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# STRUCTURE, CHEMICAL COMPOSITION AND ENZYMIC DEGRADATION OF BARLEY ENDOSPERM CELL WALLS

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# STRUCTURE, CHEMICAL COMPOSITION AND ENZYMIC DEGRADATION OF BARLEY ENDOSPERM CELL WALLS

BY

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## ABSTRACT

During the germination or malting of barley, endosperm cell walls present a barrier to the movement of enzymes that hydrolyze the endosperm starch and protein reserves. Thus the rate of disruption of the cell wall structure may in part control the rate of endosperm modification. The objectives of this study were 1) to determine the chemical composition and morphology of endosperm cell walls isolated from a Canadian malting barley (cv. Conquest), 2) to determine whether there are varietal differences in cell wall-hydrolyzing systems that may influence malting barley, and 3) to investigate the roles of endo-β1,3(4)-glucanase and endo-β1,3-glucanase in cell wall degradation.

Barley endosperm cell walls were isolated by a modification of the procedure of Mares and Stone (Aust. J. Biol. Sci. 26: 793-812 1973). Cell walls were composed almost entirely of polysaccharide consisting of 3.5% mannose, 10.9% arabinose, 17.0% xylose and 68.6% glucose. Amino acid analysis indicated that the protein associated with the cell walls did not contain hydroxyproline, but differed from total endosperm protein in containing higher proportions of several amino acids that have the potential to form linkages with cell wall carbohydrate. Scanning electron microscopy of the isolated cell wall fragments, showing the intracellular wall surfaces marked by the adpression of starch granules, indicated that the proportions of large and small granules may vary in different endosperm cells, and that some cells may not contain small starch granules.

The enzymic degradation of endosperm cell walls was investigated by treating buffered suspensions of isolated cell wall fragments with malt extracts or purified malt endo-β-glucanases and determining the amount, monosaccharide composition and molecular size distribution of the

soluble carbohydrate hydrolysis products. Scanning electron microscopy of the cell wall residues after enzyme treatments showed that the surfaces were highly disrupted, rough and pitted. Treatment of cell wall fragments with extracts of malted barleys showed that barley cultivars differ in cell wall-hydrolyzing properties. Differences in the rate and extent of hydrolysis, and differences in the molecular size distributions of solubilized cell wall carbohydrate were observed. Such variation may affect the rate of endosperm modification and thus the malting quality of barleys. Malt endo-β-xylanase activity correlated positively with extent of cell wall solubilization by malt extracts, whereas there was no apparent relation between malt endo-β-glucanase activities and cell wall breakdown. The hydroysis of arabinoxylan may be a limiting factor in cell wall degradation. Treatment of cell wall fragments with purified malt endo-β-glucanases demonstrated that endo-β1,3(4)-glucanase can hydrolyze intact cell walls and that the products of endo-β1,3-glucanase hydrolysis are large mixed-linkage β-glucans that can be degraded to low molecular weight products by endo-β1,3(4)-glucanase. Approximately 30% of the cell wall glucan was resistant to hydrolysis by malt glucanases. The results indicate that in addition to the endo-β-glucanases, two endo-β-xylanases and a mannanase are involved in cell wall degradation.

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# I INTRODUCTION

In discussing the malting of barley, MacLeod (1976) has stated that one of the most important impediments to modification is the cell wall material of the endosperm. During germination, or malting, the endosperm cell walls present a barrier to the movement of hydrolytic enzymes and must be degraded before amylolytic and proteolytic enzymes can act on the cell contents. Thus, the rate of disruption of cell wall structure may in part control the rate of endosperm modification. The hydrolysis of cell wall materials is equally important in brewing to avoid filtration problems caused by undegraded cell walls and potentially viscous cell wall polysaccharides. An understanding of the biochemical changes that occur during malting and mashing depends in part upon elucidation of the structure of the barley endosperm cell wall, and the alterations in structure that occur as a result of enzymic hydrolysis.

Barley endosperm cell walls, the non-starchy polysaccharides that comprise the walls, and the enzymes that hydrolyze cell wall polysaccharides have been investigated for many years. The relation between cell wall degradation and endosperm modification was recognized in 1890 by Brown and Morris, and it was soon concluded that changes in non-starchy polysaccharides due to enzymic degradation during malting were associated with the modification process. Subsequent studies established the structural characteristics of non-starchy polysaccarides from barley, and identified and characterized some of the enzymes that hydrolyze these polysaccharides. Recently, studies on isolated barley endosperm cell wall fragments have confirmed that the non-starchy

polysaccharides are derived from the cell walls. A review of the literature pertaining to these investigations (Thompson and LaBerge 1977) is reproduced in the Appendix.

