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Heterologous expression of cDNAs encoding barley *(Hordeum vulgare)* (1→3)-β-glucanase isoenzyme GV

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Abstract

Two cDNAs have been isolated from libraries generated from poly(A)$^+$RNA of young barley roots and leaves, using a cDNA encoding barley (1→3)-β-glucanase isoenzyme GII as a probe. Nucleotide sequence analyses and ribonuclease protection assays show that the two cDNAs differ only in the length of their 3′-untranslated regions; the corresponding mRNAs are likely to originate from a single gene by tissue-specific processing at separate polyadenylation sites. When the coding region of the cDNA is expressed in *E. coli*, the resultant protein catalyses the hydrolysis of (1→3)-β-glucan with an action pattern characteristic of a (1→3)-β-glucan endohydrolase (EC 3.2.1.39). The enzyme has been designated isoenzyme GV of the barley (1→3)-β-glucanase family.

Key words: Alternative polyadenylation site; Bacterial expression; Barley; Gene family; (1→3)-β-Glucanase isoenzyme GV

1. Introduction

The (1→3)-β-glucanases of higher plants appear to function in developmental processes such as the removal of wound or dormancy callose, pollen formation and senescence [1]. In addition, they have been classified as members of the soluble 'pathogenesis-related' (PR) proteins that are expressed in response to microbial attack [2,3]. When plants are particularly vulnerable to pathogen attack, such as in germinating grain or in floral tissues during pollination, the PR proteins may also be expressed pre-emptively in anticipation of microbial attack [4,5]. The PR function of the (1→3)-β-glucanases is probably related to their ability to hydrolyse the (1→3,1→6)-β-glucans of fungal cell walls [6,7].

In barley, Xu et al. [8] showed that the (1→3)-β-glucanase gene family encodes at least six isoenzymes, which they designated isoenzymes GI to GVI. The identities of three of the genes have been confirmed, because the corresponding isoenzymes GI to GIII have been purified and characterized [9–13]. However, isoenzymes GIV to GVI have not been purified and classification of their genes with the (1→3)-β-glucanase family has been based solely on sequence similarities [8]. Recently a barley gene encoding an additional (1→3)-β-glucanase has been described [14]. In the present work we have expressed the coding region of a cDNA for isoenzyme GV in *E. coli* and confirm that it encodes a (1→3)-β-glucan endohydrolase. Furthermore, it is suggested that the pre-mRNA encoding this isoenzyme is processed at alternative polyadenylation sites in a tissue-specific fashion.

2. Experimental

2.1. Isolation and characterization of the cDNAs

Poly(A)$^+$RNA was extracted from young roots five days after the initiation of germination and from young leaves after ten days [8]. The corresponding cDNA was ligated into lambda ZAPII arms (Strategene, La Jolla, CA, USA) with an *Eco*RI site at the 5′ end and an *Xho*I site at the 3′ end, and transfected into *E.coli* pLK-F′ cells. The phage library was screened using a ^{32}P-labelled, 1265 nucleotide pair cDNA encoding barley (1→3)-β-glucanase isoenzyme GII [10] and cDNA inserts of positive clones were rescued into plasmid pBS SK(−). The DNA was sequenced in both directions using the dideoxynucleotide chain termination procedure [15]. Computer analyses were performed with the University of Wisconsin Genetics Computer Group package [16] in the ANGIS suite of programs developed in the Department of Electrical Engineering, University of Sydney.

2.2. Ribonuclease protection assay

Plasmid DNA (1 μg) linearized with *Bam*HI was transcribed and labelled with T7 RNA polymerase in the presence of ribonucleotide triphosphates and [α-^{32}P]UTP [17]. The RNA probe, which was complementary to its corresponding mRNA,was mixed with 50 μg total RNA from 5-day-old roots or from 10-day-old leaves in 10 ml 0.04 M PIPES buffer, pH 6.4 (containing 0.4 M NaCl, 1 mM EDTA and 80% v/v formamide). After hybridizing at 42°C for 20 h, remaining single-stranded RNA was digested at 37°C for 60 min with 10 μg ribonuclease A and 1 μg ribonuclease T1 (Sigma Chemical Co., St. Louis, MO, USA) in 500 μl 10 mM Tris-HCl buffer, pH 7.5 (containing 0.4 M NaCl, 5 mM EDTA). Protected, double-stranded RNA fragments were recovered by ethanol precipitation and separated on a 6% polyacrylamide sequencing gel.

