Visualization of α-Amylase Movement and Cell Wall Breakdown During Barley Malting—Practical Application of Current Research[1]

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ABSTRACT

Two of the main events occurring during barley malting are the *de novo* synthesis and transport of α-amylase and the breakdown of the endosperm cell walls, which are rich in β-glucans. A monospecific antibody was raised against the isoenzyme group of α-amylase produced *de novo* during germination. It was thereby possible to follow the synthesis and transport of the α-amylase protein moiety immunohistochemically rather than following "amylase activity," which may be influenced by other factors. Following α-amylase localization, the sections were further treated with Calcofluor™, thus effecting a fluorescent staining of the major endosperm cell wall components. Optimized exciter and barrier filters and a new macrofluorescence microscope unit were developed to observe α-amylase and cell walls. The new method provides an accurate determination of the physiological stage of the germinating seeds. The synthesis and transport of α-amylase and the breakdown of the β-glucan-rich endosperm cell wall material were studied in maltings at the Carlsberg Breweries, and the results are compared with classical malt analyses.

Key words: *Calcofluor™, β-Glucan, Macrofluorescence microscope, Modification, Rapid malt analysis*

GERMINATION OF CEREAL SEEDS

Cereal seed germination has been widely studied. The early work of Malpighii (12,13) in 1675 and 1679 described the macroscopic structure of cereal seeds as well as anatomical changes involved in germination. Invention of the light microscope allowed closer studies of the morphological events of germination and, in the early 19th century, Mirbel (14), Poiteau (19), and Richard (20) described the changes involved in a wide variety of plant species. Toward the end of the nineteenth century, Tangl (23) suggested, on the basis of detailed microscopic observations, that the scutellum played the major role in the secretion of hydrolytic enzymes into the starchy endosperm of germinating cereal seeds. Tangl also proposed that hydrolytic enzymes, synthesized in the scutellum, were transported through the aleurone layer, thereby facilitating their distribution through the endosperm. Haberlandt (8) further investigated the role of the aleurone and presented evidence that the aleruone layer itself was capable of synthesizing hydrolytic enzymes. Haberlandt found, however, that this aleurone-mediated production of hydrolases was dependent on the presence of the embryo. Brown and Morris (1) critically reviewed Haberlandt's experiments and came to the conclusion that the scutellum alone was responsible for the production of "diastase" and that aleurone-mediated hydrolase synthesis could only occur if the embryo possessed "some mysterious power of influencing the aleurone-cells across the intermediate dead tissue of the starch-containing portion of the endosperm." This "mysterious power" remained largely unknown until the 1960s, when the presence of the plant hormone gibberellic acid was demonstrated in barley embryos. This compound could initiate synthesis of α-amylase and other hydrolases in the embryo-deprived barley endosperm and isolated aleurone cells (2,10,17,24). As a result, attention was drawn to the relationship between gibberellic acid and the aleurone and, by the end of the last decade, it was generally thought that the aleurone layer synthesized most of the enzymes responsible for the hydrolysis of the endosperm reserves (9,18). The previously well-documented action of the scutellum received less and less attention. In 1979 Akazawa's group, using histochemical techniques for the detection of amylolytic enzymes in germinating rice seeds (15), and Gibbons (5), using immunohistochemical methods to determine the transport of α-amylase in germinating barley kernels, reestablished the important role of the scutellum during the first three days of germination in these cereals. Involvement of the scutellum in other hydrolytic processes such as proteinase and ribonuclease production and transport (16) and endosperm cell wall breakdown (6) was recently described. Endosperm cell wall material was visualized by treatment of freeze-sectioned germinating barley with the commercial optical brightener Calcofluor™ White M2R New. This reagent was used by Fulcher and Wong (4) to investigate plant cell walls and was shown to react specifically with hexapyranose polymers (glucans) having a β-configuration (11).

