THE UNIVERSITY OF MANITOBA

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

ROBERTA GAIL THOMPSON

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

DOCTOR OF PHILOSOPHY

DEPARTMENT OF PLANT SCIENCE

WINNIPEG, MANITOBA

1979

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

BY

ROBERTA GAIL THOMPSON

A dissertation submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

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.

ACKNOWLEDGEMENTS

I wish to express my gratitude to the following individuals and institutions, and to thank them for their contributions to this thesis:

Dr. D.E. LaBerge, for guiding and supervising the research,

Dr. W. Bushuk, for providing direction and encouragement,

Dr. A.W. MacGregor, for producing the scanning electron micrographs, and for helpful and stimulating discussions,

Mrs. L. Morley, for typing the manuscript,

The Canadian Grain Commission, for providing laboratory facilities,

The University of Manitoba, for the award of a Fellowship, and

The National Research Council of Canada, for the award of a Postgraduate Scholarship.

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

Page

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

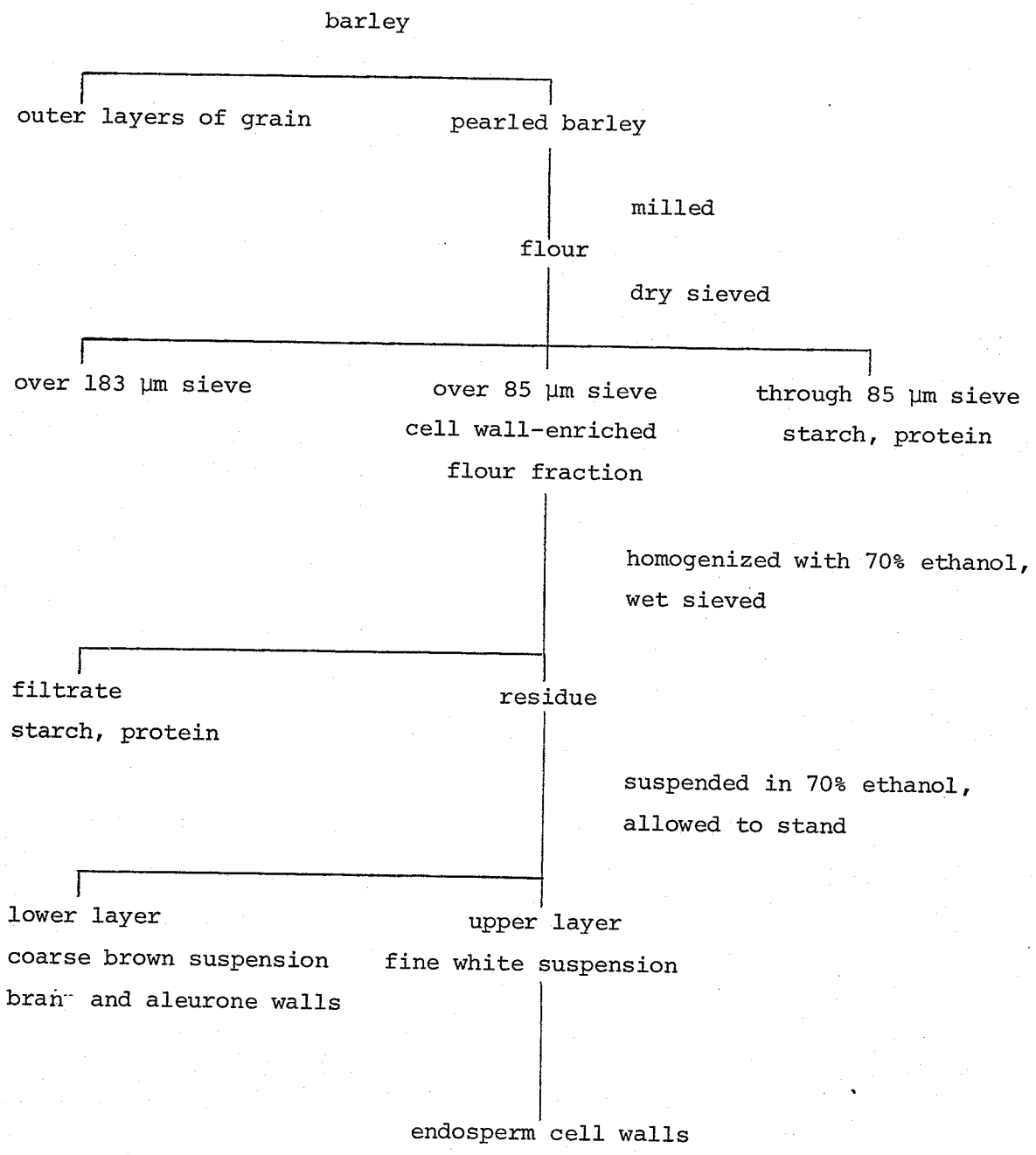
Endosperm cell walls were isolated from Conquest barley by a modification of the method described by Mares and Stone (1973a) for the isolation of wheat endosperm cell walls. The pearled, milled grain was dry sieved to give a cell wall-enriched flour fraction, the cell walls were separated from the cell 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.

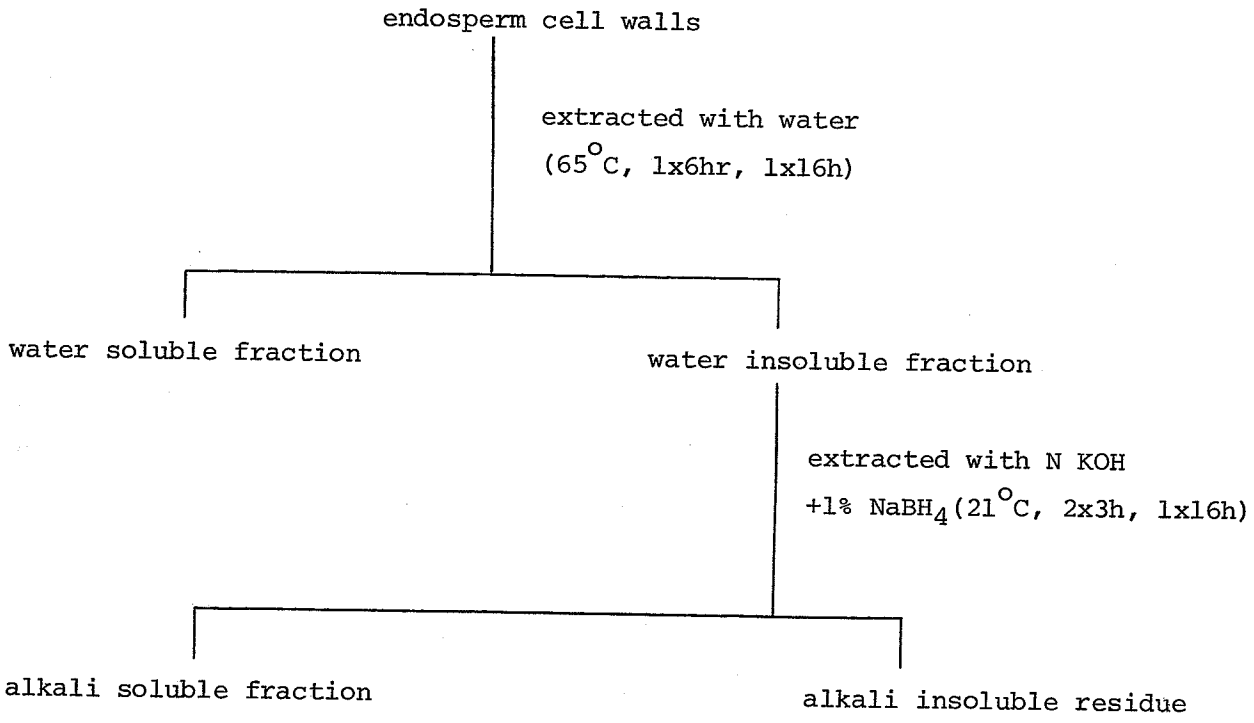
Wet sieving - The cell wall-enriched fraction was suspended in 70% aqueous ethanol, homogenized in a Waring Blendor 1 min at room temperature (21°C), and wet sieved on a stainless steel sieve having a linear pore size of 74 μm. This procedure was repeated an additional two times. The residue was washed 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.



Cell wall fragments were suspended in water (5 mg/ml) and maintained in a shaking water bath at 65°C (1 x 6 hr, 1 x 16 hr). The water-soluble extracts were separated from the residual wall material by centrifugation.

The water-insoluble cell wall residue was extracted at room temperature (21°C) with N potassium hydroxide containing 1% sodium borohydride (2 x 3 hr, 1 x 16 hr). The alkali-soluble material was obtained by centrifugation, and the supernatant solution was neutralized with perchloric acid. The resultant precipitate of potassium perchlorate was removed by centrifugation. The water and alkali extracts were each stored frozen prior to analysis.

The alkali-insoluble cell wall residue was washed with water to remove salts, and then washed with increasing concentrations of ethanol. The residue was dried under vacuum at 45°C.

The relative monosaccharide compositions of the water and alkali extracts, and the alkali-insoluble residuewere determined by ion-exchange chromatography of acid hydrolysates (Analytical Methods 4).

The molecular size distributions of water-soluble and alkali-soluble cell wall polysaccharides were investigated by gel permeation column chromatography on BioGel A50m (Analytical Methods 8). To compare the molecular size distributions of alkali-soluble glucan and arabino-xylan, portions of the fractions obtained after gel permeation chromat-ography were acid hydrolyzed and analyzed for glucose and for total reducing sugars (Analytical Methods 6 and 7). The absorbance due to glucose in the reducing sugar assay was calculated, and the absorbance due to pentose sugars was estimated by difference.

D. Hydrolysis of Isolated Barley Endosperm Cell Walls by Extracts of Malted Barley

Prior to enzymic hydrolysis, endosperm cell walls were extracted with water as follows. Cell walls (1g) were suspended in 200 ml distilled water and maintained in a shaking water bath (80 osc/min) at 65°C for 2 hr. The suspension was clarified by centrifugation, and the cell wall residue was re-extracted with water at 65°C three more times (100 ml x 2 hr, 50 ml x 2 hr, 50 ml x 18 hr). Carbohydrate in the final extract accounted for less than 1% of the weight of the original wall sample. The water-extracted cell wall residue was recovered by centrifugation, washed with increasing concentrations of ethanol, and dried under vacuum at 45°C. The relative monosaccharide composition of the water-extracted cell wall residue was determined by ion-exchange chromatography of acid hydrolysates as described in Analytical Methods 4. This water-insoluble material was the substrate used in all subsequent investigations of the effect of enzymes on the endosperm cell wall.

1. Preparation of malt extracts

Extracts were prepared in duplicate from the malts of five barley cultivars (Table 1). Malted barley (5 g) was ground in a Braun coffee grinder for 1 min. Portions (2 g) of the ground malt were extracted with 8 ml 0.2M acetate buffer (pH 5.0) in a Virtis homogenizer for 2 min. The extracts were clarified by centrifugation and dialyzed against 0.01M acetate buffer at 4°C for 24 hr. After filtration through glass wool to remove precipitated material, the dialyzed extracts were diluted to 10 ml with buffer. The relative activities of endo-β1,3-glucanase, endo-β1,3(4)-glucanase and endo-β1,4-xylanase in the malt extracts were determined by viscometric assays (Analytical Methods 9).

Table 1. Identification of barley lines.

TR No.	Pedigree	Origin
206	7118-702-13 x Klages	CDA Research Station, Winnipeg
428	Klages x S7122	University of Saskatchewan
436	Klages x S7122	University of Saskatchewan
438	Klages x S7122	University of Saskatchewan
910	HP 4388 x R/MGH2	North American Plant Breeders

2. Incubation of endosperm cell walls with malt extracts

Water-extracted cell walls (20 mg) were suspended in 9.8 ml 0.01M acetate buffer (pH 5.0) in 25 ml conical flasks. Malt extract (0.2 ml) was added, and the suspensions were maintained in a shaking water bath (80 osc/min) at 35°C. Duplicate digests were prepared with each malt extract. Control digests containing only cell walls and buffer, or malt extract plus buffer were incubated under identical conditions.

After 24 hr, the cell wall fragments were removed by filtration through glass wool, and enzyme activity was terminated by heating the solutions to the boiling point. The enzyme-solubilized cell wall components were stored frozen prior to analysis.

The rate of cell wall solubilization by each malt extract was determined in a separate experiment. Water-extracted cell walls (5 mg) were suspended in 10 ml 0.01M acetate buffer (pH 5.0) in 25 ml conical flasks and equilibrated to 35°C in a shaking water bath (80 osc/min). Malt extract (0.02 ml) was added to each suspension. Duplicate digests were prepared with each malt extract. Portions of the suspensions (1 ml) were removed after 0.2, 0.5, 1, 2, 4 and 24 hr, and added to 0.1 ml 3N sulfuric acid to terminate the reaction. The acidified suspensions were filtered immediately through glass wool to remove cell wall fragments. Control suspensions containing only cell wall and buffer, or malt extracts plus buffer were sampled in the same way.

3. Analysis of cell wall carbohydrate solubilized by malt extracts

The amount of cell wall carbohydrate solubilized by the malt extracts was estimated by the orcinol/sulfuric acid assay (Analytical Methods 5).

The relation between the activity of each of the enzymes measured

(endo-β1,3-glucanase, endo-β1,3(4)-glucanase and endo-β1,4-xylanase) and the proportion of the cell wall solublized by the malt extracts was investigated by linear regression analysis to determine correlation coefficients.

The relative monosaccharide compositions of the solubilized cell wall components were determined by ion-exchange chromatography of acid hydrolysates (Analytical Methods 4).

The molecular size distributions of solubilized cell wall carbohydrate were investigated by gel permeation column chromatography on BioGel P150 (Analytical Methods 8).

E. Hydrolysis of Isolated Barley Endosperm Cell Walls by Malt Endo-β-Glucanases

1. Isolation of endo-β1,3-glucanase and endo-β1,3(4)-glucanase from malt

Endo-β1,3-glucanase and endo-β1,3(4)-glucanase were isolated from unkilned, freeze-dried Conquest malt as described by Manners and Wilson (1976). The enzymes were purified from an extract of malted barley by ion-exchange column chromatography on DEAE-cellulose and CM-cellulose. Complete separation of endo-β1,3-glucanase and endo-β1,3(4)-glucanase activities was achieved on CM-cellulose using a linear gradient of 0.02 - 1.0M sodium acetate (pH 4.8). The elution profile was essentially the same as that reported by Manners and Wilson (1976).

2. Incubation of endosperm cell walls with malt endo-β-glucanases

Water-extracted cell walls (20 mg) were suspended in 10 ml 0.01M acetate buffer (pH 5.0) in 25 ml conical flasks. To each suspension one of the following was added: a) 0.1 ml endo-β1,3-glucanase,

b) 0.1 ml endo-β1,3(4)-glucanase, c) 0.1 ml endo-β1,3-glucanase plus 0.1 ml endo-β1,3(4)-glucanase or d) 0.1 ml water. Suspensions were maintained at 35°C in a shaking water bath (80 osc/min). At intervals of 24 hr, the suspensions were clarified by centrifugation and the supernatant solutions were heated to the boiling point to terminate enzyme activity. The cell wall residues were re-suspended in buffer with the appropriate enzyme treatments, and the procedure was repeated for four consecutive days.

In a second experiment, the cell wall-glucanase digests were prepared in duplicate as described above, maintained at 35°C for 24 hr, and then kept at 4°C for two weeks. The suspensions were clarified by centrifugation, and the cell wall residues were treated with enzymes for a further 24 hr.

After the enzyme treatments, the cell wall residues were washed with increasing concentrations of ethanol, dried under vacuum at 45°C, and examined by scanning electron microscopy. The glucanase-solubilized cell wall fractions were stored frozen prior to analysis.

3. Analysis of cell wall carbohydrate solubilized by malt endo-β-glucanases

The amount of solubilized carbohydrate was estimated by the orcinol/sulfuric acid assay (Analytical Methods 5) and expressed as a percentage of the cell wall weight.

The relative monosaccharide composition of cell wall carbohydrate that was released in the initial 24 hr period was determined by ion-exchange chromatography of acid hydrolysates (Analytical Methods 4).

To estimate the amount of cell wall glucan solubilized by the enzyme treatments, glucose in acid hydrolysates of the samples was

determined specifically with glucose oxidase (Analytical Methods 6).

The molecular size distributions of endo-β-glucanase-solubilized cell wall components were investigated by gel permeation column chromatography on BioGel P150. Fractions were analyzed for total carbohydrate and for glucose, in acid-hydrolyzed portions of the fractions, using glucose oxidase.

4. Hydrolysis of β-glucans from isolated barley endosperm cell walls by malt endo-β1,3-glucanase

Isolated endosperm cell walls (1g) were extracted three times with water (100 ml, 50 ml, 50 ml) for 30 min, in a shaking water bath (100 osc/min) at 40°C. The extracts were obtained by centrifugation and combined. The cell wall residue was re-extracted with water at 65°C by the same procedure, and the resultant cell wall residue was similarly re-extracted at 100°C.

The cell wall extracts were treated with α-amylase suspension (50 µl, hog pancreas, Sigma), and dialyzed against distilled water for 24 hr. After dialysis, the total glucan and α-glucan contents of the extracts were estimated by determining glucose (Analytical Methods 6) in total acid hydrolysates and amyloglucosidase hydrolysates respectively. The β-glucan content of the extracts was calculated by difference. The extracts were reduced in volume by rotary evaporation under vacuum at 40°C so that each solution contained 1.8 mg β-glucan/ml. The molecular size distributions of the solubilized polysaccharides were determined by gel permeation column chromatography on BioGel A50m (Analytical Methods 8).

The hydrolysis of β-glucans from the cell walls by malt endo-β1, 3-glucanase was investigated by viscometric assays. The assays were conducted using a) cell wall extracts of equivalent β-glucan concentration

and b) diluted cell wall extracts of similar initial specific viscosity (approximately 0.48).

For all the assays, a portion of the cell wall extract or diluted extract (5 ml) was mixed with 1 ml 0.6M acetate buffer (pH 5.0), and 5 ml of this solution was transferred to an Ostwald viscometer (size 200). The flow time of the solution was determined at 25°C. Endo-β1,3-glucanase (0.1 ml) was added and the flow time was measured approximately 10 times during the initial 10 to 12 min of the reaction. Enzyme activity was expressed as the rate of increase in reciprocal specific viscosity with time, $d(^{1}/\eta sp)/dt$.

III RESULTS AND DISCUSSION

A. Composition of Isolated Barley Endosperm Cell Walls

1. Isolation of the cell walls

Endosperm cell walls were isolated from Conquest barley by a procedure based upon that of Mares and Stone (1973a). The method was modified to include pin milling and dry sieving, prior to wet sieving in 70% ethanol. After pin milling, cell wall-containing flour fragments were retained on a sieve of linear pore size 85 μm, while free starch granules and starch/protein aggregates passed through the sieve. The advantage of this modification was that, using a mechanical sifter, most of the non-cell wall material was quickly eliminated, leaving only 5% of the flour sample, a cell wall-enriched fraction, to be processed by manual grinding and wet sieving. The yield from 1 kg pearled barley was 12.5 g.

2. Appearance of the cell wall preparation

The preparation consisted almost entirely of endosperm cell walls and was not contaminated by free starch granules or cytoplasmic debris (Figure 3). The majority of the cell wall fragments consisted of the walls of adjacent cells still adhering to one another. The intracellular surfaces were marked by the adpression of starch granules, while extracellular surfaces were comparatively smooth.

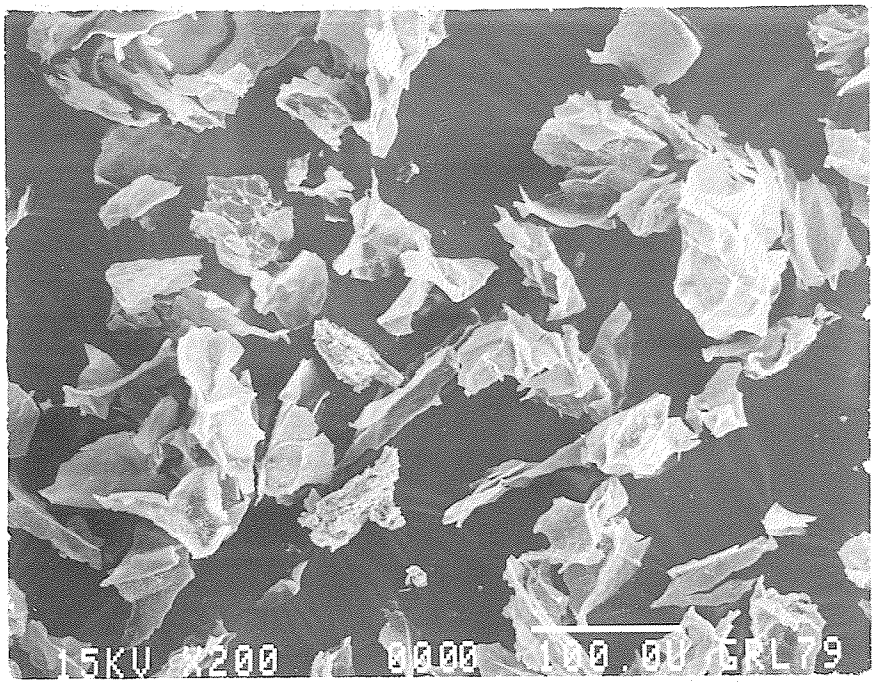
Three types of intracellular surfaces were observed. The fragment shown in Figure 4 is representative of the most frequently occurring type, in which indentations made by large granules of various sizes and shapes are surrounded by numerous small granule indentations. The surface of the same fragment is shown at higher magnifications in Figures 5 and 6.

Figure 3.

Scanning electron micrograph of isolated barley endosperm cell walls (x200).