2.3. Expression of cDNAs in E. coli

The cDNA insert was excised from the Bluescript plasmid using *Eco*RI and *Bam*HI, ligated into the expression vector plasmid pMAL-c2 (New England Biolabs, Beverly, MA, USA), and transformed into *E. coli DH5α*. Cells were grown to an optical density at 600 nm of 0.5, induced for 3 h with 1 mM IPTG and lysed by lysozyme treatment and repeated freeze/thawing. After removal of cell debris, the supernatant was passed through a column of amylose resin and bound fusion

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The sequence described in this paper can be obtained from the EMBL/Genbank databases under accession number M96939.

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protein eluted with 10 mM maltose. Following digestion of the fusion protein with Factor Xa protease, products were dialysed, separated again on the amylose resin, and concentrated.

2.4. SDS-PAGE

Protein samples were analysed on 10% SDS-polyacrylamide gels [18]. Gels were stained with Coomassie brilliant blue R-250 in 40% (v/v) methanol and 10% (v/v) glacial acetic acid and destained in the same solvent. 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 lactalbumin (M_r 14,400) were used as protein molecular size markers.

2.5. (1→3)-β-Glucanase assays

(1→3)-β-Glucanase activity was measured at 37°C using 0.2% (w/v) laminarin from *Laminaria digitata* (Sigma Chemical Co.) in 50 mM sodium acetate buffer, pH 5.5. Reducing equivalents released after 30 min by enzyme action were quantitated by the method of Somogyi [19]. One unit of activity is defined as the amount of enzyme required to release 1 μmol glucose equivalents/min. (1→3,1→4)-β-Glucanase activity was determined viscometrically, using as a substrate 0.5% (w/v) barley (1→3,1→4)-β-glucan in the same buffer. Protein was measured by the Coomassie blue method [20] or by absorbance at 280 nm.

The action pattern was examined by incubating the expressed enzyme with laminarin as described above, heating to 100°C to inactivate the enzyme, and separating aliquots on Kieselgel 60 silica gel thin layer chromatography sheets (Merck, Darmstadt, Germany) in ethyl acetate/acetic acid/water (2:2:1 by vol). Oligosaccharides and glucose were detected using the orcinol reagent.

3. Results and discussion

3.1. Isolation and sequence of the cDNAs

Single cDNA clones, designated pLQ1–1 and pLQ1–2, were isolated from the root and leaf cDNA libraries, respectively. The restriction maps of the cDNAs and sequencing strategies are shown in Fig. 1. Nucleotide sequence analysis showed that the cDNA pLQ1–1 is 1176 residues in length and includes a poly(A) tail of 21 residues. The pLQ1–2 cDNA is 1240 nucleotide pairs