Since the preparation of this review, the results of several studies concerning the amount and structure of β-glucan in barley endosperm have been reported (Anderson et al. 1978, Fleming and Kawakami 1977, Forrest 1977, Forrest and Wainwright 1977, Fulcher et al. 1977).

The purpose of the present study was to investigate the enzymic degradation of isolated barley endosperm cell walls. The specific objectives were 1) to determine the chemical composition and morphology of endosperm cell walls isolated from a Canadian malting barley, 2) to determine whether there are varietal differences in the cell wall-hydrolyzing enzyme systems of malted barleys that may influence malting quality, by examining the hydrolysis of isolated endosperm cell wall fragments by extracts of malted barleys, and 3) to investigate the roles of endo-β1,3(4)-glucanase and endo-β1,3-glucanase in cell wall degradation by examining the effect of purified malt endo-β-glucanases on isolated endosperm cell wall fragments.

# II MATERIALS AND METHODS

## A. Materials

The barley (cv. Conquest) used as the starting material for the endosperm wall preparation was kindly provided by Dominion Malting Ltd., Winnipeg, Manitoba. The malted barleys were samples of material grown at the University of Manitoba as part of the Co-operative Two-row Barley Test in 1977, and malted under standard conditions at the Canada Agriculture Research Station, Winnipeg, Manitoba.

Barley β-glucan was prepared from Betzes barley as described by Bass and Meredith (1955) for beta-polyglucoside. Wheat flour arabinoxylan was prepared according to Kulp (1968). Sodium carboxymethyl pachyman was prepared from pachyman (Sam-Ae Trading Co., Seoul, South Korea) by the method of Clarke and Stone (1962). Sodium carboxymethyl cellulose was obtained from Nutritional Biochemicals Corp. Laminarin was purchased from ICN Pharmaceuticals, Inc.

## B. Analytical Methods

### 1. Starch

The starch content of the endosperm cell wall preparation was estimated by determining the glucose produced by amyloglucosidase hydrolysis of perchloric acid - cell wall extracts.

Endosperm cell walls (5 mg) were stirred with 1 ml 95% ethanol and 9 ml 25% perchloric acid for 1 hr at room temperature (21$^{o}$C). The samples were adjusted to pH 5 with 5N sodium hydroxide and incubated with 20 µl amyloglucosidase suspension (Aspergillus niger, Boehringer-Mannheim) for 18 hr at 35$^{o}$C. The amyloglucosidase preparation did not

release glucose from barley β-glucan under these conditions.

After hydrolysis, the digests were clarified by centrifugation and diluted to 25 ml with distilled water. The cell wall residues were washed with ethanol and dried under vacuum at 45°C. Portions of the residues and solutions were treated with several drops of iodine solution to test for undegraded starch.

Portions of the amyloglucosidase hydrolysates (1 ml) were incubated with 5 ml glucose oxidase reagent (G.O.D. Period Method, Biochemical Test Combination, Boehringer-Mannheim GMBH Diagnostica) for 20 min at 35°C. Absorbance was measured at 600 nm. The glucose content of the samples was determined by reference to a standard curve prepared using solutions containing 0-60 μg glucose/ml. The glucose assay was not affected by the presence of either starch or barley β-glucan.

For comparison with the perchloric acid extraction method, the starch content of the cell wall preparation was estimated by determining the glucose produced by amyloglucosidase hydrolysis of (1) unextracted cell walls suspended in water, (2) cell walls suspended in water and heated at 100° for 15 min to gelatinize the starch, and (3) cell walls suspended in water and incubated with α-amlyase (hog pancreas, Sigma) in addition to amyloglucosidase.

The monosaccharide composition of dialyzed perchloric acid-cell wall extracts was determined by ion-exchange column chromatography of acid hydrolysates (Analytical Methods 4).

2. Nitrogen

The nitrogen content of the cell wall preparation was estimated using a modification of the procedure described by Tetlow and Wilson (1964). The method depends upon the reaction of ammonium ions with

alkaline sodium phenate in the presence of sodium hypochlorite to produce an indophenol blue complex which is measured colorimetrically.

