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Isolation and characterization of a (1→3)-β-glucan endohydrolase from germinating barley (*Hordeum vulgare*): amino acid sequence similarity with barley (1→3, 1→4)-β-glucanases

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A (1→3)-β-glucan 3-glucanohydrolase (EC 3.2.1.39) has been purified approx. 190-fold from extracts of germinating barley. The enzyme has an apparent M_r 32 000, a p*I* of 8.6, and a pH optimum of 5.6. Analysis of hydrolysis products released from the (1→3)-β-glucan, laminarin, shows that the enzyme is an endohydrolase. Sequence analysis of the 46 NH_2-terminal amino acids of the (1→3)-β-glucanase reveals 54% positional identity with barley (1→3,1→4)-β-glucanases (EC 3.2.1.73) and suggests a common evolutionary origin for these two classes of β-glucan endohydrolases. The barley (1→3)-β-glucanase also exhibits significant similarity with a (1→3)-β-glucanase from tobacco.

(1→3)-β-Glucanase; Germination; Amino acid sequence; (Barley)

1. INTRODUCTION

High levels of (1→3)-β-glucan endohydrolase (EC 3.2.1.39) are found in germinating barley [3,17], although the physiological function of the enzyme is not clear. Developmental studies show it to be present in the embryo of ungerminated grain, but activity increases markedly in the aleurone and endosperm during germination [3]. Furthermore, (1→3)-β-glucanase activity is enhanced by the phytohormone gibberellic acid both in whole grain [19] and in isolated aleurone layers [24].

In the present work we have purified a (1→3)-β-glucanase from germinating barley and detect significant similarities between its NH_2-terminal amino acid sequence and appropriately aligned NH_2-terminal sequences of both barley (1→3,1→4)-β-glucanases [29] and a (1→3)-β-glucanase from tobacco [7,20].

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2. MATERIALS AND METHODS

2.1. *Germination*

Barley (*Hordeum vulgare* L. cv. Clipper) grain was surface-sterilized in 0.2% (w/v) silver nitrate for 20 min, washed thoroughly with 0.5 M NaCl and sterile distilled water, and soaked for 24 h in sterile water containing 100 μg/ml neomycin, 10 μg/ml chloramphenicol, 100 units/ml penicillin 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 5–6 days.

2.2. *Enzyme purification*

Shoots were removed prior to homogenization of the germinated grain (3 kg dry wt) in 1.5 vol. of 50 mM sodium acetate buffer, pH 5.0 (containing 10 mM sodium azide, 10 mM EDTA, 3 mM mercaptoethanol and 3 mM phenylmethylsulphonyl fluoride). All procedures were carried out at 4°C. After 20 min incubation in homogenization buffer, insoluble material was removed by centrifugation and the fraction of the supernatant precipitated between 20 and 40% (w/v) ammonium sulphate was recovered. The precipitate was redissolved in homogenization buffer, desalted on Sephadex G-25 (Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated in the same buffer and concentrated to approx. 2.5 mg/ml protein by ultrafiltration on a YM-10 membrane (Amicon Corporation, Danvers, MA, USA). The solution was applied to a 60 ml column of Procion Blue MX4GD (Imperial Chemical Industries, Australia Operation Pty Ltd, Melbourne, Australia) [22] equilibrated in 50 mM sodium acetate buffer, pH 5.0 (con-