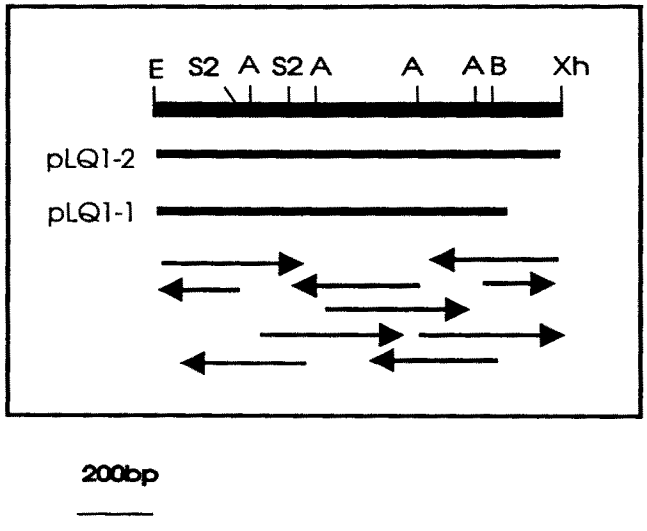

Fig. 1. Restriction map and nucleotide sequencing strategy for the cDNAs pLQ1–1 and pLQ1–2. The following abbreviations are used: A, *Acc*I; B, *Bam*HI; E, *Eco*RI; S2, *Sst*II; Xh, *Xho*I. The arrows indicate the directions and lengths of individual sequencing analyses.

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GGCACGAGCATATTTGAGCGAGCAGAGTATATTCGGGTGGTCGCCATTCATCAAAAA  57

ATGGGAGCGGTGCATGGCGTGTGCTACGGAATGGTGGGCGACAACCTGCCCTCCCGG 114
 M  G  A  V  H  G  V  C  Y  G  M  V  G  D  N  L  P  S  R
AGCGACGTGGTGCAGCTGTACAAGTCCCGCAACATCCACGCCATGCGCATCTACAAC 171
 S  D  V  V  Q  L  Y  K  S  R  N  I  H  A  M  R  I  Y  N
CCAGACCAGGAGGCCCTCACGGCCCTCCGCGGAAGCGGCATCTTCCTCATCCTCGAC 228
 P  D  Q  E  A  L  T  A  L  R  G  S  G  I  F  L  I  L  D
GTCGGCGGGGTCGACGAGGTCCGACGCCTCGGCCGTGACCCGTCATATGCCGCCGGC 285
 V  G  G  V  D  E  V  R  R  L  G  R  D  P  S  Y  A  A  G
TGGGTCCGGAGCAACGTCCAGGCCTACTACCCGGACGTCCTCATCCGGTACATCGCC 342
 W  V  R  S  N  V  Q  A  Y  Y  P  D  V  L  I  R  Y  I  A
GTCGGCAACGAGGTCCCCGCGGGCGACACGGGCATCATCCTCCTGGCCATGCAGAAC 399
 V  G  N  E  V  P  A  G  D  T  G  I  I  L  L  A  M  Q  N
GTGCACAACGCGCTGGCGTCCGCCAACCTCTCCAGCAGCATCAAGGTGTCGACGGCG 456
 V  H  N  A  L  A  S  A  N  L  S  S  I  K  V  S  T  A
GTGAGGTTCGACGTGATCACCAACTCCTTCCCGCCTTCCAGCGGCGTGTTCAGGGAC 513
 V  R  F  D  V  I  T  N  S  F  P  P  S  S  G  V  F  R  D
CCATCTGGATTAGTGCCCATCGCGCGGTTCCTGGACTCGACCGGCGCCCCGTTCCTG 570
 P  S  G  L  V  P  I  A  R  F  L  D  S  T  G  A  P  F  L
GCCAACGTGTACCCTTACTTCGCCTACCGGGACGACCGTGGCCAGAACATCCGGCTC 627
 A  N  V  Y  P  Y  F  A  Y  R  D  D  R  G  Q  N  I  R  L
AACTACGCCACGCTCCAGCCGGGCACCACGGTGAGGGACAACGGCAACGGGCTGACC 684
 N  Y  A  T  L  Q  P  G  T  T  V  R  D  N  G  N  G  L  T
TACACCAGCCTCTTCGACGCGATGGTCGACTCCATCTACGCCGCGTTGGAGAAGGCC 741
 Y  T  S  L  F  D  A  M  V  D  S  I  Y  A  A  L  E  K  A
GGCACGCCGAACGTCAGGGTGGTGGTGTCGGAGAGCGGGTGGCCGTCGGCGGGCGGG 798
 G  T  P  N  V  R  V  V  V  S  E  S  G  W  P  S  A  G  G
TTCGGGGCATCGGTGGAAAACGCGCGGAACTACAACCAGGGGCTGATTGACCACATC 855
 F  G  A  S  V  E  N  A  R  N  Y  N  Q  G  L  I  D  H  I
CGGAGCGGCACGCCGAAACGGCCAGGGGCGATCGAGACGTACATTCGCCATGTTC 912
 R  S  G  T  P  K  R  P  G  A  I  E  T  Y  I  F  A  M  F
AACGAGAACAGGAAGCCAGGGGATGAGGTGGAGAGGAACTTCGGGCTCTTCTTCCCC 969
 N  E  N  R  K  P  G  D  E  V  E  R  N  F  G  L  F  F  P
AACAAGCAACCTGTCTACCCGACAACCTTCCCCAATTAATTTTTTTTTTGCGTGGAT 1026
 N  K  Q  P  V  Y  P  T  T  F  P  N  -
CCCCAATTAATAATCGGAGGGCTTGTGAATGCTCTAACTAATTATTTTAAAAAGTCA 1083

