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ON THE LOCALISATION AND TRANSPORT OF α-AMYLASE DURING GERMINATION AND EARLY SEEDLING GROWTH OF HORDEUM VULGARE

by

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A procedure for the isolation of a germination-specific α-amylase isoenzyme from Hordeum vulgare (cv. Nordal) is described. The pure α-amylase isoenzyme was used to obtain a monospecific anti-α-amylase antibody. The antibody was subsequently used to localise α-amylase in sections of germinating barley seeds (cv. Nordal). Immunohistochemical determination of the pattern of enzyme movement during germination and early seedling growth revealed that at the commencement of germination the α-amylase protein moiety moves away from the entire face of the scutellum and intermediate crushed cell layer. As early seedling growth continues, the protein begins to be synthesised in the aleurone layer and transported away from this layer into the endosperm.

1. INTRODUCTION

The localisation and transport of hydrolytic enzymes in seeds of germinating barley (Hordeum vulgare L.) have both been widely investigated. Although as early as 1886 (21) certain theories were available to explain the biochemical changes occurring in germinating seeds of the Gramineæ, the processes have as yet not been fully explained. Certain fundamental questions, such as the role of the scutellum contra the role of the aleurone in the production of hydrolases during germination and early seedling growth, are still under discussion (18). The advent of immunohistochemical techniques suitable for use with plant tissues has allowed reexamination of the localisation and transport mechanisms in vivo rather than in isolated tissues, tissue extracts, or seeds with defined physical damage.

The experiments reported in the present work were designed to investigate the localisation and transport of one of the major hydrolytic enzymes produced during germination – the 1,4-α-D-glucan hydrolase – α-amylase (E C 3.2.1.1.) in germinating seeds of Hordeum vulgare L. cv. Nordal. Nordal was chosen as it is one of the major malting barleys in Denmark.

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2. MATERIALS AND METHODS

2.1. Chemicals

Agarose type HSB was obtained from Litex, Glostrup, Denmark. Epoxy-activated sepharose 6B and Phadebas α-amylase test kit were obtained from Pharmacia AB, Uppsala, Sweden. Blue-starch substrate was a gift from Pharmacia Diagnostica AB, Uppsala, Sweden. Schardinger-β-dextrin (β-cyclodextrin, cycloheptaamylose) and oyster glycogen were obtained from Sigma, St. Louis, USA. Anti-rabbit antibodies from swine and FITC-labelled anti-rabbit IgG antibodies from swine were obtained from Dakopatts A/S, Denmark. Monospecific anti-barley-β-amylase was a kind gift from Mr. E. D. Lund and Dr. J. Hejgaard, The Danish Polytechnical University, Lundtofte, Denmark. Freund's complete and incomplete adjuvans were obtained from Behringwerke, Marburg, Germany. β-limit dextrin was prepared from Linter starch (E. Merck, Darmstadt, Germany) by the method of UMEKI and YAMAMOTO (22). β-amylase (α-amylase free) was obtained from Serva, Heidelberg, Germany. The resulting β-limit dextrin was kindly analysed on the Sacchromat by Drs. B.S. ENEVOLDSEN and F. SCHMIDT using the method of SCHMIDT and ENEVOLDSEN (19). Sacchromat analysis revealed that the product contained 83% β-limit dextrin components.

Purified glutaraldehyde was the kind gift of Mr. E. LUND (Polytechnical University, Denmark) and of Drs. S. RASMUSSEN and P. HYDE (Carlsberg Laboratory, Denmark).

All other chemicals were Analytical Reagent grade.

2.2. Plant Material

Seeds of Hordeum vulgare L. (cv. Nordal) were obtained from Dr. J. LARSEN, Carlsberg Plant Breeding, Denmark. Barley green-malt was prepared on a rotating-drum laboratory unit. Seeds were steeped for 48 hours with two aeration periods of 8 hours, followed by 7 d of germination in the rotating drum unit at 15 °C and 98% r.h.

Germinating seed material for the immunochemical localisation experiments was obtained by pressing seeds into moist sand and germinating at 15 °C (98% relative humidity) for the appropriate time.

2.3. Immunoelectrophoresis

Rocket immunoelectrophoresis and crossed immunoelectrophoresis were performed according to WEEKE (23) using an electrophoresis buffer containing 0.073 M-Tris/HCl 0.024 M-diethylbarbituric acid and 0.00035 M-Ca-lactate (final pH 8.6). Gels were directly stained in coomassie blue R 250 according to WEEKE (23), except in the cases where amylase measurement was carried out using the method of HEJGAARD (7).

2.4. Measurement of amylase isoenzymes

Samples of column fractions were loaded into 20 µl rectangular wells on a 1.5 × 100 × 100 mm agarose gel containing electrophoresis buffer (see 2.3). The gel was subjected to a voltage of 12 $V \cdot cm^{-1}$ for 1 hour. After drying in a cold air stream the gels were placed in a solution containing 1% lintner starch in 0.02 M-Na acetate buffer pH 5.5 for 1 hour at 37 °C. The gel was stained in a solution containing 0.008 M-I_2 and 0.5 M-KI for 30 sec. The amylase activity was identified as a white zone on the pinkish-brown background.