Figure 4.

Scanning electron micrograph of the intracellular surface of an isolated endosperm cell wall fragment showing indentations due to the adpression of large and small starch granules (x1200).


15KV X200 0000 100.0U GRL79

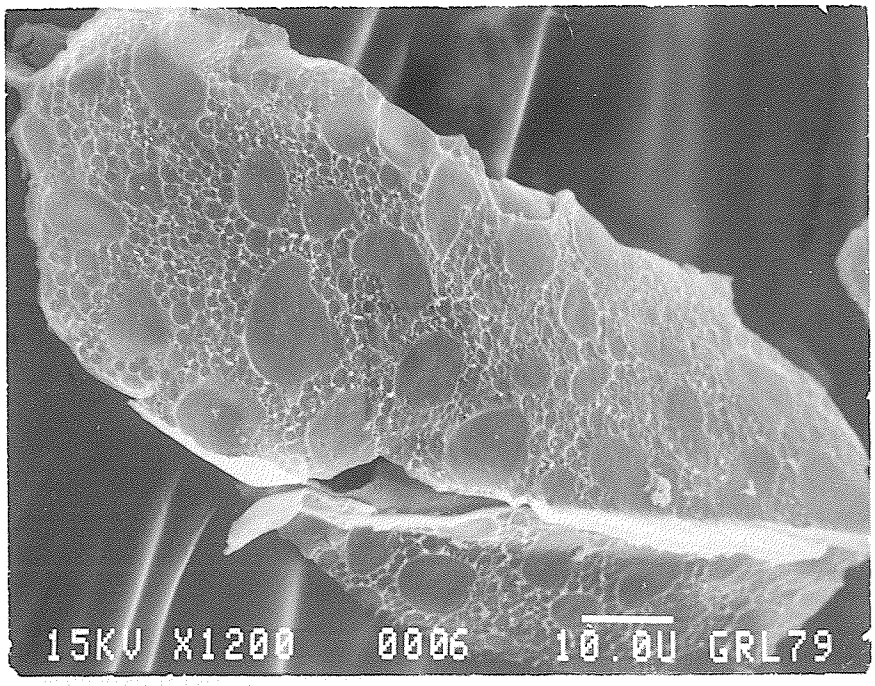
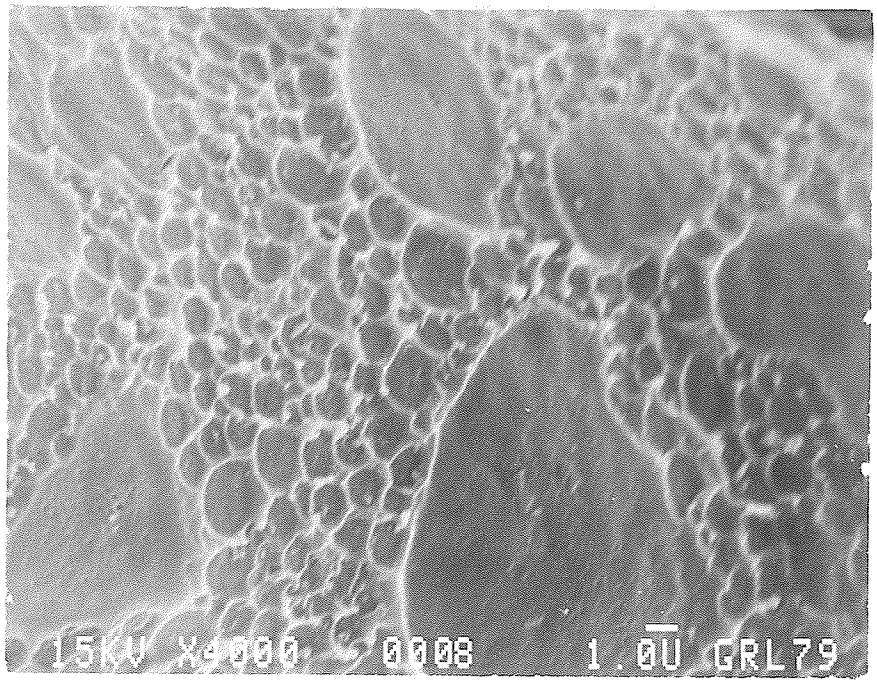

15KV X1200 0006 10.0U GRL79

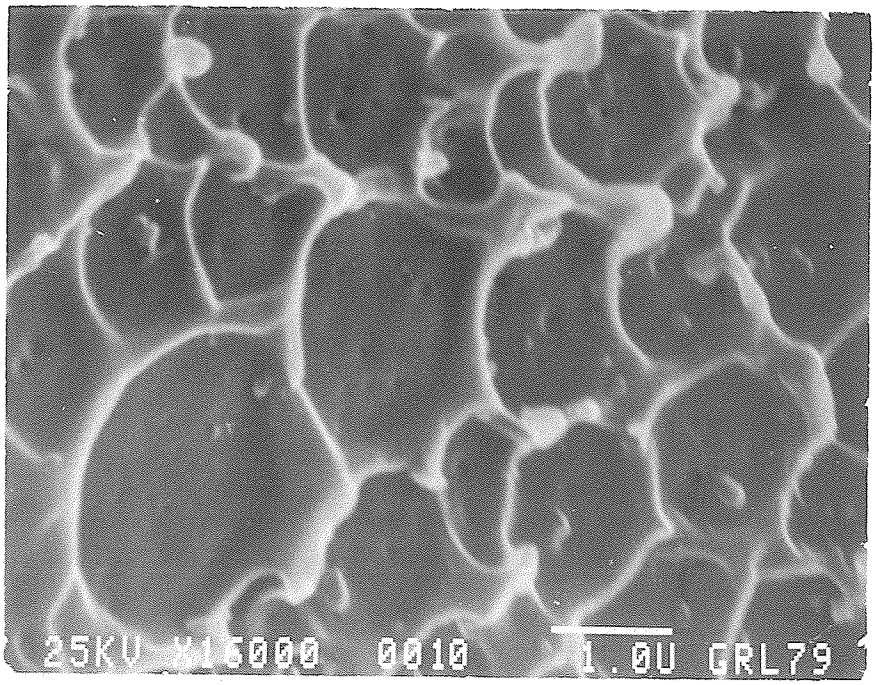
Figure 5.

Scanning electron micrograph at higher magnification (x4,000), of the cell wall fragment shown in Figure 4.

Figure 6.

Scanning electron micrograph at higher magnification (x16,000), of indentations due to the adpression of small starch granules on the intracellular surface of the cell wall fragment shown in Figure 4.


15KV X4000 0008 1.0U GRL79


25KV X16000 0010 1.0U GRL79

The surface appears to be pitted and has numerous particles adhering to it. Some of the particles appear to be continuous with the wall surface. A second type of fragment is shown in Figure 7. The large granule indentations are more uniform in size, more closely spaced, and surrounded by fewer small granule indentations. The cracked appearance of the center of the fragment may be an artifact resulting from prolonged exposure to the electron beam in the scanning microscope. Figure 8 shows the third type of intracellular surface, which is marked only by the adpression of large starch granules. Cell wall fragments having only small granule indentations were not observed.

The existence of different types of intracellular surfaces is evidence that barley endosperm cells contain variable proportions of large and small starch granules. Some cells may contain few, if any, small starch granules. MacLeod et al. (1964) observed that endosperm cells adjoining the aleurone layer contained large numbers of small granules, whereas fewer small granules were found in the inner endosperm.

Most of the cell wall fragments were free of starch, but small starch granules were observed adhering to some fragments (Figures 9 and 10). The cell wall preparation contained few, if any, large granules. Some of the small starch granules appear to be embedded in the surface of the wall.

3. Starch content

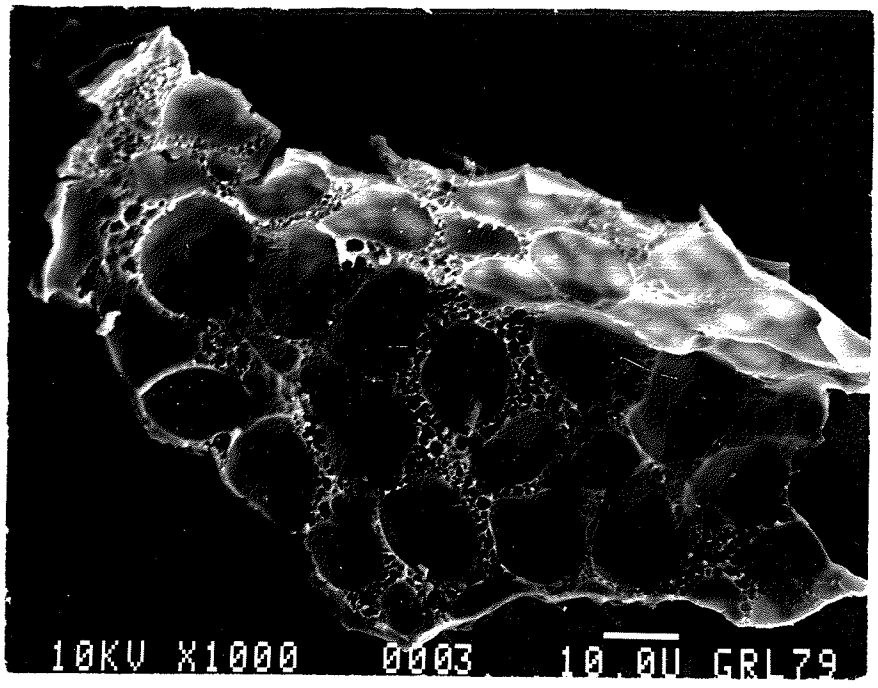
The starch content of an endosperm cell wall preparation is important for two reasons. Firstly, starch content indicates the purity of the cell wall preparation, and the effectiveness of the isolation procedure in removing the major cytoplasmic contaminant. Secondly, and more importantly, the major component of barley endosperm cell walls is a

Figure 7.

Scanning electron micrograph of the intracellular surface of an isolated endosperm cell wall fragment showing fairly uniform large starch granule indentations and few small starch granule indentations (x1000).

Figure 8.

Scanning electron micrograph of the intracellular surface of an isolated endosperm cell wall fragment having only large starch granule indentations (x1300).


10KV X1000 0003 10.0U GRL79

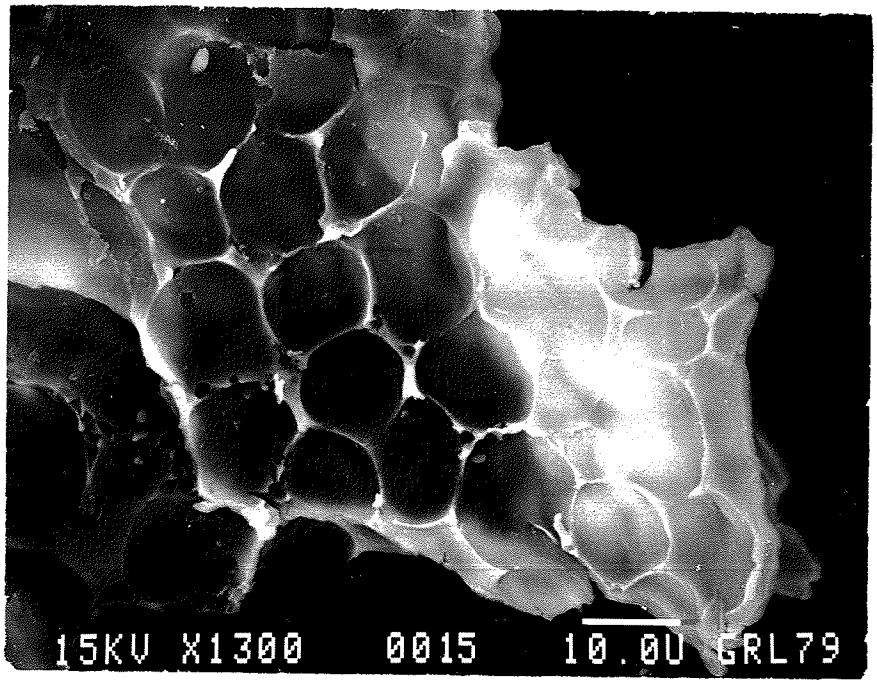
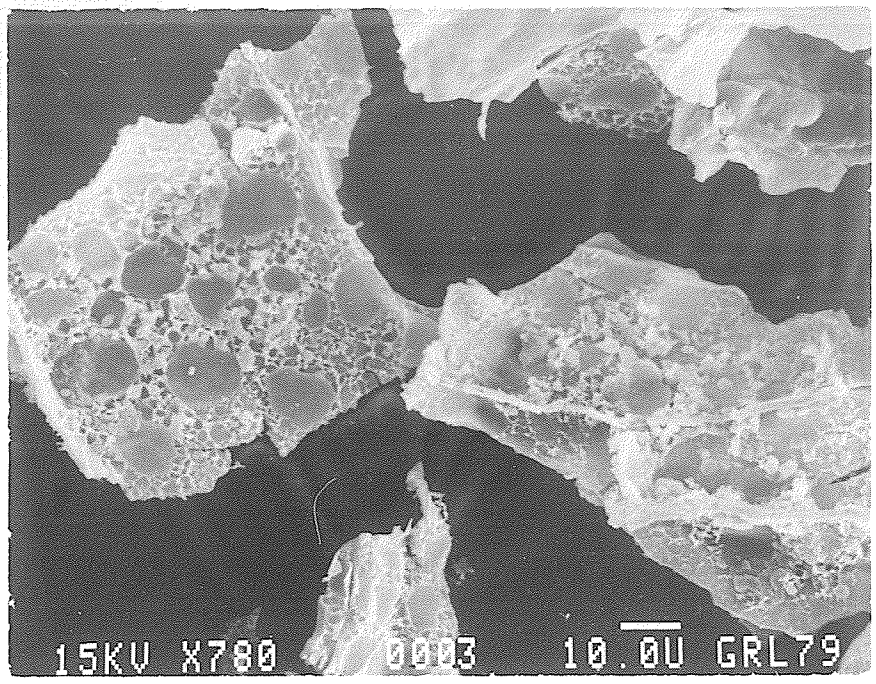

15KV X1300 0015 10.0U GRL79

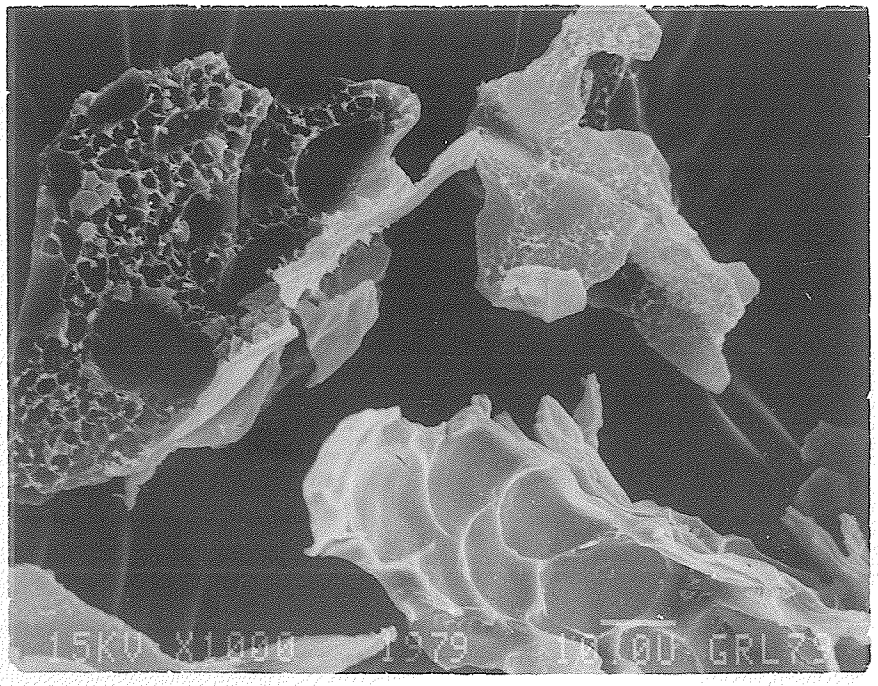
Figure 9.

Scanning electron micrograph of isolated barley endosperm cell wall fragments with small starch granules adhering to the intracellular surfaces (x780).

Figure 10.

Scanning electron micrograph of isolated barley endosperm cell wall fragments illustrating two different types of intracellular surfaces, and contamination of some cell wall fragments by small starch granules (x1000).


15KV X780 0003 10.0U GRL79


15KV X1000 1979 10.0U GRL79

glucan (Ballance 1976, Ballance and Manners 1978, Fincher 1975, Forrest and Wainwright 1977) and therefore an accurate estimate of starch associated with the cell walls is essential to the determination of the β-glucan content of the cell walls.

Four methods of detecting starch in the cell wall preparation were compared. The results are presented in Table 2. After each treatment the cell wall residues were treated with iodine solution. Undegraded starch was detected in the cell walls that had been treated with amyloglucosidase alone and in the heat-treated samples. Starch was not detected in cell wall residues after treatment with α-amylase plus amyloglucosidase, or in perchloric acid-extracted cell wall residues.

The comparatively low starch estimate obtained by treating the cell walls with amyloglucosidase alone suggests that the enzyme may have limited ability to attack intact starch granules. This conclusion is substantiated by the results of Smith and Lineback (1976) who reported that incubation of isolated wheat and corn starch granules with Aspergillus niger amyloglucosidase (16 hr, 37°C) resulted in approximately 24% and 40% conversion to glucose, respectively. After 64 hours, conversion of isolated starch granules to glucose was still incomplete.

The intermediate value of 7.9% starch obtained with the heat-treated sample may reflect incomplete gelatinization of the starch and/or the inability of the enzyme to hydrolyze small starch granules completely. Bathgate and Palmer (1972) found that the small starch granules of barley, which were the only type observed in the cell wall preparation, were more resistant to gelatinization and were associated with more protein than large starch granules. They postulated that these factors may limit the hydrolysis of small starch granules by amylolytic enzymes.

Table 2. Starch content of barley endosperm cell wall preparation, % wt/wt.

Treatment	Starch*
amyloglucosidase alone	3.2 ± .3
heat treatment + amyloglucosidase	7.9 ± .1
α-amylase + amyloglucosidase	11.7 ± .1
perchloric acid extraction + amyloglucosidase	11.7 ± .1

* mean of 3 determinations

Subsequent investigations showed that small granules were extremely resistant to attack by malt α-amylase, the maximum conversion of boiled small granules to apparent maltose being 52% (Bathgate and Palmer 1973).

The starch content of the cell wall preparation as estimated by the two remaining treatments, α-amylase plus amyloglucosidase and perchloric acid extraction prior to amyloglucosidase hyrolysis was 11.7%. The perchloric acid extraction method has the advantage of ensuring that the starch is in solution prior to enzyme treatment, thus facilitating hydrolysis.

Perchloric acid as a solvent for starch was first described by Nielsen (1943) and Nielsen and Gleason (1945). Starch is completely solubilized by 25% perchloric acid within one hour at room temperature. Hydrolysis is not detectable even if extraction time is increased to two hours. Under these conditions perchloric acid does not hydrolyze barley β-glucan. Glucose was not detected in perchloric acid-cell wall extracts prior to amylogucosidase hydrolysis.

The results indicate that amyloglucosidase hydrolysis alone gives a low estimate of starch content, and that other treatments that disrupt or dissolve the starch granules probably increase the susceptibility of the polysaccharide to amyloglucosidase hydrolysis, and thus give a more accurate estimate of starch content.

Perchloric acid extraction of the cell walls yielded very viscous solutions. Monosaccharide analysis of an acid hydrolysate of a portion of the extract indicated that 54% of the weight of the cell wall was soluble in 25% perchloric acid, and that the soluble carbohydrate was mostly glucan (Table 3).

4. Nitrogen content and amino acid composition

The nitrogen content of the endosperm cell wall preparation was

Table 3. Amount and relative monosaccharide composition of cell wall carbohydrate extracted by 25% perchloric acid, corrected for starch.

% of Total Cell Wall Carbohydrate	Relative Monosaccharide composition			
	Mannose	Arabinose	Xylose	Glucose
54	trace	6.1	15.6	78.3

0.48%. Part of the nitrogenous material may be cytoplasmic in origin, but there is evidence that protein may be a structural component of the barley endosperm cell wall (Forrest 1977, Forrest and Wainwright 1977).

The amino acid composition of the protein associated with the cell wallsis given in Table 4, and is compared with analyses of barley endosperm cell wall protein reported by Costello (1968) and pearled barley protein (Tkachuk and Irvine 1969). The amino acid compositions of the two cell wall preparations are in good agreement, but differ from total barley endosperm protein. Protein associated with the cell walls contained proportionately less glutamic acid and proline, and proportionately more of several amino acids that have the potential to form linkages with cell wall carbohydrate : aspartic acid, threonine, serine, lysine and arginine (Lamport 1970). Hydroxyproline was not detected. In many plants the cell wall protein is rich in hydroxyproline and is glycosidically linked via this residue to arabinose (Heath and Northcote 1971, Lamport 1969, Lamport and Miller 1971). However, this imino acid has not been detected in cereal endosperm cell walls (Costello 1968, Fincher 1975, Forrest 1977, Mares and Stone 1973a). A partial amino acid analysis of protein firmly associated with purified β-glucan from barley endosperm cell walls showed that glutamate, serine, leucine and aspartate accounted for 70% of the recovered amino acids (Forrest 1977). Forrest concluded that protein is a structural component of the barley endosperm cell wall but that it differs from the cell wall protein of other plants in that it does not contain hydroxyproline.

5. Monosaccharide composition of isolated cell walls and cell wall fractions

The monosaccharide compositions of the isolated endosperm cell walls and cell wall fractions are given in Table 5.