The role of the aleurone in the mediation of hydrolytic processes is seen clearly during the later stages of germination (5,6,15,16). In whole seeds (6), there is an approximately 50-hr lag before active secretion of α-amylase and cell wall hydrolases from the aleurone layer of barley. During this lag, which is independent of externally supplied gibberellic acid, dissemination of α-amylase and cell wall breakdown factors from the scutellum proceed rapidly. In a series

TABLE I
Effect of Barley Seed Size on Cell Wall Breakdown (%)

	Size Distribution[a]		
Day	Greater than 2.8 mm	2.5–2.8 mm	Less than 2.5 mm
1	0	0	0
2	8	6	15
3	9	16	32
4	36	25	55
5	40	38	58
6	65	80	96

[a] Sizes > 2.8, 2.5–2.8, and < 2.5 mm comprised 60, 33, and 6% of the sample, respectively.

[1] Presented at the 46th Annual Meeting, Minneapolis, MN, May 1980.

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of experiments with Nordal barley, the tip of the distal end of the seed was removed before germination in the presence or absence of gibberellic acid. Distal decapitation alone had no effect on the rate of α-amylase production and transport or on the rate cell wall breakdown when compared to uncut germinating seeds. The first significant effects of gibberellic acid were seen in three-day ger-

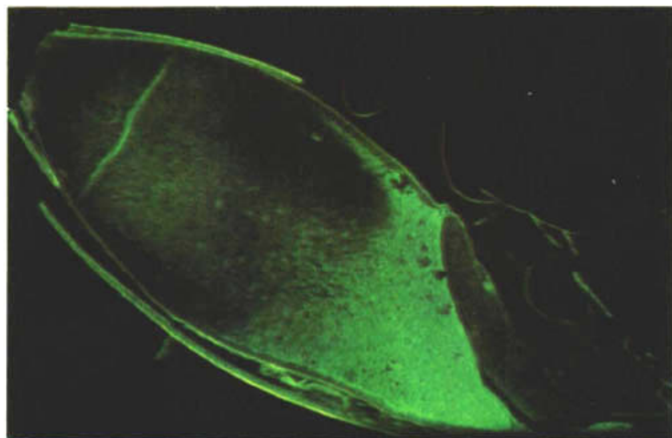

Fig. 1. Distally decapitated seed of Nordal barley germinated three days at 15° C; α-amylase located immunohistochemically as described by Gibbons (5). Exciter filter BP 450-490 nm, dichroic mirror BS 500 nm, emission filter LWP 510 nm. (×11)

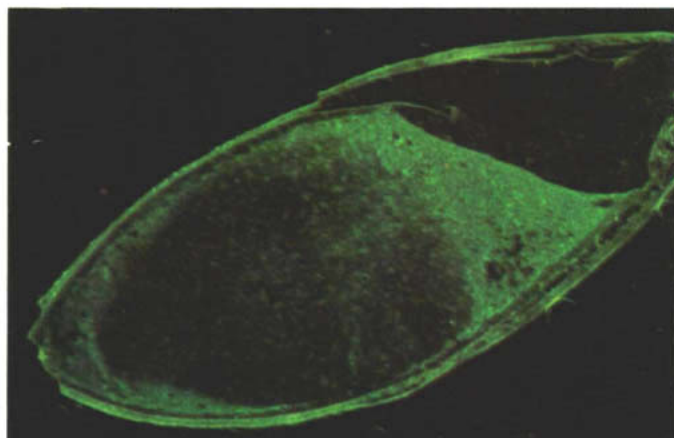

Fig. 2. Distally decapitated seed of Nordal barley germinated three days at 15° C in the presence of $10 \mu M$ gibberellic acid GA_3; α-Amylase located immunohistochemically as described by Gibbons (5). (×11)