2.5. Purification of α-amylase

Barley α-amylase was purified using the principles of SILVANOVICH and HILL (20) with several modifications as follows: Barley green-malt (cv. Nordal) was freeze-dried and milled on a Udy Cyclotec laboratory mill using a 1 mm sieve (Tecator AB, Sweden). The resulting flour was extracted by stirring in 1:3/w:v 0.2 M-Na-acetate buffer, pH 5.5 containing 0.001 M-$CaCl_2$ for 1 hour at 4 °C. Following centrifugation at 16.000 × g for 15 min at 0 °C the supernatant was filtered through 4 layers of Miracloth (Calbiochem, USA). Ethanol was added to 40% v:v and the mixture was stirred for 1 hour at 4 °C. After centrifugation at 19.000 × g for 30 min at 0 °C, the supernatant was treated with 2% w:v aqueous oyster glycogen to a final concentration of 1.88 $mg \cdot ml^{-1}$ supernatant. The ethanol concentration was readjusted to 40% v:v and following stirring for 1 hour at 4 °C, the glycogen precipitated crude α-amylase was collected by centrifugation at 48.000 × g, for 15 min at 0 °C. The precipitate was dissolved in a

small volume of extraction buffer and dialysed against the same buffer overnight at 4 °C. Undissolved material was removed by centrifugation (48.000 × g 15 min, 0 °C) and the supernatant applied to a column of cycloheptaamylose-substituted epoxy-activated sepharose 6B (prepared as described by SILVANOVICH and HILL (20)). Non-bound protein was removed from the column by washing in 0.2 M-Na acetate buffer, pH 5.5, containing 0.001 M-$CaCl_2$. The αII isoenzyme was eluted in 10 mg · ml^{-1} β-limit dextrin which was predissolved in boiling water and then added to concentrated buffer in order to obtain the desired final concentration. The α-amylase remaining on the column was eluted with 8 mg · ml^{-1} cycloheptaamylose in elution buffer. Following each run the column was washed in 1.5 M-NaCl in water and reequilibrated in start buffer.

Fractions were monitored at 280 nm for protein. α-amylase activity was measured by either the Phadebas α-amylase test and expressed as the absorbance at 620 nm (see 2.1) or the blue-starch gel diffusion assay of HEJGAARD and GIBBONS in which α-amylase activity is expressed in K(α) units (8). Selected fractions were subjected to crossed immunoelectrophoresis using anti-barley green-malt antibodies (see 2.6) or to isoenzyme analysis (see 2.4).

2.6. Preparation of antibodies

Antibodies were prepared towards total-soluble barley green-malt proteins and αII α-amylase. The antigen for total-soluble barley green-malt was prepared by grinding 20 g lyophilised green-malt (cv. Nordal) in 90 ml 0.05 M-phosphate buffer, pH 7 containing 0.5 M-NaCl for 30 min at 4 °C. Following centrifugation at 12.000 × g for 20 min the soluble supernatant was extensively dialysed against distilled water and lyophylised. The αII α-amylase antigen was obtained by extensive dialysis of β-limit dextrin eluted α-amylase (see 2.5) against distilled water followed by lyophilisation.

Antigen was dissolved in sterile aqueous 0.15 M-NaCl and mixed with an equal volume of Freunds complete adjuvans immediately prior to the first immunisation. The final concentration of antigen was 2 mg · ml^{-1} for total-green-malt and 1 mg · ml^{-1} for the αII α-amylase. The rabbits received 1 ml of the antigen/adjuvans mixture in 5 dorsal subcutaneous depots of 0.2 ml. After 14 days the immunisation was repeated, this time using Freunds incomplete adjuvans. This procedure was repeated after a further 14 days, and at 2 months after the first immunisation. At 2 months and 14 days the rabbits were tapped from the ear vein and the antibody titre evaluated by rocket immunoelectrophoresis (see 2.3). Rabbits that showed a response to antigen were then regularly immunised at 1 month's intervals and tapped 10 to 14 days after the booster immunisation.

Blood was allowed to coagulate at room temperature for 3–6 hours and after centrifugation at 10,000 × g for 20 min, the serum was decanted and $(NH_4)_2SO_4$ added to a concentration of 1.8 M. The $(NH_4)_2SO_4$-treated serum was stirred overnight at 20 °C and the centrifuged-precipitated protein dialysed overnight against 0.1 M-NaCl. After removal of undissolved protein, the immunoglobulin (IgG) fraction was purified by further dialysis and DEAE sephadex A-50 chromatography as described by HARBOE and INGILD (6).

Purified antibodies were tested by crossed immunoelectrophoresis against the appropriate antigen (see 2.3). Antibodies were stored at −25 °C.

Swine IgG was prepared from fresh swine blood using the technique described above for rabbit antibodies.

2.7. Tissue preparation

In fixation experiments the following fixation media were used: a) 2%, 4% and 10% periodate/lysine/paraformaldehyde (PLP) prepared as described by MCLEAN and NAKANE (14), b) 2%, 4%, 10% and 20% purified glutaraldehyde in 0.1 M-phosphate buffer, pH 7.4.

Fixation was carried out at 5 °C for the appropriate time, followed by washing for 6–8 hours in 0.01 Na phosphate buffer, pH 7.4 containing 0.15 M-NaCl (PBS). The fixed and washed seeds were frozen in liquid freon 22 in a liquid nitrogen bath for 90 sec and stored at −80 °C prior to sectioning.

Median longitudinal sections (15 μ) of ungerminated and germinating seeds were made on a cryostat after embedding the seeds in Tissue Tek

II (Miles Laboratories, USA). The sections were transferred to moist 1% gelatine-coated slides and fastened to the slides by drying in vacuo as described by LUND (13).

2.8. Immunohistochemical techniques

The immunohistochemical techniques used are based upon the methods devised for maturing barley kernels by LUND (13). The fixed sections were washed for 30 min in PBS (see 2.7) and treated for a further 30 min in swine IgG (see 2.6) in order to reduce non-specific binding of fluorescent antibody. The sections were then allowed to react with the appropriate antibody for 30 min. Control sections were treated with pre-immune serum IgG or a standard control rabbit IgG preparation from Dakopatts, Denmark, for the same time. The concentration of IgG used with the control sections was chosen so as to match the IgG concentration in the antigen-specific-antibody treated sections by rocket immunoelectrophoresis against a swine anti-rabbit antibody. Following specific-antibody treatment the sections were briefly washed in distilled water and allowed to stand in PBS for 30 min. Sections were then treated with fluorescein isothiocyanate (FITC) labelled anti-rabbit IgG for 30 min. Following a brief washing in distilled water and 10–15 min in PBS the sections were mounted in pH 7.4 buffered fluorescence-free glycerine.

Photomicroscopy was carried out on a Reichert Univar Microscope using epi-illumination, with selected low-fluorescence objectives (Reichert, Vienna, Austria). The exiter filter, dichroic mirror and barrier filter were designed to give maximal FITC fluorescence with minimal auto-fluorescence of the barley aleurone layer, and were custom-made by Optisk Laboratorium, Lundtofte, Denmark, as described in Results 3.4.

