Immunological determination of (1 → 3),(1 → 4)-β-D-glucan endohydrolase development in germinating barley (*Hordeum vulgare*)

I. Montgomery Stuart and Geoffrey B. Fincher*

Department of Biochemistry, La Trobe University, Bundoora, VIC 3083, Australia

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Proteins in homogenates of germinating barley were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis and, following electrophoretic transfer to nitrocellulose paper, the two (1 → 3), (1 → 4)-β-D-glucan 4-glucanohydrolases (EC 3.2.1.73) were individually quantitated in the 0–10 pmol range using crossreactive antibodies raised against either enzyme. The two enzymes develop in approximately equimolar proportions during the germination process.

Barley *β-Glucanases* *Germination* *Immunology*

1. INTRODUCTION

The two (1 → 3),(1 → 4)-β-D-glucan 4-glucanohydrolases (EC 3.2.1.73) from germinating barley exhibit almost identical kinetic and specificity properties, but differ in a number of physical and chemical properties [1,2]. In particular, enzyme I has M_r 28000 while enzyme II has M_r 33000 [1]. Based on amino acid sequence analysis it has been suggested [3] that the enzymes may be derived from different genes which originated by duplication of a common ancestral gene. The high degree of sequence homology observed [3] would explain the cross-reactivity of antibodies raised against the two enzymes [1].

The importance of (1 → 3),(1 → 4)-β-D-glucanases in the depolymerization of cell wall polysaccharides during germination and in the malting and brewing processes has led to a number of studies on the development of total (1 → 3),(1 → 4)-β-D-glucanase activity in germinating barley [4–7].

* To whom correspondence should be addressed at: Carlsberg Research Laboratories, Department of Physiology, Gamle Carlsberg Vej 10, Valby, DK-2500, Denmark

A viscometric assay using (1 → 3),(1 → 4)-β-D-glucan as substrate is usually employed, but does not allow the development of the two enzymes to be examined individually, nor does it discriminate between (1 → 3),(1 → 4)-β-D-glucanase and cellulase (EC 3.2.1.4) activity [8]. Variable amounts of cellulase, predominantly of microbial origin, are associated with germinating barley grain [9]. In addition, results of the viscometric assay can be complicated by the presence of low-M_r inhibitors of the enzymes in grain homogenates [1]. Quantitation of the levels of (1 → 3),(1 → 4)-β-D-glucanase protein is likely therefore to permit a more specific assessment of the total, potential activity of the enzymes in germinating barley.

Here, we describe the separation of soluble proteins in homogenates of germinating barley by polyacrylamide gel electrophoresis, and the use of antibodies to the (1 → 3),(1 → 4)-β-D-glucanases to quantitate simultaneously the amount of each enzyme in the grain at different developmental stages. The presence of the two enzymes in approximately equimolar proportions and the near coincidence of their development raises the possibility that expression of the genes involved is coordinated.

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2. MATERIALS AND METHODS

2.1. *Germination conditions*

Barley (*Hordeum vulgare* 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 steeped for 24 h in sterile distilled water containing 100 μg neomycin/ml, 10 μg chloramphenicol/ml, 100 units penicillin/ml and 100 units nystatin/ml [1,9]. After steeping, seeds were blotted dry, adjusted to 44% (w/w) moisture content with sterile distilled water containing antibiotics and germinated in the dark at 15°C for up to 6 days. Moisture content was maintained at 44% (w/w) throughout. The germinated grain (100 seeds) was homogenized in 100 mM sodium acetate buffer, pH 5.0 (containing 10 mM sodium azide, 10 mM EDTA, 3 mM mercaptoethanol and 3 mM phenylmethylsulphonyl fluoride) at 4°C, and after 20 min at 4°C insoluble material was removed by centrifugation [1]. The supernatant was made up to 20 ml with the same buffer and stored at −70°C. It was noted that repeated freezing and thawing led to precipitation of enzyme II from the extracts.

2.2. *Polyacrylamide gel electrophoresis*

Protein in the extract was precipitated with 7% (w/v) trichloroacetic acid, washed twice with ethanol:ether (1:1, v/v), and redissolved for sodium dodecyl sulphate–polyacrylamide gel electrophoresis (gel thickness 2 mm, 12.5% acrylamide with 3% acrylamide stacker gel) as in [10]. Known amounts of purified (1 → 3),(1 → 4)-β-D-glucanases I and II were also applied to the gels.

2.3. *Quantitation of (1 → 3),(1 → 4)-β-D-glucanases*

Proteins separated by sodium dodecyl sulphate gel electrophoresis were electrophoretically transferred from the polyacrylamide gel slabs to nitrocellulose paper (Schleicher and Schuell, Keene NH) as in [11], using a 20 mM Tris–192 mM glycine buffer (pH 8.3) containing 20% (v/v) methanol [12] and electrophoresis conditions of 30 V (170–220 mA) for 3–5 h at 4°C. After the transfer, gels were fixed and stained with Coomassie brilliant blue® (Sigma, St Louis MO) [1] to check whether any protein remained. When required, nitrocellulose papers were stained with amido black (Calbiochem, La Jolla CA) [13].

