

Purification and characterization of (1→3, 1→4)- β -glucan endohydrolases from germinated wheat (*Triticum aestivum*)

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Abstract

A (1→3, 1→4)- β -glucan 4-glucanohydrolase [(1→3, 1→4)- β -glucanase, EC 3.2.1.73] was purified to homogeneity from extracts of germinated wheat grain. The enzyme, which was identified as an endohydrolase on the basis of oligosaccharide products released from a (1→3, 1→4)- β -glucan substrate, has an apparent pI of 8.2 and an apparent molecular mass of 30 kDa. Western blot analyses with specific monoclonal antibodies indicated that the enzyme is related to (1→3, 1→4)- β -glucanase isoenzyme EI from barley. The complete primary structure of the wheat (1→3, 1→4)- β -glucanase has been deduced from nucleotide sequence analysis of cDNAs isolated from a library prepared using poly(A)⁺ RNA from gibberellic acid-treated wheat aleurone layers. One cDNA, designated λ LW2, is 1426 nucleotide pairs in length and encodes a 306 amino acid enzyme, together with a NH₂-terminal signal peptide of 28 amino acid residues. The mature polypeptide encoded by this cDNA has a molecular mass of 32085 and a predicted pI of 8.1. The other cDNA, designated λ LW1, carries a 109 nucleotide pair sequence at its 5' end that is characteristic of plant introns and therefore appears to have been synthesized from an incompletely processed mRNA. Comparison of the coding and 3'-untranslated regions of the two cDNAs reveals 31 nucleotide substitutions, but none of these result in amino acid substitutions. Thus, the cDNAs encode enzymes with identical primary structures, but their corresponding mRNAs may have originated from homeologous chromosomes in the hexaploid wheat genome.

Introduction

During the germination of cereal grains, hydrolytic enzymes secreted largely from the aleurone and scutellum catalyse the degradation and mo-

bilization of reserve carbohydrates and proteins stored in the starchy endosperm. Cell walls of the starchy endosperm limit the free diffusion of hydrolytic enzymes from the surrounding tissues and must be depolymerized before the enzymes

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers λ LW1 Z22873 and λ LW2 Z22874.

can gain access to the starch and protein substrates within the starchy endosperm cells [9]. In wheat (*Triticum aestivum*) the walls of the starchy endosperm cells are composed of ca. 70% arabinoxylan, 20% (1→3, 1→4)- β -glucan, 7% glucomannan and low levels of cellulose [2, 24]. Scanning electron microscopy has been used to monitor the disappearance of the cell walls in the starchy endosperm of wheat grains as germination proceeds, and wall degradation is associated with arabinoxylan depolymerization [10]. More recently, both (1→4)- β -xylan endohydrolase and (1→3, 1→4)- β -glucanase activities have been detected in extracts of germinated wheat grain and in the medium surrounding isolated wheat aleurone layers [20].

In the present work we have purified a (1→3, 1→4)- β -glucanase from extracts of germinated wheat grain using fractional precipitation with ammonium sulphate, ion exchange chromatography, chromatofocusing and gel filtration chromatography. This enabled the action pattern, substrate specificity and immunological properties of the enzyme to be defined, together with its pI, amino acid composition, apparent molecular mass and NH₂-terminal amino acid sequence. In addition, two near full-length cDNAs encoding wheat (1→3, 1→4)- β -glucanases were isolated and characterized. Sequence analysis of the cDNAs has allowed the complete primary structure of a wheat (1→3, 1→4)- β -glucanase to be deduced.

Materials and methods

Plant material

Wheat (*Triticum aestivum* L. cv. Millewa) grains (3 kg) were surface-sterilized by treatment with 0.5% (v/v) Tween 20 and 0.2% (w/v) silver nitrate [20]. After rinsing thoroughly, the grains were germinated in the dark for 6 days at 15 °C. Moisture content was maintained at ca. 44% (w/w) by the daily addition of sterile water containing 100 μ g/ml neomycin, 10 μ g/ml chloramphenicol and 100 units/ml nystatin. For the prep-

aration of aleurone RNA, embryoless half grains were surface-sterilized in 10% (v/v) sodium hypochlorite and rinsed thoroughly [20]. The half grains were soaked for 3 days on moist filter paper, starchy endosperm was squeezed out and 100 aleurone layers were incubated for 2 days at 26 °C in 20 ml 10 mM CaCl₂ containing 5 μ M gibberellic acid (GA₃) and 2 mM sodium acetate buffer, pH 5.5 (containing 100 μ g/ml neomycin, 10 μ g/ml chloramphenicol and 100 units/ml nystatin). The possible presence of microbial contaminants was routinely checked by plating tissue fragments or homogenates on malt extract agar, pH 5.4, and nutrient agar, pH 7.4 [20].