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taining 3 mM mercaptoethanol and 10 mM sodium azide). After unbound proteins were removed by thoroughly washing the column, the pH was adjusted to 7 with 40 mM 4-morpholineethanesulphonic acid (Mes)/10 mM Bis-Tris buffer to remove additional protein. The (1→3)-β-glucanase was eluted with the same buffer containing 1 M NaCl and fractions containing activity were pooled, dialysed exhaustively against 10 mM Hepes buffer, pH 7.0, and concentrated to 5 ml by ultrafiltration, as before. The concentrated enzyme preparation was applied to a 1 cm × 28 cm column of CM-Sepharose (Pharmacia) equilibrated in 20 mM Hepes buffer, pH 7.0. After washing to remove unbound proteins, the enzyme was eluted with 20 mM 4-morphinepropanesulphonic acid (Mops) buffer, pH 7.5, and appropriate fractions pooled and concentrated by ultrafiltration. The enzyme was applied to a 2.5 cm × 75 cm Bio-Gel P-60 column (100–200 mesh, Bio-Rad, Richmond, CA, USA) equilibrated in 25 mM sodium acetate buffer, pH 5.0, and eluted at a flow rate of 10 ml/h. Fractions rich in (1→3)-β-glucanase were examined by SDS-polyacrylamide gel electrophoresis.

Throughout the purification, (1→3)-β-glucanase activity was determined by incubating enzyme samples with 4.5 mg/ml laminarin (from *Laminaria digitata;* Sigma, St. Louis, MO) in 100 mM Mes buffer, pH 5.6 (containing 160 μg/ml bovine serum albumin), at 40°C for 10 min and measuring reducing sugars released [23].

The purity of the final preparation was examined by electrophoresis on 12.5% SDS-polyacrylamide gels [9] and protein was estimated by the method of Lowry et al. [15] using bovine serum albumin as a standard.

2.3. *Isoelectric focusing*

Purified (1→3)-β-glucanase (approx. 5 μg protein in 15 μl of 25 mM sodium acetate, pH 5.0) was subjected to isoelectric focusing on 5% (w/v) polyacrylamide gels containing 3% (w/v) ampholines (LKB, Bromma, Sweden) in the pH range 3.5–10.0. Gels were fixed and proteins stained with Coomassie brilliant blue R-250 [25]. The apparent p*I* was calculated by reference to standard proteins of p*I* 3.5–9.3 (isoelectric focusing calibrating kit, Pharmacia AB, Uppsala, Sweden).

2.4. *Substrate specificity and action pattern*

Kinetic properties (K_m, V_{max}) were determined under standard assay conditions using 0.2 μg purified enzyme in the substrate concentration range 0.375–7.5 mg/ml laminarin. Activity of the purified enzyme against barley (1→3, 1→4)-β-glucan (Biocon Australia, Boronia, Victoria, Australia), a (1→6)-β-glucan (pustulan) from *Umbilicaria pustulata* (generously provided by Professor B.A. Stone) and carboxymethylcellulose (Imperial Chemical Industries, Australia) was measured as described in [28]. To examine the action pattern, the enzyme was incubated with laminarin as described above, aliquots were removed at intervals over 2 h and the enzyme inactivated at 100°C for 5 min. The hydrolysate was applied to Kieselgel 60 thin layer chromatography sheets (Merck, Darmstadt, FRG). After developing the sheets for 2 h in ethyl acetate/acetic acid/water (2:1:1, v/v), oligosaccharides were detected by *p*-anisidine [12].

2.5. *Amino acid sequence analysis*

Automated amino acid sequence determination was performed using trifluoroacetic acid conversion chemistry [11] in an Applied Biosystems model 470A gas-liquid phase sequencer [26]. Phenylthiohydantoin derivatives of the amino acids were analysed by HPLC on a Zorbax C_8 reversed-phase column (Du Pont, Wilmington, DE, USA) eluted with a discontinuous acetonitrile gradient [30].

3. RESULTS

3.1. *Enzyme purification and properties*

The (1→3)-β-glucanase from germinating barley has been purified approx. 190-fold using ammonium sulphate fractional precipitation, Procion blue MX4GD dye chromatopgraphy, ion-exchange and gel-filtration chromatography. The specific activity of the enzyme in the initial extract of germinated grain was 0.2 mg glucose equivalents released/min per mg protein, while a value of 38 mg glucose equivalents/min per mg protein was calculated after the final step in the purification procedure. The purified enzyme was eluted as a single peak on gel-filtration chromatography, with a final yield of 600 μg protein from 3 kg germinated grain. SDS-polyacrylamide gel electrophoresis shows that the protein has an apparent M_r of 32 000 and that it is substantially free of contaminating proteins (fig.1). The pH optimum of the (1→3)-β-glucanase is 5.6 and its isoelectric point 8.6 (not shown). A single Coomassie brilliant blue-staining component was also observed during isoelectric focusing.