GCGTCTATTTACGTAGCTAGCTGGTGAATGGATGCATGCTTTGCCACGTGAATAAAA 1140

ATATATTTGTGCTTGAAAAAAAAAAAAAAAAAAAAAA....pLQ1-1           1176
..............AAACAATGTCGTAGCATATGAATGAATCTTCTTCATGAATAA  1197

ATTTATATTTCTTGTGCCAAAAAAAAAAAAAAAAAAAAAAAA.....pLQ1-2     1240
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Fig. 2. Complete nucleotide sequence and deduced amino acid sequence of cDNAs pLQ1–1 and pLQ1–2. The potential *N*-glycosylation site, the putative catalytic amino acids Glu^{239} and Glu^{296}, the *Bam*HI site and the two polyadenylation signals are underlined.

long, with a poly(A) tail of 25 residues (Fig. 2). The 5′ termini of the two clones begin at exactly the same nucleotide and their sequences are identical as far as the poly(A) tail at the 3′ end of pLQ1–1 (Fig. 2). The cDNA pLQ1–1 has a single polyadenylation sequence (AATAAA) beginning at nucleotide 1134, which is 22 nucleotides upstream from the poly(A) tail (Fig. 2). The other cDNA, pLQ1–2, is 65 nucleotides longer than pLQ1–1 at its 3′ end and has two polyadenylation signals, beginning at nucleotides 1134 and 1193; the second polyadenylation sequence is 23 nucleotides upstream from the poly(A) tail.

3.2. Tissue-specific polyadenylation patterns

In view of the different lengths of the 3′-untranslated regions of the pLQ1–1 and pLQ1–2 cDNAs (Fig. 2) and their isolation from root and leaf cDNA libraries, respectively, ribonuclease protection assays were performed in attempts to define whether or not the observed differences in polyadenylation patterns were tissue-dependent. Plasmid pLQ1–2, which contains the longer cDNA insert, was cut and linearized at the *Bam*HI site beginning at nucleotide 1023 (Fig. 2) and a ^{32}P-labelled, single-stranded, antisense RNA probe was transcribed from the T7 promoter. After hybridization of the probe with RNA preparations from roots and leaves, single-stranded RNA was removed by ribonuclease digestion.

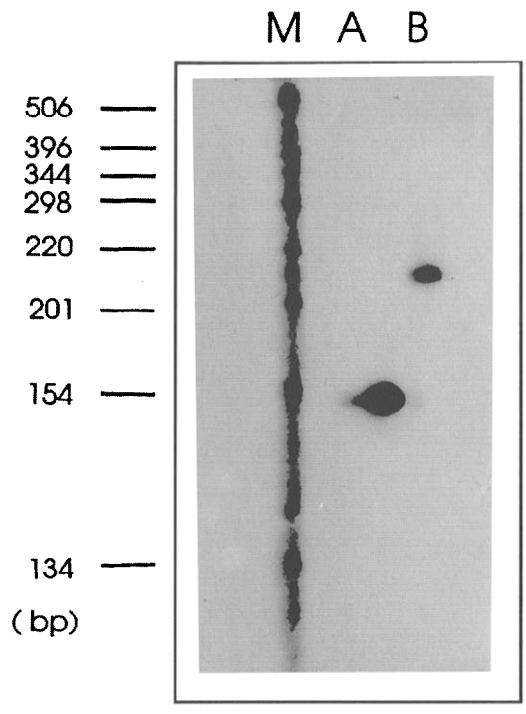

Fig. 3. Ribonuclease protection assay of mRNAs isolated from young roots and leaves. Lane 1, molecular size markers; lane 2, protected RNA fragment from root RNA; lane 3, protected RNA fragment from leaf RNA.

