe at low stringency in an attempt to isolate cDNAs encoding (1 → 3, 1 → 4)-β-glucanase isoenzyme I [15, 34]. Two distinct populations of positive clones were obtained. The first population produced strong hybridization signals and included cDNAs encoding (1 → 3, 1 → 4)-β-glucanases. The other population, of which one positive was detected in every 8000 clones screened, gave relatively weak signals. A single clone, carrying a cDNA insert of 1265 nucleotide pairs and designated λ3, was selected for nucleotide sequence analysis from the second population of positive clones. Both strands of the cDNA were sequenced, using the strategy outlined in Figure 5. A second, shorter but otherwise identical cDNA clone of approximately 900 nucleotide pairs, designated λ4, was used to overlap restriction enzyme fragments in the sequencing strategy (Fig. 5). The full length cDNA λ3 has an open reading frame extending from nucleotide 15 to 1049 (Fig. 6). The amino acid sequence deduced from nucleotides 132–251 (Fig. 6) corresponds exactly with that determined directly for the 40 NH_2-terminal amino acids of (1 → 3)-β-glucanase isoenzyme GII (Fig. 4). The region from the codon specifying the NH_2-terminal isoleucine residue at nucleotide 132 to the stop codon at nucleotide 1050 encodes a polypeptide of 306 residues with a M_r of 32333 and an estimated pI of 10.0. No Asn-X-Thr/Ser glycosylation site sequences are present. The amino acid composition deduced from this region of the cDNA is in agreement with amino acid composition obtained directly from the (1 → 3)-β-glucanase isoenzyme GII itself (Table 1). On the basis of these results, we conclude that the cDNA λ3 encodes (1 → 3)-β-glucanase isoenzyme GII.

Immediately 5′ to the codon specifying the NH_2-terminal isoleucine is a sequence representing a putative signal peptide of 28 residues (Fig. 6). Of the three in-frame methionine codons in this 5′ region, only the sequence surrounding

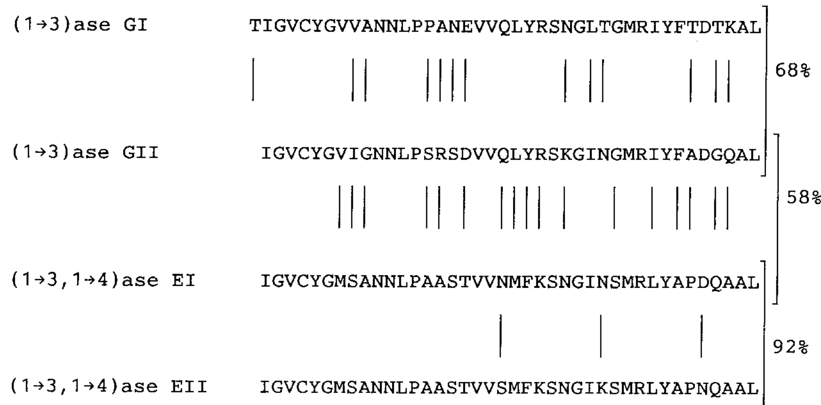

Fig. 4. Amino acid sequences of the NH_2-terminal regions of (1 → 3)-β-glucanase isoenzymes GI [21] and GII, and (1 → 3, 1 → 4)-β-glucanase isoenzymes EI and EII [39]. The lines indicate where amino acids are *different.* Standard one-letter abbreviations for amino acids are used.

the ATG codon beginning at nucleotide 48 (Fig. 6) is similar to the AACAATGGC consensus sequence which is commonly associated with ATG translation initiation codons in plant genes [24]. The coding region of the polypeptide is followed by a TAG stop codon. This constitutes part of a 216 nucleotide pair 3′ untranslated region which includes a polyadenylic acid tail of 15 residues (Fig. 6). A putative polyadenylation signal (AATAAA) begins 33 nucleotide pairs upstream from the start of the polyadenylic acid tail; the same sequence is also found 160 nucleotide pairs upstream from the polyadenylic acid tail (Fig. 6).

Codon usage

The coding region of the (1 → 3)-β-glucanase cDNA has a (G + C) content of 66% and a marked preference (95%) for the use of G or C in the wobble base position of codons (data not shown).