The isolated cell walls consist almost entirely of polysaccharide. After sequential hydrolysis in formic and sulfuric acids, 93% of the cell

Table 4. Amino acid composition of protein associated with barley endosperm cell walls, moles/100 moles amino acid.

Amino acid	Cell walls, this study	Cell walls, Costello 1968	Pearl barley, Tkachuk & Irvine 1969
Aspartic acid	8.26	8.84	5.64
Threonine	5.36	4.55	4.00
Serine	6.72	5.55	5.68
Glutamic acid	15.01	15.70	23.79
Proline	9.01	8.29	13.79
Glycine	9.27	9.34	6.82
Alanine	8.17	9.16	5.48
Valine	6.91	7.87	6.12
Cystine + Cysteine	0.77	1.79	1.66
Methionine	1.20	trace	1.34
Isoleucine	3.84	4.52	4.16
Leucine	8.00	8.69	6.96
Tyrosine	2.39	1.96	2.24
Phenylalanine	3.73	3.81	4.38
Lysine	5.09	4.08	2.80
Histidine	2.11	1.73	1.82
Arginine	4.16	4.08	3.30
Hydroxyproline	0	0	n.d.

Table 5. Monosaccharide composition and solubility of isolated barley endosperm cell walls.

	% of Total Cell Wall Carbohydrate	Relative Monosaccharide Composition			
		Mannose	Arabinose	Xylose	Glucose
Whole walls	100	3.5	10.9	17.0	68.6
65°C Water extract	43	1.8	4.9	7.9	85.4
Alkali extract	50	0	11.8	16.9	71.3
Residue	7	23.3	12.7	14.0	50.0

wall weight was recovered as monosaccharides. Cell wall carbohydrate, after correction for starch, consisted of 3.5% mannose, 10.9% arabinose, 17.0% xylose and 68.6% glucose. Compared to the monosaccharide compositions of barley endosperm cell walls isolated by similar procedures from the British cultivars, Maris Otter and Julia, the cell walls from Conquest barley contain slightly less glucose and relatively more of the other cell wall monosaccharides (Ballance and Manners 1978, Fincher 1975, Forrest and Wainwright 1977).

Structural analysis of polysaccharides isolated from barley endosperm cell walls (Ballance and Manners 1978) has confirmed that the main components are a linear β-glucan containing 1,3 and 1,4 linkages in a ratio of 3 to 7, and an arabinoxylan consisting of chains of β1,4-linked xylopyranose residues having terminal arabinofuranose units attached to C_2 and/or C_3 positions in the xylan chain. The structure of the mannose-containing polysaccharide has not been determined.

Water at 65°C extracted 43% of the cell wall, 85% of this being glucan. The water solubility of β-glucan increases with temperature (Ballance and Manners 1978, Fleming and Kawakami 1977), and therefore the relative monosaccharide compositions of the water-soluble and alkali-soluble fractions are dependent upon the temperature of the aqueous extraction. The solubility of barley polysaccharides at 65°C is of interest because this is the temperature normally used in mashing.

Potassium hydroxide (1N) containing 1% sodium borohydride extracted a further 50% of the cell wall. The alkali extract consisted of 29% arabinoxylan and 71% β-glucan, the major portion of the cell wall arabinoxylan being in this fraction. Mannose was not detected in the alkali extract.

The insoluble residue, 7% of the wall, contained glucan, arabinoxylan and a relatively high proportion of mannose (23%). This represents approximately two-thirds of the total mannose in the cell wall.

On the basis of these results, Conquest endosperm cell walls and endosperm cell walls from the British cultivars, Maris Otter and Julia, appear to have similar solubility characteristics (Ballance and Manners 1978, Fincher 1975, Forrest and Wainwright 1977). However, the results of these studies cannot be compared directly due to differences in the extraction procedures.

6. Molecular size distributions of cell wall polysaccharides

The molecular size distributions of cell wall polysaccharides were investigated by gel permeation column chromatography on Bio Gel A50m, which has an exclusion limit of 50 million daltons for globular proteins. The void volume of the column was determined using bacterial lipopolysaccharide (Cameron 1968).

Figure 11A shows the elution profiles of water-soluble and alkali-soluble cell wall polysaccharides. Both samples contain carbohydrate that is eluted at or near the void volume of the column. The elution profile of the alkali-soluble fraction shows that it is more polydisperse and contains relatively more lower molecular weight polysaccharides than the water-soluble fraction.

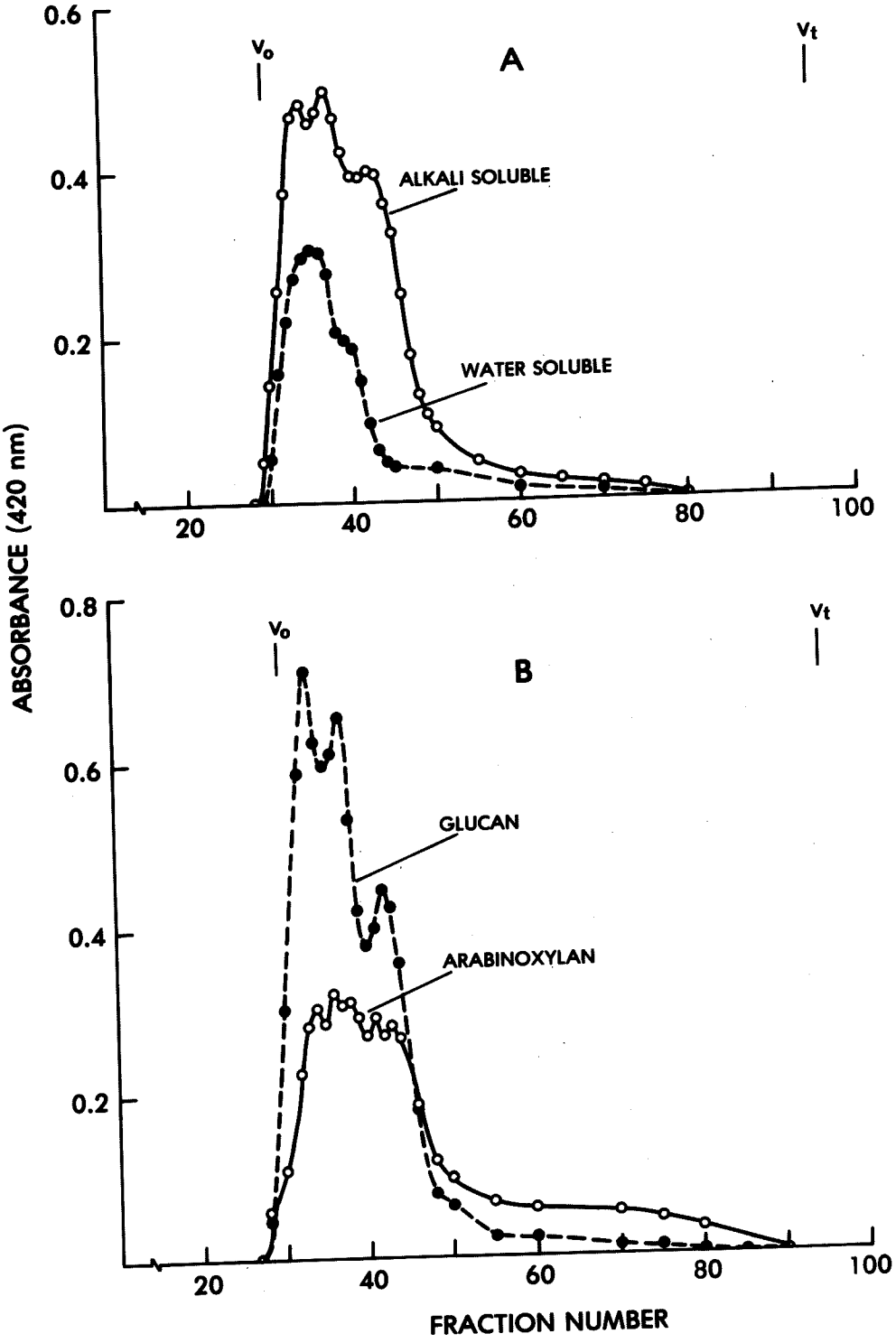
Figure 11B shows that the alkali-soluble glucan and arabinoxylan are of similar molecular size although arabinoxylan accounts for most of the lower molecular weight polysaccharide in the alkali extract.

Figure 11.

Gel permeation column chromatography of cell wall polysaccharides on BioGel A50m.

A. Elution profiles of water-soluble and alkali-soluble cell wall polysaccharides.

B. Elution profiles of alkali-soluble cell wall glucan and arabinoxylan.


A
B
V_o
V_t
ALKALI SOLUBLE
WATER SOLUBLE
GLUCAN
ARABINOXYLAN
ABSORBANCE (420 nm)
FRACTION NUMBER
0.6
0.4
0.2
0.8
20
40
60
80
100

B. Hydrolysis of Isolated Barley Endosperm Cell Walls by Extracts of Malted Barley.

The disruption of endosperm cell wall structure and hydrolysis of cell wall polysaccharides is an important process in the malting and brewing industry. If it is assumed that the endosperm cell walls present a barrier to the movement of hydrolytic enzymes during malting, the walls must be degraded before starch- and protein- hydrolyzing enzymes can act on the endosperm cell contents. In addition, undegraded cell wall fragments and the viscous solutions produced by cell wall polysaccharides can cause serious filtration problems during brewing. Therefore, one aspect of malt quality may be that all cell wall-hydrolyzing enzymes be present in the malt in sufficient quantity to ensure breakdown of the wall structure and degradation of the poly-saccharides to low molecular weight products.

To determine whether there are detectable differences in the cell wall-hydrolyzing systems of barley varieties that may influence malting quality, the cell wall-solubilizing properties of extracts of five malting barleys were investigated. Extracts of malted barley were added to buffered suspensionsof endosperm cell walls that had been extracted previously with water. The cell wall carbohydrate released into the medium was analyzed. The monosaccharide composition of the cell wall substrate material was 3.5% mannose, 11.0% arabinose, 18.8% xylose and 66.7% glucose.

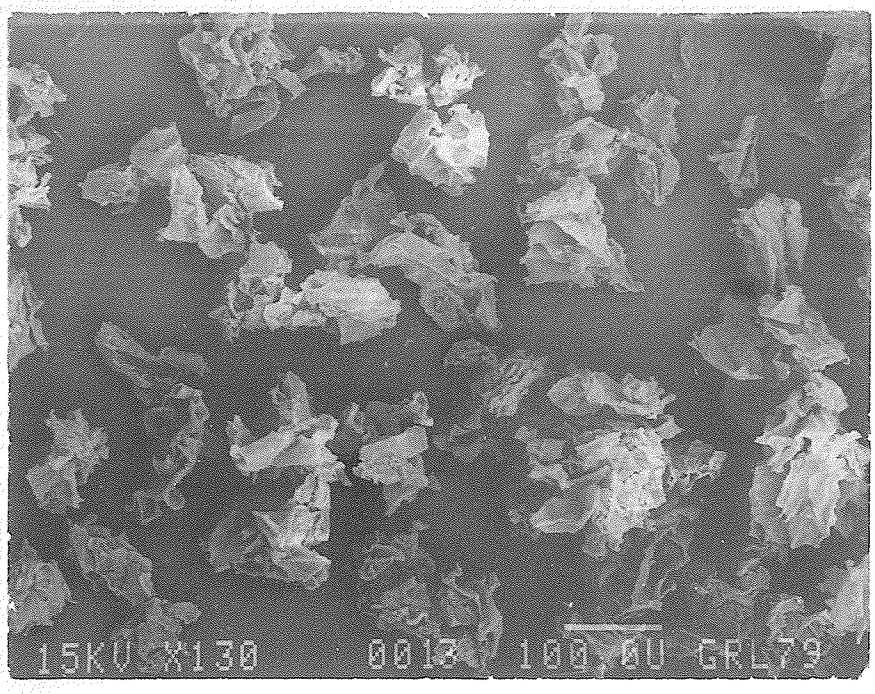
Scanning electron microscopy (Figures 12 and 13) showed that cell wall fragments that had been extracted with water exhibited much less surface detail than untreated cell walls (Figures 3-8). Indentations due to the adpression of starch granules were absent or only barely

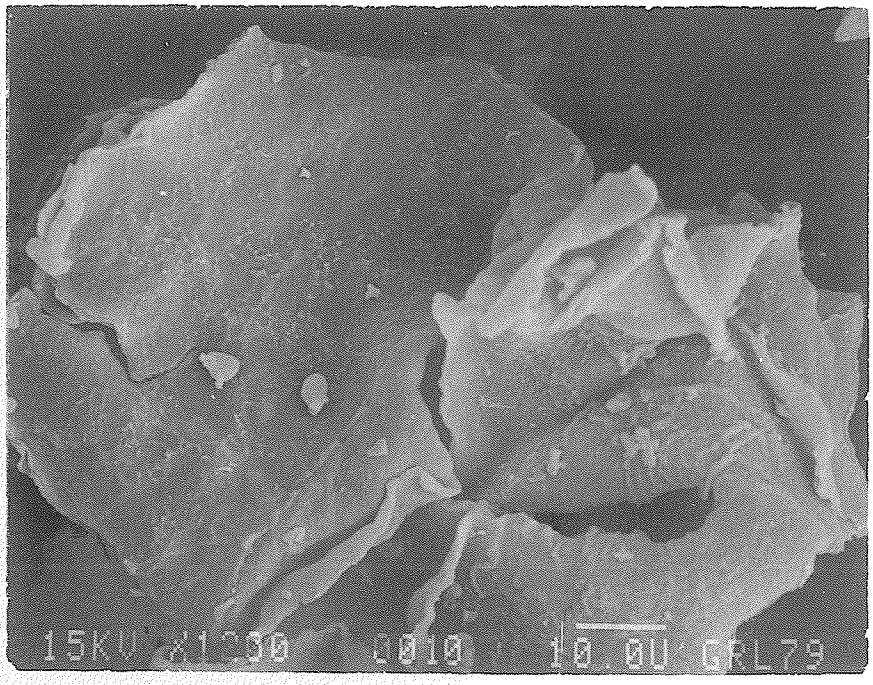
Figure 12.

Scanning electron micrograph of isolated barley endosperm cell walls after extraction with water at 65°C (x130).

Figure 13.

Scanning electron micrograph of barley endosperm cell wall fragment after aqueous extraction at 65°C (x1200).


15KV X130 0013 100.0U GRL79


15KV X1300 0010 10.0U GRL79

discernable, suggesting that the inner layer of the endosperm cell wall may be water soluble. Fincher (1975) also observed that after water extraction of barley endosperm cell walls surface indentations could no longer be seen. Mares and Stone (1973a) have proposed that layering of water-soluble polysaccharides on the surface of cell walls may be a common feature in storage tissues.

1. Extent of cell wall solubilization by extracts of malted barley

The amount of cell wall carbohydrate solubilized during 24 hour treatments with malt extracts ranged from 50% to 87% of the weight of the cell walls (Table 6). Even after release of 87% of the cell wall carbohydrate, the cell wall residue retained some structure, and the surfaces of the wall fragments had a rough, pitted appearance (Figures 14 and 15).

The variations in the rate at which carbohydrate appeared in solution when cell walls were treated with different malt extracts are shown in Figure 16. The cell wall carbohydrate solubilized by each malt extract had essentially the same monosaccharide composition. All analyses were within the range 3-4% mannose, 5-7% arabinose, 8-10% xylose and 80-83% glucose. However, expression of the amount of each monosaccharide solubilized as a percentage of the total amount of that monosaccharide in the cell wall (Table 6), indicated that there were variations in the ability of the malt extracts to release each cell wall monosaccharide.

Although 62-100% of the cell wall glucan was solubilized by the malt extracts, the other cell wall components were less readily solubilized. Less than half of the cell wall arabinoxylan was solubilized by extracts of TR 438 and TR428, which released all of the glucan and

Table 6. Amount of cell wall carbohydrate and proportion of each monosaccharide solubilized by extracts of malted barley, % (wt/wt).

Malt	Trial	Carbohydrate	Mannose	Arabinose	Xylose	Glucose
TR206	1	56	46	27	27	70
	2	50	64	23	21	62
TR910	1	69	58	41	30	85
TR436	1	74	76	42	31	91
	2	75	71	39	37	92
TR438	1	82	77	45	41	100
TR428	1	87	82	53	46	104
	2	83	74	45	38	103
correlation of % carbohydrate solubilized with monosaccharide solubilized, r*			+.954	+.971	+.923	+.999
level of significance			.01	.01	.01	.001

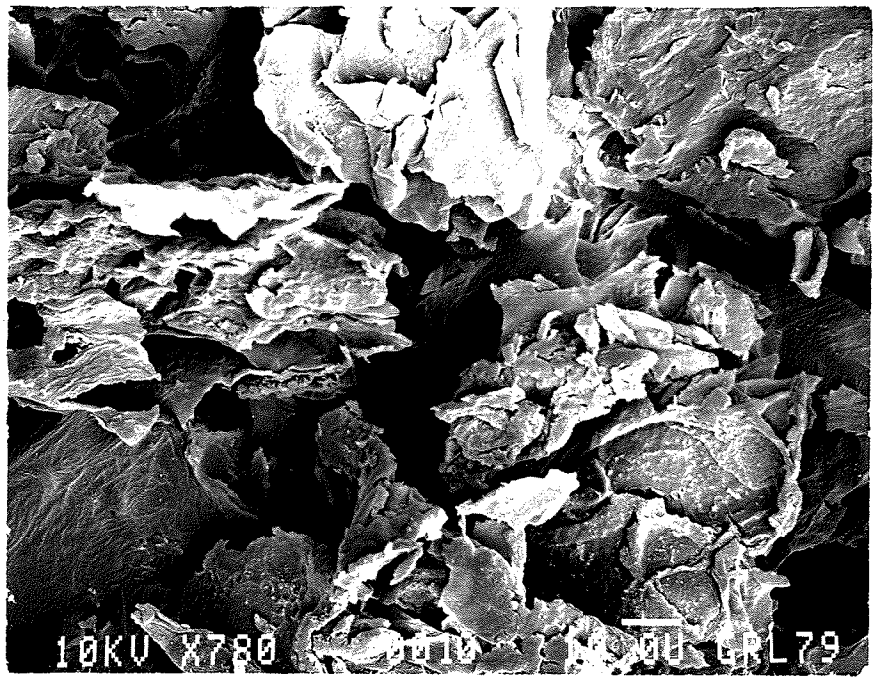
* calculated using trail 1 data only

Figure 14.

Scanning electron micrograph of water-extracted barley endosperm cell walls after 24 hr treatment with malt TR 428 extract (x780).

Figure 15.

Scanning electron micrograph, at higher magnification (x2400), of water-extracted cell wall fragments after 24 hr treatment with malt TR 428 extract.


10KV X780
10.0U GRL79

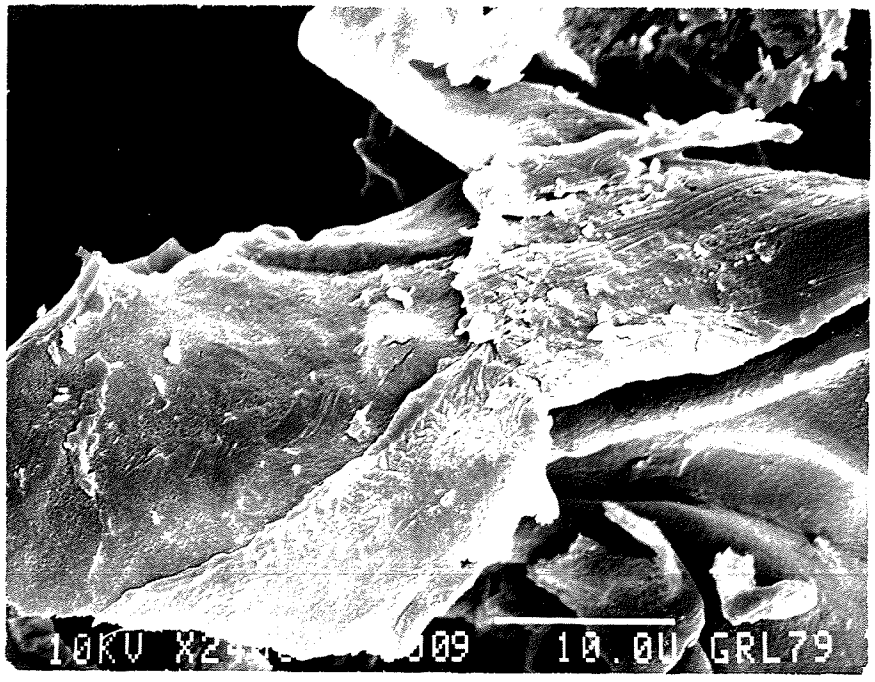
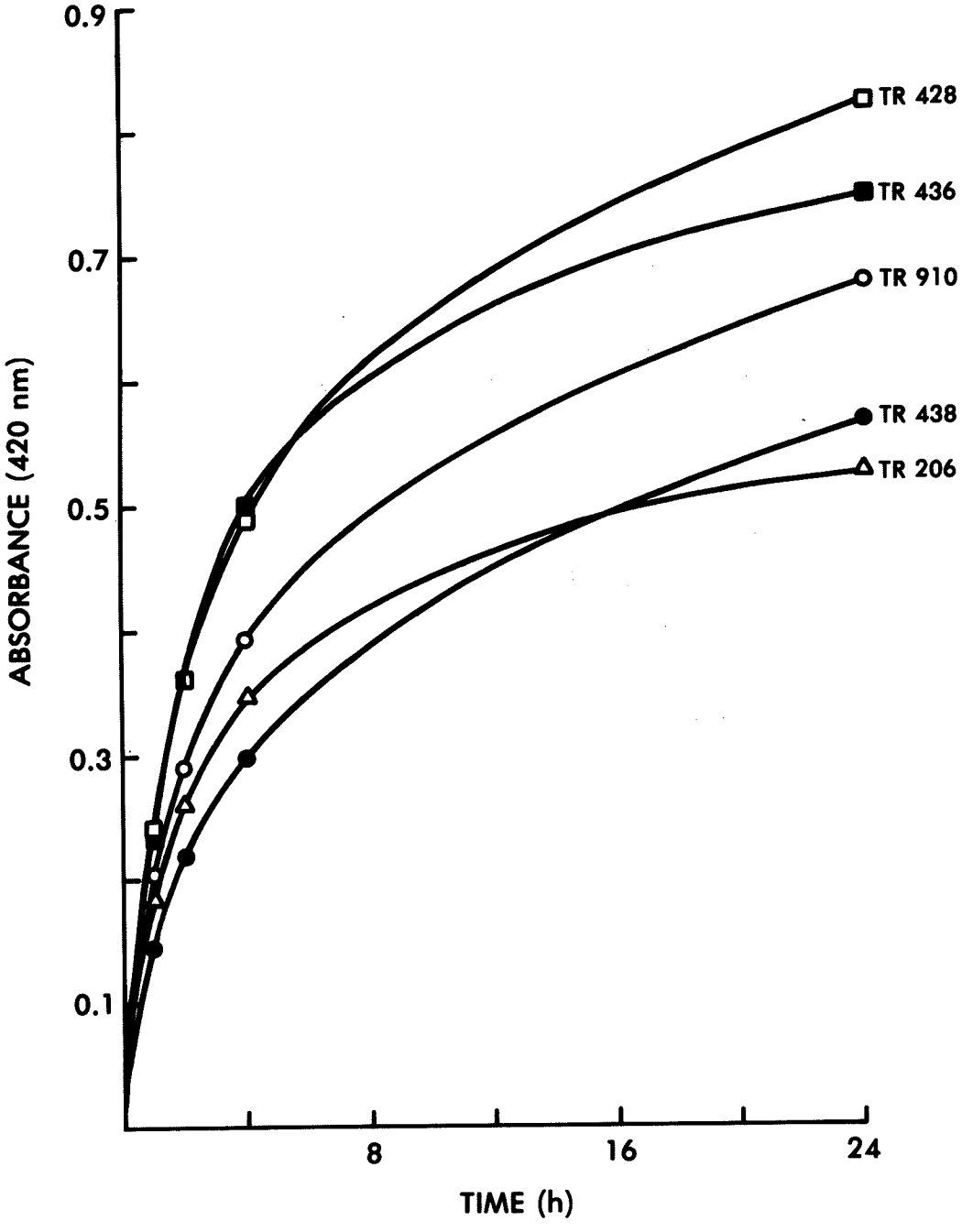

10KV X2
10.0U GRL79

Figure 16.

