Barley β-glucosidase: Expression during seed germination and maturation and partial amino acid sequences

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Unlike most of the hydrolytic enzymes that participate in endosperm mobilization, β-glucosidase of barley (Hordeum vulgare) seeds does not increase during germination, even in the presence of exogenously added gibberellic acid. However, the germination process affects the physical properties of β-glucosidase in terms of charge and apparent molecular weight. Analysis of developing barley grains shows that the enzyme is synthesized two weeks before maturation and is stored in the endosperm of the dry dormant seed. Partial amino acid sequencing of the purified β-glucosidase demonstrates significant similarity between the barley enzyme and β-glycosidases that belong to family 1 of glycosyl hydrolases.

Introduction

The hydrolysis of endosperm cell wall polysaccharides is an important process during germination of cereal grains. The degradation of the cell wall permits access of the hydrolytic enzymes, secreted from the aleurone and scutellar cells, to the starchy endosperm, where starch and protein reserves of the seed are located. Furthermore, the catalyzed cell wall polysaccharides provide a significant carbohydrate supply to the developing embryo [1,2].

The major component of the endosperm cell wall of barley grain is (1–3,1–4)-β-D-glucan which comprises 70% of the total cell wall polysaccharides [1]. The conversion of (1–3,1–4)-β-D-glucan to glucose requires the concerted action of endo-(1–3,1–4)-β-glucanases and β-glucosidases. Two isoenzymes of (1–3,1–4)-β-D-glucanase have been purified and characterised from extracts of germinating barley seeds [3,4]. These enzymes appear in the seed after the first day of germination, their level increases during the course of germination and their concentration is regulated by the plant hormone gibberellic acid [5,6,7]. In a previous publication we described the characterization of a β-glucosidase purified to homogeneity from ungerminated barley seeds [8]. This enzyme, which appears to be one of the most abundant proteins extracted from barley meal at pH 6, is a single basic polypeptide (pI > 8.5) with a \(M^+\) of 53000. It acts optimally at pH 4.5–5.0 and hydrolyzes β-glucosides (o-nitrophenyl-β-glucopyranoside and cellobiose) and less favourably β-galactosides (o-nitrophenyl-β-galactopyranoside and lactose). Following germination, the cellobiase activity of the seedling is found in extracts derived from the endosperm and is not detectable in root or shoot extracts (Karagiorgos, A., Giannakouros, T. and Simos, G., unpublished data).

In the present work, we employ enzyme assays, immunoblot and zymogram analysis to follow the expression of β-glucosidase in germinating and in maturing barley seeds. We also present partial amino acid sequences derived from purified barley β-glucosidase and compare them with sequences of other β-glycosidases.

Materials and Methods

Plant material

Barley (Hordeum vulgare var Sofia) mature, dry seeds, provided by the Cereal Institute of Thessaloniki, were surface sterilized and germinated in the dark, at 21°C, on filter paper kept moist by underlying cotton soaked in sterile distilled water [9]. When required, the

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water contained gibberellic acid at a concentration of $5 \cdot 10^{-5}$ M. Barley plants (var Clipper 71) were collected from the fields of the Agricultural School of Thessaloniki, every week over the period April 19 to June 1, 1990. First collection was one week after ear emergence and last collection one week before mowing. Ripening seeds were separated from the rest of the plant and stored until use at $-20^\circ C$.

**Enzyme extraction and purification**

Immature, dry or germinating barley seeds were triturated with a mortar and pestle in the presence of glass beads and finally homogenized in a Sorvall Omni-mixer for 2 min, with 0.1 M ammonium acetate, pH 6.0. The whole homogenate was centrifuged for 15 min at 15,000 $\times g$ and the clear supernate was employed as enzyme source. In some cases, prior to trituration, immature seeds were separated manually into husks, endosperm and embryos and germinating seeds were separated into shoots, roots and grain. Purification of $\beta$-glucosidase was carried out as previously described [8].