Discussion

Two (1 → 3)-β-glucanase isoenzymes have now been purified from extracts of germinated barley (Fig. 2, [21]). Both are basic proteins of apparent

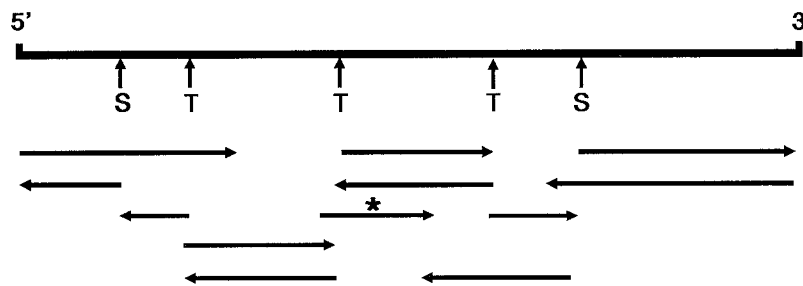

Fig. 5. Nucleotide sequencing strategy for the 1265 nucleotide pair cDNA, λ3. The *Sau*3AI (S) and *Taq* I (T) restriction sites are shown; the cDNA has *Eco* RI linkers at its 5′ and 3′ ends. Arrows indicate the length and direction of the DNA strands sequenced. The asterisk indicates sequence obtained from cDNA clone λ4, which was used to confirm the junction of the two central *Taq* I fragments. The sequence obtained from λ4 was identical with the restriction fragments of λ3.