Solubilization of cell wall carbohydrate by malt extracts.


0.9
0.7
0.5
0.3
0.1
ABSORBANCE (420 nm)
8
16
24
TIME (h)
TR 428
TR 436
TR 910
TR 438
TR 206

most of the mannose from the cell wall. This suggests that hydrolysis of arabinoxylan may be a limiting factor in cell wall breakdown.

The release of up to 82% of the cell wall mannose is evidence for the presence of a mannanase in the malt extracts. The occurrence of this type of enzyme in barley has not been reported previously.

The data for percent cell wall carbohydrate released (Table 6) are greatly influenced by the proportion of glucan solubilized in each trial, as glucan is the major cell wall component and is completely solubilized by some of the malt extracts. However, increased cell wall solubilization is not due only to greater release of glucan from the cell wall. The proportion of each monosaccharide solubilized correlates highly with total carbohydrate solubilized. The correlation coefficients are significant at the .01 level for mannose, arabinose and xylose, and at the .001 level for glucose.

2. Molecular size distributions of cell wall carbohydrate solubilized by malt extracts

The molecular size distributions of cell wall carbohydrates solubilized by malt extracts were investigated by gel permeation column chromatography on BioGel P150. The elution profiles are shown in Figures 17 and 18. The carbohydrates solubilized by TR 206, TR 910 and TR 436 (Figure 17) have similar molecular size distributions. However, the elution profiles for carbohydrate solubilized by TR 438 and TR 428 differ in having a much larger proportion of lower molecular weight products (Figure 18). Malts TR 438 and TR 428 were the most effective in solubilizing the endosperm cell walls (Table 6).

The results demonstrate that malt extracts differ in the ability to hydrolyze cell wall polysaccharides to low molecular weight products

Figure 17.

Fractionation by gel permeation column chromatography on BioGel P150 of cell wall carbohydrate solubilized by extracts of malts TR 206, TR 910 and TR 436.

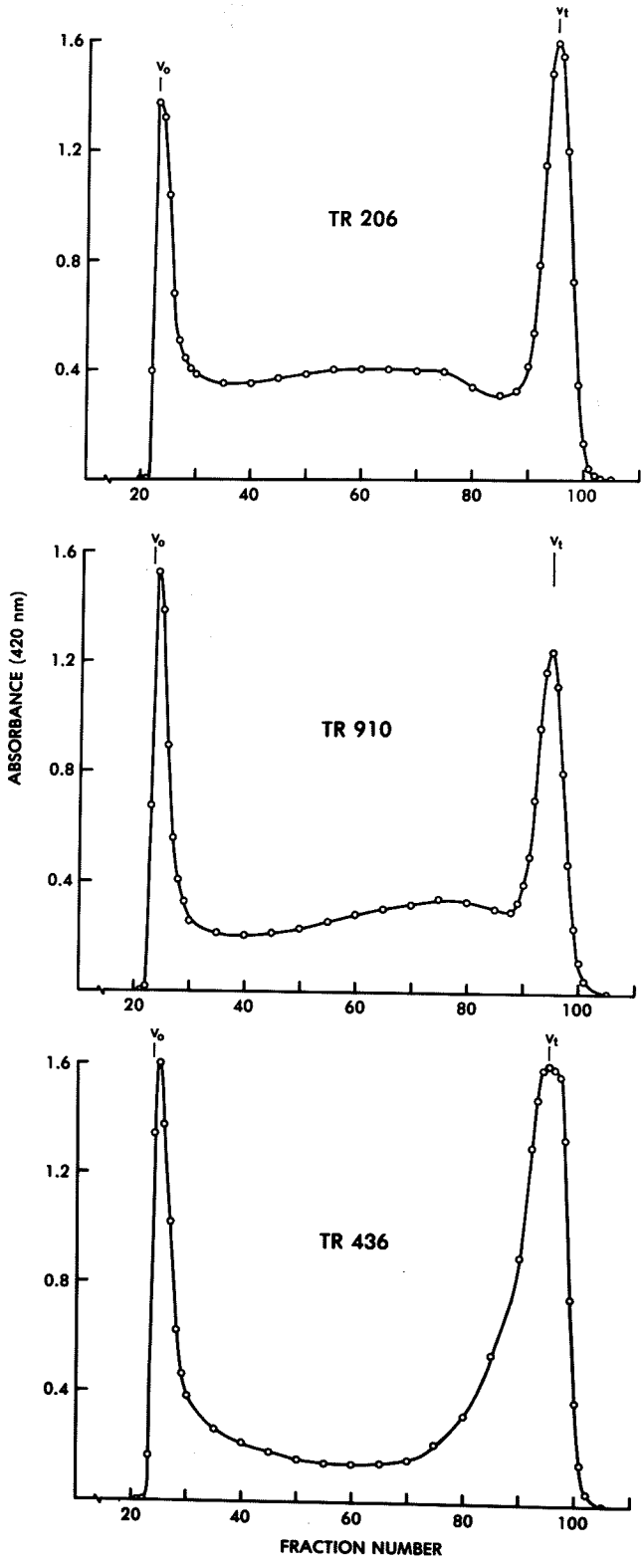
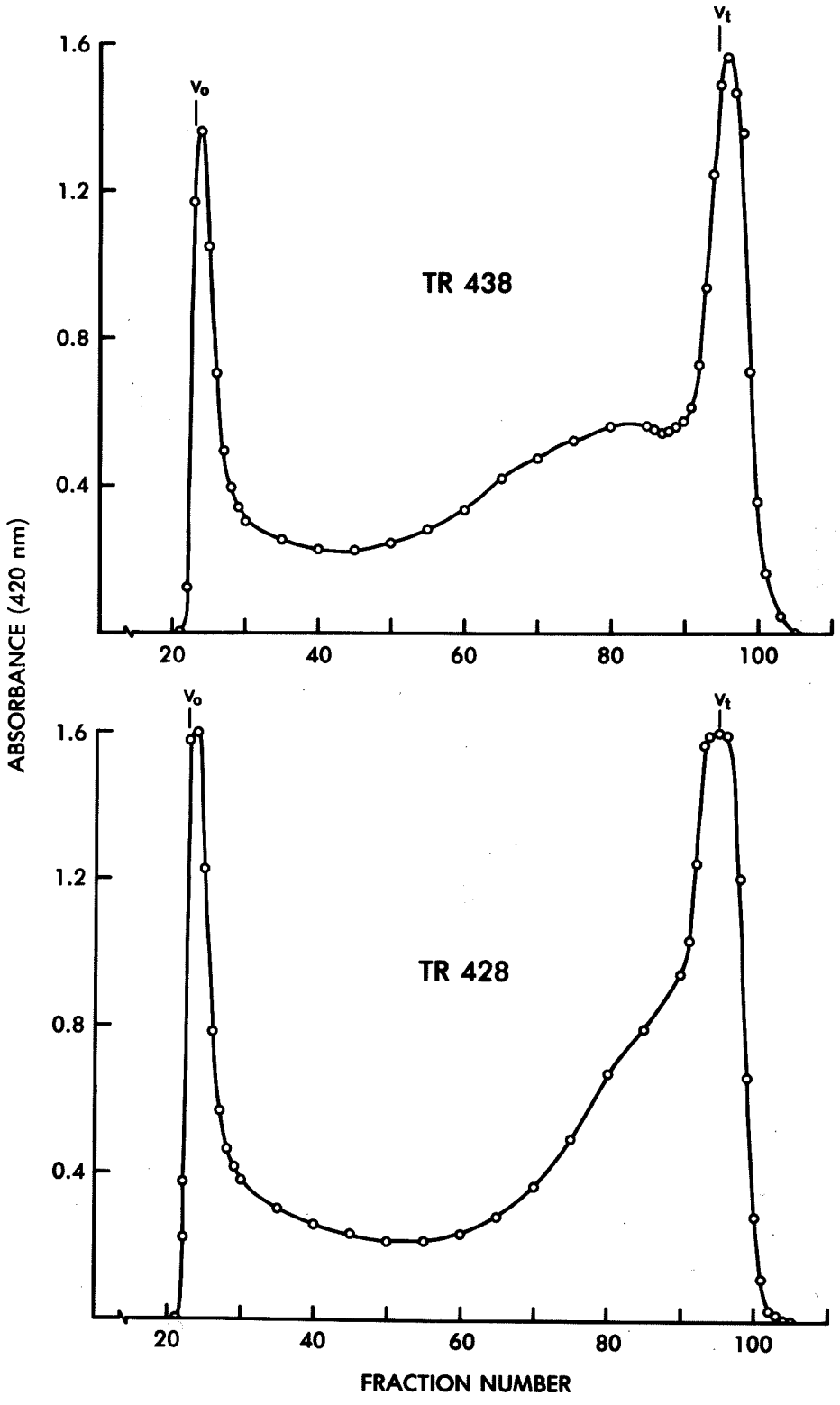

TR 206
TR 910
TR 436
V_o
V_t
1.6
1.2
0.8
0.4
20
40
60
80
100
ABSORBANCE (420 nm)
FRACTION NUMBER

Figure 18.

Fractionation by gel permeation column chromatography on BioGel P150 of cell wall carbohydrate solublized by extracts of malts TR 438 and TR 428.


TR 438
TR 428
V_o
V_t
1.6
1.2
0.8
0.4
20
40
60
80
100
ABSORBANCE (420 nm)
FRACTION NUMBER

and that, for the malts investigated in this experiment, production of a higher proportion of low molecular weight products is associated with a greater degree of cell wall breakdown. Variations in the ability of malt extracts to hydrolyze endosperm cell walls are potentially important in the brewing industry. A greater degree of cell wall breakdown leaving less cell wall residue, and hydrolysis of the solubilized cell wall polysaccharides to low molecular weight products that do not form viscous solutions would help to avoid filtration problems during lautering and would probably lead to the production of more stable beers.

3. Relation between cell wall solubilization and malt enzyme activities

The endo-β1,3-glucanase, endo-β1,3(4)-glucanase and endo-β1,4-xylanase activities of the malt extracts were determined by viscometric assays and are reported in Table 7. The five malts exhibited considerable variation in each type of enzyme activity. The endo-β1,3-glucanase activity of TR 910 was not determined due to insufficient sample.

The relation between malt enzyme activities and the proportion of cell wall carbohydrate, glucan and arabinoxylan solubilized by the malt extracts were examined by linear regression analyses. The correlation coefficients are presented in Table 8. The data indicate that there is no relation between the endo-β1,3-glucanase or endo-β1,3(4)-glucanase activity of the malt extract and the amount of cell wall carbohydrate, glucan or arabinoxylan solubilized by the malt extract. However, endo-β1,4-xylanase activity of the malt extract is positively correlated with each of these variables. Although the correlation coefficients are slightly below the .05 level of significance (.811 for 4 degrees of freedom), the fact that endo-β1,4-xylanase activity correlates positively with cell wall solubilization while endo-β-glucanase

Table 7. Endo-β1,3-glucanase, endo-β1,3(4)-glucanase and endo-β1,4-xylanase activities of malted barley, $d(^1/\eta sp)/t$.

Enzyme	Malt				
	TR206	TR910	TR436	TR438	TR428
β1,3-glucanase[1]	83	-	45	35	97
β1,3(4)-glucanase[1]	103	195	248	45	150
β1,4-xylanase[2]	50	108	66	96	116

[1] x 10^5

[2] x 10^6

Table 8. Correlation coefficients for linear regression analysis of malt β-glucanase and xylanase activities vs. % cell wall carbohydrate, glucan and arabinoxylan solubilized by malt extracts.

Enzyme	Cell Wall Carbohydrate	Glucan	Arabinoxylan
β1,3-glucanase	-.122	-.172	+.052
β1,3(4)-glucanase	-.059	-.056	-.158
β1,4-xylanase	+.744	+.733	+.790

activities do not suggests that xylanase activity may be a controlling factor in cell wall breakdown.

Barley contains several enzymes that degrade arabinoxylan. Endo-β1,4-xylanase, α-arabinofuranosidase and β-xylopyranosidase activities are present in ungerminated grain and increase significantly during germination or malting (Preece and MacDougall 1958, Taiz and Honigman 1976). The formation and release of these three types of arabinoxylan-hydrolyzing enzymes are induced by gibberellic acid (Dashek and Chrispeels 1977, Taiz and Honigman 1976). These investigators have demonstrated that degradation of isolated barley aleurone arabinoxylan by enzymes in the incubation medium of gibberellic acid-treated barley aleurone layers is primarily due to endo-enzyme activity. Xylanase activity is associated with a protein (or proteins) having a molecular weight of 29,000 (Dashek and Chrispeels 1977). It is probable that endo-β1,4-xylanase is involved in cell wall breakdown, but the ability of isolated xylanase to hydrolyze intact barley aleurone or endosperm cell walls has not been demonstrated.

The results of this experiment confirm that endo-β1,4-xylanase is present in malt, and show that there are varietal differences in xylanase activity that may influence malt quality. In addition, the positive correlation of malt endo-β1,4-xylanase activity with the amount of arabinoxylan solubilized from isolated barley endosperm cell walls (Table 8) provides evidence that endo-β1,4-xylanase is a cell wall-hydrolyzing enzyme.

The data presented in Table 8 indicate that endo-β-glucanase activities may not be limiting factors in determining the rate or extent of endosperm cell wall solubilization. Similarly, Palmer (1975) reported

that the speed of malting was not related to either the rate of development of endo-β-glucanase during malting or to the final endo-β-glucanase activity of the malt. However, purified malt endo-β1,3-glucanase (Ballance and Manners 1978) and malt endo-β1,3(4)-glucanase (Results and Discussion C) can hydrolyze isolated barley endosperm cell walls, and cause extensive solubilization of the walls. The roles of these enzymes in endosperm cell wall degradation and their effect on β-glucans isolated from endosperm cell walls are considered further in Results and Discussion C.

From Table 7, it is apparent that there are varietal differences in the endo-β1,3 glucanase, endo-β1,3(4)-glucanase and endo-β1,4-xylanase activities of malts, and that these enzyme activities occur in different proportions in different malts. For each type of activity, such variations may be due to differences in the rate of enzyme synthesis and/or differences in the total amount of enzyme produced. Presumably there is an optimal balance of cell wall-hydrolyzing enzyme activities that would result in rapid and extensive cell wall degradation during germination or malting. However, only a few cell wall-hydrolyzing enzymes have been identified, and it is probable that many other factors participate in cell wall degradation. There is evidence that proteolytic enzymes (Forrest and Wainwright 1977, Palmer 1971) and esterases (Ballance and Manners 1978, Fincher 1976, Mares and Stone 1973b) may be involved. A more detailed knowledge of the structural organization of the endosperm cell wall will be necessary before the relation between various enzyme activities and cell wall degradation can be resolved.

C. Hydrolysis of Isolated Barley Endosperm Cell Walls by Malt Endo-β-Glucanases

Recently, Ballance (1976) and Ballance and Manners (1978) have demonstrated that malt endo-β1,3-glucanase and a fungal endo-β1,4-glucanase can hydrolyze isolated barley endosperm cell walls and cause extensive solubilization of the walls. These investigators have proposed that the role of endo-β1,3-glucanase during germination may involve initial solubilization of the endosperm cell walls. The function of the enzyme has been debated for some time, as endo-β1,3-glucanase is reported to have little or no effect on isolated barley β-glucan (Ballance and Meredith 1976, Bathgate et al. 1974, Luchsinger et al. 1963, Manners and Marshall 1969, Manners and Wilson 1974), even though blocks of contiguous β1,3 linkages have been identified in barley β-glucan by chemical and enzymic methods (Bathgate et al. 1974, Fleming and Kawakami 1977, Fleming and Manners 1966, Igarashi and Sakurai 1966, Moscatelli et al. 1961). However, the length and/or frequency of β1,3 linked sequences may depend on the source of the glucan and the methods used to extract it (Bathgate et al. 1974, Fleming and Kawakami 1977). Ballance (1976) proposed that potential sites for endo-β1,3-glucanase hydrolysis may exist in the insoluble portion of the endosperm cell wall, and that the products of endo-β1,3-glucanase hydrolysis would be degraded to oligosaccharides by endo-β1,4-glucanase and endo-β1,3(4)-glucanase.

The purpose of the present study was to investigate further this hypothesis concerning the role of endo-β1,3-glucanase in endosperm cell wall degradation, and to determine the effect of malt endo-β1,3(4)-glucanase on isolated barley endosperm cell walls. Endo-β1,3(4)-glucanase is of major importance in the degradation of barley β-glucan, but its

effect on endosperm cell walls has not been reported previously.

Endo-β1,3-glucanase and endo-β1,3(4)-glucanase were isolated from unkilned, freeze-dried Conquest malt as described by Manners and Wilson (1976). The specificities and other properties of the enzymes have been reported by several investigators (Ballance and Meredith 1976, Luchsinger et al. 1963, 1965, Manners and Wilson 1974, 1976).

As isolated, the endo-β1,3-glucanase preparation caused a rapid decrease in the specific viscosity of CM-pachyman solutions, but had no effect on the specific viscosity of CM-cellulose solutions. Barley β-glucan was hydrolyzed to a limited extent. Using barley β-glucan as substrate the initial rate of viscosity reduction was approximately 1% of the rate observed using CM-pachyman solutions.

The endo-β1,3(4)-glucanase preparation rapidly reduced the specific viscosity of barley β-glucan solutions, but had no effect on the specific viscosities of solutions of CM-pachyman or CM-cellulose.

To investigate the effect of endo-β-glucanases on isolated barley endosperm cell walls, portions of the enzyme preparations were added to buffered suspensionsof cell walls that had previously been extracted with water. The cell wall carbohydrate released into the medium was analyzed.

1. Extent of cell wall solubilization by malt endo-β-glucanases

The amount of glucan solubilized by each endo-β-glucanase alone and the two endo-β-glucanases acting together was estimated by specifically determining glucose in acid hydrolysates of portions of the enzyme-solubilized cell wall carbohydrate. The results are presented in Table 9, and are expressed as a percentage of the total glucan content of the cell walls.

Table 9. Amount of cell wall glucan solubilized by endo-β-glucanases, % of total glucan.

Treatment	4-Day treatment	15-Day treatment*
Control	0	4
β1,3-glucanase	28	55 ± 1
β1,3(4)-glucanase	46	69 ± 1
β1,3-glucanase & β1,3(4)-glucanase	44	69 ± 1

* mean of 2 trials

The data demonstrate that malt endo-β1,3(4)-glucanase can hydrolyze isolated barley endosperm cell walls and cause partial solubilization of cell wall glucan, and confirm that malt endo-β1,3-glucanase is also a wall-solubilizing enzyme (Ballance 1976, Ballance and Manners 1978).

In the 4-day experiment, most of the glucan was solubilized within the initial 24 hr period. On the fourth day, less than 1% of the cell wall glucan was released from the cell walls by retreatment with the enzyme(s). Similarly, in the 15-day experiment, resuspension of the cell wall fragments with fresh enzyme(s) during the final 24 hr caused solubilization of less than 1% of the cell wall glucan.

In both the 4-day and 15-day treatments, endo-β1,3(4)-glucanase solubilized more glucan than did endo-β1,3-glucanase. However, the combined action of the two endo-β-glucanases released the same amount of glucan as did treatment endo-β1,3(4)-glucanase alone. Although cell wall degradation is no more extensive when both enzymes are used, the rate of solubilization may be increased.