**Enzyme and protein determination**

Enzyme activity was assayed with cellobiose or $o$-nitrophenyl-$\beta$-glucoside as substrate as previously described [8]. Protein was determined by the method of Bradford as modified by Bearden [10]. A unit of enzyme activity is defined as the amount of enzyme that hydrolyses 1 $\mu$mol substrate per min. Specific activity is expressed as units of enzyme activity per mg protein.

**Production of antibodies to $\beta$-glucosidase**

Antiserum to $\beta$-glucosidase was produced using purified enzyme protein [8]. To ensure the purity of the immunogen, the protein was further purified by preparative SDS-PAGE [11]. The protein band was located by incubating the gel in an ice-cold solution of 0.25 M KCl in water, excised and homogenized by passing through a syringe. A 7-month-old rabbit was injected subcutaneously with 150 $\mu$g of the electrophoretically purified enzyme which had been mixed with 1 ml complete Freud’s adjuvant. Second, third and fourth booster injections were performed subcutaneously after 30, 45 and 60 days respectively, using 100 $\mu$g of protein in incomplete Freud’s adjuvant. Blood was collected from the rabbit ear vein 10 days after the third and the fourth injection. Upon addition of glycerol to a final concentration of 10% the serum was stored in 1 ml aliquots at $-20^\circ C$.

**Electrophoresis and Western blotting**

SDS-PAGE was performed according to Laemmli [11] in 12% polyacrylamide gels. After the completion of electrophoresis the proteins were electrotransferred onto Immobilon membranes (Millipore) in 20 mM Tris-acetate buffer, pH 8.3. The membranes were saturated overnight in 4% BSA, 0.1% Tween 20 in PBS (blocking buffer) at 4$^\circ$C. The membranes were then incubated for 2 h at room temperature with anti-$\beta$-glucosidase serum (1:2000) and normal goat serum (1:200) in blocking buffer, washed three times (5 min each) with 0.1% Tween 20 in PBS and incubated further with a horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (1:2000, Sigma Chemical Co.). Isoelectric focusing on polyacrylamide gels and in situ staining for $\beta$-glucosidase were performed as previously described [8].

**Protein sequencing and sequence analysis**

Automatic sequencing of the intact protein and peptides was carried out in a pulsed gas-liquid phase sequencer [12] (Applied Biosystems model 477A) with on line detection of the PTH-amino acids by HPLC using a gradient system (Applied Biosystems, PTH analyser, model 120A). Database searches were performed with FASTA version 1.6c [13] and BLASTP version 1.2.3 [14]. Sequence databases: EMBL Release 33 [15], GenBank Release 73 [16] and SWISSPROT Release 24 [17]. Multiple alignments were performed by the PILEUP program of the GCG sequence analysis software package version 7.2 [18]. The default parameters for gap open (3.0) and gap elongation (0.1) were used. The amino acid comparison matrix was the normalized Dayhoff matrix [19]. Visualization of the multiple alignment was facilitated by the PRETTYPLOT program (courtesy of Peter Rice, EMBL).

**Results**

**Expression of $\beta$-glucosidase during seed germination**

Barley seeds were allowed to germinate for five days in the dark. Shoot and roots emerged on the third day of germination. Thirty seedlings were collected at several time intervals and extracted as described under Materials and Methods. $\beta$-glucosidase was assayed in the homogenates using cellobiose as substrate. Total enzyme activity was found to increase only moderately (40%) in the first 6 h and thereafter it remained essentially constant up to the fifth day of germination (Fig. 1A). The enzyme specific activity, however, declined steadily after the first day of germination (Fig. 1B). A similar pattern was obtained when $o$-nitrophenyl-$\beta$-glucoside was used as a substrate instead of cellobiose.

To study the effect of gibberellic acid on the expression of $\beta$-glucosidase activity, the germination of the barley seeds took place in the presence of exogenously added gibberellic acid ($5 \cdot 10^{-5}$ M in the water taken up by the seeds). No significant effect was observed on the level of $\beta$-glucosidase activity in the extracts of the seedlings, except on the fifth day of germination when
total and specific activity dropped by approx. 40% (Fig. 1). It should be mentioned that seedlings germinating in the presence of gibberellic acid acquired a faster rate of growth which led to an increase in wet weight by approx. 35% on the fifth day.