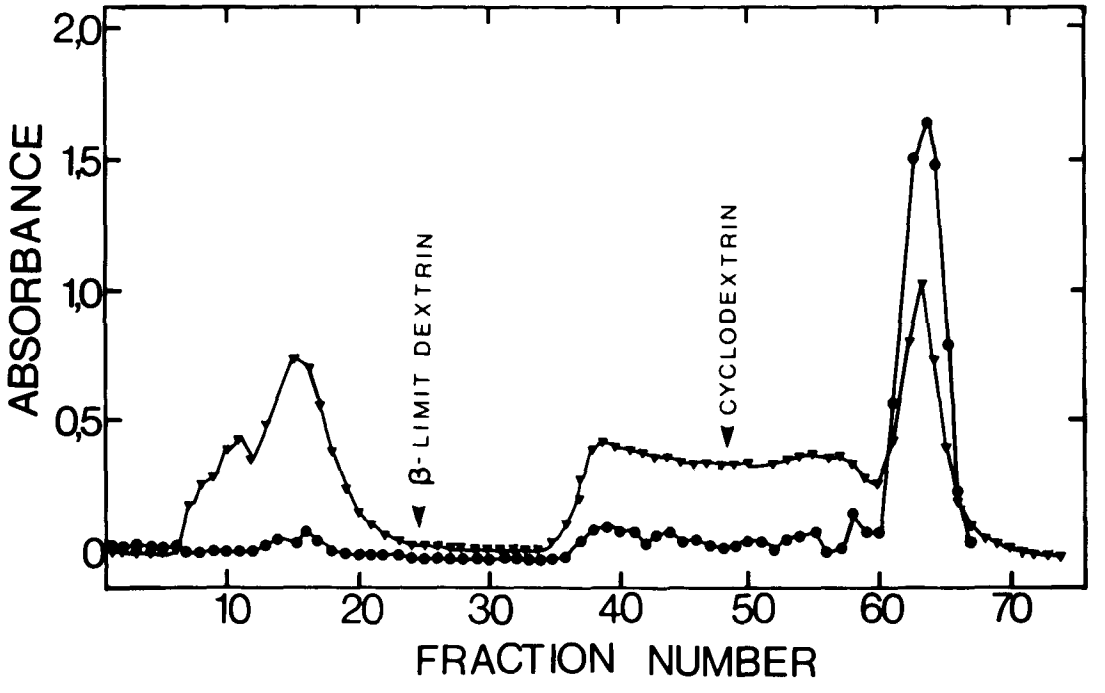


Figure 1. Separation of barley α-amylase by affinity chromatography.

The method is described in Methods 2.5. Starting material was 100 g freeze-dried barley green-malt (cv. Nordal). The column (2.6 × 7.5 cm) was loaded with 18 ml crude α-amylase and eluted at 50 ml·hour^{-1}. 5 ml fractions were collected and measured for protein (●) at 280 nm in 1 cm cuvettes. α-amylase activity (▼) was measured on 0.01 ml aliquots of each fraction by the Phadebas method and expressed as the absorbance at 620 nm in a 0.1 cm cuvette. β-limit dextrin and β-cyclodextrin (cycloheptaamylose) elution began at the points indicated in the figure.

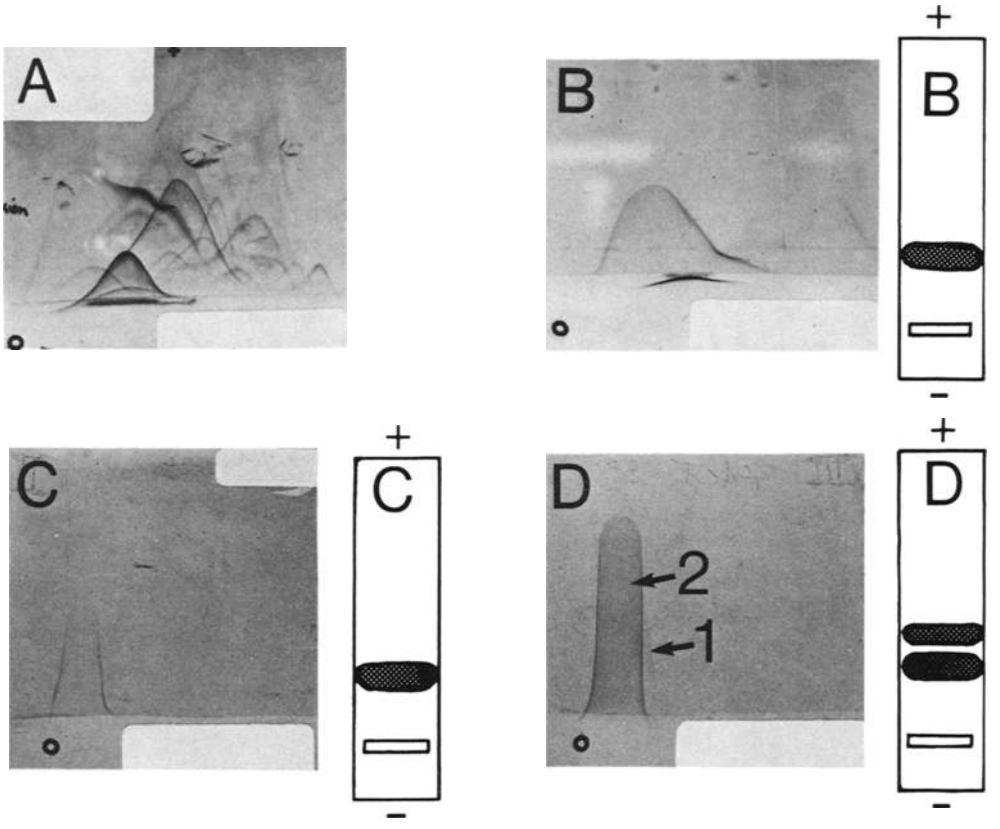


Figure 2. Immunoelectrophoretic and amylase isoenzyme characterisation of fractions following affinity chromatography.