After 15-day treatments with endo-β1,3(4)-glucanase or endo-β1,3-glucanase, the cell wall residues were examined by scanning electron miscroscopy (Figures 19-22). All of the observed surfaces were very rough and appeared to be highly disrupted. A few wall fragments of the type shown in Figures 23 and 24 were observed in the endo-β1,3-glucanase-treated cell wall residue. Some areas of the wall appeared to be entirely degraded, leaving holes of various sizes and shapes in the cell wall fragments. This type of degradation was not observed in the cell wall residue after treatment with endo-β1,3(4)-glucanase.

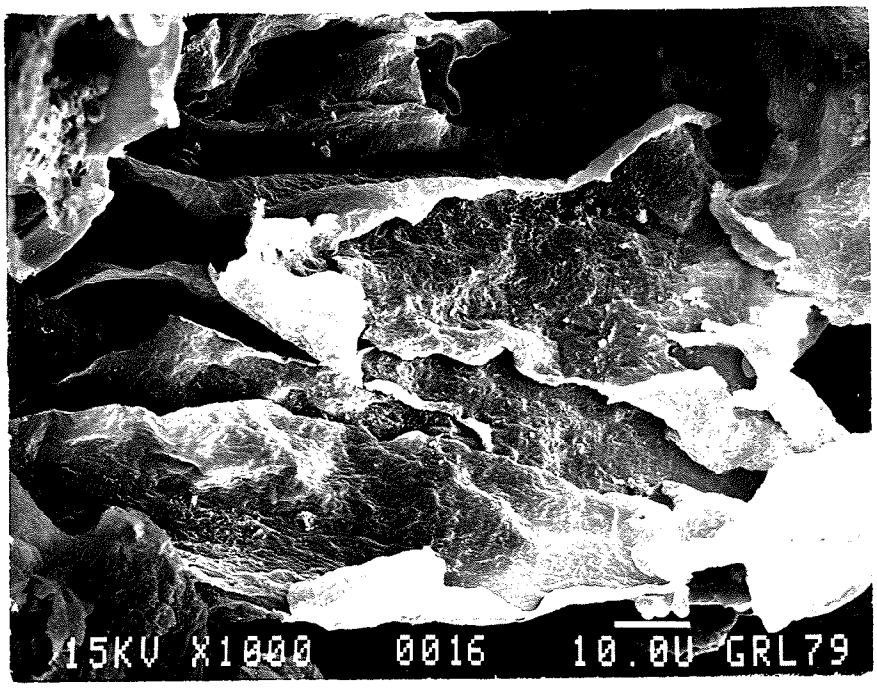
After prolonged treatment with malt endo-β-glucanases, approximately 30% of the cell wall glucan remained in the insoluble cell wall

Figure 19.

Scanning electron micrograph of isolated barley endosperm cell walls after treatment with malt endo-β1,3(4)-glucanase (x1000).

Figure 20.

Scanning electron micrograph of isolated barley endosperm cell walls treated with malt endo-β1,3(4)-glucanase (x9400).


15KV X1000 0016 10.0U GRL79

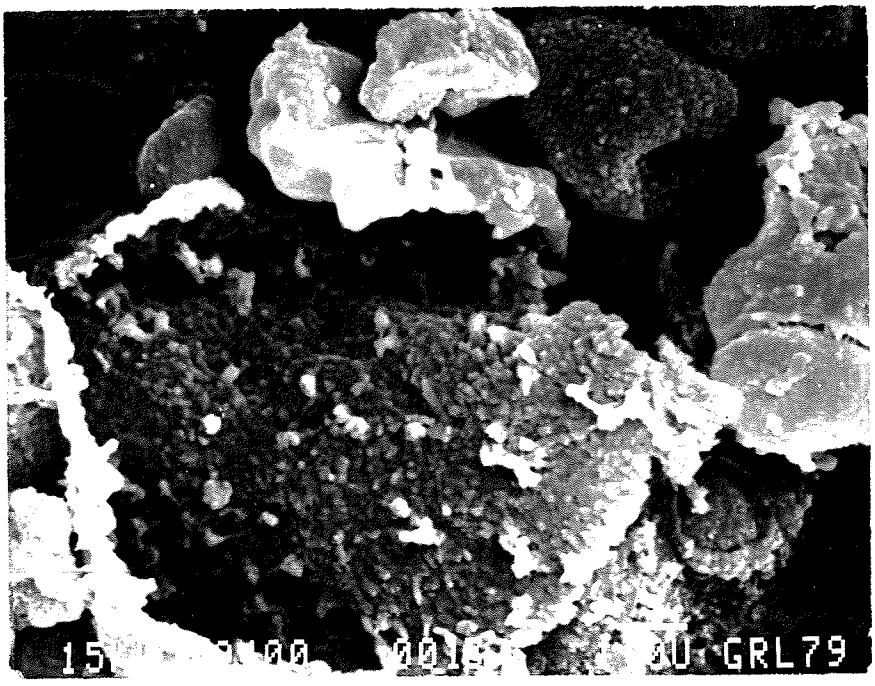
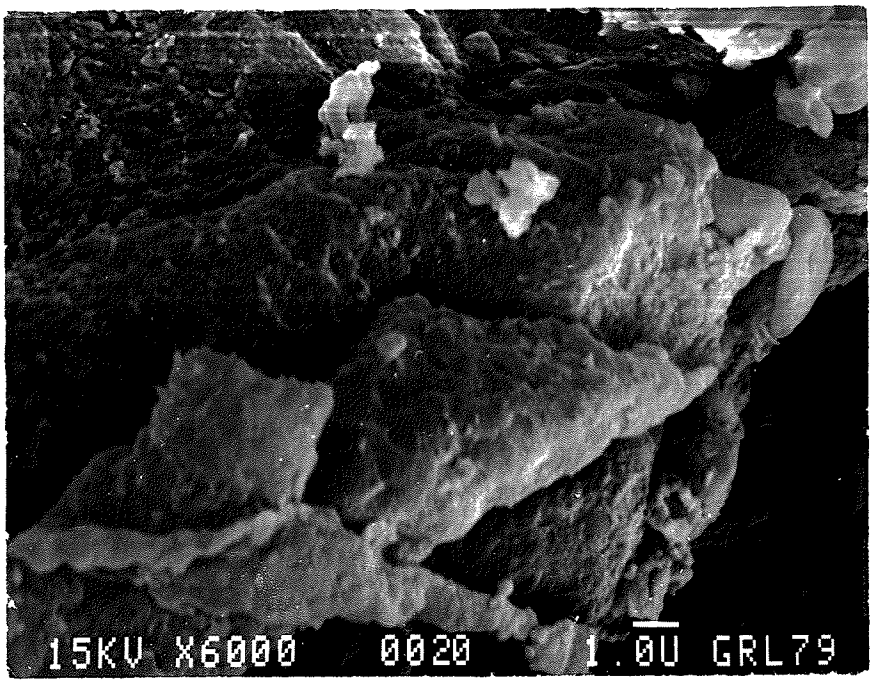

GRL79

Figure 21.

Scanning electron micrograph of isolated barley endosperm cell wall fragments after treatment with malt endo-β1,3-glucanase (x6000).

Figure 22.

Scanning electron micrograph of isolated barley endosperm cell wall fragments treated with malt endo-β1,3-glucanase (x2600).


15KV X6000 0020 1.0U GRL79

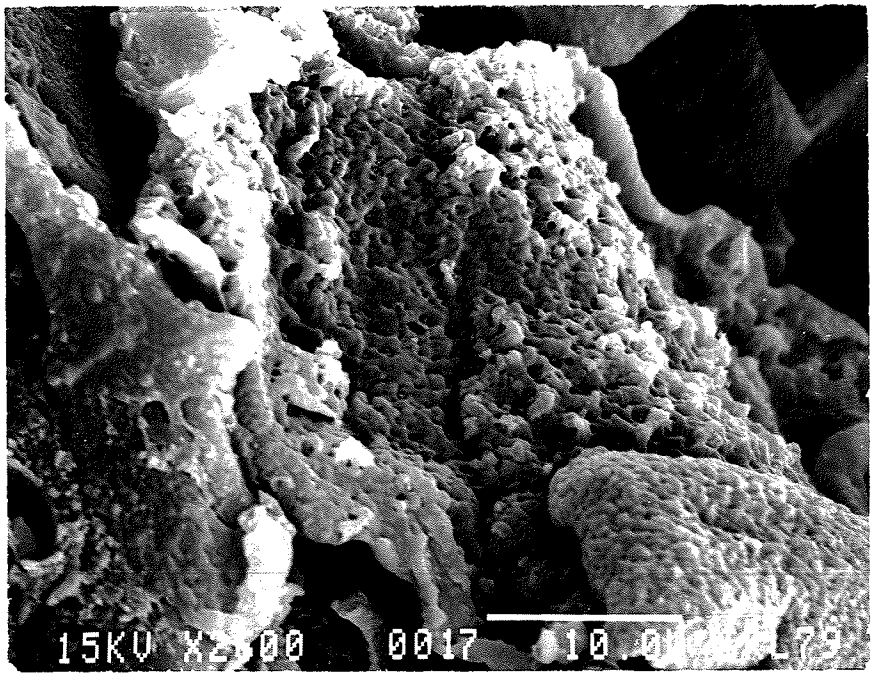
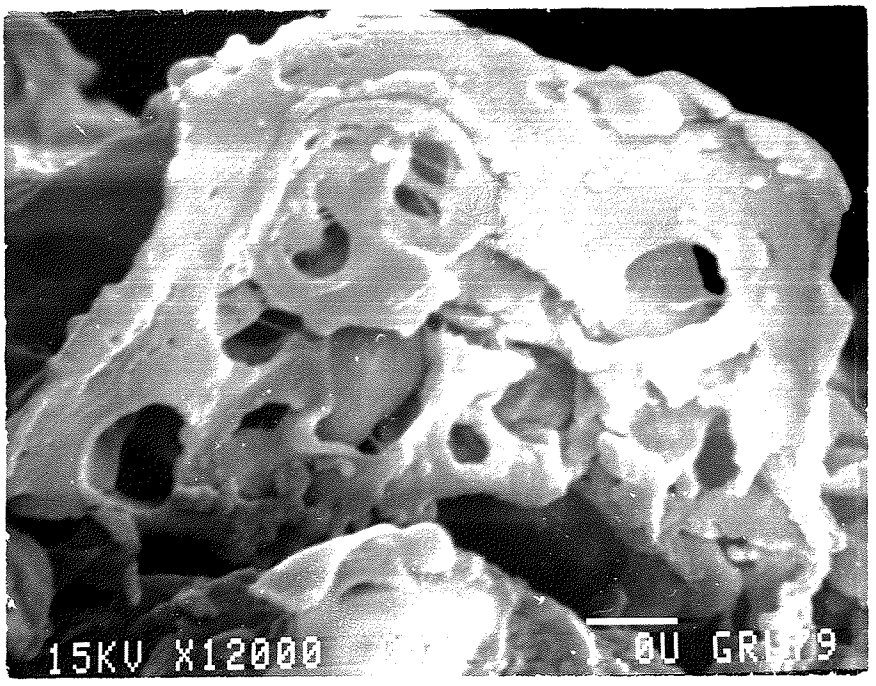

15KV X2

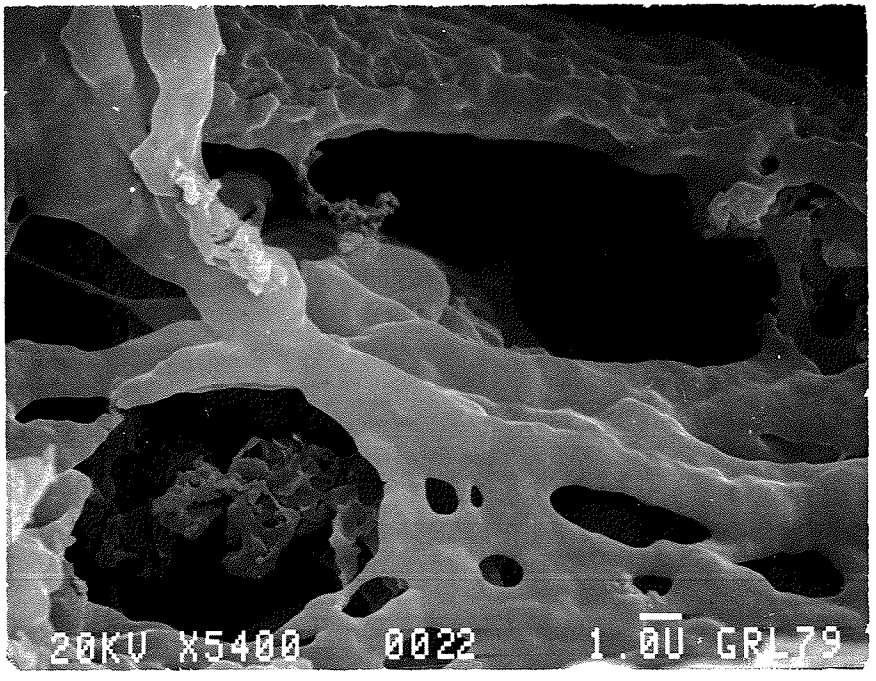
Figure 23.

Scanning electron micrograph of barley endosperm cell wall fragment treated with malt endo-β1,3-glucanase (x12000).

Figure 24.

Scanning electron micrograph of barley endosperm cell wall fragment after treatment with malt endo-β1,3-glucanase (x5400).


15KV X12000
1.0U GRL79


20KV X5400 0022
1.0U GRL79

fragments (Table 9). The reason for this resistance to endo-β-glucanase hydrolysis is unclear. The data in Table 6 indicate that treatment of isolated endosperm cell walls with crude malt extracts can solubilize all of the cell wall glucan. This suggests that some factor other than endo-β-glucanase activity is required for complete solubilization of cell wall glucan.

Forrest and Wainwright (1977) have presented evidence that protein may be an integral part of β-glucan structure. Treatment of isolated barley endosperm cell walls with the proteolytic enzyme, thermolysin, or rupture of peptide bonds by hydrazinolysis, released 96% of the glucan from the cell walls. Hydrolysis of peptide bonds may be required for complete solubilization of β-glucan. If this is the case, determination of barley β-glucan by enzymic methods (Anderson et al. 1978) may be more accurate if peptide bonds are disrupted prior to treatment with endo-β-glucanase (Wainwright 1978).

Treatment with endo-β1,3(4)-glucanase alone or with both glucanases simultaneously released approximately 60% of the total cell wall carbohydrate in the 4-day experiment, and about 70% in the 15-day experiment. Ballance (1976) and Ballance and Manners (1978) reported similar results for solubilization of endosperm cell wall carbohydrate by malt endo-β1,3-glucanase and fungal endo-β1,4-glucanase. Ballance (1976) assumed that the solubilized carbohydrate was entirely glucan, and concluded that the arabinoxylan remained in the cell wall residue. In the present study, enzyme-solubilized glucan accounted for 30% and 47% of total cell wall carbohydrate in the 4-day and 15-day experiments, respectively. These results suggest that most of the cell wall arabinoxylan was solubilized by the enzyme treatments. Monosaccharide analysis of portions of the cell wall carbohydrate solubilized by each enzyme treatment

indicated that each solution contained arabinose and xylose in addition to glucose. Mannose was not detected.

The enzyme preparations were assayed for endo-β1,4-xylanase activity using solutions of wheat flour arabinoxylan as substrate. No change in specific viscosity was observed during the initial hour of the reaction, but after 18 hr, the endo-β1,3-glucanase preparation had caused a 65% decrease in the specific viscosity of the arabinoxylan solution, whereas the endo-β1,3(4)-glucanase preparation decreased the specific viscosity by 40%. No change was observed in the specific viscosity of a control arabinoxylan solution, and there was no detectable increase of reducing sugars in the enzyme-treated arabinoxylan solutions.

It was concluded that both endo-β-glucanase preparations contained endo-β1,4-xylanase activity. The fact that the enzyme preparations reduce the viscosity of arabinoxylan solutions to different extents suggests that there may be two malt endo-β1,4-xylanases having different action patterns. The results of gel permeation chromatography of the enzyme-solubilized carbohydrate support the viscometric data and demonstrate that arabinoxylan solubilized by treatment with the endo-β1,3-glucanase preparation has a smaller average molecular size (C.2, Figures 25 A&B). These findings provide evidence that endo-β1,4-xylanase(s) from malt can hydrolyze isolated barley endosperm cell walls and cause solubilization of most, if not all, of the cell wall arabinoxylan. However, prolonged treatment with malt endo-β-glucanasesreleased only 70% of the cell wall β-glucan.

2. Molecular size distributions of cell wall carbohydrate solubilized by malt endo-β-glucanases.

As contiguous β1,3-linkages are relatively infrequent in the

β-glucan molecule, the products of endo-β1,3-glucanase hydrolysis of the cell wall would be expected to be relatively large molecules compared to the products of endo-β1,3(4)-glucanase hydrolysis. This hypothesis was investigated using gel permeation column chromatography on BioGel P150 to examine the molecular size distributions of the soluble products released from the isolated endosperm cell walls by 24 hr treatments with endo-β1,3-glucanase and endo-β1,3(4)-glucanase.

The elution profiles, presented in Figure 25, support the hypothesis. The products of endo-β1,3(4)-glucanase hydrolysis were low molecular weight oligosaccharides (Figure 25A). In contrast, the soluble products of endo-β1,3-glucanase hydrolysis had a broad molecular size distribution with a significant proportion of larger molecules as well as some low molecular weight products (Figure 25B).

The low molecular weight peak of both samples was eluted at or near the total elution volume for glucose. As the fractionation limit of the gel was approximately M.W. 1000, oligosaccharides having a degree of polymerization of six or less would not be fractionated. The low molecular weight peak of each sample consisted almost entirely of gluco-oligosaccharides. Only traces of free glucose were detected in fractions 90-100, whereas after acid hydrolysis glucose accounted for more than 95% of the total reducing sugars. This finding is consistent with the endo-action of the enzymes and indicates that the enzyme preparations did not contain β-glucosidases.

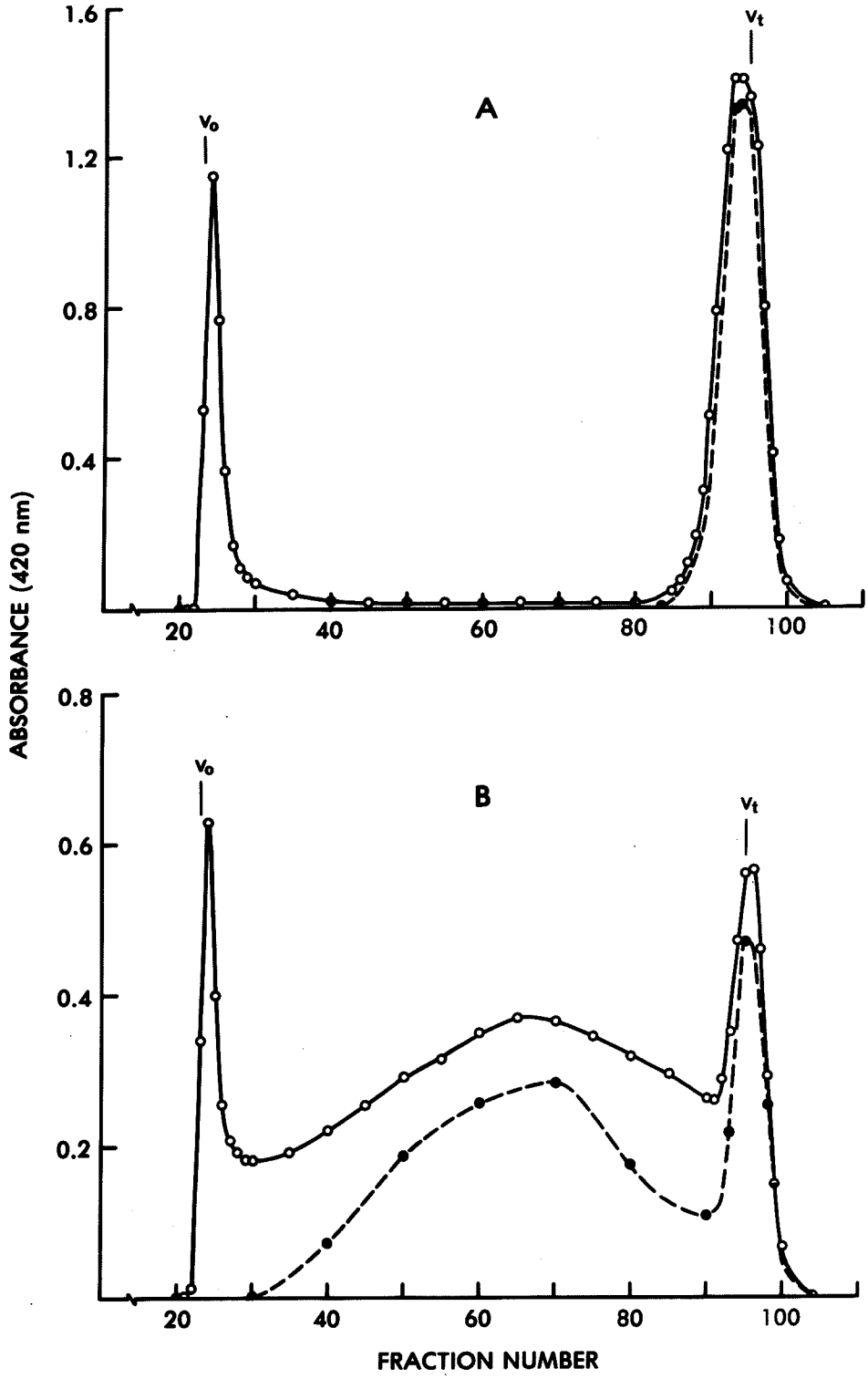
Ballance (1976) and Ballance and Manners (1978) have proposed that endo-β1,3-glucanase may be an initial cell wall-solubilizing enzyme, and that the products of endo-β1,3-glucanase hydrolysis may be degraded to smaller products by endo-β1,3(4)-glucanase. To investigate this possibility, carbohydrate released from cell wall fragments by the action

Figure 25.