Enzyme purification

Germinated wheat grains were homogenized in 50 mM sodium acetate pH 5.0, containing 10 mM sodium azide, 10 mM EDTA, 3 mM 2-mercaptoethanol and 3 mM phenylmethylsulphonyl fluoride [35]. After 30 min at 4 °C, insoluble material was removed by centrifugation and the extract fractionated with ammonium sulphate. Precipitated proteins were redissolved in 20 mM Tris-HCl pH 8.0 (containing 10 mM sodium azide, 3 mM 2-mercaptoethanol and 3 mM phenylmethylsulphonyl fluoride) and dialysed against the same buffer to remove ammonium sulphate. The extract was applied to a 5 cm × 20 cm DEAE-cellulose column (DE52, Whatman, Biosystems, Ltd.) equilibrated in 20 mM Tris-HCl buffer, pH 8.0, and unbound material was concentrated by ultrafiltration on a YM-10 membrane (Amicon Corporation), adjusted to pH 5.0 with 2 M sodium acetate and applied to a 2.5 cm × 30 cm column of CM-Sepharose (Pharmacia-LKB Biotechnology) equilibrated in 20 mM sodium acetate pH 5.0 [35]. Bound proteins were eluted with a linear 0–0.5 M NaCl gradient in the same buffer. Fractions containing (1→3, 1→4)- β -glucanase were concentrated and desalted by ultrafiltration and applied to a 0.5 cm × 70 cm chromatofocusing column containing PBE94 (Pharmacia-LKB) equilibrated in 25 mM triethylamine, pH 10 [32]. Bound proteins were eluted

with 9% polybuffer 96 (Pharmacia-LKB). Fractions containing (1→3, 1→4)- β -glucanase were concentrated by ultrafiltration and applied to a 1.25 cm $\times$ 70 cm column of BioGel P-60 (100–200 mesh, BioRad) equilibrated in 25 mM sodium acetate buffer pH 5.0 and eluted at a flow rate of 10 ml/hour.

Assay procedures

Enzyme activity was measured viscometrically at 40 °C, using 5 mg/ml barley (1→3, 1→4)- β -glucan in 50 mM sodium acetate pH 5.0 as substrate [35]. A unit of activity is defined as the amount of enzyme causing an increase of 1.0 in the reciprocal specific viscosity ($\Delta 1/\eta_{sp}$) per minute. Specific activity is expressed as the activity per mg protein. During column chromatography, (1→3, 1→4)- β -glucanase activity was determined by measuring reducing equivalents released [26, 30] after incubation with 5 mg/ml (1→3, 1→4)- β -glucan in 50 mM sodium acetate pH 5.0 for 20 min at 40 °C. Protein concentrations were measured by absorbance at 280 nm or by the colorimetric procedure of Bradford [4], using ovalbumin as a standard.

Amino acid analyses and sequences

For compositional analysis, a sample of the purified enzyme was hydrolysed for 24 h at 110 °C in evacuated, sealed tubes containing 6 M HCl and 5 mM phenol. Amino acids were quantitated using a Beckman model 6300 amino acid analyser. Automated amino acid sequence analysis was performed using trifluoroacetic acid conversion chemistry [15] in an Applied Biosystems model 470A gas/liquid phase sequencer. Phenylthiohydantoin derivatives were identified by HPLC on a Zorbax C₈ reversed-phase column (Du Pont, Wilmington, DE) eluted with a discontinuous acetonitrile gradient [39].

Gel electrophoresis and Western analysis

Purified enzymes or grain extracts were separated by sodium dodecyl sulphate polyacrylamide gel

electrophoresis (SDS-PAGE) using 2 mm thick gels containing 12.5% acrylamide and a 3% acrylamide stacker gel [12]. Gels were fixed and proteins stained with Coomassie Brilliant Blue R-250. For Western blots, proteins were transferred electrophoretically from the gels to nitrocellulose filters (Schleicher and Schuell, Keen, NH). The filters were probed with monoclonal antibodies raised against barley (1→3, 1→4)- β -glucanase isoenzymes EI and EII [17], followed by goat anti-mouse IgG-alkaline phosphatase conjugate (Sigma). Bound antibody was localized using nitroblue tetrazolium and 5-bromo-4-chloro-3-indoyl-phosphate (Sigma).

Substrate specificity and action pattern

To examine substrate specificities and action patterns, 0.2 μ g purified enzyme was incubated in 100 μ l 0.2% (w/v) substrate in 50 mM sodium acetate pH 5.0 (containing 400 μ g/ml bovine serum albumin) for 12 h at 40 °C. Reactions were stopped by heating at 100 °C for 5 min and the hydrolysates were applied to Kieselgel 60 thin-layer chromatography plates (Merck, Darmstadt, Germany). After developing the sheets for 2 h in ethyl acetate/acetic acid/water (2:1:1 v/v/v), oligosaccharides and monosaccharides were detected with the aniline-diphenylamine reagent [5]. In addition to the barley (1→3, 1→4)- β -glucan, the (1→3)- β -glucan laminarin (from *Laminaria digitata*, Sigma), the (1→6)- β -glucan pustulan (kindly provided by Professor B.A. Stone), larch (1→4)- β -xylan (Sigma) and carboxymethylcellulose (Edifas B50, ICI Australia) were incubated with the purified enzyme under the conditions described above. Cellodextrin standards were generously donated by Professor B.A. Stone.

Isolation of cDNAs

Total RNA isolated from homogenates of GA₃-treated wheat aleurone layers using the guanidinium chloride procedure was fractionated on poly(U)-Sephrose 4B (Pharmacia-LKB) as de-

scribed previously [25]. The poly(A)-enriched RNA was used as a template to synthesize cDNA using the Amersham cDNA synthesis kit. The cDNA preparation was ligated into the *Eco* RI site of bacteriophage λ gt10 and cloned in *Escherichia coli* NM514 cells [8].