Portions of endosperm cell wall (20 mg) were hydrolyzed with 3 ml concentrated sulfuric acid, 0.75 ml 30% hydrogen peroxide and selenium dioxide for 20 min. The hydrolysates were diluted to 10 ml with distilled water. Portions (1 ml) were incubated with 10 ml alkaline sodium phenate reagent and 5 ml of 1% sodium hypochlorite for 30 min at 35°C. Absorbance was measured at 630 nm. The nitogen content of the samples was determined by reference to a standard curve prepared using ammonium sulfate solutions containing 5-50 µg nitrogen/ml.

3. Amino acid analysis

Protein associated with isolated barley endosperm cell walls was hydrolyzed in 6N HCl at 110°C for 24 hr. The amino acids were eluted from a column (0.9cm x 30cm) of DC-6A resin with lithium-pico buffer system IV (Pierce Chemical Co.) and analyzed using a Beckman Model 121 automatic amino acid analyzer.

4. Hydrolysis and monosaccharide analysis

Isolated endosperm cell walls and insoluble cell wall residues were hydrolyzed with 90% formic acid at 100°C for 2 hr. The formic acid was removed by evaporation under vacuum at 40°C, and the sample was rehydrolyzed with 1.5N sulfuric acid at 100°C for 3 hr.

Soluble polysaccharides in cell wall extracts were hydrolyzed with 1.5N sulfuric acid for 2 hr at 100°C.

Monosaccharides in the acid hydrolysates were separated as negatively-charged borate derivatives by anion-exchange column chromatography on Aminex-A25 (Bio Rad Laboratories, Richmond, California).

Portions of the hydrolysates (0.1 ml) were eluted from the column (0.9cm x 16cm) with an aqueous solution of 0.38M boric acid, 0.024M sodium tetraborate and 0.01M sodium chloride, adjusted to pH 7.0 with glacial acetic acid (Verhaar and Dirkx 1977). The column was maintained at 70°C and eluted at a flow rate of 1 ml/min.

Alternately, monosaccharides were eluted from a longer column (0.6cm x 75cm) of Aminex-A25 with a gradient of increasing pH and borate ion concentration as described by LaBerge et al. (1973).

The column effluent was monitored continuously using a Technicon AutoAnalyzer for colorimetric estimation of the sugars by reaction with 0.1% orcinol in 70% sulfuric acid at 95°C (Kesler 1967).

The sugars were determined quantitatively by calculating the ratio of the area under the peak relative to the area under the curve for a known standard sugar. Areas under curves were calculated by multiplying peak height absorbance by peak width in mm at one-half peak height.

5. Carbohydrate

Carbohydrate in column fractions and in enzyme-solubilized cell wall fractions was measured colorimetrically using the Technicon Auto-Analyzer for reaction of the carbohydrate with 0.1% orcinol in 70% sulfuric acid. Standard curves were prepared using monosaccharide solutions (5-30 µg/ml) containing mannose, arabinose, xylose and glucose in the ratio 5 : 10 : 15 : 70. Results were expressed as equivalent monosaccharide.

6. Glucose

Glucose in acid hydrolysates of enzyme-solubilized cell wall carbohydrate was determined with glucose oxidase reagent (G.O.D. Period

Method, Biochemica Test Combination, Boehringer-Mannheim GMBH Diagnostica).

7. Reducing sugars

Reducing sugars were estimated by the copper sulfate/neocuproine hydrochloride method as described by Dygert et al. (1965).

8. Gel permeation column chromatography

The molecular size distributions of extracted cell wall polysaccharides and enzyme-solubilized cell wall carbohydrates were estimated by gel permeation column chromatography on BioGel A50m and BioGel P150 (BioRad Laboratories, Richmond, California).

BioGel A50m (100-200 mesh), a 2% agarose gel having an exclusion limit of 50 million daltons for globular proteins, was used to estimate the molecular size distributions of polysaccharides extracted from the cell walls with water and alkali. Samples (2-4 ml) containing 1-2 mg polysaccharide/ml were applied to the column (2.5 x 85 cm) and eluted with 0.02% sodium azide by upward flow at 20 ml/hr.

BioGel P150 (100-200 mesh) was used to estimate the molecular size distributions of cell wall carbohydrate solubilized by wall-hydrolyzing enzymes. Samples (2-4 ml) containing 1-2 mg carbohydrate/ml were applied to the column (2.5 x 82.5 cm) and eluted by upward flow with 0.1M sodium chloride at 24 ml/hr.

Both columns were operated at room temperature (21$^{o}$C). The column effluents were collected in 100 drop fractions and analyzed for carbohydrate (Analytical Methods 5). The void volumes of the columns were estimated using bacterial lipopolysaccharide (E. coli serotype No. 0127:B8, Sigma) as described by Cameron (1968). Glucose was used to estimate the total elution volume.