Gel permeation column chromatography of enzyme-solubilized cell wall carbohydrate on BioGel P150.

A. Elution progile of cell wall carbohydrate solubilized by endo-β1,3(4)-glucanase.

B. Elution profile of cell wall carbohydrate solubilized by endo-β1,3-glucanase.
   open circles - total carbohydrate
   closed circles - glucose


A
B
V_o
V_t
ABSORBANCE (420 nm)
FRACTION NUMBER
1.6
1.2
0.8
0.4
0.6
0.2
20
40
60
80
100

of endo-β1,3-glucanase was treated with endo-β1,3(4)-glucanase. Gel permeation column chromatography indicated that the larger products of endo-β1,3-glucanase action were completely hydrolyzed to low molecular weight products by endo-β1,3(4)-glucanase (Figure 26A). Similarly, if isolated cell walls were treated simutaneously with endo-β1,3-glucanase and endo-β1,3(4)-glucanase only low molecular weight products were detected in the enzyme-solubilized cell wall carbohydrate (Figure 26B). The results presented in Figure 26 support the hypothesis that endo-β1,3-glucanase may be an initial cell wall-solubilizing enzyme by demonstrating that glucan solubilized by endo-β1,3-glucanase can be hydrolyzed to oligosaccharides by endo-β1,3(4)-glucanase.

Fulcher et al. (1977) have proposed that the primary substrate for endo-β1,3-glucanase may be discrete cell wall-associated β1,3-glucan deposits which have been detected in the aleurone and endosperm tissues of barley and other grasses by fluorescence microscopy with aniline blue staining. The aniline blue-positive deposits were removed by treatment with fungal β1,3-glucanases. Such deposits may be substrates for malt endo-β1,3-glucanase, although this has not yet been demonstrated.

The results of the present study show that endosperm cell wall β-glucan is a substrate for endo-β1,3-glucanase, and that the main hydrolysis products are mixed linkage glucans that are readily hydrolyzed by endo-β1,3(4)-glucanase.

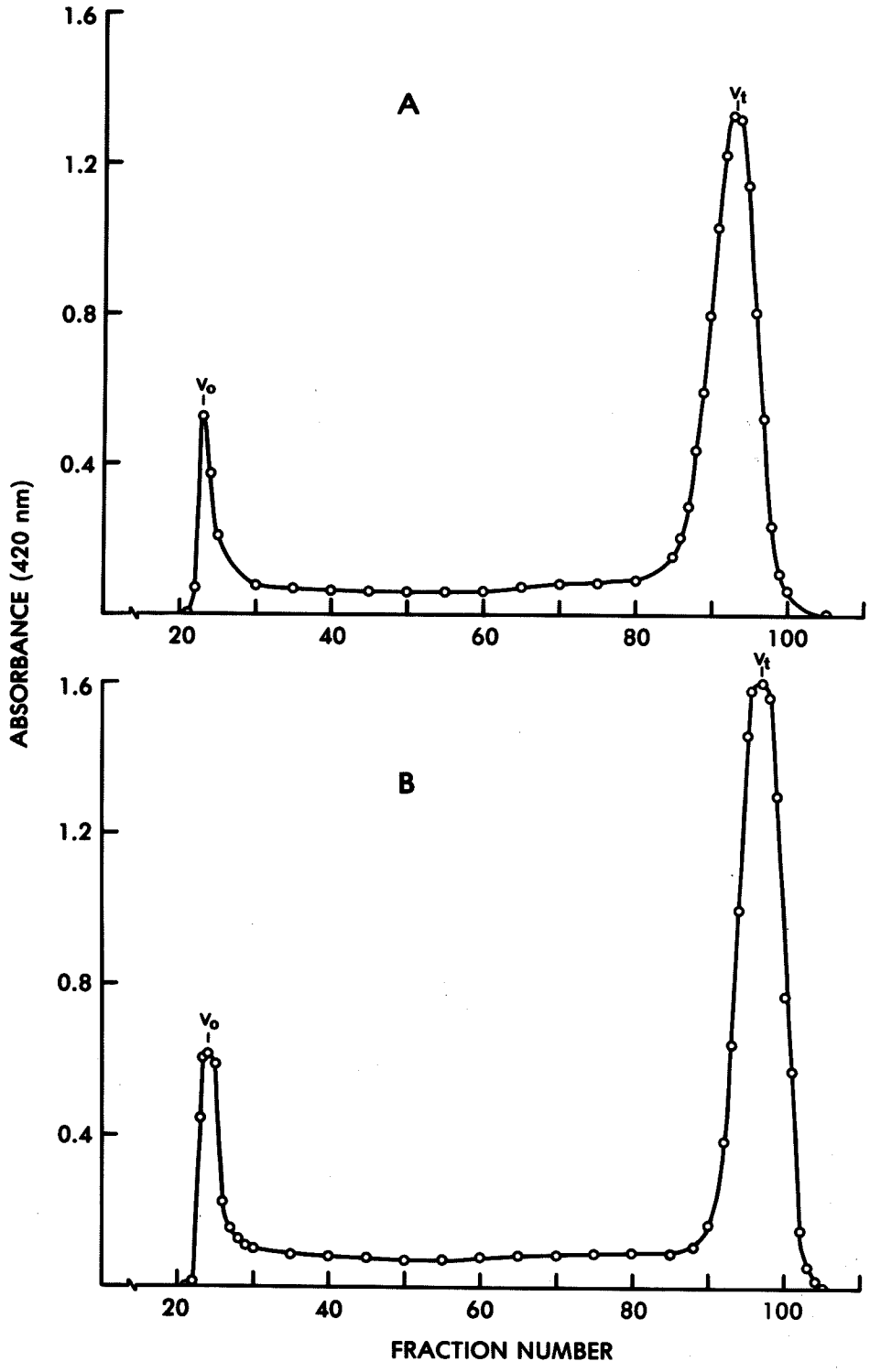
As discussed in the previous section (C.1), cell wall arabinoxylan was solubilized by treatment with the endo-β-glucanase preparations. Glucose was not detected in acid hydrolysates of the carbohydrate eluted at the void volume of the column (Figure 25), indicating that the carbohydrate which was excluded from the gel phase was entirely arabinoxylan. Figures 25A and 25B provide indirect evidence that the arabinoxylan

Figure 26.

Gel permeation column chromatography of enzyme-solubilized cell wall carbohydrate on BioGel P150.

A. Elution profile of endo-β1,3-glucanase-solubilized cell wall carbohydrate after treatment with endo-β1,3(4)-glucanase.

B. Elution profile of cell wall carbohydrate solubilized by the combined action of endo-β1,3-glucanase and endo-β1,3(4)-glucanase.


A
B
V_o
V_t
ABSORBANCE (420 nm)
1.6
1.2
0.8
0.4
20
40
60
80
100
FRACTION NUMBER

molecules solubilized by the two enzyme treatments have different molecular size distributions. The arabinoxylan solubilized by the endo-β1, 3(4)-glucanase preparation consists entirely of large molecules that are completely excluded from the gel phase (Figure 25A). Figure 25B shows that glucan accounts for only a portion of the cell wall carbohydrate fractionated by the column, indicating that the arabinoxylan solubilized by the endo-β1,3-glucanase preparation has a broad molecular size distribution, and is partly within the fractionation range of the gel.

These results suggest that malt endo-β1,4-xylanase can hydrolyze isolated endosperm cell walls and cause extensive solubilization of cell wall arabinoxylan. The different molecular size distributions of arabinoxylan molecules solubilized by the two enzyme preparations is evidence that there may be two endo-β-xylanases, having different action patterns involved in hydrolysis of cell wall arabinoxylan. This hypothesis is supported by the finding that the two enzyme preparations reduce the specific viscosity of wheat flour arabinoxylan solutions to different extents with no detectable change in reducing sugars (C.1). The results of viscometric assays and gel permeation column chromatography indicate that the hydrolysis products of the xylanase associated with the endo-β1, 3-glucanase preparation have a smaller average molecular size than the hydrolysis products of the xylanase associated with the endo-β1,3(4)-glucanase preparation.

Endo-β1,4-xylanase activity has been detected in barley and malt, and in isolated barley aleurone layers by several investigators (Dashek and Chrispeels 1977, Preece and MacDougall 1958, Taiz and Honigman 1976), and is considered to be a cell wall-hydrolyzing enzyme. In this study, most if not all of the endosperm cell wall arabinoxylan

was solubilized by malt enzyme preparations containing endo-β1,4-xylanase activities. However, malt endo-β1,4-xylanase has not been isolated or characterized, and it has not yet been demonstrated that the purified enzyme can degrade intact cell walls.

3. Hydrolysis of β-glucans from barley endosperm cell walls by malt endo-β1,3-glucanase.

Analysis of products obtained on periodate oxidative degradation has indicated that β-glucans extracted at higher temperatures may contain more and/or longer β1,3-linked sequences than β-glucan extracted at low temperature (Bathgate et al. 1974, Fleming and Kawakami 1977). Therefore, β-glucans extracted at higher temperatures may be more readily hydrolyzed by malt endo-β1,3-glucanase. This hypothesis was investigated by observing the effect of endo-β1,3-glucanase on the reciprocal specific viscosities of aqueous extracts of endosperm cell walls. Extractions were made at 40°C, 65°C and 100°C.

The viscometric assays were conducted with cell wall extracts of equivalent β-glucan concentration, and with diluted extracts having similar initial specific viscosities. During the duration of the experiment there was no change in the specific viscosities of untreated cell wall extracts or wheat flour arabinoxylan solutions treated with endo-β1, 3-glucanase. As the enzyme was free of endo-β1,4-glucanase and endo-β1, 3(4)-glucanase, but rapidly reduced the viscosity of CM-pachyman solutions, changes in the viscosities of cell wall extracts were assumed to be due to hydrolysis of β1,3-glucosidic linkages.

The data are presented in Table 10 and demonstrate the conflicting results that may be obtained with viscometric assays if the substrates used are not strictly comparable. The initial specific viscosities of

cell wall extracts of equivalent β-glucan concentration indicated that the polysaccharides had very different molecular sizes. The elution profiles obtained on gel permeation column chromatography of the cell wall extracts on Bio Gel A50m supported this conclusion (Figure 27). The differences in specific viscosities and elution profiles of the cell wall extracts probably reflect differences in the molecular size distributions of β-glucan, the major component of the extracts. However, the contribution of smaller amountsof other cell wall polysaccharides is not known.

Because of the different molecular size distributions of the substrates, viscometric assays could not give meaningful results. For equivalent concentrations, the assay would be expected to be most sensitive with the largest substrate molecules. Alternately, if the cell wall extracts are diluted to give soltuions of similar initial specific viscosity, the assay would be expected to be most sensitive with the most dilute substrate. Therefore, an apparently faster rate of increase in reciprocal specific viscosity cannot be attributed solely to increased rate of hydrolysis by endo-β1,3-glucanase. The reason for the low rate observed with the 65^{o}C extract containing 1.5 mg β-glucan/ml is unclear, but may be due to the extremely high specific viscosity of the substrate, or to intermolecular interactions of the substrate causing possible gel formation.

Differences in the number and/or length of β1,3-linked sequences in β-glucans isolated from different sources and at different temperatures have been proposed on the basis of viscometric assays with endo-β1,3-glucanase (Bathgate et al. 1974, Palmer 1975). As in the present work, the glucan substrates used in these investigations had very different molecular size distributions, a variable that invalidates comparison of

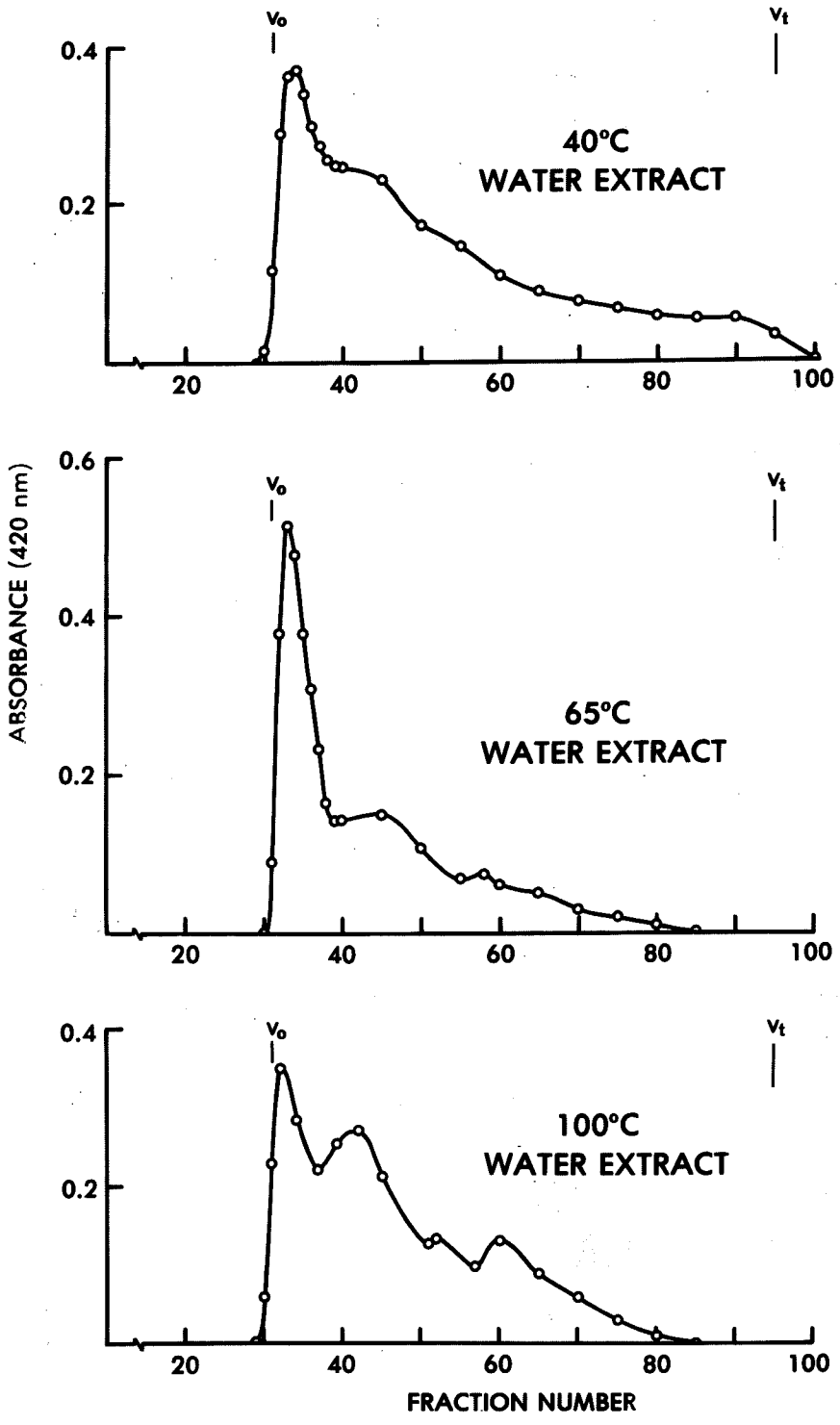
Table 10. Effect of malt endo-β1,3-glucanase on the specific viscosities of barley endosperm cell wall extracts

Extraction temperature, °C	β-glucan, mg/ml	ηsp	$d(^1/\eta sp)dt$*
40	1.5	2.096	5
65	1.5	14.286	2
100	1.5	4.237	9
40	1.5	2.096	5
65	0.6	2.137	26
100	1.1	2.053	18

* x 10^5

Figure 27.

Gel permeation column chromatography on BioGel A50m of cell wall polysaccharides extracted with water at 40°C, 65°C and 100°C.


Vo
Vt
0.4
0.2
40°C
WATER EXTRACT
20
40
60
80
100
0.6
0.4
0.2
65°C
WATER EXTRACT
ABSORBANCE (420 nm)
0.4
0.2
100°C
WATER EXTRACT
FRACTION NUMBER

the rates of endo-β1,3-glucanase hydrolysis by viscometric assays. More meaningful results might be obtained using an end group assay, which would be independent of the molecular size of the substrate. The method described by Manners et al. (1971) for determining the degree of polymerization of glucans using sorbitol dehydrogenase could be adapted to this purpose. Differences in the rate of production of end groups due to endo-β1,3-glucanase hydrolysis of various β-glucan preparations would indicate differences in the number and/or length of β1,3-linked sequences in the substrates.

IV SUMMARY

Endosperm cell walls isolated from Conquest barley were composed almost entirely of polysaccharides consisting of 3.5% mannose, 10.9% arabinose, 17.0% xylose and 68.6% glucose. The water-solubility of cell wall polysaccharides increased with extraction temperature, although extraction with water at 100°C did not dissolve all of the cell wall glucan. The polysaccharides extracted at 65°C had a larger average molecular size than polysaccharides extracted at 40°C or 100°C. In addition, alkali-soluble polysaccharides, in particular alkali-soluble arabinoxylans, had a broader molecular size distribution with a greater proportion of lower molecular weight components than did water-soluble polysaccharides. It was concluded that differences in the solubility characteristics of cell wall polysaccharides are probably not due to molecular size, but rather may reflect differences in fine structure or interaction with other cell wall components.

The isolated endosperm cell walls contained 0.48% nitrogen. Protein associated with the walls differed from total barley endosperm protein in amino acid composition and contained proportionately more of several amino acids that have the potential to form linkages with cell wall carbohydrate. The cell wall preparation did not contain hydroxyproline.

Starch associated with the cell walls was almost exclusively of the small granule type, and was relatively resistant to hydrolysis by amyloglucosidase. Dissolution of the starch with perchloric acid or treatment with α-amylase facilitated amyloglucosidase hydrolysis.

Scanning electron microscopy showed that the intracellular wall surfaces were marked by the adpression of large and small starch

granules. The various patterns left by the granules indicated that the proportions of large and small granules may vary in different endosperm cells. Some cells may contain few, if any, small granules.

Treatment of isolated barley endosperm cell walls with extracts of malted barleys showed that barley cultivars differ in cell wall-solubilizing properties. Differences in the rate and extent of hydrolysis of cell wall fragments, and differences in the molecular size distributions of solubilized cell wall carbohydrate were observed. Such variations of cell wall-solubilizing properties are likely to affect the rate of endosperm modification and may influence the malting quality of barleys. The experimental data suggest that hydrolysis of arabinoxylan by endo-β1,4-xylanase(s) may be a limiting factor in cell wall breakdown, whereas there was no apparent relation between endo-β-glucanase activities and solubilization of the cell wall.

Experiments with malt endo-β-glucanases demonstrated that endo-β1,3(4)-glucanase can hydrolyze isolated endosperm cell walls, and confirmed that endo-β1,3-glucanase is also a cell wall-hydrolyzing enzyme. Although endo-β1,3-glucanase does not appear to increase the extent of hydrolysis over that obtained with endo-β1,3(4)-glucanase alone, the effect of endo-β1,3-glucanase may be to increase the rate of cell wall breakdown. The soluble products of endo-β1,3-glucanase hydrolysis of isolated cell wall fragments 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 endo-β-glucanases,although all of the glucan was solubilized by treatment with crude malt extracts. Some other factor, perhaps proteolytic activity (Forrest and Wainwright 1977), may be required for complete solubilization of β-glucan and disruption of the

wall structure.

Scanning electron microscopy of cell wall fragments after treatment with malt extracts or endo-β-glucanases showed that the wall surfaces appeared to be very rough and pitted. Holes were observed in some of the cell wall fragments treated with endo-β1,3-glucanase. Wall structure was still apparent after relase of up to 85% of the cell wall carbohydrate by enzymic hydrolysis.

The results of the present study indicate that, in addition to endo-β-glucanases, a mannanase and two endo-β1,4-xylanases having different action patterns are involved in cell wall degradation. Other activities, such as proteolytic enzymes are also likely to be important. Isolation and characterization of the enzymes is required before their roles in cell wall breakdown can be assessed more fully. In addition, assessment of enzymic alteration in cell wall structure during germination or malting will be dependent upon the elucidation of the structural organization of components in the intact endosperm cell wall.

APPENDIX

Barley Endosperm Cell Walls: A Review of Cell Wall Polysaccharides and Cell Wall-degrading Enzymes

R. G. Thompson and D. E. LaBerge. 1977

MBAA Technical Quarterly 14: 238-243.

Abstract

This review is an attempt to summarize current knowledge of the composition and structure of endosperm cell wall of barley and of the enzymes that degrade the cell wall structure during the germination process. Contents include a summary of knowledge derived during early studies, the nature of the non-starchy polysaccharides (NSP) detected in barley endosperm tissue, a review of literature pertaining to isolation and characterization of enzymes that degrade NSP, and, finally, a summary of recent studies on isolated fragments of the endosperm cell walls of barley.

I. Introduction

The function of the cell wall in barley endosperm, as in other plant tissues, is to support the contents of the cells. This is not a static role. As barley grains develop, endosperm cell walls provide structural support, but at maturity, when endosperm cells are filled with starch and protein, structural rigidity of the wall is not of major importance. During germination, endosperm cell walls present

a barrier to the movement of hydrolytic enzymes that mobilize endosperm reserves.

Knowledge of the structure of barley endosperm cell walls has developed from an academic interest in the biochemistry and physiology of grain development, maturation and germination and also from a desire to understand and control the process whereby barley is converted to malt. For decades, it has been assumed that conversion of hard kernels of barley to friable kernels of malt is associated with physical and chemical changes of the endosperm cell wall although the structure of the cell wall and the precise nature of these changes has not yet been determined.