The cDNA library was screened by hybridization of nitrocellulose filter plaque replicas [27] with a 734 bp *Hinf* I fragment of a cDNA encoding barley (1 $\rightarrow$ 3, 1 $\rightarrow$ 4)- β -glucanase isoenzyme EII [11]. The cDNA probe was labelled to a specific activity of ca. 10^9 cpm/ μ g with deoxycytidine 5'-[α - ^{32} P]triphosphate, using random sequence hexanucleotides (Amersham Multiprime DNA Labelling System). Hybridization was for 16 h at 68 °C in 6 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl, 15 mM sodium citrate, pH 7.0), 1 $\times$ Denhardt's solution [6], 5% (w/v) dextran sulphate, 100 μ g/ml sheared herring sperm DNA and [^{32} P]-labelled cDNA that had been heated at 100 °C for 10 min [16]. Filters were washed in 6 $\times$ SSC and 0.1% (w/v) SDS for 1 h at room temperature and for 20 min at 68 °C.

Nucleotide sequence analysis

After phage DNA had been isolated from positive clones [8], cDNA inserts were excised and subcloned into plasmid pUC119 for long term storage or into the replicative form of M13 mp19 for nucleotide sequence analysis by the dideoxynucleotide chain termination procedure [28]. Smaller restriction fragments of the cDNAs were separated by electrophoresis in agarose gels, recovered from the gels with GeneClean (BIO 101) and subcloned into M13 mp19 cut with appropriate restriction enzymes.

Computer analyses

Computer analyses were performed with the Pustell Sequence Analysis Programs (International Biotechnologies, New Haven, CN) the Wisconsin Genetics Computer Group package [7] and the Staden programs (MRC Laboratories of Molecular Biology, Cambridge, UK) [31].

Results

Purification of (1 $\rightarrow$ 3, 1 $\rightarrow$ 4)- β -glucanases

Significant levels of (1 $\rightarrow$ 3, 1 $\rightarrow$ 4)- β -glucanase activity were detected in both the 20–40% (2260 units) and 40–80% (5500 units) saturated ammonium sulphate fractions of aqueous extracts from germinated wheat grain. Both fractions were subjected to the same purification procedure. The only difference observed was that two peaks of (1 $\rightarrow$ 3, 1 $\rightarrow$ 4)- β -glucanase were detected during ion exchange chromatography of the 40–80% fraction on CM-Sepharose (Fig. 1); a single peak of activity was eluted at the same position as peak 1 (Fig. 1) during purification of the 20–40% ammonium sulphate fraction. Examination of the action patterns of the enzymes in the two peaks in the 40–80% fraction revealed that peak 1 was a (1 $\rightarrow$ 3, 1 $\rightarrow$ 4)- β -glucan endohydrolase, while peak 2 was an exo- β -glucan hydrolase. Peak 2 (Fig. 1) was not further purified. When pooled fractions from peak 1 were subjected to further chromatography on a PBE94 chromatofocusing column and gel filtration chromatography on Bio-Gel P-60, single symmetrical peaks of activity were obtained (data not shown). Both enzyme fractions were eluted from the chromatofocusing

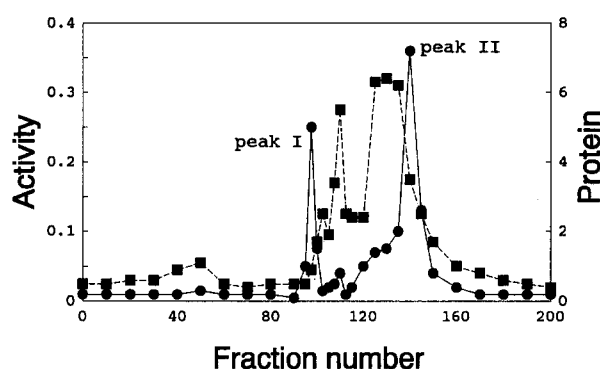


Fig. 1. Elution of the 40–80% ammonium sulphate fraction from CM-Sepharose with a 0–0.5M NaCl gradient. Activity against (1 $\rightarrow$ 3, 1 $\rightarrow$ 4)- β -glucan (A_{500nm}) is indicated by circles and protein (A_{280nm}) by squares. Peak I was subsequently shown to contain endo- β -glucanase activity and was subject to further purification. Peak II was shown to contain exo- β -glucanase activity and was not examined further here.

column at pH 8.2 in moderate yield (6.3 mg for the 20–40% fraction and 14 mg for the 40–80% fraction). Examination of the final enzyme preparations by SDS-PAGE showed the presence of single protein bands with an apparent molecular mass of 30 kDa (Fig. 2).

Action pattern and substrate specificity

The purified (1→3, 1→4)- β -glucanases exhibit an endohydrolase action pattern, as indicated by the progressive analysis of oligosaccharides released from the (1→3, 1→4)- β -glucan substrate. During the early stages of hydrolysis, both the (1→3, 1→4)- β -glucanase preparations released higher oligosaccharides but these were subsequently hydrolysed to three major oligosaccharides (Fig. 3). The smallest of the final oligosaccharides had a mobility similar to cellobiose, while the others had mobilities intermediate between cellobiose and cellotetraose. The two larger oligosaccharides had the same mobilities as 3-0- β -cellobiosyl-*D*-glucose and 3-0- β -cellotriosyl-*D*-glucose (Fig. 3), which are the major oligosaccharide products released when (1→3, 1→4)- β -glucanases hydrolyse this substrate [1, 36]. The reason for the presence of cellobiose in the hydrolysate at levels which appear to be higher than those found in the *Bacillus amyloliquefaciens* hydrolysate (Fig. 3) is believed to be related to the batch of (1→3, 1→4)- β -glucan substrate used in experiments with the wheat enzyme. The viscosity of the (1→3, 1→4)- β -glucan preparation was lower than normal [35], which is indicative of partial degradation during isolation of the polysaccharide. This in turn would result in the generation of chain ends that could be released as cellobiose following (1→3, 1→4)- β -glucanase hydrolysis. It should be noted that no contaminating proteins were detected in the preparations during gel electrophoresis or amino acid sequence analyses, so we conclude that the cellobiose did not result from the presence of contaminating enzymes.