9. Enzyme assays

Viscometric assays were used to estimate endo-β-glucanase and endo-β-xylanase activities in malt extracts and to determine the specificities of endo-β1,3-glucanase and endo-β1,3(4)-glucanase from malted barley.

Endo-β1,3-glucanase, endo-β1,4-glucanase, endo-β1,3(4)-glucanase and endo-β1,4-xylanase were assayed using carboxymethyl pachyman (0.5%), carboxymethyl cellulose (0.1%), barley β-glucan (0.5%), and wheat flour arabinoxylan (0.5%) as substrates, respectively. The polysaccharides were dissolved in 0.1M acetate buffer, pH 5.0, filtered through Whatman No. 1 paper, and equilibrated to 25°C prior to use.

For the assay, a portion of the enzyme solution (0.05-1.0 ml) was added to 6 ml substrate solution, and 5 ml of this solution was transferred immediately to an Ostwald viscometer (size 200) maintained at 25°C. The flow time was determined approximately 10 times during the initial 10-12 min of the reaction. The viscometers used for these assays had flow times from 9.3 to 9.8 sec with water. Enzyme activity was expressed as the rate of increase in reciprocal specific viscosity with time, $d(1/\eta_{sp})/dt$.

To determine the specificities of malt endo-β-glucanases, 0.1 ml portions of the enzyme solutions were incubated with the substrates for 24 hr at 25°C. Reciprocal specific viscosities of the enzyme-substrate digests and control substrate solutions were determined.

10. Scanning electron microscopy

Cell walls were mounted on metal stubs, coated with gold to a thickness of 200 nm, and examined with a JEOL JSM-35C scanning electron microscope. An accelerating voltage of 10-15 kV was used.

C. Isolation and Fractionation of Barley Endosperm Cell Walls

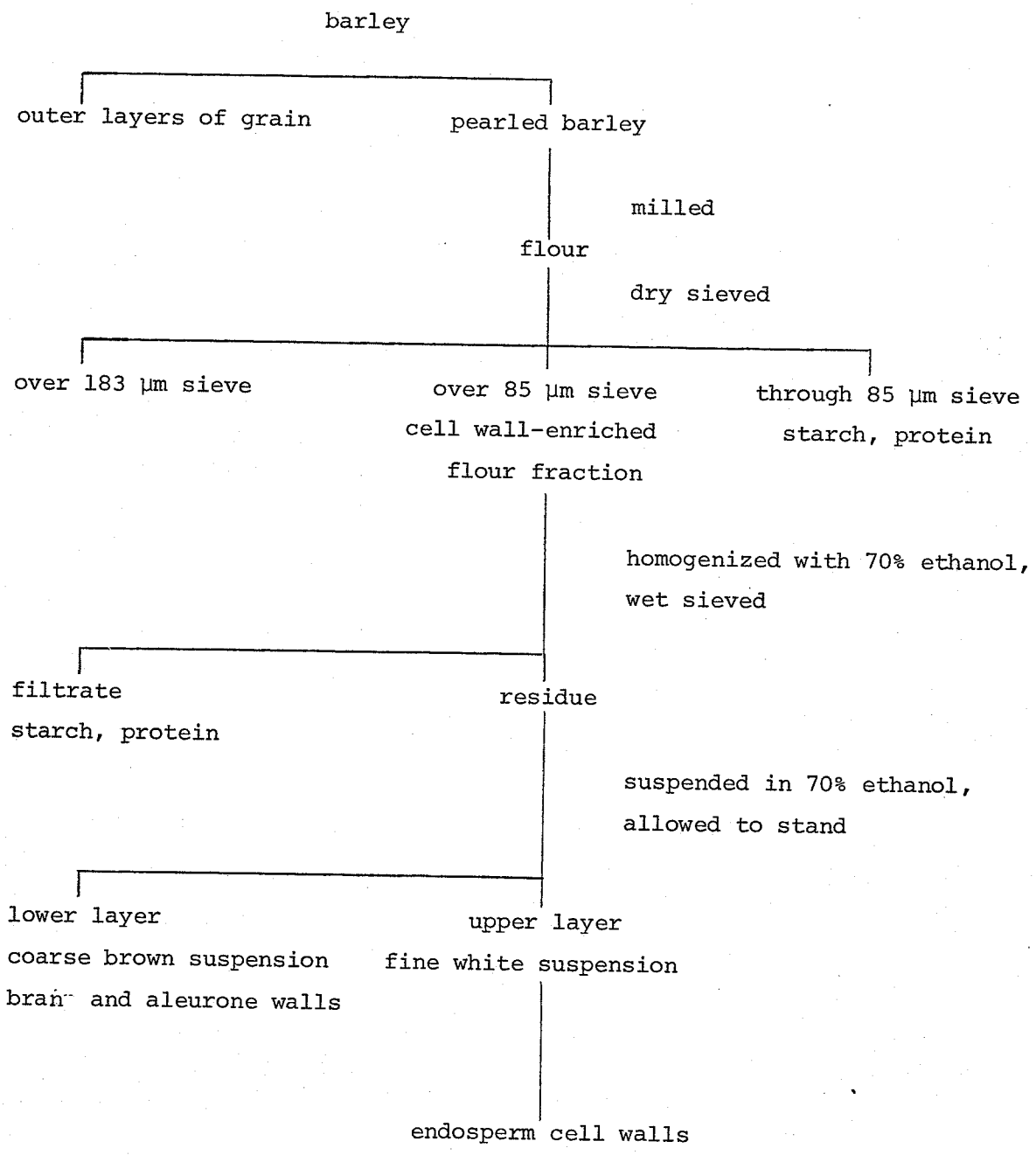
Endosperm cell walls were isolated from Conquest barley by a modification of the method described by Mares and Stone (1973a) for the isolation of wheat endosperm cell walls. The pearled, milled grain was dry sieved to give a cell wall-enriched flour fraction, the cell walls were separated from the cell contents by alternate grinding and wet sieving in 70% ethanol, and finally, non-endosperm walls were removed by differential sedimentation in 70% ethanol. The procedure is summarized in Figure 1, and details of the individual steps are given below.