In order to determine the protein levels of β-glucosidase, equivalent amounts of the extracts were subjected to Western blot analysis with immune serum raised against purified barley meal β-glucosidase. The immunoblot, shown in Fig. 2A, upper panel, reveals that the protein level of β-glucosidase remains relatively constant during the course of germination. However, after the first day, the protein band corresponding to β-glucosidase is gradually converted to a faster migrating form. The two forms differ in apparent molecular weight by 3–4 kDa. In vitro incubation of extracts derived from seeds germinated for 48 h and containing both immunoreactive β-glucosidase forms, at pH 6 or at pH 8 in the presence or absence of 0.1 mM ATP failed to produce further conversion of the higher $M_r$ form to the lower $M_r$ one (data not shown). This suggests that the conversion is taking place in vivo and is not due to proteolysis or any other modification during the extraction procedure. Germination in the presence of gibberellic acid did not produce any obvious changes in the conversion of the higher $M_r$ form of β-glucosidase to the lower $M_r$ one during the course of germination (Fig. 2A, lower panel). The amount of immunoreactive protein appears to decrease on the fifth day of germination (Fig. 2A, lower panel, lane 10) similarly to the decrease in activity observed in the presence of gibberellic acid (Fig. 1).

Analysis of equal amounts of homogenate by isoelectric focusing on polyacrylamide gel and subsequent staining for β-glucosidase activity also showed that the shift in molecular weight of barley β-glucosidase is accompanied by a change in the isoelectric pattern of the enzyme (Fig. 3, lanes 1–3). The predominant pI 8.8 form of the enzyme in the dormant seed is converted into more acidic (pI 8.2) or more basic (pI 9.4, 9.6) isoforms at the end of the germination period.

In order to characterize the nature of the lower molecular weight form of β-glucosidase that appears upon germination (termed enzyme II), five-day-old barley seedlings were extracted and β-glucosidase was purified using the established procedure for the purification of the enzyme from ungerminated seeds (en-
zyme I). Enzyme II was eluted from the ion exchange DE-52 column and the Cibacron Blue dye affinity column under the same conditions as enzyme I. Automated NH₂-terminal amino acid sequencing of the purified enzyme II allowed the determination of the first 34 residues which were identical to the first 34 residues of enzyme I (Table I, see below).

Expression of β-glucosidase during seed maturation
The results described above suggest that barley grain β-glucosidase, unlike most of the other hydrolytic enzymes that participate in endosperm mobilization, is not expressed during germination, but instead is synthesized prior to seed maturation and is stored in the endosperm of the dehydrated dormant grain. To verify this and to determine the time of synthesis of the enzyme during the development of the grain, we analyzed extracts derived from ripening seeds separated from growing barley plants, over a seven week period between ear emergence and mowing.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Partial amino acid sequence</th>
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<tbody>
<tr>
<td>Peptide 1</td>
<td>DGPNPNPEIGNTGGLSRQGFPAFVFGTAASAYO</td>
</tr>
<tr>
<td>Peptide 2</td>
<td>NGVDPQGXSIAO</td>
</tr>
<tr>
<td>Peptide 3</td>
<td>KYQPHQGRIGILLRFRXEPD</td>
</tr>
<tr>
<td>Peptide 4</td>
<td>SNGVPIGRANSXLYIVPVXGMNKAVTVYYK</td>
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<td>Peptide 5</td>
<td>RYGNPRMILSE</td>
</tr>
<tr>
<td>Peptide 6</td>
<td>LKKAIDNGAMVAGYFA</td>
</tr>
</tbody>
</table>