Double stranded RNA, which is not hydrolysed by ribonuclease, was dissociated and its size determined on polyacrylamide sequencing gels. The protected mRNA fragment from young roots was approximately 153 nucleotides in length (Fig. 3), which represents the region from nucleotides 1023–1176 in Fig. 2 and corresponds to polyadenylation at the first potential site at nucleotides 1134–1139. In contrast, the protected RNA fragment from the leaf RNA preparation was approximately 218 nucleotides in length (Fig. 3), which represents the region 1023–1240 (Fig. 2) and corresponds to polyadenylation at the second potential site at nucleotides 1193–1198 (Fig. 2).

It is likely from these results and from nucleotide sequence analyses that the two mRNAs originate from a single gene, but that 3′ processing of the primary transcript of the gene is subject to tissue-specific control. Thus, polyadenylation is effected at the first site during the synthesis of mature mRNA in roots, and at the second site in young leaves. Multiple polyadenylation sites have been detected in other plant genes [21,22] but the functional significance of tissue-specific polyadenylation has not yet been clearly demonstrated. The results might also be explained in terms of differential stability of the two mRNAs; both might be transcribed but one or other could be subject to post-transcriptional degradation in the two tissues.

3.3. Properties of the encoded protein

Nucleotide sequence analysis revealed an open reading frame extending from nucleotides 1 to 1005 (Fig. 2). The first ATG methionine codon begins at nucleotide 58. The sequence AAAAATGGG surrounding the putative initiation codon is similar to the consensus sequence AACAATGGC for translation start points in plant mRNAs [23]. The NH_2-terminal amino acid residues of the barley (1→3)-β-glucanases that have been purified correspond to amino acid positions 4 and 5 in Fig. 2 [13]. The protein encoded by the cDNA isolated here therefore appears to lack a signal peptide and indeed, no other obvious targetting signals can be detected. This leads to the conclusion that the protein is likely to be cytosolic. It should be emphasized that the NH_2-terminal residue of the mature protein can not be identified unequivocally at this stage. The presence of a Gly residue adjacent to the putative initiation Met residue suggests that the NH_2-terminal Met residue is probably removed during post-translational processing [24]. If this is the case, the mature polypeptide has 315 amino acid residues, a molecular weight of 34 kDa and an isoelectric point of 7.5. A single potential *N*-glycosylation sequence, Asn^{123}-Leu-Ser, is present.

When the amino acid sequence of the enzyme encoded by the cDNA described here (Fig. 2) is aligned with sequences from other barley (1→3)-β-glucanases, sequence identity values of 59–62% are obtained [8]. The amino acid sequence alignments are characterized by the presence of blocks of highly conserved regions, interspersed with variable regions. The three-dimensional