Green-malt extract (2A) and fractions 15 (2B), 38 (2C) and 64 (2D) of the affinity chromatography separation shown in Figure 1 were subjected to crossed immunoelectrophoresis against total-soluble barley green-malt antibodies (sections 2.3 and 2.6). Fractions 15, 38 and 64 were also analysed for amylase-isoenzymes (section 2.4). In Figure 2D the position of the heavily stained αII isoenzyme and the weaker αI isoenzyme are indicated.

3. RESULTS AND DISCUSSIONS

3.1. Purification of α-amylase

α-amylase was purified from green-malt of Hordeum vulgare-cv. Nordal as described under Materials and Methods (2.5). Cycloheptaamylose affinity chromatography of the glycogen precipitated α-amylase (Figure 1) resulted in an initial peak of unbound-protein containing a low level of α-amylase activity. The presence of α-amylase activity in this fraction could be explained by a slight overloading effect – as application of a smaller amount of the glycogen-precipitated crude α-amylase to the column completely abolished anzyme activity in this unbound-protein fraction.

Crossed immunoelectrophoresis against an anti-green-malt antibody was performed on each of the major column fractions. The starting material for the α-amylase purification is shown in Figure 2A. Immunochemical analysis of the unbound-protein fraction revealed the presence of at least 5 proteins lacking α-amylase activity (Figure 2B). This result strongly indicates that glycogen-precipitation, a highly selective technique for the purification of α-amylase from animal and human tissue (12), cannot directly be used for the preparation of pure α-amylase with germinated barley. JACOBSEN and KNOX (10) claimed that if the starting material for α-amylase purification was gibberellic acid-induced proteins secreted from isolated aleurone layers of H. vulgare cv. Himalaya, an α-amylase could be

purified by glycogen precipitation that, when injected into rabbits, induced formation of antibodies which failed to cross-react with other gibberellic acid-induced barley proteins. As to whether the antibody cross-reacted to non-gibberellic acid-induced barley proteins (i.e. endosperm, aleurone or embryonic reserve and membrane proteins) was not discussed by these authors.

Following thorough washing of the column with starting buffer (see 2.5), β-limit dextrin (10 mg · ml^{-1}) was applied to the column. β-limit dextrin elution resulted in removal of a protein fraction which exhibited α-amylase activity. Immunochemical analysis of this fraction (Figure 2C) showed the presence of only one protein which reacted against anti-barley-green-malt antibodies. This protein showed strong activity when the immunogel was tested for amylase activity as described in section 2.4. Isoenzyme analysis (Figure 2C) revealed that, based on electrophoretic mobility, this α-amylase fraction fell into the αII or »germination« α-amylase isoenzyme group of OLERED and JÖNSSON (17). It has been well established (see reviews by BRIGGS (1) and PALMER (18)) that the αII group of α-amylase isoenzymes – although separable into several components by isoelectric focussing – is synthesized de novo during germination and early seedling growth of barley. All the αII α-amylase could not be eluted with β-limit dextrin (Figure 1), and the remaining αII α-amylase was eluted together with the αI-isoenzyme of α-amylase when the column was treated with cycloheptaamylose at a concentration of 8 mg · ml^{-1} (Figure 1, Figure 2D). This αI-isoenzyme is similar to the »green« isoenzyme of OLERED and JÖNSSON (17), which is found both in immature and germinating seeds. Although the work of BØG-HANSEN and DAUSSANT (3) showed that both the αII- and αI-isoenzymes of barley α-amylase increased during germination and early seedling growth, it was clearly shown by these authors that the αII group was the major α-amylase species under these conditions.

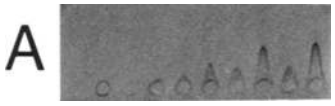
The preferential elution of the αII form of barley α-amylase with β-limit dextrin indicates that this form of the enzyme is less inhibited by cycloheptaamylose than the αI-form. This result is interesting to compare with the data of HILL and SILVANOVICH (9) who found that in the case of Triticale species, the percentage inhibition for the »germination« α-amylase (α-II) was greater than for the »green« α-amylase (α-I) at a given concentration of cycloheptaamylose. Species differences in the behaviour of the different classes of α-amylase are not however implausible.

3.2. Production of monospecific anti- αII α-amylase

As the αII form of α-amylase had been shown both to be the major α-amylase component in germinating barley, and to be synthesized de novo during germination and early seedling growth, it was decided to limit these initial experiments to localisation of this αII-form of the enzyme. Rabbits were immunised with purified αII α-amylase and the immunoglobulin G (IgG) fraction purified as described in section 2.6. In order to test the monospecificity of the antibody preparation it was necessary to show that neither did the antibody react with other soluble proteins of germinated Nordal barley, nor did it react with membrane-bound proteins, lectins, or carbohydrates of the same tissue. Only after these criteria were fulfilled could the anti-αII α-amylase antibody be successfully used in immunohistochemical studies.

The monospecificity of the antibody towards the soluble proteins of mature and germinating Nordal barley was confirmed by both rocket immunoelectrophoresis (Figures 3A and 3B) and by crossed immunoelectrophoresis (not shown).

The mature barley extract gave no immunoprecitate, either on rocket immunoelectrophoresis or crossed immunoelectrophoresis, while the germinated material showed the development of both αII α-amylase antigen activity (Figure 3A) and α-amylase activity as measured either by the blue-starch diffusion method (see section 2.5) or by starch digestion (Figure 3B). The onset of αII α-amylase production was first here measurable between 2 and 3 days of germination and it was clearly seen that only one soluble protein of the total soluble extract was reacting towards the anti-αII α-amylase antibody. This result was confirmed when soluble extracts of 7-day germinated Nordal barley were subjected to crossed immunoelectrophoresis against the αII-antibody. It is of interest to note the relative



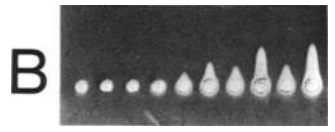


Figure 3. Development of α-II α-amylase (Figure 3A) and total amylase activity (Figure 3B) during germination and early seedling growth of barley (cv. Nordal).