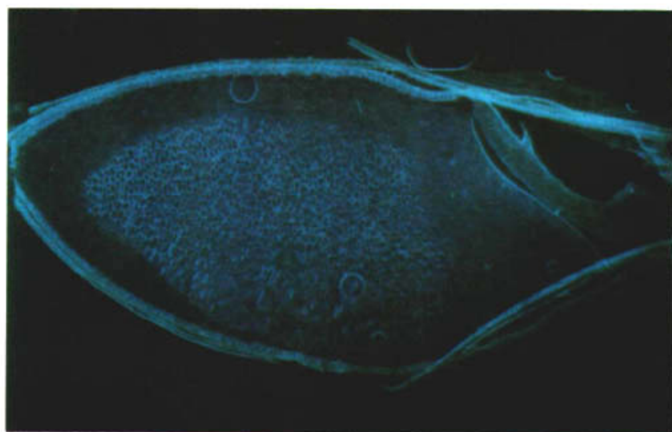

Fig. 3. Distally decapitated seed of Nordal barley germinated three days at 15° C in the presence of $10 \mu M$ gibberellic acid GA_3; cell walls stained with Calcofluor™ as described by Gibbons (6). (×11)

Fig. 4. Distally decapitated seed of Nordal barley germinated four days at 15° C; cell walls stained with Calcofluor™ as described by Gibbons (6). (×11)

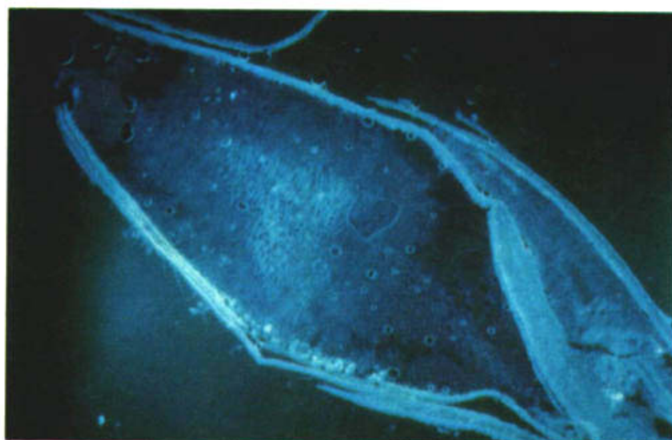

Fig. 5. Distally decapitated seed of Nordal barley germinated four days at 15° C in the presence of $10 \mu M$ gibberellic acid GA_3; cell walls stained with Calcofluor™ as described by Gibbons (6). (×11)

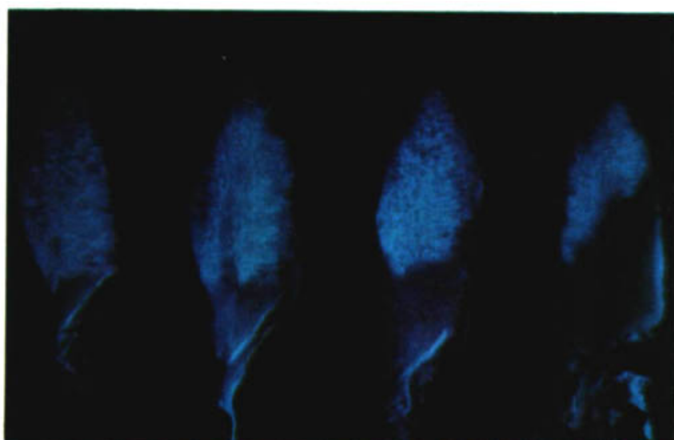

Fig. 6. Cell wall breakdown in germinating seeds of Nordal barley as seen under the macrofluorescence microscope. Half seeds stained using the rapid visulization method. Cell wall breakdown in % (left to right): 10, 20, 40, 60.

minated material. In distally decapitated control seeds (Fig. 1), immunohistochemically determined α-amylase was present in approximately 25% of the endosperm. Gibberellic acid treatment (Fig. 2), although having no significant effect on the scutellar-mediated α-amylase, stimulated aleurone-mediated α-amylase synthesis in the distal end of the seed. The same pattern was found when the endosperm cell walls were stained with Calcofluor (Fig. 3). After four days of germination, only half of the endosperm cell walls of the untreated seeds stained with Calcofluor (Fig. 4). In the gibberellic acid treatment (Fig. 5), a massive aleurone-mediated cell wall hydrolase synthesis occurred.