Neither enzyme preparation showed any activity towards laminarin, pustulan, larch xylan or CM-cellulose.

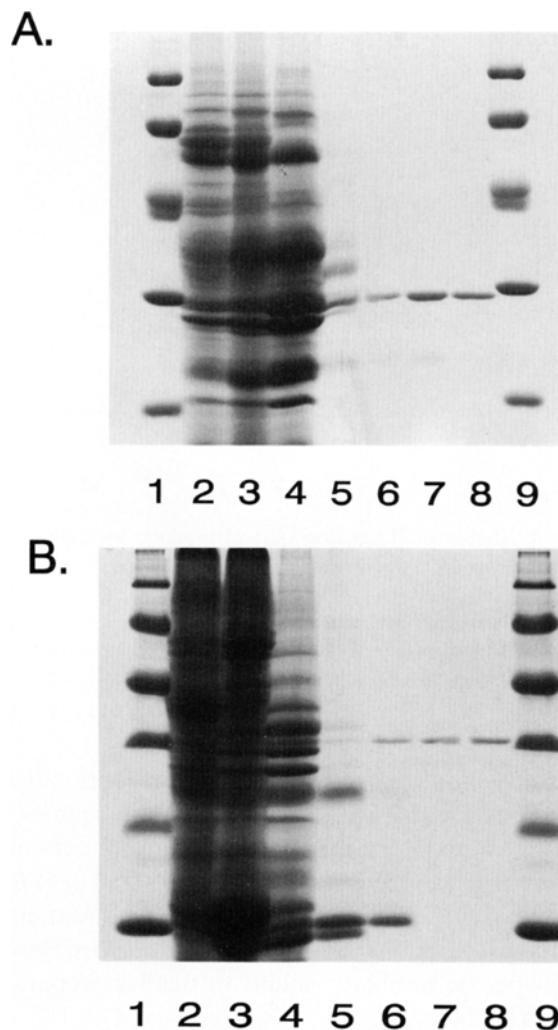


Fig. 2. SDS-PAGE of proteins during the purification of wheat (1→3, 1→4)- β -glucanase. A. 20–40% ammonium sulphate fraction. B. 40–80% ammonium sulphate fraction. In both cases lanes 1 and 9 contain standard protein markers (M_r 93 000, phosphorylase b; M_r 67 000, bovine serum albumin; M_r 43 000, ovalbumin; M_r 30 000, carbonic anhydrase; M_r 20 000, trypsin inhibitor; M_r 14 400). Lane 2 contains crude extract of the grain, lane 3 is after ammonium sulphate precipitation, lane 4 represents pooled fractions after DEAE-cellulose, lane 5 is pooled fractions after CM-Sepharose, lane 6 after chromatofocusing, lane 7 after the first BioGel P-60 gel filtration column run and lane 8 after the second pass through the same gel filtration column.

Western analysis of purified enzymes

Purified wheat (1→3, 1→4)- β -glucanase preparations were blotted from SDS gels onto nitro-

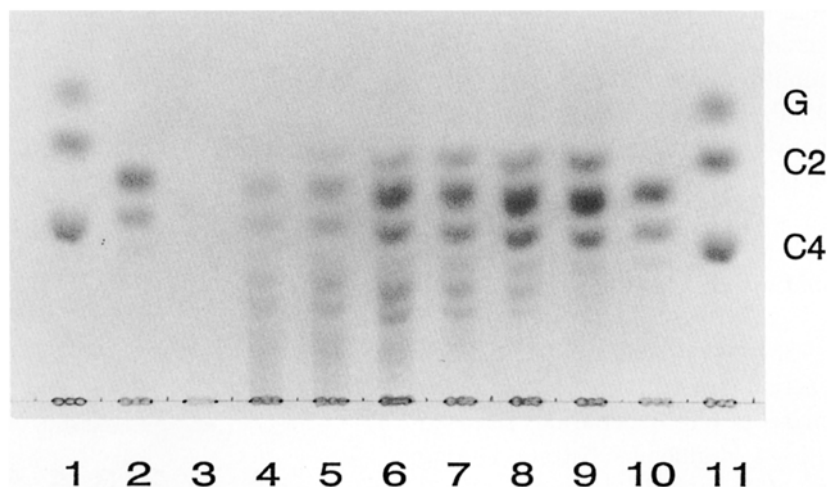


Fig. 3. Thin-layer chromatography of products released by the action of purified wheat (1→3, 1→4)- β -glucanase preparations. Lanes 1 and 11 contain standard glucose (G), cellobiose (C2) and cellotetraose (C4); lanes 2 and 10 contain the products of hydrolysis of (1→3, 1→4)- β -glucan with the *Bacillus amyloliquefaciens* (1→3, 1→4)- β -glucanase [1]; and lanes 3 to 9 contain oligosaccharides released from the substrate after 0 min, 10 min, 20 min, 40 min, 60 min, 120 min and 10 h hydrolysis with the purified wheat enzymes, respectively. Similar patterns were observed for enzyme preparations from both the 20–40% and 40–80% ammonium sulphate fractions.

cellulose and probed with monoclonal antibodies specific for barley (1→3, 1→4)- β -glucanases isoenzymes EI and EII [17]. The monoclonal antibody that recognizes barley (1→3, 1→4)- β -glucanase isoenzyme EI bound to both wheat enzyme preparations, whereas the barley isoenzyme EII-specific antibody bound to neither preparation (Fig. 4).