Seeds of mature barley and of 2, 3, 5 and 6 day germinated material were halved and the distal and embryo ends of the seed homogenised into 0.5 ml PBS buffer (section 2.7). 10 µl aliquots of the total extract were applied to 4 mm wells of a 1.5 × 100 × 100 mm gel containing 200 µl anti-αII α-amylase and subjected to rocket immunoelectrophoresis at a voltage of 1 $v \cdot cm^{-1}$ for 18 hours. The gels were first stained for amylase activity and then for protein as described in section 2.3. From left to right in both Figures 3A and 3B: Mature barley distal half; mature barley embryo half; 2 day germinated distal half; 2 day germinated embryo half; 3 day distal half; 3 day embryo half; 5 day distal half; 5 day embryo half; 6 day distal half; 6 day embryo half.

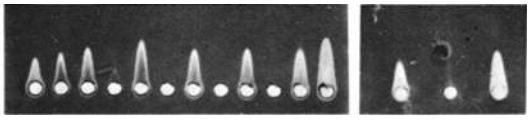


Figure 4. Effect of fixation on the release of αII α-amylase (Figure 4A) and amylase enzyme activity (Figure 4B) in 7 day germinated seeds of barley (cv. Nordal).

Germinated seeds were either fixed directly or slightly nicked with a scalpel blade and fixed. The fixation media are described in section 2.7. Following fixation for 22 hours, the seeds were first washed and then thoroughly homogenised in 1 ml of PBS and assayed for αII α-amylase and amylase enzyme activity as described for Figure 3, except that the anti-αII α-amylase content in the gel was lowered to 75 µl. Due to the number of samples it was necessary to assay the samples on two separate plates. From left to right: First gel: 2% PLP, whole seed; 2% PLP, nicked seed; 10% PLP, whole seed; 10% PLP, nicked seed; 20% glutaraldehyde, whole seed; 20% glutaraldehyde, nicked seed; 10% glutaraldehyde, whole seed; 10% glutaraldehyde, nicked seed; 4% glutaraldehyde, whole seed; 4% glutaraldehyde, nicked seed; 2% glutaraldehyde, whole seed; untreated seed; second gel: 2% glutaraldehyde, whole seed; 2% glutaraldehyde, nicked seed; untreated seed.

production of the enzyme in the embryo half of the seed was greater than in the distal end of the seed at all times. The amylase activity seen on the immuno gels corresponded exactly to the immunoprecipitate (Figures 3A and 3B).

The lack of reaction of the anti-αII α-amylase antibody towards membrane proteins, lectins, or carbohydrates is clearly seen in Figure 5C where the antibody failed to react with mature seeds of Nordal barley.

3.3. Fixation of tissue

As α-amylase is a water-soluble enzyme, it was necessary to fix the protein within the tissue. Immunohistochemical techniques involve a series of aqueous washes followed by antibody treatment, and therefore, if soluble proteins are not fixed, they can be removed from the sections before the antigen can be precipitated by antibody. The positive results on developing barley kernels of LUND (13) using the periodate-lysine-paraformaldehyde fixative of MCLEAN and NAKANE (14), and the fact that barley seeds contain large amounts of carbohydrate prompted use of this technique. A series of fixations were carried out to determine optimum times and concentrations of various fixatives. Preliminary experiments showed that at the recommended 2% paraformaldehyde concentration αII α-amylase was not fixed in germinating seeds of Nordal barley (see Methods 2.7). When concentration of paraformaldehyde was increased to 10% the fixative was effective in fixing the bulk of the αII α-amylase (Figures 4A and 4B) without significantly affecting the antigenicity of

the protein in the fixed tissue. Glutaraldehyde, even at the lowest concentration, showed reduced antigenicity of the αII α-amylase and inferior structural preservation of the carbohydrate-rich germinating barley. It was necessary however, in the case of both paraformaldehyde and glutaraldehyde to slightly abrade or nick the sides of the seed to facilitate entry of the fixative (Figures 4A and 4B).

Following a series of trials, the optimum fixation time with 10% periodate-lysine-paraformaldehyde was determined to be 18 hours at 5 °C in a shaking water bath. Following fixation, the seeds were washed for 6–8 hours in ice cold phosphate buffered saline (PBS) and frozen as described in section 2.7. The freezing technique used resulted in minimum damage to the tissue by ice crystal formation.

3.4. Localisation of α-amylase

Fluorescein isothiocyanate (FITC) conjugated antibodies are usually localised microscopically using a filter combination with an exciter filter open up to 490 nm, a dichroic beam-splitter at 500 nm and a barrier filter at 515–520. Due to the autofluorescence characteristics of the aleurone-layer of barley (MUNCK, FEIL and GIBBONS, (15)) it was found that using a standard FITC filter combination the aleurone layer autofluorescence was relatively strong and difficult to discern from that of FITC. A new filter combination was therefore designed which, by only exciting between 450 to 495 nm, eliminated the light which normally would excite the aleurone layer. Due to the resulting loss of the 434.9 nm mercury line in the excitation beam it was necessary to increase the transmission and sharpen the cutoff of both the dichroic beam-splitter and barrier filter. The resultant optimised dichroic beam-splitter showed 10% transmission at 500 nm, 50% transmission at 518 nm and 90% transmission at 528. The new barrier filter showed 10% transmission at 510 nm, 50% transmission at 519 nm and 91% transmission at 523 nm. The optimised filter combination resulted in an elimination of the major part of aleurone autofluorescence as seen in Figure 5A where only the yellow-green autofluorescence of the outer integuments can be seen.

Following a series of experiments in which seeds of germinating Nordal barley were cut into 6 pieces along different axes and the α-amylase activity measured by the blue-starch gel-diffusion assay in each piece, it was found that the α-amylase activity was most concentrated in the area immediately above the embryo and declined as the distal end of the seed was approached. For this reason it was decided that the greatest amount of information about the transport of α-amylase could be obtained using median longitudinal sections. It should be clearly stated, however, that although such sections could be extremely useful, the complete pattern of α-amylase transport can only be obtained by serial-sectioning followed by three-dimensional reconstruction of the entire germinating seed.