This review traces the development of knowledge of cell wall materials and cell wall-degrading enzymes from early studies to recent investigations of the composition and structure of isolated barley endosperm cell walls.

II. Early Studies

As early as 1890, Brown and Morris concluded that endosperm cell walls of barley were dissolved during malting with concomitant softening of the endosperm. Early investigators also recognized that non-starchy polysaccharides (NSP) were involved in cell wall degradation and endosperm modification. Polysaccharides containing arabinose and xylose were identified in barley (Brown *et al.* 1906, Lindet 1903) and in brewers' spent grains (Tollens and Stone 1888, Schulze and Tollens 1892 and Lipmann 1904, as cited in Preece 1931). Water-soluble, pentose-containing polysaccharides increased during germination, and this increase in soluble NSP was attributed to cell wall degradation (Schone and Tollens 1900, Windisch and van Waveren 1909).

In 1917, Baker and Hulton established that enzymes from green malt could degrade arabinoxylans from barley and from brewers' spent grains. Lüers and Volkamer (1928) achieved a 21-fold purification of xylanase from green malt by alcohol precipitation and adsorption on alumina. Xylanase activity increased 2.5 times during germination and decreased during kilning to lower levels than found in the original barley (Lüers and Malsch 1929).

Fink and Hartmann (1934) proposed that the increase of soluble arabinoxylan during malting be used as an index of malt modification. This method proved to be unsuitable as the amount of arabinoxylan levelled off before modification was complete (Fink 1935), and there was little varietal difference of soluble arabinoxylan contents of barleys or of malts (Ender and Schneebauer 1938).

The foregoing studies indicated that arabinoxylans were present in barley and malt and that changes of solubility due to enzymic degradation during malting were related to endosperm modification. However, in 1936, Piratzky and Piratzky and Wiecha (1937, 1938) found that a NSP consisting only of glucose was also involved in malt modification. This substance was isolated from worts of unusually high viscosity prepared from under-modified malts, and probably corresponded to the α-amylan described by O'Sullivan in 1882. This glucan could not be isolated from worts of normal viscosity prepared from well-modified malts. As enzymes from barley and malt effectively reduced wort viscosity, it was concluded that the decrease of viscosity of malt extracts observed during malting was the result of enzymic degradation of β-glucan.

The conclusion that endosperm cell walls were broken down during germination, originally expressed by Brown and Morris in

1890, was confirmed by Dickson and Shands in 1941. Photomicrographs of barley taken at various stages of germination clearly demonstrated this phenomenon. Thus, it was gradually established, over a period spanning 50 years, that the structure of barley endosperm cell walls was disrupted during germination and that at least two non-starchy polysaccharides and the enzymes that degrade these polysaccharides were involved in endosperm modification.

III. The Non-starchy Polysaccharides (NSP) of Barley and Malt

Intensive studies of NSP were initiated during the 1950's, and one of the first conclusions was a reaffirmation that changes of solubility of NSP during malting was a result of change of the endosperm cell wall structure. Preece and co-workers (1950) demonstrated that water-soluble and alkali-soluble NSP from barley and malt were similar in chemical composition. The major constituents of each NSP fraction were glucose, arabinose and xylose. β-glucan and arabinoxylan were identified in wort and beer, presumably derived from the NSP of barley (Grain Research Laboratory 1949, Preece 1951).

Initial fractionation of glucose- and pentose-containing polysaccharides from solutions was obtained using Fehling's solution and acetone (Preece *et al.* 1950). One fraction yielded only glucose after acid hydrolysis, while the other fraction had a mixture of glucose, xylose and arabinose. A more successful fractionation of the water-soluble NSP from barley and other cereals was achieved by fractional precipitation with ammonium sulfate (Preece and Hobkirk 1953, Preece and MacKenzie 1952). β-glucan was selectively precipitated

from extracts of barley by 20% ammonium sulfate. Pure arabinoxylan fractions were not obtained, but fractions containing only 6-7% glucose were recovered. An important finding was that an arabinoxylan fraction free of β-glucan could be precipitated from extracts of rye flour by 40-50% ammonium sulfate. The arabinoxylan from rye was used as a substrate in subsequent studies of the arabinoxylan-degrading enzymes of barley and malt (Preece and Hobkirk 1955, Preece and MacDougall 1958).

The water-soluble NSP of malt and the alkali-soluble NSP of barley were fractionally precipitated by ammonium sulfate in a similar manner producing pure β-glucan fractions and arabinoxylan-enriched fractions (Preece and Hobkirk 1954, Preece and MacKenzie 1952). However, comparatively low yields of NSP were obtained from malt extracts, and solutions of NSP from malt were of low viscosity compared to solutions of NSP from barley. This effect was produced by the virtual elimination of β-glucan during malting (Piratzky and Wiecha 1937, 1938). The results supported the earlier conclusion of Preece and Ashworth (1950) that the NSP of barley and malt were chemically related and that changes in solubility during malting were due to enzymic degradation.

A. Arabinoxylan

The structure of arabinoxylans from barley endosperm cell walls was investigated by Aspinall and Ferrier (1958) and Aspinall and Ross (1963). Water-soluble arabinoxylan has a main chain of β-1,4-linked xylopyranose units. Certain units of the xylopyranose main chain are singly branched having one L-arabinofuranose unit attached at either the C_2 or C_3 position and other xylopyranose units

are doubly branched, having an L-arabinofuranose unit attached at each of the C_2 and C_3 positions. More recently, Ballance (1976) found that alkali-soluble arabinoxylan from barley endosperm cell walls has a much higher proportion of doubly branched xylose residues than the water-soluble arabinoxylan studied by Aspinall and Ferrier (1958), although the two polysaccharides have similar proportions of arabinose and xylose.

The distribution of arabinose branches along the xylan main chain has not been studied for barley arabinoxylan. Investigations of the branching patterns of arabinoxylans from wheat, durum wheat and rye flours indicated that arabinose side chains occur most frequently on isolated xylose residues, less frequently on two or three consecutive xylose units and never on four or more contiguous xylose residues (Aspinall and Ross 1963, Ewald and Perlin 1959, Medcalf and Gilles 1968). Approximately 75% of water-soluble wheat flour arabinoxylan consists of fairly highly branched regions of the type described above. The branched sections are interrupted approximately every 20-25 main chain units by open regions of two to five unbranched xylose residues (Goldschmid and Perlin 1963, Perlin and Reese 1963).

The arrangement of arabinose side chains of arabinoxylans extracted from isolated aleurone cell walls of barley may control the ability of arabinoxylans to bind to other cell wall components (McNeil *et al.* 1975). Arabinoxylan that bound cellulose contained regions of at least four contiguous unbranched xylose units in the main chain, while arabinoxylan that did not bind cellulose had unbranched regions of only two adjacent xylose residues.

B. β-Glucan

The initial structural investigations of barley β-glucan were made by Aspinall and Telfer (1954). Studies of the products obtained on hydrolysis of the methylated polysaccharide revealed a linear structure of glucose linked β1,3 and β1,4 in approximately equal proportions. In this respect, barley β-glucan appeared to be similar to lichenin, a mixed-linkage β-glucan from Iceland moss (Chanda *et al.* 1957, Peat *et al.* 1957).

The arrangement of 1,3- and 1,4-linkages in barley and oat glucans was studied by Parrish and co-workers (1960). Analysis of the products obtained when the polysaccharides were hydrolyzed by a cellulase from *Streptomyces* QM B14 and laminarase from *Rhizopus arrhus* QM 1032 revealed that the two β-glucans were similar in structure. Each consisted primarily of two types of repeating units: a tetrameric unit in which a single 1,3-linkage alternated with two adjacent 1,4-linkages and a pentameric unit in which a single 1,3-linkage alternated with three consecutive 1,4-linkages. Identification of 3-O-cellobiosyl glucose and 3-O-cellotriosyl glucose as the main products of hydrolysis of barley β-glucan by purified endo-β-glucanases from *Bacillus subtilus* (Moscatelli *et al.* 1961) and germinated barley (Luchsinger *et al.* 1965) confirmed the general structure postulated by Parrish and co-workers (1960).

In addition to these repeating structures, blocks of adjacent 1,3-linkages have been identified in barley β-glucan by enzymic and chemical methods (Bathgate *et al.* 1974, Fleming and Manners 1966, Igarashi and Sukurai 1966, Moscatelli *et al.* 1961). The length of 1,3-linked sequences differs depending on the source of the glucan

and the method of extraction (Bathgate *et al.* 1974). Bathgate and co-workers (1974) proposed a model of barley β-glucan structure in which segments of the molecule consisting of the repeating units previously described are interrupted by less orderly regions that contain longer sequences of β-1,3-linkages.

IV. Enzymes That Degrade Non-starchy Polysaccharides (NSP) of Barley and Malt

Preece and Ashworth (1950) demonstrated that enzymes from green malt were capable of hydrolyzing soluble NSP of barley. Evidence that malt enzymes reduce the molecular size of barley NSP was obtained by Bass and co-workers (1952, 1953). Enzymes extracted from green malt caused a rapid decrease in the viscosity of substrate solutions. The viscosity-reducing enzymes could be separated from α-amylase by fractional precipitation with ammonium sulfate. Paper chromatography of hydrolysis products of NSP revealed that the purified preparation contained both β-glucanase and arabinoxylanase activity.

These studies confirmed earlier studies that extracts of malt contained β-glucan- and arabinoxylan-degrading enzymes. The types of activity in crude preparations could not be characterized further using mixed polysaccharide substrates. The finding that barley β-glucan and rye arabinoxylan could be obtained free of other polysaccharides by fractional precipitation of water-soluble NSP with ammonium sulfate (Preece and Hobkirk 1953, Preece and MacKenzie 1952) allowed further purification and characterization of these enzymes to proceed.

A. Arabinoxylanases

Preece and Hobkirk (1955) demonstrated that barley extracts

contained two enzymes systems capable of hydrolyzing arabinoxylan. One enzyme system rapidly decreased the viscosity of the substrate solution, was optimally active at pH 2.9 and was fairly stable to heat. A second enzyme system caused slow liberation of reducing groups, was optimally active between pH 4.6 and 5.0 and was more susceptible to thermal denaturation.

Preece and MacDougall (1958) analyzed the products of hydrolysis by quantitative paper chromatography, using extracts of barley and rye arabinoxylan as substrate. The first detectable hydrolysis product was free arabinose, indicating the presence of an arabinosidase. No other products were detected initially, suggesting that arabinose side chains blocked the action of enzymes capable of degrading the xylan main chain.

When 3-5% of the arabinose residues were liberated, xylose and xylobiose appeared almost simultaneously in the digests. The release of xylobiose before the production of higher oligosaccharides indicated the presence of an exo-xylanase enzyme that removed xylobiose units from the main chain. The simultaneous appearance of xylose suggested the presence of a xylobiase enzyme, and this was confirmed using purified xylobiose as substrate.

The presence of an endo-xylanase enzyme was confirmed by a smooth, rapid decrease in the viscosity of the enzyme-substrate digests. Oligosaccharides were not detected early in the hydrolysis period, suggesting that the initial products were of high molecular weight. The oligosaccharides detected later in the hydrolysis period were xylotriose, xylotetraose, arabinoxylobiose and tetra-, penta- and hexasaccharides containing both xylose and arabinose.

All four enzyme systems (arabinosidase, xylobiase,

exo-xylanase and endo-xylanase) were present in barley and were enhanced during malting. Kilning decreased the activity of the exo-enzymes to levels at or below those found in the original grain but had less effect on endo-xylanase activity. Similarly, heating the enzyme extracts at 70°C for 15 min inactivated the exo-enzymes, but endo-xylanase activity persisted.

No further studies of the mode of action of barley and malt arabinoxylanases have been reported. In the arabinoxylan-degrading systems of rumen micro-organisms, an arabinosidase removes arabinose from the polysaccharide before the xylan main chain is attacked (Bailey *et al.* 1962, Howard 1955). Arabinose is the first detectable hydrolysis product, and no oligosaccharides containing both arabinose and xylose are produced. However, the arabinoxylan-degrading enzymes of barley (Preece and MacDougall 1958) and of the mould *Myothecium verrucaria* (Bishop and Whitaker 1955) do liberate oligosaccharides containing both arabinose and xylose.

Several investigators have noted that arabinose is the first detectable product of arabinoxylan hydrolysis (Bailey *et al.* 1962, Howard 1955, Preece and MacDougall 1958, Schmitz *et al.* 1974). The action of an arabinosidase may be a prerequisite for hydrolysis of the xylan main chain. Perlin and Reese (1963) reported that a xylanase from *Streptomyces* QM B814 required a minimum of two adjacent unbranched xylose units in order to hydrolyze the chain, and four contiguous unbranched xylose residues were required for production of xylobiose.

B. β-Glucanases

Preece and co-workers observed the effect of barley enzymes on the viscosity and reducing power of barley β-glucan solutions and concluded that the glucanase enzyme system consisted of both

endo- and exo-enzymes, including disaccharidases (Preece *et al.* 1954, Preece and Hoggan 1956). Bass and Meredith (1955) reached a similar conclusion based on identification of glucose, cellobiose, laminaribiose and higher oligosaccharides as the products of β-glucan hydrolysis by green malt enzymes.

Separation of endo-β-glucanase activity from exo-enzymes and disaccharidases in green malt extracts was achieved by Bass and Meredith (1960) by discontinuous gradient elution from an activated charcoal column. Both fractions could hydrolyze barley β-glucan, a mixed β1,3- and β1,4-linked substrate, and laminarin, a substrate containing only β1,3-linkages. It was concluded that malt contained both endo- and exo-enzymes specific for β1,3 and β1,4 bonds.

The status of exo-glucanase activity of barley and malt extracts is not clearly defined. Cellobiase and laminaribiase activities have been demonstrated (Anderson *et al.* 1964, Manners and Marshall 1969, Preece and Hoggan 1956), but it has not been established whether these enzymes are specific disaccharidases or are capable of sequentially hydrolyzing larger substrates. Evidence of an exo-1,4-β-glucanase was obtained by Luchsinger and co-workers (1962) who found an enzyme fraction from germinated barley that increased the reducing power of a carboxymethylcellulose solution but had little effect on viscosity.

Manners and Marshall (1969) reported the separation of two β-glucosidase fractions by molecular sieve chromatography of malt extracts. Each fraction could hydrolyze β-1,3-linked glucans, β-1,4-linked glucans and barley β-glucan to glucose. As these two fractions hydrolyzed cellobiose and laminaribiose more rapidly than cellodextrin or laminarin, it was suggested that the enzymes were true β-glucosidases rather than exo-enzymes. However, the fractions

were not free of endo-β-glucanase activities, and it is possible that the combined action of endo-β-glucanase and disaccharidases, rather than β-glucosidases or exo-glucanases were responsible for complete hydrolysis of substrates. Rigorous purification and characterization of enzymes is required before meaningful conclusions can be drawn concerning the exo-β-glucanase or β-glucosidase activity of barley and malt.

Endo-β-glucanase enzymes have an important role in degrading high viscosity β-glucans during the mashing process of brewing and, as a result, have been studied more intensively than the exo-β-glucanase enzymes. The plurality of endo-β-glucanase enzymes was recognized by Luchsinger and co-workers (Luchsinger 1962, Luchsinger *et al.* 1962), who identified activities specific for β-1,3 and for β-1,4 bonds and for the mixed β-1,3- and β-1,4-linkages of barley β-glucan. The presence of these three types of endo-β-glucanase activity in barley and malt has since been confirmed by other investigators (Ballance 1973, Manners and Marshall 1969).

An endo-1,4-β-glucanase was isolated from extracts of germinated barley by ion-exchange chromatography on DEAE-cellulose (Moffa and Luchsinger 1970). The enzyme hydrolyzes CM-cellulose and barley β-glucan but had no effect on insoluble cellulose or laminarin.

An endo-β-glucanase specific for β-1,3-linkages was isolated free of other endo-β-glucanase activities by several investigators (Ballance and Meredith 1976, Luchsinger *et al.* 1963, Manners and Marshall 1969). This enzyme hydrolyzed laminarin, laminarioligosaccharides and pachyman to G_2 through G_7 oligosaccharides but had little or no effect on barley β-glucan or laminaribiose. Endo-1,3-β-glucanase hydrolyzed malt β-glucan to a limited extent presumably due to the

presence of longer sequences of β-1,3-linkages in the malt substrate (Bathgate *et al.* 1974, Manners and Wilson 1974).

Luchsinger (1962) postulated the existence of an enzyme specific for adjacent β-1,3- and β-1,4-linkages. This was confirmed by identification of 3-O-cellobiosyl glucose and 3-O-cellotriosyl glucose as the major hydrolysis products of barley β-glucan by an enzyme preparation from germinated barley (Luchsinger *et al.* 1965). Manners and Wilson (1976) have isolated an endo-1,3(4)-β-glucanase enzyme with similar properties from malt extracts. The enzyme hydrolyzes β-1,4-linkages in which glucose on the non-reducing side of the linkage is attached to the adjacent glucose at the C_3 position. The enzyme does not attack substrates containing only β-1,3 bonds (laminarin, pachyman, CM-pachyman) or only β-1,4 bonds (cellulose, CM-cellulose).

During germination, endo-1,3-β-glucanase and endo-1,3(4)-β-glucanase activities increase sharply, and increases of endo-1,4-β-glucanase and β-glucosidase activities are much lower (Ballance and Meredith 1976, Manners and Marshall 1969). Ballance (1976) proposed a model for β-glucan degradation during germination of barley. Endo-1,3-β-glucanase is specific for consecutive β-1,3-linkages, and more potential hydrolytic sites may be present in the insoluble β-glucan fraction contained in the endosperm cell wall. The main function of this enzyme may be to solubilize the endosperm cell wall allowing endo-1,3(4)-β-glucanase, the enzyme chiefly responsible for hydrolysis of β-glucan, and endo-1,4-β-glucanase to degrade the solubilized β-glucan to oligosaccharides. These, in turn, are hydrolyzed to glucose by β-glucosidase enzymes.

V. Studies on Isolated Barley Endosperm Cell Walls

Isolation of cell walls from barley endosperm and analysis of composition has confirmed the long-held assumption that non-starchy polysaccharides (NSP) of barley are derived from the cell walls. Endosperm cell walls were isolated from barley flour by Costello (1968) using an aqueous commercial batter process, while Fincher (1975) and Ballance (1976) used wet-sieving of flour suspended in 70% ethanol to avoid loss of polymeric material.

Analyses by these investigators indicate that endosperm cell walls are composed almost entirely of polysaccharides, together with 5 to 6% protein. The polysaccharides are composed of only four monosaccharides: glucose, arabinose, xylose and mannose. The absence of rhamnose, fucose and galactose and the presence of only traces of uronic acids suggests that pectic substances are of negligible importance. The monosaccharide composition as determined by these studies was 74-79% glucose, 9-11% arabinose, 11-13% xylose and 2-3% mannose.

Most of the glucose in the endosperm cell wall is in the form of a mixed linkage β-glucan. A small amount, perhaps 2 to 4%, may be present as a glucomannan, but cellulose, if present, accounts for less than 1% of the cell wall material (Ballance (1976).

Analysis of fractions obtained by successive extraction of isolated walls with water and alkali indicated that β-glucan and arabinoxylan polysaccharides are present in similar proportions in both fractions. Some mannose-containing polysaccharides were detected in the water-soluble fraction, but the bulk of this material remained in the alkali-insoluble residue.

The differences between the water-soluble β-glucans and water-soluble arabinoxylans and their alkali-soluble counterparts from

cell walls are not known but may be due to differences in molecular structure, degree of polymerization or interaction with other cell wall components. Methylation studies of water- and alkali-extracted β-glucans (Ballance 1976) and studies of urea-extracted β-glucans by periodate oxidation (Costello 1968) have shown that these β-glucan fractions contain similar proportions of 1,3 and 1,4 bonds (approximately 3 to 7). Water soluble β-glucan was shown to be more highly polymerized than alkali-soluble β-glucan (Ballance 1976). Alkali-soluble arabinoxylan from isolated endosperm cell walls (Ballance 1976) has a higher proportion of doubly branched xylose residues compared to water-soluble material (Aspinall and Ferrier 1958).

Further investigations of the polysaccharides from isolated endosperm cell walls of barley are required. Very little is known about how these polysaccharides interact with one another and with other cell wall components to form the cell wall structure.

The role of the protein in endosperm cell wall preparations is not known, due to the difficulty of determining whether protein is a structural component of the wall or a contaminating protein of cytoplasmic origin. Costello (1968) concluded that protein was bound firmly to endosperm walls as protein could not be solubilized by phenol-acetic acid-water (2:1:1), 60% aqueous chloroethanol or 3M urea. However, Ballance (1976) observed that protein occurred with starch granules on the surface of some cell wall fragments and concluded that at least part of the protein resulted from cytoplasmic contamination.

Protein is considered a structural component of plant cell walls by many investigators. Structural proteins often have a high content of hydroxyproline, and this imino acid is linked to arabinose by a glycosidic bond (Heath and Northcote 1971, Lamport 1969, 1970,

Lamport and Miller 1971). Hydroxyproline has not been detected in preparations of barley endosperm cell walls (Ballance 1976, Costello 1968, Fincher 1975). Glycoproteins other than the hydroxyproline-rich type may be part of the cell wall structure. Isolated endosperm cell walls contain high proportions of aspartic acid, serine and threonine (Costello 1968). These amino acids are found commonly in glycopeptides and glycoproteins where the carboxyl group of aspartic acid or the hydroxyl groups or serine or threonine are linked to carbohydrates.