NH₂-terminal amino acid sequences

The NH₂-terminal amino acid sequences determined directly from the purified wheat (1→3, 1→4)- β -glucanase preparations are compared with the 40 NH₂-terminal amino acids of the barley (1→3, 1→4)- β -glucanases [37] in Fig. 5. Although the sequence analysis of the wheat (1→3, 1→4)- β -glucanase purified from the 20–40% ammonium sulphate fraction did not extend as far as the '40–80%' preparation, the two enzymes have identical sequences as far as amino acid number 28. The two barley (1→3, 1→4)- β -glucanases can be distinguished at residues 20, 28 and 36 [37]. At position 20 the purified wheat (1→3,

1→4)- β -glucanase preparations have a Ser residue, as observed in the barley (1→3, 1→4)- β -glucanase isoenzyme EII, while at residue 28 an Asn is found in both wheat enzyme preparations and an Asp is present at position 36 of the '40–80%' wheat enzyme preparation (Fig. 5); the latter residues correspond with the Asn and Asp found in the barley isoenzyme EI at positions 28 and 36, respectively.

Isolation and characterization of cDNA clones

The wheat aleurone cDNA library was screened using a barley (1→3, 1→4)- β -glucanase cDNA probe [11]. Two positive clones were screened from a total of 9000 clones and are designated λ LW1 and λ LW2. The clones carried cDNA inserts of 1434 and 1426 nucleotide pairs, respectively. Both strands of each cDNA were sequenced and their complete nucleotide sequences, together with the deduced amino acid sequence, are shown in Fig. 6. The λ LW1 clone was positively identified as a (1→3, 1→4)- β -glucanase cDNA by the presence of a region beginning at

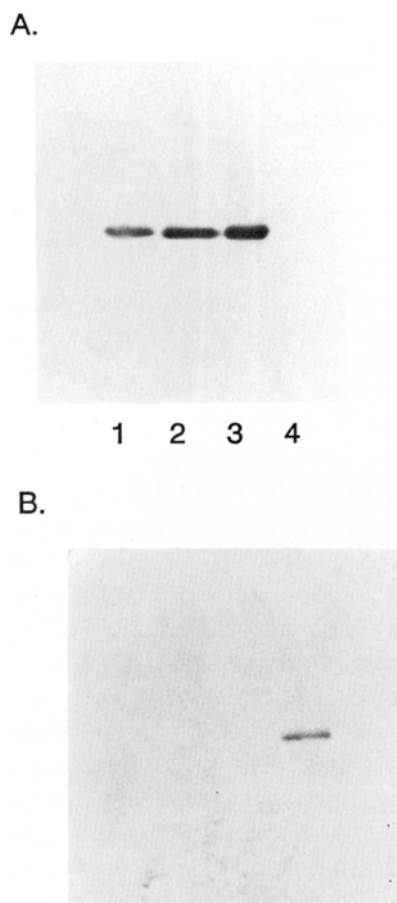


Fig. 4. Western blot analyses of purified wheat (1→3, 1→4)- β -glucanase preparations probed with monoclonal antibodies raised against barley (1→3, 1→4)- β -glucanase isoenzyme EI (A) and isoenzyme EII (B). In both cases lane 1 contains purified barley isoenzyme EI, lane 2 is wheat enzyme purified from the 40–80% ammonium sulphate fraction, lane 3 is wheat enzyme purified from the 20–40% ammonium sulphate fraction, and lane 4 is barley isoenzyme EII.

nucleotide 121 that corresponded exactly to the NH_2 -terminal amino acid sequence determined directly from the purified wheat (1→3, 1→4)- β -glucanase preparations (Fig. 6; cf. Fig. 5). The cDNA encoded a protein of 306 amino acid residues and had a 3'-untranslated region of 395 nucleotide pairs, which included a poly(A) tail of 20 residues (Fig. 6). A putative polyadenylation signal was detected 40 nucleotide pairs upstream from the poly(A) tail. However, in the region 5' to the codon for the NH_2 -terminal Ile residue,

stop codons were found in every reading frame and no sequence corresponding to a signal peptide could be detected.

The λ LW2 clone was also identified as a (1→3, 1→4)- β -glucanase cDNA on the basis of a region beginning at nucleotide 51 that corresponded exactly to the NH_2 -terminal amino acid sequence determined directly from the purified wheat (1→3, 1→4)- β -glucanase preparations (Fig. 6; cf. Fig. 5). This cDNA encodes a protein of 306 amino acid residues in which the amino acid sequence is identical to that encoded by the λ LW1 cDNA (Fig. 6). The λ LW2 cDNA has a 3'-untranslated region of 373 nucleotide pairs but no poly(A) tail. In contrast to the λ LW1 cDNA, the λ LW2 cDNA has a sequence immediately 5' to the codon specifying the NH_2 -terminal Ile that represents a putative signal peptide of 28 residues. Of the two in-frame Met codons in this 5' region, the sequence surrounding the codon beginning at nucleotide 51 (Fig. 6) is more similar to the AACAAATGGC consensus sequence commonly associated with translation initiation codons in plant genes [19, 23].

Amino acid composition

The amino acid composition determined directly from the wheat (1→3, 1→4)- β -glucanase purified from the 20–40% ammonium sulphate fraction corresponded closely with that deduced from the cDNA sequence (data not shown). This suggests that the reading frames of the cDNAs used to deduce the primary structure of the encoded enzymes were correct.