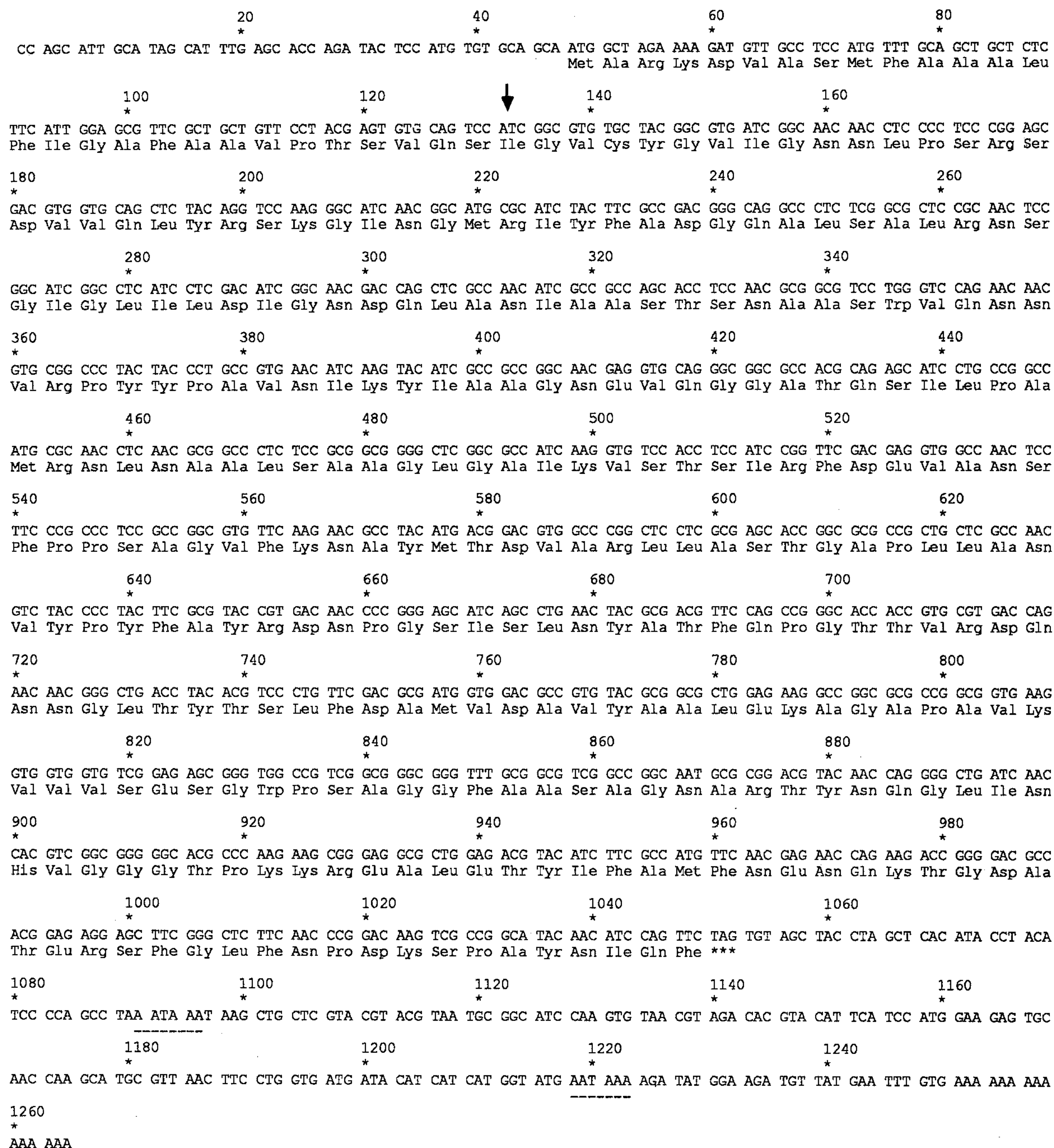

Fig. 6. The cDNA sequence and deduced amino acid sequence for barley $(1 \rightarrow 3)$-β-glucanase isoenzyme GII. Possible polyadenylation signal sequences are underlined. Arrow indicates the NH_2-terminal residue of the mature enzyme.

M_r 32000 which specifically depolymerize $(1 \rightarrow 3)$-β-glucans to laminaribiose and glucose in an endohydrolase action pattern. The complete primary structure of $(1 \rightarrow 3)$-β-glucanase isoenzyme GII has been deduced from a cDNA clone (Fig. 6). After the introduction of three gaps, an overall positional identity of 52% is obtained when the amino acid sequence is aligned with that for barley $(1 \rightarrow 3,\ 1 \rightarrow 4)$-$\beta$-glucanase isoenzyme EII [15] (Fig. 7). In regions near the COOH-termini of the proteins much higher levels of similarity are observed. Whether these con-

Table 1. Directly determined amino acid composition of $(1 \rightarrow 3)$-β-glucanase isoenzyme GII compared with the composition deduced from cDNA $\lambda 3$.

Residue	Number		Residue	Number	
	Protein*	cDNA		Protein	cDNA
Asx	39	39	Val	18	21
Thr	15	15	Met	ND	5
Ser	25	24	Ile	16	17
Glx	20	19	Leu	23	22
Pro	16	15	Tyr	15	16
Gly	32	30	Phe	13	13
Ala	44	43	His	1	1
Cys	ND	1	Lys	9	10
Trp	ND	2	Arg	11	13
			Total	298	306

* Analyses for threonine and serine corrected for 5% and 10% destruction, respectively. The numbers of residues were calculated from the molar composition assuming a total number of 298 residues, that is, residues subject to destruction (Cys, Trp, Met) were excluded from the calculation (ND, not determined).

served regions represent substrate-binding or catalytically active domains is not yet known, although the active site geometry and specificity determinants of β-glucan endohydrolases are currently under investigation [20]. In any case, the sequence similarities provide an explanation for the intitial identification of the $(1 \rightarrow 3)$-β-glucanase cDNA clones, albeit with relatively weak hybridization signals, when the cDNA library was screened using the $(1 \rightarrow 3,\ 1 \rightarrow 4)$-$\beta$-glucanase cDNA as a probe. The enzymes also share sequence similarities with a $(1 \rightarrow 3)$-β-glucanase from tobacco (*Nicotiana tabacum*) but we have no evidence for the COOH-terminal processing observed in the tobacco enzyme [3, 15, 33].