From the standpoint of brewing chemistry, a rapid version of the Calcofluor technique, if developed, would permit the progress of germination to be accurately followed during malting. This article describes modifications to the Calcofluor technique allowing rapid (90-sec) visualization of cell wall breakdown in germinating seeds of barley (Fig. 6) and in finished, kilned barley malt. It also describes a new macrofluorescence microscope developed for use in the rapid visualization technique.

SYSTEM FOR RAPID VISUALIZATION

To adapt the previously used cell wall visualization technique (6) to a rapid quality control method, the following system was developed.

Sectioning the Seeds

Dorso-ventral median longitudinally sectioned half seeds of barley green malt or kilned malt were made with a razor blade. In certain finished malt types, sectioning was improved by soaking the malt in 70% v/v aqueous ethanol overnight to reduce shattering.

Mounting

The half seeds were mounted in the holes of a modeling clay template (Fig. 7), and light pressure applied with a simple press to hold them in place.

Cell Wall Staining

Cell walls were stained for 30 sec to 1 min in 0.1% aqueous Calcofluor White M2R New (Cyanamid, Wayne, NJ). To reduce contamination, each sample was treated with fresh Calcofluor. Stock solutions of Calcofluor were made each week and stored in amber bottles to protect against light-induced breakdown of the fluorescent reagent.

Washing

The half seeds were washed for 10 sec in fresh 70% aqueous ethanol to remove excess Calcofluor and dried in a stream of dry air.

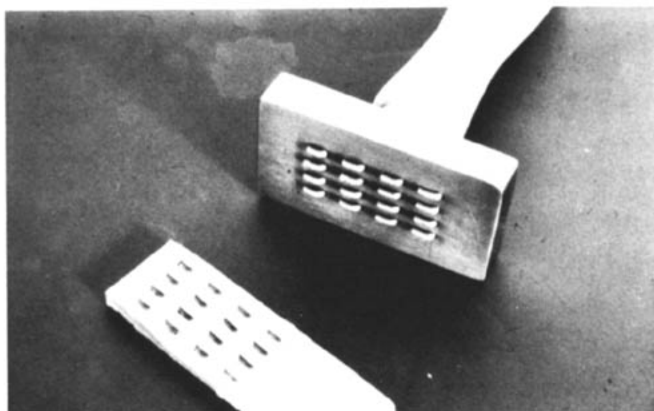

Fig. 7. Template used for embedding half seeds before cell wall staining, and the forming tool.

Counterstaining

Seeds were counterstained with 0.1% aqueous Fast Green F.C.F. (Gurr/Searle, High Wycombe, England) for 30–60 sec to increase contrast between cell walls and other endosperm components. Following counterstaining, the seeds were blotted dry to remove excess stain.

Microscopic Examination

Image analysis is the most accurate method of recording cell wall breakdown. Figure 8 shows the system used routinely at the Carlsberg Research Center. The stained seeds were examined with a Plan 2.5× (numerical aperture 0.075) objective using a Reichert Univar (or Polyvar) microscope equipped with incident fluorescence optics (SWP-400 nm exciter filter, BS 410 nm dichroic mirror, and KV-418 emission filter) and connected via a Plumbicon television camera to an Imanco Quantimet 720 image analyzer. The area of residual cell walls, as well as the total area of the endosperm, was measured with a light pen and the data collected, calculated, and recorded on a Hewlett Packard 9825 (or 9835) table top computer.[2]

Due to the slight numerical aperture of low magnification incident light microscope objectives, the visual image of the stained cell walls was extremely weak. To overcome this problem, a new macrofluorescence microscope was developed (7). The percentage of cell wall breakdown in a given sample was estimated by comparison with standards calibrated by image analysis (Figs. 6, 9, and 10). The principle of the macrofluorescence unit is seen in Fig. 11.

[2] The method was developed by S. Aastrup and C. Feil of this laboratory.

Fig. 8. Image analysis of cell wall breakdown. Left to right: Reichert Univar microscope, Quantimet 720 image analyzer, Hewlett Packard 9825 calculator.