Codon usage

The coding regions of the (1→3, 1→4)- β -glucanase cDNAs are characterized by a marked bias towards the use of G or C in the wobble base position of codons (Fig. 6). Of the 334 codons in the translated region of the λ LW2 cDNA, only 20 have A or T in the wobble base position and 8 of these codons are located in the putative signal

	1	10	20	30	40
WI_(40-80%)	IGV-YGMSANNLPAASTVVSMPK-N-IN				
WI_(20-40%)	IGV-YGMSANNLPAASTVVSMPKSNGLNSMRLYAPDQAAL				
EI	IGVCYGMSANNLPAASTVVMFKSNGLNSMRLYAPDQAAL				
EII	IGVCYGMSANNLPAASTVVSMPKSNGLIKSMRLYAPNQAAL				
			↑	↑	↑

Fig. 5. Comparison of the NH₂-terminal amino acid sequences determined from the purified wheat (1→3, 1→4)-β-glucanase preparations (designated WI and showing ammonium sulphate fraction from which the preparation originated) with those deduced from polypeptide and cDNA sequences for barley (1→3, 1→4)-β-glucanases isoenzymes EI and EII [29, 37]. The dashes in the sequences denote residues that could not be identified unambiguously. Arrows indicate differences in the sequences.

peptide region. In the 306 codons of the λLW1 cDNA that encode the mature protein, only 14 have A or T in the wobble base position.

Discussion

(1→3, 1→4)-β-Glucanases have been purified to homogeneity from extracts of germinated wheat grain. Although the enzyme preparations originate from different ammonium sulphate fractions of the initial grain extract, they exhibit similar behaviour during all purification steps, they migrate as single protein bands of apparent molecular mass 30 kDa during SDS-PAGE (Fig. 2), they have the same apparent isoelectric point (pI 8.2, data not shown) and identical NH₂-terminal amino acid sequences (Fig. 5). We are therefore unable to find any significant difference in their properties and conclude that they probably represent the same enzyme that is precipitated in both ammonium sulphate fractions. The wheat (1→3, 1→4)-β-glucanase preparations specifically hydrolyse (1→3, 1→4)-β-glucans to produce major oligomeric products which have mobilities on TLC similar to the 3-O-β-cellobiosyl-*D*-glucose and 3-O-β-celotriosyl-*D*-glucose released from the same substrate by the *Bacillus amyloliquefaciens* (1→3, 1→4)-β-glucanase (Fig. 3). No activity against other substrates was detected. The action pattern and substrate specificity of the wheat (1→3, 1→4)-β-glucanase support its classification as an

endohydrolase of the (1→3, 1→4)-β-glucan 4-glucanohydrolase group, in the Enzyme Commission category EC 3.2.1.73 (cf. [37]).

To define the complete primary sequence of the wheat (1→3, 1→4)-β-glucanases, a cDNA library was prepared from poly(A)⁺ RNA isolated from GA₃-treated aleurone layers; the secretion of (1→3, 1→4)-β-glucanase by isolated wheat aleurone layers is known to be enhanced approximately 2-fold following treatment with GA₃ [20]. Two near full-length cDNAs were screened from the library using a barley (1→3, 1→4)-β-glucanase cDNA [11] as a probe. The cDNAs may well correspond to members of the GA₃-inducible group of mRNAs described by Baulcombe and Buffard [3]. Nucleotide sequence analysis allowed the complete amino acid sequence of a wheat (1→3, 1→4)-β-glucanase to be deduced (Fig. 6). Although it is not possible to conclude that one or other of the cDNAs encode the actual (1→3, 1→4)-β-glucanase(s) purified here from germinated wheat grain, the exact correspondence of the NH₂-terminal amino acid sequences determined directly from the enzymes (Fig. 5) with those deduced from the cDNA sequence (Fig. 6) suggests that this is likely.

The cDNA designated λLW2 (Fig. 6) has a 50 bp 5'-untranslated region followed by a region that encodes a putative signal peptide of 28 amino acid residues. The initiating methionine codon is located in a sequence context which reasonably matches the plant consensus sequence [19, 23]. The primary translational products of the wheat