The results of treating median longtudinal sections of germinating Nordal barley with monospecific anti-αII α-amylase antibodies are shown in Figure 5C–5H. In the ungerminated grain (Figure 5C) αII α-amylase is absent. The yellow-green autofluorescence of the outer seed integuments corresponds to that seen in the control-antibody treatment (Figure 5A). This result is entirely consistent with previous findings (BRIGGS (1), BØG HANSEN and DAUSSANT (3), PALMER (18)). In contrast, β-amylase is found throughout the endosperm of the ungerminated grain (Figure 5B) as previously discussed by LINDERSTRØM-LANG and ENGEL (11). Immunochemical localisation of β-amylase in the endosperm of mature barley has been clearly shown by LUND (13).

After 30 hours of germination αII α-amylase is seen in the endosperm tissue immediately outside the scutellum (Figure 5D). The protein appears to move more rapidly away from the ventral region of the scutellum (i.e. distal to the scutellar vascular elements) indicating either a higher concentration of αII α-amylase or a facilitated transport of the protein in this region. A high degree of anti-αII α-amylase binding was observed in the intermediate layer – the depleted and compressed layer of cells occurring between the scutellar epithelium and the endosperm. The high concentration of αII α-amylase in this layer is seen throughout germination and early seedling growth up to 7 days (Figure 6B). The cells of this intermediate layer are apparently not capable of active metabolism as they lack any visible cell

content. They contain large amounts of residual cell wall material when stained with calcofluor or congo red and are, as such, possibly involved in funneling αII α-amylase out from its primary synthetic site.

The pattern of αII α-amylase movement after 52 hours germination (Figure 5E) indicates an outward flow of the protein in a broad front away from the scutellum. The aleurone layer at this time contains no measureable αII α-amylase antigen. After a further 24 hours αII α-amylase has filled approximately 1/3 of the endosperm (Figure 5F). The protein continues to migrate more rapidly in the ventral half of the seed, while the aleurone layer shows now a slight amylase production as can be seen by the increased level of αII α-amylase in the aleurone cells proximate to the scutellum.

After 5 days the αII α-amylase has filled over 80% of the endosperm (Figure 5G). At this stage it is now possible to observe considerable amounts of αII α-amylase in the aleurone layer. Figure 6A is a higher magnification of the aleurone layer and endosperm in an area close to the distal end of the seed. The protein is seen to first occur to the aleurone and subsequently in the endosperm. The darker (= distal) area of the micrograph is the area where the antigen is not present and it can be seen that the aleurone layer above this darker area reacts to the anti-αII α-amylase.

The αII α-amylase protein is distributed throughout the entire endosperm after 7 days (Figure 5H). By this time the entire aleurone layer is also filled with the protein. Figure 6C is a higher magnification of the aleurone of a 7-day germinated seed, showing the presence of αII α-amylase within the aleurone cells and a higher concentration of αII α-amylase in the sub-aleurone layer than in the inner endosperm. The lack of immunofluorescence in the aleurone layer in sections treated with anti-β-amylase (Figure 6D) illustrates the radical differences in distribution of these two proteins. β-amylase is evenly distributed in the endosperm and the only visible fluorescence in the aleurone cells is the yellow-green autofluorescence of the aleurone grains. A higher magnification micrograph of the scutellar/endosperm interface (Figure 6B) reveals the presence of localised areas of αII α-amylase in the scutellar parenchyma cells and a high concentration of the protein in the tips of the scutellar epithelial cells as well as in the intermediate layer of crushed cells.

The results obtained from immunohistochemical examination of the transport of αII α-amylase in median longitudinal sections of germinating Nordal barley can be summarised as follows. In the early stages of germination (0–3 days) the αII α-amylase protein appears to be synthesized at a site near to, or within, the scutellar parenchyma. αII α-amylase is subse-

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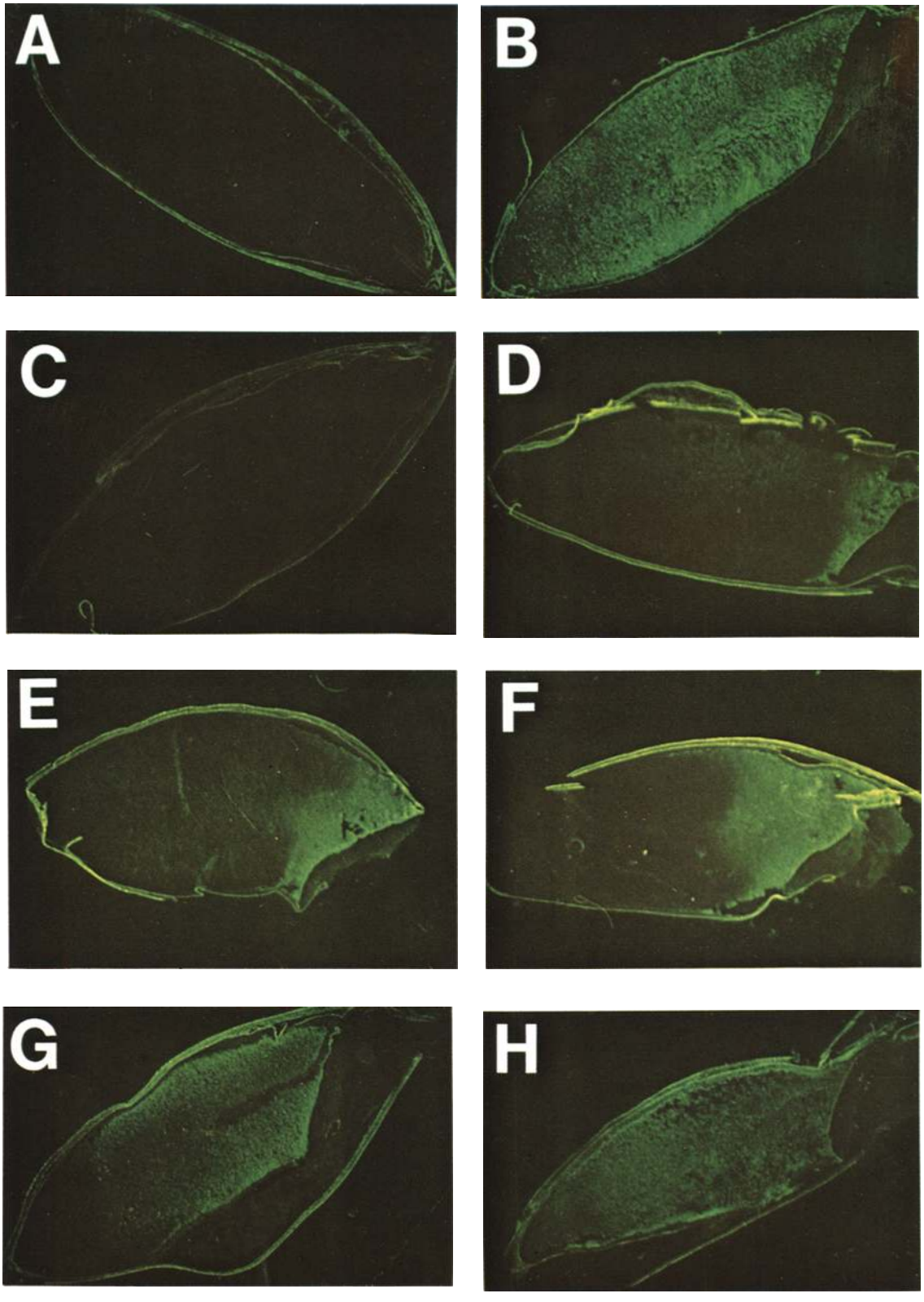
Figure 5. Immunohistochemical localisation of αII α-amylase and β-amylase in mature and germinating seeds of barley (cv. Nordal).

