

1983

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Recommended Citation

Earp, C. F.; Doherty, C. A.; Fulcher, R. G.; and Rooney, L. W. (1983) "B-Glucans in the Caryopsis of Sorghum Bicolor (L.) Moench," *Food Structure*: Vol. 2 : No. 2 , Article 9.

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β -GLUCANS IN THE CARYOPSIS OF *SORGHUM BICOLOR* (L.) MOENCH

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Abstract

Fluorescence microscopy was used to determine the location of β -glucans in sorghum. Sections from three genetically different sorghum cultivars were stained with Calcofluor or Congo Red, fluorochromes which have been reported to react with β -glucans. Autofluorescence, indicative of ferulic acid in other cereals, was observed in untreated sections. When stained sections were treated with endo- β -glucanase, fluorescence was reduced or entirely eliminated in pericarp, aleurone and endosperm cell walls. β -Glucans were isolated from the endosperm of three sorghum cultivars. When reacted with the two dyes, Calcofluor or Congo Red, precipitates formed immediately, a reaction which is similar to that produced by mixed linkage β -glucans from other cereals.

Introduction

β -Glucans are high molecular weight polymers comprised primarily of β 1,3 and 1,4 linked glucopyranosyl units. The presence of mixed linkage 1,3 and 1,4 β -glucans has been reported in the endosperm of barley (Anderson et al 1978; Wood et al 1977), oats (Wood et al 1977; Wood and Fulcher 1978), wheat (Fulcher and Wong 1980) and sorghum (Woolard et al 1976, 1977). During the brewing process, the presence of high levels of barley β -glucans can cause viscosity problems in the wort or beer (Ducroo and Delecoeur 1972). In South Africa, sorghum beer is a major industry, making sorghum β -glucans of particular interest. In the preparation of the African food *tô* (a porridge-like product), differences in stickiness have been documented by Da et al (1982). It is possible that the stickiness characteristic may be due in part to β -glucans.

Calcofluor White M2R New, an optical brightener, was used by Hughes and McCully (1975) as a stain for cell walls of higher plants. Wood and Fulcher (1978) reported that the optical brightener Calcofluor White M2R New caused intense fluorescence in barley endosperm cell walls, a major component of which is a mixed linkage β 1,3:1,4 glucan. A similar fluorescence in the endosperm cell walls occurred when sections were stained with Congo Red. Wood and Fulcher (1978) characterized a relatively specific reaction in which both Congo Red and Calcofluor precipitated mixed linkage β -glucan from oats and barley. In further studies of this reaction, Wood (1980a) reported that the strongest reaction occurred with polysaccharides containing "contiguous (1+4) β -linked D-glucopyranosyl units, such as cereal β -D-glucans, xyloglucan and substituted celluloses." Wood (1980b) demonstrated a difference in precipitation curves of the oat and barley β -glucan which he believed to be due to differences in molecular weight of the two β -glucans.

Work at Carlsberg Research Center by Gibbons (1981) and Aastrup et al (1981) has taken the basic research of Wood and Fulcher and applied this information to an industrial setting. Half kernels of barley are mounted in a modeling clay template, stained with Calcofluor and viewed in the Malt Modification Analyser developed at Carlsberg (Munck et al 1978). As cell wall

Initial paper received August 29, 1983.
Final manuscript received November 10, 1983.
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KEY WORDS: Fluorescence microscopy, β -glucans, *Sorghum bicolor*, cell walls, fluorochromes, autofluorescence

breakdown occurs, fluorescence is lost and the degree of modification in the endosperm can be expressed as % loss of fluorescence in the cell walls. The modified kernels are compared to a standard set of modified kernels.

The purpose of the work presented here was to locate and identify sorghum mixed-linkage β -glucans whose occurrence has previously been reported (Woolard et al 1976, 1977). Because the first step in germination is the breakdown of cell walls, cell wall structure of the pericarp and endosperm is important in germination studies. Therefore, identification of cell wall components could lead to a better understanding of the germination or malting process. This type of information can also aid in the comparison of sorghum kernel structure to that of other cereals. Fluorescence microscopy was used in this study to determine the location of β -glucans in sorghum. Sections were stained with either Calcofluor or Congo Red, two fluorochromes which have been reported to be specific for mixed linkage β -glucans. Fluorescence microscopy has also been used in a number of other studies to locate a wide range of specific compounds in cereals (Fulcher 1982; Fulcher and Wong 1980; Gibbons 1981) and oilseeds (Yiu et al 1982). Many phenolic compounds, such as ferulic acid, fluoresce in the blue region of the spectrum. This autofluorescence is produced when the compound is subjected to ultraviolet light and is not caused by any stain or fluorochrome. Ferulic acid has been identified as a cell wall component in wheat (Fulcher et al 1972; Fausch et al 1963) barley and oats (Fulcher and Wong, 1980), and other Gramineae family members (Harris and Hartley, 1976). Earp et al (1983) documented autofluorescence in sorghum. HPLC analysis of cell wall extracts according to the method of Hahn et al (1983), also suggested that ferulic and coumaric acids were the major phenolic acids in the cell walls of the three sorghum cultivars.