X denotes not determined amino acid

Fig. 4 demonstrates that β-glucosidase total and specific activities increase on or about the fifth week after ear emergence and reach maximal levels one week before the seed is completely ripe. Similar results were obtained using either o-nitrophenyl-β-glucoside or cellobiose as substrates except in the first two weeks when the specific activity towards the o-nitrophenyl-β-glucoside is higher, probably due to the presence of unrelated aryl-β-glucosidase(s) in the green husks. For comparative purposes, the activity of β-galactosidase was followed during seed maturation and it was found to be significantly different (Fig. 4). The course of synthesis of β-glucosidase protein follows the course of appearance of β-glucosidase activity as shown by the immunoblot in Fig. 2B. The isoelectric pattern of β-glucosidase remains the same during the last three weeks of ripening with the pI 8.8 form predominating as shown in Fig. 3, lanes 4–6. Manual separation of the ripening seeds, six weeks after ear emergence, into husks, embryo and endosperm followed by extraction, β-glucosidase activity determination and immunoblotting showed that β-glucosidase is present only in the endosperm while β-galactosidase activity is distributed in all three tissues (data not shown).
Partial amino acid sequences of barley meal β-glucosidase.

β-Glucosidase purified from barley meal was subjected to N-terminal amino acid sequencing by automated Edman degradation. A 41 residue long sequence was determined and is shown as Peptide 1 in Table I. Similarly isolated β-glucosidase, after chromatography through an HPLC Vydac C₄ column to remove minor contaminants, was digested with endoproteinase GluC and five of the resulting peptides were sequenced (Peptides 2-6, Table I). Thus, 130 amino acid residues were determined accounting for approx. 25% of the sequence of barley β-glucosidase. Searches against the GeneBank/EMBL and SwissProt sequence data bases showed that the determined partial sequences were unique. However, four of the barley β-glucosidase peptide sequences (Peptides 1, 4, 5, and 6) displayed significant similarity to amino acid sequences belonging to two cloned white clover β-glucosidases (linamarases) [20]. These two enzymes, by sequence homology, belong to Family 1 of glycosyl hydrolases which comprises β-glycosidases of prokaryote, plant and mammalian origin [21]. Fig. 5 shows an optimal alignment of all barley β-glucosidase peptide sequences with amino acid sequences of family 1 β-glycosidases: Bgls-T and Bglt-T, white clover laminarinases; Myro-B, Brassica napus myrosinase; Myro-S, Sinapis alba myrosinase; Bgls-A, agrobacterium β-glucosidase; Bglb-E, E. coli 6-phospho-β-glucosidase; Lacg-S, staphylococcal 6-phospho-β-galactosidase; Lph-H, human lactase-phlorizin hydrolase.

Fig. 5. Optimal alignment of the barley β-glucosidase peptides with amino acid sequences of family 1 β-glycosidases: Bgls-T and Bglt-T, white clover laminarinases; Myro-B, Brassica napus myrosinase; Myro-S, Sinapis alba myrosinase; Bgls-A, agrobacterium β-glucosidase; Bglb-E, E. coli 6-phospho-β-glucosidase; Lacg-S, staphylococcal 6-phospho-β-galactosidase; Lph-H, human lactase-phlorizin hydrolase.
Early reports documented the presence of $\beta$-glucosidase activity in barley as well as other cereal seeds and it has been suggested that the in vivo function of this enzyme is to participate together with endoglucanases in the degradation of $\beta$-glucan, a major cell wall polysaccharide [1,2,28,29,30]. More recently, purification of the $\beta$-glucosidase activity from barley meal led to the isolation of a single basic polypeptide with an apparent $M_r$ of 53000 [8]. This protein was shown to be one of the most abundant species accounting for at least 2.5% of the total protein extracted under the particular conditions (pH 6) from ground dry barley seeds. The purified polypeptide, when injected in rabbits, generated antibodies which could recognize specifically the 53 kDa protein in total barley extracts. These antibodies were used in the present work to determine immunologically the appearance of $\beta$-glucosidase in germinating and developing barley seeds. Our findings show that the levels of $\beta$-glucosidase protein remain constant during germination while $\beta$-glucosidase specific activity decreases. These results suggest that no further synthesis of $\beta$-glucosidase takes place upon germination. A small increase in total enzyme activity within the very first hours of imbibition can be attributed to facilitated release of the enzyme due to tissue softening. These results are in agreement with a previous report showing that barley seed $\beta$-glucosidase activity increased only very moderately (30%) during malting [29]. The state of $\beta$-glucosidase was also determined following seed germination in the presence of exogenously added giberellic acid. This treatment increases the rate of growth of seedlings and superinduces germination-specific activities [31]. Even under these conditions no significant differences were observed except on the fifth day of germination, when $\beta$-glucosidase levels suddenly dropped both in terms of protein as well as activity. This decrease probably reflects the complete decomposition of the seed endosperm which occurs earlier in the presence of giberellic acid as no $\beta$-glucosidase, measured as cellobiose hydrolyzing activity, appears in the new parts of the seedling namely in the roots and the shoot (Karagiorgos, A., Giannakouros, T. and Simos, G., unpublished data).