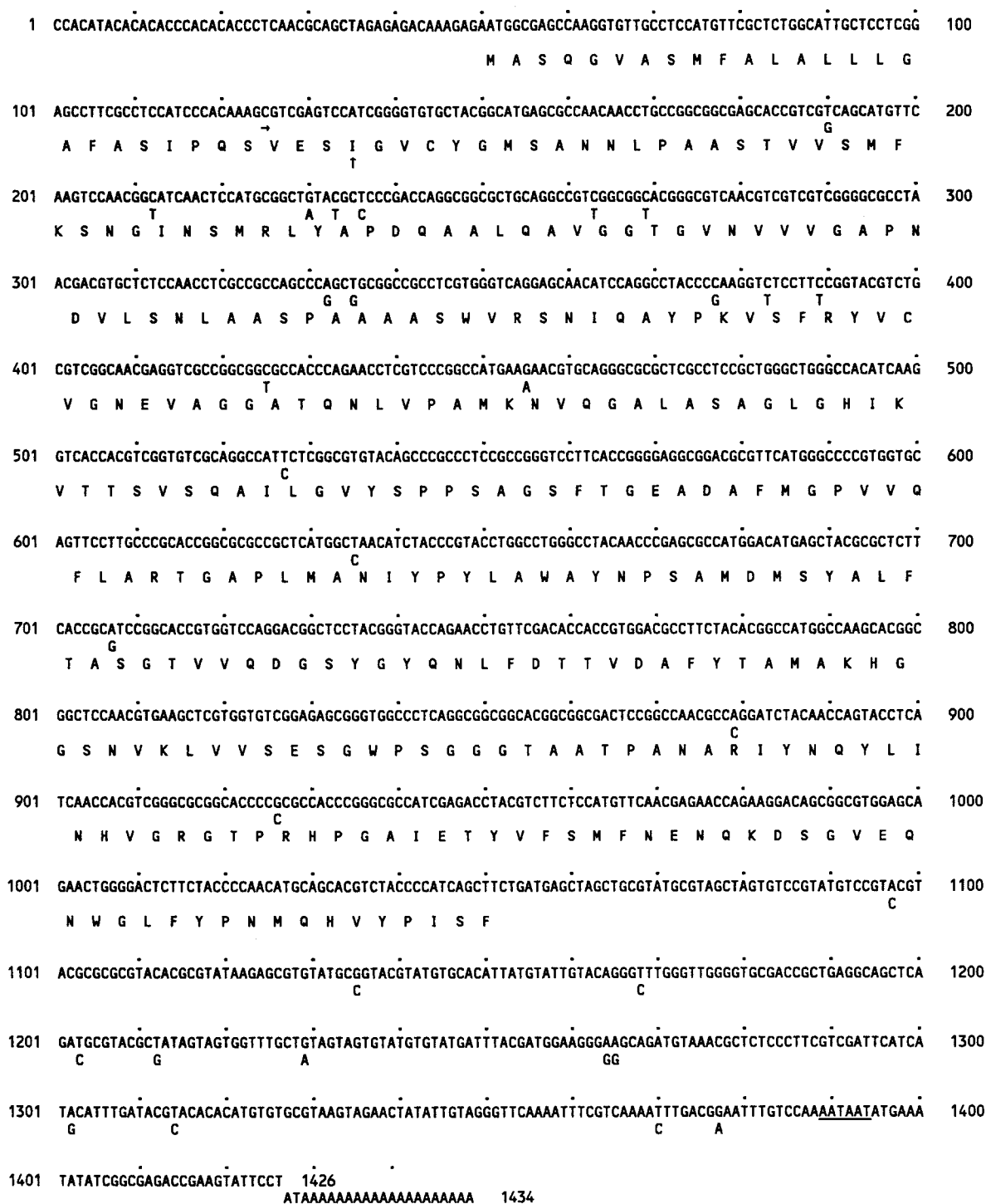


Fig. 6. Complete nucleotide sequence and derived amino acid sequence for the 1434 bp λ LW1 and the 1426 bp λ LW2 cDNAs encoding wheat (1 $\rightarrow$ 3, 1 $\rightarrow$ 4)- β -glucanase. The sequence of λ LW2 is shown on the upper lines and λ LW1 (differences only) is on the lower lines. The sequence of λ LW1 begins at nucleotide 111, because it is only after this point that the two sequences can be aligned. Thus, nucleotide 111 of λ LW1 is aligned with nucleotide 125 of λ LW2, as indicated by the horizontal arrow ($\rightarrow$). The NH₂-terminal Ile of the mature enzyme is indicated with the vertical arrow ($\uparrow$) and the putative polyadenylation signal is underlined. Standard one-letter abbreviations for amino acid residues are used.

(1→3, 1→4)- β -glucanase mRNAs would be expected to carry a leader peptide capable of targeting the polypeptide to the endoplasmic reticulum, because the mature enzyme is secreted from both aleurone and scutellar cells [20]. The signal peptide has a relatively hydrophilic NH₂-terminal region and a hydrophobic core which becomes more hydrophilic towards its COOH terminus, where there is a helix-breaking proline residue and a large polar amino acid (glutamine) (Fig. 6; [33]). The absence of a charged amino acid close to the NH₂-terminus of the signal peptide is unusual but not without precedence in eukaryotic signal peptides [33]. Immediately adjacent to the NH₂-terminal Ile residue of the mature protein is a (Val-Glu-Ser) amino acid sequence which is conserved at the signal peptide cleavage site in barley (1→3, 1→4)- β -glucanases [29] and is similar to the sequences observed in barley (1→3)- β -glucanases [16, 32].

The coding region for the mature enzyme encodes a polypeptide of 306 amino acid residues (Fig. 6). The calculated pI for the polypeptide is 8.1, which may be compared with a value of 8.2 estimated during chromatofocusing of the wheat (1→3, 1→4)- β -glucanase preparations. The calculated molecular mass for the enzyme encoded by the cDNA is 32085 Da, which is somewhat higher than the value of 30000 Da obtained for the purified enzyme during SDS-PAGE (Fig. 2). No *N*-glycosylation sites are present in the polypeptide encoded by the cDNA (Fig. 6); the (Asn¹⁷⁸-Pro-Ser) sequence is unlikely to be glycosylated because of the distorting influence of the proline residue [18].

Immediately 3' to the codon specifying the COOH-terminal Phe residue are two TGA stop codons (Fig. 6). The 3'-untranslated region of the λ LW2 cDNA is 373 bp in length and appears to stop a few nucleotides short of the poly(A) tail detected in the λ LW1 cDNA (Fig. 6).