Seeds were fixed in 10% PLP and treated with a 1/10 dilution (in PBS) of the appropriate antiserum as described in section 2.8. In all photomicrographs the sections are shown with the embryo and scutellar tissue on the right hand side, and the ventral side of the seed at the top of the picture. Figure 5A. Mature grain, treated with preimmune (control) antibody. Figure 5B. Mature grain, treated with monospecific anti-β-amylase antibody. Figure 5C. Mature grain, treated with monospecific anti-αII α-amylase antibody. Figure 5D. 30 hour germinated, anti-αII α-amylase antibody. Figure 5E. 52 hour germinated, anti-αII α-amylase antibody. Figure 5F. 76 hour germinated, anti-αII α-amylase. Figure 5G. 5 day germinated anti-αII α-amylase antibody. Figure 5H. 7 day germinated anti-αII α-amylase antibody. In all photomicrographs the magnification is 10X.

Figure 6. Immunohistochemical localisation of amylases during germination and early seedling growth of barley (cv. Nordal).

Figure 6A. Detail of aleurone-mediated αII α-amylase production after 5 days germination. The photomicrograph shows the aleurone (al) and endosperm (en) directly below it, towards the distal end of the seed (d). 100 × magnification, anti-αII α-amylase antibody. Figure 6B. Detail micrograph of the scutellum (sp-scutellar parenchyma; se-scutellar epithelium), intermediate layer of crushed cells (il) and proximate endospermal tissue (en). 7 days germination. 250 × magnification, anti-αII α-amylase antibody. Figure 6C. Detail micrograph of the aleurone (al) and sub-aleurone area of the endosperm (en) after 7 days germination. 250 × magnification, anti-αII α-amylase antibody. Figure 6D. Detail micrograph of the same area shown in Figure 6C. 7 days germination. 250 × magnification, anti β-amylase antibody.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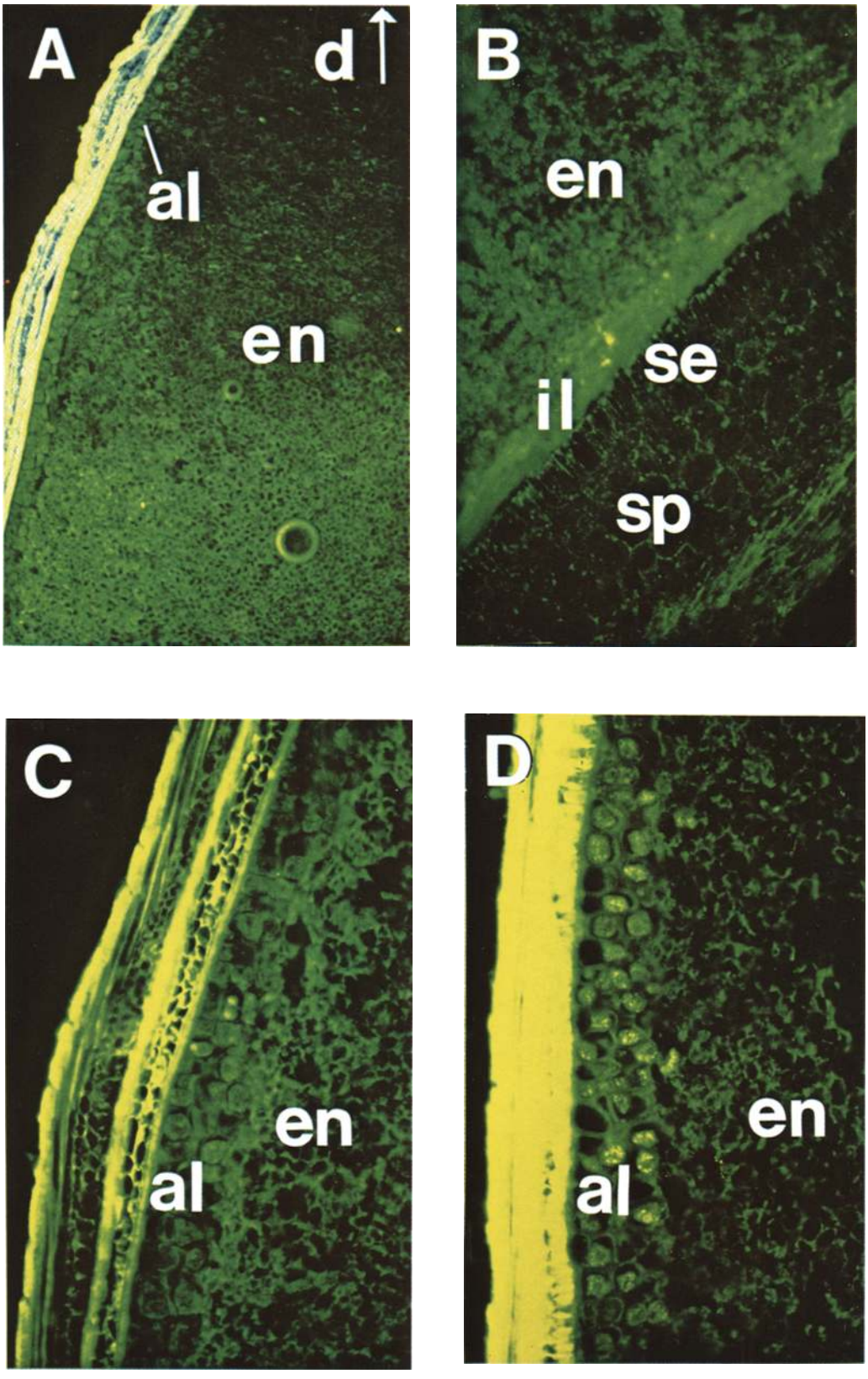
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quently rapidly transported out of the tips of the scutellar epithelial cells into the intermediate layer of crushed cells where a high concentration of the protein is observed. Transport of αII α-amylase proceeds in a broad front away from the intermediate layer into the starchy endosperm with a higher degree of transport occurring in the ventral side of the seed. After 4 days of germination the first evidence is seen of aleurone layer-mediated αII α-amylase synthesis. This aleuronic synthesis of the protein increases through to 7 days, at which stage the entire aleurone layer contains αII α-amylase. After 7 days αII α-amylase has penetrated all of the starchy endosperm. Throughout the latter phase of seedling growth the scutellum appears to continue to produce αII α-amylase, although the high concentration of the protein in the sub-aleurone layer of 7-day germinated seeds indicated that the aleurone layer contributes progressively larger amounts of α-amylase when seen in relation to the enzyme amounts emanating from the scutellum and intermediate layer.