Products released from laminarin by the barley (1→3)-β-glucanase are shown in fig.2. After 2 h hydrolysis the major products are laminaribiose and laminaritriose, with smaller amounts of glucose, laminaritetraose and higher oligosaccharides (fig.2). This action pattern is characteristic of polysaccharide endohydrolases. The enzyme preparation has no activity against either barley (1→3. 1→4)-β-glucan, as measured reductometrically (not shown) or by the release of oligosaccharides (fig.2), or carboxymethylcellulose. Furthermore, under standard assay conditions but with enzyme levels elevated 10-fold, the preparation has no activity against the (1→6)-β-glucan pustulan. The inability of the endohydrolase to cleave (1→3, 1→4)-β-glucan suggests that it is a (1→3)-β-glucan 3-glucanohydrolase and merits an enzyme classification of EC 3.2.1.39, rather than being placed with the EC 3.2.1.6 class of β-glucanases typified by the *Rhizopus arrhizus* enzyme [28].

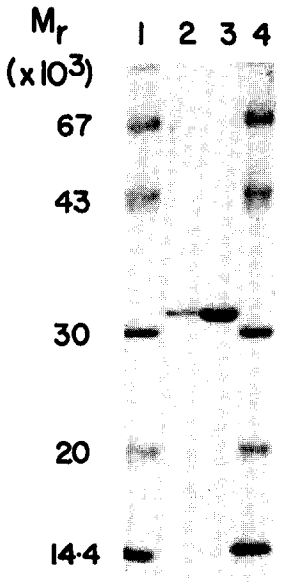

Fig.1. SDS-polyacrylamide gel electrophoresis of the purified barley (1→3)-β-glucanase. Lanes: 1 and 4, standard proteins; 2 and 3, purified enzyme (1.2 μg and 6 μg, respectively). The standard proteins are bovine serum albumin (M_r 67 000), ovalbumin (M_r 43 000), carbonic anhydrase (M_r 30 000), trypsin inhibitor (M_r 20 100) and α-lactalbumin (M_r 14 400).

Kinetic analysis revealed a K_m value of 8 mM with respect to glucose equivalents in the substrate or 0.32 mM with respect to laminarin, based on a degree of polymerization of 25 glucosyl residues. The V_{max} was 6.5×10^3 mol glucose equivalents/min per mole enzyme. However, it should be emphasised that generally it is not possible to assign single K_m and V_{max} values to polysaccharide hydrolases because of the initial heterogeneity of many polysaccharide substrates and because a range of partial degradation products, which can serve as additional substrate molecules, is present in assay mixtures.

3.2. *Amino acid sequence*

The NH_2-terminal amino acid sequence analysis enabled the first 46 residues of the (1→3)-β-glucanase to be identified. The NH_2-terminal sequences of the barley (1→3)-β-glucanase, two barley (1→3, 1→4)-β-glucanase isoenzymes [29] and a cytokinin-induced (1→3)-β-glucanase from tobacco pith [7,20] are compared in fig.3. The NH_2-terminal amino acid of the barley (1→3, 1→4)-β-glucanases is aligned with residue 2 of the barley (1→3)-β-glucanase, while the first amino acid of the tobacco enzyme has been aligned with residue 9 of the barley (1→3)-β-glucanase (fig.3). After aligning the sequences in this manner, the overall positional similarity between the barley and tobacco (1→3)-β-glucanases is 45% (residues 9–46, fig.3) and between the barley (1→3)-β-glucanase and the barley (1→3, 1→4)-β-glucanases is 54%