Excess protein absorption sites on the nitrocellulose paper were quenched by incubation in Tris–saline buffer [10 mM Tris–HCl buffer (pH 7.4), 0.9% (w/v) NaCl] containing 5% (w/v) bovine serum albumin [14] for 10 h at 4°C. Quenched papers were incubated for 1.5 h at room temperature with antibody raised in rabbits against purified enzyme I. The antibody solution was prepared by dissolving the 0–40% saturated $(NH_4)_2SO_4$ precipitate of immune serum in phosphate-buffered saline (pH 7.4) at 20 mg protein/ml, and was diluted 75-fold with Tris–saline buffer containing 5% (w/v) bovine serum albumin prior to use.

Using the procedure in [14], papers were washed and probed with 1–2 μCi ^{125}I-protein A (generously provided by Dr N.J. Hoogenraad) for 1 h at room temperature, excess ^{125}I-protein A was removed by further washing and the papers blotted dry. A second paper was routinely placed against the first during electrophoretic transfer and subsequently probed to establish that binding capacities were not exceeded. Enzymes were located by autoradiography for about 16 h on Kodak X-Omat AR film (Eastman-Kodak, Rochester NY). Radioactive bands were cut out for liquid scintillation counting.

Results are expressed as pmol enzyme/grain rather than on a protein basis, since both soluble and total protein change appreciably in germinating grain during mobilization of the endosperm.

3. RESULTS

A typical nitrocellulose paper, stained with amido black to reveal the range of soluble proteins in homogenates of germinating barley, is compared in fig.1 with an autoradiogram of a paper probed with antibody to (1 → 3),(1 → 4)-β-D-glucanase I and ^{125}I-protein A. In addition to the two enzyme bands, faint radioactive bands of higher M_r-values are detected on the autoradiogram, especially in ungerminated grain and during the first two days of germination. Significantly larger volumes of grain extract from these early stages of germination were applied to the gels in attempts to detect relatively low levels of (1 → 3), (1 → 4)-β-D-glucanases (fig.1), but the origin of the