A complete picture of the synthesis and transport of αII α-amylase during germination and early seedling growth of barley can, as stated previously, only be obtained after three-dimensional reconstruction of serial sections of the entire grain are completed. The data obtained from the present study, using median longitudinal sections of germinating Nordal barley allow, however, for comparisons to be made to the previously published theories on germination and early seedling growth. In 1886 TANGL (21) studied these processes by light microscopy and a variety of histochemical methods. Based upon observations of germinating Secale cereale, Triticum vulgare, Avena sativa, Zea Mays and Hordeum vulgare – he postulated that hydrolytic enzymes (including Diastase or α-amylase) were first secreted from the scutellar epithelium and later in germination from the aleurone layer. His observations also led him to believe that the aleurone functioned as a rapid transport pathway for the enzymes produced in the scutellum such that these hydrolytic enzymes could reach the distal area of the endosperm more quickly and subsequently be secreted from the aleurone. In 1890 HABERLANDT (5) examined the role of the aleurone more closely, using germinating seeds of Secale cereale, and came to the conclusion that this tissue was functioning as an active synthetic site for diastase (amylase) rather than as a reservoir for already produced enzyme. The work of HABERLANDT was followed up by BROWN and MORRIS (2) working mainly with barley. These authors questioned the results of HABERLANDT and came to the conclusion that the scutellum was the major source of diastase production and if the aleurone was at all involved in secretion of the enzyme then this must occur by the action of »some mysterious power« (now thought to be gibberellic acid-GA_3) which could pass from the germ, through the endosperm, to the aleurone layer. The involvement of the barley aleurone layer in production or secretion of α-amylase was confirmed in 1938 by LINDERSTRØM-LANG and ENGEL (11) using microchemical techniques. DURE (4) reinvestigated the role of the scutellum in 1960 and found that in Zea Mays, α-amylase originated exclusively from the scutellum during germination and early seedling growth, and that this α-amylase production was dependent on an interaction between scutellum and the endosperm. The majority of the results published during the last 20 years have led to the theories that the scutellum of germinating seed of Hordeum vulgare either plays only a minor role in α-amylase production (see review by BRIGGS (1)), or has no role at all in α-amylase production (see recent review by PALMER (18)) during the phase of early seedling growth (i.e. after rootlet emergence). The experiments performed prior to the establishment of these theories have been carried out with isolated tissue pieces with and without added plant hormones; seeds with damaged embryos; or by examining the microscopic or microscopic breakdown of endosperm starch and protein reserves as well as cell wall structures (»modification«).

The results shown in the present communication were obtained by immunohistochemically examining the distribution of the protein moiety of αII α-amylase in normally germinated seeds of Hordeum vulgare cv. Nordal. Germination processes could thereby be observed, in normal healthy seeds, as they occurred in vivo, without the interpretational problems involved in justifying to what degree the results obtained with tissue extracts of healthy or damaged seeds, or isolated tissue fractions, represent the normal

processes occurring within the germinating seed. An approach to the immunohistochemical method has recently been made by OKAMOTO and AKAZAWA (16) who microscopically measured total amylase activity during germination of seeds of Oryza sativa. These authors found similar results for the development and transport of both α- and β-amylase as have been here presented for αII α-amylase.

The fact that the aleurone layer of barley can produce α-amylase during germination is well established. The question remains as to the respective role of the scutellar epithelium and the aleurone layer as a source of α-amylase secretion during germination and early seedling growth. The results presented in this work indicate that until the third day of germination, the scutellum secretes the major amount of αII α-amylase. Even after 5 days the high concentration of αII α-amylase beneath the scutellum indicates that this tissue is still very actively involved in secretion of the protein.

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