Fig. 9. Examination of stained seeds with macrofluorescence microscope.

TABLE II
Comparison of Cell Wall Breakdown Percentage with Malt Analysis

	Variety and Cell Wall Breakdown (%)									
Malt Analysis	Piccolo 93.5	Dram 88.0	Keg 83.5	Aramir 80.4	Atem 79.7	Gitane 77.7	Carina 76.5	Athos 72.5	Sara 62.6	Irania 61.1
Extract yield, % db	77.7	78.7	77.8	76.7	78.6	78.8	77.2	76.0	77.0	77.2
Fine/coarse difference, % db	0.6	1.2	1.5	1.7	1.5	1.2	1.6	1.3	3.1	2.7
Kolbach index, %[a]	36	34	33	29	30	35	28	31	27	27
Apparent final attenuation, %	85.6	83.1	80.7	83.0	84.8	83.1	82.8	81.5	77.7	79.2
Diastatic power (Windisch-Kolbach)[b]	324	296	275	274	312	380	266	340	340	290
α-Amylase (30° C DU)[c]	118	70	70	74	82	88	95	80	64	71
Viscosity, cp. 20° C, 8.6° P	1.49	1.52	1.54	1.57	1.61	1.66	1.61	1.53	1.68	1.89

[a] Wort-soluble N as percent of total N.
[b] From EBC (3).
[c] SKB dextrinizing units at 30° C (21).

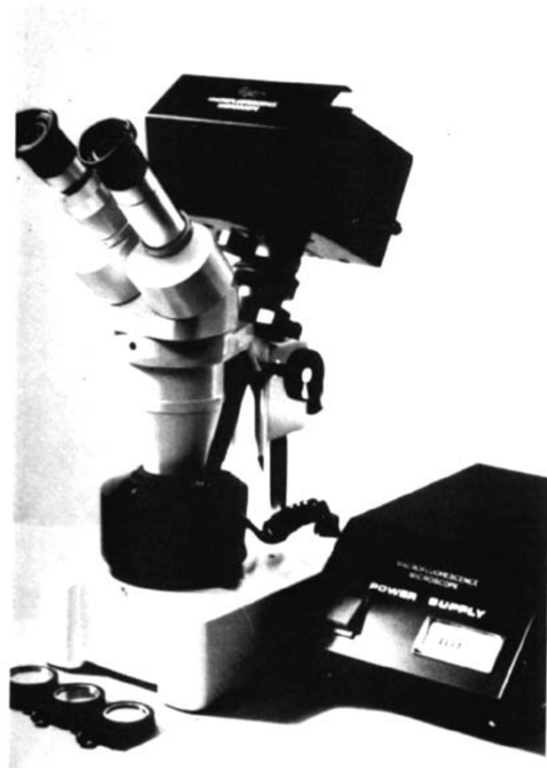

Fig. 10. Carlsberg macrofluorescence microscope and mercury lamp power supply.