Materials and Methods

Samples

Three sorghum cultivars were selected to typify differences in genetic diversity. The genetics of the three varieties as currently understood are presented below.

BTx3197 (RRyyb₁b₂B₂SS) a thick, white pericarp and no pigmented testa.

Early Hegari (RRyyB₁B₂B₂SS) a thick, white pericarp and a pigmented testa.

ATx623 X SC0103-12 (RRyyB₁b₂B₂SS) a thick, brown pericarp sorghum (genetically red) with a pigmented testa and a dominant spreader.

All samples were grown at Halfway, Texas in the Texas Agricultural Experiment Station Nursery in 1980.

Fixation and Embedding

Mature sorghum kernels were cut in halves or quarters with a sharp xylene cleaned razor blade. The half kernels were fixed in 3% glutaraldehyde in a 0.025 M phosphate buffer (pH-6.8) for 48 hr at 4°C. Fixed specimens were dehy-

drated and then embedded in glycol methacrylate according to the procedure of Feder and O'Brien (1968).

Fluorescence Microscopy

Glycol methacrylate sections of ~1 μ m were treated with Calcofluor (Biofluor, Calbiochem-Behring Corp., La Jolla, CA.) (0.01% w/v) in distilled water for 1 min or with Congo Red (0.1% w/v) in distilled water for 5 min. Excess stain was removed by washing with distilled water. Sections were air-dried, mounted in immersion oil and viewed with a Zeiss Universal Research Microscope equipped with a IIIRS epi-illuminator system and a 100W mercury arc lamp. Untreated sections and those stained with Calcofluor were examined with an exciter filter and a barrier filter combination (FC I) with maximum transmissions of 365 and > 418 nm, respectively. The exciter and barrier filters combination III (FC III) with maximum transmissions of 546nm and > 590 nm respectively were used in viewing fluorescence produced by Congo Red interactions.

Enzyme Treatment of Sections

Endo-1,3(4) β -D-glucanase from *Bacillus subtilis* was provided by the Department of Plant Science, University of Manitoba. The enzyme showed no activity towards starch, carboxymethyl cellulose, arabinoxylan or xylan. Only trace enzyme activity with laminaran was observed. When assayed with lichenin, β -glucanase activity was measured as 108 units/ml with 1 unit = 1 μ mole equivalent glucose released per minute under assay conditions. The enzyme was diluted 1:10 for microscopic use. Sections were treated with β -glucanase overnight at room temperature.

β -Glucan Isolation

Sorghum varieties were pearled using a Udy decorticating mill (Udy Corp., Fort Collins, CO) until no further pericarp removal could be achieved. Then, the pearled grain was scraped with a razor blade to remove any remaining pericarp fragments. The pearled grain was ground (through a 1mm screen) with a Udy laboratory mill (Udy Corp., Fort Collins, CO) prior to analysis. Sorghum "gum" was isolated from 5 g of ground, pearled grain using the procedure of Wood et al (1977). The gum was dissolved in 10 ml of distilled water. β -Glucans were precipitated with Congo Red or Calcofluor using the procedure of Wood and Fulcher (1978).

Results

The observations of cell walls were the same for all three sorghum cultivars. Discussion will be for sorghum in general. Untreated sections exhibited a bright blue autofluorescence in the pericarp, aleurone and endosperm cell walls. (Figs. 1, 2 and 3). In other cereals, this autofluorescence has reportedly been due to ferulic acid (Fulcher et al 1972; Fulcher & Wong 1980). In work conducted in this laboratory, ferulic acid has been shown to be the major phenolic compound in isolated cell walls from these three sorghum cultivars (Earp et al 1983). No autofluorescence was observed in the pigmented testa (Fig. 2). In Calcofluor-treated sections, an intense bluish-white fluorescence could be seen in the pericarp cell walls (Fig. 4)

and in the endosperm cell walls. In the aleurone cell walls (Fig. 5) and in the scutellar parenchyma cell walls (Fig. 6), a thin band of bluish-white fluorescence surrounded the cytoplasm. The bright blue autofluorescence can also be seen between the Calcofluor bands.