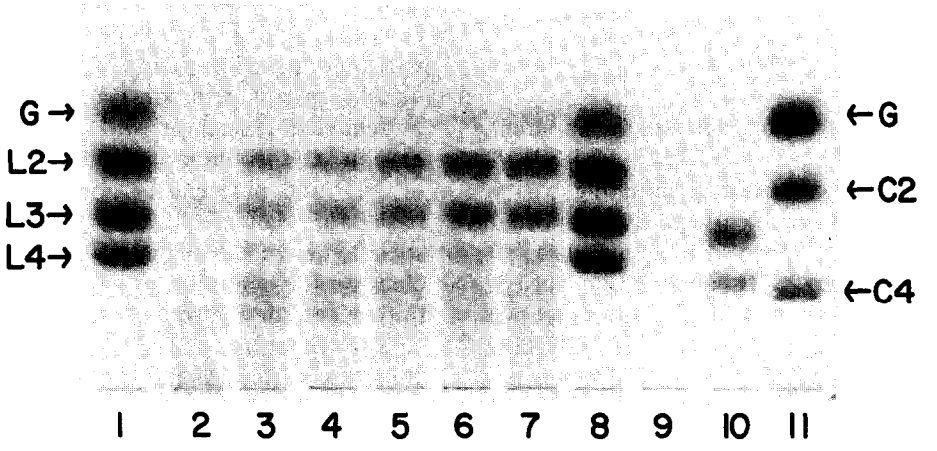

Fig.2. Thin layer chromatography of laminarin hydrolysis products. Lanes: 1, laminaridextrin standards (G, glucose; L2, laminaribiose; L3, laminaritriose; L4, laminaritetraose); 2–7, hydrolysis for 1, 10, 20, 40, 80 and 120 min, respectively; 8, laminaridextrin standards; 9, (1→3, 1→4)-β-glucan after 120 min incubation with (1→3)-β-glucanase; 10, hydrolysis products of (1→3, 1→4)-β-glucan by *Bacillus subtilis* (1→3, 1→4)-β-glucanase (major products are 3-*O*-β-cellobiosyl glucose and 3-*O*-β-cellotriosyl glucose); 11, cellodextrin standards (G, glucose; C2, cellobiose; C4, cellotetraose).

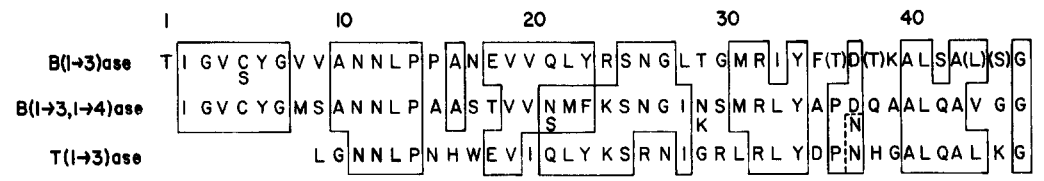

Fig.3. NH_2-terminal amino acid sequences of barley (1→3)-β-glucanase [designated B(1→3)ase], barley (1→3, 1→4)-β-glucanase [designated B(1→3, 1→4)ase] isoenzyme I, with isoenzyme II on the lower line [7,29] and tobacco (1→3)-β-glucanase [designated T(1→3)ase] [7]. Regions of amino acid sequence similarity are shown in boxes. Tentative assignments are shown in parentheses. Residue 5 of the barley (1→3)-β-glucanase may be a Cys or a substituted Ser residue.

(residues 1–46, fig.3). The ambiguity at position 5 of the barley (1→3)-β-glucanase is due to the detection of a signal eluting just prior to phenylthiohydantoin (PTH)-alanine that is characteristic of the dithiothreitol adduct of either PTH-serine or PTH-cysteine/cystine. The absence of an authentic PTH-serine signal indicated that this residue was either a substituted serine or a cysteine/cystine residue [26]. Position 5 is aligned with the cysteine/cystine at position 4 of the barley (1→3, 1→4)-β-glucanases; the barley enzymes exhibit sequence identity around this residue (fig.3). The Asn-Asn-Leu-Pro, beginning at residue 11 of the barley (1→3)-β-glucanase, and the Ala-Leu beginning at position 40, are sequences common to all three enzymes; in other regions, sequences are clearly related but no extended blocks of amino acids identical in the three enzymes are observed (fig.3).