The nucleotide sequence of the $(1 \rightarrow 3)$-β-glucanase isoenzyme GII cDNA reveals the presence of a putative signal peptide of 28 residues (Fig. 6), consistent with earlier observations that barley $(1 \rightarrow 3)$-β-glucanase is secreted from isolated aleurone layers [35]. Within this putative signal peptide, three distinct domains can be identified, in common with other eukaryotic signal peptide sequences [37]. At the NH_2-terminal end is a short hydrophilic region consisting of one acidic and two basic amino acid residues (Arg-Lys-Asp). This is followed by a hydrophobic core and, close to the COOH-terminal region, the signal peptide becomes more hydrophilic and a helix breaking proline residue is located near the NH_2-terminal residue of the mature protein (Fig. 7; cf. [37]). A residue containing a small side chain (serine) is adjacent to the NH_2-terminal residue of the mature polypeptide, as observed in other eukaryotic signal peptides [37]. The Val-Gln-Ser sequence immediately before the cleavage site (Fig. 6) differs from the Val-Glu-Ser sequence which has been deduced from four $(1 \rightarrow 3,\ 1 \rightarrow 4)$-$\beta$-glucanase cDNA and genomic clones from barley and wheat ([15], unpublished data). In two $(1 \rightarrow 3)$-β-glucanases from tobacco, both Glu-Ser [3] and Gln-Ser [33] sequences precede an isoleucine residue at the NH_2-termini of the mature proteins, but the tobacco polypeptides are cleaved at a different site during processing.

Nucleotide sequence analysis of the barley $(1 \rightarrow 3)$-β-glucanase cDNA reveals a very high $(G + C)$ content, which is partly attributable to an extreme bias for the use of G or C in the wobble base position of codons. Of the 334 codons in the translated region of the cDNA, only 22 have A or T in the wobble base position and 16 of these codons are located in the putative signal peptide region. Genes encoding α-amylase [7, 29] and $(1 \rightarrow 3,\ 1 \rightarrow 4)$-$\beta$-glucanases [15] from barley aleurone share this strong bias in codon usage; these genes are expressed at rapid rates in the germinating grain and their expression appears to be enhanced by gibberellic acid [14]. Because the rate-limiting step in the elongation cycle of protein synthesis is the stochastic search for the cognate, ternary tRNA complex for a particular codon, and differences in rates of translation are linked to tRNA availability [6, 32], it has been suggested that the bias in codon usage could confer increased translational efficiency on the corresponding mRNAs and that this would enhance the rate of expression of hydrolytic enzymes required for rapid endosperm mobilization during germination [14]. It is not yet known whether the