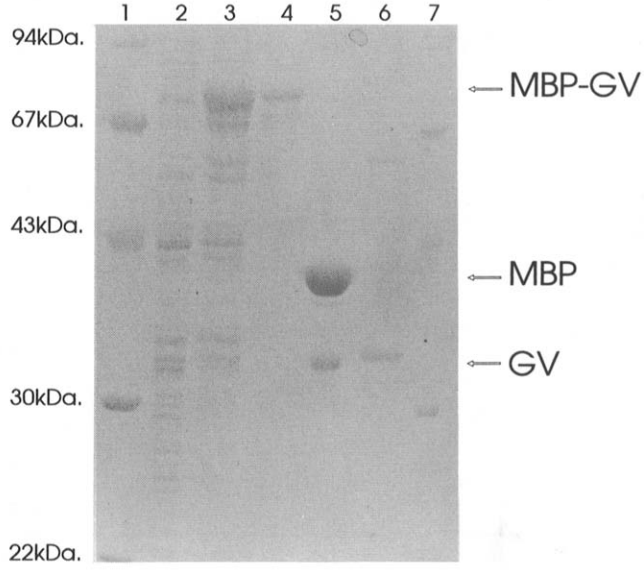

Fig. 4. SDS-PAGE of proteins expressed in *E. coli* using the pMAL-c2 expression vector. Lanes 1 and 7, molecular size markers; lane 2, cell lysate from uninduced cells; lane 3, cell lysate from cells induced with IPTG; lane 4, fusion protein eluted from the amylose resin with maltose; lane 5, fusion protein after cleavage from Factor Xa protease; lane 6, partially purified protein encoded by the pLQ1–1 cDNA, following passage of the cleavage products over the amylose resin.

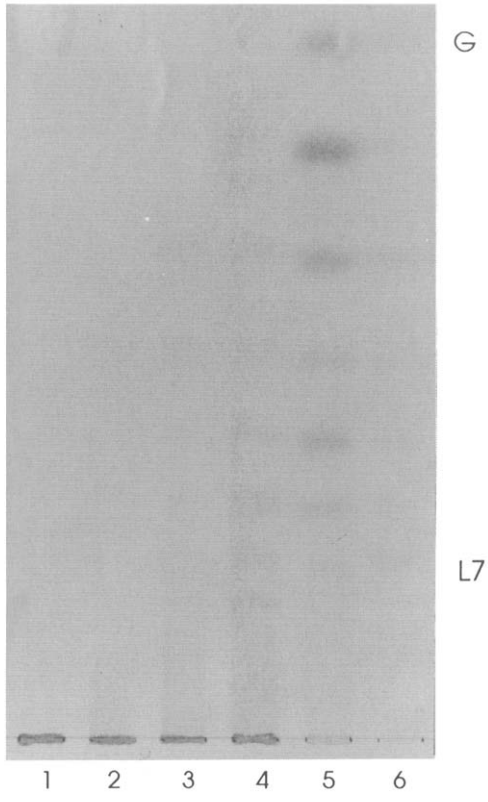

Fig. 5. Thin layer chromotography of oligosaccharide products released from laminarin by the expressed enzyme. Lane 1, laminarin without added extract; lanes 2–5, laminarin incubated for 0 min, 10 min, 30 min and 16 h with cell extracts of *E. coli* tranformed with the pMAL-c2 plasmid containing the pLQ1–1 cDNA insert; lane 6, glucose (G) and laminaridextrin standards of degree of polymerization 2–7, that is, from laminaribiose to laminariheptaose (L7).

structure of barley (1→3)-β-glucanase isoenzyme GII has recently be defined by Varghese et al. [25], who showed that the enzyme adopts an α/β barrel fold. They also predicted that other higher plant (1→3)-β-glucanases would assume α/β barrel folds similar to barley (1→3)-β-glucanase isoenzyme GII. Furthermore, recent evidence based on both chemical and crystallographic methods indicate that the catalytic amino acids in barley (1→3)-β-glucanase isoenzyme GII are Glu^{231} and Glu^{288} [25,26]. It was also noted that the positions of catalytic amino acids in the primary sequence of other plant (1→3)-β-glucanases are highly conserved; amino acid sequence alignments strongly suggest that Glu^{239} will prove to be the catalytic nucleophile in isoenzyme GV and that Glu^{296} is probably the catalytic acid [26].