The first 110 bp of the cDNA designated λ LW1 cannot be aligned with the λ LW2 cDNA (Fig. 6), nor can any sequence encoding a signal peptide be detected in this region. Instead, multiple stop codons are found in all reading frames. Immediately upstream from the point at which alignment

of the sequences begins, a sequence of CGCG-CAG¹⁰⁹ is found in the λ LW1 cDNA (data not shown). This sequence shares some similarity with the TTTGCAG↓ consensus sequence for the 3' splice junction of plant introns [14]. Furthermore, an ACTAAC motif beginning at nucleotide 78 of the λ LW1 cDNA corresponds in both position and sequence to the consensus sequence for the branch point of type II introns of plant genes [13]. On this basis it seems likely that the first 109 bp of the λ LW1 cDNA represent the sequence of an intron which was not excised from the primary transcription product. If this is the case, the cDNA provides some information on the structure of the wheat (1→3, 1→4)- β -glucanase gene. Thus, the putative intron interrupts the wheat (1→3, 1→4)- β -glucanase gene at precisely the same position into which introns are inserted in the barley (1→3, 1→4)- β -glucanase genes [21, 29, 34]. Another characteristic of the barley (1→3, 1→4)- β -glucanase genes is that the point of intron insertion in the serine codon near the 3' end of the signal peptide-coding region also marks a transition in codon usage patterns in the genes. Thus, in the 3' exon there is a strong bias towards the use of G and C in the wobble base position while in the 5' exon a more balanced codon usage is observed [29]. The effect may not be as pronounced in the wheat (1→3, 1→4)- β -glucanase genes, although examination of the λ LW2 cDNA indicates that of the 20 codons that end in A or T, 8 are located in the region which encodes the first 25 amino acids of the signal peptide (Fig. 6) and which may originate from exon 1 of the gene. In any case, the results suggest that the wheat (1→3, 1→4)- β -glucanase genes share many structural features with those of the β -glucan endohydrolase gene family from barley [38].

Beginning at nucleotide 111 of the λ LW1 cDNA and nucleotide 125 of the λ LW2 cDNA, the sequences can be aligned without the introduction of any gaps (Fig. 6). In the aligned region of approximately 1300 bp, 31 nucleotide substitutions are observed, in the coding region and also in the 3'-untranslated region. Of these, 19 are found in the region encoding the mature en-

zyme, although none of the substitutions causes an amino acid substitution. Thus, the amino acid sequences of the mature enzymes encoded by the two cDNAs are identical but the corresponding RNAs were clearly derived from different genes. The close similarity of the aligned sequences suggests that the genes may be located in homeologous chromosomes of the A, B or D genomes of the hexaploid wheat variety used in this work.

Alignment of the amino acid sequences deduced from the wheat (1→3, 1→4)- β -glucanase cDNAs (Fig. 6) with those of mature (1→3, 1→4)- β -glucanase isoenzymes EI and EII of barley [29] enables the relatedness of the wheat and barley genes to be evaluated. The two barley isoenzymes have 25 amino acid substitutions. At these positions in wheat (1→3, 1→4)- β -glucanases encoded by the cDNAs characterized here, 22 residues correspond to amino acids found in the barley isoenzyme EI and 3 correspond to those found in the barley isoenzyme EII. At these 25 positions, the amino acid residues in the wheat enzyme were the same as those found in either of the barley isoenzymes; no new substitutions are found. Furthermore, the only differences between the wheat and barley (1→3, 1→4)- β -glucanases are at the 25 positions where the barley isoenzymes themselves differ. The 3'-untranslated regions of the two barley (1→3, 1→4)- β -glucanase genes show no sequence similarity and have been used as specific probes in northern analyses to define transcription sites of the genes [29]; the 3'-untranslated region of the wheat cDNA (Fig. 6) is more than 90% identical to the barley isoenzyme EI gene. We therefore conclude that the wheat (1→3, 1→4)- β -glucanases encoded by the cDNAs isolated here are genetically related to the isoenzyme EI from barley. Additional evidence to support this conclusion is provided by the observation that the (1→3, 1→4)- β -glucanases purified from germinated wheat grain are recognised by a monoclonal antibody raised against barley isoenzyme EI, but not by a barley isoenzyme EII monoclonal antibody (Fig. 4). Southern blot analyses of DNA from wheat aneuploid lines and from wheat-barley addition

lines [22, 29] indicate that there are genes encoding (1→3, 1→4)- β -glucanases on the long arms of the group 1 and group 7 chromosomes of wheat and restriction analyses suggest that the genes encoding the enzyme(s) purified in the present work and corresponding to the cDNAs λ LW1 and λ LW2 are located on the long arm of the group 1 chromosomes of the wheat genome [22, 29].

Preliminary studies on (1→3, 1→4)- β -glucanase levels in tissue fragments isolated from wheat grains show that enzyme secretion is enhanced following treatment of aleurone layers with gibberellic acid (GA_3). Although abscisic acid (ABA) abolishes the GA_3 induction of (1→3, 1→4)- β -glucanase secretion, it does not completely inhibit enzyme secretion [20]. Indeed, relatively high levels of (1→3, 1→4)- β -glucanase are secreted from both ABA-treated and untreated wheat aleurone layers, whereas α -amylase secretion is very low after similar treatment of the layers [20]. Significant (1→3, 1→4)- β -glucanase activity is also secreted from isolated wheat scutella, where enzyme levels are comparable to those secreted from aleurone layers [20]. However, secretion of the enzyme from isolated scutella is not affected by treatment with GA_3 or ABA [20]. The availability of the cDNAs opens the way for a detailed examination of the regulation of (1→3, 1→4)- β -glucanase gene expression in germinating wheat grain, for the isolation of corresponding genes and for the elucidation of the apparently unusual responses to hormonal treatment.

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