Since the blue colors produced by autofluorescence and Calcofluor staining are very similar, Congo Red, which fluoresces red, can be used to differentiate between the ferulate produced fluorescence and that produced by mixed linkage β -glucans. When sections were treated with Congo Red, a bright red fluorescence could be seen in the cell walls of the pericarp (Fig. 7), aleurone (Fig. 8) and the endosperm (Figs. 8 and 9). Starch granules appeared red due to nonspecific staining but were not fluorescent as seen in the cell walls (Figs. 7-9). Congo Red is a histological stain that has been used for staining starch (Gurr, 1960). Starch granules in the thick mesocarp can be clearly seen (Fig. 7). Intense red fluorescence was also observed in the endocarp (cross and tube) cell walls (Fig. 7). After treatment with the β -glucanase enzyme, little or no fluorescence could be detected in the pericarp (Fig. 10), aleurone (Fig. 11) or endosperm (Fig. 12) cell walls. Figures 7 and 8 were taken of the variety without a testa (BTx3197) while Figures 10 and 11 were taken of cultivars with a testa. The testa layer appeared fluorescent after β -glucanase treatment. The source of this fluorescence has not been determined.

After following the β -glucan isolation procedure of Wood et al (1977), the resulting precipitate was dissolved in distilled water. When Calcofluor was added to the solution, a gel-like substance immediately began to drop out of solution. This precipitate was off-white in color. When Congo Red was added to the solution, a red precipitate was formed. This precipitation reaction has been described by Wood and Fulcher (1978) for both oat and barley β -glucans.

Discussion

In order to understand the effects of various processing techniques on the sorghum kernel, information concerning cell wall structure is needed. β -Glucans were first isolated and identified by Woolard et al (1976, 1977) who were interested in cell wall structure as related to sorghum germination in the production of South African beer. The use of fluorescence microscopy in this study has enabled one to identify the location of β -glucans in the sorghum kernel. Glennie et al (1983) examined cell walls of germinated sorghum grain with scanning electron and transmission electron microscopy. The authors observed extensive modification in the aleurone cell walls but no visible changes in the endosperm cell walls. In order for the sorghum endosperm to be modified during germination, some change in the cell wall structure must occur. It will be of interest to use the technique described in this paper to study germinated sorghum and to determine the fate of β -glucans during germination.

Sorghum is processed by a number of other methods to improve its nutrient availability when used as a livestock feed. Mechanical grinding improves feed efficiency markedly. This would be due to the physical action of grinding, breaking down cell wall structure, thus exposing starch and protein to enzyme attack during digestion (Hale and Theurer, 1972). Reconstitution is a process by which water is added to grain to raise the moisture level to 25 to 30%. The grain is then stored in an air-limited environment, usually for 21 days. Reconstitution is controlled germination and would be similar to the malting process in brewing. When sorghum is ground after reconstitution, feed efficiency has been reported to increase 16 to 22% over ground dry grain (Riggs and McGinty, 1970). In a microscopic study by Sullins and Rooney (1971), it was reported that reconstitution affects the kernel at the subcellular level causing a general disruption of the endosperm, particularly the peripheral endosperm. It was postulated that the disorganization may be due to enzymatic activity similar to that seen during malting where β -glucanase initiates cell wall degradation. Fluorescence microscopy using the Calcofluor and Congo Red fluorochromes would be a useful tool in determining what changes occur in cell wall structure during the processing of cereals for feed and food products.

Acknowledgements

The authors wish to thank Dr. G.M. Ballance, Department of Plant Science, University of Manitoba, for providing us with the purified endo-1,3(4) β -D-glucanase for the β -glucan enzyme study. This project was partially supported by the INTSORMIL Title XII Sorghum and Millet Research Program, supported in part by Grant AID/DSAN/XII/G-0149 from the Agency for International Development in Washington, D.C., 20253. Contribution No. 725 from Ottawa Research Station.

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Figure Captions

Fig. 1-12. Al - aleurone layer; CF - Calcofluor fluorescence; CW - cell wall; E - endosperm; EN - endocarp (cross and tube cells); EP - epicarp; M - mesocarp; SG - starch granule; SP - scutellar parenchyma; T - testa. Cultivar is indicated in parenthesis. F - filter combination.

Scale bar numbers indicate μ m.

Fig. 1. Untreated section showing intense autofluorescence in pericarp, aleurone and endosperm cell walls (BTx3197). Photographed using FC I.

Fig. 2. Untreated section showing autofluorescence in pericarp, aleurone and endosperm cell walls (ATx623 X SC0103-12). Photographed using FC I.

Fig. 3. Aleurone cells with intense autofluorescence in cell walls (Early Hegari). Photographed using FC I.

Fig. 4. Calcofluor (Biofluor) stained section showing bluish-white fluorescence produced in the mesocarp cell walls BTx3197). Photographed using FC I.

Fig. 5. Calcofluor (Biofluor) stained section showing bluish-white fluorescence in the aleurone cell wall (BTx3197). Darker blue autofluorescence can be seen between the two bands of Calcofluor-produced fluorescence. Photographed using FC I.

Fig. 6. Calcofluor produced fluorescent bands in scutellar parenchyma cell walls (Early Hegari). Photographed using FC I.