4. DISCUSSION

The purification factor of 190-fold, the apparent M_r of 32 000 and the pI of 8.6 for the (1→3)-β-glucanase purified here from germinating barley may be compared with previously reported purification factors of approx. 60 [2,16,17] and 90 [18], with an M_r of 12 800 [18] and with an isoelectric point of 9.8 [2]. Because of the differences in molecular mass and pI values, we are uncertain whether the enzyme purified in the current work corresponds with other (1→3)-β-glucanase preparations from germinating barley.

On the basis of similarities in the partial amino acid sequence and other properties, we conclude that the barley (1→3)- and (1→3, 1→4)-β-glucan endohydrolases share a common evolutionary origin. The enzymes are approximately the same size; the apparent M_r of (1→3)-β-glucanase is 32 000, while those of (1→3, 1→4)-β-glucanase isoenzymes I and II, also measured by SDS-polyacrylamide gel electrophoresis, are 28 000 and 30 000, respectively [27]. The (1→3)-β-glucanase has an additional Thr residue at its NH_2-terminus (fig.3). The enzymes are all basic proteins with pI values of 8.6, 8.5 and greater than 10 for the (1→3)- and (1→3, 1→4)-β-glucanase isoenzymes I and II, respectively [27].

Based on an observed NH_2-terminal amino acid sequence similarity of greater than 90%, it has been suggested that the barley (1→3, 1→4)-β-glucanase isoenzymes are the products of two genes which arose by duplication of a common ancestral gene [29]. It is possible that the barley (1→3)-β-glucanase(s) and (1→3, 1→4)-β-glucanases also originate from common ancestral genes which diverged earlier than the putative (1→3, 1→4)-β-glucanase gene duplication. Comparison of gene nucleotide sequences and the three-dimensional protein conformations of this group of related β-glucanases will provide futher information on their homology. Detailed information of this kind may clarify not only the molecular basis for the precise yet distinctive substrate specificities of the enzymes, but may also provide clues on the evolution of substrate specificities of polysaccharide hydrolases in general.

The function of (1→3)-β-glucanase in germinating barley has not been clearly defined. The enzyme is present in ungerminated grain [3] and rises to very high levels during germination [3,17]. A major problem in assigning a function to the (1→3)-β-glucanases has been the apparent paucity of endogenous (1→3)-β-glucan substrate within the grain. Small, wall-associated deposits of aniline-blue staining material, which is presumed

to correspond to (1→3)-β-glucan, are scattered through the endosperm, especially near the aleurone-starchy endosperm interface [1,10], but the low abundance of this material is not consistent with the high levels of (1→3)-β-glucanase synthesized in the germinating grain [8]. It has been suggested that the enzyme participates in the initial release of (1→3, 1→4)-β-glucans from barley endosperm cell walls [5], but no direct evidence for such a role is available.

Another possible role of the (1→3)-β-glucanase is to provide non-specific protection against pathogen infection, both in the quiescent grain and during germination, since (1→3)-β-glucans are major components of some fungal cell walls [4] and their degradation products can act as elicitors of defence reactions in plants [6]. This possibility is given additional weight by the recent identification of an endochitinase in barley endosperm [14]. The chitin substrate for this enzyme is not present in barley, but is an important constituent of many fungal cell walls [4]. If the barley (1→3)-β-glucanase does indeed function in a general defence strategy against pathogen attack, it is clear from the present work, where grain was germinated under sterile conditions, that fungal invasion is not a prerequisite for enzyme synthesis. Whether or not levels of (1→3)-β-glucanase in germinated barley grain increase in response to pathogen attack, as observed in other higher plant systems [13,21], remains to be investigated.

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