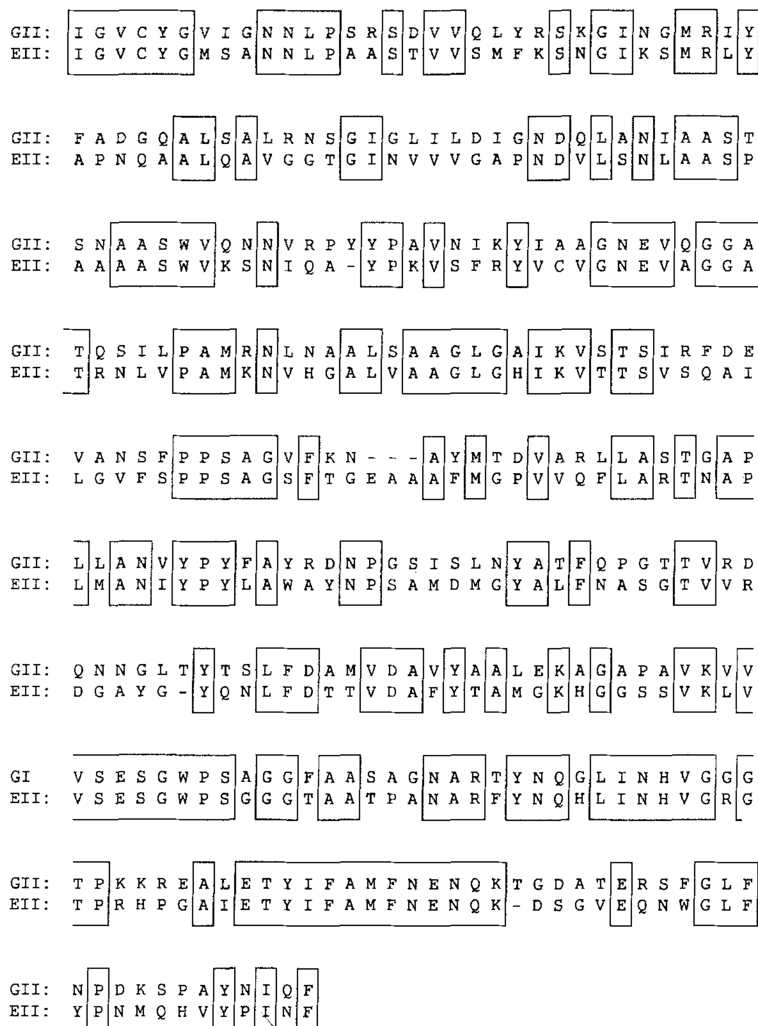

Fig. 7. Amino acid sequence similarities between barley (1 → 3)-β-glucanase isoenzyme GII and barley (1 → 3, 1 → 4)-β-glucanase isoenzyme EII. Standard one-letter abbreviations for amino acids are used.

bias in codon usage of genes expressed at rapid rates during germination is correlated with the abundance of corresponding isoaccepting tRNAs in aleurone or scutellar cells of germinating barley. Furthermore, the significance of the relatively "balanced" codon usage in the signal peptide region of the (1 → 3)-β-glucanase gene (Fig. 6) is not clear.

Both the aleurone and scutellum are likely to participate in the synthesis of the two (1 → 3)-β-glucanases in germinating barley, since isolated aleurone layers secrete (1 → 3)-β-glucanase in response to gibberellic acid [35] and the (1 → 3)-β-glucanase cDNA described here was isolated from a library prepared from scutellar mRNA. In addition, endo-(1 → 3)-β-glucanase activity in ungerminated, whole grain is located in the embryo and scutellum, while activity increases markedly in the aleurone and endosperm during germination [2]. It is not known whether tissue-specific expression of the two isoenzymes occurs in the germinated grain; there is indirect evidence for this phenomenon in barley (1 → 3, 1 → 4)-β-glucanase isoenzyme gene expression [34].

A role for the two (1 → 3)-β-glucanases in germinating barley has yet to be defined. Despite the high levels of enzyme produced, endogenous (1 → 3)-β-glucan in the grain is restricted to small

extracellular deposits scattered through the starchy endosperm [17]. The apparent paucity of endogenous substrate has led to the suggestion that the (1 → 3)-β-glucanases might participate in protection of the grain against pathogen attack [14, 21]. There is increasing evidence, albeit indirect, to support this suggestion. Firstly, (1 → 3)-β-glucanases are commonly detected amongst the soluble proteins known as "pathogenesis-related" (PR) proteins that accumulate in plants challenged with viruses, viroids, fungi or bacteria [4, 23, 27, 36]. One of two basic (1 → 3)-β-glucanases of M_r 33 500 and 34 300, designated GI and GII respectively, increase when immature pea pods are innoculated with *Fusarium solani* [26]. Secondly, other components of plant-pathogen interactive systems, such as chitinases, inhibitors of exogenous hydrolytic enzymes and protein synthesis inhibitors have been recognised in both quiescent and germinating barley, although their relationships with infection have not been established [14]. A third line of evidence has recently been obtained from near isogenic lines of barley infected with powdery mildew (*Erisiphe graminis*). In leaves of resistant plants, high levels of a (1 → 3)-β-glucanase mRNA which corresponds exactly to the (1 → 3)-β-glucanase cDNA described here are found, while in leaves of susceptible plants the mRNA is much less abundant and is induced more slowly (J.M. Manners and K.J. Scott, personal communication).

It should be emphasized that the (1 → 3)-β-glucanase isoenzymes have been purified from grain germinated under sterile conditions. If they function as part of a defence strategy and assuming our method of germination does not trigger their synthesis, the constitutive expression of (1 → 3)-β-glucanases indicates that pathogen invasion is not required for their synthesis [14, 21]. It remains to be seen whether (1 → 3)-β-glucanase levels increase upon challenging grains with microorganisms or other elicitors. The specific function of (1 → 3)-β-glucanases in plant-pathogen interactions is presumably to hydrolyse the (1 → 3, 1 → 6)-β-glucans which are major cell wall components of common pathogens of the Basidiomycetes, Ascomycetes and Oomycetes [38]. Oligomeric products of these polysaccharides can elicit other plant defence mechanisms (8, 30).