Fig. 7. Congo Red staining of the pericarp cell walls (BTx3197). Photographed using FC III.

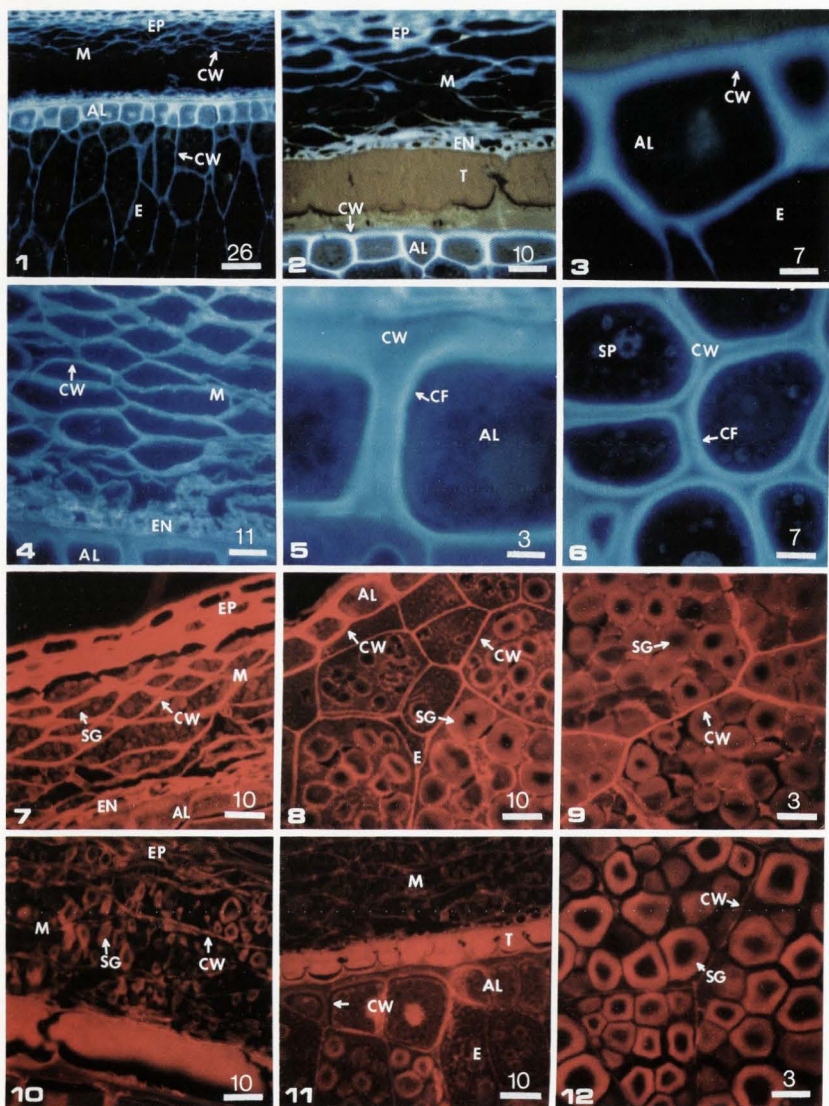
Fig. 8. Congo Red staining showing fluorescence of aleurone and endosperm cell walls. Starch granules stain red but are not fluorescent (BTx3197). Photographed using FC III.

Fig. 9. Congo Red staining showing fluorescence of endosperm cell walls (BTx3197). Photographed using FC III.

Fig. 10. After treatment with β -glucanase, the section shows little or no fluorescence in the pericarp or aleurone cell walls (ATx623 X SC0103-12). Photographed using FC III.

Fig. 11. After treatment with β -glucanase, little or no fluorescence can be seen in the aleurone cell walls (Early Hegari). Photographed using FC III.

Fig. 12. After treatment with β -glucanase, little fluorescence remains in the endosperm cell walls (Early Hegari). Photographed using FC III.



Discussion With Reviewers

E.A. Davis: The Congo Red stain appears to be brighter around the outer edges of the starch granules. Is that due to the 3-dimensional character of starch and therefore uneven focussing in the microscope?

Authors: We feel that is merely optical staining of the starch granules.

C.W. Glennie: Were the Calcofluor and Congo Red techniques tried on malted sorghum?

Authors: Not yet. We already have some germinated sorghum embedded and will be looking at it next.

C.W. Glennie: Autofluorescence, presumably due to ferulic acid is found in all endosperm cell walls (Earp et al 1983). During malting studies, I have found that the amount of endosperm cell wall decreased but the amount of ferulic did not. Did the authors find any interference from ferulic acid when the cell walls were treated with glucanase before Calcofluor treatment?

Authors: We did not see any interference from ferulic acid when the cell walls were treated with β -glucanase. The autofluorescence was still present after the β -glucanase treatment.