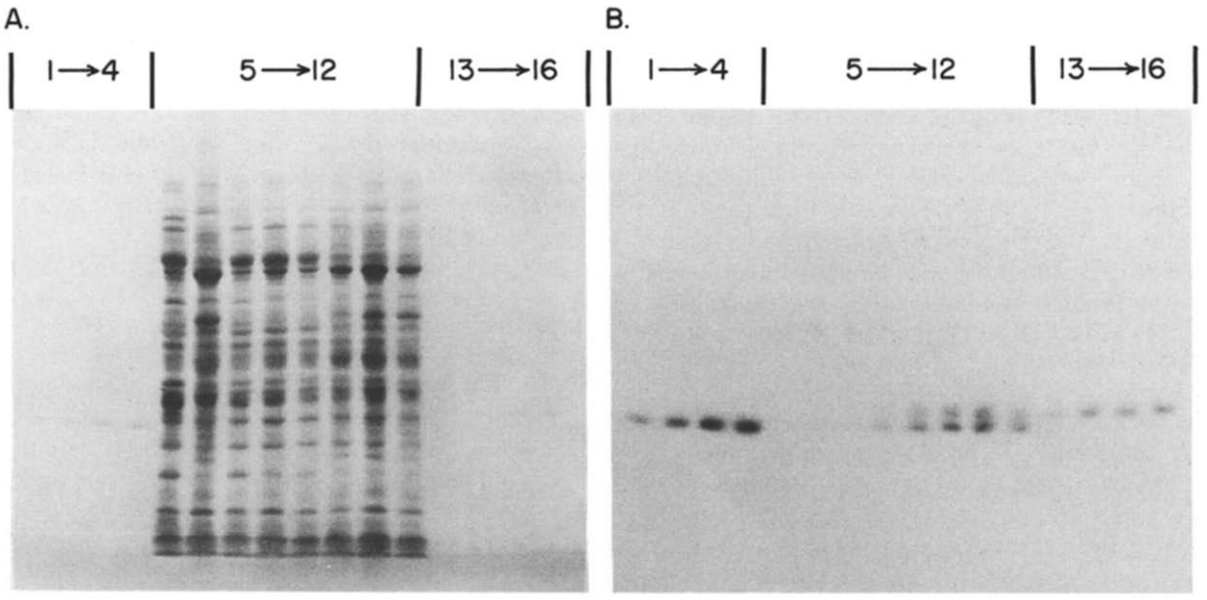

Fig.1. Separation and immunological detection of (1 → 3),(1 → 4)-β-D-glucanases in homogenates of germinating barley: (A) nitrocellulose paper stained with amido black; (B) autoradiogram of a nitrocellulose paper probed with antibody to enzyme I and ^{125}I-protein A. In both cases lanes 1–4 contain purified enzyme I standards (160–640 ng); lane 5 contains the soluble extract (60 μl) of unsteeped, ungerminated grain; lane 6 is steeped but ungerminated grain (80 μl extract); lanes 7–12 are from germinating barley extracted at 1 and 2 days (50 μl extract) and 3–6 days (30 μl extract), respectively; lanes 13–16 are purified enzyme II standards (200–750 ng).

higher M_r bands and whether they bear any relationship with the (1 → 3),(1 → 4)-β-D-glucanases remains unclear. One possibility is that they originate from antibodies directed to traces of contaminating protein in the purified (1 → 3),(1 → 4)-β-D-glucanase preparations [1].

The development of the two enzymes during germination is shown in fig.2. The antibody used to probe the nitrocellulose papers was raised against enzyme I and the greater affinity of this antibody for enzyme I is reflected in the standards (fig.2, inset). However, in both cases the amount of ^{125}I-protein A bound to each antigen–antibody complex increased linearly with the amount of enzyme applied to the gel [15] up to about 15 pmol enzyme I and 30 pmol enzyme II (fig.2), after which enzyme was detected on the second nitrocellulose paper. Thus the binding capacity of a single paper was about 0.5 μg for enzyme I and 1 μg for enzyme II. The difference is presumably related to differences in the charge and hydrophobicity of the two proteins [1,16]. Consequently, 0–10 pmol enzyme was loaded onto gels in homogenates.

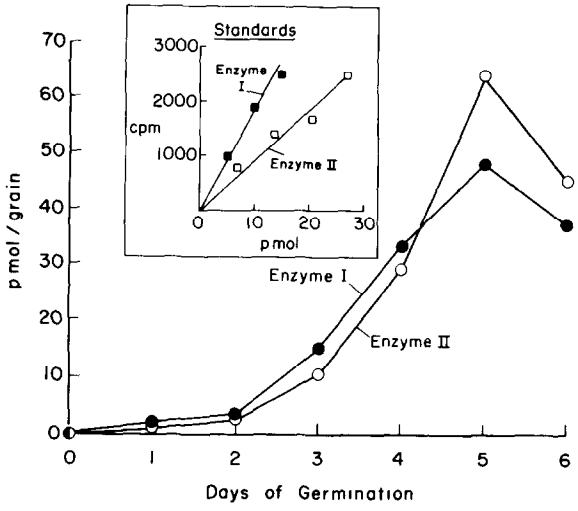

Fig.2. Development of (1 → 3),(1 → 4)-β-D-glucanases in germinating barley: (●) enzyme I; (○) enzyme II. The inset shows standard curves for enzyme I (■) and enzyme II (□) after probing nitrocellulose papers with antibodies to enzyme I and ^{125}I-protein A.

4. DISCUSSION

The difference in M_r-value between barley (1 → 3),(1 → 4)-β-D-glucanases I and II [1] enables the enzymes to be separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis and, following electrophoretic transfer to nitrocellulose paper, the enzymes may be individually quantitated to 0–10 pmol (sub-μg) using the crossreactive antibodies raised against either enzyme I or II [1]. Plant breeders or maltsters might therefore find an application for the procedure in the measurement of total, potential (1 → 3),(1 → 4)-β-D-glucanase in samples of germinating barley containing as few as 5 grains.

Although the amount of enzymic protein in the homogenates is relatively low until after 2 days germination, the two barley (1 → 3),(1 → 4)-β-D-glucanases develop in approximately equimolar proportions thereafter (fig.2). These results are consistent with the appearance of total enzyme activity as measured by viscometric assays with barley (1 → 3),(1 → 4)-β-D-glucan as substrate [5,6]. Since the antibodies would be expected to bind to zymogen forms of the enzymes and no (1 → 3),(1 → 4)-β-D-glucanase protein was extracted from ungerminated grain, the developmental patterns shown in fig.2 are compatible with de novo synthesis of the two enzymes after the initiation of germination. However, the possibility that the enzymes pre-exist in ungerminated grain as insoluble, protein-bound precursors, in a manner analogous to the cereal β-amylases [17], cannot be ruled out.

If the (1 → 3),(1 → 4)-β-D-glucanases are indeed synthesized de novo in germinating grain, their development in approximately equimolar amounts might further suggest that expression of the two genes is co-ordinated by a single regulatory process. Since information concerning the rates of synthesis, processing, translation and degradation of mRNA for the two enzymes is not available, the specific nature of the regulation can not yet be assessed.

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