Pearling - The grain was pearled in 100 g portions, for 4 min, in a Strong-Scott laboratory pearling machine. Loose husk fragments and unpearled kernels were removed by hand. The pearl represented approximately 45% of the weight of the barley sample.

Milling - The pearled barley was milled in a GRL experimental mill. The sample was passed through the second and third break rolls once, and sifted over 8xx and 72GG sieves. The overs from both sieves were passed through the reduction rolls once at setting 50, once at 45, and three times at 40, with sieving as above after each reduction. The overs from the last reduction were pin milled (Alpine Augsburg type 160z) at 16,500 rpm and sieved. Flour that did not pass the 8xx sieve (linear pore size 183 μm) contained a significant proportion of non-endosperm material and was discarded.

Dry-sieving - The flour that passed the 8xx sieve was sifted over a 15xx silk sieve (linear pore size 85 μm) using a Buhler Laboratory Sifter. Portions of the material passing the sieve, and the sieve residue were examined by light microscopy. Flour particles consisting of cell walls and adhering starch and protein were retained on the sieve,

Figure 1. Procedure for the Isolation of Barley Endosperm Cell Walls



while free starch granules and starch/protein aggregates passed through. No cell wall fragements were observed in the material passing the sieve. Much of the starch and protein adhering to the cell walls was removed by pin milling and sieving the sample for a second and third time. This process resulted in a cell wall-enriched flour fraction representing 5% of the weight of the pearled grain.

Wet sieving - The cell wall-enriched fraction was suspended in 70% aqueous ethanol, homogenized in a Waring Blendor 1 min at room temperature (21°C), and wet sieved on a stainless steel sieve having a linear pore size of 74 μm. This procedure was repeated an additional two times. The residue was washed repeatedly with 70% ethanol to remove most of the free starch and protein. The cell wall fraction was transferred to a mortar, gently ground with a pestle and wet sieved. Alternate grinding and wet sieving were repeated until no starch was observed in the sieve filtrate.

Sedimentation - The sieve residue was resuspended in 70% ethanol and allowed to stand 5 min. The upper white suspension was decanted and the process repeated until the sieve residue was divided into a fine white suspension of endosperm cell walls, and a coarser brown fraction containing a significant proportion of non-endosperm cell walls and some endosperm walls heavily contaminated with starch and protein. The clean white endosperm cell wall suspension was stored at 4°C in 70% ethanol and used for all subsequent analyses.

Drying of isolated endosperm cell walls - Prior to analysis the cell walls were washed with 95% ethanol and dried under vacuum at 45°C.

Fractionation of isolated endosperm cell walls - Isolated cell walls were fractionated by chemical extraction as shown in Figure 2.

Figure 2. Procedure for the fractionation of isolated barley endosperm cell walls.