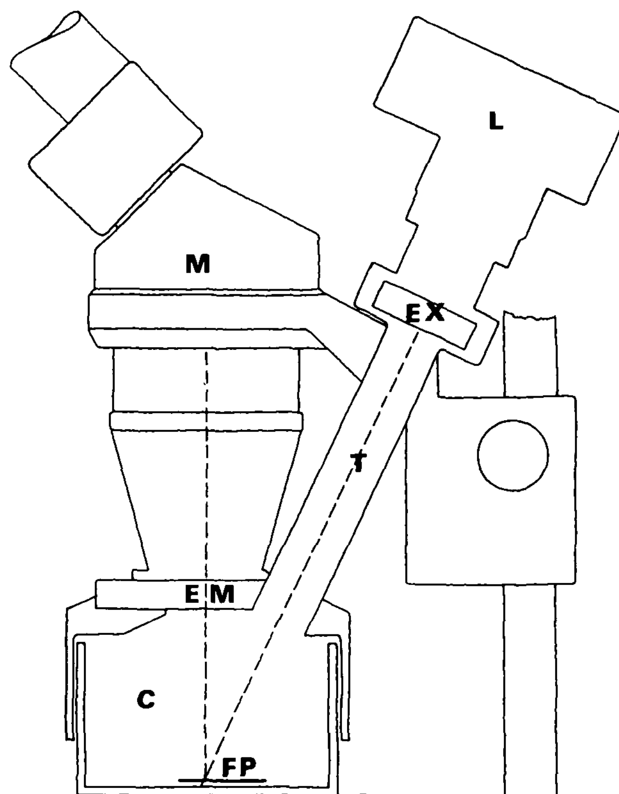

Fig. 11. Schematic diagram of macrofluorescence microscope. A standard low power stereomicroscope (M) is equipped with a light-tight telescopic chamber (C) in which the mounted stained seeds are directly illuminated through a light-tight tube (T) with a high pressure mercury lamp (L). The light beam passes through an SWP-400 nm excitation filter (EX) and the excitation wavelengths are removed with a KV 418 emission filter (EM). FP = focal plane.

Preservation of Samples

The stained and mounted half seeds can be preserved for periods of at least six months, using a thin layer of clear nail polish.

PRACTICAL APPLICATIONS

The rapid cell wall visualization technique has been routinely used at the Carlsberg Research Center since early 1980. Although statistical studies of the required sample size, reproducibility, and correlation with other malting and brewing analyses are still in progress, the preliminary results are promising.

Barley Kernel Size

The effect of barley kernel size on cell wall breakdown during germination was investigated in a sample of Nordal barley. Following sieving of the seed, three size classes (>2.8 mm; 2.5–2.8 mm; <2.5 mm) were separately germinated on moist sand at 15° C. Samples were taken daily and subjected to Calcofluor analysis. The results (Table I) illustrate clearly the rapid cell wall breakdown in small seeds. A similar conclusion was reached by Sommer (22), who employed classical malt analysis to demonstrate this well-known kernel size effect.

Barley Variety Evaluation

Evaluation of new malting barley varieties is another field in which the rapid analysis of cell wall breakdown has proved useful. Table II compares this analytical method with standard malt analysis of 10 varieties taken from the 1979 EBC barley trials. The barley was pilot-malted and subjected to malt analysis using standard EBC methods at Kvaltek, the central laboratory of The United Breweries in Copenhagen. The results are listed in the table

in order of decreasing degrees of cell wall breakdown. On the basis of the malt analyses and the behavior of the different varieties during pilot-malting, Irania and Sara were judged poor malting barleys and Piccolo and Dram the most promising. These conclusions correlate with the cell wall breakdown percentages measured on the finished malt. Interestingly, a certain degree of correspondence was apparent between cell wall breakdown and the classical malt modification variables, viscosity and fine/coarse extract difference.

Field Sprouting and Dormancy

Preharvest sprouting and dormancy can also be differentiated with the new technique. Following a standard germination test, the ungerminated kernels are subjected to cell wall breakdown analysis. Kernels that are preharvest sprouted show an area of cell wall breakdown adjacent to the scutellum, whereas the truly dormant kernels contain intact cell walls throughout the endosperm.

Method Reproducibility

Further studies are being conducted on the reproducibility of the simple method of microscopic evaluation mentioned above. In a series of experiments conducted in this laboratory, four different people were tested for their ability to judge the percentage cell wall breakdown in 100 green malt kernels varying from 0 to 100% breakdown. Stained seeds were examined in the macrofluorescence microscope. A standard set of six seeds with 10, 20, 40, 60, 80, and 90% cell wall breakdown was used as a key. Following visual evaluation, the 100 seeds were measured by image analysis. Comparison of the results showed that the human eye was excellent in judging cell wall breakdown; the values obtained by eye were within 5–7% of the actual cell wall breakdown percentage.

In conclusion, this cell wall breakdown analysis technique is a rapid method for the examination of populations of germinating barley seeds. The most important application of this new technique may be the objective assessment of green malt during malting, which could allow the maltster to determine the correct time for the start of kilning and thus avoid costly overmodification.

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