Palmer (1971) has suggested that individual endosperm cells of barley are separated from one another by an intercellular matrix of protein, rather than a middle lamella of pectic substances commonly found in primary cell walls of plant tissues. Treatment of isolated endosperm tissue with papain or trypsin will dissociate the tissue into individual cells. Weak sodium hydroxide solution also dissociated the packed endosperm cells, indicating that the intercellular protein has characteristics of glutelin-type proteins.

Rather than a direct-carbohydrate linkage, cell wall protein may be chelated to ferulic acid which, in turn, is ester-linked to polysaccharide (Lamport 1970), or polysaccharide chains may be cross-linked by dimeric ferulic acid (Geissman and Neukom 1973). Ferulic acid esterified with polysaccharide has been identified in the cell walls of rye grass (Hartley 1973, Hartley *et al.* 1976). Although ferulic acid has been detected in endosperm cell walls of barley (Fincher 1976), its role in the cell wall structure has not been investigated further.

The appearance of isolated endosperm cell walls of barley was investigated by scanning and transmission electron microscopy

(Ballance 1976, Fincher 1975). Two types of wall surface were distinguishable by scanning electron microscopy. The inner or intracellular surface was pitted, probably a result of adpression of starch granules on this surface, while the outer or extracellular surface was smooth. Fincher (1975) observed two distinct layers in cross sections of the wall and found that the indentations on the inner wall disappeared following extraction with water. These observations led to the conclusion that water-soluble polysaccharides were layered on the cell wall surface rather than distributed throughout the wall. Layering of cell wall components was not observed by Ballance (1976). Transmission electron microscopy of endosperm cell walls following successive extraction with water and alkali revealed the multinet microfibrillar nature of the residual wall structure (Ballance 1976, Fincher 1975).

Ballance (1976) demonstrated that endo-1,3-β-glucanase from malted barley and a fungal endo-1,3(4)-β-glucanase could hydrolyze isolated barley endosperm cell walls and cause extensive degradation. The combined action of these enzymes removed most of the mixed linkage β-glucan, but the structure of the cell wall was still apparent.

Treatment of cell-wall fragments by arabinoxylanase enzymes has not been investigated. Palmer (1975) has suggested that the order in which hydrolytic enzymes attack the cell wall may be controlled by the arrangement and chemical properties of the arabinoxylans and β-glucans in the cell wall. Arabinoxylanase enzymes probably have a key role in cell wall degradation. The endosperm cell wall serves as a barrier to migration of enzymes during malting, and slow-malting barleys may lack sufficient enzyme activities to quickly disrupt the cell wall structure.

Summary

Although some of the non-starchy polysaccarides and NSP-degrading enzymes have been identified and characterized to varying degrees, our understanding of the process of endosperm modification is far from complete. Much further work is required to delineate the structure of the endosperm cell wall of barley and to determine the manner in which this structure is altered enzymically during the germination process.

LITERATURE CITED

ANDERSON, M.A., COOK, J.A. and STONE, B.A. 1978. Enzymatic determination of 1,3: 1,4-β-glucans in barley grain and other cereals. J. Inst. Brew. 84: 233-239.

ASPINALL, G.O. and FERRIER, R.J. 1958. Cereal gums. III. Constitution of an arabinoxylan from barley flour. J. chem. Soc. 638-642.

ASPINALL, G.O. and ROSS, K.M. 1963. The degradation of two periodate oxidized arabinoxylans. J. chem. Soc. 1681-1686.

ASPINALL, G.O. and TELFER, R.G.J. 1954. Cereal gums. I. The methylation of barley glucans. J. chem. Soc. 3519-3522.

BAILEY, R.W., CLARKE, T.J. and WRIGHT, D.E. 1962. Carbohydrases of the rumen ciliate Epidinium ecaudatum (Crawley). Action on plant hemicellulose. Biochem. J. 83: 517-523.

BAKER, J.L. and HULTON, H.F.E. 1917. Evidence of the existence in malt of an enzyme hydrolyzing the furfuroids of barley. J. chem. Soc. 111: 121-130.

BALLANCE, G.M. 1973. Cytolytic enzymes in barley and malt. M. Sc. Thesis, University of Manitoba, Winnipeg.

BALLANCE, G.M. 1976. Studies on cereal polysaccharides and enzymes. Ph.D. Thesis, Heriot-Watt University, Edinburgh.

BALLANCE, G.M. and MANNERS, D.J. 1978. Structural analysis and enzymic solubilization of barley endosperm cell walls. Carbohydr. Res. 61: 107-118.

BALLANCE, G.M. and MEREDITH, W.O.S. 1976. Purification and partial characterization of an endo-β1,3-glucanase from green malt. J. Inst. Brew. 82: 64-67.

BASS, E.J. and MEREDITH, W.O.S. 1955. Enzymes that degrade barley gums. III. Studies of beta-polyglucosidases of green malt. Cereal Chem. 32: 374-381.

BASS, E.J. and MEREDITH, W.O.S. 1960. Enzymes that degrade barley gums. VII. Fractionation by column chromatography. Proc.Am. Soc. Brew. Chem. 38-47.

BASS, E.J., MEREDITH, W.O.S. and ANDERSON, J.A. 1952. Enzymes that degrade barley gums. I. Isolation from a bacterial source. Cereal Chem. 29: 262-272.

BASS, E.J., MEREDITH, W.O.S. and ANDERSON, J.A. 1953. Enzymes that degrade barley gums. II. Preparations from barley and green malt. Cereal Chem. 30: 313-322.

BATHGATE, G.N. and PALMER, G.H. 1972. A reassessment of the chemical structure of barley and wheat starch granules. Die Stärke 24: 336-341.

BATHGATE, G.N. and PALMER, G.H. 1973. The *in vivo* and *in vitro* degradation of barley and malt starch granules. J. Inst. Brew. 79: 402-406.

BATHGATE, G.N., PALMER, G.H. and WILSON, G. 1974. Action of endo-β-1, 3-glucanases on barley and malt β-glucans. J. Inst. Brew. 80: 278-285.

BISHOP, C.T. and WHITAKER, D.R. 1955. Mixed arabinose-xylose oligosaccharides from wheat straw xylan. Chem. Ind. 119.

BROWN, H.T. and MORRIS, G.H. 1890. Researches on the germination of some of the gramineae. J. chem. Soc. 57: 458-528.

BROWN, H.T., ESCOMBE, F., McMULLEN, A. and MILLAR, J.H. 1906. Water soluble polysaccharides of barley and malt. Trans. Guiness Res. Lab. 1: 312 (Abstract J. Inst. Brew. 13: 83, 1907).

CAMERON, J.A. 1968. Bacterial lipopolysaccharide as a void volume marker for agarose gel permeation chromatography. J. Chromatogr. 37: 331-332.

CHANDA, N.B., HIRST, E.L. and MANNERS, D.J. 1957. A comparison of isolichenin and lichenin from Iceland moss (Cetraria islandica). J. chem. Soc. 1951-1958.

CLARKE, A.E. and STONE, B.A. 1962. β-1,3-Glucan hydrolases from the grape vine and other plants. Phytochem. 1: 175-188.

COSTELLO, P.R. 1968. Preparation of cereal endosperm cell walls with structural studies on the wall components. M. Sc. Thesis, University of Melbourne, Melbourne.

DASHEK, W.V. and CHRISPEELS, M.J. 1977. Gibberellic-acid-induced synthesis and release of cell-wall-degrading endoxylanase by isolated aleurone layers of barley. Planta 134: 251-256.

DICKSON, J.G. and SHANDS, H.L. 1941. Cellular modification of the barley kernel during malting. Proc. Am. Soc. Brew. Chem. 4: 1-10.

DYGERT, S., LI, L.H., FLORIDA, D. and THOMA, J. 1965. Determination of reducing sugar with improved precision. Anal. Biochem. 13: 367-374.

ENDER, C. and SCHNEEBAUER, F. 1938. Analytical judgment of malt modification. I. Woch. Brau. 55: 73-78, 81-86. (Abstract J. Inst. Brew. 44: 342-344, 1938).

EWALD, C.M. and PERLIN, A.S. 1959. The arrangement of branching in an arabino-xylan from wheat flour. Can. J. Chem. 37: 1254-1259.

FINCHER, G.B. 1975. Morphology and chemical composition of barley endosperm cell walls. J. Inst. Brew. 81: 116-122.

FINCHER, G.B. 1976. Ferulic acid in barley cell walls: A fluorescence study. J. Inst. Brew. 82: 347-349.

FINK, H. 1935. Modification of malt. Woch. Brau. 52: 265-267. (Abstract J. Inst. Brew. 41: 449, 1935)

FINK, H. and HARTMANN, J. 1934. Pentosans in the malting of barley. Woch. Brau. 51: 273-276. (Abstract J. Inst. Brew. 40: 425, 1934)

FLEMING, M. and KAWAKAMI, K. 1977. Studies of the fine structure of β-D-glucans of barleys extracted at different temperatures. Carbohydr. Res. 57: 15-23.

FLEMING, M. and MANNERS, D.J. 1966. A comparison of the fine structure of lichenin and barley glucan. Biochem. J. 100: 4P-5P.

FORREST, I.S. 1977. The role of protein in the structure of barley endosperm cell walls. Biochem. Soc. Trans. 5: 1154-1156.

FORREST, I.S. and WAINWRIGHT, T. 1977. The mode of binding of β-glucans and pentosans in barley endosperm cell walls. J. Inst. Brew. 83: 279-286.

FLUCHER, R.G., SETTERFIELD, G., McCULLY, M.E. and WOOD, P.J. 1977. Observations of the aleurone layer. II. Fluorescence microscopy of the aleurone-sub-aleurone junction with emphasis on possible β1,3-glucan deposits in barley. Aust. J. Plant Physiol. 4: 917-928.

GEISSMAN, T. and NEUKOM, H. 1973. Composition of the water soluble wheat flour pentosans and their oxidative gelation. Lebensmittel-Wissenschaft und Technologie 6: 59-62. (Chem. Abstr. 79: 30641h, 1973)

GOLDSCHMID, H.R. and PERLIN, A.S. 1963. Interbranch sequences in the wheat arabinoxylan. Selective enzymolysis studies. Can. J. Chem. 41: 2272-2277.

GRAIN RESEARCH LABORATORY. 1949. Twenty-third annual report. pp 31-32

HARTLEY, R.D. 1973. Lignin-carbohydrate linkages in plant cell walls. I. Carbohydrate esters of ferulic acid as components of cell walls of Lolium multiflorum. Phytochem. 12: 661-665.

HARTLEY, R.D., JONES, E.C. and WOOD, T.M. 1976. Carbohydrates and carbohydrate esters of ferulic acid released from cell walls of Lolium multiflorum by treatment with cellulolytic enzymes. Phytochem. 15: 305-307.

HEATH, M.F. and NORTHCOTE, D.H. 1971. Glycoprotein of the wall of sycamore tissue-culture cells. Biochem. J. 125: 953-961.

HOWARD, B.H. 1955. Hydrolysis of the soluble pentosans of wheat flour and Rhodymenia palmata by ruminal microorganisms. Biochem. J. 67: 643-651.

IGARASHI, O. and SAKURAI, Y. 1966. Non-starchy polysaccharides of the endosperm of naked barley. II. The periodate oxidative degradation of F-1 β-glucan prepared from the endosperm of naked barley. Agr. Biol. Chem. 30: 642-645.

KESLER, R.B. 1967. Rapid quantitative anion-exchange chromatography of carbohydrates. Anal. Chem. 39: 1416-1422.

KULP, K. 1968. Enzymolysis of pentosans of wheat flour. Cereal Chem. 45: 339-350.

LaBERGE, D.E., MacGREGOR, A.W. and MEREDITH, W.O.S. 1973. Changes in the free sugar content of barley kernels during maturation. J. Inst. Brew. 79: 471-477.

LAMPORT, D.T.A. 1969. The isolation and partial characterization of hydroxyproline-rich glycopeptides obtained by enzymic degradation of primary cell walls. Biochem. 8: 1155-1163.

LAMPORT, D.T.A. 1970. Cell wall metabolism. Ann. Rev. Plant Physiol. 21: 235-270.

LAMPORT, D.T.A. and MILLER, D.H. 1971. Hydroxyproline arabinosides in the plant kingdon. Plant Physiol. 48: 454-456.

LINDET, L. 1903. The carbohydrates of barley and their transformations during germination (malting). C.R. Acad. Sci. (Paris) 137: 73. (Abstract J. Inst. Brew. 9: 498, 1903).

LUCHSINGER, W.W. 1962. The mechanism of action of malt beta-glucanases. II. Separation and characterization of malt endo-beta-glucanases. Cereal Chem. 39: 225-235.

LUCHSINGER, W.W., CHEN, S.-C. and RICHARDS, A.W. 1965. Mechanism of action of malt β-glucanases. 8. Structures of products formed during hydrolysis of barley β-D-glucan by A_{11}-endo-β-glucanase. Arch. Biochem. Biophys. 112: 524-536.

LUCHSINGER, W.W., FERRELL, W.J. and SCHNEBERGER, G.L. 1963. The mechanism of action of malt beta-glucanases. IV. The preparation and properties of laminarinase from germinated barley. Cereal Chem. 40: 554-563.

LUCHSINGER, W.W., HOU, E.J. and SCHNEBERGER, G.L. 1962. The mechanism of action of malt beta-glucanases. III. The number and types of beta-glucanases present in germinated barley. Proc. West. Va. Acad. Sci. 34: 51-58. (Chem. Abstr. 58: 10472d 1963).

LÜERS, H. and MALSCH, L. 1929. Alteration of the hemicellulose-splitting enzyme during the germination and kilning process. Woch. Brau. 46: 163-164. (Abstract J. Inst. Brew. 35: 297-298, 1929).

LÜERS, H. and VOLKAMER, W. 1928. Malt enzyme (cytase) which decomposes hemicelluloses. Woch. Brau. 45: 83-87. (Abstract J. Inst. Brew. 34: 287, 1928).

MacLEOD, A.M. 1976. Malting-the ultimate? MBAA Tech. Quart. 13: 193-198.

MacLEOD, A.M., JOHNSON, C.S. and DUFFUS, J.H. 1964. Ultra-structure of caryopses of the Gramineae I. Aleurone and central endosperm of Bromus and barley. J. Inst. Brew. 70: 303-307.

MANNERS, D.J. and MARSHALL, J.J. 1969. Carbohydrate metabolizing enzymes. XXII. The β-glucanase system of malted barley. J. Inst. Brew. 75: 550-561.

MANNERS, D.J., MASSON, A.J. and STURGEON, R.J. 1971. An enzymic method for the determination of the degree of polymerization of glucans. Carbohydr. Res. 17: 109-114.

MANNERS, D.J. and WILSON, G. 1974. Purification and properties of an endo-(1→3)-β-D-glucanase from malted barley. Carbohydr. Res. 37: 9-22.

MANNERS, D.J. and WILSON, G. 1976. Purification of malted-barley endo-β-D-glucanases by ion-exchange chromatography: some properties of an endo-barley-β-D-glucanase. Carbohydr. Res. 48: 255-264.

MARES, D.J. and STONE, B.A. 1973a. Studies on wheat endosperm. I. Chemical composition and ultrastructure of the cell walls. Aust. J. biol. Sci. 26: 793-812.

MARES, D.J. and STONE, B.A. 1973b. Studies on wheat endosperm. II. Properties of the wall components and studies on their organization in the wall. Aust. J. biol. Sci. 26: 313-330.

McNEIL, M., ALBERSHEIM, P., TAIZ, L. and JONES, R.L. 1975. The structure of plant cell walls. VII. Barley aleurone cells. Plant Physiol. 55: 64-68.

MEDCALF, D.G. and GILLES, K.A. 1968. Structural characterization of a pentosan from the water-insoluble portion of durum wheat endosperm. Cereal Chem. 45: 550-556.

MOFFA, D.J. and LUCHSINGER, W.W. 1970. Endo-beta-1,4-glucan hydrolase from germinated barley. Cereal Chem. 47: 54-63.

MOSCATELLI, E.A., HAM, E.A. and RICKES, E.L. 1961. Enzymatic properties of a β-glucanase from Bacillus subtilus. J. biol. Chem. 236: 2858-2862.

NIELSEN, J.P. 1943. Rapid determination of starch. An index to maturity in starchy vegetables. Ind. Eng. Chem. Anal. Ed. 15: 176-179.

NIELSEN, J.P. and GLEASON, P.C. 1945. Rapid determination of starch. Factors for starches and comparison with acid and enzymic hydrolysis methods. Ind. Eng. Chem. Anal. Ed. 17: 131-134.

O'SULLIVAN, C. 1882. Alpha-beta-amylan: constituents of some cereals. J. chem. Soc. 41: 24-32.

PALMER, G.H. 1971. Modes of action of gibberellins during malting. Proc. Eur. Brew. Conv., 13th Congress, Estoril, 59-71.

PALMER, G.H. 1975. Influence of endosperm structure on extract development. Proc. Am. Soc. Brew. Chem. 33: 174-180.

PARRISH, F.W., PERLIN, A.S. and REESE, E.T. 1960. Selective enzymolysis of poly-β-D-glucans, and the structure of the polymer. Can. J. Chem. 38: 2094-2104.

PEAT, S., WHELAN, W.J. and ROBERTS, J.G. 1957. The structure of lichenan. J. chem. Soc. 3916-3924.

PERLIN, A.S. and REESE, E.T. 1963. Dimensions of the substrate site involved in the enzymolysis of a polysaccharide. Can. J. Biochem. Physiol. 41: 1842-1846.

PIRATZKY, W. 1936. Viscosity of malt worts. Woch. Brau. 53: 105. (Abstract J. Inst. Brew. 42: 264, 1936).

PIRATZKY, W. and WIECHA, G. 1937. Changes in the viscosity of malt worts due to enzyme action. Woch, Brau. 54: 145-148. (Abstract J. Inst. Brew. 43: 311, 1937).

PIRATZKY, W. and WIECHA, G. 1938. Amylan. Woch. Brau. 55: 97-100. (Abstract J. Inst. Brew. 44: 339-340, 1938).

PREECE, I.A. 1931. Studies on hemicelluloses. The hemicelluloses of brewers' grains. J. Inst. Brew. 37: 409-413.

PREECE, I.A. 1951. Non-starchy polysaccharides of barley, and their influence on wort composition. Proc. Eur. Brew. Conv., 3rd Congress, Brighton, 213-227.

PREECE, I.A., AITKEN, R.A. and DICK, J.A. 1954. Non-starchy polysaccharides of cereal grains. VI. Preliminary study of the enzymolysis of barley β-glucosan. J. Inst. Brew. 60: 497-507.

PREECE, I.A. and ASHWORTH, A.S. 1950. Cytolysis in germinating barley. II. Preliminary study of enzyme relationships. J. Inst. Brew. 56: 40-47.

PREECE, I.A., ASHWORTH, A.S. and HUNTER, A.D. 1950. Cytolysis in germinating barley. I. Some barley and malt polysaccharides. J. Inst. Brew. 56: 33-40.

PREECE, I.A. and HOBKIRK, R. 1953. Non-starchy polysaccharides of cereal grains. II. Higher molecular gums of common cereals. J. Inst. Brew. 59: 385-392.

PREECE, I.A. and HOBKIRK, R. 1954. Non-starchy polysaccharides of cereal grains. V. Some hemicellulose fractions. J. Inst. Brew. 60: 490-496.

PREECE, I.A. and HOBKIRK, R. 1955. Non-starchy polysaccharides of cereal grains. VII. Preliminary study of pentosan enzymolysis. J. Inst. Brew. 61: 393-399.

PREECE, I.A. and HOGGAN, J. 1956. Enzymatic degradation of cereal hemicelluloses. I. Observations on the β-glucanase system and its development during malting. J. Inst. Brew. 62: 486-496.

PREECE, I.A. and MacDOUGALL, M. 1958. Enzymatic degradation of cereal hemicelluloses. II. Pattern of pentosan degradation. J. Inst. Brew. 64: 489-500.

PREECE, I.A. and MacKENZIE, K.G. 1952. Non-starchy polysaccharides of cereal grains. I. Fractionation of barley gums. J. Inst. Brew. 58: 353-362.

SCHMITZ, J.F., McDONALD, C.E. and GILLES, K.A. 1974. Arabinoxylanases and cellulases of wheat. Cereal Chem. 51: 809-821.

SCHÖNE, A. and TOLLENS, B. 1900. The behaviour of the pentosans of seeds during germination. J. Landwirts. 48: 349-354. (Abstract J. Inst. Brew. 7: 288, 1901).

SMITH, J.S. and LINEBACK, D.R. 1976. Hydrolysis of native wheat and corn starch granules by glucoamylases from Aspergillus niger and Rhyzopus niveus. Die Stärke 28: 243-248.

TAIZ, L. and HONIGMAN, W.A. 1976. Production of cell wall hydrolyzing enzymes by barley aleurone layers in response to gibberellic acid. Plant Physiol. 58: 380-386.

TETLOW, J.A. and WILSON, A.L. 1964. An adsorptiometric method for determining ammonia in boiler feed water. The Analyst 89: 453-463.

THOMPSON, R.G. and LaBERGE, D.E. 1977. Barley endosperm cell walls: A review of cell wall polysaccharides and cell wall-degrading enzymes. MBAA Tech. Quart. 14: 238-243.

TKACHUK, R. and IRVINE, G.N. 1969. Amino acid compositions of cereals and oilseed meals. Cereal Chem. 46: 206-218.

VERHAAR, L.A.T. and DIRKX, J.M.H. 1977. The analysis of mixtures containing glucose, fructose and mannose. Carbohydr. Res. 53: 247-249.

WAINWRIGHT, T. 1978. Barley analysis and malt quality. MBAA Tech. Quart. 15: 184-189.

WINDISCH, W. and van WAVEREN, G. 1909. The pentosans during malting and mashing. Woch. Brau. 26: 581-585. (Chem. Abstr. 4: 947, 1910).