The (1 → 3)-β-glucanases share similarities with the (1 → 3, 1 → 4)-β-glucanases at the amino acid sequence level, in their pI and M_r values, in their

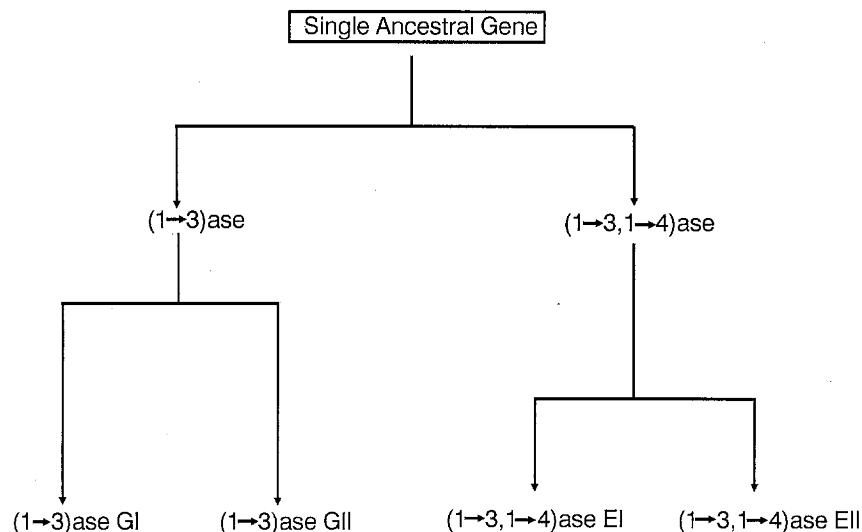

Fig. 8. Possible evolutionary route of (1 → 3)-β-glucanases and (1 → 3, 1 → 4)-β-glucanases found in germinating barley grain. The putative duplication of the ancestral (1 → 3)-β-glucanase gene is shown to have occurred earlier than the duplication of the (1 → 3, 1 → 4)-β-glucanase gene, since the (1 → 3)-β-glucanase isoenzymes are not as similar as the (1 → 3, 1 → 4)-β-glucanase isoenzymes (68% cf. 93%, Fig. 4).

specificities for related β-glucans and in the similar codon usage of their genes. While it seems likely that the genes for each pair of isoenzymes arose by gene duplication and divergent evolution of a single ancestral gene, it is also plausible that the original barley $(1 \rightarrow 3)$-β-glucanase and $(1 \rightarrow 3, 1 \rightarrow 4)$-β-glucanase genes themselves evolved from a single gene (Fig. 8). If this were the case, one might argue that the $(1 \rightarrow 3, 1 \rightarrow 4)$-β-glucanases preceded the $(1 \rightarrow 3)$-β-glucanases, on the basis of their central role in the degradation of cell walls during endosperm mobilization. Alternatively, an ancestral enzyme capable of hydrolysing both $(1 \rightarrow 3)$- and $(1 \rightarrow 3, 1 \rightarrow 4)$-β-glucans might have evolved through subtle changes in its substrate-binding domains into the two functionally distinct, specialized classes of β-glucanases now found in barley grain. It is of interest in this connection that the $(1 \rightarrow 3)$-$(1 \rightarrow 3, 1 \rightarrow 4)$-β-glucan 3(4)-glucanohydrolase (EC 3.2.1.6) from *Rhizopus arrhizus* can depolymerize both $(1 \rightarrow 3)$- and $(1 \rightarrow 3, 1 \rightarrow 4)$-β-glucans [1]. Specialization in this way might provide competitive advantages by permitting differential regulation of β-glucanase gene expression. Thus, $(1 \rightarrow 3)$-β-glucanase genes expressed in dormant grain [2] could help protect the grain against fungal invasion at this critical phase in the plant's life cycle without prematurely depolymerizing the $(1 \rightarrow 3, 1 \rightarrow 4)$-β-glucans which represent the major wall component of the starchy endosperm [13].

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Note added in proof

Following the submission of this manuscript, a paper by G.M. Ballance and I. Svendsen entitled 'Purification and amino acid sequence determination of an endo-1,3-β-glucanase from barley' appeared (Carls Res Commun 53: 411–419 (1988)). We are pleased to note that except for a single substitution, the amino acid sequence determined by these authors using Edman degradation corresponds exactly to that deduced from our cDNA clone λ3.

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