Although the germination process did not have a significant effect on the amount of $\beta$-glucosidase present in the seed, the molecular properties of the enzyme were detectably altered in terms of apparent $M_r$ and $pI$. After the first day of imbibition $\beta$-glucosidase (enzyme I) was gradually converted into a faster migrating polypeptide on SDS-PAGE which became the predominant form (enzyme II) at the end of the germination period (fifth day). This shift in $M_r$ was accompanied by a change in the relative intensity of the $pI$ isoforms of the enzyme as detected by zymograms of isoelectrically focused polyacrylamide gels. Two possible explanations can be offered for this observation: (1) enzyme II may be a newly synthesized protein recognized by the antibody raised against enzyme I and therefore closely related to enzyme I or (2) enzyme II represents a modified form of enzyme I and this modification is a result of the germination process. To address this problem, enzyme II purified from 5 day-old barley seedlings was subjected to N-terminal amino acid sequencing. A 34 amino acid long sequence was determined and it was found to be identical to the N-terminal sequence of enzyme I. This result suggests that enzyme I and enzyme II represent products of the same gene and that enzyme II is a modified form of enzyme I. The exact nature of this modification is under study at present and our preliminary results suggest that it is the result of limited proteolysis of the C-terminal part of the protein. The function of this modification is not clear yet, but we can speculate that it triggers the in vivo release of the enzyme in a manner similar to that of $\beta$-amylase [32,33].

Addressing the question of when $\beta$-glucosidase is synthesized, we analyzed extracts derived from developing barley seeds over a period of seven weeks prior to maturation. The results of this analysis showed that $\beta$-glucosidase synthesis is initiated approx. three weeks before maturation and is completed within two weeks. The newly synthesized protein shares the same $M_r$ and $pI$ isoform pattern as the protein stored in the endosperm of the dry mature seed.

In summary, the pattern of $\beta$-glucosidase expression is rather unusual. The synthesis and secretion of most of the hydrolytic enzymes involved in barley seed endosperm mobilization is induced upon germination and regulated by giberellic acid [2]. Best studied examples of this type of regulation include the a-amylase [33,34] and the endo(1-3),(1-4)-$\beta$-d-glucan endohydrolase isozymes [2,6]. On the other hand, $\beta$-glucosidase resembles $\beta$-amylase [32,33] in that both species are abundant proteins of the endosperm, are synthesized during the development of the grain and are stored in the resting seed until they are utilized upon germination. The question remains open whether $\beta$-glucosidase serves an additional function during grain development or whether it can be considered also as a
soluble storage protein, as it has been suggested for β-amylase [33].

About 25% of the total barley β-glucosidase (enzyme I) primary structure was determined by protein sequencing of the N-terminal and five proteolytically derived peptides. The sequencing revealed a homology between the barley protein and other cloned plant β-glucosidases and unequivocally confirmed the enzymic classification of the protein. Furthermore, according to the sequence information, barley seed β-glucosidase belongs to family 1 of glycosyl hydrolases [21] also referred to as BGA enzyme family [35]. This large gene family of β-glycosidases includes β-galactosidases and phospho-β-glycosidases from as diverse organisms as archaebacteria, bacteria, plants and mammals. The so far sequenced plant members of this family are two white clover β-glucosidases [20], a *Brassica napus* myrosinase and a *Sinapis alba* myrosinase [36]. A distinctive feature of this family is its low specificity with respect to the C4 configuration which means that several members of this family are active on both β-glucosides and β-galactosides. This property is also shared by the barley β-glucosidase as it has significant activity towards lactose and synthetic β-galactosides [8].

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