3.4. Heterolcgous expression of the cDNA

The enzyme encoded by the cDNAs isolated here shares sequence similarities with both (1→3)-β-glucanases and (1→3,1→4)-β-glucanases [8,13], and although Xu et al. [8] concluded that the cDNA encodes a (1→3)-β-glucanase, direct evidence to support this suggestion was not available. The cDNA was therefore inserted into the expression plasmid pMAL-c2 and expressed in *E. coli*. The pMAL-c2 vector directs expression of a fusion protein consisting of a maltose-binding protein (MBP), a linker region containing a Factor Xa protease cleavage site, and the protein encoded by the inserted DNA [27]. The fusion protein can be purified in a single step by affinity binding to a column of amylose resin, and elution with maltose. Following cleavage with Factor Xa protease, the MBP can be removed by again passing the mixture over the amylose resin column.

When the cDNA isolated here is inserted in the pMAL-c2 vector, induction of the fusion protein with IPTG is clearly seen (Fig. 4). The fusion protein has an M_r of approximately 78 kDa, which is consistent with the size of the MBP (42 kDa) and the expressed enzyme (35 kDa). The fusion protein appears to be almost completely cleaved by the protease and, although low levels of contaminating polypeptides can be detected, the expressed protein can be substantially purified in a single step on the amylose resin (Fig. 4).

The fusion protein, the cleaved fusion protein/enzyme mixture and the purified enzyme defined by the cDNA isolated in the present study all catalyse the hydrolysis of the (1→3)-β-glucan, laminarin, but have no activity against barley (1→3,1→4)-β-glucan. The specific activities of the three fractions were 16 units/mg protein, 16 units/mg and 29 units/mg, respectively. Although the values are comparable with the specific activity of isoenzyme GIII purified from extracts of young barley seedlings (40 units/mg), they are significantly lower than purified isoenzymes GI and GII (240–280 units/mg) [13].

It should be noted that insertion of the entire cDNA into the vector resulted in the addition of an NH_2-terminal extension of approximately 23 amino acid residues on the expressed protein, but the enzyme was active nevertheless. This can be predicted from the crystal structure of the (1→3)-β-glucanase isoenzyme GII, in which the NH_2-terminus is located on the surface opposite the substrate-binding cleft [25] and would be unlikely to interfere with substrate binding. It is also likely that the NH_2-terminal extension facilitates Factor Xa protease cleavage of the fusion protein. As the extension gets shorter cleavage becomes more difficult, presumably because of steric occlusion of the Factor Xa protease cleavage site (L. Chen, P.B. Høj, A.J. Harvey and G.B. Fincher, unpublished data).

Analysis of hydrolysis products released from laminarin showed that oligosaccharides with degrees of polymerization in the range 5–10 were initially detected and that the shorter oligosaccharides laminaribiose and laminaritriose, together with glucose, accumulated as hydrolysis proceeded (Fig. 5). This pattern of product release from the polysaccharide is characteristic of an endohydrolase and we conclude that the enzyme encoded by the cDNAs pLQ1–1 and pLQ1–2 can be classified as a (1→3)-β-glucan glucanohydrolase (EC 3.2.1.39). We

have designated the enzyme barley (1→3)-β-glucanase isoenzyme GV.

3.5. Enzyme function

The function of the (1→3)-β-glucanase isoenzyme GV in barley is not yet clear. Although cDNAs encoding the enzyme were isolated from both root and leaf libraries, Northern blot analyses suggest that the mRNA levels in these tissues are low [8]. The apparent absence of a signal peptide, vacuolar targetting sequence or any other obvious cellular targetting motif suggests that the enzyme is cytosolic in origin. This contrasts with the majority of (1→3)-β-glucanases from higher plants, which appear to be deposited in vacuoles or in the extracellular space [28,29]. A cytosolic location for the barley (1→3)-β-glucanase isoenzyme GV is difficult to reconcile with a role in callose turnover during normal growth and development or during wounding, because callose is normally deposited in the extracellular space [1]. However, if it is indeed involved in protection against pathogen invasion, it might act as a second line of defence should cells be ruptured during microbial penetration of the tissue. It should be emphasized that pathogen invasion is not essential for expression, because the poly (A)$^+$RNA used for cDNA synthesis was isolated from the roots and leaves of plants grown under sterile conditions; if the enzyme participates in a larger defence strategy it must be expressed pre-emptively to protect these tissues.

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